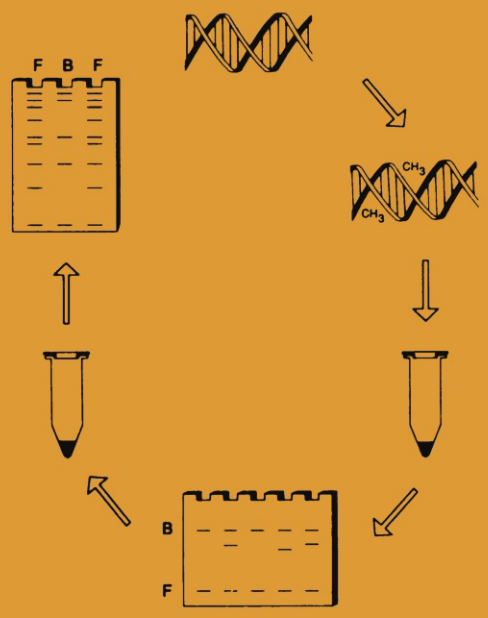
BioMethods

Series Editors
H.P. Saluz
M.M. Becker



J.P. Jost
H.P. Saluz
(Eds.)

A Laboratory Guide to In Vitro Studies of Protein-DNA Interactions

Birkhäuser

BioMethods
Vol. 5

Series Editors:

Dr. H. P. Saluz
IRBM
Via Pontina Km 30,600
I–00040 Pomezia (Roma)/Italy

Dr. M. M. Becker
Dept. of Biological Sciences
University of Pittsburgh
Pittsburgh, PA 15260 / U.S.A.

A Laboratory Guide to In Vitro Studies of Protein-DNA Interactions

edited by
J.P. Jost
H.P. Saluz

Birkhäuser Verlag
Basel · Boston · Berlin

Volume Editors Addresses:

Dr. Jean-Pierre Jost
Friedrich Miescher-Institut
Postfach 2543
CH–4002 Basel / Switzerland

Dr. Hans-Peter Saluz
IRBM
Via Pontina Km 30.600
00040 Pomezia (Rome) / Italy

Cover drawing by Jean-Pierre Jost

Library of Congress Cataloging-in-Publication Data

A Laboratory guide to in vitro studies of protein-DNA interactions /
edited by J.P. Jost, H.P. Saluz.
p. cm. – (BioMethods ; vol. 5)
Includes bibliographical references and index.

1. DNA-protein interactions-Research-Methology.
I. Jost, J.P. (Jean-Pierre), 1937– . II. Saluz, H.P., 1952–
III. Series.
QP624.75.P74L33 1991
574.87'3282–dc20

CIP-Titelaufnahme der Deutschen Bibliothek

A laboratory guide to in vitro studies of protein-DNA interactions /
ed. by J.P. Jost ; H.P. Saluz. – Basel ; Boston ;
Berlin : Birkhäuser, 1991
(BioMethods ; Vol. 5)

NE: Jost, Jean-Pierre [Hrsg.]; GT

Softcover reprint of the hardcover 1st edition 1991
P.O. Box 133
CH–4010 Basel, Switzerland

ISBN 978-3-7643-2627-2 ISBN 978-3-0348-7561-5 (eBook)
DOI 10.1007/978-3-0348-7561-5

"Du point de vue de l'homme c'est l'échelle d'observation qui crée le phénomène. Chaque fois que nous changeons l'échelle d'observation, nous rencontrons des phénomènes nouveaux."

P. Lecomte du Noüy
L'homme et sa destinée 1948

Table of Contents

List of the Contributing Authors

Adhya Sankar
Laboratory of Molecular Biology, Division of Cancer Biology, Diagnosis and Centers, National Cancer Institute, NIH, Bethesda, ND 20892, U.S.A.
Becker Michael M.
Department of Biological Sciences, University of Pittsburgh, PA 15260, U.S.A.
Burkhoff Amanda M.
Department of Chemistry, Johns Hopkins University, Baltimore, Maryland 21218, U.S.A.
Corman Natalie
Friedrich Miescher-Institut, P.O. Box 2543, CH–4002 Basel, Switzerland.
Fogerty Scott
Pharmacia P-L Biochemical Inc., 2202 North Bartlett Avenue, Milwaukee, WI 53202, U.S.A.
Grindley Nigel D.F.
Department of Molecular Biophysics and Biochemistry, Yale University, School of Medicine, 333 Cedar Street, New Haven, CT 06510, U.S.A.
Heiser William
Pharmacia P-L Biochemical Inc., 2202 North Bartlett Avenue, Milwaukee, WI 53202, U.S.A.
Hemmings Brian
Friedrich Miescher-Institut, P.O. Box 2543, CH–4002 Basel, Switzerland.
Hofsteenge Jan
Friedrich Miescher-Institut, P.O. Box 2543, CH–4002 Basel, Switzerland.
Hughes Melya J.
IRBM Via Pontina KM 30,600, 00040 Pomezia (Roma), Italy.
Jiricny Josef
IRBM Via Pontina KM 30,600, 00040 Pomezia (Roma), Italy.
Jolly James
Pharmacia P-L Biochemical Inc. 2202 North Barlett Avenue, Milwaukee, WI 53202, U.S.A.
Jost Jean-Pierre
Friedrich Miescher-Institut, P.O. Box 2543, CH–4002 Basel, Switzerland.
Levin Judith R.
Department of Chemistry, Johns Hopkins University, Baltimore, Maryland 21218, U.S.A.
Liang Haimin
Friedrich Miescher-Institut, P.O. Box 2543, CH–4002 Basel, Switzerland.
Matthias Patrick
Friedrich Miescher-Institut, P.O. Box 2543, CH–4002 Basel, Switzerland.

McEwan Iain J.
Department of Medical Nutrition and Center for Biotechnology, Karolinska University Hospital, Novum, S–141 86 Huddinge, Sweden.
Müller Michael M.
Institut für Molekularbiologie II, Universitat Zürich, Hönggerberg, CH–8046 Zürich, Switzerland.
Pawlak André
Friedrich Miescher-Institut, P.O. Box 2543, CH–4002 Basel, Switzerland.
Reudelhuber Tim
Metabolic Research Unit, University of California, San Franscisco, CA 94143, U.S.A.
Ramanujam Ponnusamy
Pharmacia P-L Biochemical Inc., 2202 North Bartlett Avenue, Milwaukee, WI 53202. U.S.A.
Rimphanitchayakit Vichien
Department of Biochemistry, Faculty of Sciences, Chulalongkorn University, Phyathai, Bangkok 10330, Thailand.
Saluz Hanspeter
IRBM Via Pontina KM 30.600, 00040 Pomezia (Roma), Italy.
Schaffner Walter
Institut für Molekularbiologie II, Universitat Zürich, Hönggerberg, CH–8046 Zürich, Switzerland.
Schaufele Fred
Metabolic Research Unit, University of California, San Fransisco, CA 94143, U.S.A.
Shaw Elliot
Friedrich Miescher-Institut, P.O. Box 2543, CH–4002 Basel, Switzerland.
Stone Stuart R.
Friedrich Miescher-Institut, P.O. Box 2543, CH–4002 Basel, Switzerland.
Ten Heggeler-Bordier Beatrice
Institut de Biologie Animale, Université de Lausanne, Bâtiment de Biologie, CH–1015 Lausanne, Switzerland.
Toth Miklos
Howard Hughes Medical Institute, Department of Molecular Biology, Princeton University, NJ 08544, U.S.A.
Tullius Thomas D.
Department of Chemistry, Johns Hopkins University, Baltimore, Maryland 21218, U.S.A.
Von der Ahe Dietmar
Forschungsgruppe für Hämostaseologie, Abteilung Hämostaseologie und Transfusionsmedizin, Kerckhoff-Klinik, Sprudelhof 11, 6350 Bad Manheim, Germany.

Wang Zhou
Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260, U.S.A.
Wahli Walter
Institut de Biologie Animale, Université de Lausanne, Bâtiment de Biologie, CH–1015, Lausanne, Switzerland.
Wallace Andrew
IRBM Via Pontina KM 30.600, 00040 Pomezia (Roma), Italy.
West Brian L.
Metabolic Research Unit, University of California, San Fransisco, CA 94143, U.S.A.
Zwieb Christian
Department of Molecular Biology, University of Texas, Health Center, P.O. Box 2003, Tyler, TX 75710, U.S.A.

Acknowledgements

We are grateful to many colleagues, both here in the Friedrich Miescher-Institut and elsewhere, without whose help this book would have been impossible to write. We are especially indebted to Ms Yan-Tsim Jost for the correction and text processing of the book and to Ms Inge Obergfoell for her excellent photographic work. Finally we would like to thank Dr. Pat King for carefully correcting the manuscript.

J.P. Jost and H.P. Saluz
Basel and Rome 1991

I Introduction

A Safety Considerations

Many techniques described here involve a number of hazards, such as high electrical current and voltage, radioactivity and highly toxic chemicals. It is absolutely essential that the instructions of equipment manufacturers be followed, and that particular attention be paid to the local and federal safety regulations.

B Introduction

The expression of prokaryotic and eukaryotic genes has been shown most often to be regulated at the level of mRNA synthesis. Thanks to the rapid development of methods for dissecting DNA sequences, *cis*-acting regulatory elements such as promoters and enhancers have been recognised. More recently, the widely expressed intuition that discrete sequences within these elements constitute binding sites for sequence-specific binding proteins has been confirmed, especially through the use of "footprinting" assays (for examples, Galas and Schmitz, 1978). This and similar assays have already resulted in the recognition, isolation and analysis of DNA-binding proteins for several genes. Excellent reviews exist of the structural studies on these transcription regulatory proteins and related DNA elements (for example, Glover, 1989 and Johnson and McKnight, 1989), to which the reader is referred for detailed information. To set the scene for applications of the techniques described in this volume, only the barest outline of previous studies is presented here.

Protein-DNA interactions are dependent on very specific tertiary configurations of the binding protein which allow the closest contact with the DNA helix. Recognition occurs via hydrogen bonding and both ionic and hydrophobic interactions, especially within the major groove. The energy of binding is enhanced by the formation of protein dimers and tetramers, and by the palindromic character of the recognised DNA sequences. Based upon specific motifs in the primary protein sequence, five families of DNA-binding proteins are recognised in bacteria, fungi, animals and, probably, in higher plants:

1 *Helix-turn-helix* – These proteins consist of two opposed -helices joined by a turn, the C-terminal helix being the recognition helix.

2 *Homeodomains* – The eukaryote homeobox, which shares features of the helix-turn-helix motif, has a highly conserved 180-bp, 60 amino acid sequence, the

homeodomain. Many homeodomain proteins are now known, and these proteins have been shown to bind to DNA in vitro.

3 *Zinc finger* – This large group of proteins shows considerable overall structural diversity but has in common a zinc requirement for DNA binding and transcriptional activity. The binding domain contains four cysteine and/or histidine residues, and these proteins also have features related to the helix-turn-helix motif.

4 *Leucine zipper* – The zipper is a 30–35 amino acids region containing 5 leucines spaced 7 amino acids residues apart. It is devoid of proline and makes an alpha helix of 8–10 turns. The zipper is a protein dimerization motif. The associated DNA binding region lies immediately N terminal of the zipper. The two alpha helices interact in a parallel fashion by hydrophobic interactions between the leucines. Regularity enables formation of heterodimers. Such proteins bind to DNA in a frontal fashion with recognition sites being short inverted repeats.

5 *Helix-loop-helix* – Some proteins analysed more recently consist of two α-helices joined by a variable spacer with a tendency to loop.

It is already clear that these five categories of proteins do not represent all possible motifs for protein-DNA interactions and that work will continue on identifying DNA-binding proteins and defining the DNA binding elements. At the same time, the task becomes increasingly and automatically one of finding out how such interactions lead to changes in rates of transcription.

The basic information needed to understand exactly how proteins interact with DNA requires knowledge of both the structure of the protein and the precise contact points between the protein and the DNA. The purpose of this book is to address the second question by describing a wide variety of techniques ranging from enzymatic footprinting and electron microscopy, to the use of special vectors and the chemical modification of DNA. In addition, we thought it appropriate to include a special section on techniques which exploit protein-DNA interactions on solid supports to isolate factors or to select clones expressing a specific protein. Because of the hazards arising from the use of radioactively labelled DNA probes, we are pleased to include a chapter on the new generation of probes labelled by chemiluminescence. Finally, the Appendix gives a further series of simple techniques which should generally facilitate the work of the molecular biologist.

J.P.J.
H.P.S.

C Bibliography

Galas, D.J., and Schmitz, A., DNAase footprinting: a simple method for the detection of protein-DNA binding specificity. Nucleic Acids Res. 5 (1978) 3157–3170.

Glover, C.V.C. (III), Sequence-specific protein-DNA recognition by transcriptional regulatory proteins. Plant Molecular Biology Reporter 7 (1989) 183–207.

Johnson, P.F., and McKnight, S.L., Eukaryotic transcriptional regulatory proteins. Annu. Rev. Biochem. 58 (1989) 799–839.

II The Study of Protein-DNA Interactions by Deoxyribonuclease I Footprinting

Dietmar von der Ahe

Contents

A Introduction

In prokaryotes and eukaryotes, the binding of proteins to specific DNA sequences is critical to the regulation of many cellular processes: replication, recombination and transcription. Gene expression in eukaryotic cells is controlled by transregulatory factors which modulate transcription initiation of a particular gene or gene network (Ptashne, 1986 and 1988; Schaffner et al., 1988; Schaffner, 1988; Maniatis et al., 1987). Over the years, numerous biologically important genes have been cloned, and many investigators are now interested in the regulation of these genes in particular cells and in response to specific environmental or developmental signals (Renkawitz, 1989). During the last decade, numerous DNA sequences responsible for this regulation have been identified, and this has naturally led to the detection and characterisation of proteins binding to these regulatory sequences (Dynan and Tjian, 1985).

The most direct and accurate information about DNA sequences bound by specific proteins comes from DNase I protection analysis. The DNase I protection or footprinting assay was developed as a qualitative technique to locate protein-binding sites on DNA (Galas and Schmitz, 1978). In parallel to the isolation and purification of the regulatory DNA-binding proteins, this method was used as a quantitative assay of the binding strength and affinities of proteins to their binding sites (Brenowitz et al., 1986a and 1986b). This led to a better understanding of the nature of protein-DNA interactions and the mechanism of gene regulation (Ptashne, 1986 and 1988; Schleif, 1989; Echols, 1986). In the following years, the method was extended to crude fractions or semi-purified nuclear proteins (Fujimura et al., 1986; Wildeman et al., 1986; Barrera-Saldana et al., 1985), thus providing an assay for use during purification (Briggs et al., 1986). The purpose of using crude nuclear extracts is to locate particular binding activity and not to characterise the strength of the binding and affinity. Such qualitative use of DNase I footprinting is not as clean as with purified proteins.

The basis of the DNase I protection assay is the protection of the phosphodiester backbone of DNA from DNase-I-catalysed hydrolysis afforded by bound proteins, followed by the separation of the hydrolysis products on denaturing DNA-sequencing gels, and visualisation of the binding sites by autoradiography. In the following, I will present the conditions which allowed me to detect several protein-binding sites in the regulatory regions of the urokinase-type plasminogen activator gene and other eukaryotic genes (von der Ahe, 1986, 1988 and 1990). Only crude or semi-purified nuclear protein preparations were used in these experiments. The chapter is divided into five parts: one-end labelling and purification of a DNA fragment containing a protein-binding site; protein-DNA binding; exposure of the DNA-protein complex to DNase I; separation of the reaction products on a sequencing gel and autoradiography; critical parameters/trouble-shooting.

B One-end Labelling and Purification of a DNA Fragment

1 Enzymes, Buffers and Materials

- Restriction endonucleases
- Plasmid DNA containing protein-binding site(s)
- TE Buffer (10 mM Tris-HCl, 1 mM NaEDTA) pH 8.0
- Aqueous (α-^{32}P) dNTPs, (α-^{32}P)ddATP and/or (γ-^{32}P)ATP, 3000 to 6000 Ci/mmol
- 100% and 70% ice-cold ethanol
- Klenow fragment of *E. coli* DNA polymerase I
- 10× Klenow buffer: 500 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 10 mM DTT, 500 µg BSA/ml
- Terminal transferase
- 5× Tailing buffer: 500 mM sodium cacodylate pH 7.0, 5 mM $CoCl_2$, 0.5 mM DTT, 0.25 mg BSA/ml
- 0.5 mM dNTPs
- CsCl
- Agarose
- 10×TBE-buffer, pH 8.3 (890 mM Tris-HCl, 890 mM boric acid, 2 mM NaEDTA)
- Regulated water bath
- Agarose gel tray
- Gel combs for analytical and preparative agarose gels
- Power supply

The genomic sequences containing the putative protein-binding site(s) are subcloned into the polylinker of one of numerous plasmid vectors, e.g. of the pUC series. Plasmid DNA is prepared by the alkaline lysis method followed by CsCl gradient centrifugation (for details of standard procedures see Sambrook et al., 1989).

2 Step-by-Step Procedure

> Digest 5–15 pmol plasmid DNA with the appropriate restriction enzyme that generates a 3'-recessed or 3'-protruding end, 30 to 100 base pairs (bp) from the first protein-binding site (Fig. 1).

> Extract the DNA twice with chloroform/isoamyl alcohol,

ethanol precipitate the DNA on dry ice for 10 min and pellet the DNA. Wash the pellet two times with 0.5 ml cold 70% ethanol and dry in a SpeedVac concentrator. Dissolve the pellet in 20 µl TE buffer.

> 3'-labelling by Klenow fill-in reaction: add 20 µl restricted plasmid DNA (5 pmoles), 5 µl 10× Klenow buffer, 5 µl aqueous (^{32}P) dNTPs (50 µCi), 2 U Klenow fragment, sterile water up to 50 µl; mix gently and incubate 25 min at room temperature; load the whole volume onto a G-50 (medium) column to remove unincorporated nucleotides and collect the peak fractions (200 µl); ethanol precipitate the DNA, wash twice with 70% ice-cold ethanol, dry for 30 min in a SpeedVac concentrator and dissolve the pellet in 20 µl TE buffer.

> Alternatively, 3'-labelling by terminal transferase: add to a 1.5-ml reaction tube: 20 µl restricted DNA (5 pmoles) 10 µl 5× tailing buffer1 µl 50 U/µl of terminal transferase 10 µl (α-^{32}P) ddATP (100 µCi) and sterile water to 50 µl; incubate 60 min at 37°C and continue as indicated for the above Klenow fill-in reaction.

> Alternatively, 5'-labelling by T_4 polynucleotide kinase (for details see Chapters IV or VIII).

> Calculate the incorporation of the labelled nucleotides (usually 60–80% of the theoretical figure). From the specific radioactivity of the ^{32}P dNTPs estimate the molar concentrations of the DNA and the putative binding site(s).

> Digest the labelled plasmid DNA with a second enzyme to generate a restriction fragment labelled only at one end and on one strand (Fig. 1). The second restriction site should generate a 150–800 bp fragment containing the specific protein–binding sites. The second digest usually needs more enzyme activity (2-fold excess).

> Extract the DNA twice with chloroform/isoamyl alcohol, ethanol precipitate the DNA on dry ice for 10 min and pellet the DNA. Wash the sediment twice with 0.5 ml cold 70% ethanol and dry in a SpeedVac concentrator. Dissolve the DNA pellet in 20–40 µl loading buffer.

> Purify the one-end labelled DNA fragment: load 20–40 µl of DNA (5 pmoles) into a preparative slot of an agarose gel

and separate the labelled fragments; calculate the time of electrophoresis from the molecular weight of the DNA fragments.

> Cut out the desired band; for this purpose it is not necessary to stain the gel by ethidium bromide because the DNA is radiolabelled. Wrap the gel in a single layer of Saran wrap and expose it to an X-ray film for 5–10 min. Use the autoradiogramme as a template for excising the appropriate band from the gel.

> Electro-elute the DNA in 0.5× TBE buffer (standard procedure, Sambrook et al., 1989).

> Purify and concentrate the labelled DNA by ion-exchange (DEAE-cellulose; Elutip) or reverse-phase (Nensorb) chromatography. It is essential to remove impurities to obtain high quality footprints.

> Pool the peak fractions (200 µl) and ethanol precipitate the DNA. For recovery of small quantities of labelled DNA, it is advisable to centrifuge for 30–60 min at 4°C in a microfuge, or using a SS-34 Sorvall rotor at 17000 rpm for 15 min at 4°C.

> Dissolve the DNA in TE buffer (20000 to 40000 cpm/µl). To avoid quantitative radiochemical nicking, store DNA at 4°C (*do not freeze*) and use it within a week.

C Protein-DNA Binding Reaction and Exposure of the Protein-DNA Mixture to DNase I

1 Material, Buffers and Solutions

- Deoxyribonuclease I (DNase I; EC. 3.1.4.5.) from Worthington; stock solution in storage buffer at 1 mg/ml
- DNase storage buffer: 50 mM Tris-HCl, pH 7.2
- DNase I stop solution: 30 mM EDTA, 0.25% (w/v) SDS, 0.5 mg/ml Proteinase K
- Assay Buffer A (1×): 20 mM Hepes pH 7.9, 100 mM KCl, 0.1 mM NaEDTA, 6 mM $MgCl_2$, 2 mM DTT, 17% (v/v) glycerol

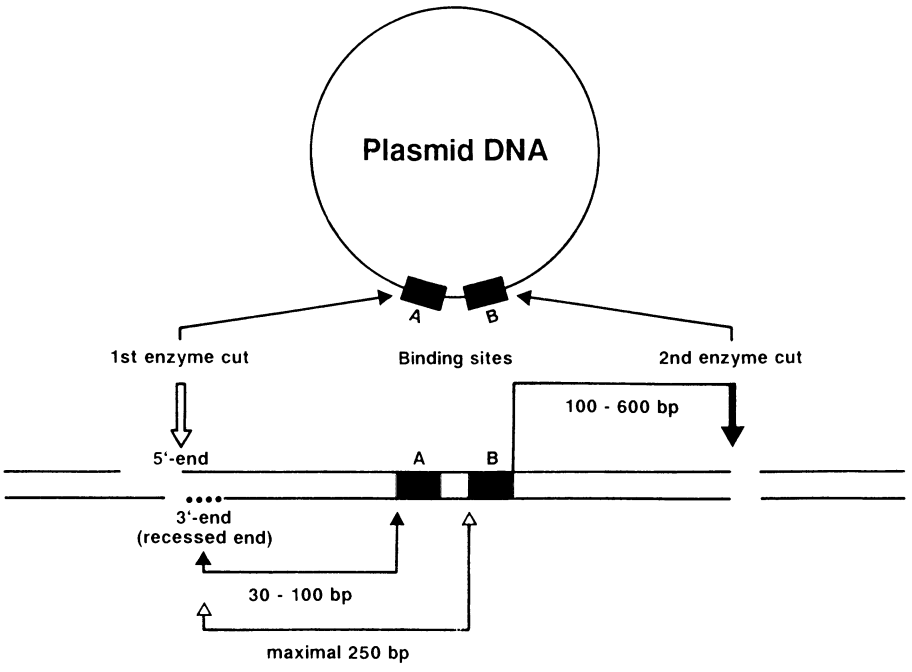


Fig. 1: Principle of the preparation of one-end labelled DNA fragments for the DNase I protection assay: The diagram shows the correct positioning of protein-binding sites from the restriction cuts. This procedure gives a single end-labelled fragment with a proximal binding site not further than 30 bp and a distal binding site not further than 250 bp from the labelled end. Black boxes represent the binding sites and dots indicate (^{32}P)dNTP incorporation by Klenow fill-in reaction.

- Assay Buffer B (10×): 100 mM Hepes pH 7.9, 0.5 mM NaEDTA, 10 mM DTT, 60% glycerol (v/v)
- Buffered phenol (0.1 M Tris-HCl, pH 8.0)
- Buffered phenol: chloroform/isoamylalcohol (1:1)
- Formamid loading buffer: 95% (v/v) formamid, deionised (Amberlite, Biorad), 50 mM TBE pH 8.3, 1 mM NaEDTA, 0.1% (w/v) xylene cyanol, 0.1% (w/v) bromophenol blue
- Dry ice
- Siliconised 1.5-ml microcentrifuge tubes

2 Step-by-Step Procedure for the Protein-DNA Binding Reaction

> Before starting, calculate the number of binding reactions. An upper limit is given by the number of sequencing gel lanes.

> Start the binding reaction by adding appropriate, diluted, crude protein solution to the binding-buffer system, including ^{32}P-labelled DNA fragments and unspecific competitor DNA.

Standard reaction mixture, add consecutively:
Nuclear extract (5–80 µg), made up to 10 µl with buffer A (1×)

Buffer B (10×)	2 µl
poly(dI-dC) (1 mg/ml)	2–3 µl
^{32}P-DNA (0.01–0.1 pmol)	1 µl

Make up total volume to 20 µl with sterile water

> Gently mix each tube, microcentrifuge briefly and incubate samples in a water bath at 25°C for 25 min.

3 Step-by-Step Procedure for the Treatment of Protein-DNA Mixture with DNase I

> Before starting, prepare an excess of DNase I stop solution (Proteinase K solution must always be used fresh) and leave it at room temperature.

> Prepare an excess of DNase I dilutions and keep on ice (thaw the enzyme on ice)

DNase I is diluted in two steps:

1st dilution: 1:30 in sterile water (control 1:100 in sterile water)

2nd dilution: the first dilution is further diluted 1:5 in 50 mM MgCl/25 mM CaCl

> Pipette exactly 5 µl diluted DNase I from the second dilution step into the binding reaction tube (final volume: 25 µl); mix gently by pipetting up and down three times (no vortex).

> After a 2-min incubation at 25°C, rapidly add 75 µl of DNase I stop solution to the reaction tube, vortex vigorously and place the tube into a 42°C water bath for 30–45 min.

> Extract twice with an equal volume (100 µl) of phenol: chloroform/isoamyl alcohol (1:1)

> Centrifuge for 10 minutes at room temperature, recover the aqueous phase and ether extract twice.

> Ethanol precipitate the DNA, microcentrifuge for 20 min and rinse the pellet with 70% ethanol. Alternatively, centrifuge in a SS-34 Sorvall rotor at 17000 rpm for 15 min at 4°C.

> Dry the pellet in a SpeedVac concentrator (2–3 min).

> Dissolve the DNA in 3 µl formamid loading buffer, ensuring a quantitative resuspension. It may be difficult to get the DNA pellet and loading buffer into physical contact. A high microcentrifuge speed and the use of siliconised tubes facilitate the formation of good pellets in the bottom of the tube. Incomplete recovery and resuspension of DNA will result in substantial variations in the sequence ladders, and the different lanes will not be comparable.

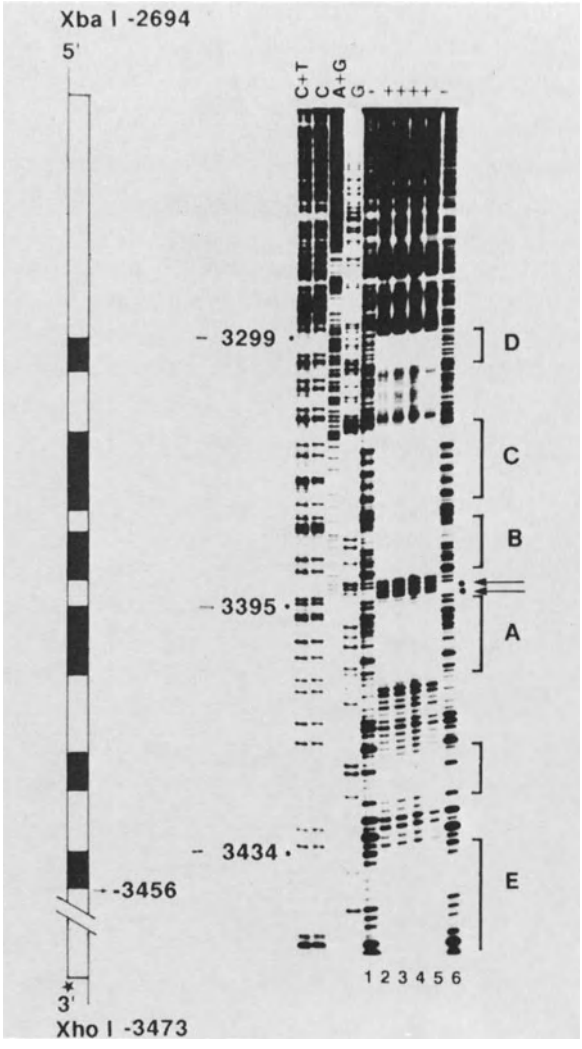


Fig. 2: DNase I protection analysis: The strategy of protein-binding site mapping is diagrammatically shown on the left. The 779-bp XhoI-XbaI fragment (nucleotides –3473 to –2694), overlapping the remote cAMP inducible enhancer of the urokinase-type plasminogen activator gene (von der Ahe et al., 1990), was 3'end labelled at the ShoI site (asterisk indicates the label). Two fmoles (300000 cpm) of the labelled probe were incubated with 3 µg poly (dI-dC) and 40 µg (lanes 2 and 3) or 60 µg (lanes 4 and 5) crude nuclear protein repared from LLC-PK_1 pig kidney epithelia cells (von der Ahe et al., 1988) and subjected to DNase I digestion. The reaction conditions are described in the text. The protected areas are specified with brackets and indicated (A to E). Some margins are indicated at the left. In the lanes C+T, D, A+G and G the sequence pattern (Maxam and Gilbert technique) of the respective fragment (lower strand) is shown. Dots and arrows indicate protein-induced DNase I hypersensitive sites. Numbers refer to the distance from the initiation of transcription.

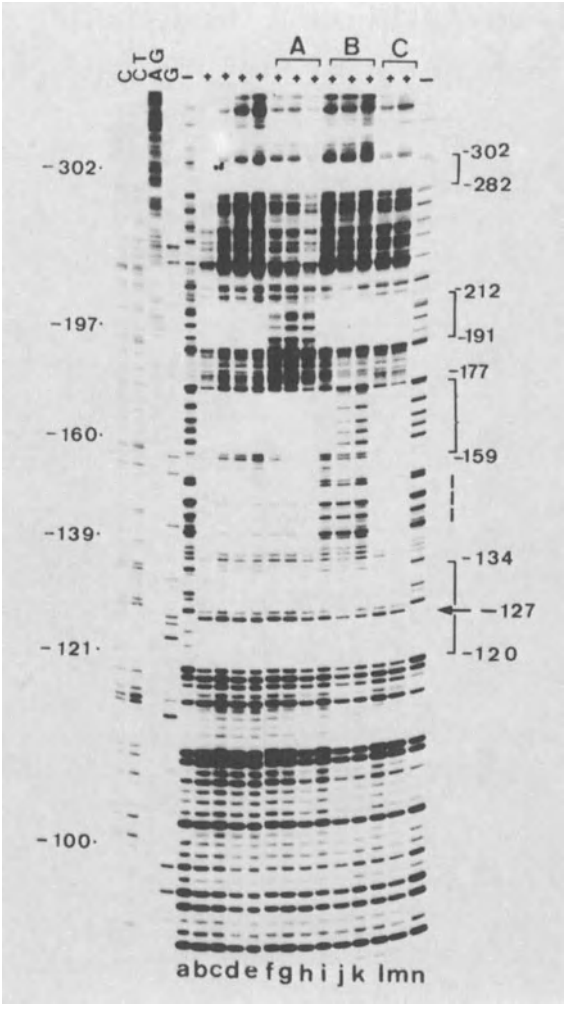


Fig. 3: DNase I protection analysis and specific competition by oligonucleotides: The Sst I-Mbo II fragment (nucleotides –61 to –481) partially overlapping the promoter of the porcine gene for the regulatory subunit type I of protein kinase A (R_I) (Nowak et al., 1987). The DNA fragment was one-end labelled at the Sst I site (–61) by terminal transferase and (α-^{32}P)ddATP. The labelled probe was incubated without (lanes a and n) and with (lanes b to m) 40 µg crude nuclear protein. The reaction conditions are described in the text. The protected regions are indicated by brackets. Lanes A, B and C show competition with the oligonucleotide corresponding to the region –177 to –159. C: unspecific competition by a random 40-mer oligonucleotide (unpublished results). In lanes G, G+A, C+T and C, the sequence pattern of the respective fragment is shown. The arrow indicates protein-induced DNase I hypersensitive sites (B.H. Hemmings and D. von der Ahe, unpublished results).

D Separation of the Reaction Products on a Sequencing Gel and Autoradiography

1 Material and Buffers

- Sequencing gel unit: gel stand, pair of glass plates 40×20×0.5 cm, 0.4-mm spacers, 0.4-mm combs, high-voltage power supply
- Formamid loading buffer: 95% (v/v) formamid, deionised, 50 mM TBE pH 8.3, 1 mM NaEDTA, 0.1% (w/v) xylene cyanol, 0.1% (w/v) bromophenol blue
- 20% Acrylamide solution (0.5× TBE): 96.5 g acrylamide, 3.35 g methylene-bis-acryl, 25 ml 10× TBE pH 8.3, make up to 500 ml with sterile water
- Urea mix: 233.5 g urea, 25 ml 10× TBE pH 8.3, make up to 500 ml with sterile water

2 Step-by-Step Procedure

> Prepare and pre-run a polyacrylamide DNA sequencing gel (for standard procedure see Sambrook et al., 1989). The percentage of acrylamide depends on the size of the fragments to be separated, i.e. on the distance between the first binding site and the labelled end (Fig. 1); in most cases 5–8% is optimal.

> During pre-running of the gel, heat the samples at 90°C for 3 min, followed by immediate quenching in wet ice. Do not heat above 90°C.

> After heating up the gel (50–55°C), disconnect the power supply, flush the slots with 1× TBE buffer and load the samples into the slots. Do not use shark-tooth combs for forming the slots because of the bleeding effect; use combs with 5-mm spacing and 6-mm-wide lanes. Load the samples with micropipette tips or self-made 0.3-mm glass capillaries (siliconised).

> Continue electrophoresis until the most proximal binding site to the labelled nucleotide reaches the last third of the gel.

> As DNA size standards, use the Maxam and Gilbert chemical sequence reaction products of the same labelled DNA fragment (Maxam and Gilbert, 1980).

> After electrophoresis, transfer the gel to a Whatman 3 MM paper of the same size, cover with Saran wrap and place on the support of the gel dryer.

> Apply vacuum and heat at 80°C for 2 h.

> Autoradiograph the dried gel with Kodak X-Omat AR film and an intensifying screen at –70°C. With 10000 to 20000 cpm loaded per lane and 40%–50% of the DNA nicked at least once, an exposure of 12–24 h will be required.

E Critical Parameters/Trouble-Shooting

1 Quality of Crude Nuclear Protein Fractions:

The conditions for nuclear extract preparation depend on the source of the nuclei, and must be determined empirically (Digman and Roeder 1983; von der Ahe et al., 1988). There are two problems to consider:

a) Prevent histone contamination by avoiding high salt extraction of the nuclei/chromatin. The relative histone content of the nuclear fraction may be determined by a southwestern binding assay (for details see von der Ahe et al., 1988).

b) Depending on the source of nuclei, the crude fractions may contain high nuclease activity, leading to considerable nicking of the DNA during the binding reaction. This must be tested by a control experiment without added DNase I enzyme. It is difficult to separate this nuclease activity by simple chromatography steps, e.g. heparin-sepharose.

2 Non-specific/Specific Competitor DNA and Concentration of Specific Binding Sites:

The protein-DNA binding assay and DNase I treatment described above was designed for labelled DNA fragments in the range of 150–800 bp. Within this size range, the results were reproducible without changing the equilibrum conditions. To obtain a clear DNase I footprint with crude fractions, it is necessary to develop conditions that allow binding of the protein(s) to a specific site avoiding non-specific DNA binding. Depending on the proteins, it will be necessary to include and adjust substantially the amounts of competitor DNA in the reaction mixture and/or vary the salt concentration. As non-specific carrier DNA, synthetic polynucleotide poly

(dI-dC) exhibits lower affinities for site-specific DNA-binding proteins than other carriers such as calf thymus or *E. coli* DNA. To find out the optimal binding conditions for a given nuclear extract and a given DNA fragment, a series of pilot experiments should be performed. Pilot DNase I footprint experiments and calculations of the DNase I concentration are outlined in Table I. It is advisable also to vary the nuclear extract concentrations, because the concentration of specific DNA-binding proteins can vary greatly from factor to factor and from different extracts. In order to verify the position of a specific binding site, use a corresponding oligonucleotide as specific DNA competitor. Ideally, this DNA competitor should abolish the DNAse I footprint without altering the overall DNA ladder (Fig. 3).

3 DNase I Enzyme Concentration:

The DNase I concentration must be determined empirically for every type of experiment, depending on nuclear extract, DNA fragment and DNase I enzyme preparation. The assay conditions (buffers, competitor DNA) and repeated freezing and thawing of DNase I greatly influence the enzyme activity. For experiments with either highly purified proteins, or crude nuclear fractions, the average frequency of single-stranded nicks introduced into the DNA should be one per DNA fragment. Given a random distribution of nicks and fragments, 50% of the fragments will contain nicks and 50% will be intact DNA. This can be easily determined by comparing the unnicked DNA not exposed to DNase I (top band of the gel following electrophoresis) and the DNA exposed to DNase I (sequence ladder Fig. 3). The distribution of the number and average number of nicks per DNA fragment can be calculated by Poisson's statistics. It should be emphasised that DNase I enzyme activity is partially inhibited by many crude nuclear fractions. In contrast to the footprint-titration assay with purified proteins, it is difficult to produce a proper control. The best control would be the binding reaction of the specific DNA fragment with a crude fraction depleted of the specific DNA-binding activity. In general, this means extensive purification of the protein. The only solution to this problem is to vary the DNase I concentration within an experiment to find DNase I concentrations that produce an even ladder of (40–60%) nicked DNA fragments (Table I).

4 Assay Buffer Conditions:

There are two limitations to the broad range of different protein-DNA binding conditions: the binding affinity of the specific protein factors and the requirements for the enzymatic activity of DNase I (outlined above). Temperature, pH and salt concentrations required for such experiments should be determined in preliminary experiments by gel retardation assays or by nitrocellulose-filter-binding assays (Henninghausen and Lubin, 1987).

Table I: Assay Conditions for DNase I Footprinting (a, b)

		µl poly (dI-dC) (1 mg/ml)	DNase I/µg/ml (c)
A	1	0.5	0.4
	2	1.0	0.4
	3	2.0	0.4
	4	4.0	0.4
	5	6.0	0.4
	6	8.0	0.4
B	1	0.5	0.8
	2	1.0	0.8
	3	2.0	0.8
	4	4.0	0.8
	5	6.0	0.8
	6	8.0	0.8
C	1	0.5	1.2
	2	1.0	1.2
	3	2.0	1.2
	4	4.0	1.2
	5	6.0	1.2
	6	8.0	1.2
D	1	0.5	1.6
	2	1.0	1.6
	3	2.0	1.6
	4	4.0	1.6
	5	6.0	1.6
	6	8.0	1.6
E	1	0.5	2.4
	2	1.0	2.4
	3	2.0	2.4
	4	4.0	2.4
	5	6.0	2.4
	6	8.0	2.4

a) 30 µg of crude nuclear proteins used
b) Reaction volume was 20 µl
c) Final concentration (see dilution procedure p. 7)

5 Time Considerations:

Allow 2 days for preparing the one-end labelled DNA fragment (not labour intensive), 6 h for footprint titration and 4 h for electrophoresis. The footprint titration and preparation of the samples for sequencing gel electrophoresis are labour intensive. A further 24–48 h is required to obtain the final results, making a total of 5 days.

F Bibliography

Barrera-Saldane, H., Takahashi, K., Vigneron, M., Wildeman, A., Davidson, I., and Chambon, P. All six GC-motifs of the SV 40 early upstream element contribute to promoter activity *in vivo* and *in vitro*. EMBO J. 4 (1985) 3839–3849.

Brenowitz, M., Senear, D.F., Shea, M.A., and Ackers, G.K. Footprint titrations yield valid thermodynamic isotherms. Proc. Natl Acad. Sci USA 83 (1986) 8462–8466.

Brenowitz, M., Sebear, D.F., Shea, M.A., and Achers, G.K. Quantitative DNase I footprints titration: a method for studying protein-DNA interactions. Meth. Enzymology 130 (1986) 132–181.

Briggs, M.R., Kadonaga, J., Bell, S.P., and Tjian, R. Purification and biochemical characterization of the promoter-specific transcription factor Sp 1. Science 234 (1986) 47–52.

Digman, J.D., Lebovitz, R.M., and Roeder, R.G. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. 11 (1983) 1475–1489.

Dynan, W.S., and Tjian, R. The promoter-specific transcription factor Sp 1 binds to upstream sequences in SV 40 early promoter. Cell 35 (1983) 79–87.

Echols, H. Multiple DNA-protein interactions governing high-precision DNA transactions. Science 233 (1986) 1050–1056.

Fujimura, F.K. Nuclear activity from F9 embryonal carcinoma cells binding specifically to the enhancer of wild-type polyoma virus and PyEC mutant DNAs. Nucleic Acids Res. 14 (1986) 2845–2861.

Galas, D., and Schmitz, A. DNase I footprinting: a simple method for the detection of protein-DNA binding specificity. Nucleic Acids Res. 5 (1978) 3157–3170.

Henninghausen, L. and Lubon, H. Interaction of protein with DNA *in vitro*. Methods in Enzymology 152 (1987) 721–735.

Maniatis, T., Goodbourn, S., and Fischer, J. Regulation of inducible and tissue-specific gene expression. Science 236 (1987) 1237–1245.

Maxam, A.M., and Gilbert, W. Sequencing end labeled DNA with base specific chemical cleavage. Method Enzymology 65 (1980) 499–560.

Nowak, I., Seipel, K., Schwarz, M., Jans, D., and Hemmings, B.A. Isolation of a cDNA and characterization of the 5'-flanking region of the gene encoding the type I regulatory subunit of the cAMP-dependent protein kinase. Eur. J. Biochem. 167 (1987) 27–33.

Ptashne, M. Gene regulation by proteins acting nearby and at a distance. Nature 322 (1986) 697–701.

Ptashne, M. How eukaryotic transcriptional activators work. Nature 335 (1988) 683–689.

Renkawitz, R. Tissue specific gene expression. VCH publishers, Suite 909, 220 East, 23rd Street, New York (USA) 1989.

Sambrook, J., Fritsch, E.F., and Maniatis, T. Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, New York): Cold Spring Harbour Laboratory, 1989.

Schaffner, G., Schirm, S., Müller-Baden, B., Weber, F., and Schaffner, W. Redundancy of information in enhancers as a principle of mammalian transcription control. J. Mol. Biol. 201 (1988) 81–90.

Schaffner, W. A hit-and-run mechanism for transcriptional activation? Nature 336 (1988) 427–428.

Schleif, R. DNA binding by proteins. Science 241 (1988) 1182–1187.

von der Ahe, D., Renoir, J.M., Buchou, T., Beaulieu, E.E., and Beato, M. Receptors for glucocorticosteroid and progesterone recognize distinct features of a DNA regulatory element. Proc. Natl. Acad. Sci., USA 83 (1986) 2817–2821.

von der Ahe, D., Pearson, D., Nakagawa, J., Bhanu, R., and Nagamine, Y. Multiple nuclear factors interact with promoter sequences of the urokinase-type plasminogen activator gene. Nucleic Acids Res. 16 (1988) 7527–7544.

von der Ahe, D., Pearson, D., and Nagamine, Y. cAMP regulation of urokinase-type plasminogen activator gene: cooperative macromolecule interaction on a cAMP responsive region and a role of protein phosphorylation. Nucleic Acids Res. 18 (1990) 1991–1999.

Wildeman, A.G., Zenke, M., Schatz, C., Winzerith, M., Grundstrom, T., Matthes, H., Takahashi, K., and Chambon, P. Specific protein binding to the simian virus 40 enhancer *in vitro*. Mol Cell. Biol. 6 (1986) 2098–2105.

III Exonuclease III Protection Assay for Specific DNA-Binding Proteins

Jean-Pierre Jost

Contents

A Introduction

The exonuclease III from *E. coli* catalyses the stepwise 3' to 5' removal of mononucleotides from double-stranded DNA carrying a 3' OH end (Richardson et al, 1964). The products of a complete digest of the linear duplex are two single-stranded molecules, each approximately half the length of the original duplex with only a small amount of complementary sequence between them at their 3' ends (see Fig. 1). These single strands remain resistant to further degradation by the enzyme. At 20°C in the presence of 1 mM $MgCl_2$ and 0.5 units of exonuclease III per µl of reaction mixture (units defined by Richardson et al, 1964), the rate of degradation is approximately 800–1000 nucleotide ends per hour (Smith, 1979). Exonuclease III has other enzymatic activities, including 3' ribonuclease H activity and endonuclease activity specific for apurinic DNA (Rogers and Weiss, 1980).

Exonuclease III from *E. coli* has been used to locate proteins or complexes thereof on specific regions of DNA. The protection of the bound DNA upon digestion with exonuclease III locates the site of protein binding. This method has been employed to map the binding sites of a number of purified prokaryotic and eukaryotic DNA-binding proteins (Siebenlist et al., 1980; Shalloway et al., 1980; Chan and Lebowitz, 1983; Wu, 1985; Von der Ahe et al., 1985; Riley and Weintraub, 1978). In addition, exonuclease III has been used in combination with other enzymes for in situ studies of protein-DNA interactions in chromatin (Wu, 1984 a/b). Finally, exonuclease III was used to study the precise position of nucleosomes in chromatin (Zhang et al., 1983; Igo-Kemenes et al., 1980; Chao et al., 1979; Hörz and Zachau, 1980; Prunell and Kornberg, 1982). In the present protocol, we will only consider the interaction of DNA with proteins from crude or semi-purified cellular extracts. The strategy used to study the footprint on a given sequence of end-labelled DNA is shown in Fig. 1. It consists of binding the protein to double-stranded 5' end-labelled DNA (only one of the strands is labelled) under stringent conditions. In addition, the protein-DNA complex and the unbound DNA are treated in parallel with different concentrations of exonuclease III. After stopping the reaction, the DNA is either directly extracted and analysed on a sequencing gel, or in certain cases the free and bound DNA are first separated and then isolated and analysed on sequencing gel.

1 Labelling and Purification of the Oligonucleotide

The oligonucleotide should be chosen so that the binding site of the protein is not in the middle, because it is difficult to distinguish between the small region of double-stranded structure on the free DNA and the binding site of the protein on the DNA (see Fig. 1). A 40-mer oligonucleotide is sufficient for such experiments. Using restriction fragments, it is advisable to cut the DNA first with one enzyme,

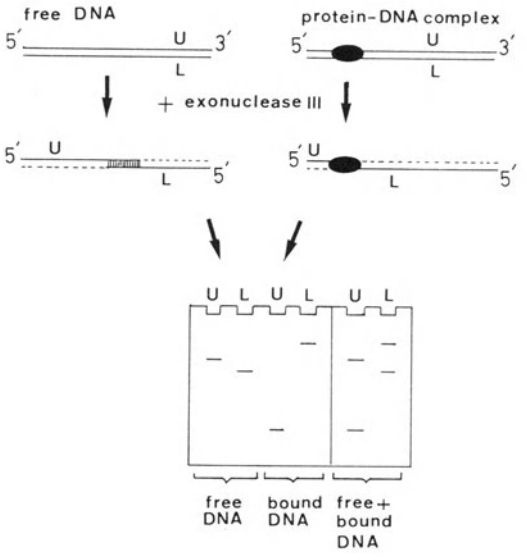


Fig. 1: Principle of the exonuclease III protection for specific DNA-binding proteins: On the upper left, naked DNA digested by exonuclease III; on the right, the exonuclease digest of a protein-DNA complex. The digestion of the naked DNA proceeds symmetrically and terminates once the two enzymes on each DNA strand meet in the centre of the double-stranded DNA. On the other hand the digestion of the protein-DNA complex, will proceed until progression of the enzyme is stopped by the protein bound to the DNA. After purification of the reaction product, DNA is separated on a sequencing gel. U and L means upper and lower DNA strands.

end-label the 5' end with polynucleotide kinase (as described in Chapter IV) and then cleave with a second restriction enzyme. This generates a suitable restriction fragment labelled at one end. One should keep in mind that the choice of the restriction enzyme is important for the subsequent digestion with exonuclease III. For example, the restriction enzymes Aat II, Apa I, Hae II, Kpn I, Sac I and others that generate a four-base protrusion at the 3' end will not be digested by exonuclease III (Henikoff et al., 1984). The end-labelled restriction fragment is then separated and extracted from a low-melting-temperature agarose gel as outlined in Chapter VIII.

Since it is known that exonuclease III has the capacity to degrade DNA at nicks within the duplex at a rate comparable to the degradation of 3' termini of linear duplex (Masamune et al., 1971), it is advisable to purify the oligonucleotides on a preparative sequencing gel. One strand of the oligonucleotide is labelled at its 5' end by the polynucleotide kinase reaction, as described in Chapter VIII. The labelled oligonucleotide is then annealed with its complementary non-labelled strand.

2 The Protein-DNA Binding Reaction

One prerequisite for obtaining a clean and clear exonuclease III footprint is to have a very specific protein-DNA complex. This should first be tested by gel-shift assays. For a given amount of oligonucleotide, one needs to titrate both the amount of protein and non-specific competing DNA. Optimally only one complex should be obtained under conditions in which the amount of unbound oligonucleotide is not limiting. As non- specific competing DNA we routinely take purified, sonicated *E. coli* DNA or poly (dI-dC). It should also be noted that free histones bind with very high affinity to DNA and block the progression of exonuclease III. For this reason it is not advisable to substitute a high salt, crude cellular extract for the nuclear extracts (Wu, 1985). Since the exonuclease III reaction must be carried out in the presence of 1–5 mM $MgCl_2$, it is necessary first to test first by gel-shift assay the effect of $MgCl_2$ on the formation of the protein-DNA complex and on the non-specific degradation of labelled DNA by contaminating nucleases in the extract. At the end of the reaction the exonuclease III activity is blocked by means of EDTA and SDS, if the free and bound DNA are to be extracted together, or by EDTA alone, if the free and bound DNA must be extracted separately. It is further advisable to test by gel-shift assay or by nitrocellulose-filter assay whether the addition of EDTA to the reaction mixture affects the stability of the protein-DNA complex.

B Materials and Methods

1 Buffers and Enzyme

- 10× Binding buffer: 1 M NaCl, 100 mM Tris HCl pH 8.0, 10 mM EDTA
- Sonicated and purified *E. coli* DNA or poly (dI-dC).
- 50 mM $MgCl_2$ containing an appropriate concentration of exonuclease III
- Exonuclease III obtained from Biofinex, 1724 Praroman, Switzerland
- Stop buffers: 15 mM EDTA pH 8.0 or 20 mM EDTA pH 8.0, 1% SDS
- Distilled phenol containing 0.5% hydroxyquinoline, saturated with 1 M Tris pH 8.0
- Nitrocellulose filters, Schleicher & Schuell, BA 85 0.45 μm, diameter 9 mm
- 0.5 M Ammonium acetate pH 7.5, 0.1% SDS, 1 mM EDTA
- Proteinase K from Boehringer Mannheim

2 Step-by-Step Procedure for Assembling the Binding Reaction

> To a silicone treated Eppendorf tube add consecutively: 10 µl of 25% glycerol in water

> 2 µl of 10× binding buffer (1M Nacl, 100 mM Tris-HCl pH 8, 10 mM EDTA)

> 2 µl (1 µg) of *E. coli* DNA (purified and sonicated) as non-specific competing DNA

> 5 µl of end-labelled DNA substrate (approx. $1–3.10^5$ cpm)

> 2 µl of protein extract (1–5 µg protein).

> Incubate at 20°C for 15 to 30 min and then start the exonuclease III reaction.

3 The Exonuclease III Reaction

Definition: One unit of exonuclease III is the enzyme activity which causes the release of 1 nmole of acid-soluble nucleotides within 30 min at 37°C from sonicated calf thymus DNA (Richardson et al, 1964).

To the reaction mixtures at room temperature add 2 µl of 50 mM $MgCl_2$ containing the appropriate concentration of exonuclease III. In our case we used 2, 4, 8 and 12 units of exonuclease III per incubation mixture of 20 µl. Care must be taken when adding the exonuclease III-$MgCl_2$ mixture so as not to dissociate the protein-DNA complex. Mix gently with the tip of the micropipette. Incubate 15 min at 20°C and stop the reaction by adding either an equal volume (20 µl) of 15 mM EDTA, pH 8, if the sample has to be further processed for the separation of the free and bound DNA, or an equal volume (20 µl) of 20 mM EDTA in 1% SDS if the sample is to be immediately extracted with phenol.

C Separation of the Reaction Product on Nitrocellulose Filters

Use small Schleicher & Schuell nitrocellulose filters BA 85 0.45 µm, diameter 9 mm and a filtration device with vacuum regulation. Handle only one filter at a time.

1 Step-by-Step Procedure for the Nitrocellulose-Filter Binding Assay

> At room temperature pre-soak the filters in a small volume of 1× binding buffer containing the appropriate concentration of non-specific competing DNA (the same as for the binding test).

> Put the wet filter on the glass, fritted surface of the filtration device and apply very low vacuum.

> Slowly pipette the reaction mixture onto the pre-wetted filter. Wash the filter twice slowly with 1 ml of cold binding buffer containing non-specific competing DNA.

> Place the nitrocellulose filter in an Eppendorf centrifuge tube containing 250 µl of 0.5 M ammonium acetate pH 7–7.5, 0.1% SDS, 1 mM EDTA, 10–15 µg of tRNA carrier and 50–100 µg of proteinase K.

> Incubate at 37°C for 20 min.

> Extract once with phenol and once with chloroform.

> Add 3 volumes of ethanol and chill at –80°C for 10 min.

> Centrifuge 10 min at 15000 rpm in a SS-34 Sorvall rotor.

> Wash sample by centrifugation with cold 90% ethanol, 10% water.

> Dry sample in a SpeedVac concentrator.

2 DNA Size Standards

Take the G+C and the A+T sequence reaction product of the end-labelled oligonucleotide as DNA size standards.

3 Separation of the Reaction Product on a Sequencing Gel

For the separation of 40-mer templates we use a 40-cm-long 20% polyacrylamide sequencing gel (Maxam and Gilbert, 1980). The same amount of radioactivity (~10000 cpm) is loaded in each lane. To resolve the fragments between positions 2 and 40, the bromophenol blue marker must migrate 15 cm.

D An Example

An example of a cytoplasmic *trans*-acting factor binding to the third intron of the avian vitellogenin gene (Jost et al, 1987) is seen in Fig. 2. The protein factor was eluted from a Heparin-Sepharose column (0.35 M KCl fraction). After ammonium sulphate precipitation, the protein was dissolved in the binding buffer and dialysed for 3 h against the same buffer.

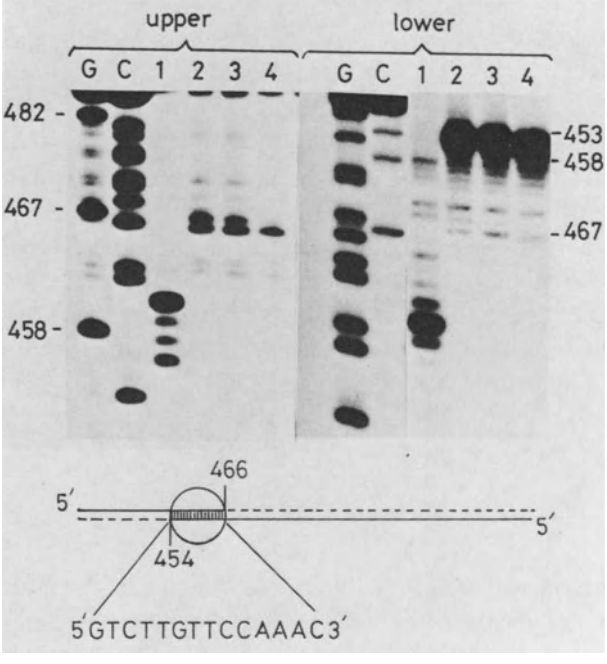


Fig. 2: Exonuclease III footprinting experiments: The strategy of the binding-site mapping is diagrammatically represented below the autoradiogramme. The DNA duplexes, labelled at the 5' end of either the upper or the lower strand, were incubated with the protein factor, and then subjected to exonuclease III digestion. The duplex labelled in the upper strand was digested up to nucleotide 466 (dotted line), while the duplex labelled on the lower strand was digested up to nucleotide 454. Lane 1 represents exonuclease III digests in the absence of added proteins. Exonuclease III concentrations were as follows: 100 U/ml (lane 2); 200 U/ml (lane 1 and 3); 400 U/ml (lane 4). G and C lanes represent the guanine- and cytidine-specific sequencing tracks, respectively. The sequence of the upper strand of the synthetic duplex is 5' GGCCATGTCTTGTTCCAAACGCACCAACCAACACTGAATTC 3' (Jost et al., 1987).

E The most Common Problems and Their Solutions

- *No radioactivity remaining after purification of the exonuclease-III-treated DNA*
  This could be due either to a gross contamination of the preparation with various nucleases, or to the use of the wrong labelling procedure. This would be the case if the filling-in reaction with the Klenow fragment of polymerase I was used, resulting in the labelling of the 3' end of the oligonucleotide or restriction fragment.
- *There is no difference in the footprint between free and bound DNA*
  This could be due to a very weak affinity of the protein for the DNA (check this point by gel-shift assay), or to the precise overlapping of the protein-DNA footprint with the exonuclease-III- resistant part in the middle of the oligonucleotide. Avoid protein binding in the middle of the oligonucleotide by choosing another synthetic oligonucleotide or restriction fragment.
- *No digestion by exonuclease III*
  The enzyme may be inactive or you forgot to add Mg^{++} to the reaction mixture.
- *A complex footprint patterns*
  This indicates binding of more than one protein to the oligonucleotide and that these proteins are in a different mole ratio. Make sure by gel-shift assay that you have only one major band of protein-DNA complex and not several bands of different strengths. If the latter is the case, you may need to purify the protein, or increase the amount of competitor DNA in the reaction.

F Bibliography

Chao, M.V., Gralla, J., and Martinson, H.G. DNA sequence directs placement of histone cores on restriction fragments during nucleosome formation. Biochemistry 18 (1979) 1068–1074.

Chan, P.T., and Lebowitz, J., The coupled use of footprinting and exonuclease III methodology for RNA polymerase binding and initiation. Application for the analysis of three tandem promoters at the central region of colicin E1. Nucleic Acids Research 11 (1983) 1099–1116.

Henikoff, S. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene 28 (1984) 351–359.

Hörz, W., and Zachau, H.G., Deoxyribonuclease II as a probe for chromatin structure. I Location of cleavage sites. J. Mol. Biol. 144 (1980) 305–327.

Igo-Kemenes, T., Omori, A., and Zachau, H.G., Non-random arrangement of

nucleosomes in satellite I containing chromatin of rat liver. Nucleic Acid Research 8 (1980) 5377–5390.
Jost, J.P., Saluz, H.P., Jiricny, J., and Moncharmont, B., Estradiol dependent transacting factor binds preferentially to a dyad symmetry structure within the third intron of the avian vitellogenin gene. J. Cellular Biochemistry 35 (1987) 69–82.
Masamune, Y., Fleishman, R.A., and Richardson, C.C., Enzymatic removal and replacement of nucleotides at single-strand breaks in deoxyribonucleic acid. J. Biol. Chem. 246 (1971) 2680–2691.
Maxam, A.M., and Gilbert, W., Sequencing end-labelled DNA with base-specific chemical cleavages. Methods Enzymology 65 (1980) 499–560.
Prunell, A., and Kornberg, R.D., Variable center to center distance of nucleosomes in chromatin. J. Mol. Biol. 154 (1982) 515–523.
Richardson, C.C., Lehman, I.R., and Kornberg, A. A deoxyribonucleic acid phosphatase-exonuclease from *Escherichia coli*. II Characterization of the exonuclease activity. J. Biol. Chem. 239 (1964) 251–258.
Riley, D., and Weintraub, H., Nucleosomal DNA is digested to repeats of 10 bases by exonuclease III. Cell 13 (1978) 281–293.
Rogers, S.G., and Weiss, B., Exonuclease III of *Escherichia coli* K-12 and AP endonuclease. Methods in Enzymology 65 (1980) 201–211.
Shalloway, D., Kleinberger, T., and Livingstone, D.M., Mapping of SV 40 DNA replication origin binding sites for the SV 40 T antigen by protection against exonuclease III digestion. Cell 20 (1980) 411–422.
Siebenlist, U., Simpson, R.B., and Gilbert, W., *E coli* RNA polymerase interacts homologously with two different promoters. Cell 20 (1980) 269–281.
Smith, A.J.H., The use of exonuclease III for preparing single-stranded DNA for use as a template in the chain terminator sequencing method. Nucleic Acid Res. 6 (1979) 831–848.
Von der Ahe, D., Janich, S., Scheidereit, C., Renkawitz, R., Schütz, G., and Beato, M., Glucocorticoid and progesterone receptors bind to the same sites in two hormonally regulated promoters. Nature 313 (1985) 706–709.
Wu, C., An exonuclease protection assay reveals heat-shock elements and TATA box DNA-binding proteins in crude extracts. Nature 317 (1985) 84–87.
Wu, C., Activating protein factor binds in vitro to upstream control sequences in heat shock chromatin. Nature 311 (1984a) 81–84.
Wu, C., Two protein-binding sites in chromatin inplicated in the activation of heat shock genes. Nature 309 (1984 b) 229–234.
Zhang, X.Y., Fittler, F., and Hörz, W., Eight different highly specific nucleosome phases on alpha satellite DNA in the African green monkey. Nucleic Acid Res. 11 (1983) 4287–4307.

IV The Proteolytic Clipping Band-Shift Assay of Protein-DNA Complexes

Melya J. Hughes, Haimin Liang and Jean-Pierre Jost

Contents

A Introduction

Proteases have been used in many instances to characterise different domains of transcription factors. For example, the purified glucocorticoid receptor has been characterised by partial proteolytic digestion into an immunogenic domain in the N-terminal region, a central DNA-binding domain and a C-terminal ligand-binding domain (Gustafsson et al., 1987 and references therein). Selective proteolysis has been used to define DNA-binding domains of the yeast transcription factor tau (Marzouki et al., 1986) and the 5S RNA transcription factor III A (TFIIIA) (Smith

et al., 1984, Miller et al., 1985). More recently Schreiber et al. (1988) have refined the analysis of protein-DNA complexes by combining partial proteolytic digestion with a gel-shift assay, the so-called proteolytic clipping band-shift assay.

The basic principle of the assay (see Fig. 1) involves the binding of protein to its radioactively labelled DNA site and then subjection of the complex to partial digestion by specific proteases (Schreiber et al., 1988). The bound and unbound DNA are separated on a non-denaturating polyacrylamide gel and autoradiographed. Each protein gives a characteristic pattern with a given protease. This technique allows the distinction of closely related proteins binding to the same or different DNA-binding sites. The method was used to show unambiguously that the purified non-histone protein 1 (NHP1) binds to different DNA sequences (Hughes and Jost, 1989). A major advantage of the technique is the rapid collection of information about possible relationships between DNA-binding proteins present in

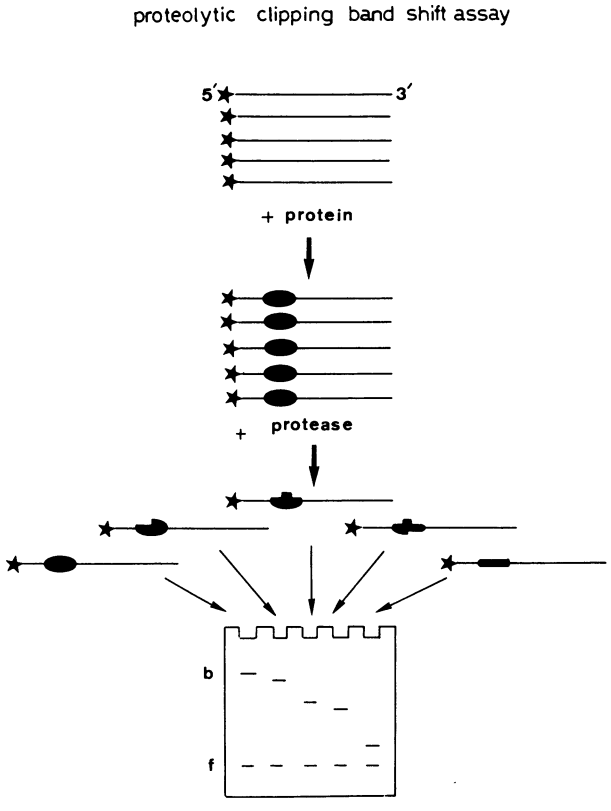


Fig. 1: The proteolytic clipping band-shift assay: The stars represent the 5' end labelling of the oligonucleotide. b and f mean bound and free DNA, respectively.

a crude extract. The same technique might also be extended to analysis of recombinant proteins from bacterial lysates and their comparison with the native proteins from nuclear extracts. In specific cases where the protein has a relatively low affinity for the DNA or RNA, it is possible to cross-link the protein to the bromodeoxyuridine-substituted nucleic acid by UV light and then subject the protein-nucleic acid complex to proteolytic clipping. Recently it has been shown that the migration of the proteolytically clipped protein in the gel-shift assay correlates to the logarithm of its molecular weight (Bading, 1988, Schreiber et al., 1988).

B Substrate Specificity of Different Commonly Used Proteolytic Enzymes

Trypsin specifically cleaves peptide bonds on the C-terminal side of lysine and arginine residues (-Lys-X- and -Arg-X-). However, when X is proline, the bond is almost completely resistant to cleavage. Trypsin is maximally active in the pH range 7–9, and should be made up in 10 mM HCl to prevent autocatalysis. The enzyme can be stored frozen in solution for a few weeks,and should be diluted with water just before use. As a serine protease, trypsin is rapidly and irreversibly inactivated by diisopropylphosphorofluoridate (DFP) and phenylmethylsulphonyl fluoride (PMSF). A concentration of 0.5–1 mM of PMSF is sufficient to inhibit the enzyme. Care must be taken when handling these highly toxic compounds. Alternatively it is possible to use soybean trypsin inhibitor; ratio of 4 mg of soybean inhibitor 1 mg of trypsin is sufficient to inactivate the enzyme. Commercially available trypsin (e.g. Worthington, Boehringer; Wilkinson, 1986) may be contaminated with small amounts of chymotrypsin. This latter enzyme can be inactivated by treatment with L-(1-tosylamido-2-phenyl)-ethylchloromethyl ketone (TPCK). TPCK-treated is also commercially available (as above).

Thrombin cleaves the bonds on the C-terminal side of arginine residues (-Arg-X-). In fibrinogen, the natural substrate X is alanine, arginine, aspartic acid, cysteine or valine. Thrombin is active at pH 8, and the conditions of digestion are the same as for trypsin. Thrombin is a serine protease and can be irreversibly inhibited by PMSF and DFP. The enzyme is commercially available (e.g. Calbiochem, Miles Labs, Sigma, Boehringer; Wilkinson, 1986).

Staphylococcus aureus V8 protease specifically cleaves the peptide bonds C-terminal to glutamic acid residues -Glu-X-, unless X is proline or glutamic acid. The V8 protease is active over the pH range of 3.5–9.5 and has two optima at pH 4.0 and pH 7.8. As a serine protease, V8 protease is inhibited by DFP and PMSF. This enzyme is commercially available (e.g. Miles Labs, Boehringer; Wilkinson, 1986).

Chymotrypsin cleaves primarily at the peptide bond C-terminal to aromatic or large hydrophobic residues of the type-H-X-, where H is tyrosine, phenylalanine,

tryptophan or leucine. Bonds are resistant to cleavage when X is proline. Chymotrypsin has an optimum at pH 7, and as a serine protease it can be inhibited by DFP and PMSF.

Clostripain is a sulfhydryl protease isolated from *Clostridium histolyticum*. The major specificity of clostripain is cleavage at the C-terminal side of arginine residues (-Arg-X-). The optimal pH is approximately 7.7 and the enzyme has an absolute requirement for a sulfhydryl reagent (e.g. 1–10 mM DTT). Clostripain is commercially available from Worthington and Boehringer (Wilkinson, 1986).

C Materials and Methods

1 Materials and Buffers

- 10 × Kinase buffer: 0.5 M Tris HCl pH 7.6, 0.1 M $MgCl_2$, 50 mM DTT
- T4 Polynucleotide kinase (Biofinex Praroman, Switzerland or Boehringer)
- Sephadex G-50 (medium)
- 10 × Klenow buffer: 0.5 M Tris HCl pH 7.5, 0.1 M $MgCl_2$, 2 mM DTT
- Klenow large fragment of polymerase I (Biofinex, Praroman, Switzerland)
- 10 × Binding buffer: 1 M Tris HCl pH 8.0, 50 mM EDTA, 20 mM β-mercaptoethanol
- *E. coli* DNA (purified and sonicated) 500 ng/µl
- V8 protease (4 mg/ml) (Boehringer)
- Trypsin (10 mg/ml in 10 mM HCl) (Boehringer)
- (γ-^{32}P)ATP, 3000 Ci/mmole (Amersham)
- 10 × TBE buffer: 0.89 M Tris base, 0.89 M boric acid, 0.02 M EDTA pH 8.3
- Phenylmethylsulphonyl fluoride (PMSF) 10 mM in 25% glycerol
- Bovine serum albumin (enzyme grade) 5 mg/ml in water (Gibco-BRL)

2 Step-by-Step Procedure for the Preparation of the Labelled Synthetic Oligonucleotides

> To a silicone-treated Eppendorf tube add consecutively 10–20 pmol of oligonucleotide (60–120 ng of a 20-mer, upper or lower strand) in 5 µl water, 2 µl of 10-× kinase buffer, 3–6 µl of (γ-^{32}P)ATP (30–60 µCi, 3000 Ci/mmole, i.e. 10–20 pmoles of ATP). Make up the volume to 20 µl with water. Add 10 units (1 µl) of T4 polynucleotide kinase and incubate 30 min at 37°C.

> Stop the reaction by heating to 90°C for 5 min.

> Add 10–20 pmol of the complementary strand.

> Heat the mixture to 90°C for 2 min and allow to cool slowly to room temperature over 30 min (annealing of the complementary DNA strands will occur).

> Add about 10 µg of tRNA as carrier.

> Separate the labelled double-stranded oligonucleotide from the non-incorporated nucleotides by centrifugation through a spun column of Sephadex G-50 (see Appendix).

> After centrifugation at 4000 rpm for 15 min, determine the specific radioactivity of the probe. Repeated ethanol precipitations can be used instead of spun column chromatography.

Note:
Alternatively, if a restriction DNA fragment or an oligonucleotide duplex have a 3' recessed end, it is possible to label with the fill-in reaction of polymerase I (Klenow large fragment).

> To a silicone-treated Eppendorf tube add consecutively 5 µl of 100–200 ng of double-stranded DNA, 2 µl of 10× Klenow buffer, and 50 µCi of the appropriate deoxyribonucleotide triphosphate (3000 Ci/mmole).

> Make up the volume to 20 µl with water. Add 1 unit of Klenow enzyme and incubate for 5 min at room temperature.

> Stop the reaction with 50 µl of 0.5 M EDTA, heat at 70°C for 2 min and cool slowly.

> Separate the labelled DNA from the non-incorporated nucleotides by a spun column of Sephadex G-50 (see Appendix).

3 Step-by-Step Procedure for the Assembly of Reactions and Proteolytic Digestion

Note:
Nuclear extracts and fractions thereof are frequently prepared in the presence of protease inhibitors such as aprotinin, PMSF, benzamidine (which reversibly inhibits arginine-specific pro-

teases) or a cocktail of several other inhibitors. In this case, the presence of free inhibitors in the extracts could inactivate the serine proteases used for the clipping reaction. Therefore, it is advisable to prepare the extracts first in the presence of PMSF (0.5–1 mM), which has a short half-life in aqueous solution but irreversibly inactivates the serine proteases present in the nuclear extracts. The extracts are then dialyzed against the appropriate binding buffer in the absence of PMSF or in cases where the protein is sensitive to dialysis, additional protease may be added as determined by titration.

> To a silicone treated Eppendorf tube (for a total volume of 10 µl) add consecutively: 0.2–1 × 10^5 cpm (40 fmol) of end-labelled oligonucleotide, 0.2–5 µg of non-specific competing DNA (*E. coli* DNA), 2 µl of 25% glycerol in water, and 1–5 µg of nuclear protein.

Note:
The optimal incubation conditions (the buffer, concentrations of protein and non-specific competing DNA) must be determined empirically. Highly purified protein may be stabilised by the addition of a few microgrammes of bovine serum albumin (enzyme grade). The amount of bound DNA should exceed that of free DNA in the absence of protease, since with progressive proteolysis the binding of the protein to the oligonucleotide can become weaker.

> Incubate for 20 min at 20°C.

> Add 1 µl of the appropriate dilution of the protease (the protease solutions are diluted with water just before use). For example, the amounts of V8 protease used for titration with 5 µg protein were 0, 0.25, 0.5 and 2.5 units. A control sample with protease alone should be included.

> After incubation for 10 min at room temperature, inhibit the protease (in the case of a serine protease) by the addition of 0.5 µl of 10 mM PMSF in 25% glycerol (optional). Mix gently with the micropipette tip but do not vortex the reaction mixture.

Note:
Alternatively, it is possible to add the protease directly to the binding reaction before incubation. In this case, the total incubation time should not exceed 10 min. At the end of the

reaction, the sample should be immediately loaded onto the polyacrylamide gel without addition of dye or protease inhibitor since the protein-DNA complex is separated from proteases by electrophoresis.

4 Separation of the Reaction Product on a Polyacrylamide Gel

We generally use polyacrylamide gels of 4–5% (acrylamide: bisacrylamide 29 : 1) prepared in 0.25× TBE. The dimensions of the gel are 24 cm × 18 cm × 0.5 mm. Carry out electrophoresis at room temperature for 1 h using 0.25× TBE and constant current at 20 mA. Dry the polyacrylamide gel onto Whatman paper 3mm and subject to autoradiography.

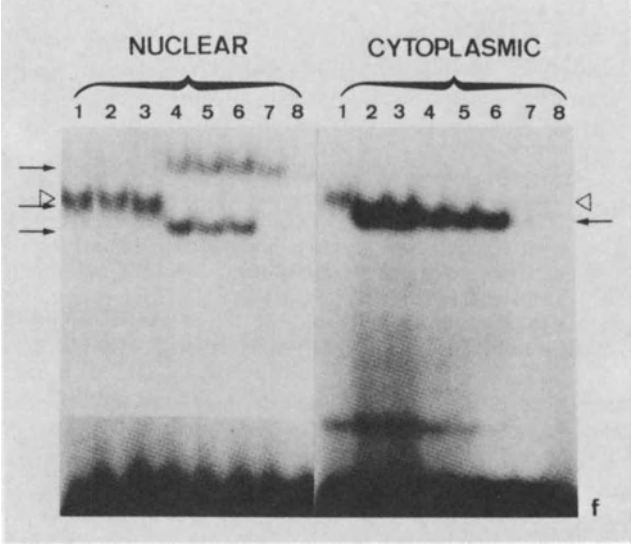


Fig. 2: Proteolytic clipping band-shift assay: Protein-DNA complexes formed between nuclear or cytoplasmic extracts and the single-stranded oligonucleotide 5' TCACCTTCGCTATG3' were subjected to limited proteolysis with trypsin (lanes 1–8 represent: no enzyme, 0.02, 0.04, 0.08, 0.2, 0.4, 2 and 10 units of enzyme, respectively. Undigested complex (△) and proteolytic products (→) are indicated (Feavers et al., 1989). Reprinted with permission from J. Biol. Chem. 264 (1989) 9114–9117, copyright 1990, American Society for Biochemistry and Molecular Biology.

D An Example:

Fig. 2 shows the proteolytic clipping band-shift assay of a cytoplasmic and a nuclear protein binding specifically to single-stranded 5'TCACCTTCGCTATG 3'DNA. As can be seen in the presence of increasing concentrations of trypsin, the clipping patterns obtained for the nuclear and cytoplasmic proteins are different, suggesting that these are two different proteins (Feavers et al., 1989). In the second case (Fig. 3), we show that identical clipping patterns are obtained with V8 protease for a given protein (NHP1) binding to different DNA sequences. This would indicate that the proteins binding to these different DNA substrates are most likely identical.

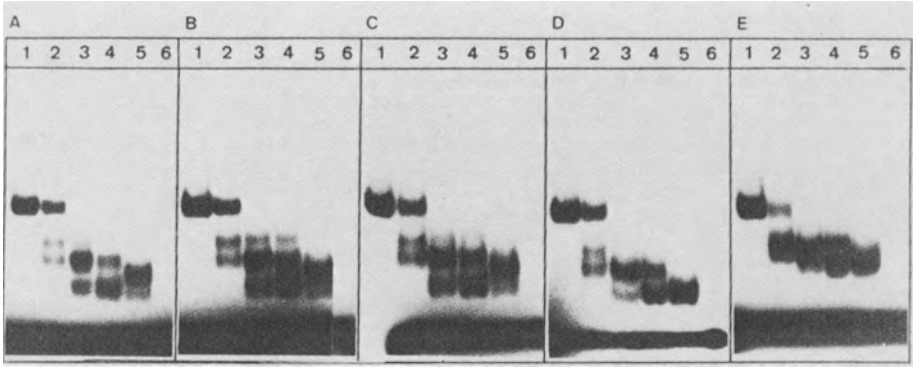


Fig. 3: Protein binding to double stranded oligonucleotides containing the binding sites of NHP1, NHP2, NHP3 and NHP4 give identical patterns in proteolytic clipping band-shift assays: Purified NHP1 was incubated with different oligonucleotide substrates in the presence of increasing amounts of *Staphylococcus aureus* V8 protease and 5 µg bovine serum albumin (enzyme grade). Panel A, B, C, D and E correspond to gel-shift assays with the estrogen response element (ERE), ERE containing 5- methylcytosine, the NHP2 binding site, the NHP3 binding site and the NHP4 binding site, respectively. In each panel, lane 1 is the gel-shift assay carried out in the absence of V8 protease, lanes 2, 3, 4 and 5 are with 0.1, 0.5, 1 and 10 µg V8 protease, respectively, and lane 6 is a gel-shift assay with 5 µg V8 protease in the absence of NHP1. The samples were incubated for 10 min at room temperature before electrophoresis on a 5% native polyacrylamide gel. The lower band is free DNA, the upper bands are bound DNA (Hughes and Jost, 1989). Reprinted with permission from Nucleic Acids Res. 17 (1989) 8511–8520, copyright 1990, Oxford University Press.

E The Most Common Problems and their Solutions

- *No cleavage of the protein-DNA complex with proteases*
  This could mean either that the enzyme is inactive or that there are traces of free PMSF or other protease inhibitors in the protein preparation. Test the activity of the enzyme on a model substrate in the buffer to be used for the binding reaction. Try to use different concentrations of proteases or increase the incubation times.
- *The digestion of the protein-DNA complex is too rapid even with the lowest concentrations of proteases*
  Dilute the DNA-binding protein by adding a few microgrammes of enzyme-grade bovine serum albumin.
- *The signals are very weak after overnight autoradiography*
  The specific radioactivity of the probe may be too low, or there is too little protein factor in the preparation. In this latter case, it may be necessary to purify the factor further. The conditions for the binding reaction may not be optimal.

F Bibliography

Bading, H., Determination of the molecular weight of DNA-bound protein(s) responsible for gel electrophoretic mobility shift of linear DNA fragments exemplified with purified viral myb protein. Nucleic acids research, 16 (1988) 5241–5248.

Feavers, I.M., McEwan, I.J., Liang, H. and Jost, J.P., An estradiol-dependent protein from chicken liver binds single stranded DNA and RNA., J. Biol. Chem., 264 (1989) 9114–9117.

Gustafsson, J.A., Carlstedt-Duke, J., Poellinger, L., Okret, S., Wikström, A.C., Brönnegard, M., Gillner, M., Dong, Y., Fuxe, K., Cintra, A., Häfstrand, A. and Agnati, L., Biochemistry, Molecular biology and physiology of the glucocorticoid receptor. Endocrine Reviews 8 (1987) 185–234.

Hughes, M.J. and Jost, J.P., The ubiquitous nuclear protein, NHP1 binds with high affinity to different sequences of the chicken vitellogenin II gene. Nucleic Acid Res., 17 (1989) 8511–8519.

Marzouki, N., Camier, S., Ruet, A., Moenne A. and Sentenac, A., Selective proteolysis defines two DNA binding domains in yeast transcription factor tau. Nature, 323 (1986) 176–178.

Miller, J., McLachlan, A.D. and Klug, A., Repetitive zinc-binding domains in the protein transcription factor IIIA from Xenopus oocyte. EMBO J., 4 (1985) 1609–1614.

Schreiber, E., Matthias, P., Müller, M.M. and Schaffner, W., Identification of a novel lymphoid specific octamer binding protein (OTF-2B) by proteolytic clipping band-shift assay (PCBA), EMBO J., 7 (1988) 4221–4229).

Smith, D.R., Jackson, I.J., Brown, D.D., Domains of the positive transcription factor specific for the Xenopus 5S RNA gene. Cell, 37 (1984) 645–652.

Wilkinson, J.M., Fragmentation of polypeptides by enzymatic methods. In Practical Protein Chemistry. A handbook edited by A. Darbre. John Wiley & Sons. Chichester, New York, Brisbane, Toronto, Singapore (1986) 121–148.

V Visualising Intimate Protein-DNA Contacts and Altered DNA Structures with Ultravioltet Light

Michael M. Becker and Zhou Wang

Contents

A Introduction

The use of light to footprint DNA (photo-footprinting) was first introduced in 1980 (Becker and Wang, 1984). The simplest photo-footprinting technique, termed ultraviolet (UV) footprinting, uses UV photons as the sole footprinting agent, and has several advantages over other techniques. First, because UV light readily penetrates cells, footprinting analysis can be carried out directly on living cells (Becker and Wang, 1984; Becker, et al. 1989; Selleck and Majors, 1987; Axelrod and Majors, 1989). Second, because the quantum yield of a UV photoreaction is generally insensitive to changes in salt, solvent or temperature, UV footprints of DNA can be obtained in vitro under a very wide range of environmental conditions. Third, because UV light readily damages DNA, footprinting analysis can be conveniently carried out in the laboratory using simple transilluminators as light sources. Under these low levels of UV irradiation, protein-DNA complexes are not dissociated (Wang and Becker, 1988; Becker, et al., 1989) and subtle structural alterations along the DNA helix can readily be footprinted (Becker and Wang, 1989a/b). As summarised in Table 1 and discussed previously (Becker and Wang, 1989b), there is an excellent correlation between the ability of an agent to alter the UV photoreactivity and the torsional flexibility of DNA. This correlation reflects two important properties of UV photoreactions. First, the majority of UV photoproducts result from photodimerisation reactions between adjacent bases. Second, before adjacent bases can dimerise, they must rotate into a geometry which allows photoreactive double bonds on adjacent bases to form four members rings. As discussed previously (Becker and Wang, 1989b), the absorption of a UV photon cannot simultaneously induce the geometrical changes required for adjacent bases to dimerise. Rather, upon the absorption of a UV photon, only those thermally excited bases photoreact that are in a geometry allowing easy formation of a photoproduct during excitation. Thus, agents which inhibit the torsional flexibility of DNA, e.g. the B→A transition, sequence-specific protein-DNA contacts, and triple-strand formation (Lyamichev, et al., 1990), inhibit the UV photoreactivity of DNA. Agents which enhance the torsional flexibility of DNA, e.g. melting or kinking of the double helix, and premelting of rigid dAdT tracts, enhance UV photoreactivity. Finally, agents which do not alter the torsional flexibility, e.g. salt, organic solvents, temperature, and chromosonal protein-DNA interactions, have little effect little UV photoreactivity of DNA. Thus, in contrast to other footprinting methods, UV footprinting can be used to detect the changes in torsional flexibility of individual bases along the DNA helix caused by intimate sequence-specific contacts in protein-DNA complexes (Wang and Becker, 1988). As described previously, UV-footprinting patterns can be generated by breakage of the DNA backbone at the sites of UV damage using chemical reactions (Becker and Wang, 1984) or through the termination by UV photoproducts of DNA polymerase-catalysed synthesis of complementary DNA (Wang and Becker, 1988; Becker et al., 1989).

In this chapter, we describe the use of chemical reactions to reveal UV-footprinting patterns of the *Xenopus* TF IIIA transcription factor bound to a sea urchin 5S DNA fragment (Wang and Becker, 1988). Our results demonstrate that intimate contacts between the zinc fingers of the TF111A factor and DNA can readily be detected by UV light. The basic steps involved in this research consist of binding a protein to a DNA fragment uniquely end labelled with ^{32}P, UV irradiation of the complex and a protein-free DNA control, introduction of strand breaks at the sites of UV damage with a series of chemical reactions, visualisation of strand breakage patterns using autoradiography on a sequencing gel, and, finally, quantitative analysis of the UV-footprinting patterns by microdensitometry (Fig. 1).

B Labelling and Purification of a DNA Fragment

There are four ends which can be labelled on a given DNA fragment. The choice of the labelled end should satisfy two conditions: First, because pyrimidines form UV photoproducts more readily than purines, the labelled strand should be more pyrimidine rich than its complement. As discussed previously, adjacent pyrimidines in double-stranded DNA, or purines flanked on their 5' side by two or more pyrimidines, are photoreactive; all other bases are less photoreactive (Becker and Wang, 1989b). Second, the labelled end of the DNA fragment should be separated from the putative protein-binding site an appropriate distance so that UV photoproducts over the protein-binding region can be well resolved on a sequencing gel. End labelling of a DNA fragment can be carried out according to Maniatis et al., (1982). The uniquely end-labelled DNA fragments are subjected to electorphoresis on a polyacrylamide gel, the radioactive band after location by autoradiography is sliced out of the gel, and the resultant DNA is purified either by electro-elution (Maniatis et al., 1982) or elutrap purification (Becker et al., 1989). Alternatively, the fragment can be purified from low-melting agarose (Maniatis et al., 1982; See also Chapter VIII).

C The Protein-DNA Binding Reaction

Because protein-free DNA generally reacts more readily with footprinting reagents than protein-bound DNA, it is essential that protein-binding sites be fully occupied during the footprinting reaction. It is advisable to use low ionic polyacrylamide gel electrophoresis (Fried and Crothers, 1981) or DNase I footprinting to establish conditions for maximal occupancy.

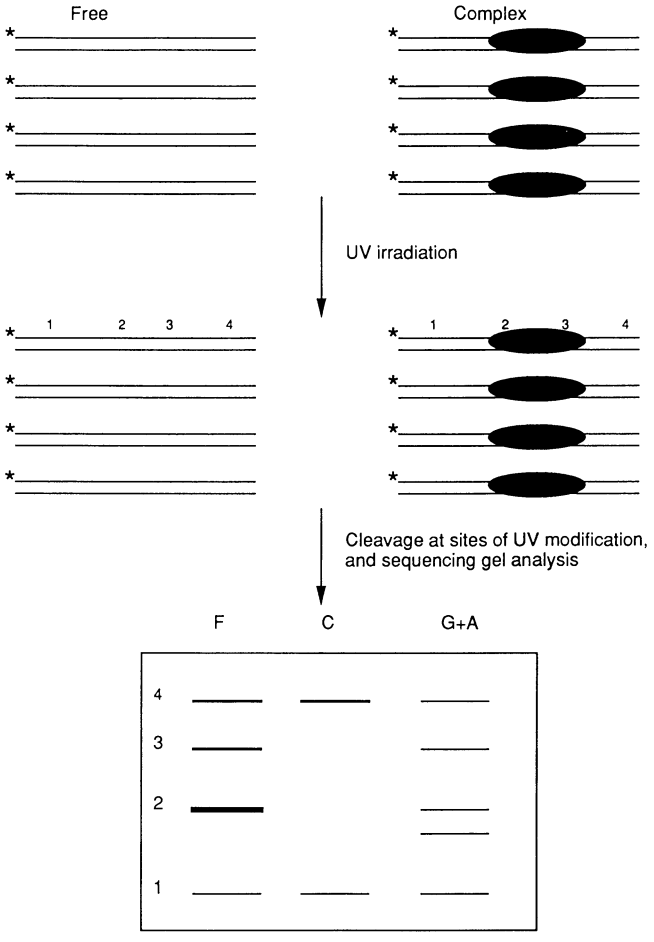


Fig. 1: Principle of probing DNA-protein interactions with UV light: DNA is labelled at one unique end (*). Protein-free DNA (Free, or F) or protein-bound DNA (Complex, or C) are irradiated with UV light. The DNAs are then purified, strand cleavages introduced at the sites of UV modification, and DNA fragments separated on a sequencing gel. The numbers 1,2,3 and 4 represent the potential sites for UV-photoproduct formation on the labelled strand and the black dots are the UV photoproducts. Sites 2 and 3 are located in the protein-binding region. Photoadducts formed in the unlabelled strand are omitted since they cannot be visualised on the sequencing gel. G+A is the DNA depurination pattern (Maxam and Gilbert, 1980) used as a DNA size marker.

D UV Irradiation of a Protein-DNA Complex

UV irradiation can be conveniently carried out with a 254-nm transilluminator commonly found in molecular biology laboratories. An irradiation dose of 12 mW/cm^2 for 10–20 s is sufficient to damage DNA for footprinting analysis.

1 Equipment: A TS-40 or similar UV transilluminator (UVP. San Gabriel, CA USA)

2 Step-by-Step Procedure

> Remove the top cover of the UV transilluminator revealing the bare bulbs. Invert and raise the transilluminator so that the bulbs are approximately 4 cm above the bench.

> Warm up the transilluminator for approximately 2 min and switch it off just before use.

> Place the sample to be irradiated in a quartz cuvette or in an opened Eppendorf tube, or spot the sample onto a glass or plastic surface.

> Place the sample under the centre of the transilluminator and irradiate for 10–20 s.

E Breakage of the DNA Backbone at the Sites of UV Damage

The chemical reactions used to break the DNA backbone at the sites of UV photoproducts include deamination, $NaBH_4$ reduction, and acidic aniline cleavage.

1 Buffers and Reagents

- Sevag: 50% TE saturated phenol, 48% chloroform, 2% isoamylalcohol
- 10% SDS
- 95% Ethanol
- TE (10 mM Tris pH 8.0, 1 mM EDTA)
- Deamination buffer (1 mM Tris pH 7.6, 5 mM NaCl)

- $NaBH_4$ reduction buffer (5 mM KPO_4 pH 8.3, 5 mM NaCl, 50 mM thymidine)
- $NaBH_4$
- Sequence loading buffer (Maxam and Gilbert, 1980)
- 3 M NaOAc pH 7.0
- 0.4 M NaOAc pH 5
- 7.5 M NH_4OAc
- Salmon sperm DNA (phenol extracted, ethanol precipitated)
- Acidic aniline pH 4.5; acidic aniline is prepared from distilled aniline and acetic acid. It should be distilled under reduced pressure and can be stored for months in the dark under nitrogen as a colourless solid at –20° to –78°C. An acidic aniline solution at pH 4.5 can conveniently be prepared by adding 910 µl of aniline and 60 µl of glacial acetic acid to 8.2 ml of water. The pH of the solution is then adjusted to pH 4.5 by adding approximately 200 µl of glacial acetic acid and monitoring the stirred solution with a pH meter.

2 Step-by-Step Procedures

Purification of UV-Irradiated Protein-DNA Complexes

> After UV irradiation, add 15 µl of 10% SDS and TE to a final volume of 300 µl. Then, add 300 µl Sevag to each sample.

> Vortex vigorously.

> Spin the sample in an Eppendorf centrifuge for 3 min.

> Transfer the aqueous layer to a new Eppendorf tube.

> Add 300 µl of Sevag and vortex vigorously.

> Spin the sample in an Eppendorf centrifuge for 3 min.

> Transfer the aqueous layer to a new Eppendorf tube.

Ethanol Precipitation of the DNA

> Add 5 µl salmon sperm DNA (2 µg/µl) to each sample.

> Add 7.5 M NH_4OAc to each sample to a final concentration of 2.5 M.

> Add 2.5 volumes of 95% ethanol.

> Chill the samples in an ethanol-dry ice bath (–78°C) for 10 min.

> Spin the samples in an Eppendorf centrifuge for 10 min.

> Withdraw the supernatant with a Pasteur pipette (leave approximately 50 µl liquid behind to avoid disturbing the DNA pellet).

> Add 1 ml 95% ethanol.

> Invert the tubes 10 times.

> Spin the tubes for 1 min.

> Withdraw the supernatant as before.

> Dry the samples in a SpeedVac concentrator.

> Resuspend the DNA pellets in 300 µl TE buffer.

> Add 30 µl of 3 M NaOAc solution to each sample.

> Add 750 µl of 95% ethanol and vortex.

> Chill in an ethanol-dry ice bath as before to precipitate the DNA. Then repeat the spin, rinse, and dry steps as described above.

Deamination Reaction

> Resuspend purified, ethanol-precipitated DNA pellets in 300 µl of deamination buffer.

> Incubate the DNA samples in a water bath at 50°C for 5 h in the dark.

> Add 30 µl of 3 M NaOAc and 750 µl of 95% ethanol to precipitate the DNA. The precipitation is carried out as described above.

$NaBH_4$ Reduction Reaction

> Resuspend the DNA pellets in 50 µl of $NaBH_4$ reduction buffer.

> Cool to 0°C.

> Add 50 µl of an ice-cold $NaBH_4$ reduction solution containing 1 mg freshly dissolved $NaBH_4$ to each sample. Because $NaBH_4$ decomposes in water it may be necessary occasionally to spin down the $NaBH_4$ reduction solution in an Eppendorf centrifuge before adding it to the samples.

> Vortex the samples briefly.

> Spin briefly in an Eppendorf centrifuge.

> Allow the samples to stand undisturbed and open to the air at 4°C in the dark for 16 h.

> Slowly add 200 µl of 0.4 M NaOAc pH 5.0 solution to stop the $NaBH_4$ reduction.

> Allow the solution to stand at room temperature for 1 hour and vortex occasionally.

> Add 750 µl of 95% ethanol to precipitate the DNA according to the procedures described previously.

> Resuspend the rinsed and dried pellets in 300 µl of 0.4 M NaOAc pH 5.0.

> Allow the samples to sit at room temperature for 1 h with occasional vortexing.

> Add 750 µl of 95% ethanol to precipitate DNA as before.

Acidic Aniline Cleavage Reaction

> Resuspend the rinsed and dried pellets in 50 µl of 1 M aniline pH 4.5.

> Heat at 60°C for 25 min in the dark.

> Freeze in an ethanol-dry ice bath.

> Lyophilise to dryness in a SpeedVac concentrator.

> Resuspend the DNA in 100 µl of double-distilled water.

> Freeze and lyophilise to dryness.

> Add 100 µl of double-distilled water and repeat the lyophilisation.

> Resuspend the dried DNA samples in 300 µl TE.

> Add 30 µl of 3 M NaOAc and 750 µl of 95% ethanol to precipitate the DNA.

> Repeat the ethanol precipitation once more.

> Resuspend the rinsed and dried DNA samples in 100 µl of double-distilled water.

> Spin the samples in an Eppendorf centrifuge for 15 min to bring down any insoluble materials.

> Transfer 90 µl of each sample to a new Eppendorf tube, leaving behind any insoluble precipitate.

> Count 3 µl of each sample in a scintillation counter to determine their relative DNA concentrations.

> Freeze and lyophilise the samples to dryness.

> Resuspend all samples in a sequencing loading solution such that the specific radioactivity of all samples is the same. Resuspend the sample with the lowest radioactivity in 6 µl.

F Sequencing-Gel Analysis of the UV-Footprinting Pattern

The sequencing gel we use to visualise the strand-breakage patterns is a 50-cm-long, 0.8-mm-thick 8% denaturing polyacrylamide gel (Maxam and Gilbert, 1980). Although thinner gels give somewhat better resolution, 0.8-mm-thick gels are easier to handle. The sequencing gel is subjected to electrophoresis until its temperature reaches 55°C. At this point the samples to be analysed are boiled for 4 min in sequence loading buffer and then loaded onto the gel; each lane should have the same amount of radioactivity.

Fixing and drying the footprinting gel for autoradiography

1 Step-by-Step Procedure

> Following electrophoresis, separate the two glass plates of the sequencing gel; the gel will stick to one of the plates.

> Immerse the gel, while still attached to the glass plate, in 10% acetic acid for 30 min with gentle shaking.

> Remove the 10% acetic acid solution and soak the gel in water for 10–20 minutes. Remove the water.

> Add enough water to cover the gel.

> Place two layers of Whatman 3 MM paper of appropriate size onto the gel.

> Cover the Whatman papers and the gel with another sequencing glass plate, flip over the sandwich and carefully remove the top glass plate.

> Cut the gel so that it fits into a slab gel dryer. Cover the trimmed gel with Saran wrap and trim to size.

> Place the Saran wrap-covered gel with the two layers of Whatman paper onto the metal support of the slab dryer and cover with the rubber sheet of the dryer.

> The gel will be dry after 2 h under vacuum at 80°C.

> Expose the gel on a pre-flashed X-ray film.

G Quantitation of the UV-Footprinting Result

To visualise intimate contacts between DNA and a protein, it is necessary to quantitatively analyse the UV-footprinting pattern. The quantitation includes densitometric scanning of the UV-footprinting patterns, assignment of the DNA sequence on the scans, and measurement of the alteration of UV- photoproduct formation by protein-bound DNA.

1 Equipment

- Densitometer (E-C Apparatus Co.)
- Chart recorder (The Recorder Co.)
- Kodak diffuse optical density standard

2 Step-by-Step Procedure for the Scanning of the UV-Footprinting Patterns

> Warm up the densitometer and chart recorder for 60 min.

> Adjust the densitometer so that it responds linearly to optical density using a Kodak optical density standard.

> Place an autoradiogramme with the UV-footprinting patterns onto the densitometer.

> Adjust the densitometer so that the background of the autoradiogramme equals zero on the chart recorder.

> Adjust the densitometer so that the strongest UV-photoproduct band gives full-scall deflection on the chart recorder, this manipulation ensures the highest sensitivity to the UV-footprinting patterns over the protein-binding region.

> Scan each lane on the autoradiogramme.

3 Assignment of the DNA Sequence on the Autoradiogramme

Maxam-Gilbert sequencing patterns of an end-labelled DNA fragment can be used as DNA size markers. When compared to Maxam-Gilbert sequencing standards, the UV-footprinting pattern of a 5'-end-labelled DNA migrates with an apparent 1-bp increase in length (Wang and Becker, 1988). In contrast, the pattern of a 3'-end-labelled DNA migrates the same as Maxam-Gilbert sequencing standards (Becker and Wang, 1984).

4 Quantitation of the Densitometric Scans

To measure the alteration of UV photoreactivity induced by protein binding, it is necessary to compare the UV-footprinting pattern of the protein-DNA complex to the UV-footprinting pattern of protein-free DNA (Fig. 2). Since the chemical reactions used to cleave the DNA backbone at the sites of UV damage also cleave unirradiated DNA, it is necessary to subtract from UV-footprinting patterns any contribution from unirradiated DNA. To accurately quantitate the inhibition of UV-photoproduct formation, it is necessary to overlay and match the densitometric scans of unirradiated DNA, UV-irradiated protein-bound DNA, and UV-irradiated protein-free DNA. Since the same amount of radioactivity was loaded in each lane of the sequence gel, the intensities of all lanes are directly comparable and can be overlaid on one piece of paper. When overlaying the different densitometric scans, it is essential that the scans of unirradiated DNA, UV-irradiated protein-bound DNA, and UV-irradiated protein-free DNA be the same intensity over regions of DNA which are not UV photoreactive. Fig. 3 shows an example of such an overlay. It should be noted that the scan of strand-breakage patterns of unirradiated DNA and UV-irradiated DNA do not match well at the top of the lanes because the amount of strand cleavage in unirradiated DNA is lower than that in UV-irradiated DNA. The extent of UV inhibition induced by protein binding is expressed as the ratio bound/free, where bound is the intensity of photoproduct formation at a particular base in the presence of the bound protein minus the intensity of the same base observed in the absence of light. A similar calculation in the absence of protein binding yields the free quantity. Fig. 4 shows the quantitation of the TF IIIA UV-footprinting pattern.

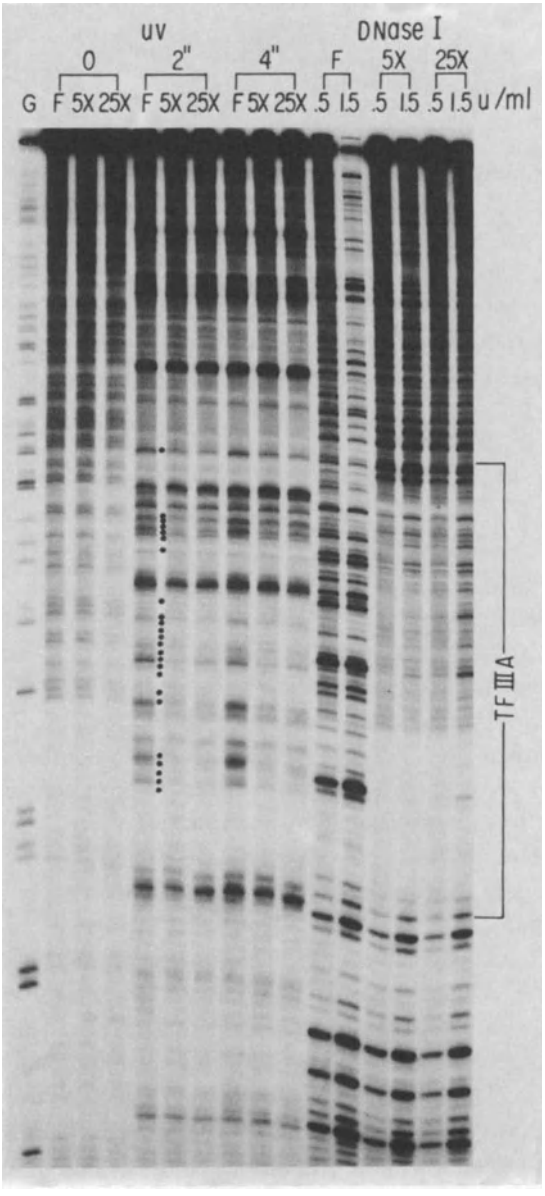


Fig. 2: UV- and DNase I-footprinting analysis of *Xenopus leavis* TFIIIA-5S sea urchin gene complex: DNA in the absence of TFIIIA (F; free) or in the presence of 5 M excess (×5) or 25 M excess (×25) TFIIIA is digested with DNase I or irradiated with 0,2 or 4 seconds of 270-nm light (42×10^{-3} W/cm^2; Wang and Becker, 1988).
This dose is equivalent to 10–20 s irradiation with a 254-nm UV transilluminator. Sites of altered UV photoproduct formation due to TFIIIA binding are denoted by solid circles. The drop in intensity observed midway in both UV and DNase I footprinting is due to a salt gradient moving through the gel. The 5S gene was uniquely end-labelled with ^{32}P on the lower strand.

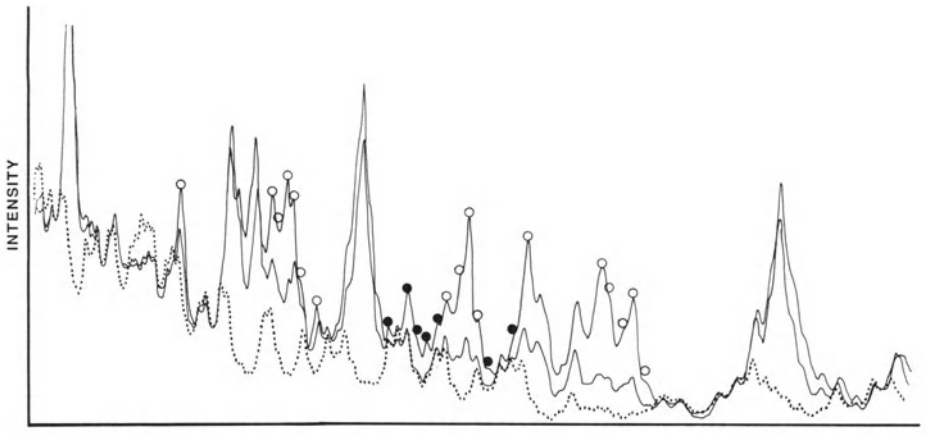


Fig. 3: Densitometric scans of the TFIIIA-5S sea urchin gene complex probed with UV light: The upper trace is DNA irradiated for 2 s in the absence of TFIIIA and the middle trace is DNA irradiated in the presence of TFIIIA. The lower (dotted) trace shows the strand breakage of unirradiated DNA which has been carried through the same chemical reactions used to induce strand breakage at UV photoproducts in the upper two traces. Circles correspond to protected bases denoted on the autoradiograph in Fig. 2; when filled, these circles denote strongly protected bases.

5 Interpretation of the TFIIIA UV-Footprinting

In Fig. 4 the UV footprinting of the TFIIIA protein is compared to previous chemical and enzymatic footprintings obtained by others. The UV footprint of the TFIIIA protein consists of seven regions of intimate protein contact separated by regions of DNA which are not in contact with the protein. Each site of intimate contact identified by UV light is also detected by chemical and enzymatic probes. These sites of intimate contact are postulated to be those sites along the DNA which are bound by the zinc fingers of the TFIIIA protein (Wang, 1990).

H The Most Common Problems and Their Solutions

– *Smear in the UV-footprinting lanes*
  This could be due to residual aniline, impurities generated during the chemical reactions, or excess salt in the samples. In the first case, make sure that samples are frozen during lyophylisation and use a high vacumm to remove aniline. In

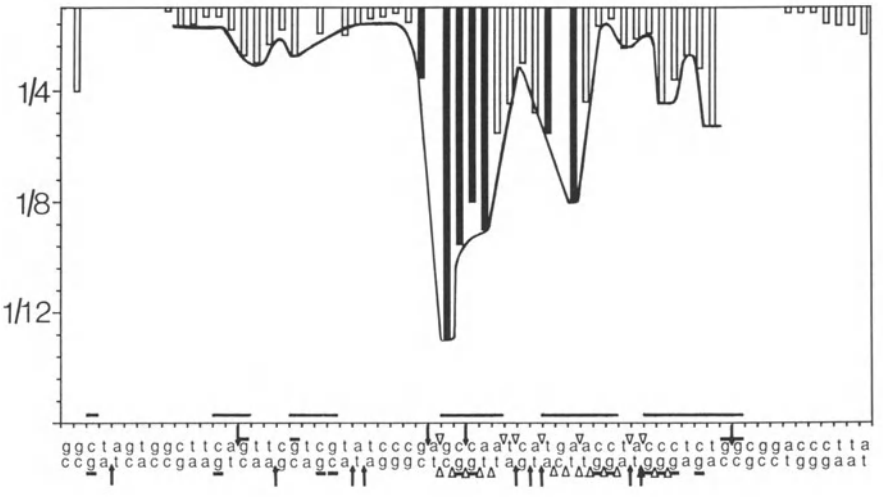


Fig. 4: Quantitation of the UV-footprinting results of the TFIIIA-5S gene complex, and comparison of the protein-DNA contacts detected by UV light to those detected by other footprinting methods: The autoradiograph in Fig. 2 was quantitated by microdensitometry. Inhibition of photoproduct formation is expressed as the ratio bound/free (see text for details of this calculation). Solid regions of the histogram denote strongly protected bases whose protection is equal to or greater than the indicated ratio. Filled bars (—) above or below guanine residues represent DNA protected from methylation by TFIIIA *in vitro* (Fairall et al., 1986). A subset of these bars occurs at guanine residues which interfere, when methylated, with the binding of TFIIIA (Sakonju and Brown, 1982). Open triangles (△) show phosphate residues that, when methylated, prevent the binding of TFIIIA (Sakonju and Brown, 1982), or which are protected from micrococol nuclease (MNase) digestion (Fairall et al., 1986). Arrows (↓↑) show cutting sites of MNase, DNase I and DNase II on the TFIIIA-5S DNA complex (Fairall et al., 1986). Bold lines above the sequence assignment indicate putative intimate contacting sites between TFIIIA and the 5S DNA.

the second case, extract the samples once with Sevag and precipitate them twice with ethanol. Rinse the DNA pellets carefully with ethanol before drying.

- *UV-specific strand breakage is weak throughout the lanes of UV-irradiated samples*
  In this case, the DNA is under-irradiated with UV light.
- *UV-specific strand breakage is strong at the lower part of the sequencing gel but weak at the top part of the gel*
  In this case, the DNA is over-irradiated with UV light.
- *Both the UV- and DNase I-footprinting patterns of a DNA-binding protein are weak*
  This could be due to dissociation of the complex by non-specific binding of the complex to the tube. One way to solve this problem is to coat the tube with gelatin

(1 mg/ml) before use. Allow the gelatin solution to sit in the tube one hour and then rinse with water.

I Bibliography

Axelrod, J.D. and Majors, J. An improved method for photofootprinting yeast genes *in vivo* using Taq polymerase. Nucl. Acids Res. 17 (1989) 171–183.

Becker, M.M. and Wang, J.C. Use of light for footprinting DNA *in vivo*, Nature 309 (1984) 682–687.

Becker, M.M. and Wang, Z. B → A transitions within a 5S ribosomal RNA gene are highly sequence-specific. J. Biol. Chem. 264 (1989a) 4163–4167.

Becker, M.M. and Wang, Z. Origin of ultraviolet damage in DNA. J. Mol. Biol. 210 (1989b) 429–438.

Becker, M.M. and Wang, Z., Grossman, G.G., and Becherer, K.A. Genomic footprinting in mammalian cells with ultraviolet light. Proc. Natl. Acad. Sci. USA 86 (1989) 5315–5319.

Becker, M.M., Lesser, D., Kurpiewski, M., Barranger, A., and Jen-Jocobson, L. Ultraviolet footprinting accurately maps sequence-specific contacts and DNA kinking in the EcoRI endonuclease-DNA complex. Proc. Natl. Acad. Sci. USA 85 (1988) 6247–6251.

Fairall, L., Rhodes, D., and Klug, A. Mapping of the sites of protection on a 5S gene by the *Xenopus* transcription factor IIIA: A model for the interaction. J. Mol. Biol. 192 (1986) 577–591.

Fried, M. and Crothers, D.M. Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. Nucl. Acids Res. 23 (1981) 6505–6525.

Lyamichev, V.I., Frank-Kamenetskii, M.D., and Soyfer, U.N. Protection against UV-induced pyrimidine dimerization in DNA by triplex formation. Nature 344 (1990) 568–570.

Maxam, A.M. and Gilbert, W. Sequencing end-labelled DNA with base specific chemical cleavage. Methods Enzymology 65 (1980) 497–560.

Maniatis, T., Fritsch, E.F., and Sambrook, J. Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Lab.) Cold Spring Harbor, N.Y. (1982).

Sakonju, S. and Brown, D.D. Contact points between a positive transcription factor and the *Xenopus* 5S RNA gene. Cell 31 (1982) 395–405.

Selleck, S.B. and Majors, J.E. *In vivo* DNA-binding properties of a yeast transcription activation protein. Mol. Cell Biol. 7 (1987) 3260–3267.

Wang, Z. Detection of genetic regulatory processes within the chromosomes of living cells with ultraviolet light. Ph.D. Thesis (1990).

Wang, Z. and Becker, M.M. Selective visualization of gene structure with ultraviolet light. Proc. Natl. Acad. Sci. USA 85 (1988) 654–658.

Agent	Torsional flexibility	UV photoreactivity
Proteins		
1. Chromosomal		
–Nucleosomes –H1	Unaltered	Unaltered[1] (< 2- to 3-fold)
2. Sequence-specific		
–Lac repressor –E.coli RNAP –Eco RI –TF IIIA	Inhibited	Inhibited[2] (5- to 20-fold)
Structural changes		
1. Melting 2. Premelting of rigid dAdT tracts	Enhanced	Enhanced[3] (2- to 10-fold)
3. B → A	Inhibited	Inhibited[4] (5- to 20-fold)
Environment		
1.Salt (0–250 mM) 2.Ethanol (0–60%)	Unaltered	Unaltered[5]

1) Wang and Becker, 1988.
2) Becker and Wang, 1984; Becker, et al.,1988; Wang and Becker, 1988.
3) Becker and Wang, 1989b.
4) Becker and Wang, 1989a.
5) Becker and Wang, 1989a; Wang, 1990.

VI UV Cross-Linking of Protein to Bromouridine-Substituted RNA

Iain J. McEwan

Contents

A Introduction

Protein-nucleic acid interactions play a fundamental role in the control of gene expression, both at the transcriptional and post-transcriptional level. The aim of present research is to understand how the interplay of DNA- and/or RNA-binding proteins determines the level of specific gene products in a given cell or tissue. An important step in this research is the full characterisation of putative regulatory factors, which requires that sufficient amounts of these proteins are available for analysis. As an initial step in the characterisation, the covalent cross-linking of protein to nucleic acid is a powerful tool that complements the widely used gel-shift assay and DNA-footprinting techniques. There are now several techniques for the cross-linking of protein to nucleic acids, involving chemical means such as formaldehyde, glutaraldehyde or 1-ethyl-3(3-dimethyl-aminopropyl) carbodiimide, physical means such as UV-light irradiation, or a combination of the two involving the use of psoralens or bromouridine-substituted nucleic acid (for a review see

Welsh and Cantor, 1984). UV-light, either directly or with bromouridine-substituted nucleic acid, has been used to address a variety of problems, such as the binding of *trans*-acting factors to their cognate DNA sequences (Chodosh et al., 1986; Hughes et al., 1989; McEwan, unpublished data), and the binding of proteins to RNA (Moore et al., 1988; Feavers et al., 1989; Muller et al., 1989). In this chapter, a protocol is described for cross-linking protein(s) to bromouridine-substituted RNA synthesised *in vitro*. UV cross-linking to bromouridine-substituted DNA is described in Chapter VII. Fig. 1 shows an autoradiograph of proteins cross-linked to the 5' untranslated region of the chicken vitellogenin gene. This procedure allowed the identification of a specific hormone dependent protein of molecular mass 71 kDa (Feavers et al., 1989). It is important to remember that certain steps (see also below), for example the time of UV irradiation, may need to be adapted to the equipment and to the problem being studied.

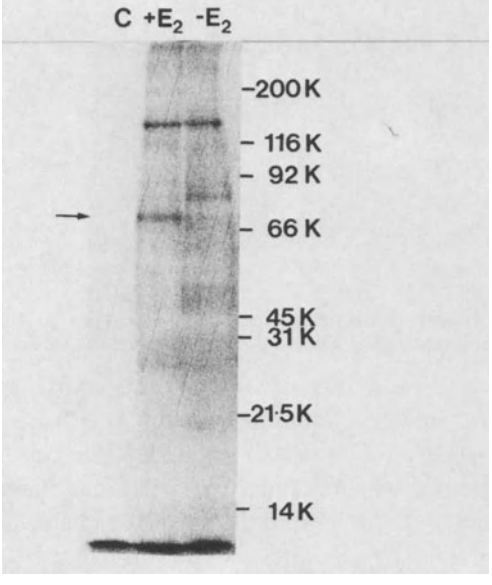


Fig. 1: Proteins from oestradiol-induced (+E_2) and uninduced (-E_2) cytoplasmic extracts UV cross-linked to bromouridine-substituted RNA: The arrow indicates an oestradiol-dependent RNA-binding protein of M_r 71000. A binding reaction in the absence of cross-linking is included as a control (C). Reprinted with permission from J. Biol. Chem. 264 (1989) 9114–9117, copyright 1990, the American Society for Biochemistry and Molecular Biology.

B Materials and Methods

1 Materials and Buffers

- Template DNA (see below)
- ATP, GTP, CTP, UTP, 5Br.UTP (10- mM stock solutions)
- [^{32}P]CTP (3000 Ci/mmol, Amersham)
- Human placental ribonuclease inhibitor (RNasin, 40 U/µl; Promega Biotec)
- T7 RNA Polymerase (20 U/µl; Boehringer, Mannheim)
- Acrylamide (2× recrystallised; Serva, Heidelberg)
- Bisacrylamide (2× recrystallised; Serva, Heidelberg)
- Ammonium persulphate
- N,N,N',N'-Tetramethylenediamine (TEMED)
- Urea (Analytical grade)
- Glycerol
- Phenol/chloroform (saturated with water)
- Sonicated *E.coli* DNA
- *E.coli* tRNA
- Ribonuclease A (RNase A; Boehringer, Mannheim)
- Ribonulease T_1 (RNase T_1; Boehringer, Mannheim)
- Molecular weight marker proteins for SDS-PAGE (Bio-Rad)
- Ethanol
- Methanol
- Acetic acid
- Water baths (37°C, 90°C)
- Silicone treated Eppendorf tubes
- Eppendorf centrifuge
- Sorvall centrifuge and SS-34 rotor or equivalent
- Glass plates (18 × 24 cm)
- Small glass plate (7 × 10 cm), silicone treated
- Plastic spacers and gel combs (0.5- and 1.0-mm-thick)
- Electrophoresis power supply
- Micro-Collodion bags (Sartorius)
- UV-light source (254 nm)
- Gel dryer (Model 483 slab dryer; Bio-Rad)
- X-ray film (Kodak)
- 10× TBE Buffer (pH 8.2)
  0.89 M Tris base
  0.89 M Boric Acid
  0.02 M EDTA
- 10× RNA polymerase buffer (pH 7.5)

0.40 M Tris-HCl
0.06 M $MgCl_2$
0.04 M Spermidine
10 μM Dithiothretol (DTT)

- STOP buffer
  12.4 mM SDS
  64.3 mM Sodium acetate
  0.7 mM EDTA
- 10× Protein-RNA binding buffer
  500 mM Hepes (pH 8.0)
  100 mM $MgCl_2$
  800 mM KCl
  1 mM EDTA
- Formamide sample buffer
  94% Formamide
  0.01% Bromophenol blue
  0.01 mM EDTA
- 4× Lower gel buffer (pH 8.8)
  1.5 M Tris-HCl
  0.4% (w/v) SDS
- 4× Upper gel buffer (pH 6.8)
  0.5 M Tris-HCl
  0.4% (w/v) SDS
- SDS sample buffer
  12.5% (v/v) Upper gel buffer (4×)
  2.0% (w/v) SDS
  10.0% (v/v) Glycerol
  2% β-Mercaptoethanol (added just prior to use)
- 10× SDS-PAGE running buffer (pH 8.3)
  0.25 M Tris
  1.92 M Glycine
  0.05 M SDS

2 Step-by-Step Procedures

a) *Synthesis of RNA Substrate*

The sequence of interest should be cloned into a suitable vector (i.e. pSPT18 or 19, Pharmacia LKB Biotechnology Inc.) and linearised with an appropriate restriction endonuclease. The steps involved are outside the scope of the present discussion (see Krieg and Mellon, 1987 for details), and the linearised plasmid will simply be referred to here as "template DNA".

> To a silicone-treated Eppendorf tube add consecutively:
2 µl template DNA (5 µg/µl)
2 µl 10× RNA polymerase buffer
1 µl NTP mix without CTP (to final concentrations of 500 µM ATP and GTP, and 250 µM UTP and Br.UTP)
2 µl [^{32}P]CTP (20 µCi)

Note:
The choice of labelled nucleotide should reflect the occurrence of that base in the binding site being studied.

1 µl RNasin (2 U/µl)
9 µl distilled water
2 µl T7 RNA polymerase (2 U/µl)

> Mix reagents well.

> Incubate at 37°C for 20 min.

> Add 1 µl of NTP mix (ATP, GTP, CTP, UTP).

> Continue the incubation for a further 20 min (= chase).

> Add 140 µl of STOP buffer.

> Extract with 0.5 volumes phenol/chloroform (pH 5).

> Centrifuge for 5 min in an Eppendorf centrifuge.

> Carefully remove the aqueous phase.

> Add 5 µg *E.coli* DNA and 2.5 volumes ethanol.

> Precipitate in an ethanol/dry ice bath for 15 min.

> Centrifuge in a Sorvall at 15000 rpm for 20 min.

> Resuspend the sample in formamide sample buffer and heat for 2–3 min at 90°C.

> Purify the RNA on an 8% denaturing polyacrylamide gel.

> Recover the labelled RNA from the gel by electro-elution into distilled water using micro-collodion bags.

> Add 0.1 volumes of 3 M sodium acetate/ 0.5 M EDTA and 2.5 volumes of ethanol; leave at –20°C overnight.

> Centrifuge in a Sorvall as above.

> Resuspend samples in distilled water.

> Determine recovered radioactivity.

b) *Protein-RNA Binding Reaction*

> To a silicone-treated Eppendorf tube add consecutively:
10 µl 25% Glycerol (12.5% final concentration)
2.5 µl 10× RNA binding buffer
2.0 µl tRNA (0.25 µg/µl)
Labelled RNA (250000 cpm = 0.7 ng RNA)
10 µg protein (i.e. partially purified cytoplasmic extract from hen liver)

> Mix components well.

> Incubate on ice for 30 min.

c) *UV-light Irradiation*

> Pipette samples as droplets onto a small silicone-treated glass plate and keep on ice.

> Irradiate for 10 min* at a distance of 17 cm from the UV source (2 Philips UV tubes, TUV 15WG15T8, with a UV flux (254 nm) of 0.39 mW/cm^2 at 22 cm; Saluz and Jost, 1987)

> Replace samples in Eppendorf tubes.

> Add 4 µg of tRNA, and the enzymes RNaseA and RNaseT_1 to a final concentration of 1 µg/µl and 2.5 U/µl respectively, and incubate at room temperature for 30 min*.

> Add 2–3 µl SDS sample buffer and heat for 3 min at 90°C.

**Note*:
It is advisable first to carry out a time course to determine the optimal time of UV irradiation. Determination of the period and temperature at which the RNase digestion of a given

sample is carried out is also critical. The conditions described above were optimal for studying the binding of a single-stranded nucleic acid-binding protein from hen liver cytoplasmic extracts to RNA corresponding to the start of the chicken vitellogenin gene (Feavers et al., 1989).

d) *SDS-PAGE and Autoradiography*

> Prepare a 10% polyacrylamide SDS gel:

Resolving gel
8.30 ml 30% acrylamide:bisacrylamide stock (29.2% : 0.8%)
6.30 ml 4× lower gel buffer
3.75 ml glycerol
6.70 ml distilled water
35 µl 40% ammonium persulphate
35 µl TEMED

Stacking gel
1.70 ml 30% acrylamide:bisacrylamide stock (29.2%:0.8%)
2.50 ml 4× upper gel buffer
5.80 ml distilled water
16 µl 40% ammonium sulphate
16 µl TEMED

> Resolve cross-linked products by SDS-PAGE at 200–300V constant voltage (10 volts/cm). Continue electrophoresis until the bromophenol blue has migrated at least 12 cm into the resolving gel.

> After electrophoresis, fix and stain the gel in methanol : acetic acid : water (50:10:40) containing 0.1% commassie blue for 10–20 min at room temperature.

> Destain by shaking in several changes of destain buffer, methanol : acetic acid : water (20:10:70). Alternatively, the gel can be electro-destained at a current of 4–5 Amps for 5–10 minutes.

> Dry the gel in a slab-gel dryer (Bio-Rad Model 483) at a setting of 80°C for 1 h.

> Expose the dried gel directly on X-ray film (XAR-5 or XomatS, Kodak)

Acknowledgments

I am grateful to Dr. Ian Feavers (N.I.B.S.C., London) for providing template DNA for these studies and for stimulating discussions on protein-nucleic acid interactions. I am also indebted to Dr. Jean-Pierre Jost (F.M.I., Basel), in whose laboratory this work was carried out, for his encouragement and enthusiasm.

C Bibliography

Chodosh, L.A., Carthew, R.W., and Sharp, P.A. A single polypeptide possesses the binding and transcription activities of the adenovirus major late transcription factor. Mol.Cell.Biol. 6 (1986) 4723–4733.

Feavers, I.M., McEwan, I.J., Liang, H. and Jost, J.P. An estradiol-dependent protein from chicken liver binds single-stranded DNA and RNA. J.Biol.Chem. 264 (1989) 9114–9117.

Hughes, M.J., Liang, H., Jiricny, J., and Jost, J.P. The purification and characterization of a protein from HeLa cells that binds with high affinity to the estrogen response element, GGTCAGCGTGACC. Biochemistry 28 (1989) 9137–9142.

Krieg, P.A. and Melton, D.A. *in vitro* RNA synthesis with SP6 RNA polymerase. Methods Enzym. 155 (1987) 397–415.

Moore, C.L., Chen, J., and Whoriskey, J. Two proteins crosslinked to RNA containing the adenovirus L3 (poly A) site require the AAUAAA sequence for binding. EMBO J. 7 (1988) 3159–3169.

Muller,E.W., Neupert, B., and Kuhn, L.C. A specific mRNA binding factor regulates the iron-dependent stability of cytoplamic transferin receptor mRNA. Cell 58 (1989) 373–382.

Saluz, H.P. and Jost, J.P. Optimized genomic sequencing as a tool for the study of cytosine methylation in the regulatory region of the chicken vitellogenin II gene. Gene 42 (1987) 151–157.

Welsh, J. and Cantor, C.R. Protein-DNA cross-linking. Trends in Biochem. 9 (1984) 505–508.

VII Molecular Weight Determination of DNA-Binding Proteins by UV Cross-linking Combined with SDS Polyacrylamide Gel Electrophoresis

Melya J. Hughes, Haimin Liang and Jean-Pierre Jost

Contents

A Introduction

It was first shown by Smith (1962) that UV-light irradiation of *E. coli* decreased the amount of DNA that could be extracted by sodium dodecylsulphate in the presence of 0.5 M KCl. The remaining DNA was cross-linked with protein, forming insoluble complexes. Later Smith et al. (1966, 1968, 1969, 1970) showed that UV-light caused formation of a covalent linkage between cysteine and uracil (5-s-cysteine-6-hydrouracil), poly U, poly C and thymine. Among L-amino acids, the most reactive with uracil are phenylalanine, tyrosine and cysteine (Smith, 1969). The photochemical attachment of specific proteins to DNA could be later improved by using bromodeoxyuridine-substituted DNA (Lin and Riggs, 1979). UV irradiation of BrdU-substituted DNA leads to debromination and the subsequent production of highly reactive uracilyl radicals (Smith and Hanawalt, 1969). Proteins, therefore, will be cross-linked more readily to BrdU-DNA than to unsubstituted DNA. The formation of such stable complexes between the radioactively labelled DNA and the protein enables estimation of the molecular weight of the protein in relatively crude preparations. This method has been used with success to determine the molecular weight of specific proteins binding to double-stranded DNA, single-stranded DNA and RNA (Hillel and Wu, 1978, Chodosh et al., 1986, Jiricny et al., 1988, Feavers et al., 1989).

In cases where the native protein is composed of several poly-peptides of different molecular weight, UV cross-linking can be used to identify which poly-peptide binds to the DNA (Hughes et al., 1989).

The basic principle of the technique is shown in Fig. 1. One of the prerequisites for such an experiment is to know exactly where the protein binds to the DNA. This can be determined by different techniques outlined in other chapters. As seen in Fig. 1, one strand of the binding site is either synthesised enzymatically by primer extension or chemically with an automated synthesiser. In the first case, the radioactively labelled nucleotide and the BrdUTP are incorporated during the synthesis of the complementary DNA strand. In the second case, BrdUTP is incorporated by chemical reactions during the synthesis of the oligonucleotide, and the 5'end is labelled using the polynucleotide kinase reaction. When using such end-labelled DNA, the UV-treated protein-DNA complex cannot be digested with DNase, therefore the synthetic oligonucleotide should be as short as possible and just cover the DNA-binding site. After UV irradiation, the stable protein-DNA complex is separated on a denaturating SDS polyacrylamide gel with suitable molecular weight standards.

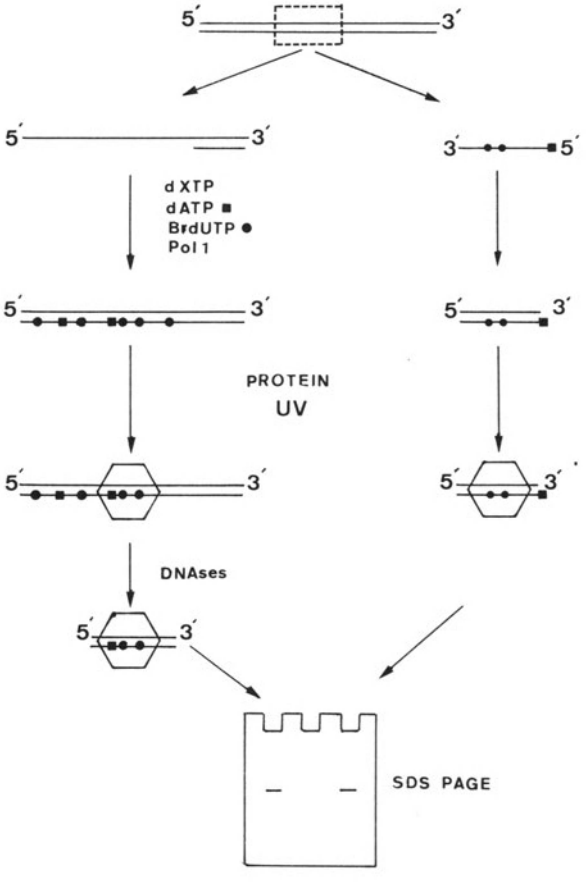


Fig. 1: Flow diagram of the different steps involved in UV cross-linking: The left column represents the approach with primer extension and subsequent trimming of the protein-DNA complex with nucleases; the right column represents the chemical synthesis of the binding site substituted with bromodeoxyuridine.

Choice of the Oligonucleotide

To obtain unambiguous results the oligonucleotide should bind only the protein to be studied. The binding site of the protein can be determined by looking at the sequence protected by the protein against DNAse I digestion, (see Chapter II) or by missing-contact probing (see Chapter VIII). Fig. 2 shows an example of missing-contact probing of a protein with its target-DNA oligonucleotide. The results show that the binding of the protein requires two thymidine residues which are going to be replaced by bromodeoxyuridine in the synthetic oligonucleotide. Non- specific protein-DNA interactions can be minimised by competition with non-specific DNA such as total sonicated *E. coli* DNA or poly (dI-dC).

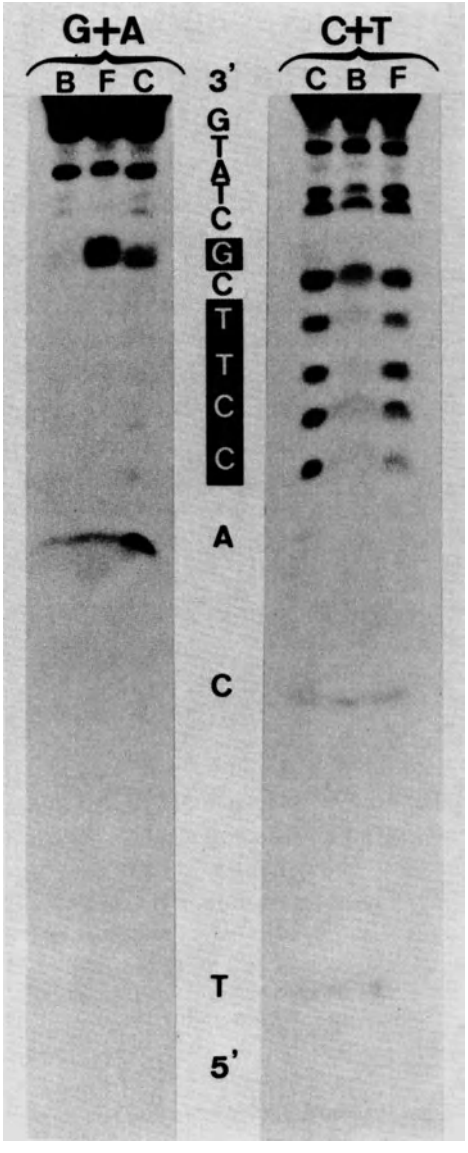


Fig. 2: Determination of the precise DNA-binding site of a protein by depurination and depyrimidination interference experiments (in our case the single-stranded DNA-binding protein was isolated from liver cytoplasm): The G-A and C-T reactions are shown for the protein-bound (B), free (F) and control (C) DNA. The latter represents the free DNA in the absence of added protein. The sequence of the oligonucleotide is shown and the basis critical for complex formation are indicated with boxes (Feavers et al, 1989). Reprinted with permission from J. Biol. Chem. 264 (1989) 9114–9117, copyright 1990, The American Society for Biochemistry and Molecular Biology.

B Synthesis of BrdUTP-Substituted Oligonucleotides

In the first procedure outlined in Fig. 1, two oligonucleotides are required for the synthesis of the substrate: an oligonucleotide containing the protein-binding site and a shorter oligonucleotide that acts as a primer and contains sequences that are not required for the protein binding. The Klenow fragment of *E. coli* polymerase I or Sequenase (version 2, mutated cloned enzyme, United States Biochemicals), which has no 3'-5' exonuclease activity, can be used for the filling-in reaction. For the second approach, short oligonucleotides covering just the binding site and containing BrdU are synthesised chemically. After purification, the oligonucleotide is labelled at the 5' end using polynucleotide kinase.

1 Materials and Buffers

- 10× Klenow buffer: 500 mM Tris HCl pH 7.5, 100 mM $MgSO_4$, 1 mM DTT
- 5× Sequenase buffer: 200 mM Tris HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl
- Purified synthetic 6 to 20-mer primer, 0.15 µg/µl in water
- Purified 25 to 45-mer oligonucleotide complementary to the primer, 0.5 µg/µl in water
- Klenow fragment of *E. coli* DNA polymerase I (Biofinex, Praroman, CH 1724, Switzerland)
- Cloned Sequenase (version 2, United States Biochemicals)
- Unlabelled dNTPs (sequencing grade), each stock solution at 2 mM
- 5-bromodeoxyuridine triphosphate, 2 mM stock solution (kept in the dark at –20°C)
- (α-^{32}P)dATP or (α-^{32}P)dCTP, 3000 Ci/mmole (Amersham)
- (γ-^{32}P)ATP, 3000 Ci/mmole (Amersham)
- Spun-column of Sephadex G-50 medium

2 Step-by-Step Procedure

The Filling-in Reaction using Klenow Polymerase

> Anneal equimolar amounts of complementary oligonucleotides. For example, add 1 µl of the 9-mer (0.15 µg) and 1 µl of the 25-mer to 30-mer (0.4 µg) to 14 µl of water in a silicone-treated tube. Heat to 90°C for 5 min and allow to cool slowly to room temperature.

> Add 1 µl each of dCTP, dGTP and 5-bromodeoxyuridine triphosphate (each at 80 µM final concentration).

> Add 3 µl (30 µCi) of (α-^{32}P)dATP, 2.5 µl of 10× Klenow buffer and 0.5 µl (3 units) of Klenow fragment of *E. coli* DNA polymerase in a total volume of 25 µl.

> Incubate 15 min at room temperature.

> Add 1 µl of dATP (2mM stock) and incubate a further 5 min at room temperature.

> Stop reaction by adding 1 µl of 0.5 M EDTA.

> Separate the oligonucleotides from free nucleotides by centrifugation through a spun column of Sephadex G-50 (medium). Determine the amount of radioactivity incorporated into an aliquot.

The Sequenase Reaction (Saluz and Jost, 1990)

> To a sterile, silicone-treated Eppendorf tube add 6 µl of 5× Sequenase buffer, 1 µl of the 25-mer to 30-mer (0.5 µg) and 1 µl of a complementary 9-mer (0.14 µg). The complementary oligonucleotides are chosen so that after annealing, the duplex has a 3' recessed end.

> Mix well and centrifuge for a few seconds in a microfuge.

> Place tube on a float in a small glass beaker (50 ml) containing 40 ml of water at 80°C and cool on the bench to room temperature within 20–30 min.

> Spin the sample for a few seconds in a microfuge and place the tube in ice.

> Add 4 µl each of sequencing grade dCTP, dGTP and 5-bromodeoxyuridine triphosphate from 2 mM stock solutions (final concentration 250 µM).

> Add 10 µl (100 µCi) of (α-^{32}P)dATP (3000 Ci/mmole) (or sufficient to complete synthesis).

> Add 1 µl (13 units) of Sequenase (version 2).

> Mix, spin briefly and incubate for 20 min at 23°C.

> Stop the reaction by adding 1 µl of 0.5 M EDTA.

> Separate the oligonucleotide from the free nucleotides by centrifugation through a spun column of Sephadex G-50 (medium).

C Complex Formation, UV Irradiation and Nuclease Treatment

The labelled substrate containing the 5-bromodeoxyuridine (~100000 cpm) is incubated with the protein to form a complex. Conditions should be chosen which allow the protein to bind at least 50% of the substrate, and sufficient *E. coli* DNA should be present to hinder non-specific binding to the oligonucleotide in gel-shift assays. Example: Protein (5 µg) containing NHP1 purified by Sephacryl S-300, heparin Sepharose and Mono Q was cross-linked in the presence of 1 µg of *E. coli* DNA (see Fig.4). In the case of affinity-purified NHP1, 5 ng of the protein was incubated with its substrate in the presence of 200 ng of *E. coli* DNA and 5 µg of bovine serum albumin, which stabilises the highly purified protein.

1 Materials and Buffers

- 10× Binding buffer: 1 M Tris HCl pH 8, 50 mM EDTA 20 mM β-mercaptoethanol, 1 mM $ZnCl_2$ (The buffer requirements may differ between proteins.)
- Germicidal UV lamp, Philips TUV 15 Watts, G 15 T8
- Enzyme grade bovine serum albumin (BRL)
- 0.5 M EDTA, pH 8.0
- 25% Glycerol in water
- Sonicated *E. coli* DNA 6 µg/µl
- 100 mM $CaCl_2$
- 100 mM $MgSO_4$
- Deoxyribonuclease I (Worthington) 5 mg/ml in water
- Micrococcal nuclease (Boehringer) 10^4 units/ml, diluted 1:10 just before use
- SDS sample buffer: 0.062 M Tris HCl pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue

2 Step-by-Step Procedure

> Mix labelled oligonucleotide (40 fmol in 1 µl) with 2 µl of 10× binding buffer, 10 µl of 25% glycerol, 0.2–10 µg protein in a total volume of 20 µl.

> Incubate the reaction mixture for 20 min at room temperature (20°–22°C).

> Spot the reaction mixture onto a clean, silicone-treated glass plate, or parafilm, which is placed on ice.

> UV irradiate the droplet for 2, 5, 10 or 20 min at a distance of about 22 cm from the UV lamps. We used two Philips TUV 15 Watt G 15 T8 germicidal UV lamps.

> Using a "pipettman", transfer the droplets back into the Eppendorf tube. If the DNA-binding site has been synthesised by primer extension, the protruding DNA of the protein-DNA complex needs to be digested with nucleases as described below.

> Add 2 µl of 100 mM $MgSO_4$ and 2 µl of 100 mM $CaCl_2$.

> Mix well and add 0.5 µl (5 µg) of deoxyribonuclease I and 1 µl of micrococcal nuclease (1 unit).

> Incubate at 37°C for 30 min.

> Stop the reaction by adding 5 µl of SDS PAGE sample buffer.

D Separation of the Protein-DNA Complex by SDS-PAGE

1 Materials and Buffers

- Protein molecular weight (MW) standard for SDS-PAGE (Bio-Rad); or Rainbow MW standards (Amersham). This latter standard consists of coloured methylated proteins.
- Stacking gel buffer stock solution: 0.5 M Tris HCl, pH 6.8. Dissolve 6 g of Tris base in 40 ml of water. Titrate to pH 6.8 with 1 M HCl (about 48 ml) and bring the total volume to 100 ml with water. Filter the solution and keep it at 4°C.
- Resolving buffer stock solution: 3 M Tris HCl, pH 8.8. Dissolve 36.3 g Tris base

in 48 ml of 1 M HCl, check pH and make up volume to 100 ml with water. Filter and store at 4°C.

- Reservoir buffer: 10× stock solution: 0.25 M Tris, 1.92 M glycine, 1% SDS (pH 8.3). Dissolve consecutively 30.3 g Tris base, 144 g glycine and 10 g SDS in water and make up to 1 l with water. Store solution at room temperature.
- Glass plates 24 × 18 cm with 0.5 mm spacers and combs; for mini-slab gel: glass plates, 10 × 8 cm with corresponding 0.75-mm (1.5-mm for large volume) spacers and combs
- Coomassie blue R250, 0.1% solution in water-methanol-acetic acid (5:5:2 by volume).
- Destaining solution: water-methanol-acetic acid (7:2:1 by volume)
- Stock of 30% acrylamide - bisacrylamide (30 : 0.8)
- SDS sample buffer: 0.0625 M Tris HCl pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue

Table 1 summarises the requirements for the preparation of discontinuous SDS polyacrylamide gels (Laemmli, 1970, Hames and Rikwood, 1982). Proteins with a molecular mass between 40 and 100 KDa were routinely analysed on 10% SDS polyacrylamide gels.

Table 1

Stock solutions	3.75% Stacking gel	Resolving gel % acrylamide for 100 ml					
		20	17.5	15	12.5	10	7.5
30% acrylamide-bisacryl (30:0.8)	2.5 ml	66.6	58.3	50	41.6	33.3	25
stacking gel buffer	5.0						
resolving gel buffer		12.5	12.5	12.5	12.5	12.5	12.5
10% SDS	0.2	1	1	1	1	1	1
1.5% ammonium persulphate	1.0	5	5	5	5	5	5
water	11.3	14.8	23.1	31.5	39.8	48.1	56.5
TEMED	0.015	0.05	0.05	0.05	0.05	0.05	0.05

The final concentrations of buffers are: stacking gel, 0.125 M Tris HCl pH 6.8; resolving gel, 0.375 M Tris HCl pH 8.8; reservoir buffer, 0.025 M Tris, 0.19 M glycine pH 8.3.

2 Step-by-Step Procedure

> Assemble the clean glass plates and spacers.

> Prepare the resolving gel mixture according to Table 1.

> Add TEMED and pour the gel between the glass plates. Leave a space of approximately 4 cm from the top of the 24-cm plate for the stacking gel. Overlay the polymerising gel solution with 1× resolving buffer or butanol.

> Upon polymerisation, pour out the remaining buffer, rinse with water and overlay the resolving gel with the stacking gel (for composition see Table 1).

> Insert the comb in the stacking gel and allow the gel to polymerise.

> After polymerisation, carefully remove the comb and wash the wells with a stream of reservoir buffer.

> Mix 5 µl of SDS PAGE sample buffer with 10 µl of reaction mixture.

> Heat sample to 90°C for 1 min, centrifuge in an Eppendorf microfuge and load immediately onto the SDS gel.

> Do the same with 0.5 µl of the Bio-Rad protein-size markers mixture.

> Carry out electrophoresis overnight at room temperature with constant voltage 60V using SDS reservoir buffer.

> For the mini-gels, carry out electrophoresis for 45–60 min at constant current (35 mA) using the same SDS reservoir buffer as above.

> After electrophoresis, stain the gel with Coomassie blue R250 (0.1%) in water-methanol-acetic acid (5:5:2) for 20–30 min.

> Destain the gel in water-methanol-acetic acid (7:2:1) in a destaining apparatus with constant voltage (70 volts) for 6 min.

> If the gel does not need to be stained, fix it for 30 min in the destaining solution.

> Place the gel on a Whatman paper pre-wetted with the destaining solution and dry the gel on a slab dryer for at least

50 min at 80°C. If the gel has over 12% polyacrylamide, fix it in 3% w/v glycerol, in water for at least 3 h before drying at 80°C for 2 h.

> After drying the gel, carry out autoradiography on a pre-flashed X-ray film with an intensifying screen at –70°C (Saluz & Jost, 1990).

E An Example

Figure 3A shows the kinetics of UV cross-linking of a liver cytoplasmic protein to single-stranded DNA and Fig. 3B shows the same for NHP1 binding to the double-stranded estrogen response element (Hughes et al., 1990). In both cases the protein-DNA complex was separated on a 10% SDS polyacrylamide gel. For the single-stranded DNA-binding protein, the oligonucleotide was synthesised with an Applied Biosystem model 380 A synthesiser and purified on a 20% polyacrylamide sequencing gel. In the second case (Fig. 3B), the oligonucleotide was synthesised

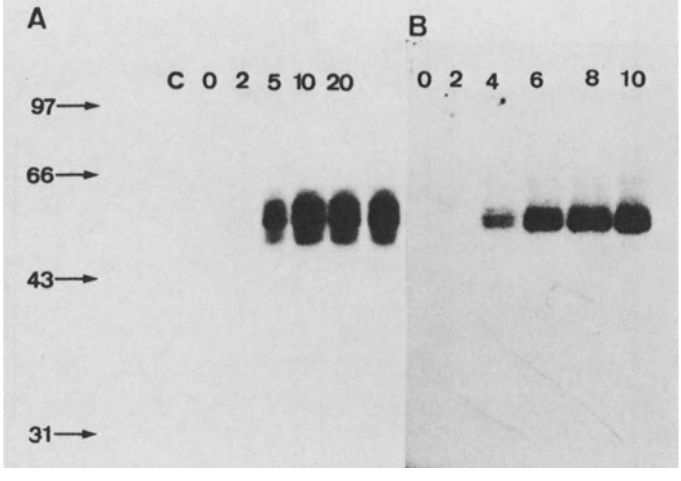


Fig. 3: A- Kinetics of UV irradiation of a bromodeoxyuridine-substituted synthetic oligonucleotide 5' XCACCXXCGCXAXG3' (where X is T or BrdUTP 50:50) with the cytoplasmic protein-single-stranded DNA complex: The sequence of the binding site was determined by missing-contact probing (see Fig. 2). B- Kinetics of UV irradiation of a bromodeoxyuridine-substituted double-stranded estrogen response element (synthesised by the filling-in reaction) with non-histone protein I (NHP1) from HeLa cells. After UV cross-linking, the protein-DNA complex was digested with nucleases and separated on a SDS-polyacrylamide gel.

by primer extension; after protein-DNA complex formation and UV irradiation, the protruding DNA was trimmed by DNAse I and micrococcal nuclease. Figure 4 shows the effect of nuclease digestion on the UV-cross-linked NHP1 with the estrogen response element.

F The Most Common Problem and Its Solution

During the staining procedure the most common problem is contamination of the gel with free labelled oligonucleotides, and this can be avoided by cutting off the bottom of the gel containing the oligonucleotides.

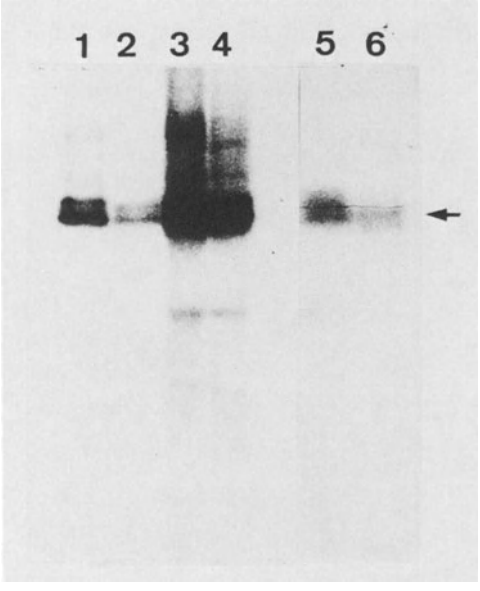


Fig. 4: UV cross-linking of the affinity chromatography-purified NHP1 to the estrogen response element: UV cross-linking was carried out as indicated in the protocol. Lane 1 shows distinct bands corresponding to the 85 KDa polypeptide cross-linked to the wild-type avian estrogen response element. Lane 2 shows a faint band corresponding to polypeptide cross-linked to the mutant oligonucleotide lacking the GCG at the centre of the wild-type palindrome. NHP1 binds only weakly to this oligonucleotide (Hughes et al, 1989). Size markers are given in KDa. UV cross-linking of the partially purified NHP1 to the wild-type and mutant ERE in the absence of nuclease treatment is shown in lanes 3 and 4. Lanes 5 and 6 show the same experiment carried out in the presence of nucleases.

G Bibliography

Chodosh, L.A., Carthew R. W. and Sharp, P.A. A single polypeptide possesses the binding and transcription activities of the Adenovirus major late transcription factor. Molecular and Cellular Biol. 6 (1986) 4723–4733.

Feavers, I.M., McEwan, I.J., Liang, H. An estradiol dependent protein from chicken liver binds single-stranded DNA and RNA. J. Biol Chem. 264 (1989) 9114–9117.

Hames, B. D. and Rickwood, D. Gel electrophoresis of proteins: a practical approach. Eds B.D. Hames and D. Rickwood. IRL PRESS, Oxford/Washington DC (1981).

Hillel, Z. and Wu, C.W. Photochemical cross-linking studies on the interaction of *Escherichia coli* RNA polymerase with T7 DNA. Biochemistry 17 (1978) 2954–2961.

Hughes, M.J., Liang, H., Jiricny, J. and Jost, J.P. Purification and characterization of a protein from HeLa cells that binds with high affinity to the estrogen response element. Biochemistry 28 (1989) 9137–9142.

Jiricny, J., Hughes, M., Corman, N. and Rudkin, B. A human 200 KDa protein binds selectively to DNA fragments containing GT mismatches. Proc. Natl. Acad. Sci. USA 85 (1988) 8860–8864.

Lin, S.Y. and Riggs. Photochemical attachment of lac repressor to bromodeoxyuridine substituted lac operator by ultraviolet radiation. Proc. Natl. Acad. Sci. USA 71 (1974) 947–951.

Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227 (1970) 680–685.

Saluz, H.P. and Jost J.P. A laboratory guide for *in vivo* studies of DNA methylation and protein DNA interactions. Birkhäuser Verlag Basel-Boston-Berlin (1990).

Smith, K.C. Dose-dependent decrease in extractability of DNA from bacteria following irradiation with ultraviolet light or with visible light plus dye. Biochem. Biophys. Res. Commun. 8 (1962) 157–163.

Smith, K.D. and Aplin, R.T. A mixed photoproduct of uracil and cysteine (5-S-Cysteine-6-hydrouracil). A possible model for the *in vivo* crosslinking of deoxyribonucleic acid and protein by UV light. Biochemistry 5 (1966) 2125–2130.

Smith, K.C. and Meun, H.C. Kinetics of the photochemical addition of 5-S-cysteine to polynucleotide and nucleic acids. Biochemistry 7 (1968) 1033–1037.

Smith, K.C. Photochemical addition of amino acids to ^{14}C uracil. Biochem. Biophys. Res. Commun. 34 (1969) 354–357.

Smith, K.C. A mixed photoproduct of thymine and cysteine: 5-S-cysteine, 6-hydrothymine. Biochem. Biophys. Res. Commun. 39 (1970) 1011–1016.

Smith, K.C. and Hanawalt, P.C. Molecular photobiology inactivation and recovery. pp. 57–84 Academic Press, New York/London 1969.

VIII Interaction of Proteins with Partially Depurinated and Depyrimidinated Oligonucleotides (Missing-Contact Probing of Protein-DNA Interactions)

Haimin Liang, André Pawlak and Jean-Pierre Jost

Contents

A Introduction

The procedure first reported by Majors (Majors, 1976, cited by Brunelle and Schleif, 1987) and developed by Brunelle and Schleif (1987) involves the binding of a defined protein to a partially depurinated or depyrimidinated end-labelled oligonucleotide (see Fig. 1). From the mixture of modified DNA oligonucleotides, the protein only binds to those where the bases required for the specific amino acid base contact are intact. As seen in Fig. 1, bases 3 and 4 are important for binding of the protein, whereas bases 1, 2 and 5 may be removed without affecting formation of the protein-DNA complex. This situation is reflected in the final PAGE analysis on a sequencing gel. However, meaningful interpretation of the data from such an experiment assumes that the absence of a base causes no significant structural alteration of the DNA. The essential steps involved in the procedure are shown in the flow diagram in Fig. 2. separation of the protein-DNA complex by gel-shift assay, the DNA is extracted from the gel and subjected to β-elimination with piperidine. The final reaction products are further separated on a polyacrylamide sequencing gel, which is subsequently autoradiographed on an X-ray film.

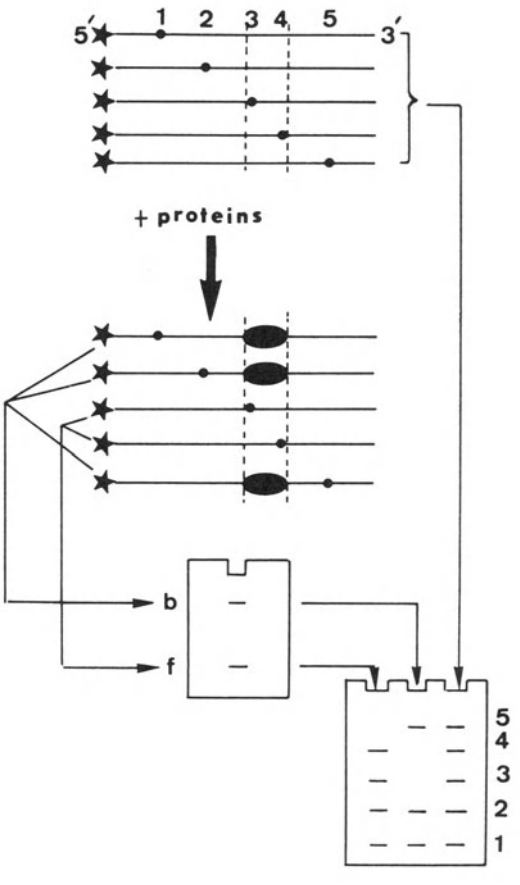


Fig. 1: The principle of missing-contact probing of DNA-protein interactions: Partially modified (black dots) end-labelled oligonucleotides reacted with a given, specific protein. Protein-DNA complex (B) is separated from the non-reacted free DNA (f) by means of a gel-shift assay. After extraction from the gel, the DNA is cleaved at the missing bases with piperidine and the fragments are separated by means of a polyacrylamide sequencing gel.

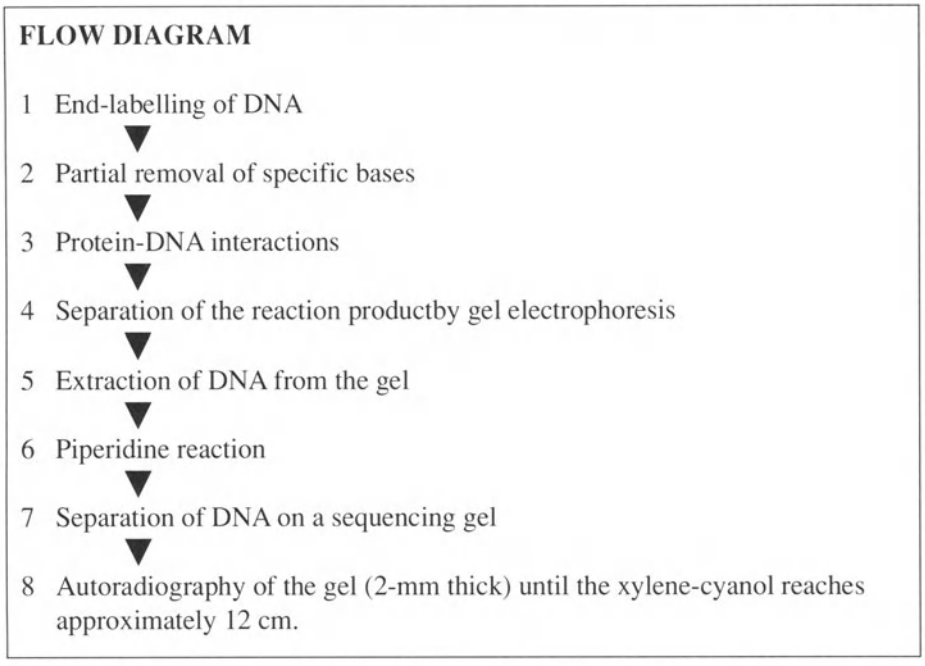


Fig. 2: Flow diagram of the missing-contact probing of DNA-protein interactions.

B Materials and Methods

1 Preparation of the End-Labelled Oligonucleotide

a) *Solutions and Enzymes*

- T4 Polynucleotide kinase, 5 units per µl
- [γ-^{32}P]ATP, specific activity 3000 Ci/mmole
- 10× Kinase buffer: 0.5 M Tris HCl pH 7.6, 0.1 M $MgCl_2$, 0.05 M dithiothreitol, 1 mM spermidine, 1 mM EDTA
- Sequencing tracking dye: 94% formamide, 10 mM EDTA, 0,05% xylene-cyanol, 0.05% bromophenol blue
- 10× TBE: 890 mM Tris, 890 mM boric acid, 20 mM EDTA (pH not adjusted)
- 0.5 M Ammonium acetate, 0.1 mM EDTA (pH not adjusted)
- 3 M sodium acetate, 1 mM EDTA pH 5
- Ultrafree-MC-Millipore filter, 0.45 µm, diameter 10 mm

b) *Step-by-Step Procedure*

Throughout all experiments use silicone-treated Eppendorf tubes.

> To an Eppendorf tube add 0.1–0.5 µg of unphosphorylated oligonucleotide, 1.5 µl of 10× kinase buffer, 50 µCi of [γ-^{32}P]ATP, and water to a final volume of 15 µl. Mix and add 1 µl (5 units) of T4 polynucleotide kinase.

> Mix and incubate 30 to 45 min at 37°C.

> Stop the reaction by adding 25 µl of sequencing dye, heat for 5 min at 90°C, and then chill in ice. Separate the labelled oligonucleotide on a preheated 20-cm-long, 8% polyacrylamide, urea sequencing gel. Use 1× TBE as buffer and run the gel at 55 mA, constant current, (the gel is 2-mm-thick) until the xylene-cyanol reaches approximately 7 cm.

> Remove one plate from the gel, put radioactive markers on a small piece of filter paper and place them on the gel for orientation.

> Place Saran wrap on top of the gel and expose it to an X-ray film for a few minutes.

> Cut out the radioactive band corresponding to the full-length oligonucleotide and cut the slice of gel into smaller pieces.

> Put the gel pieces into an Eppendorf tube, add 0.5 ml of 0.5 M ammonium acetate, 0.1 mM EDTA and shake the tube at room temperature for about 2 h with an Eppendorf mixer. Vortex the tube and take out the radioactive solution. Add another aliquot of 0.5 M ammonium acetate, 0.1 mM EDTA and shake for a further hour at room temperature.

> Pool the solutions, remove acrylamide debris by filtration through an Ultrafree-MC Millipore filter and precipitate DNA in the presence of 10 µg of carrier tRNA with three volumes of ethanol at –80°C for 10 min.

> Centrifuge DNA at 15000 rpm in a SS-34 Sorvall rotor for 10 min. Dissolve precipitate into 100 µl of 0.3 M sodium acetate, 1 mM EDTA and precipitate again with three volumes of ethanol as indicated above.

> Dry the DNA in a SpeedVac concentrator and dissolve in a small volume of sterile water.

> To the labelled DNA add a slight excess of the complementary unlabelled oligonucleotide.

> Heat at 80°C for a few minutes and allow to cool down slowly to room temperature. The duplex DNA is ready for the selective removal of bases.

Alternative Procedure for the Extraction of DNA From the Polyacrylamide Gel

> Cut out the acrylamide slice containing the labelled DNA.

> Crush it by forcing it through a 1- or 2.5-ml syringe without a needle.

> Add four volumes/weight of 0.5 M ammonium acetate, 1 mM EDTA and heat 1 h at 65°C.

> Do one phenol extraction and centrifuge to sediment the gel pieces in the phenol phase.

> Remove residual phenol by a chloroform extraction.

> Optional: pass the water phase through an Ultrafree-MC-Millipore filter.

> Add three volumes of ethanol. Quick chill at –80°C for 10 min.

> Centrifuge for 10 min at 15000 rpm in a SS-34 Sorvall rotor.

> Wash pellet by centrifugation with cold 90% ethanol.

> Dry the pellet in the SpeedVac concentrator.

2 Partial Depurination and Depyrimidination of DNA

This procedure works well for oligonucleotides of any size, down to a lowest limit of approximately 15 base pairs. Table 1 gives a summary of the reaction that we describe below.

a) *Solutions and Chemicals*
- Sonicated *E. coli* DNA (purified)
- DMS buffer: sodium cacodylate 50 mM pH 8, 1 mM EDTA
- Dimethylsulphate (DMS) (Any DMS used for Maxam & Gilbert DNA-sequencing reactions is suitable.)
- DMS stop buffer: 1.5 M sodium acetate pH 7, 1 M mercaptoethanol
- 3 M Sodium acetate pH 7, 1 mM EDTA

- TE buffer: 10 mM Tris HCl pH 8, 1 mM EDTA
- 2 M NaCl
- Formic acid, 98–100%, 'pro analysis' grade
- HZ (hydrazine) stop buffer: 0.3 M sodium acetate pH 7.5, 1 mM EDTA
- Hydrazine (HZ) (any HZ used for Maxam & Gilbert DNA-sequencing reactions is suitable)
- Piperidine

Note:
Dimethylsulphate and hydrazine should be handled only in a well ventilated fume hood.

Table 1 Summary of the base-specific chemical reactions according to Maxam and Gilbert (1980).

REACTION	DEPURINATION DEPYRIMIDINATION	BASE DISPLACEMENT	STRAND SCISSION
depurination of guanine	dimethylsulfate	heat to 90° at pH 7	piperidine
depurination of guanine + adenine	formic acid	formic acid	piperidine
depyrimidination of thymine + cytosine	hydrazine	n-butanol	piperidine

b) *Step-by-Step Procedures for:*

Depurination at Guanine Residues

> Take 30 µg of sonicated, purified *E. coli* DNA and 2–5 × 10^6 cpm of end-labelled DNA in 5 µl water. Add 180 µl DMS buffer.

> Chill on ice for 10 min.

> Add 20 µl of 2% dimethylsulpfate prepared in DMS buffer. A 2% solution of DMS is at the limit of solubility in water.

> Incubate for 5 min at 20°C.

> Stop the reaction with 50 µl of DMS stop buffer.

> Add 750 µl of cold ethanol, mix well.

> Quick chill at –80°C for 10 min.

> Centrifuge 10 min (4°C) at 15000 rpm in a SS-34 Sorvall rotor.

> Remove the supernatant and dissolve the pellet in 250 µl of sodium acetate pH 7.0, 1 mM EDTA.

> Precipitate with three volumes of cold ethanol as described above.

> Rinse the DNA pellet with 70% cold ethanol by centrifugation and dry in a SpeedVac concentrator.

> Dissolve the pellet in 25 µl of 10 mM Tris-HCl pH 8, 1 mM EDTA.

> Add 5 µl of 2 M NaCl.

> Heat the sample for 10 min at 90°C (to remove the modified guanosine without breaking the phosphodiester bond).

> Let the sample cool slowly to room temperature (for annealing of the modified oligonucleotides).

> Precipitate the DNA with ethanol as described above and centrifuge for 10 min at 15000 rpm in a SS-34 Sorval rotor.

> Rinse the pellet with 90% cold ethanol by centrifugation and dry the sample in a SpeedVac.

> Dissolve the sample in a small volume of water.

> The sample is ready for the binding test.

Depurination of the Adenine and Guanine Residues

> Dissolve 30 µg of purified *E. coli* DNA and 2–5 × 10^6 cpm of end-labelled DNA in 10 µl of water, and chill on ice.

> Add 24 µl of formic acid and incubate for 2.5 min at 20°C.

> Stop reaction with 200 µl of ice cold HZ stop buffer.

> Precipitate the DNA with three volumes of ethanol.

> Quick chill at –80°C for 10 min and centrifuge at 15000 rpm for 10 min in a SS-34 Sorvall rotor.

> Dissolve the pellet in 250 µl of 0.3 M sodium acetate, 1 mM EDTA, pH 7 and precipitate with ethanol as indicated above.

> Wash the DNA pellet with 70% ethanol by centrifugation.

> Dry the sample in a SpeedVac concentrator.

> Dissolve the sample in a small volume of water.

> The sample is ready for the binding test.

Depyrimidination of the Thymine and Cytosine Residues

> Dissolve 30 µg of purified, sonicated *E. coli* DNA with 2–5 $\times 10^6$ cpm of end-labelled oligonucleotide in 25 µl of water.

> At room temperature, add 15 µl of hydrazine and incubate for 30 min at 20°C.

> Add 1 ml of n-butanol and mix vigorously by vortexing for 10–15 s.

> Centrifuge 5 min at room temperature in a microfuge.

> Dissolve the DNA pellet in 250 µl of 0.3 M sodium acetate, 1 mM EDTA, pH 7.

> Add three volumes of ethanol. Quick chill at –80°C for 10 min and centrifuge for 10 min at 15000 rpm in a SS-34 Sorvall rotor.

> Repeat the above ethanol precipitation.

> Wash the DNA pellet with 70% cold ethanol by centrifugation.

> Dry the pellet in a SpeedVac concentrator and dissolve the DNA in a small volume of water.

> Sample is ready for the binding test.

Important Remark

The reaction times given for depurination and depyrimidination of DNA were optimal under our laboratory conditions. The reaction time must be adjusted according to the quality of the reagents. Take aliquots of the depurinated or depyriminated, end-labelled oligonucleotides and subject them to the piperidine reaction. Separate the reaction product on a polyacrylamide sequencing gel, loading about 10000 cpm per lane. After overnight exposure to a X-ray film, the sequence ladder should be clearly visible with the unreacted DNA at the top (see the example in Fig. 3).

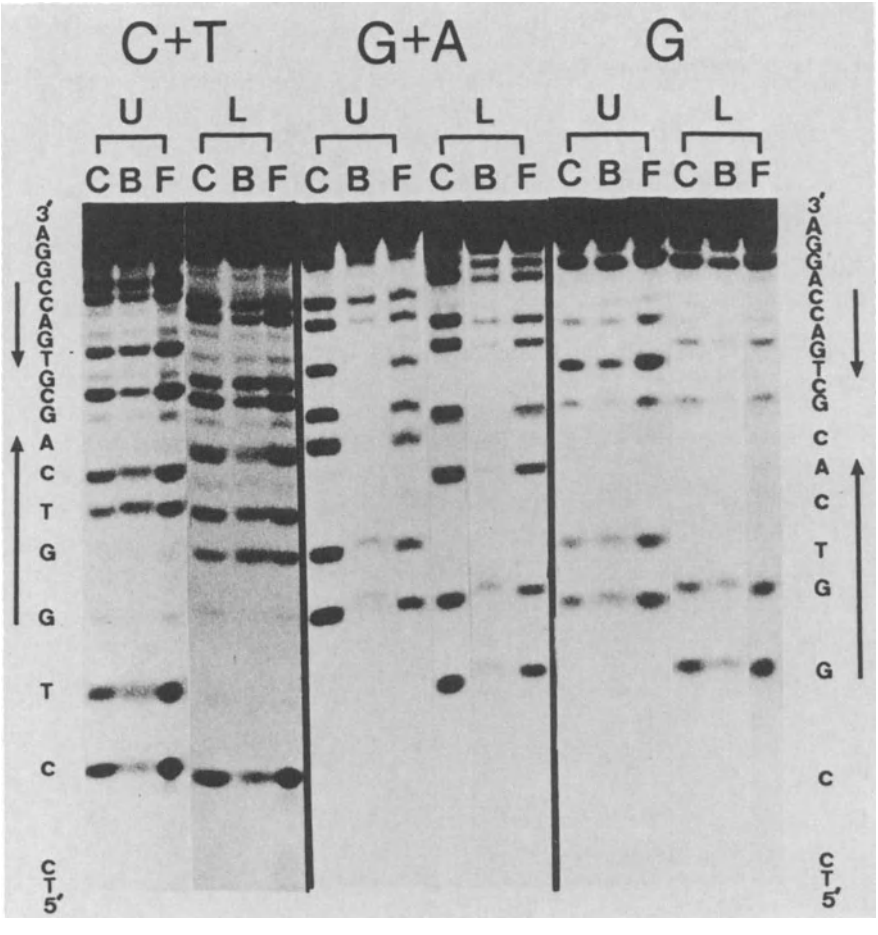


Fig. 3: An example of missing-contact probing of chicken NHP1 with avian estrogen response element (ERE): The results of C+T, G+A and G reactions are shown for the upper (U) and the lower (L) strand of the ERE. The bound DNA (B) eluted from the NHP1-ERE complex can be compared with control DNA (C) and the pretreated, free DNA (F). Reprinted with permission from Biochemistry 28 (1989) 9137–9142. Copyright 1990 American Chemical Society.

3 Protein-DNA Interaction

- Determine the amount of radioactivity of labelled, modified DNA per µl.
- In appropriate binding buffer, use $1–5 \times 10^5$ cpm of labelled oligonucleotide, in 10–20 µl of incubation mixture.
- The amount of appropriate protein and non-specific, competing DNA, either *E. coli* DNA or poly (dI-dC), must be determined in preliminary experiments. Keep in mind that your labelled DNA already contains a total of 30 µg *E. coli* DNA. Calculate how much carrier you already have per 10^5 cpm.
- The composition of the incubation buffer must be optimised for your own protein, and should contain, if possible, approximately 10% glycerol (for loading of the gels!). At the end of the incubation, add 2–4 µl of 0.05% bromophenol blue dye in 10% glycerol as a marker.
- The incubation time and temperature should be tested for each protein.

4 Separation of the Reaction Product by Gel Shift Assay

a) *Buffers and Chemicals*

- 10× TBE: 890 mM Tris HCl, 890 mM boric acid, 2 mM EDTA, pH not adjusted
- 10× TAE: 100 mM Tris pH 8, 30 mM sodium acetate, 10 mM EDTA. Low-melting-temperature agarose, Bio Rad

b) Step-by-Step Procedures for:

Mini Gels of 1% Low-Melting-Temperature Agarose

> Prepare a solution of 1% low-melting-temperature agarose in 1× TBE or 1× TAE or 0.25× TBE buffers.

> Let the solution cool to approximately 45°C.

> Pipette 23–25 ml of the solution onto a 10 × 7 cm glass plate fitted with the appropriate slot former.

> When the gel has set, load the sample and run for 40 min at 100 volts with 1× TBE or 1× TAE or 0.25× TBE buffer.

Vertical Slab Gel (1% Low-Melting-Temperature Agarose)

> We generally use glass plates of 22 × 18 cm with 0.2-cm-thick plastic or teflon spacers.

> Prepare 1% low-melting-temperature agarose in 1× TAE

buffer. Let it cool to approximately 45°C, pour the gel, place the comb and let the gel solidify.

> After loading the gel, run it in 1× TAE buffer with 30–40 mA (constant current) at room temperature. Change the buffer at least once.

> Run the gel until the bromophenol blue dye has migrated to approximately 10 cm from the top of the gel.

> At the end of the electrophoresis, remove the upper glass plate.

> Place radioactive markers onto the gel for orientation.

> Cover the gel with Saran wrap and expose it on a X-ray film for a few hours, or overnight at 4°C if necessary.

Vertical Slab Gel (4% Polyacrylamide Gel)

Polyacrylamide gels at 4% with different cross-linking can also be used for the separation of protein-DNA complexes from free DNA. We routinely separate protein-DNA complexes (oligonucleotides between 12 and 50 bp) on a 4% polyacrylamide gel with a acrylamide/bisacrylamide ratio of 29 : 1. For larger DNA fragments of up to 500 bp, we use a 4% polyacrylamide gel with a acrylamide/bisacrylamide ratio of up to 80 : 1. In all cases we use 1× TAE buffer. As for agarose gels, electrophoresis is carried out with 30–40 mA (constant current) at room temperature and at least one buffer change. In special cases gels may be run at 4°C.

5 Recovery of Free and Bound DNA from the Low-Melting-Temperature Agarose and from Acrylamide Gels

a) *Buffer and Chemicals*

- Redistilled phenol containing 0.1% hydroxyquinoline, saturated with 1 M Tris HCl pH 8
- 3 M Sodium acetate pH 5
- 0.5 M Ammonium acetate, 1 mM EDTA (pH not adjusted)

b) Step-by-Step Procedure for:

Agarose Gels

> After autoradiography cut out the bands corresponding to the specific protein-DNA complex and the free DNA.

> Add four volumes/weight (or more) of 20 mM Tris HCl pH 8, 1 mM EDTA to the slice in an Eppendorf centrifuge tube or a Corex centrifuge tube.

> Heat to 65–70°C for 10 min.

> Add an equal volume of phenol and extract the agarose by vortexing at room temperature.

> Separate the phases by centrifugation in a microfuge.

> Put the aqueous phase plus interphase in a further Eppendorf tube.

> Repeat the phenol extraction at least four times.

> Extract the residual phenol with chloroform, and centrifuge.

> Remove the aqueous phase without the residual interphase.

> Add 0.1 volumes of 3 M sodium acetate pH 5 and approximately 15 µg of carrier tRNA.

> Precipitate with three volumes of cold ethanol at –80°C for 10 min.

> Centrifuge at 15000 rpm (4°C) for 10 min in a SS-34 Sorvall rotor.

> Wash the DNA pellet with cold 90% ethanol by centrifugation.

> Dry the pellet in a SpeedVac concentrator.

It is advisable to use a hand monitor to follow the recovery of the labelled DNA during the whole extraction procedure.

Polyacrylamide Gels

For this electro-elution procedure, the DNA should not be smaller than 20 nucleotides.

> Cut out the appropriate gel slice with a sterile scalpel blade.

> Secure one end of a lenth of pretreated dialysis tubing with

a dialysis clip. Place the rectangular gel piece inside the dialysis tubing.

> Add 200–300 µl of electrophoresis buffer (in this case 1× TAE).

> Remove air bubbles and close the tubing with a second clip.

> Place in a minigel electrophoresis tank with the longest side of the gel nearest the cathode.

> Add an appropriate volume of 1× TAE buffer.

> Run the electrophoresis for 30 min at 40 mA then reverse the current for 20 s to remove any DNA stuck to the dialysis tubing.

> Remove the buffer from the dialysis tubing and ethanol precipitate by adding 0.1 volumes of 3 M sodium acetate and 1 µl of Dextran T40 (10 mg/ml).

> Add 2.5 volumes of cold ethanol, place at –70°C for 1 h and centrifuge at 15000 rpm in a SS-34 Sorvall rotor for 30 min at 4°C.

6 Piperidine Reaction

The recovered free and bound DNA and an aliquot of the original modified DNA is subjected to β-elimination by means of piperidine treatment.

a) Step-by-Step Procedure

> Dissolve the pellet in 100 µl of 1 M piperidine (add 100 µl of piperidine to 900 µl of water.

> Incubate at 90–95°C for 30 min.

> Freeze the sample in dry ice and lyophilise under high vacuum.

> Dissolve the pellet in 100 µl of water, freeze in dry ice and lyophilise again.

> Dissolve the pellet in 100 µl of 50% ethanol/water and evaporate in a SpeedVac concentrator.

> Repeat the previous step at least twice and dissolve the pellet in 5 µl of sequencing dye.

> Measure the radioactivity (Cerenkov) of each tube and adjust the aliquots to the same radioactivity.

7 Separation of the Reaction Products on Sequencing Gel and Autoradiography

Conventional, denaturing, sequencing gels serve our purpose. If the labelled DNA fragments to be analysed are between 15 and 35 bp long, use a 40-cm-long, 20% polyacrylamide gel containing 6 M urea. Load 8000–10000 cpm per lane for an overnight exposure on X-ray film. On a 20% polyacrylamide gel with an acrylamide/bisacrylamide ratio of 29 : 1, it will be possible to read the sequence between nucleotide 2 and 40 when the bromophenol blue migrates 15 cm from the top of the gel. After electrophoresis the gel is either directly exposed to X- ray film or dried and then exposed. Drying the gel is recommended when using low counts or 35 S-labelled nucleotides.

Fixing and Drying the Gel for Autoradiography

- Take off the upper glass plate. Cover the gel with Saran wrap and squeeze out any trapped air bubbles.
- Mark the piece of gel to be fixed directly on the Saran wrap. Cut out the piece of gel with the Saran wrap and place it in a shallow, plastic dish containing a mixture of 10% methanol, 10% acetic acid and 80% water for gels of up to 15% polyacrylamide, or a mixture of 3% glycerol w/v, 40% methanol, 10% acetic acid, 47% water for gels of 15% polyacrylamide and above.
- Remove the Saran wrap incubate the gel by shaking slowly at room temperature. Treat for 30 min gels below 15% polyacrylamide, and at least 3 h for gels above 15% polyacrylamide.
- Remove the solution.
- Place a piece of Whatman 17 paper of the appropriate size onto the gel and turn the plastic dish upside down whilst holding the gel with the other hand.
- Cover the gel with Saran wrap, cut it to the appropriate size and squeeze out any trapped air bubbles.
- Lay a piece of Whatman 3 MM paper on the porous gel support of the slab dryer and place the gel assembly on top of the paper.
- Apply vacuum and cover with the sealing gasket.
- The gel will be dry after 1 to 2 h under vacuum at 80°C.
- Before exposing the dried gel to X-ray film, renew the Saran wrap. (This is essential if you wish to obtain a clean background!).

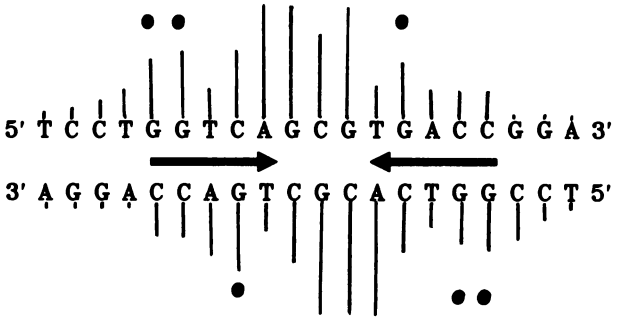


Fig. 4: Summary of the contact points of NHP1 with the ERE. The diagram summarises (trend analysis) the results shown in Fig. 3. The lengths of the vertical bars indicate the importance of the corresponding base for the binding of NHP1. Reprinted with permission from Biochemistry 28 (1989) 9137–9142. Copyright 1990 American Chemical Society.

C An Example

Fig. 3 shows an example of missing-contact probing of the non-histone protein 1 (NHP1) that binds specifically to the avian estrogen response element (ERE) (Hughes et al., 1989). The results may be interpreted either by scanning the autoradiograph of the gel or by trend analysis as shown in Fig. 4.

D The Most Common Problems and Their Solutions

- *One sequence lane has very weak signals throughout when compared with the corresponding controls*
  This could be due to different amounts of counts loaded onto the gel. Make sure that you pipette the same amount of radioactivity into each slot. A second cause could be insufficient depurination or depyrimidination of the DNA. In this case although the same number counts may have been pipetted onto the gel the majority will stay on the top of the sequence. To correct this fault, change the chemicals (DMS, hydrazine, formic acid) or increase the time of reaction.
- *Smear in the sequence lanes*
  This could be due to traces of piperidine or excess of salt in the preparation. In the first case, use a high vacuum to remove the piperidine, and in the second case,

wash the DNA pellet carefully with ethanol both before drying and following solubilisation in the sequence dye.

- *No band is present upon gel shift separation of the reaction product*
  This could be due to over-reaction of the labelled DNA during depurination or depyrimidination. Test this by treating a small aliquot of modified DNA with piperidine, followed by electrophoresis on a sequencing gel. This will tell you whether the DNA over-or under-reacted with DMS, hydrazine or formic acid. In the ideal case, you should still have some unmodified oligonucleotides on the top of the sequence, and the sequence should have a nice, even spread of either A+G or C+T on the sequence ladder (see Fig. 3).

E Bibliography

Brunelle, A. and Schleif, R.F., Missing contact probing of DNA-protein interactions. Proc. Natl. Acad. Sci. USA 84 (1987) 6673–6676.

Hughes, M.J., Liang, H., Jiricny, J. and Jost, J.P., Purification and characterization of a protein from HeLa cells that binds with high affinity to the estrogen response element GGTCAGCGTGACC. Biochemistry 28 (1989) 9137–9142.

Maxam A.M. and Gilbert, W., Sequencing end labeled DNA with base-specific chemical cleavage. Method Enzymology 65 (1980) 499–560.

IX The Study of Protein-DNA Contacts by Ethylation Interference

Vichien Rimphanitchayakit and Nigel D.F. Grindley#*

Contents

A Introduction

The ethylation interference procedure initially developed by Gilbert and his colleagues (Majors, 1977; Siebenlist and Gilbert, 1980) provides a valuable tool for the analysis of DNA-protein interactions. The procedure identifies those backbone phosphates that are in close contact with a DNA-binding protein by determining which ethylated phosphates inhibit binding of the protein. The chief requirement for this procedure is a satisfactory mehtod for separating DNA-protein complexes from unbound DNA. If the protein of interest is an enzyme that acts upon DNA (for example, a restriction enzyme, a recombinase, or even a polymerase), the procedure

can be used to identify phosphate ethylations that interfer with activity, provided the product can be separated from the substrate (Falvey and Grindley, 1987). A major advantage of the ethylation interference procedure is that it is independent of the DNA sequence of the binding site; all phosphates in the vicinity of the binding site are analysed equally, giving an unbiased picture of the interaction, clearly showing, for example, localised surfaces of interaction.

The reagent used for ethylation of backbone phosphates is ethylnitrosourea (ENU), a known chemical mutagen and carcinogen. The experimental procedure, in outline, is as follows: A DNA substrate (usually an end-labelled DNA fragment) is partially ethylated by treatment with ENU. Alkylated DNA is incubated with the protein of interest, and then either complexes are separated from unbound DNA, or enzymatic products are separated from the substrate. The distribution of ethylated phosphates in the two fractions (complexes and free DNA, or substrate and products) are then analysed by alkali cleavage of the ethylated positions, followed by electrophoresis on a standard DNA-sequencing gel and autoradiography. Ethylated phosphates that interfere with the binding or activity of a protein show up as more intense bands in the free (or substrate) DNA lane, and as blank spaces in the complexed (or product) DNA lane in sequencing-gel analysis (Fig. 1). Interference can be interpreted as either steric hindrance or disruption of ionic interactions between DNA and protein. Although the majority of ENU-mediated ethylations of DNA (about 70%) occur on the backbone phosphates, most of the rest are on the purines, with guanine being alkylated about five times more frequently than adenine (Sun and Singer, 1975). These alkylations of the bases can also interfere with protein interactions, either directly or as the result of formation of an apurinic site through loss of the labile ethylated base. Signals resulting from base ethylation do not usually cause difficulty in interpretation of the data from an ENU ethylation experiment, but one should keep an eye open for the occasional oddity (see the section on Interpretation of the Data).

B Methods

1 Labelling and Purification of the DNA Fragment

We recommend the use of a short (less than 200 bp) DNA fragment with the DNA-binding site(s) at least 25 bp from the labelled end. A short fragment facilitates the subsequent separation of complexes from free DNA, increasing the resolution between bound and unbound species and reducing the time required for electrophoresis. Placing the binding site more than 25 bp from the labelled end results in co-migration (over the region of interest) of the two alternative products of alkali

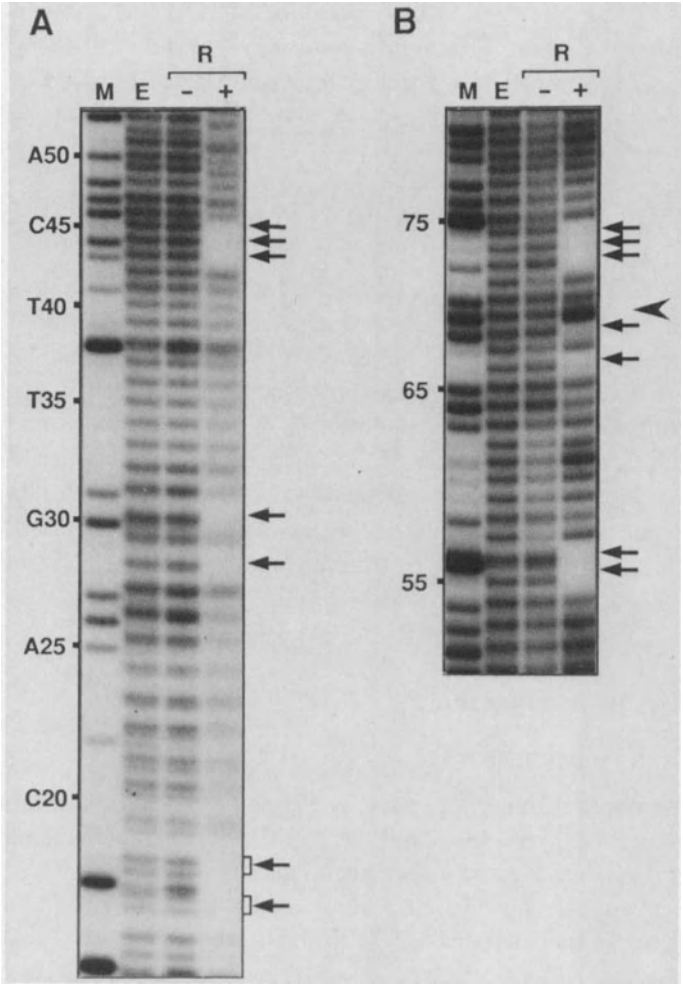


Fig. 1: Analysis of the interaction between γ δ resolvase and DNA by phosphate ethylation interference:
A. DNA containing binding site II.
B. DNA containing site III
Lanes are as follows:
M: marker lane with G > A sequencing reaction (Maxam and Gilbert, 1980); E: ENU-ethylated DNA (starting material); R+ and R–: resolvase – complexed and unbound DNA from the binding reaction. Numbers indicate the distance from the labelled end. Arrows indicate the positions of ethylated phosphates that strongly interfere with resolvase binding. In A, note that fragments less than about 20 bases in length migrate as doublets, showing separation of the two products of alkali cleavage at the positions of ethylated phosphates. At the positions of purines (especially G), a third band with slightly faster mobility is also visible – a result of ethylation of the base. The arrow head in B indicates an intense band in the R+ lane that results from enhanced binding of resolvase to a DNA fragment with a particular purine modification (an apurinic site or alkylated purine).

cleavage at ethylated phosphates (see Fig. 1). One end of the DNA fragment should be either 5'- or 3'-end-labelled with ^{32}P to high specific activity, and purified by native polyacrylamide gel electrophoresis. Detailed procedures are described in Chapters II, IV, VIII, and X.

2 Ethylnitrosourea Ethylation of the Labelled DNA Fragment

ENU is highly toxic due to mutagenic and carcinogenic effects. All procedures involving manipulation of ENU or the ENU-alcohol solution should be performed in a well-ventilated fume hood. A face mask, protective gloves and coat should be worn. Furthermore, any contaminated materials should be discarded safely in a separate container for further detoxification and disposal. We have found for some unknown reason that a portion of the radioactive DNA cannot be ethanol precipitated after ENU treatment, and up to 30–40% of radioactivity remains attached to the plastic microcentrifuge tube. We recommend that excess labelled DNA be used, although it is possible that the use of silanised microcentrifuge tubes would alleviate this problem.

Step-by-Step Procedure

> In a screw-capped, plastic tube add solid ENU (obtainable from Pfaltz and Bauer, Inc. or Sigma Chemical Co.) to 0.3 ml of 95% ethanol at 50°C until saturated. The final volume should be approximately 1 ml. This solution may be stored at –20°C and re-used several more times, provided the solution is saturated at 50°C before each use.

> Resuspend approximately 0.5–2 µg of end-labelled DNA fragment in 100 µl of 50 mM sodium cacodylate, pH 8.0 in a 1.5 ml microcentrifuge tube.

> Pre-warm the DNA solution to 50°C and add 100 µl of saturated ENU solution. Mix immediately.

> Incubate the mixture at 50°C for 45–60 min.

> Precipitate the DNA by adding 20 µl of 3 M sodium acetate and 550 µl of cold 95% ethanol. Freeze on dry ice (10 min) and spin in a microcentrifuge for 5 min at room temperature.

> Resuspend the DNA in 250 µl of 0.3 M sodium acetate and re-precipitate the DNA with three volumes of 95% ethanol. This step is repeated three times to remove excess ENU.

> Rinse the final DNA pellet with 70% ethanol and vacuum dry the DNA.

> Resuspend in a small volume of 10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA (TE).

3 Formation and Separation of DNA-Protein Complexes

Precise conditions for forming complexes between a DNA-binding protein and the modified DNA will depend largely on the particular protein under investigation. We recommend using native polyacrylamide gel electrophoresis, i.e. a gel-shift procedure (Fried and Crothers, 1981; Garner and Revzin, 1981), for separating protein-DNA complexes from free DNA, although the nitrocellulose-filter-binding procedure has also been used with satisfactory results (Siebenlist and Gilbert, 1980). Many protein-DNA interactions result in more than a single discrete complex – a large advantage of the gel-shift procedure is that different complexes are readily separable and can then be analysed individually. We aim to use sufficient protein to complex about 50% of the DNA in a binding reaction. With this amount of complex, useful data is obtained both from the complexed and from the unbound DNA, since the latter will exhibit bands of significantly increased intensity at the positions of inhibitory ethylated phosphates. However, one should avoid driving too high a proportion of the DNA into complexes, since shadow bands will start to appear at the positions of inhibitory ethylations, reducing the inhibitory signal. Our experience suggests that DNA from the ethylation procedure may have less affinity than unmodified DNA for a binding protein. In addition, the concentration of binding site(s) in the binding reaction (approximately micromolar) is likely to be considerably higher than the dissociation constant of the protein-DNA interaction, requiring stoichiometric quantities of protein rather than a specific concentration. It is necessary, therefore, to determine empirically the amount of protein required to bind about 50% of the DNA.

A typical experiment would be performed as follows. (Various alternative separation conditions are described in Chapters III, IV, VIII, X, and XV.)

Step-by-Step Procedure

> Use a portion of the modified DNA to determine the amount of protein required to drive about 50% of the DNA into complexes.

> Set up a 20-µl binding reaction with labelled, ethylated DNA in a suitable buffer (e.g. 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) with non-specific

carrier DNA [e.g. 2 µg of sonicated calf thymus DNA or poly (dI-dC)], 2 µg of BSA, and an appropriate amount of DNA-binding protein.

> Incubate the mixture for 10–30 min at 37°C (or as required for the chosen protein) and for 5 min at room temperature.

> During the incubation, pre-run the native polyacrylamide gel at 7.5 V/cm for 15 min. A 1.5-mm-thick gel is preferred.

> At the end of the incubation period, place 4 µl of dye solution (sample buffer containing 5% Ficoll and 1% xylene cyanol FF) onto the side of the tube above the reaction mixture.

> Carefully tap down the dye solution and gently load the mixture while the gel is running, using intramedic polyethylene tubing connected to a Hamilton microsyringe via an 18 gauge syringe needle. Be careful not to touch the running buffer.

> Carry out electrophoresis at 7.5 V/cm at room temperature (conditions are for a gel in 100 mM Tris-borate, pH 8.3, 1 mM EDTA), until the dye marker (xylene cyanol FF) has migrated the appropriate distance. For a 5% gel, xylene cyanol FF co-migrates with DNA fragments of about 260 bp. The gel may be operated at 4°C, which stabilises the complex but extends the running period.

> Detach one plate and cover the gel with plastic wrap. Mark reference points with fluorescent ink.

> Identify the locations of DNA bands by autoradiography using Kodak XAR or a similar fast X-ray film. Ideally, the complexed DNA band should be visible within 30 min exposure in order to have sufficient labelled DNA for subsequent analysis.

> Cut out the desired bands (complexes and free DNA); minimising the size of the gel slice at this stage generally results in better electrophoretic behaviour of the DNA in the analysis to follow.

> Elute the DNA using either electro-elution or crush-and-soak methods (see Chapters VIII and X).

> Phenol extract the DNA solution and ethanol precipitate the DNA.

> Rinse the DNA pellet with 70% ethanol and vacuum dry.

> Resuspend the DNA pellet in 10 mM sodium phosphate pH 7, containing 1 mM EDTA. Keep the volume low, especially if the DNA fraction has low radioactivity. The sample may be stored at –20°C but should be analysed as soon as possible.

4 Analysis of Ethylated DNA

The samples to be analysed are: the complexed and free DNA from the binding reaction (both recovered from the polyacrylamide gel) and a sample of the ethylated DNA that was used in the binding reaction. In addition, a marker lane is required – we have generally found that the labelled DNA fragment processed through the Maxam-Gilbert A+G (acid depurination) sequencing reaction (see Chapter VIII) provides a very satisfactory marker, particularly since these bands will mark the positions of bands resulting from ethylated purines, a minor product of ENU-mediated DNA ethylation.

Step-by-Step Procedure

> To 10 µl DNA (in 10 mM sodium phosphate, pH 7.0, 1 mM EDTA) add 1 µl 1.5 M NaOH for all DNA samples. Mix, quick spin and incubate at 90°C for 30 min in well-sealed containers to prevent evaporation of contents.

> Spin down the contents of the tube.

> Determine the radioactivity in each tube (by counting the entire tube by Cerenkov radiation or by removing a small sample).

> Adjust the volume with TE so that the radioactivity per µl is the same for each sample.

> To 2 µl of each sample add 2 µl of sequencing dye solution (formamide containing 10 mM EDTA, 0.1% xylene cyanol FF and 0.1% bromophenol blue). Mix, quick spin and boil for 1–2 min.

> Analyse the samples by polyacrylamide gel electrophoresis (acrylamide:bis-acrylamide, 20:1) in 50 mM Tris-borate pH 8.3, 1 mM EDTA and 8 M Urea. An 8–10% gel is recommended for best resolution.

> The remainder of the alkali cleavage samples may be stored at –20°C but a white precipitate may form. Be sure to mix the stored samples thoroughly before re-using.

C Interpretation of the Data

The results of two typical phosphate ethylation interference experiments analysing the interaction between the γδ resolvase and two of its binding sites in *res* (Rimphanitchayakit et al., 1989) are shown in Figs. 1A and B. In both R+ lanes (in which the resolvase-DNA complexes are analysed) blank spaces are clearly visible in the "phosphate ladder", indicating that ethylation of these particular phosphates inhibits the formation of resolvase-DNA complexes. In Fig. 1B, bands in the R- lane (unbound DNA recovered from the DNA-protein binding reaction) adjacent to the blank spaces are significantly enhanced relative to the neighbouring bands that result from non-inhibitory phosphate ethylations.

Precise identification of the inhibitory phosphate ethylations is obtained by reference to the marker lane. As can be seen from Figs. 1A and B, bands in lanes M (G > A DNA-sequencing reaction; Maxam and Gilbert, 1980) run in between the bands that form the phosphate ladder. Cleavage of ethylphosphotriesters by heating in alkali occurs at two alternative positions, either 5' or 3' to the modified phosphate. For 5' end-labelled DNA, the *labelled* products are terminated with 3' hydroxyls or 3' ethylphosphates; for 3'end-labelled DNA the *labelled* products are terminated with 5' hydroxyls or 5' ethylphosphates. In both cases the hydroxyl-terminated fragment runs a little slower than the same fragment terminated with an ethylphosphate. Electrophoresis resolution of these two products is clearly seen with fragments of less than 20 bases (see Fig. 1B). However, for fragments greater than

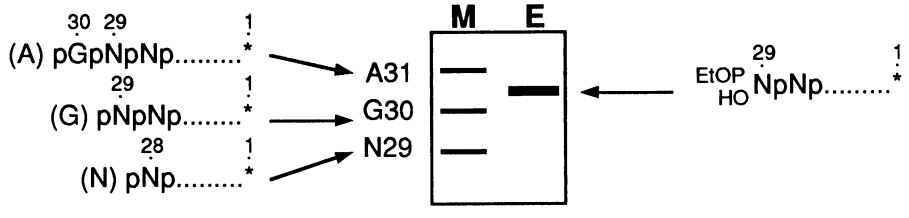


Fig. 2: Diagram showing interpretation of data: A portion of a gel (lanes M and E) is drawn showing the composition of the ends of the cleaved fragments. The bases in parentheses are those at which the sequence-specific modification occurred (lane M), and are absent in the cleaved DNA product.

25 bases, the two products co-migrate. The relation between marker bands and the phosphate ladder is illustrated schematically in Fig. 2. The "phosphate" band that migrates between the marker bands "G30" and "A31" (i.e. the bands that result from base-specific cleavage at G30 and A31, respectively) results from ethylation of the phosphate between G30 and base 29 (where nucleotide 1 is the labelled end). This phosphate is the 5' phosphate of G30 if the fragment is 5' labelled but the 3' phosphate of G30 if the fragment is 3' labelled.

As mentioned in the Introduction, ENU also ethylates purines (particularly guanines), albeit with reduced efficiency. Examples of bands resulting from purine alkylation are clearly visible in Figs. 1A and B; these, like the bands of the marker lane (M), are terminated with a phosphate and run in between the bands resulting from ethylation of phosphates. Because of their distinguishable mobilities, the cleavage products of phosphate and base alkylations are not usually confusable. However, Fig. 1B does illustrate one artifact resulting from base alkylation: the lane analysing the protein-DNA complex contains a single band of increased intensity (shown by the arrowhead). Careful inspection indicates that this band migrates just ahead of the ethylphosphate band, precisely co-migrating with a band in the marker lane (M). Our interpretation is that alkylation of this A, or its loss to form an apurinic site, enhances resolvase binding, selectively enriching the complexed DNA with this relatively rare, modified form.

D Problem Solving

It is not uncommon to find that the final DNA samples run poorly on the sequencing gel and that the bands appear rather smeared. If the DNA samples have sufficient radioactivity, this problem is most easily solved by simply diluting the samples and re-running the sequencing gel. Dilute samples (with correspondingly longer exposure times) generally give cleaner results than concentrated samples and short exposure. Starting out the procedure with enough radio-labelled DNA of high specific activity alleviates most problems, since the final DNA samples can be substantially diluted, thus reducing the concentration of salts, unpolymerised acrylamide etc. which cause poor electrophoretic behaviour. Vershon et al. (1987) have obtained good results by dissolving the DNA from eluted complexes (and unbound DNA) in 100 µl of 10 mM Tris-HCl pH 7.5, 1 mM EDTA and then adding 100 µl of 0.3 M KOH, 10 mM EDTA for the cleavage step. Following the 90°C incubation for 30 min., the solution was neutralised (100 µl of 0.3 M HCl) and the DNA was ethanol precipitated before analysis on a DNA-sequencing gel.

E Bibliography

Falvey, E. and Grindley, N.D.F. Contacts between γ δ resolvase and the γ δ res site. EMBO J. 6 (1987) 815–821.

Fried, M. and Crothers, D.M. Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. Nucl. Acids Res. 9 (1981) 6505–6525.

Garner, M.M. and Revzin, A. A gel electrophoresis method for quantifying the binding of proteins of specific DNA regions: application to components of the *Esherichia coli* lactose operon regulatory system. Nucl. Acids Res. 9 (1981) 3047–3060.

Majors, J. PhD Thesis (1977) Harvard University Cambridge, MA.

Maxam, A. and Gilbert, W. Sequencing end-labelled DNA with base-specific chemical cleavages. Meth. Enzymol. 65 (1980) 499–560.

Rimphanitchayakit, V., Hatfull, G.F., and Grindley, N.D.F. The 43 residue DNA binding domain of γδ resolvase binds adjacent major and minor grooves of DNA. Nucl. Acids Res. 17 (1989) 1035–1050.

Siebenlist, U. and Gilbert, W. Contacts between *Escherichia coli* RNA polymerase and an early promoter of phage T 7. Proc. Natl. Acad. Sci., USA 77 (1980) 122–126.

Sun, L. and Singer, B. The specificity of different classes of ethylating agents towards various sites of HeLa cell DNA *in vitro* and *in vivo*. Biochemistry 4 (1975) 1795–1802.

Vershon, A.K., Liao, S.M., McClure, W.R., and Sauer T. Bacteriophage P_{22} Mnt Repressor, DNA binding and effects on transcription *in vitro*. J. Mol. Biol. 195 (1987) 311–322.

X The Study of Protein-DNA Interactions by Methylation Interference

Josef Jiricny and Natalie Corman

Contents

I Introduction

The DNA-binding proteins identified to date can be subdivided into several classes. The first group contains proteins that bind to DNA mainly by electronic interactions in a non-specific way, such as histones. These are positively charged polypeptides, which interact with the negatively charged phosphates of the sugar-phosphate backbone of the nucleic acid. Such an interaction takes place mainly on the surface of the molecule, leaving the major groove largely free and thus available for complexing with other proteins. The second class includes structure-specific polypeptides, such as mismatch-binding proteins. These recognise specific distortions in the DNA structure and bind irrespective of their sequence context. The third and largest class comprises sequence-specific DNA binding proteins.

How proteins recognise a particular DNA sequence or structure is currently the subject of intensive studies. Knowing the points of contact between the DNA and

the protein can provide us with invaluable information about the nature of the interaction, as well as about the protein itself. One way to establish the contact points is to use the so-called methylation-interference method developed by Siebenlist and Gilbert (1980). Labelled DNA fragments containing the protein-binding site are first partially modified with dimethyl sulphate (DMS) and then incubated with the protein in the presence of non-specific, competitor DNA. The DMS treatment generates a mixture of oligonucleotide duplexes where each chain is methylated only once on average, either on guanine or on adenine. The method is based on the fact that the protein will bind only to those duplexes in which the base modification does not interfere with site recognition. Separation of the protein/DNA mixture is carried out by non-denaturing polyacrylamide gel electrophoresis (band-shift assay). The bound, "shifted" DNA will contain only that subpopulation of the modified duplexes where the methylation of the base did not interfere with the protein binding. The free, "unshifted" band will contain all the modified species. Extraction and cleavage of the DNA, followed by denaturing polyacrylamide gel electrophoresis, will show missing bands in the "shifted" pattern at the sites important for protein binding (see Fig. 1). The other figures serve to demonstrate what the differences in the methylation interference pattern can tell us about the nature of the DNA-binding protein. In Fig. 2, only a very small footprint is apparent for a G/T mismatch-binding protein. This is consistent with the protein being able to bind to a structural distortion within any sequence context. Fig. 3 shows an example of a sequence-specific DNA binding protein; the transcription factor Sp1 produces a footprint which indicates that all G residues within the consensus recognition sequence are required for binding.

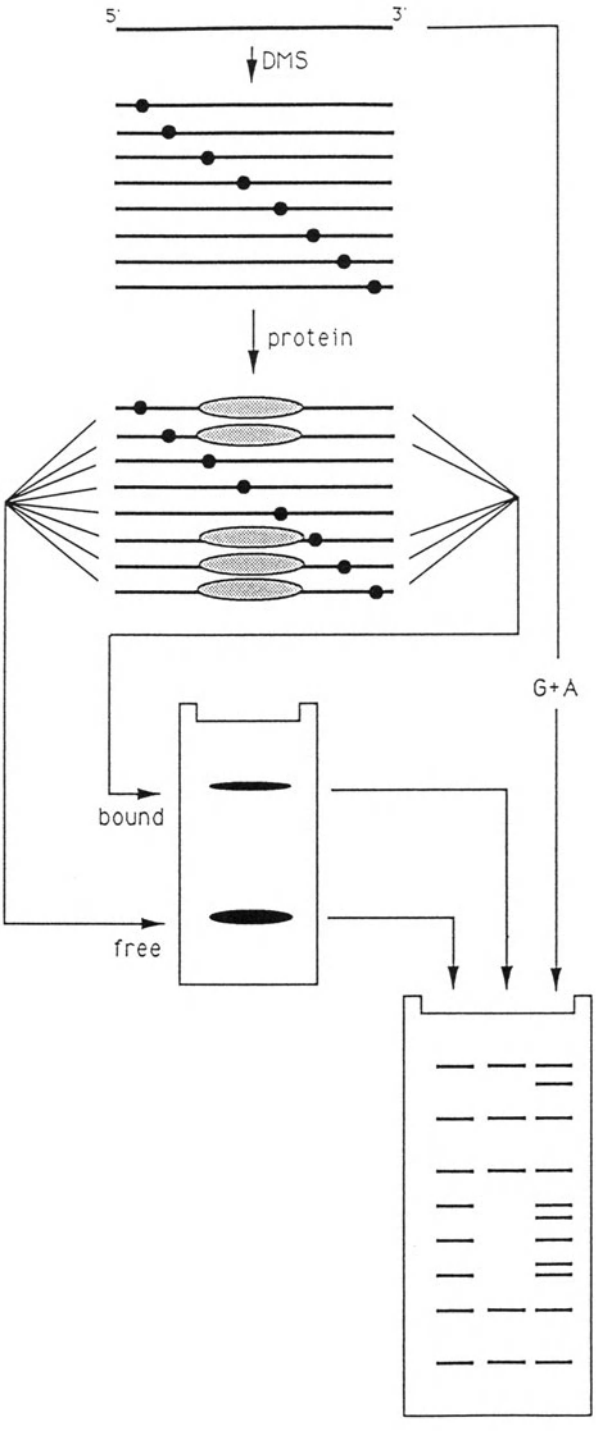


Fig. 1: Schematic representation of a methylation interference experiment.

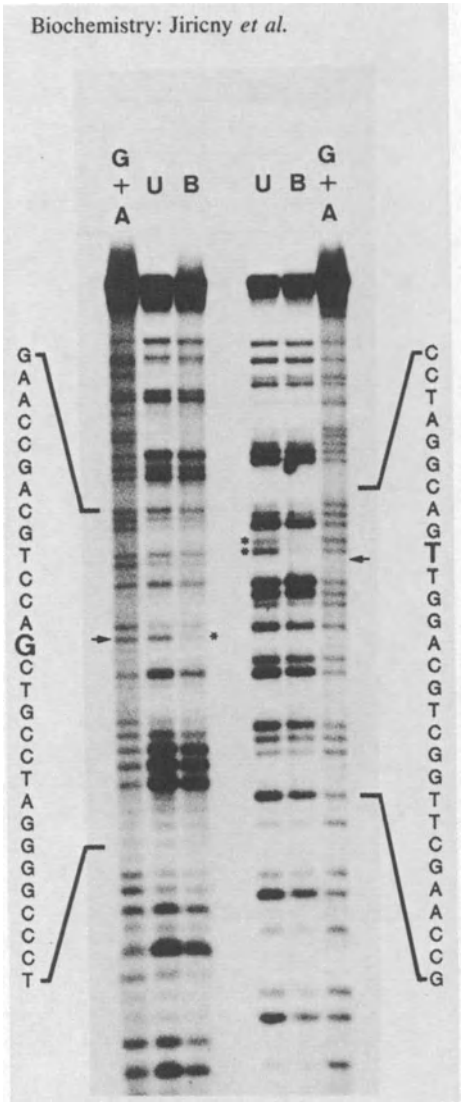


Fig. 2: Methylation interference footprint of a G/T mismatch-binding protein present in extracts from HeLa cells: A synthetic 90-mer substrate was incubated with a Mono-Q FPLC fraction containing the DNA-binding activity. Parts of the sequences are shown along the gel, with the mismatched nucleotides shown in larger letters. The position of the mismatch is denoted by arrows. Asterisks mark the positions of the missing G- or A-specific bands. Lanes: G+A, purine-specific cleavage pattern of the unmodified 90-mer with formic acid (Zoller and Smith, 1983); U, G>A-specific cleavage pattern of the methylated 90-mer unbound in the band-shift assay; B, G>A-specific cleavage pattern of the methylated 90-mer recovered from the retarded band in the band-shift assay. The oligonucleotide duplexes were labelled with ^{32}P on the 5'-end of either the G-containing strand (the three left lanes) or the T-containing strand (the three right lanes).

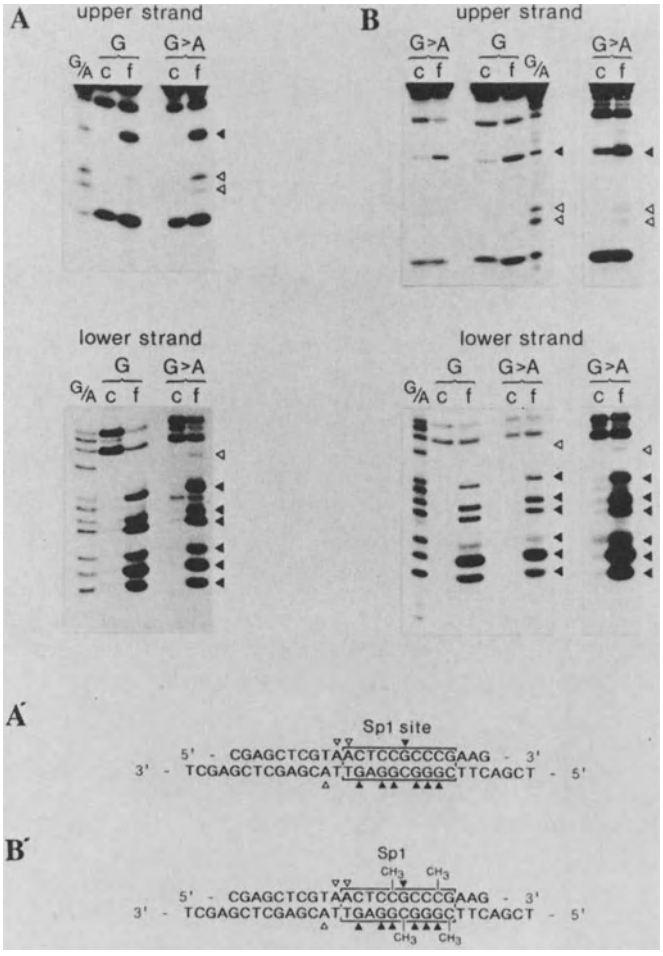


Fig. 3: Chemical modification experiments identify purine bases interacting with Sp1 factor: (*A*) Both strands of the SpNOMET oligonucleotide carrying an unmethylated Sp1 recognition sequence were end-labelled and analysed separately by a chemical modification assay ("methylation interference", Siebenlist and Gilbert, 1980) using HeLa cell nuclear extracts. (Lanes **c**) Complexed form of the DNA from the lower or upper strand; (lanes **f**) free, non-shifted DNA from the same binding reaction. Two different cleavage reactions were performed: one that cuts only at modified guanine residues (**G**) and another that shows up the guanines and the adenines (**G>A**). A purine-specific (**G/A**) sequence reaction indicates the position of the corresponding bases in the oligonucleotides. The lanes to the far right in *B* depict longer exposures of the GA reaction. (*B*) The same procedure as in *A* was performed with the oligonucleotide containing the CpG methylated Sp1 recognition sequence. (*A'*) and (*B'*) summarise the interference data on the unmethylated and methylated Sp1 recognition sequence. The triangles indicate the purines whose modification interferes with protein-DNA interaction; solid and open triangles show strong and weaker interference, respectively. The Sp1 binding site is bracketed. Reprinted with permission from Genes and Dev. 2 (1988) 1127–1135, copyright 1990 Cold Spring Harbor Laboratory, N.Y. 11724, USA.

B Materials and Methods

1 Material and Chemicals

- Oligonucleotide solutions, 5 pmole/µl in water
- Oligonucleotide solutions, 0.5 pmole/µl in water
- 10× Linker kinase buffer (700 mM Tris.HCl pH 7.6, 100 mM $MgCl_2$, 50 mM DTT)
- T4 Polynucleotide kinase (Boehringer Mannheim, 9u/µl)
- [γ-^{32}P]ATP (Amersham, 3000 Ci/mmole)
- 10× Annealing buffer (100 mM Tris.HCl pH 8, 100 mM $MgCl_2$)
- t-RNA solution: 10 mg/ml in sterile water
- 0.3 M NaOAc, pH 6
- Ethanol
- Dimethyl sulphate (Merck)
- DMS buffer (50 mM sodium cacodylate pH 8, 1 mM EDTA)
- DMS stop solution (1.5 M NaOAc pH 6, 1 M mercaptoethanol, 0.1 mg/ml carriert-RNA)
- 0.3 M NaOAc pH 6, 0.1 mM EDTA, 25 µg/ml carriert-RNA
- Poly d(I-C) (Pharmacia, 1 mg/ml)
- 50% Glycerol
- 10% Polyethylene glycol 20000 (PEG, Merck)
- Sequencing dye (90% formamide, 0.01% bromophenol blue, 0.01% xylene cyanol FF)
- Urea dye (5 M urea, 0.01% bromophenol blue, 0.01% xylene cyanol FF)
- 6% Non-denaturing polyacrylamide gel: 6 ml 30% acrylamide stock (acrylamide:bis-acrylamide ratio 29:1), 3 ml 10× TEA buffer (400 mM Tris.acetate pH 7.5, 10 mM EDTA), water to 30 ml; then add 300 µl 10% ammonium persulphate (APS), 30 µl TEMED
- 8% Denaturing polyacrylamide gel: 13.3 ml 30% acrylamide stock (acrylamide:bis-acrylamide ratio 29:1), 5 ml 10× TBE buffer (890 mM Tris.borate pH 8.3, 20 mM EDTA), 21 g urea; make up to 50 ml with water, then add 300 µl 10% APS, 30 µl TEMED.
- Bovine serum albumin (BSA, 10 mg/ml)
- Gel-elution buffer (500 mM ammonium acetate pH 6, 5 mM EDTA)
- Phosphate buffer (10 mM sodium phosphate pH 7, 1 mM EDTA)
- 1 M NaOH
- 0.1 M Acetic acid

2 Step-by-Step Procedures

a) *Oligonucleotide Labelling*

> Mix:
Oligonucleotide solution 2 µl (10 pmol)
10× linker kinase buffer 2 µl
[γ-^{32}P]ATP 3 µl (10 pmol)
T4 polynucleotide kinase 1 µl (9 u)

> Add 12 µl of water

> Incubate the total reaction mixture of 20 µl in a 1.5-ml Eppendorf tube for 30 min at 37°C.

> Heat to inactivate the enzyme (5 min at 80°C).

> Cool in ice and spin shortly in a microfuge.

b) *Annealing of Oligonucleotides*

> Mix:

kinased oligonucleotide	4 µl (2 pmole)
unlabelled complementary strand	5 µl (2.5 pmole)
10× annealing buffer	1 µl
	10 µl total

> Heat in a water bath at 80°C for 5 min, then allow to cool to room temperature for 10–20 min.

> Spin shortly in a microfuge

> Add:

NaOAc	240 µl
t-RNA	1 µl
cold ethanol	750 µl

> Chill in dry ice for 15 min, spin in a microfuge for 15 min, decant supernatant, spin again (shortly) and withdraw remaining traces of liquid with a drawn-out capillary.

> Dissolve pellet in NaOAc, 250 µl

> Add cold ethanol, 750 µl

> Chill in dry ice for 15 min, spin in a microfuge for 15 min and remove liquid as above.

> Wash the pellet with 70% ethanol, 500 µl.

> Spin in a microfuge (1 min) and withdraw liquid as above.

> Dry the pellet in a SpeedVac (5 min) and resuspend in 5 µl sterile water.

This experiment should be carried out in duplicate, once with the upper strand labelled and once with the lower strand labelled.

c) *DMS Modification*

> Add together:

methylated duplex (see above)	5 µl
t-RNA	2 µl
DMS buffer	200 µl

> Mix and add DMS, 1 µl.

> Mix and incubate at 37°C for 1.5 min.

> Add DMS stop buffer, 50 µl.

> Add ethanol, 750 µl.

> Chill in dry ice for 15 min and spin for 15 min in a microfuge.

> Withdraw liquid as described above and repeat ethanol precipitation twice more, followed by one wash with 70% ethanol.

> Resuspend the dried pellet in 20 µl sterile water.

d) *Band-Shift Experiments*

The procedure described below was used in the methylation interference study of the G/T mismatch-binding protein. Although generally applicable, the binding buffer may have to be altered to give optimal complex formation. The amount of non-specific competitor DNA, such as the poly d(I-C) used in this case, also needs to be adjusted. As a rule of thumb, we use 1 µg poly d(I-C) per 10 µg total protein. Make the stock as follows:

methylated duplex	1 µl (0.1 pmole)
poly d(I-C) (1 mg/ml)	5 µl
10× binding buffer	50 µl
water	85 µl
50% glycerol	100 µl
10% PEG	100 µl
BSA (10 mg/ml)	20 µl

> Dispense 20-µl aliquots into separate Eppendorf tubes and add 5 µl of the protein fraction (FPLC Mono-Q in this case).

> Incubate at room temperature for 30 min and then load onto a 6% non-denaturing polyacrylamide gel (20 × 20 cm, 1-mm-thick, 0.5-cm slot width).

> Run the gel at 10 V/cm until the bromophenol blue marker dye (loaded in an adjacent slot) has migrated approximately 7 cm.

> Cover the gel with Saran wrap and expose to a Kodak XAR-5 film at 4°C for approximately 1 h.

> Develop the film, superimpose on the gel and cut out the shifted and the unshifted bands (as thin as possible).

> Put the gel slices into a 15-ml polypropylene, Falcon tube and add a minimum volume of gel-elution buffer to cover the gel (approximately 0.5 ml).

> Shake overnight at 37°C.

> Remove buffer, extract once with 0.5 volumes of phenol/chloroform and twice with an equal volume of n-butanol.

> Add 20 µg t-RNA and three volumes of cold ethanol, and chill in dry ice for 15 min.

> Spin for 15 min in a microfuge.

> Remove liquid and repeat the ethanol precipitation and 70% ethanol wash as described above.

> Dry the pellet in a SpeedVac (5 min).

e) *Cleavage of the Methylated DNA*

DMS modifies guanine and adenine residues in an approximate ratio of 9:1. The modified oligonucleotides can be cleaved at all methylated guanines with hot piperidine or at methylated guanines and adenines with hot alkali. Although both methods work well, the latter provides more information, as well as giving cleaner gels (at least in our hands). Both cleavage methods are described below; for more detailed information, consult the work of Zoller and Smith (1983). This also applies to the reference G+A reaction (see the Figures).

Piperidine Cleavage (G only)

> Add 1 M piperidine (100 µl) and heat at 90°C for 30 min.

> Lyophilise

> Add 10 µl water, making sure to get all the counts into solution; transfer to a fresh tube and lyophilise (repeat at least twice more).

> Add sequencing dye (2–3 µl), heat (90°C, 3 min), chill in ice, spin shortly in a microfuge and load onto an 8% denaturing polyacrylamide gel (20 × 40 cm, 0.3-mm-thick, 0.5-cm slot width).

> Run fairly hot (25 v/cm) until the bromophenol blue marker dye has migrated approximately two-thirds of the gel length.

> Fix and dry the gel and expose at –80°C to a Kodak XAR-5 film overnight.

NaOH Cleavage (G>A)

> Resuspend the pellet in 90 µl 10 mM sodium phosphate pH 7, 1 mM EDTA to get all counts into solution, then chill on ice and spin shortly in a microfuge.

> Add 1 M NaOH (10 µl), mix and heat at 90°C for 10 min.

> Chill on ice and add 0.1 M acetic acid (100 µl), 10 µg t-RNA (1 µl) and 600 µl cold ethanol.

> Chill on dry ice for 15 min and spin in a microfuge for 15 min.

> Wash the pellet with 70% ethanol, dry in a SpeedVac and resuspend in 2–3 µl urea loading dye.

> Heat (90°C, 3 min), chill on ice, spin shortly and load onto a gel as described above.

C Bibliography

Höller, M., Westin, G., Jiricny, J., and Schaffner, W. Sp1 transcription factor binds DNA and activates transcription even when the binding site is CpG methylated. Genes and Dev. 2 (1988) 1127–1135.

Jiricny, J., Hughes, M., Corman, N, and Rudkin, B.B. A human 200 KaDa protein binds selectively to DNA fragments containing G/T mismatches. Proc. Natl. Acad. Sci., USA 85 (1988) 8860–8864.

Siebenlist, U. and Gilbert, W. Contacts between *Escherichia coli* RNA polymerase and an early promoter of phage T7. Proc. Natl. Acad. Sci. USA, 77 (1980) 122–126.

Zoller, M. and Smith, M. Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. Methods in enzymology 100 (1983) 468–500.

XI Using the Chemistry of the Hydroxyl Radical to Determine Structural Details about DNA and Protein-DNA Complexes

Judith R. Levin, Amanda M. Burkhoff and Thomas D. Tullius

Contents

A Introduction

The hydroxyl radical can be used as a highly sensitive chemical probe to study the structure of DNA (Tullius, 1989) and DNA complexed with proteins (Tullius & Dombroski, 1986; Tullius et al., 1987) or drug molecules (Churchill et al., 1990). The hydroxyl radical cleaves the DNA strand by abstracting a hydrogen atom from a deoxyribose along the DNA backbone. For structural studies, the major advantage of using the hydroxyl radical over large endonucleases, such as DNase I, stems from the radical being very small and highly reactive. In contrast to DNase I, the hydroxyl radical cleaves the DNA backbone non-specifically and at each position along the DNA molecule. The hydroxyl radical is generated by allowing the EDTA complex of iron(II) to react with hydrogen peroxide: $[Fe(EDTA)]^{2-} + H_2O_2 \rightarrow [Fe(EDTA)]^{1-} + \cdot OH + OH^-$. In this reaction an electron from iron (II) EDTA serves to reduce and break the O–O bond in hydrogen peroxide, giving as products iron(III)EDTA, the hydroxide ion, and the neutral hydroxyl radical. Sodium ascorbate is present to reduce the iron(III) product to iron(II), thereby establishing a catalytic cycle and permitting low (micromolar) concentrations of iron(II)EDTA to be effective in cleaving DNA. A consequence of this scheme is that the concentrations of the three chemical species (iron(II)EDTA, hydrogen peroxide and sodium ascorbate) may be varied to optimise the generation of the hydroxyl radical under different solution conditions, e.g. to compensate for the presence of radical scavengers in the binding buffer of a protein-DNA complex. In this chapter, we will describe the basic protocol for hydroxyl radical cleavage of DNA, as well as protocols that allow one to measure the helical periodicity of DNA (Rhodes & Klug, 1980; Tullius & Dombroski, 1985; Hayes et al., 1990) to detect footprints (Tullius & Dombroski, 1986), and to collect missing-contact information (Hayes & Tullius, 1989) for protein-DNA complexes.

B Materials and DNA Preparation

1 Solutions

Cutting reagents

(A): 0.1 mM $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$/0.2 mM EDTA*
(prepared by mixing equal volumes of 0.2 mM $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ and 0.4 mM EDTA)

(B): 10 mM Sodium ascorbate*

(C): 0.3% H_2O_2*

Other solutions
0.2 M EDTA
50 mM Tris-HCl buffer, pH 8.0
0.3 M Sodium acetate
0.1 M Thiourea
100% Ethanol
70% Ethanol
0.5 mg/ml tRNA
TE: 10 mM Tris · HCl (pH 8.0), 1 mM EDTA
Formamide-dye mixture: 80% (v/v) deionised or recrystallised formamide, 50 mM Tris-borate (pH 8.3), 1 mM EDTA, 0.1% (w/v) xylene cyanole, 0.1% (w/v) bromophenol blue
10× TBE: per litre, use 109 g Trizma base, 55 g boric acid, 18.6 g Na_2EDTA

Solutions for preparing the calcium phosphate precipitate
50 mM $CaCl_2$ in 50 mM Tris · HCl (pH 8.0)
80 mM K_2HPO_4 in 50 mM Tris · HCl (pH 8.0)

Note:
All solutions are prepared with water purified by a Milli-Q system (Millipore) in order to remove contaminants, including all organics.

*These solutions should be prepared immediately before use.

2 DNA Preparation

- Prepare plasmid DNA using any standard lysis procedure, followed either by chromatography or CsCl/ethidium bromide gradient purifications. Avoid exposing the DNA to light when using the ethidium bromide method, since nicking of the DNA strands can occur, causing a significant amount of background cleavage. In every experiment using hydroxyl radical, a sample of untreated DNA should be run on the sequencing gel as a control. This sample should show no detectable DNA breakage.
- Use standard procedures, such as those used for preparation of DNA for Maxam-Gilbert sequencing (Maxam & Gilbert, 1980), to generate a fragment of double-stranded DNA uniquely end-labelled with radioactive phosphorus at one end of one strand (either a 5' or 3'end).
- Purify the labelled DNA fragment by electrophoresis on a native polyacrylamide gel. Isolate the labelled DNA fragment from the gel using any standard technique, such as crush and soak (Maxam & Gilbert, 1980) or electro-elution.
- Store the labelled DNA in TE at a final concentration of ~10 Kdpm/µl for use in

standard hydroxyl radical experiments (sections C-E below), or at ~100 Kdpm/µl for immediate use in missing-nucleoside experiments (section F).
- Using the labelled DNA fragment, perform a Maxam-Gilbert sequencing reaction (Maxam & Gilbert, 1980) for one of the DNA bases, for use as a set of size markers on the sequencing gel. We usually use the guanine-specific (dimethylsulphate) reaction.

C The Hydroxyl Radical Experiment

The suggested conditions for the hydroxyl radical reaction are designed so that at most one cleavage event occurs per DNA molecule. This criterion is satisfied for most of the DNA in the sample when greater than 70% of the sample remains full length after cleavage (see Section H, 1) (Brenowitz et al., 1986).

1 Step-by-Step Procedure

> a: In a 1.5-ml Eppendorf tube dilute ~100 Kdpm of end-labelled DNA with 50 mM Tris · HCl (pH 8.0) so that the final volume equals 70 µl.

> b: Place onto the inner wall of the Eppendorf tube a 10 µl drop of each of the cutting reagents A, B and C.

> c: To start the cutting reaction, let the three reagents combine and then mix with the DNA solution.

> d: After 1 min stop the cutting reaction by adding stop reagents: 0.1 M thiourea (10 µl), 0.2 M EDTA (32 µl), 5 mg/ml tRNA (1 ul) and 0.3 M sodium acetate (200 µl).

> e: Precipitate the DNA in the reaction by adding ~900 µl of 100% ethanol and spinning in a microfuge at 4°C for 20–30 min to pellet the DNA.

> f: Remove the supernatant with a pipette or an aspirator.

> g: Resuspend the pellet in 200 µl of 0.3 M sodium acetate.

> h: Precipitate the DNA by adding 500 µl of 100% ethanol and spinning in a microfuge at 4°C for 20–30 min.

> i: Wash the DNA pellet with 70% ethanol (~500 µl) and spin again for 5–10 min in the microfuge.

> j: Remove the supernatant and dry the DNA pellet in a SpeedVac concentrator (Savant). Air drying the pellet is also acceptable if a SpeedVac is unavailable.

> k: DNA pellets can be stored at –20°C until ready to run on a denaturing acrylamide gel (see section G for details).

D Cleaving DNA Bound to a Precipitate of Calcium Phosphate to Determine Helical Periodicity

> Mix equal volumes of 50 mM $CaCl_2$ and 80 mM K_2HPO_4 to generate a suspension of calcium phosphate microcrystals.

> Combine 40 µl of the calcium phosphate suspension, 1 µg of unlabelled carrier DNA, and labelled DNA (~100 Kcpm) to give a total volume of 70 µl.

> Let the DNA bind to the precipitate for at least 1 h at room temperature before starting the hydroxyl radical cleavage experiment.

> Cleave the bound DNA with the hydroxyl radical by the procedure described above in section C, Steps b-k.

E Protein-DNA Interactions

It is assumed in the following protocol that optimal conditions for binding the protein to its DNA-binding site (concentration of protein and DNA, buffer conditions, temperature, kinetics and non-specific DNA requirements) have previously been determined. This is often best achieved by determining the conditions that lead to a good footprint using DNase I.

1 Titrations for Determining Reaction Conditions

Sometimes the reagents and buffers used for protein-DNA systems reduce the rate of cleavage of DNA by hydroxyl radical (e.g. glycerol scavenges · OH). Therefore, it is necessary to determine empirically the reagent concentrations necessary for

optimal cutting of the DNA. The following variables are suggested for consideration:

Vary the Fe(II)EDTA concentration with a constant amount of DNA. This can be done without protein in the reaction, although protein storage buffer should be included.

Vary the amount of ascorbate present in the reaction mix, once a concentration of Fe(II)EDTA is determined. Since some proteins are sensitive to hydrogen peroxide, increasing its concentration is not advised.

Vary the amount of protein to optimise occupancy of the binding site.

2 Controls and Considerations

Add protein and protein storage buffer to the reaction mix so that a constant concentration of all storage buffer ingredients is maintained. Storage buffers for proteins often contain high concentrations of glycerol which is known to inhibit cutting by the hydroxyl radical. Therefore, one must keep the final concentration of glycerol in the reaction mix constant, even while varying the protein concentration. The binding of some proteins to DNA is adversely affected by one or more of the reagents used to generate the hydroxyl radical (Tullius et al., 1987; Vrana et al., 1988). To test for this possibility, it is advisable to perform a DNase I footprinting reaction in the presence and absence of the hydroxyl radical-generating reagents. If the degree of DNase I protection is diminished in the presence of the reagents, perform the DNase I experiment in the presence of each of the individual reagents, to determine those causing the problem. With this information in hand, it may be possible to adjust the reagent concentrations so as to achieve adequate cutting without significantly inhibiting binding of the protein to the DNA. The concentrations of each of the reagents (Fe(II)EDTA, hydrogen peroxide, and sodium ascorbate) may be varied over a wide range and still permit cleavage of DNA (Tullius et al., 1987; Vrana et al., 1988). The concentrations mentioned in this chapter should be considered as a starting point for optimisation of the conditions for each new protein-DNA complex.

3 A Step-by-Step Protocol for Footprinting with Hydroxyl Radical

> a: In a final volume of 70 µl of the appropriate binding buffer, combine 50–200 Kdpm of end-labelled DNA containing the binding site for the protein of interest, 0–0.5 µg of non-specific DNA, and the appropriate amount of protein.

> b: Incubate at the appropriate temperature and for an adequate time to allow binding of the protein to the DNA.

> c: Proceed with the hydroxyl radical reaction as described in steps b – g in section C.

> d: Extract protein with 200 µl of phenol/chloroform (1:1 mixture).

> e: Precipitate DNA from the aqueous phase by adding 500 µl of 100% ethanol and spinning for 20–30 min in a microfuge at 4°C.

> f: Wash the DNA pellet with 70% ethanol and dry in a SpeedVac.

F Missing-Nucleoside Experiment

The missing-nucleoside experiment (Hayes & Tullius, 1989) identifies gaps in the DNA molecule that interfere with binding to such a degree that the protein and DNA no longer migrate as a complex on a non-denaturing gel. End-labelled DNA is treated with hydroxyl radical prior to binding with protein in order to determine which nucleosides make essential contacts with protein.

1 Hydroxyl Radical Reaction

Follow the procedure in Section C, using enough DNA to allow for ~500 Kdpm/lane on the band-shift gel (see below). This hydroxyl radical-treated DNA will be referred to below as "gapped DNA".

Note: When preparing labelled DNA for this experiment, store at 100 Kdpm/ul.

2 Binding Protein to DNA, and Separation of Free DNA from the Protein-DNA Complex on a Native Polyacrylamide Gel

A mobility-shift gel is used to separate the bound and unbound populations of gapped DNA. At least four sample lanes will be loaded on this gel: 1) Gapped DNA with no protein (should run as a single band of free DNA); 2) One or more lanes of gapped DNA with added protein (e.g. varying protein concentration); 3) Ungapped DNA without protein; 4) Ungapped DNA with protein. Allow at least 500 Kdpm/lane for samples 1 and 2. Samples 3 and 4 serve as controls for potential effects of gapped DNA on protein binding and complex mobility. Since no bands will be excised from these control lanes, fewer dpm may be used (200 Kdpm is sufficient).

- Work out experimental conditions for optimising the separation of protein-bound from free DNA on a mobility-shift gel (Garner & Revzin, 1981; Fried & Crothers, 1981) (i.e. acrylamide concentration, ratio of acrylamide to bisacrylamide, contents of gel running buffer, temperature and duration of electrophoresis).
- Follow steps a and b of section E (3) to bind the protein to the DNA. Use 500 Kdpm per lane of gapped DNA, and 200 Kdpm per lane of untreated DNA. It is better to reduce the volume of the reaction mix described in section E (3) by half (i.e. a final volume of 35 µl rather then 70 µl), so that the sample will easily fit into one well on the mobility-shift gel.
- Add 0.2 volumes of 30% glycerol, mix gently, and load the sample onto the mobility-shift gel. Dye is not included in the loading buffer since it might disrupt the protein-DNA complex. If desired, tracking dyes can be loaded in lanes not containing experimental samples.
- After running the gel under the predetermined conditions, remove one glass plate of the assembly and cover the gel with Saran wrap.
- Expose the gel to film for 1 h, taking care to mark the gel and film to facilitate later alignment.
- Let the film dry and then align with the gel. Cut out bands corresponding to free DNA, unbound DNA and bound DNA.
- Elute the DNA from the gel slices. It is advisable to add 10 µg/ml tRNA to the elution buffer to act as carrier, since the amount of DNA here is much less than in a typical labelling gel.
- After precipitation of the DNA with ethanol and washing of the DNA pellet with 70% ethanol, dry the DNA samples and proceed with the sequencing gel (Section G).

G Gel Electrophoresis

1 Preparation of the Gel

- Siliconise the larger of the two glass plates with Sigmacoat (Sigma) to prevent the gel from sticking to both plates when transferring to paper (see Drying the gel below).
- Gel spacers can either be of a constant thickness (0.35 mm) or a wedge shape that varies from 0.25 mm at the top to 0.75 mm at the bottom.
- Prepare the mixture for a denaturing polyacrylamide gel containing 50% w/v urea in 1× TBE buffer. The percentage acrylamide used will depend on the distance of the binding site from the labelled end of the DNA.
- Pour the gel and allow to polymerise.
- Prerun the gel at constant power (60–65 W) until the gel temperature (monitored with a thermometer taped to one of the plates) is at least 45°C.

2 Preparation of the Sample and Running the Gel

- Redissolve DNA samples in 3 µl of formamide-dye mixture.
- Heat samples at 90–100°C for 3–5 min to denature the strands. One of the samples should be the products of a Maxam-Gilbert, guanine-specific sequencing reaction to serve as size markers for the gel.
- Place the DNA samples immediately on ice.
- Load samples onto the prewarmed polyacrylamide gel.
- Run the gel at constant power (60–65 W) for sufficient time to resolve the DNA region of interest.

3 Drying the Gel

- Remove the gel from the apparatus.
- Remove one glass plate from the gel assembly.
- Place a piece of Whatman 3MM paper on the gel and smooth down using gloved hands.
- Invert the assembly so that the gel and glass plate are on top of the paper. Carefully pull the paper away from the plate, peeling the gel along with it.
- Cover the gel with Saran wrap and dry with a standard gel drier.

H Densitometric Analysis of Hydroxyl Radical Patterns

1 Interpretation of Patterns

Naked DNA:

a) Expose the gel to X-ray film for ~10 min to evaluate the amount of cutting, by comparing the intensities of the strong, full-length bands in each of the experimental samples to the intensity of the band in the lane containing untreated DNA. As mentioned above, the intensities of the full-length bands in the experimental samples should be at least 70% of the intensity of the untreated DNA band, to ensure single-hit cleavage kinetics.

b) Expose the gel to X-ray film to reveal the hydroxyl radical cleavage pattern. Since every nucleotide in the DNA molecule gives a cleavage signal, and very little of the DNA is cleaved at all (<30%), longer exposures than are usual for DNase I footprints are often necessary. Variations in band intensities in the cleavage pattern may be subtle and uninterpretable by simple visual inspection. Densitometry is advised for detailed analysis of hydroxyl radical patterns.

Footprints:

a) Use a densitometer to scan the lanes in the autoradiograph in which DNA samples cleaved in the presence and absence of protein were run. The lane containing control DNA that was not treated with hydroxyl radical should be scanned if background subtraction is planned.

b) Compare scans of lanes that contained the protein-DNA complex with the scan of the lane that contained free DNA. If the footprint is not obvious, a subtraction plot may be necessary (Vrana et al., 1988). To do this, transfer the data points for each scan to a spreadsheet programe on a computer so that the data for bound and free DNA can be aligned. The data for the free-DNA sample is then subtracted from the data for the protein-DNA-complex sample, to correct for slight non-uniformity of DNA cleavage by the hydroxyl radical. This is necessary only in cases where the naked DNA has a particularly complex hydroxyl radical cleavage pattern, or where the footprint is weak.

Missing nucleoside experiment:

A low-intensity or missing band on the sequencing gel in the lane containing DNA that was bound to protein, or a high-intensity band in the lane containing unbound DNA, identifies a nucleoside that is important to formation of the protein-DNA complex. Conversely, uncontacted nucleosides within the binding site also yield a positive experimental signal, but with the opposite pattern of band intensity.

Thus the lanes that contain bound and unbound DNA, fractionated from the same sample, give complementary information. This is in contrast to protection (footprinting) methods, which give a negative result for bases not involved in protein contact.

a) Scan the lanes in the autoradiograph that correspond to the samples containing free DNA (from lane 1 of the mobility shift gel), unbound DNA, and bound DNA (both from lane 2 of the mobility-shift gel).

b) Bands that are reduced in intensity in the bound-DNA lane (compared to the free-DNA lane), and are enriched in the unbound-DNA lane, represent nucleosides that make contacts essential for formation of a stable protein-DNA complex.

I Bibliography

Brenowitz, M., Senear, D.F., Shea, M.A., and Ackers, G.K. Quantitative DNase footprint titration: A method for studying protein-DNA interactions. Meth. Enzymol. 130 (1986) 132–181.

Churchill, M.E.A., Hayes, J.J., and Tullius, T.D. Detection of drug binding to DNA by hydroxyl radical footprinting. Relationship of Distamycin binding sites to DNA structure and positioned nucleosomes on 5S RNA genes of Xenopus. Biochemistry 29 (1990) 6043–6050.

Fried, M., and Crothers, D.M. Equilibria and kinetics of *lac* repressor-operator interactions by polyacrylamide gel electrophoresis. Nucl. Acids. Res. 9 (1981) 6505–6525.

Garner, M.M., and Revzin, A. A new gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: Application to components of the *Escherichia coli* lactose operon regulatory region. Nucl. Acids Res. 9 (1981) 3047–3060.

Hayes, J.J., and Tullius, T.D. The missing nucleoside experiment: A new technique to study recognition of DNA by protein. Biochemistry 28 (1989) 9521–9527.

Hayes, J.J., Tullius, T.D., and Wolffe, A. The structure of DNA in a nucleosome. Proc. Natl. Acad. Sci. (USA) 87 (1990) 7405–7409.

Maxam, A.M., and Gilbert, W. Sequencing end-labeled DNA with base-specific chemical cleavages. Meth. Enzymol. 65 (1980) 499–560.

Rhodes, D., and Klug, A. Helical periodicity of DNA determined by digestion. Nature 286 (1980) 573–578.

Tullius, T.D. Structural studies of DNA through cleavage by the hydroxyl radical. Nucleic Acids and Molecular Biology 3 (1989) 1–12.

Tullius, T.D., and Dombroski, B.A. Iron(II) EDTA used to measure the helical twist along any DNA molecule. Science 230 (1985) 679–681.

Tullius, T.D., and Dombroski, B.A. Hydroxyl radical "footprinting": high-resolution information about DNA-protein contacts and application to lambda repressor and cro protein. Proc. Natl. Acad. Sci. (USA) 83 (1986) 5469–5473.

Tullius, T.D., Dombroski, B.A., Churchill, M.E.A., and Kam, L. Hydroxyl radical footprinting: A high-resolution method for mapping protein-DNA contacts. Meth. Enzymol. 155 (1987) 537–558.

Vrana, K.E., Churchill, M.E.A., Tullius, T.D., and Brown D.D. Mapping functional regions of transcription factor TFIIIA. Mol. Cell. Biol. 8 (1988) 1684–1696.

XII Detection of Unusual DNA Structures and DNA-Protein Interactions by Diethylpyrocarbonate Carbethoxylation

Miklos Toth

Contents

A Introduction

Diethylpyrocarbonate (DEPC), which carbethoxylates purines in the N-7 position, is a useful probe to detect altered or unusual DNA structures (Wells, 1988). DEPC reacts strongly with Z-DNA, particularly with nucleotides in the *syn* configuration. DEPC also reacts with purine/pyrimidine sequences, like (AG)n · (CT)n. Hyperreactivity is thought to be due to the different backbone conformations of (AG)n · (CT)n, related to either differences in base stacking or triple-stranded structure (H-DNA) of these sequences. Purine/pyrimidine stretches, like (G)n · (C)n or (A)n · (T)n can also be recognised by DEPC. Since DEPC can react with DNA structures, it can also be used as a footprinting reagent to detect DNA-protein interactions. Proteins which bind to DEPC-reactive DNA structures can prevent the chemical reaction between DEPC and DNA at the contact points, as demonstrated by Runkel and Nordheim (1986) with Z-DNA and anti-Z-DNA antibodies. The DNA structure and DNA-protein interactions can be determined by the DEPC chemical reaction not only in vitro but also in vivo (in situ) in the native chromatin structure. DEPC-hyperreactive and footprinting techniques may be particularly useful in studying DNA sequences involved in recombination, transcriptional regulation and replication.

B DEPC-reactive-site Mapping and Footprinting in vitro (Table 1, Steps 1–6)

For DNA structural analysis, a labelled DNA fragment is reacted directly with DEPC. In the footprinting method, the DNA is pre-incubated with proteins (cellular extracts, purified DNA-binding proteins, antibodies etc.) prior to reacting with DEPC. DNA is then cleaved by piperidine at the modified positions and fragments are separated on a sequencing gel. Strong bands at certain nucleotide positions represent DEPC-reactive residues, while missing bands in footprints indicate the presence of protein at those residues.

Step-by-Step Procedures:

1 End-Labelling of DNA

The DNA molecules can be radiolabelled enzymatically at the 3' or 5' termini (Sambrook et al., 1989; see also Chapter VIII). Since DNA fragments with labelled single ends are required, the Klenow fragment of *E. coli* DNA polymerase I is especially useful, because it can fill only recessed 3' termini of double-stranded DNA molecules. If both termini of the DNA molecules are labelled, the DNA fragment can be cleaved and the appropriate subfragment isolated. The 5' termini of DNA fragments can be labelled with bacteriophage T4 polynucleotide kinase.

2 Pre-incubation of DNA with Protein

In this reaction, proteins with sequence-specific DNA affinities are allowed to bind to the DNA. Extracts prepared from isolated nuclei (Dignam et al., 1983) or from whole cells (Manley et al., 1980, see also Appendix) can be used directly. These extracts can also serve as starting material for the purification of DNA-binding proteins. The pre-incubation reaction is carried out in a volume of 20 µl. The labelled probe (5000–10000 cpm/sample) is incubated with different amounts of protein in assay buffer. The amount of protein must be optimised, and extracts with high protein concentrations (10 µg/ml) are generally needed. The reaction mixture also contains poly(dI-dC), usually 1 µg per reaction. The DNA is incubated at 30°C for 10–20 min. The assay buffer is 10 mM Tris HCl pH 7.6, 5 mM $MgCl_2$, 100 mM KCl, 2 mM dithiothreitol (DTT), 50 µg/ml bovine serum albumin (BSA).

Note:
In a special application, Runkel and Nordheim (1986) used anti-Z-DNA monoclonal antibodies in the pre-incubation reaction (see Fig. 1). The pre-incubation step is omitted if DNA structure is analysed.

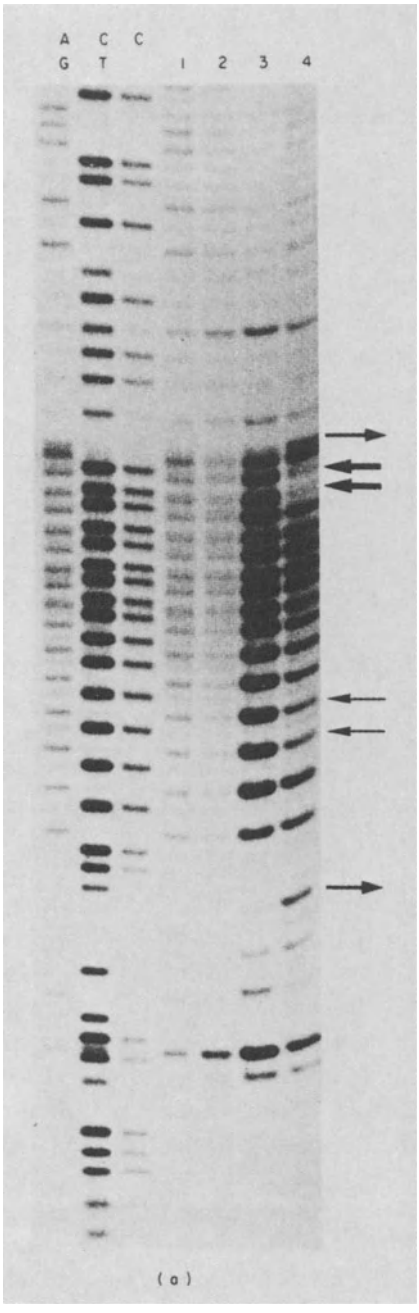


Fig. 1: DEPC footprint of antibody Z22 over the extended $d(C\text{-}G)_{16}$ Z-DNA antigen: Denaturing polyacrylamide gel analysis of DEPC footprinting patterns. Plasmid pLP_{32} was relaxed (lanes 1 and 2) or supercoiled to $-\sigma$ (δ) = 0.060 (lanes 3 and 4) and treated with DEPC in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of antibody Z22. The footprinting pattern on the upper strand is shown. The three sequencing lanes on the left are marked according to the Maxam and Gilbert (1980) sequencing reactions. Arrows at the right of the sequence column indicate purine residues of antibody-induced protection (arrow pointing left) or induction (arrows pointing right) of DEPC hyperreactivity. (*Note*: the significance of the strong band at the bottom of lanes 2 to 4 is not understood at present). From J. Mol. Biol. 189 (1986) 487–501, reprinted with the permission of A. Nordheim and Academic Press, copyright 1990.

3 DEPC Reaction

The DNA which has been pre-incubated with protein is treated with 1 µl of 10-fold diluted DEPC in water (final concentration is 0.5%, mix by vigorous vortexing) at 30°C for 20 min. Then samples are diluted with 0.2 ml of 0.3 M sodium acetate pH 5.2, and 0.1 mM EDTA containing 5 µg tRNA. Samples are further processed by phenol and chloroform extraction to remove proteins prior to the final precipitation with three volumes of ethanol. If the DNA is not pre-incubated with protein, the reaction is carried out in 20 µl of 50 mM sodium cacodylate pH 7.1, 1 mM EDTA.

Note:
Sometimes circular plasmid is used for the DEPC reaction in order to detect supercoil-dependent structural distorsions in DNA. In this case, the probe has to be labelled following DEPC treatment by first cleaving the plasmid with an appropriate restriction enzyme.

4 Piperidine Reaction

Piperidine treatment of DNA at high temperature (> 85°C) may result in a G ladder on the sequencing gel, therefore the DEPC-treated DNA sample is incubated in 100 µl of 1 M piperidine at 80–83°C for 30 min. Then samples are lyophilised, redissolved in 100 µl water and lyophilised again. The DNA is dissolved in 5 µl 95% formamide containing xylene cyanol and bromphenol blue (each 0,025%). Samples are incubated at 95°C for 2 min, quickly chilled on ice and loaded onto a sequencing gel.

5 Separation of Reaction Products on a Sequencing Gel and Autoradiography

Conventional sequencing gel (polyacrylamide gel containing 6 M urea; Maxam and Gilbert, 1980) is used in analysis and mapping DEPC hyperreactive sites or in footprint experiments to determine the protected positions. The concentration of acrylamide depends on the range of sequences to be analysed (see Tables in Appendix). In parallel with the DEPC-treated

samples, dimethylsulphate-(DMS)-treated and labelled DNA (G ladder) serves as a marker. Other chemical sequencing reactions can also be performed (Maxam and Gilbert, 1980). The gel is dried and autoradiographed as for a standard sequencing gel.

C DEPC as a Probe for Structural Alterations of DNA and DNA-Protein Complexes in vivo (Table 1, Steps I–VII)

The small size of DEPC allows its use as an in situ probe for DNA structural alterations and DNA-protein complexes in eukaryotic cells (Toth and Doerfler, unpublished results). The focus of this book is DNA-protein interactions in vitro, therefore the in situ DEPC reaction is only briefly summarised here. The method is very similar to the in vivo DMS footprinting protocol (Church and Gilbert, 1984; Saluz et al., 1986, 1988; Saluz and Jost, 1987, Toth et al., 1989, 1990), except that DEPC is used as a chemical probe. The cells in monolayer cultures can be treated at room temperature for as long as 15 min in Dulbecco medium (D-MEM) containing upto 0.5% DEPC. The pH of the medium might shift towards the acidic during the incubation, and it is possible to supplement the medium with Hepes buffer (20 mM final concentration) to stabilise the pH. Isolated nuclei treated with DEPC can also be used (Glaser et al., 1990). Other parts of the protocol are identical to the in vivo DMS, footprinting method (Table 1, Steps II–VII).

Table 1

DEPC reactions			
	A *in vitro*		B *in vivo*
1	End labelling of DNA	I	Isolation of cell nuclei (or whole cells are used)
2	DNA is incubated with proteins	II	DEPC reaction, isolated total DNA is digested with restriction enzyme
3	DEPC reaction	III	Piperidine treatment
4	Piperidine treatment	IV	Separation of DNA on a sequencing gel
5	Separation of DNA on a sequencing gel	V	Transfer of DNA onto nylon membrane by electroblotting
6	Autoradiography	VI	Hybridisation with strand-specific probe
		VII	Autoradiography

D Bibliography

Church, G.M. and Gilbert, W. Genomic sequencing. Proc. Natl. Acad. Sci. USA 81 (1984) 1991–1995.

Dignam, J.D., Lebovitz, R.M., and Roeder, R.G. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucl. Acids Res. 11 (1983) 1475–1489.

Glaser, R.L., Thomas, G.H., Siegfried, E., Elgin, S.C.R., Lis, J.I. Optimal heat-induced expression of the *Drosophila* hsp26 gene requires a promoter sequence containing (CT)n · (GT)n repeats. J. Mol. Biol. 211 (1990) 751–761.

Manley, J.L., Fire, A., Cano, A., Sharp, P., and Gefter, M.L. DNA-dependent transcription of adenovirus genes in a soluble whole-cell extract. Proc. Natl. Acad. Sci. USA 77 (1980) 3855–3859.

Maxam, A.M. and Gilbert, W. Sequencing end-labelled DNA with base specific chemical cleavage. Method Enzymology 65 (1980) 499–560.

Runkel, L. and Nordheim, A. Chemical footprinting of the interaction between left-handed Z-DNA and anti-Z-DNA antibodies by diethylpyrocarbbonate carbethoxylation. J. Mol. Biol. 189 (1986) 487–501.

Saluz, H.P., Jiricny, J., and Jost, J.P. Genomic sequencing reveals a positive correlation between the kinetics of strand-specific DNA demethylation of the

overlapping estradiol/glucocorticoid receptor binding sites and the rate of avian vitellogenin mRNA synthesis. Proc. Natl. Acad. Sci USA 83 (1986) 7167–7171.

Saluz, H.P. and Jost, J.P. A laboratory guide to genomic sequencing, Birkhäuser, Basel (1987).

Saluz, H.P., Feavers, I.M., Jiricny, J., and Jost, J.P. Genomic sequencing and *in vivo* footprinting of an expression-specific DNase-I hypersensitive site in avian vitellogenin II promoter reveal a demethylation of a mCpG and a change in specific interactions of protein with DNA. Proc. Natl. Acad. Sci. USA 85 (1988) 6697–6700.

Sambrook, J., Fritsch, E.F., and Maniatis, T. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press (1989).

Toth, M., Lichtenberg, U., and Doerfler, W. Genomic sequencing reveals a 5-methylcytosine-free domain in active promoters and the spreading of preimposed methylation patterns. Proc. Natl. Acad. Sci. USA 86 (1989) 3728–3732.

Toth, M., Muller, U., and Doerfler, W. Establishment of de novo DNA methylation patterns. Transcription factor binding and deoxycytidine methylation at CpG and non-DpG sequences in an integrated adenovirus promoter. J. Mol. Biol. 213 (1990) 673–683.

Wells, R.D. Unusual DNA structure. J. Biol. Chem 263 (1988) 1095–1098.

XIII Electron Microscope Visualisation of Protein-DNA Complexes

Béatrice ten Heggeler-Bordier and Walter Wahli

Contents

A Introduction

Electron microscope (EM) examination of protein-DNA complexes was possible long before various powerful biochemical approaches such as the band-shift and footprinting assays were developed. Together these different techniques are of special interest for the mapping of proteins recognising specific target sites on DNA molecules. Electron microscopy allows the fast but relatively low-resolution localisation of binding sites, even on molecules several kilobases long; the biochemical mapping methods offer high resolution but are limited to short DNA fragments. The most successful method of visualising nucleic acids (DNA and RNA molecules, DNA-RNA hybrids) was first reported more then 30 years ago by Kleinschmidt and Zahn (1959). It consists of the spreading of a mixture of negatively charged nucleic acids and cytochrome c, a basic low-molecular-weight protein, via a ramp at a water-air interface. The unaggregated nucleic acid molecules are held in the surface-denatured film of protein with which they are associated. Cytochrome c

increases the diameter of the nucleic acid molecules to 15–20 nm. After spreading, the nucleic acid-protein film is adsorbed to a support membrane on a grid, contrasted by shadowing with heavy metals and observed in the EM. However, examination of specific protein-DNA complexes is not possible by this approach, since the proteins specifically associated to DNA will be obscured by the cytochrome c layer. To overcome this problem, Dubochet et al. (1971) developed a method that involved changing the surface charge of carbon-coated grids to increase their affinity for nucleic acids. Grids treated by the glow-discharge method in the presence of amylamine (pentylamine) are positively charged and interact well with nucleic acids by direct adsorption from solution (Lang and Mitani, 1970). This and other protein-free methods (Williams, 1974; Vollenweider et al., 1975) allow routine observation of proteins with molecular masses of over 50 KDa bound to nucleic acids, as first demonstrated by the mapping of *E. coli* RNA polymerase in the early promoter region of T7 DNA (Bordier and Dubochet, 1974).

The procedures described below were successfully used in our laboratory to analyse the interaction of transcription factors and RNA polymerase II with the

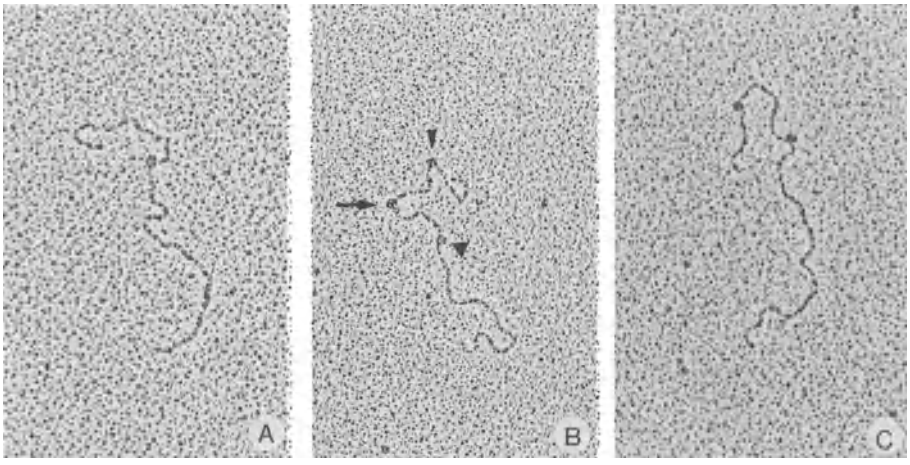


Fig. 1: Binding of RNA polymerase II and estrogen receptor at a 5'-end region DNA fragment of the vitellogenin gene B2: A) DNA restriction fragment containing the promoter region of the gene B2 with a bound RNA polymerase II molecule at the transcription initiation site. The protein-DNA binding reaction was performed in the presence of a HeLa whole-cell extract, the dinucleotide ApU specific for the gene B2 transcription start site, and RNA polymerase II. B) Same DNA fragment as in A after incubation in an estrogen-induced, frog liver, nuclear extract in the presence of estrogen and the dinucleotide ApU. Complexes are observed at three different sites: the estrogen response element (ERE, arrowhead), the transcription initiation site (arrow), and at a specific site within the first intron (triangle). C) Same as in B but without the dinucleotide ApU and thus the complex formed by RNA polymerase II in B (arrow) is missing. All specimens were rotary shadowed with Pt/Pd.

estrogen-regulated vitellogenin gene promoter region. Fig. 1A shows a RNA polymerase II ternary transcription complex (Ackerman et al., 1983) formed in vitro at the transcription start site of the *Xenopus laevis* vitellogenin gene B2 promoter (ten Heggeler and Wahli, 1985). The formation of this stable complex is dependent on cellular factors present in a HeLa whole-cell extract, as well as RNA polymerase II and at least two nucleotides complementary to sequences at the transcription initiation site. Stable protein-DNA complexes can also be assembled in vitro at the 5'-end region of the same gene, using extracts of nuclei from estrogen-induced frog liver producing vitellogenin, and visualised by EM (Fig. 1B; ten Heggeler-Bordier et al., 1987). Complexes at three different sites can be identified (Fig. 1B): the transcription initiation site (arrow), the estrogen-response element (arrowhead), and within the first intron (triangle). The complex at the transcription start site is not stable in the absence of the promoter-specific dinucleotide (see Fig. 1C) and thus represents the ternary transcription complex (Fig. 1B, arrow). The occurrence of the two other complexes is enhanced by estrogen and reduced by tamoxifen, an antagonist of estrogen. The latter effect is reversed by an excess of hormone,

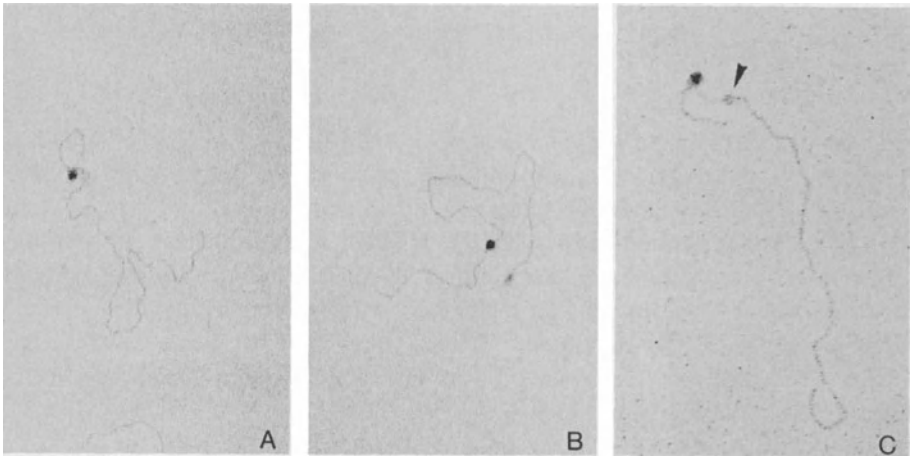


Fig. 2: Estrogen-receptor-specific immuno-labelling of protein-DNA complexes in the vitellogenin gene B2 promoter region: A) Protein-DNA complex immuno-labelled with estrogen receptor monoclonal antibody-Protein A/Gold and localised at the estrogen response element. Stained with saturated uranyl acetate. B) Same type of receptor-DNA complex but at a specific site within the first intron of the gene B2. Stained with saturated uranyl acetate. C) Estrogen receptor immuno-labelling of a protein-DNA complex at the estrogen response element next to an unlabelled, ternary transcription initiation complex (arrowhead). After staining with saturated uranyl acetate, this sample was slightly shadowed with Pt/Pd. The DNA template used here was a restriction fragment of the vitellogenin gene B2 5'-end region but 0.8 kb longer than the fragment used in the experiments of Fig. 1

suggesting that the estrogen receptor is present in the complexes or involved in their formation. That the estrogen receptor is part of these two complexes can be demonstrated by immuno-electron microscopy, using the indirect, colloidal gold, immunological method with a monoclonal antibody specific to the estrogen receptor. The antibody is found linked to the two complexes as identified by its interaction with Protein A/Gold (Figs. 2A and B), but not to the ternary transcription initiation complex (Fig. 2C, arrowhead; ten Heggeler-Bordier et al., 1988). Thus, this observation demonstrates the direct participation of the receptor in the estrogen-dependent complexes. Immuno-electron microscopy using similar procedures has also been used to localise the T-antigen on simian virus 40 DNA (Harper et al., 1984), to visualise nucleoprotein structures at the origin of replication of the bacteriophage λ (Dodson et al., 1989), and to show protein-mediated joining of distant recombination sites at a recombinatorial enhancer sequence by the Hin protein of *Salmonella* (Heichman and Johnson, 1990).

Below, we decribe in detail the experimental procedures used to obtain and visualise the different protein-DNA complexes presented above.

B Protein-DNA Binding Reaction and Specimen Preparation

It is useful to have a restriction site map for the DNA region used as template. Analysis of the distribution of protein-DNA complexes on several or at least two overlapping fragments from the same region is indeed required for the mapping of the complexes visualised. The salt concentration in the binding and washing reactions is very important and the optimal concentration varies from one protein to another, mainly depending on the affinity of the proteins for their cognate DNA-binding sites. The concentrations given below were optimised for the visualisation of the estrogen receptor and RNA polymerase II and may need to be adjusted for the observation of other protein-DNA interactions.

1 Materials and Solutions

- Glow discharge apparatus (Balzers, Liechtenstein or home made)
- DNA template fragments, if possible no longer than 6 kb and at a concentration of 125 ng/μl
- Nuclear or whole-cell extract at 3–5 μg protein/μl (if purified proteins are used, much less protein is needed)
- Non-specific competitor DNA, pBR 322 or poly (dI-dC) at a concentration of 100 ng/μl

- 120 mM Hepes, pH 7.9
- Glycerol (ultra pure)
- 1 mM EDTA
- 10 mM DTT
- 100 mM Creatine phosphate
- 5×10^{-8} M 17β-Estradiol (only for estrogen receptor mapping)
- 70 mM $MgCl_2$
- 1 M KCl
- 5 mM Mixture of the first two or three nucleotides complementary to sequences at the transcription initiation site or 5 mM of a corresponding dinucleotide (only for RNA polymerase II binding)
- Sepharose 2B
- Siliconised glasswool
- 200-µl Eppendorf pipette tips
- 30 mM Triethanolamine (TEA)-HCl pH 7.9, 500 mM KCl, 10 mM $MgCl_2$ and 0.1% gluteraldehyde
- Eppendorf tubes
- Carbon-coated, electron microscopy grids
- Amylamine (also called pentylamine), Fluka

2 Step-by-Step Procedure

> Mix the following components (final concentrations or amounts) in the order given below:
  - 20 mM Hepes, pH 7.9 (0.6 µl of 120 mM)
  - 15% glycerol (0.5 µl of 100%)
  - 0.08 mM EDTA (0.3 µl of 1 mM)
  - 80 mM KCl (0.3 µl of 1 M)
  - 0.8 mM DTT (0.3 µl of 10 mM)
  - 50 ng poly (dI-dC) or pBR322 DNA (0.5 µl of 100 ng/µl)
  - Nuclear extract: 3–5 µg of protein (1 µl of 3–5 µg/µl)

> Pre-incubate this reaction mixture for 15 min on ice to allow non-specific binding of proteins to poly(dI-dC) or pBR 322 DNA.

> Bring the volume to 6 µl by adding (final concentrations and amount):
  - 7 mM $MgCl_2$ (0.6 µl of 70 mM)
  - 10 mM creatine phosphate (0.6 µl of 100 mM)
  - 5×10^{-9} M 17β-estradiol (estrogen receptor mapping; 0.6 µl of 5×10^{-8} M)

- 0.4 mM dinucleotides or nucleotides (for RNA polymerase II mapping, 0.5 µl of 5 mM)
- 25 ng of specific DNA template (0.2 µl of 125 ng/µl)

For the first mixed components (see above), the final concentrations and amount of protein are now 12 mM Hepes pH 7.9, 0.5 mM DTT, 8% glycerol, 50 nM EDTA, 50 mM KCl and 1.6–2.7 µg protein from the nuclear extract.

> Incubate the whole mixture for 15 min at 30°C.

> After the 15-min incubation at 30°C, dilute the sample with one volume of 30 mM TEA-HCl pH 7.9, 1 M KCl and 10 mM $MgCl_2$ and continue the incubation for an additional 5 min at 30°C. This step is necessary to release non-specific bound proteins from the DNA, and the molarity of KCl must be adjusted, depending on the stability of the protein-DNA complexes to be studied.

> During these incubations the Sepharose 2B minicolumn should be prepared. Place a very small plug of siliconised glasswool into a 200-µl Eppendorf tip, which is then filled completely with Sepharose 2B, and equilibrate with 30 mM TEA-HCl pH 7.9, 0.5 M KCl, 10 mM $MgCl_2$ and 0.1% gluteraldehyde. This column is used to separate unbound proteins from protein-DNA complexes. The KCl concentration is the same as above, adjusted according to the stability of the complexes.

> Fractionate the mixture on the Sepharose 2B minicolumn in 30 mM TEA-HCl pH 7.9, 0.5 M KCl, 10 mM $MgCl_2$ and 0.1% gluteraldehyde.

> Collect one-drop fractions (approximately 20 µl). The majority of the complexes come out in fractions number 7 and 8. Under the conditions given above, the peak fractions contain an appropriate concentration of DNA for the specimen preparation for EM observation (see below).

The complexes will be fixed during fractionation, because the buffer contains 0.1% gluteraldehyde. Do not try to fix the complexes before loading the column when using nuclear or whole-cell extracts. The concentration of proteins is very high in the binding reaction mixture and large aggregates of fixed proteins will form and block up the column. If working with purified proteins, much less material is used and thus fixation

with gluteraldehyde is possible before the chromatographic step (which may be omitted if very little protein is used in the protein-DNA binding reaction).

Specimen Preparation for Electron Microscopy

> Samples of 5 µl are deposited on previously positively charged carbon films according to the protein-free spreading method of Dubochet et al. (1971).

> Wash the grids after 30–60 s by floating them on two drops of double-distilled water.

> Stain the grids by floating them on 1% uranyl formate for 1 min (Brack and Pirotta, 1975).

> Blot dry on filter paper with the specimen side down.

> Rotary shadow the specimen with Pt/Pd at an angle of 6°.

C Immuno-electron Microscopy of Protein-DNA Complexes

1 Materials and Solutions

- Same as for protein-DNA interactions
- Monoclonal antibodies (here the estrogen receptor H222 antibody, Abbot Laboratories) or IgGs against the protein of interest
- Protein A/Gold 5 nm (Janssen Life Products)

2 Step-by-Step Procedure

> Prepare the protein-DNA binding-reaction mixture as described above. The specific antibodies are added (5 µl of H222 antibodies) to the reaction mixture as the last component, i.e. after addition of the specific DNA template (see above).

> Incubate for 30 min at room temperature, and load the reaction mixture onto a Sepharose 2B column in an Eppendorf pipette tip (see above) equilibrated with 30 mM TEA-HCl pH 7.9 and 60 mM KCl.

> Pool the two peak fractions (40 µl).

> Add 2 µl of Protein A/Gold 5 nm conjugate and incubate the mixture for an additional 30 min at room temperature.

> Bring the reaction mixture to 0.5 M KCl (molarity to be adjusted according to the stability of the complexes).

> Leave for 5 min at room temperature to allow dissociation of non-specific protein-DNA interactions.

> Separate protein-DNA complexes from unbound Protein A/Gold by gel filtration as described above, in a column equilibrated with 30 mM TEA-HCl pH 7.9, 10 mM $MgCl_2$, 0.5 M KCl and 0.1% gluteraldehyde.

Specimen Preparation for Electron Microscopy

> Deposit a 5-µl sample from the selected fraction onto a previously positively charged carbon film.

> Adsorb the protein-DNA complexes for 1 min (time may vary according to the DNA concentration in the sample).

> Wash the grids by floating on two drops of distilled water.

> Stain the sample with saturated, aqueous uranyl acetate for 10 s and blot the grid dry on filter paper, specimen side down.

> Observe without shadowing for high resolution microscopy (see Figs. 2A, B). For routine examination, the specimen can be lightly rotary shadowed at an angle of 6° with Pt/Pd to increase the contrast (see Fig. 2C).

D Bibliography

Ackerman, S., Bunick, D., Zandomeni, R., and Weidmann, R. RNA polymerase II ternary transcription complexes generated *in vitro*. Nucleic Acids Res., 11 (1983) 6041–6064.

Bordier, C. and Dubochet, J. Electron microscopic localization of the binding sites of *Eschericha coli* RNA polymerase in the early promoter region of T7 DNA. Eur. J. Biochem. 44 (1974) 617–624.

Brack, C., and Pirotta, V. Electron microscopic study of the repressor of Bacteriophage *lambda* and its interaction with operator DNA. J. Mol. Biol. 96 (1975) 139–152.

Dodson, M., McMacken, R., and Echols, H. Specialized nucleoprotein structures of the origin of replication of bacteriophage *lambda*. J. Biol. Chem. 264 (1989) 10719–10725.

Dubochet, J., Ducommun, M., Zollinger, M., and Kellenberger, E. A new preparation method for dark field electron microscopy of biomacromolecules. J. Ultrastruct. Res. 35 (1971) 147–167.

Harper, F., Florentin, Y., and Puvion, E. Localization of T-antigen on simian virus 40 minichromosomes byimmuno-electron microscopy. EMBO J. 3 (1984) 1235–1241.

ten Heggeler, B. and Wahli, W. Visualization of RNA polymerase ternary transcription complexes formed *in vitro* on a *Xenopus laevis* vitellogenin gene. EMBO J. 4 (1985) 2269–2273.

ten Heggeler-Bordier, B., Hipskind, R., Seiler-Tuyns, A., Martinez, E., Corthésy, B., and Wahli, W. Electron microscopic visualization of protein-DNA interactions at the estrogen responsive element and in the first intron of the *Xenopus laevis* vitellogenin gene. EMBO J. 6 (1987) 1717–1720.

ten Heggeler-Bordier, B., Claret, F.X., and Wahli, W. Immuno-electron microscopic identification of human estrogen receptor-DNA complexes at the estrogen responsive element and in the first intron of a *Xenopus* vitellogenin gene. J. Mol. Biol. 204 (1988) 217–220.

Heichman, K.A. and Johnson, R.C. The Hin invertosome: Protein-mediated joining of distant recombination sites at the enhancer. Science 249 (1990) 511–517.

Kleinschmidt, A.K. and Zahn, R.K. Ueber Deoxyribonucleinsäure Molekülen in Protein-Mischfilmen. Z. Naturforschung 14b (1959) 770.

Lang, D. and Mitani, M. Simplified quantitative electron microscopy of biopolymers. Biopolymers 9 (1970) 373.

Vollenweider, H.J., Sogo, J.M., and Koller, T. A routine method for protein-free spreading of double- and single-stranded nucleic acid molecules. Proc. Natl. Acad. Sci. USA 72 (1975) 83–87.

Williams, R.C. Use of polylysine for adsorption of nucleic acids and enzymes to electron microscope specimen films. Proc. Natl. Acad. Sci. USA 74 (1974) 2311–2315.

XIV Qualitative and Quantitative Studies of Protein-DNA Interactions by Gel Mobility-Shift Assay

Stuart R. Stone, Melya J. Hughes, Jean-Pierre Jost

Contents

A Introduction

The nitrocellulose-filter-binding assay has been widely used to determine equilibrium and rate constants for the binding of proteins to DNA in prokaryotic systems (Riggs et al., 1968, 1970a, 1970b). The electrophoretic separation of protein-DNA complexes on polyacrylamide or agarose gels provides an alternative to the nitrocellulose-filter-binding assay (for review see Hendrickson, 1985; Revzin, 1989). The electrophoretic procedure has the advantage that it can be used with relatively crude nuclear or cellular extracts where more than one protein may bind to the DNA fragment (see later for a discussion of this aspect). The method also allows the study of proteins that interact weakly with DNA. The detection limits of the assay are not known exactly; proteins with a binding constant as high as 10^{-7} M have been observed (Hendrickson, 1985). Among the first proteins found to alter the mobility of DNA on agarose or polyacrylamide gels were the protein covalently bound to the end of the adenovirus DNA, and the proteins that interact with *Bacillus subtilis* phage DNA (Sharp et al, 1976; Ito et al., 1976). Later, a nucleic-acid-binding assay utilising gel electrophoresis was used to study the ternary complexes formed by *E. coli* RNA polymerase, a DNA fragment and a nascent RNA chain (Chelm and Geiduschek, 1979). The basis of the so-called gel retardation or gel mobility-shift assay is the reduction in the movement of a DNA molecule through a non-denaturing gel (such as agarose or polyacrylamide) when it carries bound protein(s). This basic technique, combined with chemical modifications or removal of specific bases in the DNA, has yielded precise information on DNA-protein interactions. Gel retardation is equally well suited to the study of single- or double-stranded, linear or circular DNA, as well as RNA.

B Qualitative Aspects of the Gel Mobility-Shift Assay

1 Types of Gel

A 4–5% polyacrylamide gel with a ratio of acrylamide : bisacrylamide of 30 : 1 is recommended for proteins with a molecular mass of 15–500 KDa and DNA fragments 12–600 bp in length. High molecular weight DNA fragments can be separated on gels with the same percentage acrylamide but with a ratio of acrylamide : bisacrylamide of 50 : 1 or even 80 : 1 (see the example given in Fig. 1). In general, such gels are rather difficult to handle and give much broader bands than "normal" 30 : 1 gels. Figs. 1 and 2 show the gel-shift assay of the same protein using a 4%

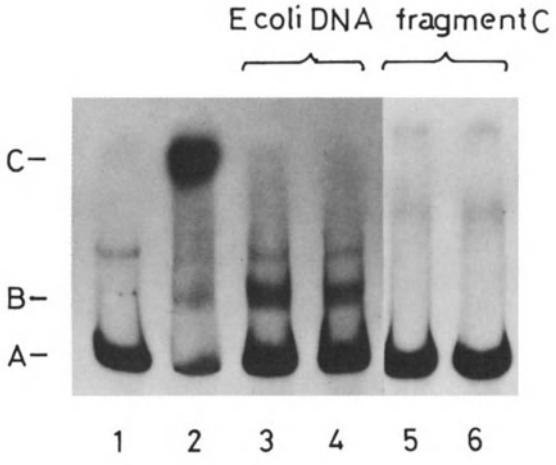


Fig. 1: Gel mobility-shift assay on a 4% polyacrylamide gel (acrylamide : bisacrylamide, 80 : 1): Lane 1 : labelled DNA restriction fragment of 560 bp. Lane 2 : protein-DNA complex in the absence of non-specific competing DNA. Lanes 3 and 4 are the same as lane 2 but with a 1000- or a 2000-fold-weight excess of non- specific competing DNA (*E. coli*) respectively. Lanes 5 and 6: the same as lane 2 but with, a 50- or a 100-fold-weight excess of the non- labelled restriction fragment respectively. Band A: unbound DNA. Band B: specific DNA-protein complex. Band C: non-specific DNA-protein complex (Jost et al., 1987). Reprinted with permission from John Wiley & Sons, Inc., all rights reserved.

polyacrylamide gel 80 : 1 (Fig. 1) and 30 : 1 (Fig. 2), respectively. In addition, Fig. 2 shows that large differences in the size of DNAs used for the gel-shift assays (25–125 KDa are not reflected by corresponding differences in the mobility of the protein-DNA complexes (in the present case, the protein is approximately 70 KDa). These results suggest that the distance migrated by the protein-DNA complex is dictated by the size and the conformation of the protein rather than by the size of the DNA. However, in certain cases the rate of migration through the gel will also depend on the degree of bending in the DNA (Wu and Crothers, 1984; Crothers, 1987). The rate of migration of bent or curved DNAs and their protein complexes will depend on the position of the bend on the DNA. For example, a complex bent in the centre of the DNA fragment moves considerably more slowly than the corresponding complex with a bend near one end (Wu and Crothers, 1984). The best way to acertain whether complex formation leads to bending of the DNA is to use a series of circularly permutated DNA fragments with the bend in various positions (see Chapter XIX). The use of agarose or polyacrylamide (1.5–2.5%) mixed with agarose (0.5%) gels is recommended for interactions in which several proteins bind simultaneously to the DNA, or for circular DNA (McEntee et al., 1981).

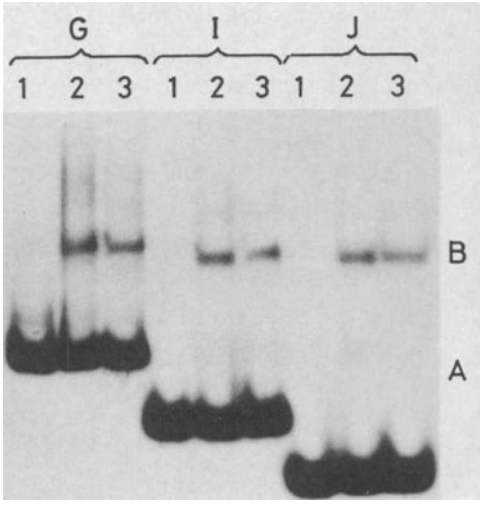


Fig. 2: Gel mobility-shift assay of the same protein as in Fig. 1 but on a 4% polyacrylamide gel (acrylamide : bisacrylamide 30 : 1) and with DNA fragments of different size: G has 190 bp, I: 83 bp and J: 39 bp. The binding assay was made in the presence of 0.1 ng of labelled DNA, 3 µg of protein and (lane 2) a 1000-fold- or (lane 3) a 2000-fold- weight excess of *E. coli* DNA as non-specific competitor. Band A: unbound DNA. Band B: protein-DNA complex (Jost et al., 1987). Reprinted with permission from John Wiley & Sons, Inc., all rights reserved.

2 Buffers

The following buffers have been frequently used for the gel retardation assays: TBE : 90 mM Tris borate, 2.5 mM EDTA, pH 8.3, and TEA buffer: 40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, pH 8.0. TBE buffers should be used with caution since borate ions can interact with proteins and give artifactual, double bands (Cann, 1966). Electrophoresis buffers should be of low ionic strength, because at high salt concentration the protein-DNA complex carries only a small fraction of the current and does not migrate very far. A high salt concentration may also increase heating during electrophoresis. On the other hand, a very low salt buffer may lead to an excessive broadening of the bands (Cooper, 1977). Furthermore, a buffer concentration that is too low may result in a shift in the pH through electrolysis of the buffer, severely interfering with the stability of the protein-DNA complex.

anode: $H_2O \rightarrow 2\ e^- + 2\ H^+ + 1/2\ 0_2$, pH decreases

cathode: $2\ e^- + 2\ H_2O \rightarrow 2\ OH^- + H_2$, pH increases

In special cases, low pH is required for the separation of the protein-DNA complex from free DNA, e.g. for the *E. coli* trp repressor with its operator, where the separation of the complex from free proteins requires a buffer of pH 6 (Carey, 1988).

In order to achieve an optimal protein-DNA interaction, it may be necessary to determine if any cofactors, such as Mg^{++}, Zn^{++}, cAMP or hormones, are required for complex formation. Such cofactors can then be added to the assay mix, the gel and even to the running buffer.

3 DNA Binding of Proteins in Crude Extracts

When the labelled DNA fragment is added alone to a cell or nuclear extract, virtually all the radioactive DNA is found at the top of the gel, bound in very large complexes with many different proteins (see Fig. 1, Lane 1). In such cases, it is necessary to add non-specific competing DNA, such as *E. coli* DNA, calf thymus DNA or poly d(I-C), to the reaction mixture. If the protein binds preferentially to sequences containing methylated cytosine, it is advisable not to use calf thymus DNA as a non-specific competitor. The optimal amount of non-specific competing DNA to be added to the incubation mixture is determined experimentally using a series of reactions with increasing amounts of non-specific competing DNA. In such experiments, the level of radioactivity in complexes at the top of the gel diminishes while the intensity of the specific complex(es) and the free DNA band rise. If too much non-specific DNA is added, it will compete for the specific factor of interest and the level of the complex will decrease.

Further information on a specific protein-DNA complex can be obtained by competition assays with oligonucleotides containing the mutated binding sequences or containing modified bases such as methylcytosine (see Fig. 3). The protein composition of the complex can be analysed by cutting out the band corresponding to the protein-DNA complex, electro-eluting the protein, and analysis on a SDS polyacrylamide gel. The presence of other proteins in the extract that migrate at the same position as the specific complex could complicate this analysis. In certain cases, this difficulty can be circumvented by using non-specific DNA of very large size or by partial purification of the extract with conventional procedures. The possibility always exists that the DNA used specifically binds more than one protein.

Some proteins in crude extracts may aggregate, causing many different complexes to smear down the gel or remain on the top. In such cases, the use of non-ionic detergents, such as Nonidet NP40, in the binding buffer may eliminate this problem. The detergent may also stabilise proteins (Hendrickson and Schleif, 1984). The use of glycerol in the buffer is also recommended for unstable proteins.

Composition of the Binding Buffer

Ideally, one should try to reproduce the in vivo conditions for the binding of a protein to DNA or RNA. Since this is impossible, one of the best choices is to use the conditions for in vitro transcription. For a given incubation time and temperature (for example, 20 min. at 20°C), determine the concentration dependence on monovalent (Na^+, K^+) and divalent cations (Mg^{++}, Zn^{++}). For certain proteins low

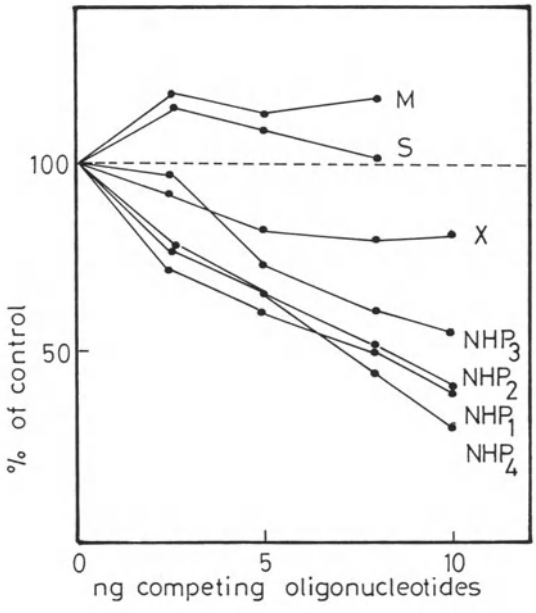


Fig. 3: Cross-competition band-shift assay of the protein-DNA complex, NHP1-ERE, with oligonucleotides containing the binding site of NHP2, NHP3 and NHP4: The upper strand of the estrogen response element (ERE) is indicated by SS, whereas M and X are the mutated ERE lacking the GCG in the middle of the palindrome, and the *Xenopus* vitellogenin ERE, respectively. Five nanogrammes of the ^{32}P-ERE oligonucleotide were incubated with 4 µg of partially purified NHP1 from HeLa cells and increasing concentrations of unlabelled oligonucleotides. The reaction product was separated on gels and the protein-DNA complexes were cut out and counted for radioactivity. (100% represents the radioactivity in the protein-DNA complex in the absence of competing oligonucleotides). (Hughes et al., 1989). Reprinted with permission from Nucleic Acids Res. 17 (1989) 8511–8520; copyright 1990 Oxford University Press.

concentrations of polyamines (spermidine 0.5 mM, spermine 0.15 mM), dithiothreitol, non-ionic detergents, glycerol or cofactors such as cAMP, ATP, GTP may be required for stable binding to DNA. Once the best binding conditions are established, the optimal amount of non-specific competing DNA to be added to the system for a given protein concentration should be ascertained, and the time-dependence of the binding reaction should be examined.

4 Loading the Gel and Electrophoresis

It is very important that the amount of DNA-protein complex in solution remains unchanged during loading of the gel and subsequent electrophoresis. Differences in the composition of the binding buffer and the gel running buffer could alter protein binding during loading and entry of the complex into the gel. To avoid this problem, it is possible to preload the wells of the gel with the binding buffer (for vertical gels use a concentration of glycerol lower than the sample buffer). As an alternative, it is possible to add more non-specific competitor DNA to the sample just before loading the gel; this will block any additional binding of the protein to the DNA. It is interesting to note that complexes dissociating in a few minutes or even seconds in binding buffer can be subjected to electrophoresis for a few hours without substantial dissociation. A possible explanation is that the affinity of the protein for the DNA is increased in the buffers of low ionic strength used for the electrophoresis, but the "caging" effect of the gel provides an alternative explanation (Hendrickson, 1985; Revzin, 1989).

C Quantitation of Protein-DNA Interactions: Theory and Data Analysis

1 Determination of the Dissociation Constant of a Protein-DNA Complex and the Concentration of Active Protein

The binding of a protein P to labelled DNA (D) to form a DNA-protein complex can be represented by the following scheme:

$$D + P \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} DP$$

where k_{on} and k_{off} are the association and dissociation rate constants for the process. If the binding stoichiometry of the protein to the oligonucleotide is 1:1, then the dissociation constant (K_d) of the DNA-protein complex will be given by:

$$K_d = \frac{[P]_f [D]_f}{[DP]} \tag{1a}$$

$$= \frac{([P]_t - [DP])([D]_t - [DP])}{[DP]} \quad (1b)$$

$$= \frac{([P]_t - [DP])\,[D]_f}{[DP]} \quad (1c)$$

where $[P]_f$, $[D]_f$, $[DP]$, $[P]_t$ and $[D]_t$ are the concentrations of free active protein, free DNA, DNA-protein complex, total active protein and total DNA, respectively. It should be noted that the equilibrium constant (K_{eq}) for the interaction is the inverse of the dissociation constant (K_d), i.e. $K_d = 1/K_{eq}$. The dissociation constant can be related to the association (k_{on}) and dissociation (k_{off}) rate constants by Eq. 2:

$$K_d = \frac{k_{off}}{k_{on}} \quad (2)$$

If the total concentration of DNA ($[D_t]$) is in vast excess ($[D_t] > 10\times[P_t]$), then Eq. 1b reduces to:

$$K_d = \frac{([P]_t - [DP])[D]_t}{[DP]} \quad (3)$$

Eq. 1c and 3 are useful for data analysis; they can be rearranged to give Eqs. 4a and 4b, respectively.

$$[DP] = \frac{[D]_f\,[P]_t}{K_d + [D]_f} \quad (4a)$$

$$[DP] = \frac{[D]_t\,[P]_t}{K_d + [D]_t} \quad (4b)$$

These equations have the same form, but it should be noted that Eq. 4b can only be applied when the concentration of DNA is at least 10 times that of the protein. In practice, Eq. 4a is more useful since $[DP]$ and $[DP]_f$ can be estimated after ectrophoresis by cutting out and counting the bands corresponding to both the protein-DNA complex and the free DNA. The amount of complex formed ($[DP]$) at various concentrations of DNA can be used to estimate both the dissociation constant for the protein-DNA complex (K_d) and the total concentration of active DNA-binding protein ($[P]_t$) in the assay. This can be done by fitting the data directly to Eq. 4a, or using the computer programe given in the Appendix to this chapter, or graphically by using a linear transformation of Eq. 4a such as a Scatchard plot. For a Scatchard plot, Eq. 4a is rearranged to give:

$$\frac{[DP]}{[D]_f} = \frac{[P]_t}{K_d} = \frac{[DP]}{K_d} \tag{5}$$

A plot of $[DP]/[D]_f$ against $[DP]$ yields a straight line with a slope of $-1/K_d$ and an intercept on the X-axis of $[P]_t$. Other methods have also been used to determine the concentration of active protein and the value of K_d (Rigg et al., 1970; Fried and Crothers, 1981, Garner and Revzin, 1981; von Hippel and Berg, 1986; Chodosh et al., 1986).

2 Determination of the Dissociation Rate Constant (k_{off})

An estimate of the dissociation rate constant k_{off} can be obtained by forming the protein-DNA complex with radioactive oligonucleotide and then adding a large excess of unlabelled oligonucleotide (50- to 100-fold) to sequester the protein as it dissociates from the labelled DNA. The dissociation rate constant can be determined from the rate of disappearance of radioactivity from the protein-DNA complex. This rate will be given by Eq. 6:

$$-\frac{d[DP*]}{dt} = k_{off}[DP*] \tag{6}$$

where $[DP*]$ is the concentration of labelled protein-DNA complex and t is time. Integration of Eq. 6 yields:

$$[DP*] = [DP*]_0 \exp(-k_{off}t) \tag{7}$$

where $[DP*]_0$ is the concentration of protein-DNA complex at zero time. Eqs. 8a and b are linear transformations of Eq. 7.

$$\ln \frac{[DP*]}{[DP*]_0} = -k_{off}t \tag{8a}$$

$$\log \frac{[DP*]}{[DP*]_0} = \frac{-k_{off}t}{2.303} \tag{8b}$$

An estimate for k_{off} can be determined either by fitting the data to Eq. 7 by nonlinear regression using the computer programe, or graphically by plotting the logarithm or the natural logarithm of $[DP*]/[DP*]_0$ against time. The slope of this plot will be $-k_{off}/2.303$ when logarithms are plotted or $-k_{off}$ for the natural logarithm plot. In both computer and graphical analyses, the raw data in terms of cpm in the protein-DNA band can be used without calculating the molar concentration of the complex.

3 Determination of the Association Rate Constant (k_{on})

An estimate of the association rate constant k_{on} can be obtained by measuring the amount of the protein-DNA complex formed as a function of time. The rate of complex formation will be given by Eq. 9:

$$\frac{d[DP]}{dt} = k_{on}[D][P] - k_{off}[DP] \qquad [D]_t \gg [P]_t \tag{9}$$

If the experimental conditions are chosen such that $[D]_t \gg [P]_t$, then Eq. 9 can be written:

$$\frac{d[DP]}{dt} = k_{on}[D]_t([D]_t - [DP]) - k_{off}[DP] \tag{10a}$$

$$= k'([D]_t - [DP]) - k_{off}[DP] \tag{10b}$$

where k' is the apparent first-order rate constant for the formation of DP at a particular concentration of oligonucleotide and is equal to $k_{on}[D]_t$. The treatment of the data is greatly simplified if it is possible to adjust the concentration of oligonucleotide such that it is much greater than the K_d for the protein-DNA complex. In this case, the interaction of the oligonucleotide with the protein in practice becomes irreversible and Eq. 10 reduces to Eq. 11:

$$\frac{d[DP]}{dt} = k'([P]_t - [DP]) \tag{11}$$

Integration of Eq. 10b and use of the relationship $k'[P]_{eq} = k_{off}[DP]_{eq}$, where $[P]_{eq}$ and $[DP]_{eq}$ are the concentrations of D and P at equilibrium, yields Eq. 12. The more straight-forward integration of Eq. 11 gives Eq. 13:

$$[DP] = [DP]_{eq}[1 - \exp(-k''t)] \tag{12}$$

$$[DP] = [P]_t[1 - \exp(-k't)] \tag{13}$$

where k" equals $k_{on}[D]_t + k_{off}$. Linear forms of Eq. 12 and 13 can be obtained by taking logarithms of both sides and rearranging to yield Eqs. 14 and 15, respectively.

$$\ln \frac{[DP]_{eq} - [DP]}{[DP]_{eq}} = -k''t \tag{14a}$$

$$\ln ([DP]_{eq} - [DP]) = \ln ([DP]_{eq}) - k''t \tag{14b}$$

$$\log ([DP]_{eq} - [DP]) = \log ([DP]_{eq}) - \frac{k''t}{2.303} \quad (14c)$$

$$\ln \frac{[P]_t - [DP]}{[P]_t} = -k't \quad (15a)$$

$$\ln ([P]_t - [DP]) = \ln ([P]_t) - k't \quad (15b)$$

$$\log ([P]_t - [DP]) = \log ([P]_t) - \frac{k't}{2.303} \quad (15c)$$

Determination of an estimate for k_{on} is obviously simpler when a concentration of oligonculeotide much greater than the K_d of the complex can be used. For practical purposes, a concentration at least ten times the K_d should be sufficient. In this case, the data can be fitted to Eq. 13 using the computer programe, or by using the linear transformation given in Eqs. 15b or 15c. In the latter case, ln-($[P]_t$-[DP]) or log ($[P]_t$ – [DP]) is plotted against time and the slope of the plot is –k'/2.303 when logarithms are used and –k' with the natural logarithm plot. Once again, the raw data in terms of cpm in the protein-DNA band can be used without calculating the molar concentration of the complex in both computer and graphical analyses.

It should be noted that the value of k' obtained from the analyses must be divided by $[D]_t$ in order to obtain an estimate for k_{on}.

In some cases, however, it may not be possible to use a concentration of oligonucleotide that is much greater than the K_d. There may be several reasons for such a problem. The interaction may be weak (a high K_d value) and it may not be practical to use concentrations of oligonucleotide in excess of the K_d value. Alternatively, the reaction of the oligonucleotide with the protein may become too fast to measure at high concentrations, and concentrations of oligonucleotide equal to or less than the K_d value may have to be used in order to obtain accurate estimates of k_{on}. In any case, the apparent first-order rate constant (k") for the interaction should be determined at several concentrations of DNA; the value for k_{on} can then be determined from the relationship $k'' = k_{on}[D]_t + k_{off}$. Estimates for k" can be obtained by fitting the data to Eq. 12 by nonlinear regression using a computer programe. It is not advisable to graph the data according to Eq. 14 in order to obtain an estimate of k". Such a plot depends very heavily on an accurate estimate of $[DP]_{eq}$, which is often difficult to obtain. It is, therefore, advisable to use a Guggenheim plot to obtain estimates of k". This plot will not be described here and the reader is referred to Cornish-Bowden (1976).

D Quantitation of Protein-DNA Interactions: Methods

1 Materials and Buffers

- 10× Klenow buffer (500 mM Tris HCl pH 7.2, 100 mM $MgSO_4$, 1 mM DTT
- dTTP, dCTP, dGTP (2 mM stock solutions)
- 5× Binding buffer (0.5 M Tris HCl pH 8, 25 mM EDTA, 10 mM β-mercaptoethanol, 0.5 mM $ZnCl_2$ containing 1 µg/ml of *E. coli* DNA
- Bovine serum albumin, enzyme grade (10 mg/ml)
- Klenow fragment of *E. coli* DNA polymerase (Biofinex Praroman, Switzerland (7 U/µl)
- 0.5 M EDTA pH 8
- Loading dye: 25% glycerol in binding buffer, 0.05% bromophenol-blue
- Purified protein factor
- Scintillation fluid
- 4% or 5% Polyacrylamide gel (30 : 1) in 1× TEA buffer
- 10× TEA buffer (400 mM Tris HCl pH 8, 200 mM sodium acetate, 20 mM EDTA)

2 Synthesis of the Labelled DNA Substrate

- Complementary oligonucleotides (10 pmol) containing the protein binding site and 5' overhanging ends are annealed by heating to 90°C for 3 min and allowing to cool slowly to room temperature. The overhanging ends should contain complementary bases to the ^{32}P-labelled dNTP(s) used during the fill-in.
- The annealed oligonucleotides are then mixed with 2 µl of 10× Klenow buffer, 1 µl each of dCTP, dGTP and dTTP, 3 µl of (α-^{32}P)-dATP (3000 Ci/mmole, 10 mCi/ml) and 0.5 µl Klenow fragment of DNA polymerase (3–4 units).
- Incubate the mixture for 10 min at room temperature and stop the fill-in reaction by the addition of 1 µl of 0.5 M EDTA.
- The oligonucleotide is then separated from the free nucleotides by centrifugation through a spun column of Sephadex G-50 (Maniatis et al., 1982, see also Appendix).

A specific activity of 3000 Ci/mmole is equivalent to 6.6×10^{15} dpm/mmole (2.2×10^6 dpm is equivalent to 1 µCi). One fmole of double-stranded oligonucleotide, having 2 fmoles of ^{32}P label, would emit 13 200 dpm. The concentration of oligonucleotide can be measured accurately by detecting the amount of radioactivity with scintillation counting. However, the specific activity must be corrected for the half-life of the isotope.

3 Determination of the Concentration of Active Binding Protein and the Dissociation Constant (K_d)

The concentration of active binding protein ($[P]_t$) and the dissociation constant of the complex(K_d) is determined by titrating a fixed amount of protein with increasing concentrations of oligonucleotide. A curve similar to that shown in Fig. 4 should be obtained. The saturation point for the curve gives the value of $[P_t]$. An accurate estimate of $[P_t]$ is only obtained when levels of oligonucleotide that are much greater than the value of K_d are used, i.e. only when the plateau in Fig. 4 is well defined. The estimates for $[P]_t$ and K_d are obtained by fitting the data to Eq. 4a using the computer programe or a Scatchard plot (Eq. 5) as outlined above in the data analysis section.

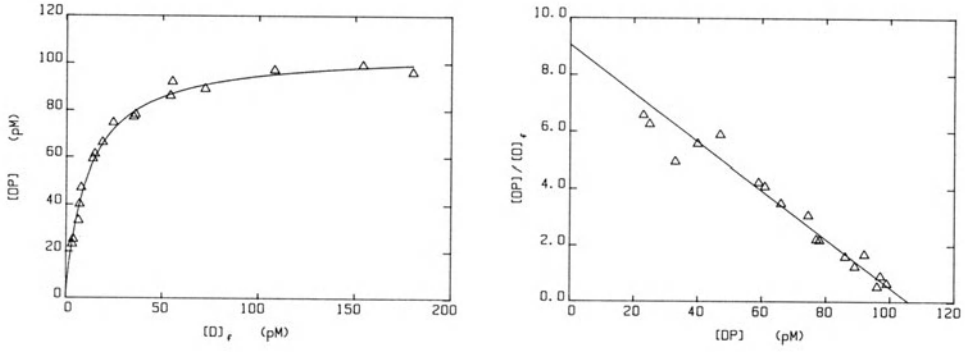


Fig. 4: Equilibrium binding constant of NHP1 with the estrogen response element: Purified protein eluted from the oligonucleotide affinity column (0.3 ng) was titrated with increasing concentrations of ^{32}P-labelled ERE in the presence of 1 ng of *E. coli* DNA in a total volume of 5 µl. After a 15-min incubation at room temperature, the complex was separated from the oligonucleotide on a 5% native polyacrylamide gel. After autoradiography, the free and bound oligonucleotides were cut out of the gel and assayed for radioactivity (Hughes et al., 1989). The titration curve is shown in the upper panel. Analysis of these data according to Eq. 4a using the computer programe yielded the following values for the parameters: $[P]_t$ = 106 ± 2 pM, K_d = 11.7 ± 0.7 pM and K_{eq} = 8.6 ± 0.5 × 10^{10} M^{-1}. The curve in the upper panel was drawn using these values. The lower panel shows the data in the form of a Scatchard plot (Eq. 5); the line was drawn using the above values for the parameters in Eq. 5.

Step-by-Step Procedure

> In a series of silicone treated Eppendorf tubes, add to the binding buffer a constant amount of protein extract in a volume of 1 µl (the amount will largely depend on the degree of purification of the protein).
Bovine serum albumin (ca. 0.5 mg/ml) can be included in order to stabilise the protein.

> Add a constant amount of non-specific competing DNA (determined by gel-shift assays).

> Add increasing concentrations of the labelled DNA in a volume of 4 µl. For the initial experiment, try a wide range (10^{-14} to 10^{-8} M ; about 10^{-19} to 10^{-13} moles in 4 µl).

> Incubate at 20°C for 20 min.

> Add 1 µl of loading buffer without dye.

> Load reaction mixture onto a 4% or 5 % native polyacrylamide gel (30 : 1) in 0.25× TBE buffer.

> Put loading buffer with 0.05% bromophenol blue in adjacent slots.

> Separate by electrophoresis at 20 mA (constant current) for about 1 h or until the bromophenol blue migrates to about 7 cm from the top.

> Take off the top glass plate, put radioactive markers on the gel for orientation, cover with Saran wrap and expose to an X-ray film at 4°C.

> Cut out the bands corresponding to the bound and free DNA with a razor blade. A blank piece of gel is also removed to indicate the background level of radioactivity. Some investigators find it more convenient to work with dried gels; in this case, the radioactive bands and a blank piece of gel are simply cut out of the dried gel using a razor blade or scissors. Each band is put into a scintillation vial with 8 ml scintillation fluid and the ^{32}P content measured by liquid scintillation counting for 10 min/sample. Longer or shorter times of counting may be used depending on the amount of radioactivity in the bands.

> The initial titration should be carried out to determine the concentrations of binding protein and oligonucleotide to be

used for the determination of $[P]_t$ and K_d. The total amount of active protein added will determine the amount of radioactivity incorporated into the band corresponding to the complex and should be adjusted to give a satisfactory level of incorporation. The amount of oligonucleotide added should be sufficient to ensure that the plateau in the curve (bound vs total) is reached (see Fig. 4). The oligonucleotide and/or protein concentration should be varied so that ideally two to three points are at the plateau, and two-three points are at the start of the curve, and the majority of the rest of the points are in the region of maximum curvature just before the curve reaches the plateau. These are the most important points for the determination of the K_d.

> After suitable titration has been obtained, the K_d can be calculated by fitting the data using the computer programe or a Scatchard plot (see data- analysis section).

Remarks

Much tighter bands are obtained if the native gel (0.5-mm-thick) is run in 0.25-× TBE. The tighter bands result in a smaller piece of gel being excised, which reduces quenching during liquid scintillation counting.

4 Determination of the Dissociation Rate Constant k_{off}

The dissociation rate constant is determined by first forming a protein-DNA complex and then adding an excess of the non-labelled DNA substrate to sequester the protein as it dissociates from the labelled DNA substrate (Hendrickson, 1985). The dissociation rate constant is determined by following the disappearence of the original complex with time. As an example, a protein with a K_d of 10^{-10} M will be taken. The active protein P (at a final concentration of 10^{-12} M) is incubated with 10^{-10} M final concentration of labelled DNA. The incubation time should be sufficient to reach binding equilibrium. Measurement of dissociation can then be initiated by the addition of a 60-fold molar excess of the non-labelled oligonucleotide diluted in the binding buffer. At regular intervals (every 5 or 10 min depending on the protein and the binding temperature) aliquots are taken and chilled on ice prior to electrophoresis in a low-salt gel at 4°C. The data are then analysed using the computer programe or by plotting the results according to Eq. 8 (see data-analysis section).

Step-by-Step-Procedure

> Dilute the active protein into binding buffer to give a final concentration of 10^{-12} M in 200 µl.

> Add the labelled substrate DNA to a final concentration of 10^{-10} M with an appropriate amount of non-specific competing DNA, if needed.

> Incubate for 20 min at 20°C.

> Save 20 µl for the zero-time control and put it on ice.

> Add a 50- to 100-fold molar excess of the non-labelled substrate oligonucleotide diluted in binding buffer.

> Continue the incubation at 20°C; at regular intervals (5 or 10 min) take 20 µl aliquots and put them on ice.

> At the completion of the time course, add 5 µl of the loading buffer to each sample. Load an aliquot onto a 5% polyacrylamide non-denaturating gel in 1× TEA or 0.25× TBE in the cold room and separate the protein-DNA complex from the free DNA.

> Put radioactive markers onto the gel for orientation and autoradiograph.

> Cut out the band corresponding to the protein-DNA complex and determine the radioactivity as indicated above. Correct the data for any dilutions made, e.g. the zero-time point was taken before competing DNA was added and must be corrected for this dilution.

> Estimate k_{off} by fitting the data to Eq. 7 using the computer programe or by plotting the results according to Eq. 8 (see data-analysis section). Analysis of the data given in Fig. 5 yielded a value for k_{off} of $1.01 \pm (0.02) \times 10^{-3}$ s^{-1}. It should be noted that the standard errors of parameters obtained from progress-curve data often underestimate the true error (Cornish-Bowden, 1976) and, thus, do not necessarily reflect the accuracy of the data.

> An accurate estimate of k_{off} is only obtained if the reaction is followed for several half-lives. Therefore, the length of the time course should be adjusted such that a significant decrease in the amount (ca. 90%) of labelled complex is

observed during the time course. The sampling interval can then be adjusted such that about ten points are obtained.

> If the kinetics of dissociation are very slow, the samples should be separated on the gel immediately upon their removal from the incubation mixture.
> Alternatively, the reaction could be started in separate tubes at different times, and all tubes stopped at the same time in order to achieve different reaction times.

5 Determination of the Association Rate Constant k_{on}

The association rate constant is determined by measuring the fraction of complex formed as a function of time. Below we describe a k_{on} measurement for a protein with a K_d of 10^{-10} M.

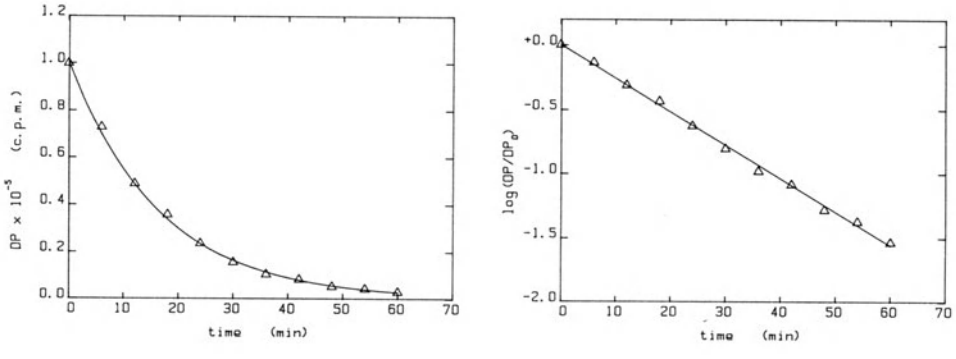


Fig. 5: Determination of the dissociation rate constant (k_{off}): The data were simulated using the experimental conditions outlined in the Step-by-Step Procedure. It was assumed that the radioactivity of the complex at zero time was 1×10^5 cpm and that the value of k_{off} was 1×10^{-3} s^{-1}. A random, normally distributed error of 5% was introduced into the simulated data (Pollard, 1977). The data were analysed according to Eq. 7 and the curve drawn shows the fit that was obtained. Values of $1.01 \pm 0.02 \times 10^{-3}$ s^{-1} and $1.01 \pm 0.01 \times 10^5$ cpm were obtained for k_{off} and $[DP]_0$, respectively. In the lower panel, the data are plotted according to Eq. 8b; the line was drawn using the above values for k_{off} and $[DP]_0$.

Step-by-Step-Procedure

The reaction mixture is assembled on ice.

> For a total volume of 200 µl, add the active protein to a final concentration of about 10^{-12} M in the binding buffer and 0.5 mg BSA/ml.

> Add an appropriate amount of non-specific competing DNA.

> Take out a 20 µl aliquot at zero time and keep on ice.

> Add the labelled oligonucleotide substrate to a final concentration of 10^{-9} M. The concentration of labelled oligonucleotide used should ideally be at least ten times higher than the K_d value, as this facilitates analysis of the data (see data-analysis section).

> Incubate at 20°C and retrieve 20 µl aliquots at regular intervals (for example every 5 min); chill the aliquots on ice. Mix with a 50-fold molar excess of the non-labelled oligonucleotide substrate to quench the association of the protein with the DNA.

> Separate free and bound DNA by gel electrophoresis in the cold room and process the gels as indicated above.

> Fit the data to Eq. 13 by using the computer programe or plot the data according to Eqs. 15b or 15c to obtain an estimate of k_{on} as described in the data-analysis section. If it was not possible to use a concentration of oligonucleotide in excess of the value of K_d, then the data should be fitted to Eq. 12. In this case, an estimate of the concentration of the complex at equilibrium will be needed. From the data of Fig. 6, a value of $1.03\ (\pm 0.08) \times 10^{-3}\ s^{-1}$ was estimated for the apparent first-order rate constant k'. The value of k' divided by the concentration of DNA gave a value for k_{on} of $1.03\ (\pm 0.08) \times 10^{6}\ M^{-1}s^{-1}$.

> Once again it should be noted that an accurate estimate of k' is only obtained if the reaction is followed for several half-lives, i.e. the reaction should be followed until at least 90% of the active binding protein has been converted into the DNA-protein complex.

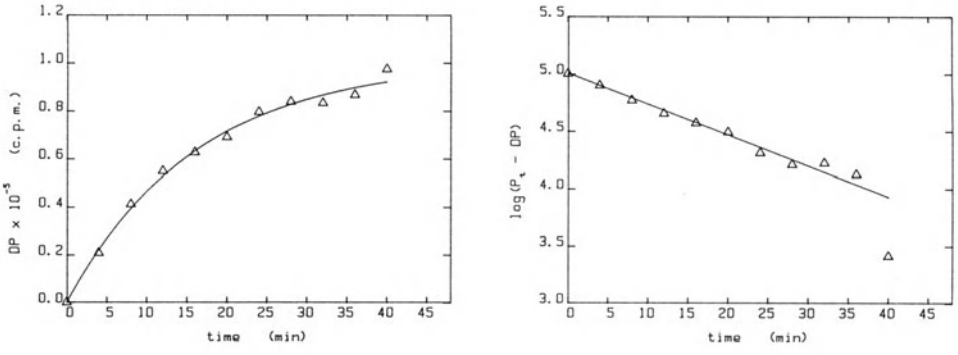


Fig. 6: Determination of the association rate constant (k_{on}): The data were simulated using the experimental conditions outlined in the Step-by-Step Procedure. It was assumed that the total radioactivity that could be bound by the protein at equilibrium was 1×10^5 cpm and that the value of k' was 1×10^{-3} s^{-1}. Once again, a random, normally distributed error of 5% was introduced into each simulated data point (Pollard, 1977). The data were analysed according to Eq. 13 and the curve drawn shows the fit that was obtained. Values of $1.03 \pm 0.08 \times 10^{-3}$ s^{-1} and $1.01 \pm 0.03 \times 10^5$ cpm were obtained for k' and $[P]_t$, respectively. In the lower panel, the data are plotted according to Eq. 15c; the line was drawn using the above values for k' and $[P]_t$.

6 Comparison of K_d, k_{on} and k_{off}

For a protein-DNA interaction in which the complex is formed in a single step and also dissociates in one step, the values obtained for K_d, k_{on} and k_{off} are not independent but are related to each other by Eq. 2, i.e. $K_d = k_{off}/k_{on}$. Thus, if values of all three parameters are obtained, the experimentally determined value of K_d can be compared with the one calculated from the ratio k_{off}/k_{on}. A discrepancy between the experimentally determined and the calculated values suggests the incorrect estimation of one of the parameters, and one or more of the values should be redetermined. If, however, the experimenter is confident that the values obtained are correct, a discrepancy between the calculated and experimentally determined values of K_d probably indicates that the formation and dissociation of the complex require more than one step.

E Bibliography

Cann, J.R. Multiple electrophoretic zone arising from protein-buffer interactions. Biochemistry 5 (1966) 1108–1112.

Carey, J. Gel retardation at low pH resolves trp repressor DNA complexes for quantitative study. Proc. Natl. Acad. Sci. USA 85 (1988) 975–979.

Chelm, B. and Geiduschek, E. Gel electrophoretic separation of transcriptional complexes: an assay for RNA polymerase selectivity and a method for promotor mapping. Nucleic Acids Res. 7 (1979) 1851–1867.

Chodosh, L. A., Carthew, R. W. and Sharp, P. A. A single polypeptide possesses the binding and transcription activities of the adenovirus major late transcription factor. Mol. Cell. Biochem. 12 (1986) 4723–4733.

Cooper, T. G. Electrophoresis, pp 194–233 (1977). In Tools of Biochemistry, John Wiley & Sons, New York.

Cornish-Bowden, A. Principles of Enzyme Kinetics (1976), Butterworths, London.

Crothers, D.M. Gel electrophoresis of protein-DNA complexes. Nature 325 (1987) 464–465.

Duggleby, R.G. A nonlinear regression program for small computers. Anal. Biochem. 110 (1981) 9–18.

Fried, M. and Crothers, M. Equilibrium and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. Nucleic Acids Res. 9 (1981) 6505–6525.

Garner, M.M. and Revzin, A. A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the *Escherichia coli* lactose operon regulatory system. Nucleic Acids Res. 9 (1981) 3047–3060.

Hendrickson, W. and Schleif, R. Regulation of the *E. coli* L-arabinose operon studied by gel electrophoresis DNA binding assay. J. Mol. Biol. 178 (1984) 611–628.

Hendrickson, W. Protein-DNA interaction studies by the gel electrophoresis-DNA binding assay. Bio Techniques 3 (1985) 198–207.

Hughes, M.J., Liang, H. Jiricny, J. and Jost, J.P. Purification and characterization of a protein from HeLa cells that binds with high affinity to the estrogen response element GGTCAGCGTGACC. Biochemistry 28 (1989) 9137–9142.

Hughes, M.J. and Jost, J.P. The ubiquitous nuclear protein, NHP1 binds with high affinity to different sequences of the chicken vitellogenin II gene. Nucleic Acids Res. 17 (1989) 8511–8520.

Ito, J., Kawamura, F. and Uanofsky, S. Analysis of phi 29 and phi 15 genomes by bacterial restriction endonuclease, EcdRI and HpaI. Virology 70 (1976) 37–91.

Jost, J.P., Saluz, H.P., Jiricny, J., and Moncharmont, B. Estradiol-dependent trans-acting factor binds preferentially to a diad-symmetry structure within the third intron of the avian vitellogenin gene. J. Cell. Biochem. 35 (1987) 69–82.

Maniatis, T., Fritsch, E. F. and Sambrook, J. Molecular cloning. A laboratory manual (1982) 545 p, Cold Spring Harbor Laboratory.
McEntee, K., Weinstock, G.M. and Lehman, I.R. Binding of the rec A protein of *Escherichia coli* to single- and double-stranded DNA. J. Biol. Chem. 256 (1981) 8835–8844.
Pollard, J.H., A Handbook of Numerical and Statistical Techniques (1977), Cambridge University Press, Cambridge.
Revzin, A. Gel electrophoresis assays for DNA-protein interactions. Biotechniques 7 (1989) 346–355.
Riggs, A.D., Bourgeois, S. and Cohn, M. On the assay isolation and characterization of the lac repressor. J. Mol. Biol. 34 (1968) 361–364.
Riggs, A.D., Suzuki, H. and Bourgeois, S. Lac repressor-operator interaction I. Equilibrium studies. J. Mol. Biol. 48 (1970) 67–83.
Riggs, A.D., Bourgeois, S. and Cohn, M. The lac repressor-operator interaction III kinetics studies. J. Mol. Biol 53 (1970) 401–417.
Sharp, P., Moore, C. and Haverty, J. The infectivity of adenovirus 5 DNA-protein complex. Virology 75 (1976) 442–456.
Von Hippel, P. and Berg, O.G. On the specificity of DNA-protein interactions. Proc. Natl. Adad. Sci. USA 83 (1986) 1608–1612.
Wu, M. and Crothers, D. The locus of sequence-directed and B-protein-induced DNA binding. Nature 308 (1984) 509–513.

F Computer Programs for the Analysis of Binding and Kinetic Data

The following three computer programes have been written in BASIC for the analysis of data to obtain binding constants as well as kinetic constants for the association and dissociation of protein-DNA complexes. The programes were originally based on the non-linear regression programe written by Duggleby (1981). The programes should run with most forms of BASIC. The authors have tested BASICA, GW-BASIC, TURBO-BASIC and QUICK-BASIC. There are undoubtedly more efficient and more elegant ways of writing programes capable of achieving the same results. However, similar programes have been used over a number of years in one of our laboratories (S.R.S.) for the analysis of many different types of data. Therefore, a listing of the programe is provided for the readers who may be interested in statistical analysis of their data. Three programes were used for the analysis of data: BIND was used to analyse binding data according to Eq. 4, RATEOFF was used to determine the dissociation rate constant (analysis according to Eq. 7), and RATEON was used for the determination of association rate constant (analysis according to Eq. 13). Non-linear regression analysis requires starting estimates for the parameters. In the programes, starting estimates are obtained by linear regression using one of the linear transforms of the equations given in the text. The data are then graphed on the screen and the fit to the data using these starting estimates is shown. The researcher is then given the chance to alter the starting estimates if he feels that linear regression has provided the wrong ones; occasionally, non-linear regression programes will fail if the initial estimates are too inaccurate. After the initial estimates are accepted, the programes performs the non-linear regression analysis, prints out the results and graphs the data on the screen to show the fit obtained. The following is a listing for BIND:

```
10 DEF FNBIND(B1,B2,D)=B1*D/(B2+D)' B1=Pt: B2=Kd: D=D
20 DIM Y(200),D(200),A(9,10)
30 ON ERROR GOTO 3000
40 INPUT "Enter the name of the datafile";DFILE$
50 OPEN DFILE$ FOR INPUT AS #1' read data from data file
60 LINE INPUT #1, TITLE$
70 INPUT #1, NPTS
80 FOR I=1 TO NPTS
90 INPUT #1, D(I),Y(I)
100 NEXT I
110 CLOSE #1
120 NP=2' calculate the initial estimates
130 FOR I=1 TO NP+1
140 FOR J=1 TO NP+2
150 A(I,J)=0
```

```
160 NEXT J
170 NEXT I
180 FOR I=1 TO NPTS
190 WT=1' set weighting if required
200 X(1)=1
210 X(2)=Y(I)
220 X(3)=Y(I)/D(I)
230 FOR K=1 TO NP
240 FOR L=1 TO NP+1
250 A(K,L)=A(K,L)+X(K)*X(L)*WT
260 NEXT L
270 NEXT K
280 NEXT I
290 GOSUB 2000
300 B(1)=-A(1,1)/A(2,1)
310 B(2)=-1/A(2,1)
320 NCALL=1
330 GOSUB 4000' graph the data and correct initial estimates
340 IT=0' if necessary
350 IT=IT+1' begin the iterations
360 IF IT>15 THEN 930
370 FOR I=1 TO NP+1
380 FOR J=1 TO NP+2
390 A(I,J)=0
400 NEXT J
410 NEXT I
420 S2=0
430 FOR I=1 TO NPTS
440 WT=1' set weighting if required
450 Z=Y(I)-FNBIND(B(1),B(2),D(I))
460 X(1)=D(I)/(B(2)+D(I))
470 X(2)=-B(1)*D(I)/(B(2)+D(I))^2
480 X(3)=Z
490 FOR K=1 TO NP
500 FOR L=1 TO NP+1
510 A(K,L)=A(K,L)+X(K)*X(L)*WT
520 NEXT L
530 NEXT K
540 S2=S2+Z^2*WT
550 NEXT I
560 GOSUB 2000' solve the normal equations
570 C=0
580 FOR I=1 TO NP' correct the parameters and test for conver-
gence
590 C=C+ABS(A(I,1)/B(I))
600 NEXT I
610 FOR I=1 TO NP
```

```
620 B(I)=B(I)+A(I,1)
630 NEXT I
635 IF (B(1)<0) OR (B(2)<0) THEN PRINT "** Pt or Kd < 0 **":
DV$="Y": GOTO 120
640 IF C>.00001 THEN 350
650 FOR I=1 TO NP' run has converged - print the results
660 FOR J=2 TO NP+1
670 A(I,J)=A(I,J)*S0(I)*S0(J-1)
680 NEXT J
690 NEXT I
700 V=S2/(NPTS-NP)
710 OPEN "lpt1:" FOR OUTPUT AS #1
720 PRINT #1,TAB(15) TITLE$
730 PRINT #1,:PRINT #1, "Data were taken from ";DFILE$
740 PRINT #1,:PRINT #1, "final values . . ."
750 PRINT #1,
760 PRINT #1," Pt = ";B(1);" +/- ";SQR(A(1,2)*V)
770 PRINT #1," Kd = ";B(2);" +/- ";SQR(A(2,3)*V)
780 PRINT #1," Keq = ";1/B(2);" +/- ";SQR(A(2,3)*V/B(2)^4)
790 P2$="####.## ####.### ####.### +###.### ####.### ####.###"
800 PRINT #1,:PRINT #1,
810 PRINT #1," D DP(obs) DP(cal) diff DP(obs)/D DP(cal)/D"
820 PRINT #1,
830 FOR I=1 TO NPTS
840 YHAT=FNBIND(B(1),B(2),D(I))
850 PRINT #1,USING P2$; D(I),Y(I),YHAT,Y(I)-
YHAT,Y(I)/D(I),YHAT/D(I)
860 NEXT I
870 PRINT #1,:PRINT #1,"variance = ";V;" with ";NPTS-NP;"
degrees of freedom"
880 PRINT #1,CHR$(12)
890 CLOSE #1
900 NCALL=2
910 GOSUB 4000
920 END
930 OPEN "lpt1:" FOR OUTPUT AS #1
940 PRINT #1,"iteration limit exceeded"
950 PRINT #1,
960 CLOSE #1
970 GOTO 650
2000 REM
2010 REM matrix solution subroutine
2020 REM
2030 FOR I=1 TO NP
2040 S0(I)=1/SQR(A(I,I))
2050 NEXT I
2060 S0(NP+1)=1
```

```
2070 FOR I=1 TO NP
2080 FOR J=1 TO NP+1
2090 A(I,J)=A(I,J)*S0(I)*S0(J)
2100 NEXT J
2110 NEXT I
2120 S1(NP+1)=-1
2130 A(1,NP+2)=1
2140 FOR I=1 TO NP
2150 FOR J=1 TO NP
2160 S1(J)=A(J,1)
2170 NEXT J
2180 FOR J=1 TO NP+1
2190 FOR K=1 TO NP
2200 A(K,J)=A(K+1,J+1)-S1(K+1)*A(1,J+1)/S1(1)
2210 NEXT K
2220 NEXT J
2230 NEXT I
2240 FOR I=1 TO NP
2250 A(I,1)=A(I,1)*S0(I)
2260 NEXT I
2270 RETURN
3000 REM Error handling subroutine
3010 IF (ERR=53) OR (ERR=75) OR (ERR=76) THEN 3020 ELSE 3030
3020 PRINT "File not found; try again": RESUME 40
3030 IF ERR=71 THEN PRINT "Disk drive not ready; try again": RE-
SUME 40
3040 IF ERR=62 THEN PRINT "Data not entered correctly":END
3050 IF (ERR=5) OR (ERR=6) OR (ERR=11) OR (ERR=7) THEN 3060
ELSE 3080
3060 INPUT "Program appears to have diverged; press ENTER to
continue";R$
3070 DV$="Y": SCREEN 0: RESUME 120
3080 IF (ERR=57) THEN 3090 ELSE 3100
3090 CLOSE #1: INPUT "Printer probably not ready";R$: RESUME 710
3100 ON ERROR GOTO 0
3110 RESUME
4000 REM
4010 REM graphics subroutine
4020 REM
4030 IF NCALL=2 THEN 4210
4040 IF DV$="Y" THEN 4660
4050 XMAX=0
4060 YMAX=0
4070 FOR I=1 TO NPTS
4080 IF D(I)>XMAX THEN XMAX=D(I)
4090 IF Y(I)>YMAX THEN YMAX=Y(I)
4100 NEXT I
```

```
4110 PRINT:PRINT "largest value of D is ";XMAX
4120 PRINT:PRINT "largest value of Y is ";YMAX
4130 PRINT:PRINT "Enter maximum values for the X and Y axes
(XMAX,YMAX)"
4140 INPUT XMAX,YMAX
4150 PRINT:PRINT "Enter scaling intervals for the X and Y axes
(XINT,YINT)"
4160 INPUT XINT,YINT
4170 XSCALE=480/XMAX
4180 YSCALE=188/YMAX
4190 XINT1=XSCALE*XINT
4200 YINT1=YSCALE*YINT
4210 SCREEN 2
4220 CLS
4230 KEY OFF
4240 LINE(47,3)-(47,191)' draw the axes
4250 LINE(47,191)-(527,191)
4260 DRAW "BM47,191"
4270 FOR I=XINT TO XMAX STEP XINT' put the intervals on the axes
4280 DRAW "R="+VARPTR$(XINT1)
4290 DRAW "NU2"
4300 NEXT I
4310 DRAW "BM47,191"
4320 FOR I=YINT TO YMAX STEP YINT
4330 DRAW "U="+VARPTR$(YINT1)
4340 DRAW "NR6"
4350 NEXT I
4360 LOCATE 25,6
4370 FOR X=0 TO XMAX STEP XINT' label the axes
4380 IF X=0 THEN 4400
4390 LOCATE 25,60*X/XMAX+5
4400 PRINT X;
4410 NEXT X
4420 LOCATE 24,2
4430 FOR Y=0 TO YMAX STEP YINT
4440 IF Y=0 THEN 4460
4450 LOCATE 23-22*Y/YMAX,2
4460 PRINT USING "###.##";Y;
4470 NEXT Y
4480 FOR I=1 TO NPTS' plot the points
4490 X1=D(I)*XSCALE+47
4500 Y1=191-Y(I)*YSCALE
4510 CIRCLE (X1,Y1),4
4520 NEXT I
4530 DRAW "bm47,191"
4540 FOR X=0 TO D(NPTS) STEP D(NPTS)/100' draw the curve
4550 Y=FNBIND(B(1),B(2),X)
```

```
4560 Y1=191-Y*YSCALE
4570 X1=X*XSCALE+47
4580 LINE -(X1,Y1)
4590 NEXT X
4600 IF NCALL=2 THEN 4730
4610 LOCATE 1,15
4620 PRINT "Do you want to change the values of the parameters
(y/n)";
4630 INPUT CHANGE$
4640 SCREEN 0
4650 IF (CHANGE$="n") OR (CHANGE$="N") THEN RETURN
4660 PRINT :PRINT "Current values of the parameters are . . ."
4670 PRINT
4680 PRINT " Pt = ";B(1)
4690 PRINT " Kd = ";B(2)
4700 PRINT :PRINT "Enter new values for Pt and Kd"
4710 INPUT B(1),B(2)
4720 GOTO 4210
4730 LOCATE 1,15
4740 PRINT "Enter <RETURN> to return to the alpha mode";
4750 INPUT R$
4760 SCREEN 0
4770 RETURN
```

The programe RATEOFF that is used to estimate the dissociation rate constant (k_{off}) is basically the same as the above programe.

```
10 DEF FNRATE(B1,B2,T)=B1*EXP(-B2*T)' B1=DP0: B2=k'
20 DIM Y(200),T(200),A(9,10)
30 ON ERROR GOTO 3000
40 INPUT "Enter the name of the datafile";DFILE$
50 OPEN DFILE$ FOR INPUT AS #1' read data from data file
60 LINE INPUT #1, TITLE$
70 INPUT #1, NPTS
80 FOR I=1 TO NPTS
90 INPUT #1, T(I),Y(I)
100 NEXT I
110 CLOSE #1
105 PRINT: INPUT "Enter the concentration of DP at zero time";B1
120 NP=1' calculate the initial estimates
130 FOR I=1 TO NP+1
140 FOR J=1 TO NP+2
150 A(I,J)=0
160 NEXT J
170 NEXT I
180 FOR I=1 TO NPTS
```

```
185 IF T(1)=0 THEN B(1)=Y(1) ELSE B(1)=B1
190 WT=1
200 X(1)=T(I)
210 X(2)=LOG(Y(I)/B(1))
230 FOR K=1 TO NP
240 FOR L=1 TO NP+1
250 A(K,L)=A(K,L)+X(K)*X(L)*WT
260 NEXT L
270 NEXT K
280 NEXT I
290 GOSUB 2000
300 B(2)=-A(1,1)
310 NP=2
320 NCALL=1
330 GOSUB 4000' graph the data and correct initial estimates
340 IT=0' if necessary
350 IT=IT+1' begin the iterations
360 IF IT>15 THEN 930
370 FOR I=1 TO NP+1
380 FOR J=1 TO NP+2
390 A(I,J)=0
400 NEXT J
410 NEXT I
420 S2=0
430 FOR I=1 TO NPTS
440 WT=1
450 Z=Y(I)-FNRATE(B(1),B(2),T(I))
460 X(1)=EXP(-B(2)*T(I))
470 X(2)=-B(1)*T(I)*EXP(-B(2)*T(I))
480 X(3)=Z
490 FOR K=1 TO NP
500 FOR L=1 TO NP+1
510 A(K,L)=A(K,L)+X(K)*X(L)*WT
520 NEXT L
530 NEXT K
540 S2=S2+Z^2*WT
550 NEXT I
560 GOSUB 2000' solve the normal equations
570 C=0
580 FOR I=1 TO NP' correct the parameters and test for conver-
gence
590 C=C+ABS(A(I,1)/B(I))
600 NEXT I
610 FOR I=1 TO NP
620 B(I)=B(I)+A(I,1)
630 NEXT I
640 IF C>.00001 THEN 350
```

```
650 FOR I=1 TO NP' run has converged - print the results
660 FOR J=2 TO NP+1
670 A(I,J)=A(I,J)*S0(I)*S0(J-1)
680 NEXT J
690 NEXT I
700 V=S2/(NPTS-NP)
710 OPEN "lpt1:" FOR OUTPUT AS #1
720 PRINT #1,TAB(15) TITLE$
730 PRINT #1,:PRINT #1, "Data were taken from ";DFILE$
740 PRINT #1,:PRINT #1, "final values . . ."
750 PRINT #1,
755 P1$ = "& #.###^^^^ & #.###^^^^"
760 PRINT #1, USING P1$; " DP0(entered) = ";B1
770 PRINT #1, USING P1$; " DP0(det'd) = ";B(1);" +/-
";SQR(A(1,2)*V)
780 PRINT #1, USING P1$; " k' = ";B(2);" +/- ";SQR(A(2,3)*V)
790 P2$="####.## #.###^^^^ #.###^^^^ +#.###^^^^"
800 PRINT #1,:PRINT #1,
810 PRINT #1," t DP(obs) DP(cal) diff "
820 PRINT #1,
830 FOR I=1 TO NPTS
840 YHAT=FNRATE(B(1),B(2),T(I))
850 PRINT #1,USING P2$; T(I),Y(I),YHAT,Y(I)-YHAT
860 NEXT I


lines 870 - 4060 are identical to those in BIND


4070 FOR I=1 TO NPTS
4080 IF T(I)>XMAX THEN XMAX=T(I)
4090 IF Y(I)>YMAX THEN YMAX=Y(I)
4100 NEXT I
4110 PRINT:PRINT "largest value of D is ";XMAX
4120 PRINT:PRINT "largest value of Y is ";YMAX
4130 PRINT:PRINT "Enter maximum values for the X and Y axes
(XMAX,YMAX)"
4140 INPUT XMAX,YMAX
4150 PRINT:PRINT "Enter scaling intervals for the X and Y axes
(XINT,YINT)"
4160 INPUT XINT,YINT
4170 XSCALE=480/XMAX
4180 YSCALE=188/YMAX
4190 XINT1=XSCALE*XINT
4200 YINT1=YSCALE*YINT
4210 SCREEN 2
4220 CLS
```

```
4230 KEY OFF
4240 LINE(47,3)-(47,191)' draw the axes
4250 LINE(47,191)-(527,191)
4260 DRAW "BM47,191"
4270 FOR I=XINT TO XMAX STEP XINT' put the intervals on the axes
4280 DRAW "R="+VARPTR$(XINT1)
4290 DRAW "NU2"
4300 NEXT I
4310 DRAW "BM47,191"
4320 FOR I=YINT TO YMAX STEP YINT
4330 DRAW "U="+VARPTR$(YINT1)
4340 DRAW "NR6"
4350 NEXT I
4360 LOCATE 25,6
4370 FOR X=0 TO XMAX STEP XINT' label the axes
4380 IF X=0 THEN 4400
4390 LOCATE 25,60*X/XMAX+5
4400 PRINT X;
4410 NEXT X
4420 LOCATE 24,2
4430 FOR Y=0 TO YMAX STEP YINT
4440 IF Y=0 THEN 4460
4450 LOCATE 23-22*Y/YMAX,2
4460 PRINT USING "#.#^^^^";Y;
4470 NEXT Y
4480 FOR I=1 TO NPTS' plot the points
4490 X1=T(I)*XSCALE+47
4500 Y1=191-Y(I)*YSCALE
4510 CIRCLE (X1,Y1),4
4520 NEXT I
4530 DRAW "bm47,191"
4540 FOR X=0 TO T(NPTS) STEP T(NPTS)/100' draw the curve
4550 Y=FNRATE(B(1),B(2),X)
4560 Y1=191-Y*YSCALE
4570 X1=X*XSCALE+47
4580 LINE -(X1,Y1)
4590 NEXT X
4600 IF NCALL=2 THEN 4730
4610 LOCATE 1,15
4620 PRINT "Do you want to change the values of the parameters
(y/n)";
4630 INPUT CHANGE$
4640 SCREEN 0
4650 IF (CHANGE$="n") OR (CHANGE$="N") THEN RETURN
4660 PRINT :PRINT "Current values of the parameters are . . ."
4670 PRINT
4680 PRINT " DP0 = ";B(1)
```

```
4690 PRINT " k' = ";B(2)
4700 PRINT :PRINT "Enter new values for DP0 and k'"
4710 INPUT B(1),B(2)
4720 GOTO 4210
4730 LOCATE 1,15
4740 PRINT "Enter <RETURN> to return to the alpha mode";
4750 INPUT R$
4760 SCREEN 0
4770 RETURN
```

The programe RATEON for the estimation of the association rate constant (k_{on}) is identical to RATEOFF with the following substitutions:

```
10 DEF FNRATE(B1,B2,T)=B1*(1-EXP(-B2*T))' B1=Pt: B2=k'


105 PRINT: INPUT "Enter the concentration of P at zero time";B1
120 NP=2' calculate the initial estimates
130 FOR I=1 TO NP+1
140 FOR J=1 TO NP+2
150 A(I,J)=0
160 NEXT J
170 NEXT I
180 FOR I=1 TO NPTS
185 IF Y(I)>B1 THEN 280
190 WT=1
200 X(1)=1
210 X(2)=T(I)
220 X(3)=LOG(B1-Y(I))
230 FOR K=1 TO NP
240 FOR L=1 TO NP+1
250 A(K,L)=A(K,L)+X(K)*X(L)*WT
260 NEXT L
270 NEXT K
280 NEXT I
290 GOSUB 2000
300 B(2)=-A(2,1)
310 B(1)=B1

460 X(1)=1-EXP(-B(2)*T(I))
470 X(2)=B(1)*T(I)*EXP(-B(2)*T(I))


4680 PRINT " Pt = ";B(1)
4690 PRINT " k' = ";B(2)
4700 PRINT :PRINT "Enter new values for Pt and k'"
```

The data is entered in the same format for each of the three programes:

line 1: title

line 2: number of points (npts)

line 3 to line 3 + npts: the data points, one pair per line with the pair separated by a comma, i.e. for binding data, the free and bound concentrations should be separated by a comma; for rate data, the time and concentration should be separated by a comma.

Below is the set of data plotted in Fig. 4. Analysis of these data should yield the values for $[P]_t$ and K_d given in the text.

Binding NHP1 to ERE
17
3.5,23
4.0,25
6.7,33
7.2,40
8,47
14,59
15,61
19,66
24.5,74.5
35,77
36,78
54,86
72,89
55,92
108,97
154,99
180,96

XV Diagonal Gel Mobility-Shift Assays for the Resolution of Multi-Subunit Complexes Binding to Regulatory Elements of Specific Genes

Fred Schaufele, Brian L. West, Jean-Pierre Jost and Tim Reudelhuber

Contents

I Introduction

In gel mobility-shift assays, it often occurs that a number of different protein-DNA complexes are formed with a single labelled oligonucleotide. These different complexes may represent either: i) different proteins interacting with different, overlapping sites within the oligonucleotide, ii) a family of different proteins interacting with exactly the same site within the oligonucleotide, iii) differential modifications of a single protein binding to a single site, iv) multimeric complexes interacting with a single binding site through a common DNA-binding protein, or v) some combination of the above possibilities.

Whether the factor(s) present within each of the different gel-shifted complexes interacts with exactly the same binding-site nucleotides can be readily determined by observing if each complex is affected by the same series of point mutations within the site, and whether the methylation interference pattern (Chapter X) or the DNase I footprints (Chapter II) are identical for each complex isolated from the gel. Furthermore, cross-linking the protein to the DNA component present within each of the different complexes followed by SDS gel electrophoresis (Chapter VII) identifies whether each complex contains DNA-binding protein(s) of different or similar sizes. To further investigate the composition of such complexes, a rapid and simple technique, the diagonal, gel mobility-shift assay, was developed (Fig. 1). Briefly, the technique is a two-dimensional gel shift in which the gel-shift pattern formed in the first dimension is further resolved by electrophoresing that pattern perpendicular to the original direction of electrophoresis. Assume for the moment that three complexes were formed during electrophoresis in the first dimension (Fig. 1A). If each complex were to represent the binding of completely different-sized, monomeric proteins to the DNA, then each complex should have exactly the same mobility in the second dimension as it did in the first. Thus, the most slowly migrating complex would also migrate most slowly in the second dimension, and a fast migrating complex would also migrate more quickly in the second dimension; each complex would, therefore, migrate on a single straight line diagonal to the two directions of electrophoresis (Fig. 1B).

The diagonal line would also be observed even if each of the complexes were to represent different, but very stable, multimeric complexes interacting with the same DNA-binding site. However, if the conditions of electrophoresis were such that those multi-subunit complexes were quite unstable, then nucleoprotein complexes would be observed which no longer lie on the diagonal line, since some of the complexes would fall apart and thus migrate differently in the second dimension than in the first dimension (Fig. 1C). As it is the DNA-binding subunit which is labelled by virtue of its binding to the radiolabelled DNA, only the sequence-specific, DNA-binding subunit is followed by this assay. Furthermore, signals representing the dissociation of a multimeric subunit into smaller multi-subunit structures may also occur and would be represented by a second distinct signal (in addition to the signal from the

minimal DNA-binding complex) migrating below the diagonal line (Fig. 1D). The diagonal gel mobility-shift technique was used to investigate the composition of a series of complexes binding to a single site within the rat growth-hormone gene promoter (Schaufele et al. 1990). The pattern observed (Fig. 1E) was indicative of a series of multi-subunit complexes interacting with the binding site through a minimal DNA-binding subunit common to at least five different complexes (arrow, Fig. 1E). A schematic diagram of the diagonal gel-shift results, indicating the minimal composition of each complex, is presented in Fig. 1F.

B Materials and Methods

1 Materials and Buffers

- Glass plates 20 × 20 cm
- Spacers and slot formers 1-mm-thick
- Stock solution of 29:1 acrylamide:bisacrylamide in water
- 10× Running buffer: 67 mM Tris (8.1 g/l), 10 mM EDTA (3.7 g/l), 33 mM sodium acetate (4.5 g/l); pH 7.5 adjusted with acetic acid (ca. 4–5 ml/l)
- Pump and gel apparatus for recirculating buffer
- pH Indicator strips, non bleeding, pH 0–14 (Merck)
- Gel dryer

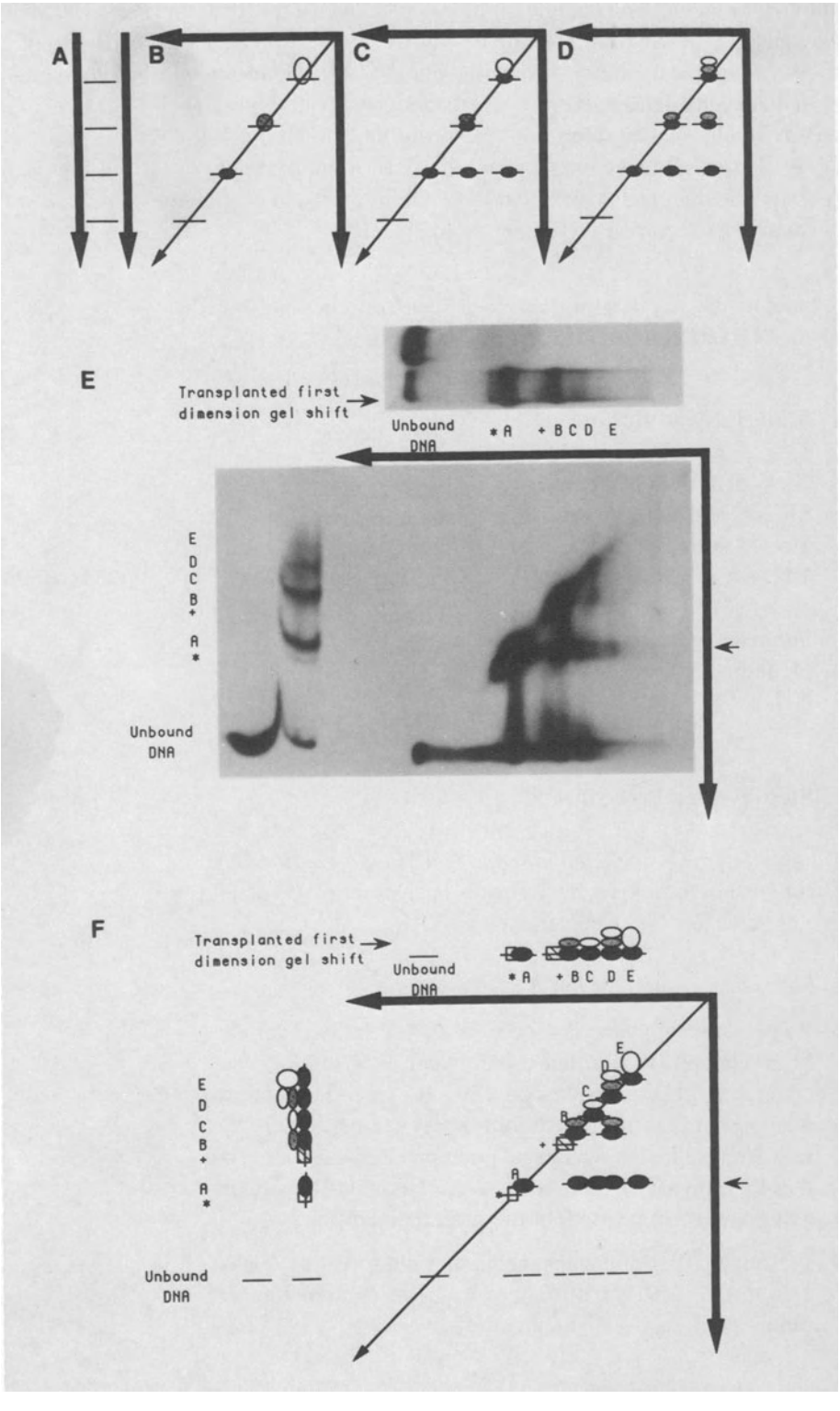
2 Step-by-Step Procedure

a) *Labelling of Oligonucleotides and Gel Mobility-Shift Assay*
These general techniques have already been described in detail in other chapters (see Chapters IV and VIII).

b) *First Dimension Gel-Shift Assay*

> Prepare two polyacrylamide gels (4% 29:1 acrylamide: bisacrylamide) in 1× running buffer and allow to cool in the cold room (gel dimensions: 20 × 20 × 0.1 cm). One gel (into which a comb is inserted to form slots) will be used for the first dimension; the second gel (with no slots and poured so that the polyacrylamide lies 1–2 cm below the top of the glass plate) will be used for the second dimension.

> Pre-run the first-dimension gel in the cold room for at least 1 h at 125–150 V (constant voltage) in recirculating 1× running buffer.


A
B
C
D
E
Transplanted first dimension gel shift
Unbound DNA
*A
+BCDE
EDCB+A*
Unbound DNA
F
Transplanted first dimension gel shift
Unbound DNA
*A
+BCDE
EDCB+A*
Unbound DNA

> Incubate DNA with the protein preparation (see Appendix), split into two and load onto two different slots. One lane will be used as a marker for the running of the first dimension. The second lane will be excised and run in the second dimension; the slots on either side of this lane should be loaded with 0.05% xylene cyanole and 0.05% bromophenol blue (in 10% glycerol) to facilitate the localisation of the lane to be excised.

> Continue running the gel at 125–150V until the bromophenol blue has migrated 7–10 cm from the top of the gel (running time: ca. 3 h).

> Approximately 1 h from the finish of the first-dimension gel, start pre-running the second-dimension gel, also at 125–150V with recirculating buffer.

c) *Second Dimension Gel-Shift Assay*

Two different procedures are presented for applying the first-dimension gel shift to the second-dimension gel. In Procedure 1, the gel slice containing the first dimension is placed on top of an already prepared second gel, whereas in Procedure 2, the second-dimension gel is poured around the gel slice. Both procedures have been used successfully in diagonal gel-shift assays (see Figs. 1 and 4)

Fig. 1: Hypothetical patterns formed when a lane from a gel shift (A,) is excised, placed horizontally over a second, identical gel and electrophoresed in the second dimension: They include: B, a diagonal, if each complex represents individual monomeric proteins of differing electrophoretic mobilities, C, a diagonal and signals of a common electrophoretic mobility in the second dimension, if the complexes are partially unstable multi-subunit entities which bind DNA through a common DNA-binding subunit; or D, the same pattern as in C, except that some higher-order complexes contain another subunit in common, in addition to the same DNA-binding subunit; E, Actual diagonal gel shift of the GHF3 factor binding to its site within the rat growth-hormone promoter. The observed pattern displays components of each of the above possibilities: *, +, and GHF3-A are solely located on the diagonal and of differing electrophoretic mobilities (as in Fig. 1B); GHF3-A, -B, -C, -D and -E contain a common DNA-binding subunit (as in Fig. C); GHF3-B and GHF3-D contain an additional subunit in common (as in Fig. 1D); F, Schematic representation of the gel shown in Fig. 1E with all of the signals labelled for clarification. This experiment was performed using Procedure 1. Reprinted with permission from J. Biol. Chem. 265 (1990) 14592–14598, copyright 1990 American Chemical Society.

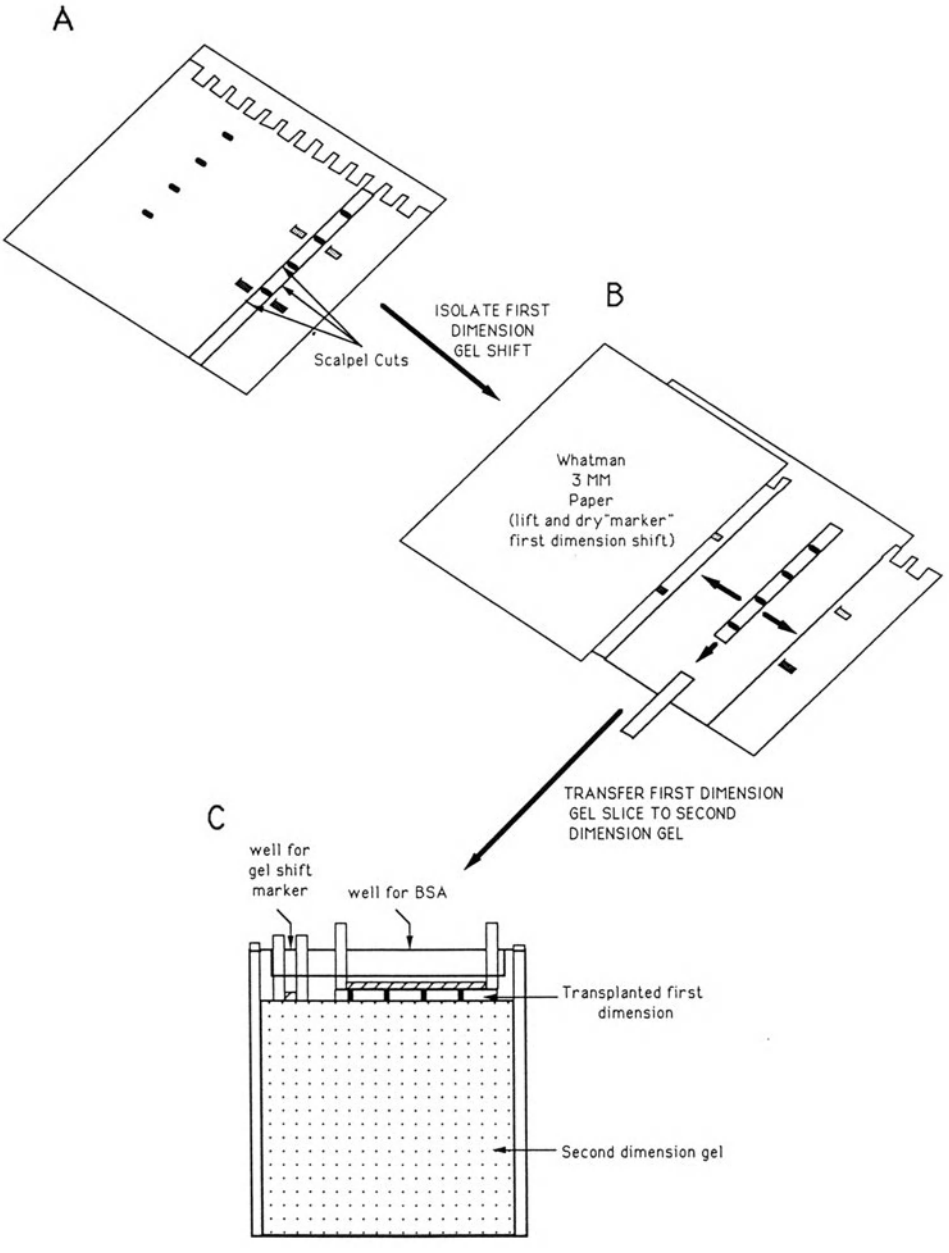


Fig. 2: Transfer of the gel slice from the first dimension to the second-dimension gel (Procedure 1): The first-dimension gel shift is localised by the xylene cyanole and bromophenol blue marker dyes in the adjacent lanes, cut out with a scalpel blade and a ruler (A), then isolated from the rest of the gel (B). The gel slice is then slid gently into the slot so that it now lies horizontally on top of the second-dimension gel (C). The applied current is thus perpendicular to the original direction of electrophoresis and will generate the patterns discussed in Fig. 1.

Procedure 1

> After electrophoresis in the first dimension, remove the top plate from the gel. Precise location of the lane containing the first-dimension gel-shift is facilitated by the xylene cyanole and bromophenol blue markers present on either side of the lane (Fig. 2A). These markers are used to align a ruler so that the first dimension can be excised using a scalpel blade.

> Remove the adjacent gel pieces from the glass plate leaving behind the single lane containing the gel shift. This is better than trying to pick up the single lane from the glass plate, since stretching of the polyacrylamide during handling will cause distortion of the gel shift pattern. The second "marker" first-dimension lane is best removed with minimal distortion by placing a piece of dry Whatman 3MM paper on top of it and lifting the 3MM paper with the attached gel (Fig. 2B). Care should be taken not to touch the dry 3MM paper to the lane which is to be transferred to the second gel. The "marker" first-dimension gel shift is then dried on a gel dryer and exposed to X-ray film.

> Wet the gel slice remaining on the glass plate with 1× running buffer, as this makes it much easier to slide the gel off of the glass plate and into the (wetted) second-dimension gel (Fig. 2C). Using gel spacers, gently squeeze the first-dimension gel shift into the space between the two glass plates until the gel slice lies flat on top of the second dimension. This procedure requires some patience during the first attempts but the art of squeezing the gel slice into the second dimension without destroying the gel slice is easily mastered.

> Just before stopping the first-dimension gel shift, another incubation of labelled DNA/protein should be set up. This will be used as a marker lane on the second-dimension gel. In order to form this slot, two lightly greased gel spacers should be inserted into the top of the gel (Fig. 2C) into which the protein/DNA incubation will be loaded.

> Gel spacers are also inserted over the ends of the transplanted first-dimension gel slice (Fig. 2C) in order to i) hold the gel slice in place and against the second-dimension gel until the complexes have migrated into the second gel, and

ii) act as a slot into which 300 µl of the standard gel-shift incubation buffer containing 75 µg of BSA and 3 µg of poly (dI-dC) is laid over the first-dimension gel slice. By way of explanation, gel mobility-shift assays using small amounts (nanogramme quantities) of purified protein are usually unsuccessful unless carrier protein is added to the reaction. This effect is probably a consequence of non-specific binding of protein to the gel matrix and/or disruptive effects caused by the electric current. A similar situation is faced during the running of the second dimension, and this phenomenon may in fact be helpful in the dissociation of the multi-subunit complexes during this dimension.

> The second-dimension gel shift is electrophoresed at 125–150V in the cold room with recirculating buffer for approximately the same length of time as the first-dimension gel. The top glass plate is then removed, the gel transferred to a piece of Whatman 3MM paper, dried and autoradiographed.

Procedure 2

> Locate and cut out the lane containing the first-dimension gel shift as described in Procedure 1. Remove the surrounding gel (Fig. 3A).

> Cut a piece of used X-ray film to a size slightly larger than the gel slice and place the X-ray film on top of the gel slice (Fig. 3B). The gel slice will stick firmly to the X-ray film.

> Transfer the X-ray film with the attached gel slice to a clean glass plate. Apply a thin layer of silicone grease (Bayer, medium viscosity) to plastic spacers and place them on both sides of the glass plate. Without removing the piece of X-ray film, cover with a second, slightly longer silicone-treated glass plate (Fig. 3C) and seal the bottom of the assembly with strong tape. The glass plates should also be held firmly together with metal clamps just where the spacers are sandwiched between the glass plates. Place an appropriate slot former on the side of the first-dimension gel (Fig. 3D).

> Put the entire assembly in a slanted position and pour the acrylamide solution along the edge between the glass plates (arrow, Fig. 3E) until the solution reaches to the top (avoid the introduction of air bubbles). Place the gel in a vertical position and let it polymerise at room temperature for 10–

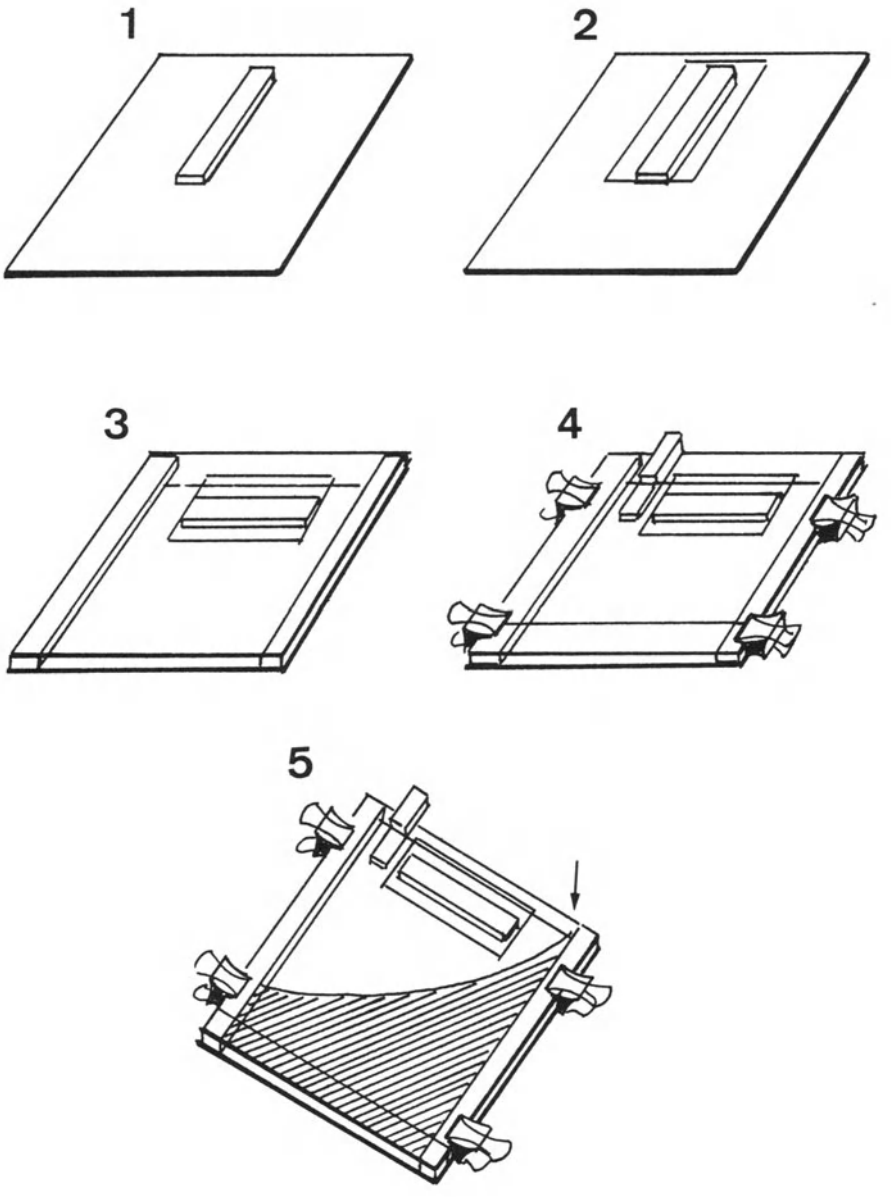


Fig. 3: Diagram showing the setting up of the second-dimension gel: For the details, see the Step-by-Step description for Procedure 2.

20 min. Transfer the gel into the electrophoresis apparatus in the cold room. The gel is then electrophoresed under the same conditions as used for the first dimension.

Figure 4 shows an example obtained with Procedure 2. Protein-DNA complex (a) was completely unstable in the second dimension whereas complex (b) dissociated only partially from the same piece of DNA. This suggests that the affinity of the oligonucleotide was probably greater for the factor(s) present in complex (b) than for those in complex (a) under those conditions. The dissociation products detected for both complexes were only free DNA and (unlabelled) proteins; no intermediate complexes were observed. The experiment was carried out in the cold room with a 5% polyacrylamide gel (29:1) using either 1× TEA buffer (6.7 mM Tris pH 8, 1 mM EDTA, 3 mM sodium acetate; panel A) or 0.25× TBE buffer, panel B (10× TBE buffer is 0.89 M Tris base, 0.89 M boric acid, 0.02 M EDTA, pH 8.3).

C Important Note:

Since no stacking gel is utilised, it is best to keep the width of the first-dimension gel to a minimum in order to get clearer resolution of the signals in the second dimension. This is best achieved by cutting the first-dimension gel-shift slice into a number of thinner slices; we typically cut a 7.5-mm-wide lane into three approximately 2.5-mm-wide gel slices. This has the added advantage of being able to use each of these gel slices to play with some of the parameters of the second dimension (for example adding detergents to the second-dimension gel to try to aid the dissociation of the multi-subunit complexes).

D Other Considerations

The protocol presented here is just one method by which the diagonal gel mobility-shift assay could be performed. Depending upon the equipment available and the characteristics of the investigated factor(s), the protocol may be modified. The glass plates need not be of the indicated size and the voltage required for the electrophoresis should be altered accordingly. If no pumps for recirculating the buffer are available, simply change the running buffer every 30–40 min during electrophoresis to avoid a shift in the pH (the pH of the buffer can be tested by adding a non-bleeding,

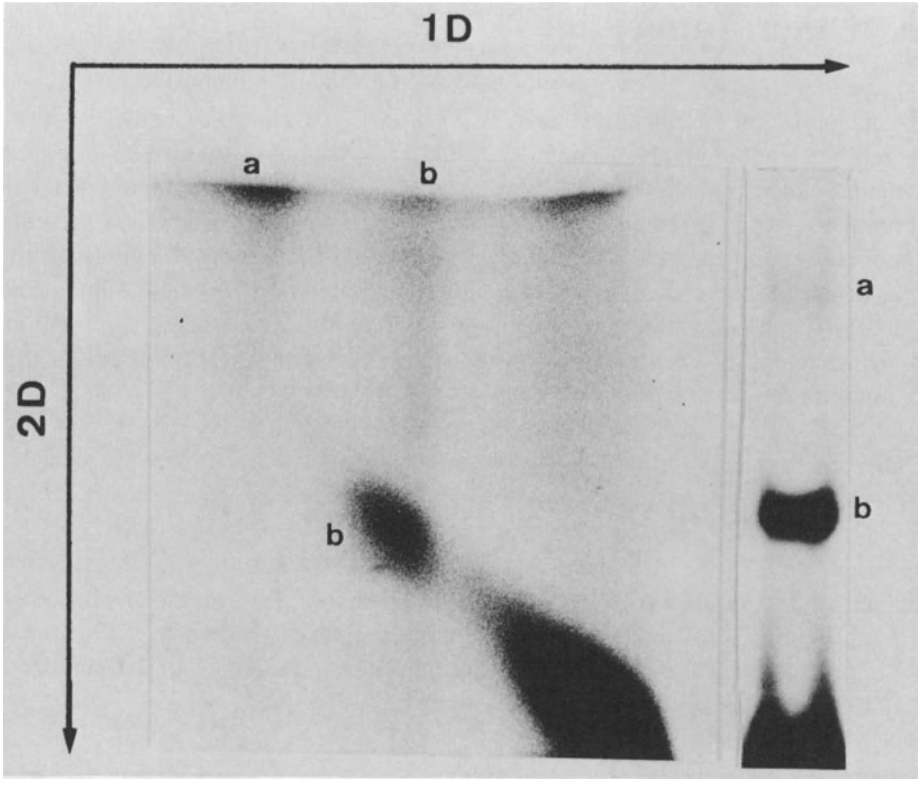


Fig. 4: An example of a pattern obtained with Procedure 2: a and b are protein-DNA complexes; c is the free DNA. The same nuclear extract and labelled DNA was used in the gels of panels A and B, which differed in using 1× TAE and 0.25× TBE, respectively, as buffer systems. 5% polyacrylamide gels were used in both dimensions.

pH-indicator strip to the upper and lower tank). If the complex appears to be stable at room temperature, the procedure may be run on the bench instead of in the cold room. If neither bromophenol blue nor xylene cyanole affect the binding of the protein to the DNA, these dyes may be included in the lane in which the gel shift is run (instead of adjacent lanes). The length of electrophoresis can be varied according to the distance that the complexes are separated, in order to optimise the resolution.

E Other Applications

The diagonal gel mobility-shift assay was originally developed to analyse a series of multi-subunit complexes binding to a single site within the rat growth-hormone gene promoter (Schaufele et al., 1990, see Fig. 1). It could also be used to resolve a number of complexes binding to different sites on a larger promoter fragment, particularly with an eye to investigating any heirarchical order of binding. It may also be used for investigating whether any agents added into the second-dimension gel may specifically affect the mobility of any one of the complexes, information which may aid the choice of reagents for the selective purification of one of the complexes.

F Bibliography

Schaufele, F., Cassill, J.A., West, B.L., and Reudelhuber, T. Resolution by diagonal gel mobility shift assays of multisubunit complexes binding to a functional important element of the rat growth hormone gene promoter. J. Biol. Chem. 265 (1990) 14592–14598.

XVI A Rapid Gel-Shift Technique to Analyse DNA-Protein Interactions using Phastsystem™

Ponnusamy Ramanujam, Scott Fogerty, William Heiser and James Jolly

Contents

I Introduction

1 Gel-Shift Assay

DNA-binding proteins have been implicated in several cellular functions such as transcription, translation, replication and viral integration (Mitchell & Tijan, 1989). Significant progress in our understanding of DNA-protein and RNA-protein interactions has been achieved by the popular gel- or mobility-shift assay. The assay is based on the differential migration of free nucleic acid versus protein-bound nucleic acid molecules through native polyacrylamide and agarose gels. In this technique, ^{32}P-end-labelled DNA molecules containing a specific target sequence are incubated with a cell- or protein extract. Electrophoresis of the resulting products separates fast-moving, unbound (free) DNA from slower-moving (bound) DNA complexed with specific DNA-binding proteins. A number of recent reviews on the gel-shift assay have been published (Crothers, 1987; Garner & Revzin, 1986; Hendrickson, 1985; Revzin, 1989). Novel applications of this technique have led to the discovery of numerous *trans*-acting transcription factors (Wingender, 1988). More recent research has been directed towards purification, characterisation and cloning of these nucleic-acid binding proteins to elucidate their structure and function (Singh et al., 1988; 1989).

While the gel-shift assay is very simple in concept and powerful in terms of the information it offers, in practice it can involve using many gels. Typically, this electrophoretic separation takes several hours, with cumbersome steps involving gel preparation and careful gel manipulation. These steps can become tedious when many binding parameters are to be tested or when many fractions are to be assayed during the purification of a DNA-binding protein. Because these studies require considerable time and technical expertise, fast and accurate gel-mobility assays would expedite DNA-binding protein research. We have previously described a rapid gel-shift technique called PhastShift™ (Ramanujam et al., 1990), which combines the convenience of pre-cast gels with microprocessor-controlled electrophoretic separation using PhastSystem™.

2 PhastSystem™

PhastSystem™ developed at Pharmacia LKB Biotechnology (Fig. 1) is an automated electrophoresis system capable of separating proteins, oligonucleotides and PCR products in a rapid and reproducible manner (Brewer et al., 1986; McOsker et al., 1989). It offers the advantages of programable software, pre-cast gradient and homogeneous gel media, and fast separation time.

Fig. 1: Pharmacia PhastSystem and Accessories

B Materials and Methods

1 DNA and Cell Extracts

An oligonucleotide (82-mer) including the Epstein-Barr virus nuclear antigen (EBNA-1) binding site [5'-TAGCATATGCTA -3' (Rawlins et al., 1985)] and an oligonucleotide (63-mer) containing the octomer (Oct-1) binding site [5'-ATG-CAAAT-3' (Singh et al., 1989)] were synthesised on Gene Assembler® Plus from Pharmacia LKB. All target sites used in the binding reactions were duplexed DNA-labelled at an internal nucleotide using Klenow fragment of DNA polymerase I (Maniatis et al., 1982).

HeLa cell nuclear extract was prepared according to Dignam et al. (1983). A recombinant clone expressing the DNA-binding domain of EBNA-1 was kindly provided by Dr. Harinder Singh (Singh et al., 1988). A crude protein extract containing EBNA-1 was prepared by a modification of the method described by

Rawlins et al. (1985). Briefly, induced cells were pelleted and resuspended in 0.1 volumes of the original culture volume with 50 mM Tris-HCl (pH 7.5 at 22°C), 10 mM EDTA, and 1 mM PMSF (phenylmethylsulphonyl fluoride). The cells were disrupted by sonication on ice, then mixed with 0.2 volumes of 5 M NaCl. After an incubation on ice for 15 min, cell debris were pelleted by centrifugation, and an equal volume of glycerol was added prior to storage at –70°C. The protein concentration in the extracts was determined by the method of Bradford (1976).

2 DNA-Protein Binding Reaction

Typical binding reactions involved mixing ^{32}P-end-labelled DNA fragments or oligonucleotide (0.1–1 ng, ~10000 cpm) and 1 µg of poly (dI-dC) · poly (dI-dC) (Pharmacia LKB) with protein extract (0.1–5 µg) in binding buffer adjusted to a total volume of 20 µl. The binding buffer contained 10 mM Tris-HCl (pH 7.5 at 22°C), 50 mM NaCl, 0.5 mM dithiothreitol (DTT) for assaying Oct-1, and 20 mM Tris-HCl (pH 7.5 at 22°C), 100 mM NaCl, 5 mM $MgCl_2$, 1.0 mM DTT for assaying EBNA-1. Samples were incubated at 22°C for 20 min, then 2 µl of 10× loading dye (250 mM Tris-HCl, pH 7.5 at 22°C, 0.2% xylene cyanol, 0.2% bromophenol blue and 40% glycerol) was added. A sample of 1 µl was removed for analysis by electrophoresis on PhastSystem.

C Separation of DNA-Protein Complexes using PhastSystem

1 Step-by-Step Procedure for Programing the PhastSystem

The specific electrophoretic conditions used to programe PhastSystem (Pharmacia LKB) are outlined in Table 1. Except for reducing the temperature to 4°C and increasing the pre-electrophoresis to 100 Vh, use the same separation method recommended for native polyacrylamide gel electrophoresis (PhastSystem™ Separation Technique File, Native-PAGE no. 120, Pharmacia LKB, Biotechnology Inc. Piscataway, NJ 08855, USA and Uppsala, Sweden).

Table 1

PhastSystem Program					
1. Programe PhastSystem (code No.18-1600) using the following electrophoretic separation method*:					
SAMPLE APPL.		DOWN AT	1.2		0 Vh
SAMPLE APPL.		UP AT	1.2		2 Vh
SEP 1.1	400 V	10.0 mA	2.5 W	4°C	100 Vh
SEP 1.2	400 V	1.0 mA	2.5 W	4°C	2 Vh
SEP 1.3	400 V	10.0 mA	2.5 W	4°C	268 Vh
**Note* PhastSystem method 1 is given as an example					

> The programe contains three steps; each step specifies electrophoretic running conditions for voltage, current, power, temperature and time in volt hours.

> The first step (SEP 1.1) is a pre-electrophoresis step. This sets the ionic conditions within PhastGel: ions from the buffer strips migrate through the stacking gel, which achieves a pH of approximately 8.6.

> The second step (SEP 1.2) controls automatic sample application onto the gel. SAMPLE APPŁ. DOWN AT 1.2 refers to Method 1 and step 2 when the sample applicator will be lowered at the end of step 1 (at the end of 100 Vh, the pre-electrophoresis step). 2 Vh will elapse before the sample applicator is raised from the gel.

> The final step (SEP 1.3) gives the actual electrophoretic separation parameters. The samples are separated at the set parameters of step 3.

2 Step-by-Step Procedure for Fast Gel-Shift:

PhastShift™ using PhastSystem™

PhastGel buffer contains 0.112 M acetate, 0.112 M Tris (pH 6.4) and PhastGel Native Buffer Strips contain 2% agarose in 0.88 M L-alanine and 0.25 M Tris (pH 8.8). During the initial part of the separation, while the gel and buffer strips form a discontinuous system, the gel is run at constant current and the voltage increases with time. After approximately 100 volt

hours, the buffer strips and the gel form a continuous system and PhastGel is run at a constant voltage of 400 V. We have found that these electrophoretic conditions maintain the DNA-protein complex and separate the free and bound DNA molecules that we have studied.

> Use pre-cast 8–25% gradient acrylamide gels (PhastGel Gradient 8–25, Code No. 17-0542) for most gel-shift assays. For gel-shift assays involving small oligonucleotides (less then 50 base pairs), use 20% acrylamide gels (PhastGel Homogeneous 20, Code No. 17-0624).

> Use PhastGel Native Buffer Strips (Code No. 17-0517) for all separations. Caution: Do not use PhastGel SDS Buffer Strips containing SDS.

> Layer the DNA-protein binding reaction mixture onto parafilm wells made with the PhastGel sample-well stamp.

> Apply samples (4, 1, 0.5 or 0.3 µl per well) using the appropriate PhastGel sample applicator.

> Perform electrophoresis using the programed PhastSystem (Table 1).

> Stop electrophoresis when the blue tracking dye almost reaches the lower buffer strip.

> Cover the PhastGel with protective film or Saran wrap.

> Autoradiograph gels using an intensifying screen for 2 h at –70°C or overnight at room temperature.

Note:
Modifications in the electrophoresis parameters and buffer strips may be required to optimise separation of free and bound target DNA. See Modifications section.

D PhastShift

We have tested several published DNA-binding protein systems to demonstrate rapid gel-shift assays using pre-cast PhastGels and PhastGel Buffer Strips with PhastSystem. Using this system, the products of the DNA-binding reaction were conveniently separated from free DNA in less than 1 h (Ramanujam et al., 1990).

PhastShift™. Fig. 2 is an example of a 82-bp Epstein-Barr Virus EBNA-1-binding oligonucleotide complexed with EBNA-1 cloned protein separated on PhastSystem using a pre-cast, ultra-thin 20% homogeneous acrylamide gel (PhastGel Homogeneous 20, Pharmacia LKB) as outlined in the Step-by-Step-Procedure. Addition of EBNA-1 extract results in slower migration of some of the 82-bp target DNA (Lane 2).

Unlabelled target DNA added to the binding reaction competes with the labelled DNA for binding, causing a reduction in the amount of labelled DNA in the retarded band (Lane 3). As expected, unlabelled oligomer which contains the binding site for the DNA-binding protein Oct-1 does not compete with binding to the labelled EBNA-1 target DNA (Lane 4).

Fig. 3 is an example of a 63-bp Oct-1 binding oligonucleotide complexed with octomer-binding proteins separated on PhastSystem using a pre-cast, ultra-thin 20% homogeneous acrylamide gel (PhastGel Homogeneous 20, Pharmacia LKB) as outlined in the Step-by-Step Procedure.

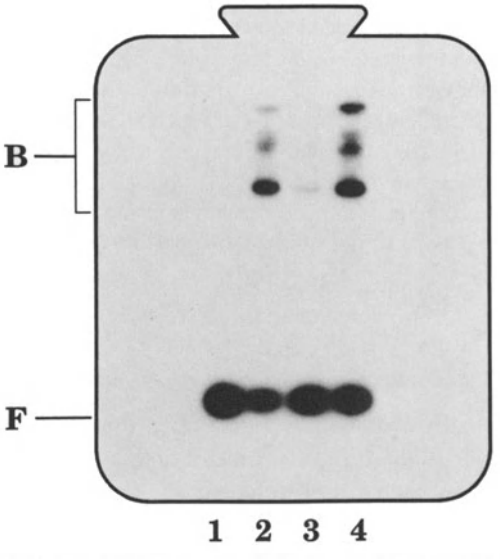


Fig. 2: PhastShift using PhastSystem: Binding of EBNA-1 to its substrate EBNA oligonucleotide. EBNA-1 protein extract, EBNA-1 target sequences and binding reaction conditions were as described in Materials and Methods. A sample of 1 µl was loaded per lane for analysis by electrophoresis on PhastSystem using PhastGel Homogeneous 20 and was performed as described in the Step-by-Step Procedure. EBNA-1-bound oligonucleotide (B) and free target (F) are as indicated. Lane 1, no extract; Lane 2, EBNA-1 extract; Lane 3, competition assay with 30 ng of unlabelled EBNA oligonucleotide; Lane 4, competition assay with 30 ng of a 63-base Oct-1 binding oligonucleotide. Autoradiography was for 1 h at room temperature.

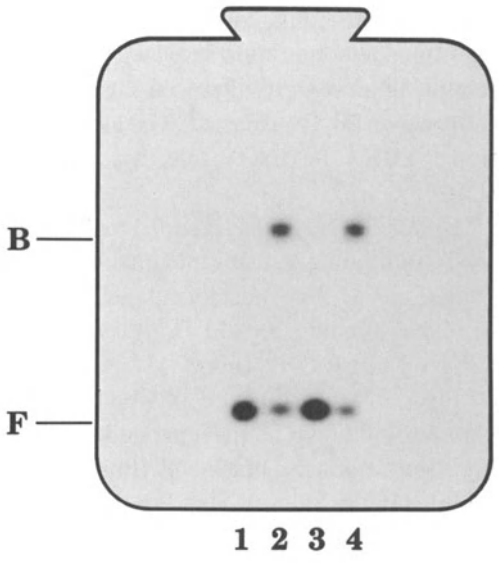


Fig. 3: PhastShift using PhastSystem: Detection of Oct-1 and other octomer-binding proteins using Oct-1 DNA and HeLa extract, with reactions analysed by PhastShift. All lanes contained 0.05 ng of labelled Oct-1 target DNA. Lane 1, no extract; Lane 2, HeLa extract; Lane 3, same as Lane 2 but with 30-fold excess unlabelled Oct-1 DNA competing for binding; and Lane 4, same as Lane 2 but binding with a 30-fold excess of EBNA-1 target DNA which does not contain a binding site for Oct-1. Reactions were performed according to the conditions described in Materials and Methods. A sample of 1 µl was loaded per lane for analysis by electrophoresis on a PhastSystem using PhastGel Homogeneous 20 and was performed as described in the Step-by-Step Procedure. Autoradiography was for 1 h at room temperature. Bound octomer-binding protein complexes (B) and free target DNA (F) are as indicated.

Sequence specificity of binding is typically demonstrated by competition experiments such as those shown in figs. 2 and 3. In these figures, lanes 1 and 2 show the positions of free and protein-bound DNA. In lane 3, a 30-fold excess of unlabelled target DNA has been added to the gel-shift reaction. This cold target DNA competes with the labelled target for EBNA-1 or Oct-1 binding, thus reducing the amount of labelled target which is retarded in the gel. In lane 4, unrelated DNA (not to be confused with non-specific competitor DNA, which is included in all binding reactions to block binding of target to non-specific binding proteins) has been added to the reaction. Since this unrelated DNA (Oct-1 in Fig. 2 and EBNA-1 in Fig. 3) has no effect on the shifted band (compare lanes 2 and 4), these results demonstrate that binding to the labelled target DNA is sequence specific. Evidence of sequence specificity of binding can be further strengthened by performing experiments with

target DNA carrying mutations in the binding sequence. The horizontal electrophoresis separation using PhastSystem was completed in less than 30 min. The results obtained with PhastSystem were comparable to those obtained by standard vertical gel electrophoresis (Ramanujam, 1990).

E Modifications

Successful separation of DNA-protein complexes is dependent upon several factors such as buffer composition, pH, salt concentration, presence of non-specific competitor, gel conditions, and the intrinsic conformation of the complex itself (Fried, 1989). Conventionally, these parameters have been experimentally determined to optimise the required separation on a standard gel system. Large sample volumes and generous gel area for clear detection are advantages available with standard gels. However, the separation of bound vs free DNA and the information obtained using PhastGel are sufficiently optimal for the DNA-protein interactions we have studied.

The pre-electrophoresis step programed in the separation method of PhastSystem effectively replaces the ionic conditions of PhastGel with those of the buffer strips. During PhastShift, the buffer and ionic conditions of the gel are predominantly determined by the buffer strips. Hence, modifications in the pH and ionic strength may be achieved by preparing alternate buffer strips [in 2% Agarose IEF, (Code No. 17-0468)] to optimise conditions for complexes that are not separated using PhastGel Native Buffer Strips.

F Advantages

The advantages of PhastShift is its convenience, fast separation time, and small sample size, combined with the same high resolution of standard vertical gel electrophoresis. Some advantages of using PhastSystem are given in Table 2.

The added advantages of using PhastSystem for gel-shift assays include microprocessor-controlled separation parameters such as temperature, sample application and power supply setting. Other advantages include the small size of the gels ($43 \times 50 \times 0.45$ mm): since they occupy less space on autoradiography films, many gels can be exposed on a single sheet of film, giving more information per autoradiogram. PhastGel is easy to handle and store compared with standard gels, which often shrink and distort after electrophoresis or which require drying onto a solid support prior to autoradiography. Simultaneous electrophoretic separation of two PhastGel gels with 16 lanes (0.5 or 1 µl per well) or 24 lanes (0.3 µl per well) usually takes less than 1 h.

Table 2

Advantages of Gel-shift assays using PhastSystem	
Condition	*PhastSystem*
Size (mm)	43 × 50 × 0.45
High resolution	Yes
Electrophoresis time	~ 30 min
Microprocessor control	Yes
Gel casting required	No
Buffer circulation	No
Number of gels per 8 hours	25
Number of samples per 8 hours	200
Sample volume (µl)	< 4
Number of gels per 8 × 10 X-ray film	16
Homogeneous gels	Yes
Gradient gels	Yes
Easy handling	Yes
Gel drying	No

G Applications

Further potential applications of PhastSystem for DNA-protein interaction studies include the recent western blotting capability (PhastTransfer™) to perform south-western blotting (Miskimins et al., 1985), rapid separation of restriction fragments and generation of free and protein-bound DNA for footprinting analyses (Singh et al., 1988). Recently, two-dimensional gel electrophoresis to determine molecular weights of DNA-binding proteins and to resolve multimer-binding complexes have become very useful (Gray et al., 1990; Schaufele et al., 1990, see also Chapter XV). Two-dimensional electrophoresis is easily performed on PhastSystem by cutting out a strip of sample lane from a one-dimensional PhastGel and simply layering the strip onto another PhastGel. Appropriate SDS or native buffer strips may be used for the second-dimension electrophoresis. In addition, PhastShift technique can be conveniently used for studies of RNA-protein interactions.

Simplicity, speed and small assay volumes make PhastShift using Pharmacia PhastSystem a convenient methodology for studing nucleic acid/protein interactions. Using this method, we have performed DNA-binding protein experiments and obtained autoradiography results within just 3 h. We believe that PhastShift will facilitate rapid and accurate gel-shift assays for nucleic-acid binding proteins. Other PhastSystem applications and references are described in the PhastSystem reference list (Pharmacia LKB Biotechnology In., PhastSystem™ References List. Piscataway, NJ 08855, USA and Uppsala, Sweden).

H The Most Common Problems and Their Solutions

- *No separation of DNA-protein complexes*
  This could either be due to the presence of the protective film still on PhastGel or the use of PhastGel Buffer Strips containing SDS.
- *Failure to detect DNA-protein complexes*
  This could be due to a variety of problems associated with the optimal conditions for DNA-protein interactions. Choice of appropriate percentage of polyacrylamide gel based on the size of the target DNA may be important. Adjusting the binding conditions for ionic strength, cell extract, type and amount of non-specific competitor may be useful. Optimisation of other conditions of the binding reaction such as pH, temperature and electrophoresis along with the inclusion of BSA, glycerol, DTT, non-ionic detergents and EDTA as additives and protectants also may be helpful.
- *Target DNA does not enter the gel*
  This is most often the result of either non-sequence-specific proteins binding to the target DNA, or aggregation of the protein-DNA complexes. The former may be eliminated by optimising the type and amount of synthetic polymers or natural DNAs [e.g. poly (dI-dC) · poly (dI-dC) or sonicated calf thymus DNA] included in the binding reaction. The latter may be overcome by including non-ionic detergents such as NP-40 or Triton X-100 in the binding reaction; disruption of protein aggregates have been shown to help DNA-protein complexes enter the gel.
- *Free target DNA migrates off the gel*
  Monitor electrophoresis carefully and stop the separation when the dyes have migrated two-thirds of the way down the gel.
- *Degradation of target DNA*
  This may be due to nuclease or free-radical activity in the cell extract. Include a chelator such as EDTA in the binding reaction . Further purification of crude cell extracts may be required to eliminate nuclease activity.
- *High background on gel*
  This may be caused by the presence of unicorporated dNTPs in the sample. These are easily removed by gel filtration using Sephadex G-25 or Sephadex G-50.

I Bibliography

Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72 (1976) 248–254.

Brewer, J., Grund, E., Hagerlid, P., Olsson, I., and Lizana, J. Fast separation and visualization of proteins in a new system for electrophoresis and isoelectric focusing. Electrophoresis 86 (1986) 226–229.

Crothers, D.M. Gel electrophoresis of protein-DNA complexes. Nature 325 (1987) 464–465.

Dignam, J.D., Martin, P.L., Shastry, B.S., and Roeder, R.G. Eukaryotic gene transcription with purified components. Methods in Enzymology 101 (1983) 582–598.

Fried, M.G. Measurement of protein-DNA interaction parameters by electrophoresis mobility shift assay. Electrophoresis 10 (1989) 366–376.

Garner, M.M., and Revzin, A. The use of gel electrophoresis to detect and study nucleic acid-protein interactions. Trends in Biochemical Sci. 111 (1986) 395–396.

Gray, T.A., Gumucio, D.L., and Collins, F.S. A two-dimensional electrophoresis system for DNA-binding proteins. Technique 2 (1990) 147–154.

Hendrickson, W. Protein-DNA interactions studied by the gel electrophoresis-DNA binding assay. BioTechniques 3 (1985) 198–207.

Maniatis, T., Fritsch, E.F., and Sambrook, J. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory (1982), Cold Spring Harbor, New York.

McOsker, P., Sofer, G., and Lepinske, M. An automated electrophoresis system for protein purity monitoring and analysis of oligonucleotides and PCR amplification products. American Biotechnology Laboratory May (1989) 36–40.

Miskimins, W.K., Roberts, M.P., McClelland, A., and Ruddle, F.H. Use of a protein-blotting procedure and a specific DNA probe to identify nuclear proteins that recognize the promoter region of the transferrin receptor gene. Proc. Natl. Acad. Sci. USA 82 (1985) 6741–6744.

Mitchell, P.J., and Tjian, R. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science 245 (1989) 371–378.

Pharmacia LKB Biotechnology Inc., PhastSystem™ References List.

Piscataway, NJ 08855, USA and Uppsala, Sweden.

PhastSystem™ Separation Technique File. Native-PAGE. No. 120.

Pharmacia LKB Biotechnology Inc., Piscataway, NJ 08855, USA and Uppsala, Sweden.

Ramanujam, P.S., Fogerty, S., Heiser, W., and Jolly, J. Fast gel electrophoresis to analyze DNA-protein interactions. BioTechniques 8 (1990) 556–563.

Rawlins, D.R., Milman, G., Hayward S.D., and Hayward, G.S. Sequence-specific

DNA binding of the Epstein-Barr Virus nuclear antigen (EBNA-1) to clustered sites in the plasmid maintenance region. Cell 42 (1985) 859–868.

Revzin, A. Gel electrophoresis assays for DNA-protein interactions. BioTechniques 7(1989) 346–355.

Schaufele, F., Cassell, J.A., West, B.L., and Reudelhuber, T. Resolution by diagonal gel mobility shift assays of multisubunit complexes binding to a functionally important element of the rat growth hormone gene promoter. J. Biol. Chem. 265 (1990) 14592–14598.

Singh, H. LeBowitz, L.H. Baldwin, Jr., A.S., and Sharp, P.A. Molecular cloning of an enhancer binding protein: isolation by screening of an expression library with a recognition site DNA. Cell 52 (1988) 415–423.

Singh, H., Clerc, R.G., and LeBowitz, J.H. Molecular cloning of sequence-specific DNA binding proteins using recognition site probes. BioTechniques 7 (1989) 252–261.

Wingender, E. Compilation of transcription regulating proteins. Nucl. Acids Res. 16 (1988) 1879–1902.

XVII Purification of Sequence-Specific DNA-Binding Proteins by Affinity Chromatography

Melya J. Hughes, Jean-Pierre Jost and Josef Jiricny

Contents

A Introduction

DNA-affinity chromatography was first used by Kadonaga and Tjian (1986) for the purification of the transcription factor, Sp1. Oligonucleotides containing the protein-binding site are phosphorylated, ligated and covalently bound to Sepharose. Nuclear proteins are then incubated with the affinity matrix in the presence of non-specific competing DNA, and the protein binding specifically to the oligonucleotides is eluted with high salt. This method has been used widely for the

purification of a variety of DNA-binding proteins (e. g. Fletcher et al., 1987; Prywes and Roeder 1987; Bagchi et al., 1987; Sawadogo et al., 1988; Lenardo et al., 1988; Lichtensteiner and Schibler 1989).

B Materials and Methods

1 Solutions and Enzymes

- CNBr-activated Sepharose 4B or Sepharose CL2B (Pharmacia)
- Cyanogen bromide (Aldrich)
- N,N-Dimethylformamide, analytical grade (Aldrich)
- T4 Polynucleotide kinase (Biofinex, 5U/µl)
- 10× Linker kinase buffer: 0.7 M Tris.Cl pH 7.6, 0.1 M $MgCl_2$, 50 mM DTT
- [γ-^{32}P]ATP, specific activity 3000 Ci/mmole (Amersham)
- ATP (20 mM) pH7
- 10× Ligase buffer: 0.66 M Tris.Cl pH 7.6, 50 mM $MgCl_2$, 50 mM DTT
- T4 Ligase (Biofinex, 2 Weiss units/µl)
- Ethanolamine · HCl
- HE buffer: 25 mM Hepes, 1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), pH 7.65 at room temperature (A 100-mM stock solution of PMSF in ethanol is kept at –20 °C and is diluted just before use.)
- Regeneration buffer: 5 mM Tris.Cl pH 7.6, 2.5 M NaCl, 0.5 mM EDTA
- Storage buffer: 10 mM Tris.Cl pH 7.6, 0.3 M NaCl, 1 mM EDTA, 0.02% NaN_3

Synthesis of affinity matrix
The affinity matrix is synthesised essentially as described by Kadonaga and Tjian (1986). The oligonucleotides contain the minimal binding site (determined by a combination of functional assays and binding studies) for the protein of interest and 5'GATC sticky ends.

2 Step-by-Step Procedure

a) *Preparation of Oligonucleotides for Coupling to CNBr-Activated Sepharose*

> Dry the oligonucleotides (220 µg of upper or lower strand) in a SpeedVac and redissolve in 70 µl water.

> Mix an aliquot (10 pmol) of each strand, 10 pmol (γ-^{32}P)ATP (33 µCi) and 2 µl 10× linker kinase buffer with water to give a final volume of 19 µl.

> Add 1 µl T4 polynucleotide kinase (5 units) to each Eppendorf tube, mix and incubate for 30–45 min at 37°C.

> Meanwhile mix the remaining oligonucleotides (220 µg in 70 µl of each strand) with 10 µl 20 mM ATP and 10 µl 10× kinase buffer. After addition of 10 µl T4 polynucleotide kinase, incubate the mixture at 37°C for 30–45 min.

> Stop the kinase reactions by heating to 95°C for five minutes, then mix the oligonucleotides (labelled and non-labelled) together and allow to anneal.

> Add 0.1 volumes 3 M Na acetate pH 5.2 and 3 volumes of ethanol. Quick chill at –80°C for 10 min.

> Centrifuge for 10 min in an Eppendorf centrifuge at 4°C.

> Wash the pellet by centrifugation with 200 µl cold 80% ethanol.

> Dry the pellet in a SpeedVac concentrator.

> Dissolve the pellet in 80 µl water and add 10 µl 10× ligase buffer and 5 µl 20 mM ATP.

> Add 5 µl T4 ligase and incubate overnight at 12°C.

> Perform Phenol-chloroform extraction.

> Ethanol precipitate as above.

> Dissolve the pellet in 100 µl water and analyse aliquot on a 1% agarose gel (see Fig. 1).

> Determine the amount of radioactivity incorporated in the oligonucleotides.

b) *Preparation of CNBr-Activated-Sepharose*

> Wash 10 ml Sepharose CL-2B with 500 ml of water in a sintered-glass filter.

> Transfer the Sepharose as a 50% (v/v) slurry in water into a glass beaker containing a magnetic flea and equilibrate to 15°C in a water bath.

> Working in a fume hood, dissolve CNBr (1.1 g; 10 mmol) in 2 ml N,N-dimethylformamide and add slowly (dropwise) over 1 min to the Sepharose.

> CNBr releases HCN gas. Waste and glassware that has been in contact with CNBr should be treated with NaOH.

> Add 1.8 ml 5 M NaOH dropwise to the resin over 10 min. The pH of the reaction should not exceed 10.

> Stop the reaction by the addition of 100 ml ice-cold water and wash over a sintered-glass filter with gentle suction. Do not allow the resin to dry.

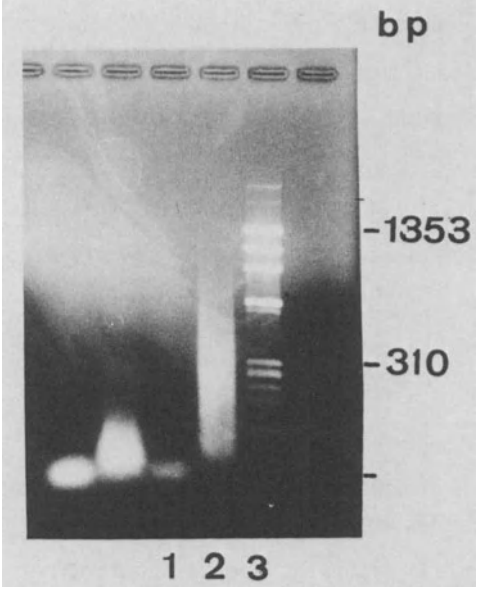


Fig. 1: Concatenised oligonucleotides for the synthesis of the affinity matrix: The oligonucleotides containing the binding sites for NHP2 and NHP3 (Hughes and Jost, 1989) (lane 1)were each phosphorylated and ligated as described in the Methods section. The concatenised oligonucleotides (lane 2) were analysed on a 1% agarose gel.

> The CNBr-activated resin is then washed with 300 ml ice-cold water and 100 ml 10 mM potassium phosphate pH 8.

> Resuspend the matrix in 4 ml 10 mM potassium phosphate pH 8 in a siliconised Erlenmeyer flask.
The oligonucleotides can also be coupled to CNBr-Sepharose 4B.

> Pre-swell 2.5 g (dry weight) CNBr-Sepharose 4B in water.

> Wash successively with 500 ml 1 mM HCl, 1 l water, 400 ml 10 mM potassium phosphate pH 8 in a sintered glass funnel.

> Resuspend matrix in 4 ml 10 mM potassium phosphate pH 8 in a siliconised Erlenmeyer flask.
Alternatively, a universally primed Sepharose can be used to link the specific oligonucleotides to the matrix. A palindromic oligonucleotide containing a cohesive end is covalently linked via its loop (containing an activated T residue) to a Sepharose matrix. The double-stranded oligonucleotides containing the sequence of the protein-binding site and the complementary cohesive end are then ligated simultaneously to each other and to the covalently linked palindromic oligonucleotide (Arcangioli et al., 1989).

c) *Coupling of Ligated Oligonucleotides to CNBr-Sepharose.*
The activated Sepharose is immediately used for coupling the DNA as follows

> Add the ligated oligonucleotides and shake on a rotary shaker for 16 h at room temperature.

> Transfer the slurry to a sintered-glass filter and collect the filtrate.

> Wash the resin with 200 ml water followed by 100 ml 1 M ethanolamine · HCl pH 8.

> Resuspend the matrix in 7 ml 1 M ethanolamine.HCl pH 8.

> Shake on a rotary shaker for 6 h at room temperature.

> Wash the resin with 100 ml each of 10 mM potassium phosphate pH 8, 1 M potassium phosphate pH 8, 1 M KCl, water and storage buffer.

> Store at 4°C in storage buffer.

Determine the amount of radioactivity present in the washes so that the amount of oligonucleotides bound to the matrix can be estimated. The incorporation is typically greater than 70%.

DNA affinity resins typically have low loading capacities (50–100 µg/ml). The concentration of covalently bound DNA on affinity latex particles has been shown recently to be six times higher than on DNA-Sepharose (Kawaguchi et al., 1989).

d) *DNA-Affinity Chromatography*
DNA-binding proteins have been purified from both whole cell and nuclear extracts. Before purification by affinity chromatography, the crude extract is enriched for the specific DNA-binding protein of interest by a combination of ammonium sulphate precipitation, gel filtration and ion-exchange chromatography. These steps not only reduce the amount of non-specific proteins but also decrease the volumes of protein solution to be handled. Thus, for example, for the purification of the non-histone protein, NHP1 (Hughes et al., 1989), the HeLa whole cell extract was first precipitated with ammonium sulphate and subjected to chromatography on Sephacryl S-300, heparin Sepharose and MonoQ-FPLC prior to the DNA affinity step. The optimal purification strategy for other DNA-binding proteins should be determined empirically, in particular, the choice of buffer, salt concentration for loading and elution, and the amount and type of competitor used for affinity chromatography. Synthetic double-stranded poly (dI-dC) and poly (dA-dT) or sonicated *E. coli*, calf thymus and salmon sperm DNAs or their combination have all been used successfully as competitor DNAs. Sufficient competitor DNA is added to reduce non-specific protein-DNA interactions without decreasing the specific binding of protein to the affinity matrix. Conditions for affinity chromatography can be optimised by using multiple 1-ml columns.

Step-by-Step Procedure

All operations are performed at 4°C.

> Equilibrate 4 ml of affinity matrix with 10 volumes HE buffer containing 0.15 M KCl.

> Adjust the salt concentration of the sample to 0.15 M KCl and incubate 10 min on ice with *E. coli* DNA at 100 ng/µg protein.

> Incubate the sample with the resin in a 15-ml Falcon tube with end-over-end mixing for 30 min at 4°C.

Alternatively, pour the affinity matrix into an Econo-column (BioRad) to give a bed volume of 1 ml. After equilibration with 10 volumes HE buffer containing 0.15 M KCl, load the sample with a flow rate of 15 ml/hour (i. e. under gravity).

> After the batchwise incubation, pour the matrix into a 10-ml syringe plugged with glass wool and collect the flow-through fraction.

> Wash the column with 5 volumes HE buffer containing 0.1 M KCl and elute proteins with 1 volume each of HE buffer containing 0.2, 0.3, 0.6 and 1 M KCl.

> Store fractions at –70°C after taking aliquots for assaying (band-shift assay, footprinting), analysis by SDS-PAGE, and UV cross-linking. The latter is important to show that the polypeptide(s) purified bind(s) specifically to the DNA sequence of interest.

> Wash the affinity matrix with 10 volumes of regeneration buffer and 10 volumes of storage buffer.

Purification may be improved by first passing the sample over a non-specific-DNA resin before chromatography on the specific-DNA Sepharose. Optimal purification may be achieved by multiple cycles over the specific-DNA Sepharose using different binding sites for different passes. This eliminates unwanted proteins that might fortuitously bind to one resin. The amount of competitor DNA should be decreased for consecutive passes. An alternative to the DNA affinity column is to react biotinylated binding-site DNA in the presence of competing DNA with a partially purified protein in solution and passing the mixture over a streptavidin-agarose column (Chodosh et al., 1986, Francillard et al., 1989). Recently, this technique has been modified by using magnetic DNA affinity purification (Gabrielsen et al., 1989). The biotinylated DNA interacts strongly with the streptavidin and the DNA-binding protein can be released by eluting with high salt. This technique has the advantage that binding conditions can be optimised in solution. However, non-specific adsorbance to streptavidin-agarose can be higher than on Sepharose and the protein of interest may mask the biotin groups of the oligonucleotides, thereby inhibiting the interaction with streptavidin and reducing the yield of purified protein.

C An Example

Fig. 2 shows a gel-shift assay of NHP1 present in protein fractions eluted from the DNA affinity column. Most of the specific DNA-binding activity was eluted with 0.3 and 0.6 M KCl. Fig. 3 shows a silver-stained SDS-polyacrylamide gel of protein at different stages of the purification. Lane 3 shows that upon purification by DNA-affinity chromatography, the protein consists of two polypeptides of 75 and 85 kDa. UV cross-linking experiments have shown that only the 85-kDa polypeptide binds directly to the DNA (Hughes et al., 1989).

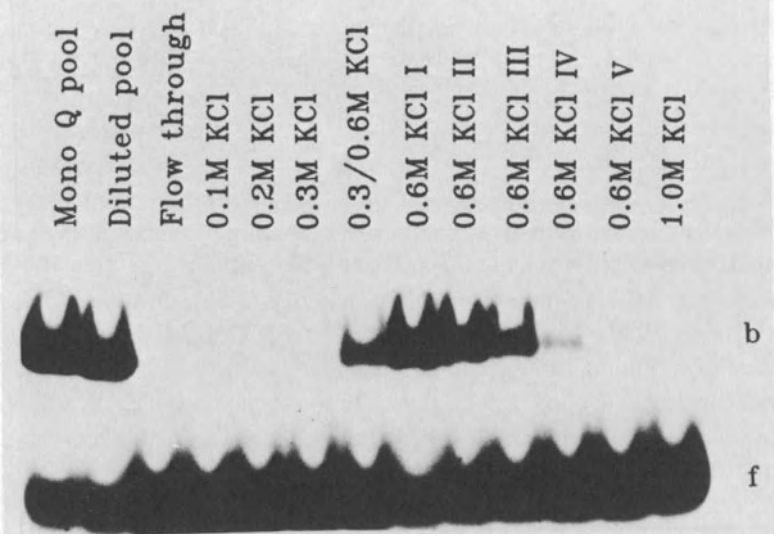


Fig. 2: Gel shift assay of the protein fractions eluted from the DNA affinity column: Chromatography was carried out as described in Materials and Methods. Gel-shift assays were carried out by incubating 0.5 ng of end-labelled oligonucleotide with 5 µl of protein fraction in the presence of 750 ng *E. coli* DNA. The labels above the gel indicate the fraction from which the protein was taken for the gel-shift assay. Bands b and f represent bound and free DNA respectively. Reprinted with permission from Biochemistry 28 (1989) 9137–9142, copyright 1990 American Chemical Society.

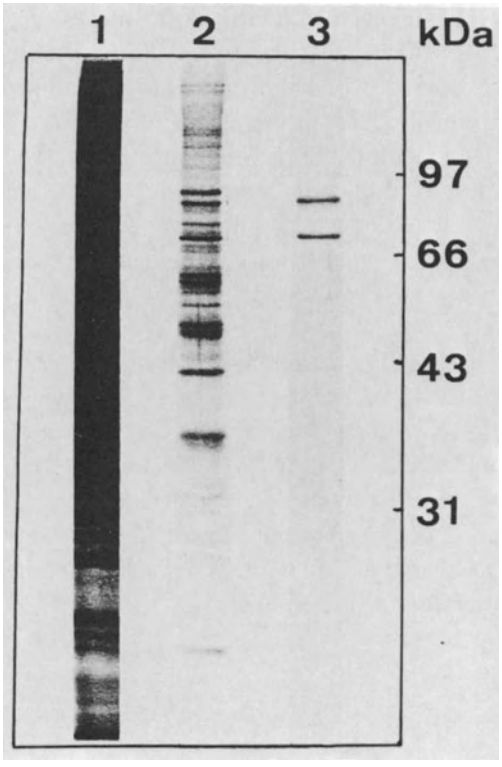


Fig. 3: SDS-polyacrylamide gel electrophoresis at different stages of the purification: Fractions were subjected to 10% SDS-polyacrylamide gel electrophoresis and stained with silver. Lane 1, 2 µg heparin-Sepharose fraction; lane 2, 0.4 µg Mono Q fraction; lane 3, 100 µl (~100 ng) out of 1 ml of the purified protein fraction from the affinity column. The latter fraction was precipitated with a final concentration of 15% trichloroacetic acid and then washed with acetone before loading onto the gel. Size markers are indicated in kDa. Reprinted with permission from Biochemistry 28 (1989) 9137–9142, copyright 1990 American Chemical Society.

D The Most Common Problems and Their Solution

– For efficient concatenation of the oligonucleotides, it is important to precipitate the oligonucleotides after the kinase reaction. If the ligation products are too short, binding of the protein to the affinity matrix may be blocked because of steric hindrance. A high degree of polymerisation is of advantage if the protein binds cooperatively to the DNA.

- *After the coupling reaction, most of the oligonucleotides are in the washes*
  The activation of the Sepharose with CNBr may have been unsuccessful. During the preparation of CNBr-activated Sepharose, the temperature (15°C) and pH (<10) of the reaction mixture should be controlled, otherwise the CNBr-activated Sepharose will be destroyed before the addition of the oligonucleotides. The manufacturer's guidelines should be followed when commercial preparations are used. The CNBr-activated-Sepharose should be used immediately for the coupling reaction.
- *After assaying the protein fractions eluted from the DNA affinity column, no DNA-binding activity is seen*
  Gel-shift assays with purified protein should be carried out in the presence of much lower amounts of competitor DNA and perhaps in duplicate with no competitor DNA, since any other DNA-binding proteins that may bind non-specifically to the affinity matrix would be eluted with a lower salt concentration. The protein may be stabilised during the assay by the addition of bovine serum albumin. If most of the DNA-binding activity is found in the flow-through fraction, the amount of competitor DNA may be too high. The optimal amount should be determined by numerous small-scale experiments.

E Bibliography

Arcangioli, B., Pochet, S., Sousa, R., and Huynh-Dinh, T. Preparation and use of a universal primed Sepharose for the purification of DNA-binding proteins. Eur. J. Biochem. 179 (1989) 359–363.

Bagchi, M. K., Tsai, S. Y., Tsai, M-J., and O'Malley, W. Purification and characterization of chicken ovalbumin gene upstream promoter transcription factor from homologous oviduct cells. Mol. Cell. Biol. 7 (1987) 4151–4158.

Chodosh, L. A., Carthew, R. W., and Sharp, P. A., A single polypeptide possesses the binding and transcription activities of the adenovirus major late transcription factor. Mol. Cell. Biol. 6, (1986) 4723–4733.

Fletcher, C., Heintz, N,. and Roeder, R. G. Purification and characterization of OTF-1, a transcription factor regulating cell cycle expression of a human histone H2b gene. Cell 51 (1987) 773–781.

Gabrielsen, O.S., Hornes, E., Korsnes, L., Ruet, A., and Oyen, T.B. Magnetic DNA affinity purification of yeast transcription factor tau – a new purification principle for the ultrarapid isolation of near homogenous factor. Nucleic Acids Research 17 (1989) 6253–6267.

Hughes, M. J., Liang, H., Jiricny, J., and Jost, J.-P. Purification and characterization of a protein from HeLa cells that binds with high affinity to the estrogen response element, GGTCAGCGTGACC., Biochemistry, 28 (1989) 9137–9142.

Hughes, M. J. and Jost, J.-P., The ubiquitous nuclear protein, NHP1, binds with high

affinity to different sequences of the chicken vitellogenin II gene., Nucleic Acids Research, 17 (1989) 8511–8520.

Kadonaga, J.T. and Tjian, R. Affinity purification of sequence-specific DNA binding proteins. Proc. Natl. Acad. Sci. USA, 83 (1986) 5889–5893.

Kawaguchi, H., Asai, A., Ohtsuka, Y., Watanabe, H., Wada, T., and Handa, H. Purification of DNA-binding transcription factors by their selective adsorption on the affinity latex particles. Nucleic Acids Research 17 (1989) 6229–6240.

Leblond-Francillard, M., Dreyfus, M., and Rougeon, F. Isolation of DNA-protein complexes based on streptavidin and biotin interaction. Eur. J.Biochem, 166 (1987) 351–355.

Lenardo, M. J., Kuang, A., Gifford, A., and Baltimore, D., NF-kB protein purification from bovine spleen: Nucleotide stimulation and binding site specificity., Proc. Natl. Acad. Sci. USA 85 (1988) 8825–8829.

Lichtensteiner S., and Schibler , U. A glycosylated liver-specific transcription factor stimulates transcription of the albumin gene. Cell 57 (1989) 1179–1187.

Prywes, R. and Roeder, R. G. Purification of the c-*fos* enhancer-binding protein. Mol. Cell. Biol. 7 (1987) 3482–3489.

Sawadogo, M., Van Dyke, M. W., Gregor, P. D., and Roeder, R. G. Multiple forms of the human gene-specific transcription factor USF. J. Biol. Chem. 263 (1988) 11985–11993.

XVIII Cloning of Sequence-Specific DNA-Binding Proteins by Screening λ cDNA Expression Libraries with Radiolabelled Binding-Site Probes

Patrick Matthias, Michael M. Müller and Walter Schaffner

Contents

A Introduction

The isolation of DNA-binding, transcription factors has been facilitated by a novel strategy that depends on the functional expression in *E. coli* of high levels of the DNA-binding domain (DBD) of the factor studied. This procedure (Singh et al., 1988, Vinson et al., 1988), which is essentially a modification of the original antibody screening of λ gt11 libraries (Young & Davis 1983), can be outlined as follows (see Fig. 1): A cDNA expression library is constructed in an inducible prokaryotic expression vector such as the bacteriophage vectors λ gt11 or λ ZAPII. Both vectors produce proteins fused with the N-terminal portion of β-galactosidase, and in each case synthesis of the fusion protein is under the control of the *lac* repressor. After plating of the library, the phage plaques are blotted onto filters which had previously been soaked in a solution of IPTG, a lactose analogue. IPTG inactivates the *lac* repressor, and thus induces in situ expression of the fusion proteins from the λ recombinants. The presence of the desired fusion protein is then detected by incubating the filters with a radiolabelled DNA probe containing the binding site for the desired factor, under conditions comparable to those used for footprint or gel-retardation experiments. After washing and autoradiography of the filters, the phages deemed positive are eluted and replated. Parallel filters are then prepared, induced for protein expression and probed with either the genuine binding-site probe, or, ideally a point-mutated version of it known to abolish binding in vitro (Fig. 1). If this initial screening suggests that the right phage has been identified, an additional test of the binding specificity should be performed. For this, the phage is lysogenised in the appropriate protease-deficient *E. coli* strain (usually Y1089, Huynh et al., 1985) and a protein extract is prepared from an induced culture. This extract should contain large amounts of the fusion protein and can be used for gel-shift, footprint or methylation interference assays. This thus allows one to directly compare the DNA binding specificity of the protein encoded by the λ recombinant to that of the original factor from the nuclear extract (Singh et al., 1988). A number of parameters which are likely to affect the outcome of such an experiment are discussed briefly below.

1 cDNA Library

Because most transcription factors are relatively low-abundance proteins, the quality of the cDNA library to be screened is of paramount importance. The success of the method does not depend on having full-length cDNAs, but rather on having cDNAs that encode the DBD of the desired factor. Since the DBD of a factor can be localised anywhere in the polypeptide, it has been argued that libraries generated by random priming should be used (Singh et al., 1989). However, in practice, libraries generated by oligo-dT priming of the RNA also appear suitable, as internal

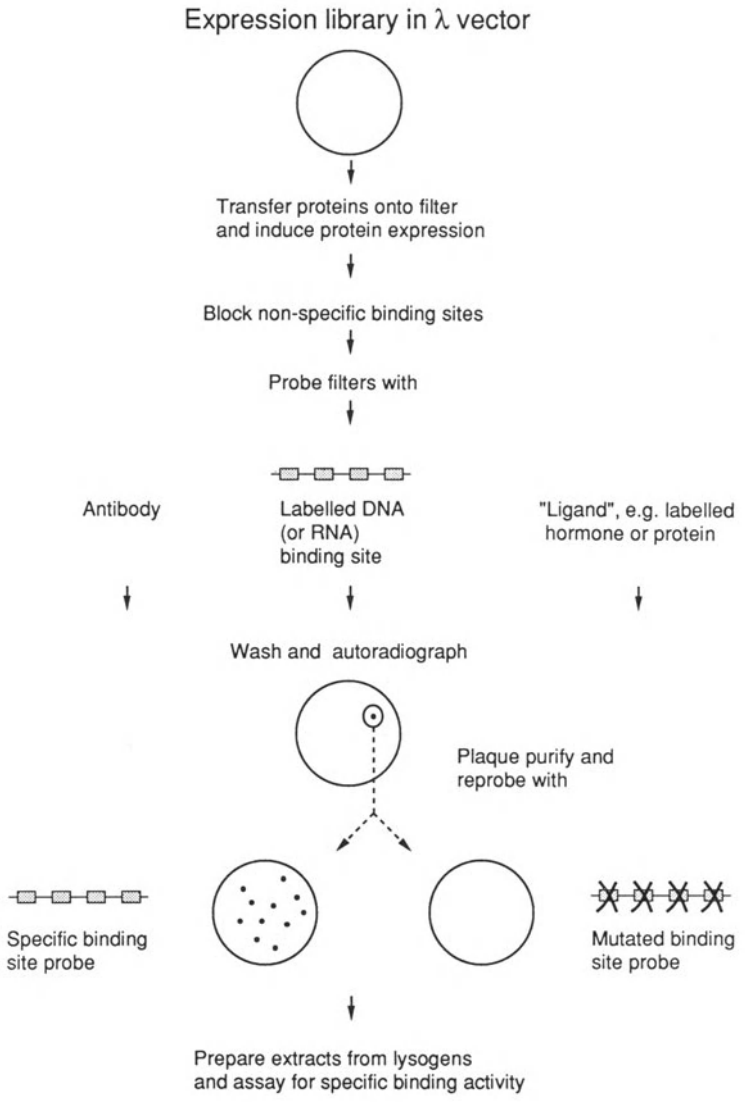


Fig. 1: General scheme of the procedure to isolate sequence-specific DNA-binding proteins from a λ expression library: The library is represented by a Petri dish and the various steps are indicated. The probe depicted consists of a labelled DNA fragment containing four binding sites for its cognate factor; an almost identical probe with all four binding sites mutated is also shown. As is apparent from the diagram, this procedure is essentially a variation of the original screening procedure with antibody probes (left part, 3). Another variation of the original method is to use a labelled ligand (such as a hormone or a bacterially expressed protein) to identify receptors or partner proteins in an expression library. This approach has already been successful in several cases (see Macgregor et al., 1990; Sikela and Hahn, 1987). This general scheme could also be used to identify sequence-specific RNA-binding proteins by using a labelled RNA probe.

priming at A-rich stretches often occur in such libraries (see e.g. Müller et al., 1988). The use of a directional cDNA-cloning procedure should be considered, as it doubles the theoretical number of in-frame recombinants to one out of three. Finally, it is most important that a primary, unamplified library be used for the initial screening. Each round of amplification can lead to selective loss of phages, and this appears to be particularly the case with phages encoding DNA-binding proteins which often are somewhat toxic to the host (for example, we have observed that cDNAs for oct-2 were very much under-represented in several amplified cDNA libraries).

2 Probe

Careful preparation of the probe is crucial to ensuring that the right factor is isolated. The probe should have a very high specific activity and be prepared in such a way that it is absolutely free from labelled single strands, which could give a high background (most eukaryotic cells contain single-stranded DNA-binding proteins in abundance!).

Vinson et al.(1988) and Staudt et al.(1988) have shown that the use of a probe containing multiple binding sites greatly increases the signal intensity, presumably by allowing one DNA fragment to simultaneously bind to several protein molecules, and thereby stabilise the interaction. Fig. 2 presents an example of the increase in signal obtained with a multimerised probe. One way of preparing such a multimerised probe is simply by ligating up an oligonucleotide containing a binding site with DNA ligase and labelling the resulting mix by nick-translation (Fig. 2). An alternative which we prefer because it produces a more defined probe, is to multimerise the binding site oligonucleotide and to clone it conveniently in a polylinker-containing plasmid (e.g, Bluescript or pUC). The oligomerised DNA sequence is then reclaimed from the plasmid by restriction digestion and radiolabelled by fill-in with Klenow polymerase; to increase the specific activity, the incubation can first be done very briefly in the absence of deoxynucleotides. After labelling, the probe is gel-purified and used for the binding reaction. The availability of a similar (i.e. multimerised), but point-mutated probe is highly desirable in order to unequivocally test the binding specificity (see Fig. 1).

3 Filters

Nitrocellulose filters have been used successfully to clone cDNAs for various factors (e.g. H2TF-1/NF-$_k$B, Oct-2, Oct-1, E47, C/EBP, Singh et al., 1988; Müller et al., 1988; Sturm et al., 1988; Murre et al., 1989; Vinson et al., 1988). However, in the case of IRF-1, a factor binding to the β-interferon gene promoter, detection could only be achieved by using nylon membranes (e.g. Nytran,; Migamoto et al.,

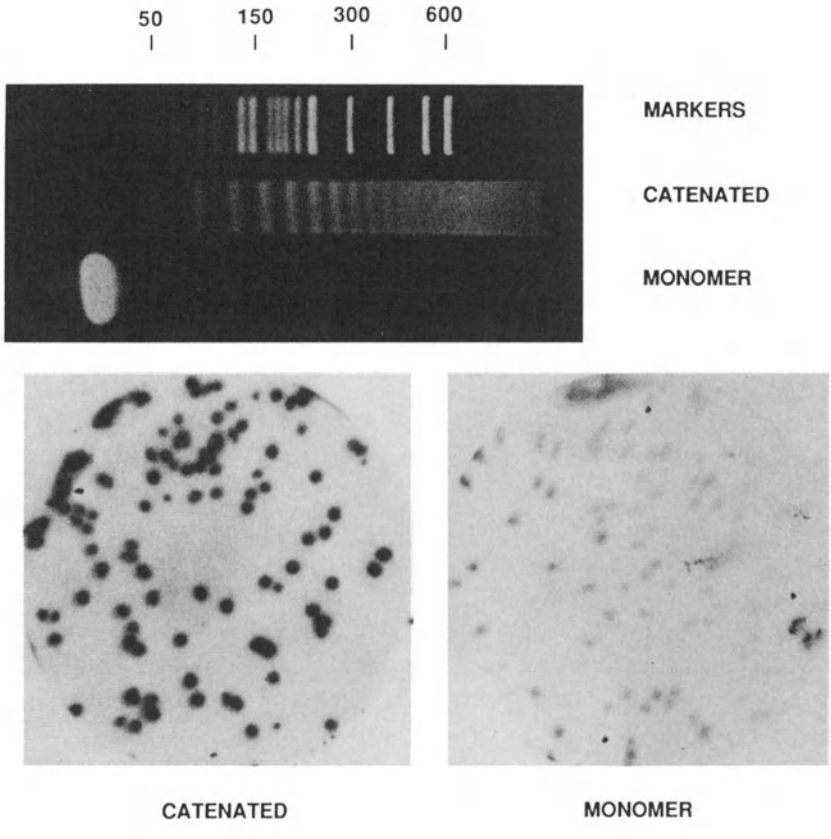


Fig. 2: Influence of binding site multimerisation on in situ detection of sequence-specific DNA binding: Two Petri plates were inoculated with bacterial cells that had been infected with about 100 pfu of L20, a bacteriophage containing a cDNA for the factor C/EBP. Filter replicas were prepared and processed by denaturation/renaturation through guanidium hydrochloride. One filter was probed with a catenated C/EBP binding site; the other filter was probed with a monomeric C/EBP binding site. The autoradiographic images produced by the two filters are shown at the bottom. The top shows the ethidium bromide stained images of monomer and catenated C/EBP binding sites probes following sizing on a polyacrylamide electrophoresis gel (from Genes and Development 2 , 801–806, 1988 reprinted with the permission of S. McKnight).

1988). The signal for the H2TF-1/NF-$_k$B factor (Singh et al., 1988) was also much stronger when a nylon membrane was used (T. Fujita, personal communication). Thus it appears that for certain proteins the type of membrane used may be very critical. Vinson et al. (1988) have shown that the signals of protein-DNA interactions can be greatly increased by subjecting the protein-containing filters to denaturation/renaturation cycles with guanidium hydrochloride. These denaturation/renatu-

ration cycles may increase the signal by facilitating correct folding of the fusion protein or by helping dissolve insoluble precipitates which could prevent binding of the DNA probe. Moreover, this procedure allows the preparation of duplicate filters from the same plate. This feature greatly facilitates identification of the genuine factor. However, not every factor may tolerate the rather harsh denaturation/renaturation treatment. Thus, both the type of filter most suitable for a particular factor as well as the factor's tolerance to denaturation/renaturation may be tested by performing Southwestern blot experiments (Miskimins et al., 1985; Hübscher, 1987) with nuclear extracts, using various types of membranes and subjecting them to diverse regimens. The resulting information can then be used to perform the library screening accordingly.

4 Screening

The binding conditions used for the screening itself should closely match those known to be optimal for e.g. gel-retardation assays. In particular, if a given factor requires a metal ion for binding (e.g. Mg^{2+}, Zn^{2+}) it should be added to all buffers. The nature and amount of competitor DNA present during the binding reaction is also crucial for identification of the correct recombinant. A combination of poly (dI-dC) and denatured calf thymus DNA (similar to what we use for gel-retardation assays) seems best.

The entire procedure requires a very long working day, especially if duplicate filters are prepared and if the filters are processed through the denaturation/renaturation cycles. Therefore, the binding reaction can be allowed to proceed overnight, although considerably shorter incubation times would be sufficient. To minimise dissociation of the protein-DNA complexes, the binding and washing reactions can be performed at 4°C instead of room temperature (Vinson et al., 1988).

B Materials and Methods

The step-by-step procedure presented below is adapted from a Swiss Commission for Molecular Biology (SKMB) course which we organised at the Institute for Molecular Biology II, University of Zürich in October, 1989. The aim of the course was to demonstrate the potential of this procedure by (re)cloning the B-cell-specific transcription factor Oct-2 from a B-cell cDNA library. The probe used consisted of a cloned DNA fragment containing four tandem copies of the adjacent heptamer/octamer sites as they are found in the immunoglobulin, heavy chain, variable region promoter. Because both the heptamer and the octamer site bind octamer factors this probe in effect contains eight binding sites. An almost identical probe having point mutations in the heptamer/octamer sites served as a control for

the specificity of the binding reaction. Both probes were prepared from plasmid clones (Müller et al., 1988), end-labelled to high specific activity with Klenow polymerase and purified by gel electrophoresis. For convenience, 150 × 15-mm Falcon Petri dishes were used throughout and the volumes given below are for such plates. 2×10^4 plaque forming units (pfu) were inoculated per plate. As a positive control, a λ recombinant containing a cDNA for Oct-2 (λ OB-C, Müller et al., 1988) was spiked in some of the library samples. The screening for Oct-2 was done using nitrocellulose filters which had been subjected to denaturation/renaturation cycles with guanidium hydrochloride.

1 Preparation of the Labelled DNA Probe

The starting material is a plasmid containing multimerised copies (e.g. Huynh et al., 1985; Macgregor et al., 1990; Sikela et al., 1987) of the binding site of interest. This plasmid has been restricted with the appropriate enzymes, such that the fragment containing the binding sites (100–150 bp in length) is liberated with as little polylinker DNA as possible. The DNA has been phenol extracted, ethanol precipitated and resuspended in TE buffer at a concentration of 2 pmole 5' end/µl.

a) *Buffers*
10× Fill-in buffer: 500 mM NaCl, 100 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 20 mM DTT

100× dNTPs: 20 mM solution of each dATP, dCTP, dGTP, or dTTP in TE

b) *Step-by-Step Procedure*

> In an Eppendorf tube pipette consecutively: 10 µl probe DNA (20 pm 5' ends), 10 µl 10× fill-in buffer, 55 µl water.

> Add 10 units Klenow polymerase (2 µl).

> Incubate 1 min on ice.

> Immediately add 1 µl of each dCTP, dGTP, dTTP and 20 µl of ^{32}P-labelled dATP (5000 Ci/mmole, 200 µCi). Incubate at room temperature for 45 min.

> Add 1 µl of cold dATP (20 mM solution) and incubate at 37° for 15 min.

> Stop the reaction by adding 0.5 volumes of ammonium acetate 7.5 M and 2 volumes of 100% ethanol. Incubate on dry ice for 15 min.

> Spin 15 min in an Eppendorf centrifuge, wash the pellet, air-dry briefly and resuspend in gel-loading buffer. Load onto a 5% polyacrylamide gel.

> After electrophoresis, visualise fragments by autoradiography (3–4 min).

> Cut out the region corresponding to the probe fragment and transfer into an Eppendorf tube. With flame-sealed blue tip crush the acrylamide piece and add 0.8 ml elution buffer (500 mM NaCl, 50 mM Tris pH 7.5, 1 mM EDTA). Elute by shaking vigorously on a platform shaker for several hours.

> Extract twice with phenol-chloroform and ethanol precipitate (include 5 µg poly (dI-dC) as carrier for quantitative recovery).

> Spin, wash the pellet, air-dry briefly and resuspend in TE. Determine radioactivity incorporation by scintillation counting. Probes prepared under these conditions should have a specific activity well above 2×10^7 cpm/pmole (because some internal labelling takes place due to the very brief incubation in the absence of nucleotides) and the total incorporation should be $3–5 \times 10^8$ cpm.

2 Plating of the Library

a) *Chemicals and Buffers*

- Phage broth, per l : 5 g NaCl, 5 g yeast extract, 10 g bactotryptone, 10 ml 1 M Tris-HCl pH 7.5, 10 ml 1 M $MgSO_4$. Dispense solution into 500 ml bottles and sterilise by autoclaving. Just before use, add 1 : 100 dilution of a sterile solution of 20% maltose (0.2% final concentration). Add 1 : 1000 dilution of a sterile solution of Ampicilin (50 mg/ml in water).
- SM solution, per l: 5.8 g NaCl, 2 g $MgSO_4 \cdot H_2O$, 50 ml 1 M Tris-HCl pH 7.5, 2% gelatin. Aliquot into bottles and autoclave.
- Top agarose: Prepare exactly like phage broth and add 7 g agarose per l (0.7% final concentration). No maltose or Ampicillin is needed for the top agarose. Aliquot into 100 ml bottles and autoclave.
- Phage plates, per l: 5 g NaCl, 5 g yeast extract, 10 g trytone, 10 ml 1 M Tris-HCl pH 7.5, 10 ml 1 M $MgSO_4$, 15 g agar (1.5% final concentration). Sterilise by autoclaving. Cool to 60°C and pour under sterile conditions (about 80 ml per 150 × 15-mm Petri dish).
- Binding buffer: (10×) 200 mM Hepes pH 7.9, 30 mM $MgCl_2$, 400 mM KCl.

(After ten fold dilution with water, add DTT to a final concentration of 1 mM (from a stock solution of 1 M DTT.)
- IPTG: Isopropyl-β-D-thiogalactopyranoside in water (10 mM made fresh)
- 6 M Guanidium hydrochloride (Gu-HCl) in 1× binding buffer
- Carnation instant, non-fat, milk powder (*Note*: US-made non-fat milk powder such as Carnation contains no fat. European-made powder milk contains some fat. This difference may or may not have an influence on the success of the binding reaction.)

b) *Step-by-Step Procedure*

Plating of the Library

> Transfer 50 ml of an overnight culture of *E. coli* Y1090 into a 50-ml Falcon conical tube.

> Pellet the bacteria by low speed centrifugation in a clinical centrifuge.

> Resuspend the bacteria in 25 ml sterile, cold 10 mM $MgSO_4$ and store the suspension at 4°C. These $MgSO_4$ bacteria can be used for about 8 days (after which the efficiency drops very sharply!).

> Melt the top agarose in a microwave oven and then cool down to about 45°C; put in a 45°C waterbath.

> In a 10-ml snap-cap Falcon tube pipette 250 µl of $MgSO_4$ Y1090 bacteria and 100 µl of the proper phage dilution (such that approximately 1.5×10^4 pfu are plated on each 150 × 15-mm dish).

> Adsorb the phages for 15 min at 37°C.

> Remove phage plates from the 42°C incubator just before doing the plating.

> To the snap-cap containing the *E. coli* and phages slowly add 10 ml of top agarose, letting it run down the side of the tube. Close the cap tightly and immediately invert the tube three times. Pour the mixture and distribute it evenly onto the pre-warmed plates. Leave at room temperature until the top agarose has solidified (5–10 min).

> Incubate at 42°C for 3 to 4 h. Plaques should be clearly visible by 4 h.

3 Screening of the Library

a) *Filters*

- Immerse the filters for 20 min into a solution of 10 mM IPTG. Then blot the filters on Whatman 3 MM paper and transfer them rapidly onto the phage plates.
- Incubate the plates at 37°C in a forced-air incubator for 4–6 h.
- Before removing the filters mark them by sticking a needle dipped in Indian ink at various locations around the periphery of the plate, following an asymmetrical pattern.
- Lift the filters from the plates with Millipore forceps and transfer them onto a 3MM filter paper with the plaques facing upwards. Let them dry for 15 min at room temperature. Lay down a second, IPTG-impregnated filter on each plate and mark similarly with Indian ink at the same locations as the initial marks. Further incubate at 37°C for 2 h. All subsequent procedures should take place at 4°C.
- After 15 min of air drying, place the filters in a small dish containing 200 ml of 6 M Gu-HCl in 1× binding buffer. Incubate with gentle shaking for 10 min.
- Pour off the Gu-HCl wash into a 500 ml graduated cylinder. Dilute by half (to 3 M) with 1× binding buffer. Pour into a glass baking dish and transfer filters to the solution one at a time, making sure each filter is allowed complete exposure to the new 3 M dilution of Gu-HCl. Shake gently for 5 min.
- Repeat the above-described 100% dilution process four more times, followed by another two rinses in binding buffer alone; each time for approximately 5 min.

Incubation	1	2	3	4	5	6	7	8
Gu-HCl	6M	3M	1.5M	0.75M	0.38M	0.19M	none	none

- Place the filters into the blocking buffer consisting of 1× binding buffer supplemented with 5% Carnation instant, non-fat, dry milk powder (Miskimins et al., 1985). Shake gently at 4°C for 30 min.
- Rinse the filters in 1× binding buffer containing 0.25% milk powder.

Note: The second set of filters should be processed until the end of the denaturation/renaturation procedure, after which they can be left at 4° overnight in the buffer containing 0.25% milk. Alternatively, the binding reaction can be set up immediately also with these filters.

b) *Binding Reaction and Washing of the Filters*

- Add the ^{32}P-labelled DNA probe to 1× binding buffer supplemented with 0.25% milk powder at a minimum concentration of 1× 10^6 cpm/ml. Also add poly (dI-dC) to 10 µg/ml and denatured calf thymus DNA to 0.2 µg/ml. Use 50 ml of binding solution per six nitrocellulose filters (132 mm) in a glass crystallisation dish. Incubate at 4°C for at least 2 h (for convenience, incubate overnight).

- On the next day, remove the radioactive solution, rinse two times with 50 ml of 1× binding buffer containing 0.25% dry milk (to remove most of the radioactivity). Wash filters for 5 min at 4°C in 1000 ml of 1× binding buffer with 0.25% dry milk.
  Repeat the washing twice.
- Decant the wash buffer. Wrap the filters between layers of Saran wrap, fix on a stiff support, mark asymmetrically with fluorescent ink and expose to Kodak XAR-5 film overnight.
- If not already done, similarly process the second set of filters (binding for ca. 4 h only, then washing and exposure). If positive plaques are identified, the corresponding region of the top agarose is cut out with an inverted blue tip and the phages are eluted for 2–3 h in 500 µl of SM buffer. This phage lysate can then be used by replating some of it (a dilution!) on fresh plates. The entire procedure is then repeated, and this time a binding reaction is also performed with the control probe having the mutated binding site (see Fig. 1).

C Bibliography

Clerc, R.G., Corcoran, L.M., LeBowitz, J.H., Baltimore, D., and Sharp, P.A. The B-cell specific Oct-2 protein contains POU box and homeo box-type domains. Genes Dev. 2 (1988) 1570–1581.

Hübscher, U. Double replica southwestern. Nucl. Acids Res. 15 (1987) 5486.

Huynh, T.V., Young, R.A., and Davis, R.W. Constructing and screening cDNA libraries in λ gt10 and λ gt11. DNA cloning, Vol. 1: A Practical Approach, pp. 49–78. Ed D.M. Glover. IRL PRESS, Oxford 1985.

Macgregor, P.F., Abate, C., and Curran, T., Direct cloning of leucine zipper proteins: Jun binds cooperatively to the DRE with DRE-BP1. Oncogene 5 (1990) 451–458.

Miskimins, W.K., Roberts, M.P., McClelland, A., and Ruddle, F.H. Use of a protein-blotting procedure and a specific DNA probe to identify nuclear proteins that recognise the promoter region of the transferrin receptor gene. Proc. Nat. Acad. Sci. USA 82 (1985) 6741–6744.

Miyamoto, M., Fujita, T., Kimura, Y., Maruyama, M., Harada, H., Sudo, Y., Miyata, T., and Taniguchi, T. Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-β gene regulatory elements. Cell 54 (1988) 903–913.

Müller, M.M., Ruppert, S., Schaffner, W., and Matthias, P. A cloned octamer transcription factor stimulates transcription from lymphoid-specific promoters in non-B cells. Nature 336 (1988) 544–551.

Murre, C.M., Schonleber-McCaw, P., and Baltimore, D. A new DNA binding and

dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD and myc proteins. Cell 56 (1989) 777–783.
Sikela, J.M., and Hahn, W.E. Screening an expression library with a ligand probe: Isolation and sequence of a cDNA corresponding to a brain calmodulin-binding protein. Proc. Natl. Acad. Sci. USA 84 (1987) 3038–3042.
Singh, H., Clerc, R.G., and LeBowitz, J.H. Molecular cloning of sequence-specific DNA binding proteins using recognition site probes. Biotechniques 7 (1989) 252–261.
Singh, H., Lebowitz, J.H., Baldwin, A.S. Jr., and Sharp, P.A. Molecular cloning of an enhancer binding protein: isolation by screening of an expression library with a recognition site DNA. Cell 52 (1988) 415–423.
Staudt, L.M., Clerc, R.G., Singh, H., Lebowitz, J.H., Sharp, P.A. and Baltimore, D. Cloning of a lymphoid specific cDNA encoding a protein binding the regulatory octamer DNA motif. Science 241 (1988) 577–580.
Sturm, R.A., Das, G., and Herr, W. The ubiquitous octamer protein Oct-1 contains a POU domain with a homeo subdomain. Genes Dev. 2 (1988) 1559–1569.
Vinson, C.R., La Marco, K.L., Johnson, P.F., Landschulz, W.H. and McKnight, S.L. In situ detection of sequence-specific DNA binding activity specified by a recombinant bacteriophage. Genes Dev. 2 (1988) 801–806.
Young, R.A. and Davis, R.W. Efficient isolation of genes by using antibody probes. Proc. Natl. Acad. Sci. USA 80 (1983) 1194–1198.

XIX Detection of DNA Bending by Gel Electrophoresis: Use of Plasmid Vectors

Christian Zwieb, Jin Kim and Sankar Adhya

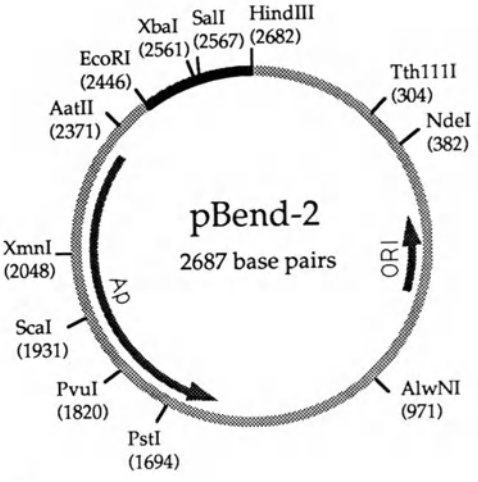
Contents

A Introduction

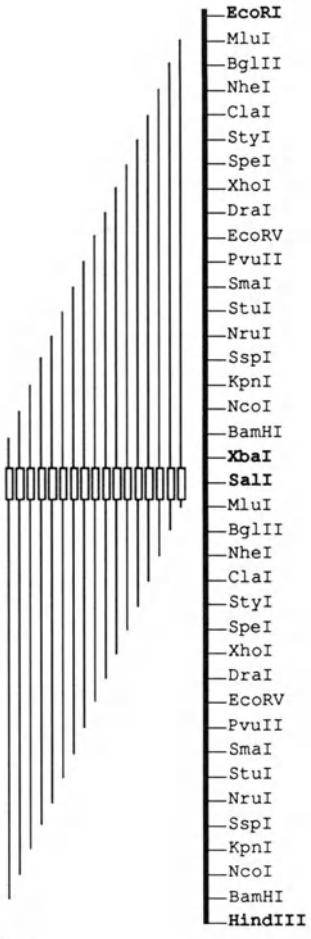
Bend in a segment of the DNA backbone, whether intrinsic or induced by binding of a sequence-specific protein, helps formation of DNA-multiprotein complexes; DNA-protein or protein-protein contact required for biologically effective compaction of the complexes is facilitated. Such bending can be easily monitored by gel electrophoresis, making use of the change in electrophoretic mobility of a contorted DNA fragment. The mobility varies according to the site and degree of bending (Wu and Crothers, 1984); a bend in the middle of the DNA fragment reduces the mobility more than a bend at either end. For analysis of DNA bending using change in mobility, the DNA sequence is inserted at one cloning site of a plasmid vector carrying two identical DNA segments with many restriction sites in a direct repeat spanning a central region containing the cloning sites (Kim et al., 1989). The plasmid is then cleaved at the directly repeated restriction sites, giving many DNA fragments of identical size, in which the segment of bent axis, intrinsic or protein-induced, is

---

Fig. 1: A. Structure of bending vector pBend2: The plasmid is a derivative of pBR322 from which the tetracycline resistance portion between the EcoRV andPvuII site has been removed. The region between the restriction sites for EcoRI and HindIII (shown in black) contains two DNA segments in direct repeat, flanking the unique cloning sites XbaI and SalI for the insertion of a bending site. The details of the construction of pBend2 have been described (Kim et al., 1989). The map illustrates the position of the ampicillin resistance gene (Ap) and the origin of DNA replication (ORI). Restriction sites of those enzymes that cut the molecule once are shown; the location of their cut sites in bp appear in brackets. B. Schematic representation of the 236 bp segment located between the restriction sites EcoRI and HindIII in pBend2 (shown as a bold line): Two unique cloning sites XbaI and SalI are present in the centre as well as seventeen duplicated 6-bp restriction sites which generally occur rarely in DNA. We note that the following seven of the seventeen sites have limited use: BglI: two sites in the repeat of pBend2; one additional site corresponding to position 3482 of pBR322 co-ordinates. ClaI: two sites in the repeat of pBend2; no sites in the pBR322-part. ClaI sites are methylated in most *E. coli* hosts, the use of this enzyme is limited to prior growth of the plasmid in a methylation-defective (dam^-) host. StyI: two sites in the repeat of pBend2, but StyI will also cut the two NcoI-sites present in the repeat. Can be used under partial digestion conditions. No site in the pBR322 part. DraI: two sites in the repeat of pBend2; three additional sites corresponding to positions 3232, 3251 and 3943 of pBR322 co-ordinates. SspI: two sites in the repeat of pBend2; one additional site corresponding to position 4170 of pBR322. KpnI: two sites in the repeat of pBend2; no site in the pBR322 part. RsaI also cuts at these sites, yet three additional sites present corresponding to positions 164, 2282 and 3847 of pBR322. The other ten of the seventeen restriction enzymes have only two sites each in the repeat of pBend2 and none in pBR322. C. Nucleotide sequence of the EcoRI-HindIII insert in pBend2 with the unique cloning sites XbaI and SalI flanked by duplicated (direct) restriction sites MluI, BglII, NheI, ClaI, StyI, SpeI, XhoI, DraI, EcoRV, PvuIII, SmaI, StuI, NruI, SspI, KpnI, NcoI and BamHI in that order.



A



B

```
  EcoRI  MluI  BglI  NheI  ClaI  StyI  SpeI  XhoI  DraI  EcoRV
  GAATTCACGCGTAGATCTGCTAGCATCGATCCATGGACTAGTCTCGAGTTTAAAGATATC
  1         11        21        31        41        51

   PvuII  SmaI  StuI  NruI  SspI   KpnI  NcoI       BamHI  XbaI
  CAGCTGCCCGGGAGGCCTTCGCGAAATATTGGTACCCCATGGAATCGAGGGATCCTCTAG
 61         71        81        91        101       111

     SalI  MluI  BglI  NheI  ClaI  StyI  SpeI  XhoI  DraI  EcoRV
  AGTCGACACGCGTAGATCTGCTAGCATCGATCCATGGACTAGTCTCGAGTTTAAAGATAT
121        131       141       151       161       171

     PvuII  SmaI  StuI  NruI  SspI  KpnI  NcoI      BamHI  HindIII
  CCAGCTGCCCGGGAGGCCTTCGCGAAATATTGGTACCCCATGGAATCGAGGGATCCAAGCTT
181        191       201       211       221       231       241
```

C

located in a permutated order. The intrinsic or protein-bound, contorted DNA fragments are subjected to gel electrophoresis. Finally, the degree by which the DNA fragment bends from linearity at the bending site is calculated from an arbitrary relationship between degree of bending and relative electrophoretic mobility of fragments (Thompson and Landy, 1988). Structural details of a bending vector, pBend2, used in the protocol for analysis of a bending is shown in Fig. 1. Note that for analysis of intrinsic DNA bends, the formation of protein-DNA complexes should be omitted.

B Cloning of a DNA-bending Site into the Bending Vector pBend2

To study protein-induced DNA bending, the binding site for the protein of interest must be inserted into the XbaI or SalI restriction site of pBend2 (Fig. 1A). An insert may be derived from a restriction fragment, or obtained with advantage by the synthesis of two complementary oligonucleotides forming the duplex binding site. The oligonucleotides can be designed with "sticky" ends compatible with the XbaI and/or SalI sites. If the oligonucleotides are already available with "blunt" ends, filling-in of the pBend2 XbaI or SalI site is required. The shorter the size of the inserted DNA segment containing the bending formation, the more accurate is the determination of the bend. A size of 20–50 bp is recommended.

1 Reagents and Enzymes

- 1× TE: 10 mM Tris · HCl pH 7.8, 1 mM EDTA, pH 8.0
- 500 mM EDTA, pH 8.0
- 7.5 M NH_4 acetate
- 2 mM dNTPs
- 3 M Na acetate
- 100 mM NaCl
- 5 mM ATP
- Agarose
- 10× Concentrated fill-in buffer: 200 mM Tris · HCl pH 7.5, 100 mM NaCl, 6 mM β-mercapto-ethanol
- DNA polymerase (Klenow) 100 units/17 µl, e.g. BRL 8012SA
- T4 Polynucleotide kinase 10 units/µl, e.g. BRL 8004SA
- 5× Ligase buffer: 250 mM Tris · HCl, pH 7.6, 50 mM $MgCl_2$, 50 mM DTT
- T4 DNA ligase, 1 unit/µl, e.g. BRL 5224SB

- Ethanol
- LB-Ampicillin (100 µg/ml) agar plates
- Restriction enzymes and their digestion buffers

2 Step-by-Step Procedure

a) *Preparation of pBend2 Vector DNA*

> To 1.5-ml Eppendorf tube add 25 µl of pBend2 DNA (25 µg) purified by cesium chloride gradient.

> Add 55 µl water, 10 µl 10× concentrated digestion buffer for XbaI or SalI, 10 µl (100 units) restriction enzyme XbaI or SalI.

> Incubate at 37°C for 2 h.

> Check a small aliquot of the digestion on a 1.5% agarose gel to verify the complete linearisation of the plasmid DNA.

> Add 3 µl EDTA pH 8.0, 100 µl ice-cold TE, 100 µl ice-cold 7.5 M NH_4 acetate.

> Keep on ice for 10 min.

> Spin in a table-top centrifuge for 10 min to sediment the restriction enzyme.

> Add the supernatant (containing DNA) to 600 µl ethanol.

> Keep at –70°C for 20 min.

> Spin in a table-top centrifuge for 10 min to sediment DNA.

> Wash the pellet with 500 µl 80% ethanol.

> Spin in a table-top centrifuge for 5 min.

> Dry the pellet in a vacuum centrifuge.

> Dissolve the DNA in 250 µl TE.

> To fill-in the sticky end generated by SalI or XbaI, add 10 µl DNA to an 1.5-ml Eppendorf tube.

> Add 2 µl 10× concentrated fill-in buffer, 1 µl of 2 mM dNTPs, 6.5 µl water, 0.5 µl DNA polymerase (Klenow).

> Incubate at room temperature for 10 min and on ice for 10 min.

> Add 1 µl 500 mM EDTA pH 8.0, 40 µl ethanol, 2 µl 3 M Na acetate, pH 6.0.

> Incubate 20 min at –70°C to precipitate the DNA.

> Spin, wash the pellet with 80% ethanol and dry as described above.

> Store linearised vector DNA at –20°C.

b) *Preparation of the Bending Site*

> Two complementary oligonucleotides which form the desired bending site and have proper restriction ends (XbaI, SalI or blunt ends) are synthesised chemically. Oligonucleotides up to about 30 bases long usually require no further purification.

> Dissolve oligonucleotides separately at a concentration of 200 µg/ml.

> To an Eppendorf tube add 180 µl 1× TE containing 100 mM NaCl.

> Add 10 µl of each oligonucleotide.

> Incubate in a beaker with boiling water for 3 minutes.

> Place the beaker in a cold room to allow annealing of the two oligonucleotides by slow cooling to 4°C.

> Store annealed oligonucleotides at –20°C.

c) *Insertion of Bending Site into Linearised pBend2 DNA*

> Add to an Eppendorf tube 2 µl of annealed oligonucleotides.

> Add 3 µl 5× ligase buffer (this buffer is potent for a polynucleotide kinase reaction).

> Add 1 µl 5 mM ATP, 8.5 µl water, 0.5 µl T4 polynucleotide kinase.

> Incubate for 10 min at 37°C and place sample on ice.

> Add 3 µl 5× ligase buffer, 1 µl linearised vector DNA, 1 µl 5 mM ATP, 9 µl water, 1 µl T4 DNA ligase.

> Incubate at 15°C for several hours or overnight.

> Store the samples at 4°C.

> Transform competent *E. coli* cells (any transformation pro-

ficient *E. coli* strain should work), plate on LB plates containing 100 µg/ml ampicillin and incubate at 37°C.

> Next day streak transformants for single colony isolation on LB plates containing 100 µg/ml ampicillin.

> Incubate at 37°C until colonies appear.

> Prepare plasmid DNA on a small scale from individual colonies and digest with restriction enzymes EcoRI and HindIII.

> Separate the DNA fragments on a 2% agarose gel.

> Select plasmids which give a large EcoR1-HindIII fragment caused by the insertion of the bending site.

> Confirm the insertion of the proper DNA segment and determine the orientation by DNA sequencing.

> Purify plasmid DNA by centrifugation in a cesium chloride gradient.

C Considerations when Selecting Restriction Sites to Generate Permutated DNA Fragments

pBend2 provides 17 restriction sites which can be used to produce fragments of identical length in which the bending site is closer to the ends or in the centre (Fig. 1B). To determine if a certain degree of DNA bending occurs, it is advisable initially to use only two or three of the restriction sites: MluI or BamHI (which place the bending site closer to the ends) and EcoRV or PvuII (which position the bending site in the middle region). The general considerations for selecting a restriction site are: (1) The enzyme should not cut in the bending site. (2) Preferably there are no additional sites in the plasmid. (3) The mobility of a bent DNA on the electrophoresis gel should not interfere with another restriction fragment generated by such an enzyme. In this regard, some cautionary notes are included in the legend to Fig. 1B.

For protein-induced bending, unlabelled DNA can be used when sufficient protein is available, and it is not necessary to purify the individual fragments. The remainder of the plasmid DNA will be well separated from the DNA-protein complex on polyacrylamide gels. When the amount of protein is limited, it is advisable to purify and isolate the fragments after electrophoresis on agarose or polyacrylamide gels. The fragments can then be labelled individually by the T4 kinase reaction with γ^{32}P-ATP, or other standard labelling procedures, before use in a protein-binding reaction.

1 Reagents and Enzymes

– See above

2 Step-by-Step Procedure for the Digestion of the pBend2 Construct

> Add to an Eppendorf tube 100 µl plasmid DNA (100 µg) purified by caesium chloride.

> Add 30 µl 10× buffer (depending on restriction enzyme used), 160 µl water, 10 µl restriction enzyme (100 units).

> Incubate overnight at 37°C and place sample on ice.

> Add 150 µl ice-cold 7.5 M NH_4 acetate.

> Leave on ice for 15 min to precipitate the restriction enzyme.

> Spin 10 min in a bench-top centrifuge.

> Add the supernatant to 900 µl ethanol, mix and place at –70°C for 20 min.

> Spin, wash the pellet with 80% ethanol and dry as described above.

> Dissolve the pellet in 50 µl water and store at –20°C.

D Analysis of DNA-protein Complexes by Polyacrylamide Gel Electrophoresis

The conditions for the formation of DNA-protein complexes vary from one protein to another and need to be worked out before a bending experiment is carried out. It is advantageous if a protein for which the DNA-bending angle is known can be used as a control for comparison on the same gel. A protein concentration should be chosen which will result in sufficient complex formation, leaving some unbound DNA. This is usually determined in a preliminary titration experiment using a range of protein concentrations. About 10 µg of digested plasmid DNA in a single polyacrylamide gel lane is required to visualise both complexed and free DNA by ethidium bromide (100 µg/ml) staining. Much less DNA and protein can be applied if labelled DNA is used. The sample procedure given below is for Gal repressor

operator complexes (Zwieb et al., 1989), and the results from a typical bending experiment are shown in Fig. 2.

1 Reagents and Enzyme

- Acrylamide/bisacrylamide
- Other reagents appropriate for binding of protein to DNA and subsequent electrophoresis.

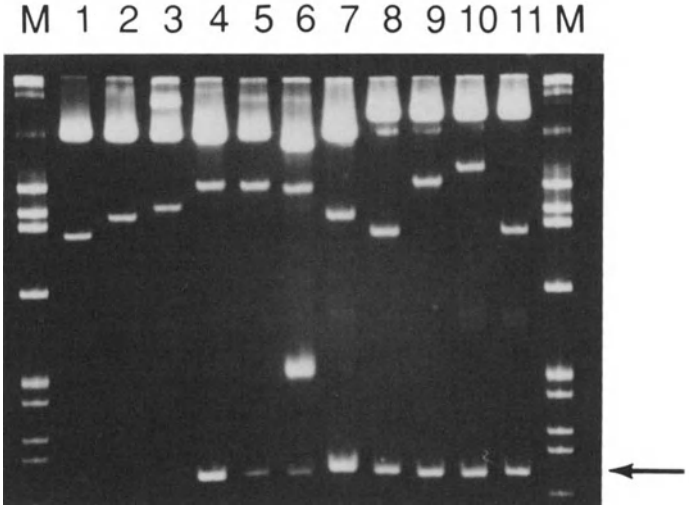


Fig. 2: Results of a typical bending experiment with Gal repressor protein: Analysis of protein-DNA complexes by polyacrylamide gel electrophoresis and staining of the DNA with ethidium bromide. The pBend-construct containing a Gal repressor binding site (operator, O_E) was digested individually with restriction endonucleases MluI (lane 1), BglII (lane 2), NheI (lane 3), PvuII (lane 4), StuI (lane 5), SspI (lane 6) or BamHI (lane 7). Enough Gal repressor was added to form the complexes, as shown by the bands of intermediate but varying mobility from lane to lane, achieving half-maximal binding, i.e. to have some unbound DNA left; bands marked by arrow. The material near the top involves the Gal repressor. The extra fragment present in lane 6 is because of the presence of an additional SspI site in the vector. Lanes 8 to 11 show the mobility difference between operator, O_E (lanes 8 and 9) and another Gal repressor binding site (operator, O_i) (lanes 10 and 11, Zwieb et al., 1989). DNA was digested with MluI (lanes 8 and 11) or EcoRV (in lanes 9 and 10). Lanes "m" show DNA markers from a HaeIII digest of ∅× 174 DNA.

2 Step-by-Step Procedure

> Prepare an 8% polyacrylamide gel with 20 ml 40% acrylamide/0.8% bisacrylamide, 6 ml glycerol, 2 ml 1 M HEPES pH 8.3, 40 µl 250 mM EDTA pH 7.5, in a total volume of 100 ml. The reservoir buffer is prepared by mixing 10 ml 1 M HEPES (pH 8.3) and 400 µl EDTA (pH 7.5) in a total volume of 1 l.

> For the binding reaction, add to a 1.5-ml Eppendorf tube at room temperature 1.5 µl 5× binding buffer (50 mM Tris · HCl pH 7.6, 50 mM $MgSO_4$, 0.5 mM EDTA, 0.5 mM DTT, 250 µg/ml BSA).

> Add 2 µl DNA (cold or end-labelled), 4.5 µl 40% glycerol, and 2 µl Gal repressor protein (diluted appropriately in 1× binding buffer immediately before use).

> Mix the sample gently with the tip of the pipette (do not vortex).

> Leave the sample at room temperature for 10 min.

> Load samples directly onto the 8% polyacrylamide gel without the addition of tracking dyes.

> Run the electrophoresis at room temperature and 200 V for 5 h.

> After electrophoresis, stain the gel with ethidium bromide solution (100 µg/ml) or prepare the gel for autoradiography by drying.

E Interpretation of Results

1 Determination of the Bending Angle

The bending angle α of a DNA fragment is defined as the angle by which a segment of the DNA departs from linearity, as shown in Fig. 3. This change assumes a value of 0° for a straight duplex, and α can be estimated from gel mobilities using an empirical relationship between degree of bending and relative, electrophoretic mobility retardation caused by bending (Thompson and Landy, 1988). Since the mobility of a rigid DNA fragment is related to its end-to-end distance, the latter

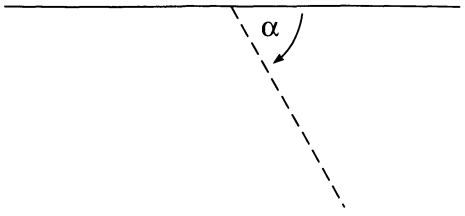


Fig. 3: The angle, α, in degrees by which a DNA straight rod bends from linearity.

equals L · cos (α/2), with L being the length of the unbent DNA. The end-to-end distance of a fragment bent at one end will be virtually the same as L. Thus, $\mu_M/\mu_E = L \cdot \cos(\alpha/2)/L = \cos(\alpha/2)$, where μ_M = mobility of the complex with protein bound in the middle, and μ_E = mobility of the complex with the protein bound at one end. It must be noted that values calculated with this equation may be different from absolute bending angles, since factors other than the end-to-end distance may influence the mobility of protein-bound and unbound DNA fragments. The apparent bending angles of O_E and O_I induced by repressor are 97° and 112°, respectively.

2 Determination of the Site of Bending

The pBend2 vector can also be used to determine the approximate position of the bend in the protein-binding site (Wu and Crothers, 1984). By the use of several restriction fragments, enough data points can be obtained to plot accurately the positions of the bending sequence (in base pairs measured from one end of the DNA fragment) versus the mobilities of the protein-DNA complexes. The minimum of the curve will coincide with the centre of the bend. A theoretical plot of this nature is shown in Fig. 4. If several bends are introduced by the protein, only an average position of the bends is given. A single, sharp bend at one position and a smooth, curve over a larger DNA region would not be distinguished.

F Common Problems and their Solutions

Failure to insert a bending site into pBend2 vectors
One common reason is that the pBend2 DNA is not linearised completely, resulting in a large number of transformants due to the presence of supercoiled and circular plasmid DNA. In this case, alter the DNA-enzyme ratio in favour of the enzyme, and confirm complete digestion by electrophoresis of a small aliquot of the DNA.

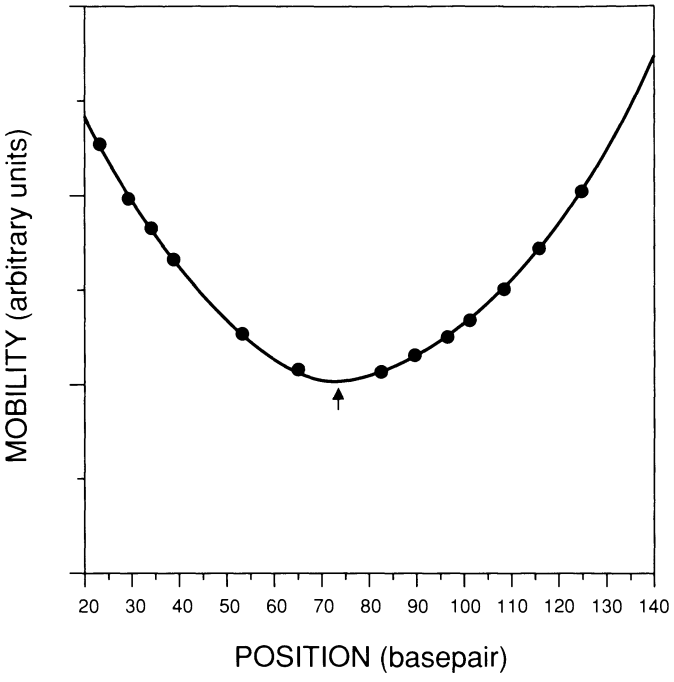


Fig. 4: The relative mobility of DNA-protein complexes versus the location of the binding site in the permutated fragments. The arrow indicates the approximate bending site.

Cloning also fails if the oligonucleotides are of poor quality. They should be checked by polyacrylamide gel electrophoresis. Large oligonucleotides should be first purified. Titration of the input of duplexed DNA will help to find conditions which allow successful insertion of the bending site.

No complex formation

The binding conditions need to be worked out individually for each protein. Variation of the protein concentration is necessary to determine the concentration required for an appropriate amount of complex formation.

Unexpected additional bands on the polyacrylamide gel

This can be a problem especially when radioactively labelled DNA is used. Use less enzyme or shorter digestion periods for restriction of the plasmid DNA. Make sure to include a control without added protein. Another disadvantage in using labelled DNA is the variable amount of radioactivity incorporated into the fragments by the T4 polynucleotide kinase depending on the restriction enzyme used. This problem is overcome by loading the gel with aliquots of the binding reactions adjusted for the efficiencies of labelling of the fragments.

G Bibliography

Kim, J., Zwieb, C., Wu, C., and Adhya, S. Bending of DNA by gene-regulatory proteins: construction and use of a DNA bending vector. Gene 85 (1989) 15–23.

Thompson, J.F., and Landy, A. Empirical estimation of protein-induced DNA bending angles: application to site-specific recombination complexes. Nucl. Acids Res. 20 (1988) 9687–9705.

Wu, H.-M., and Crothers, D.M. The locus of sequence-directed and protein-induced DNA bending. Nature (London) 308 (1984) 509–513.

Zwieb, C., Kim, J., and Adhya, S. DNA bending by negative regulatory proteins: Gal and Lac repressors. Genes and Development 3 (1989) 606–611.

XX The Use of Amplification for in vitro Footprinting Experiments

Hanspeter Saluz

Contents

A Introduction and Flow Diagrams

1 The Use of DNA Amplification for in vitro Footprinting

There are often situations where the availability of biological material for in vitro footprinting experiments, i.e. proteins or DNA, is limited. In both cases, it may be helpful to increase the quantity of DNA by either linear (Fig. 1) or exponential amplification (Fig. 2). In this chapter, I will describe how these two techniques of DNA amplification can be used as an aid to in vitro footprinting procedures.

In the situation where only a small amount of protein is available (a few cells, small extract etc.), the in vitro footprinting experiment is performed as usual but with correspondingly less protein and DNA. After the footprinting reactions the DNA is isolated and then linearly amplified as indicated in the flow diagram (Fig. 1).

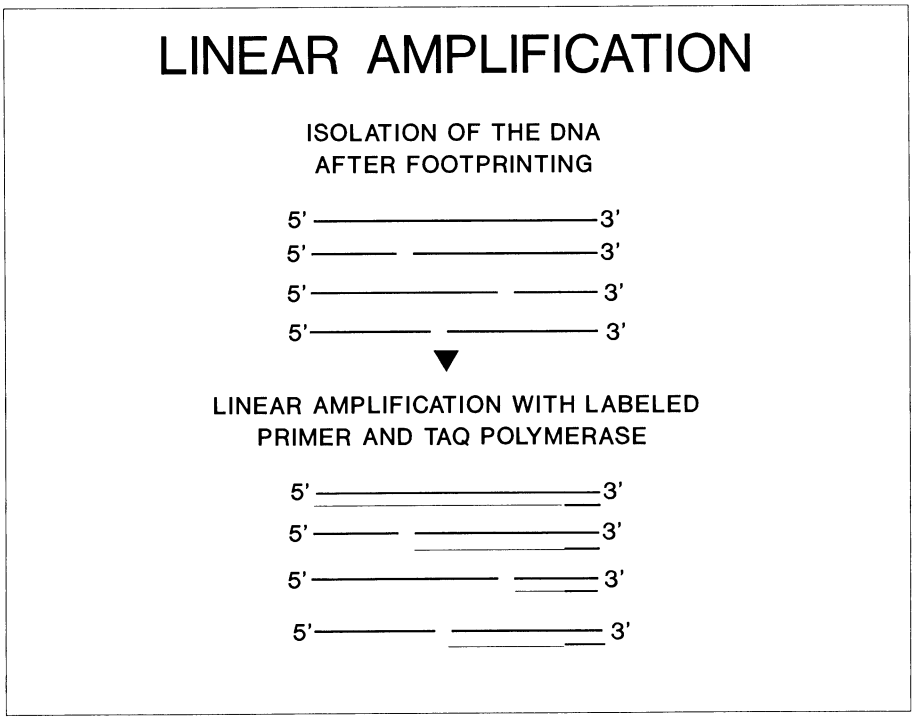


Fig. 1: Flow diagram of the linear amplification of DNA.

If only a few DNA molecules are available, the target sequence can be exponentially amplified (Fig. 2) before it is used for the in vitro footprinting experiments. By this means, millions of copies can be obtained from very low quantities of the DNA target sequence and tedious cloning work can be circumvented. It is possible to use any kind of DNA polymerase for the amplification, but since the procedure involves a heat denaturation step after each cycle, for practical reasons I will only describe a method where the thermostable DNA polymerase from *Thermus aquaticus* (Taq polymerase) is used. This is extremely convenient since only a single addition of enzyme is necessary at the start of the amplification process. If other thermolabile polymerases are used, a fresh aliquot must be added after each denaturation step. Moreover, the potential for introducing artifacts is reduced since higher temperatures, hence more stringent conditions, can be employed. Increasing the stringency of the reaction influences the choice of the primer. I describe the use of relatively long (i.e. approximately 20-mer) primers which guarantee high fidelity of amplification; they are short enough to allow calculation of the melting tempera-

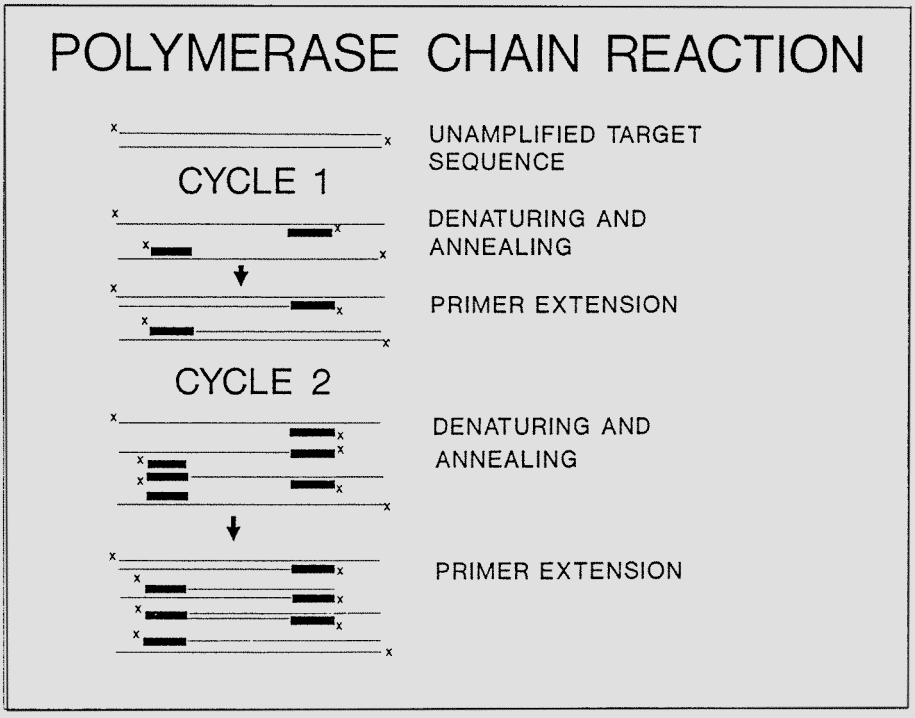


Fig. 2: Flow diagram of the exponential amplification of DNA (polymerase chain reaction).

ture according to the empirical rules of Suggs et al. (1981), which are valid for oligonucleotides of up to 21 base-pairs in length. This avoids the need for determining complex melting curves with expensive apparatus. The Suggs' rule is very simple and the melting temperature (T_m) is calculated by adding 4°C for each G or C plus 2°C for each A or T, respectively. The annealing temperature is obtained by subtracting approximately 4°C from the calculated T_m. To guarantee an optimal stringency during exponential amplification, both primers should be chosen with similar, melting temperatures.

Several procedures (Saiki et al.,1988; Ehrlich, 1989; Saluz and Jost 1989, 1990;) to label the amplified target fragments are conceivable. Here I will only describe the use of an end-labelled primer since this gives clean and sufficiently strong signals in a relatively straightforward way. If good signal strength cannot be obtained by this method, internally labelled primers or filling-in reactions should be used. Such procedures have been described in detail elsewhere (Saluz and Jost 1989, 1990) and will not be discussed further here.

2 Using Linear Amplification for in vitro Footprinting Where the Amount of Protein (Extract) is Limited

a) *The Oligonucleotide Primer*

The primer must be complementary to the 3' end of the target sequence to be studied, since DNA polymerase from *Thermus aquaticus* elongates a DNA strand in the 5' to 3' direction. Furthermore, it should be noted that incorporation of a guanosine or a cytosine at the 3' end of the amplification primer may influence the annealing kinetics of the end to be elongated by Taq polymerase (the free energy of guanosine and cytosine is greater than that of adenosine or thymidine). A very important parameter for a precise amplification is the annealing temperature of the oligonucleotide primer with its template. This is performed approximately 2–4°C below the Tm.

As already mentioned above, for in vitro footprinting experiments it is usually sufficient to prepare sequencing primers (Huibregtse and Engelke, 1986) by direct end-labelling with DNA kinase and (γ-^{32}P)ATP.

b) *Linear Amplification of Footprinted DNA*

In this technique, the footprinting procedures are followed by selective, linear amplification with the thermostable DNA polymerase from *Thermus aquaticus*, and the reaction products are directly separated on a sequencing gel. This approach allows the use of very small quantities of protein for in vitro footprinting. However, the success of such a procedure is highly dependent on the quality of the Taq polymerase. With the availability of cloned Taq polymerases it should be possible to obtain the best possible quality of enzymes. The recombinant enzyme can be purchased from Perkin-Elmer Cetus and United States Biochemical Corporation.

As already indicated by Saiki et al. (1985), I also find that the number of enzyme units used per reaction mixture influences the quality of the amplification. I now routinely use 2–5 units of Taq polymerase per 100 µl of reaction mixture. The reaction efficiency decreases with the number of amplification cycles, and for reliable copying of the DNA fragments a total of 25–30 cycles should be optimal. The amount of footprinted DNA used for one reaction is dependent on the copy number of the target sequence, number of amplification cycles, and the quality of the dNTPs and polymerase.

The buffer used for the Taq polymerase reaction influences the efficiency and quality of the linear amplification. Several buffers were tested (Saluz & Jost, 1989; 1990) as described in the different PCR protocols. For copying short DNA fragments (up to 300–400 nucleotides) as used in in vitro footprinting experiments (size exclusion of a common sequencing gel: approximately. 300–400 nucleotides), good results were obtained (Saluz & Jost, 1990) with the buffer described by BIOLABS. Oste (1989) recommends optimisation of the magnesium concentration whenever the amount of DNA template or concentration of primers and dNTPs are changed. However, the higher the magnesium concentration the higher will be the melting temperature of the double-stranded DNA, which is going to make the denaturation step more difficult. This may play an important role in the study of DNA with a high G+C-content.

Too low a concentration of magnesium resulted in much weaker signals, even when the annealing temperature was adjusted. The influence of magnesium on the melting temperature was tested with different oligonucleotidess (Saluz & Jost, 1990). For example, the melting temperature of the double-stranded 27-mer oligo was 68°C when using 6.7 mM magnesium. Decreasing the magnesium concentration to 1.3 mM resulted in a melting temperature of 64°C. The sequence signals were much lower, even when the annealing conditions were adjusted. The best experience was had with 6.7 mM Mg^{2+} and an annealing temperature 2–4°C below the Tm. Very weak signals were obtained when Mg^{2+} ions were replaced by Mn^{2+}.

c) *Purification of the Reaction Products*

It seems that certain enzyme preparations contain additives, such as enzyme stabilisers, which may interfere with the electrophoretic separation of the amplification products (resulting in smears within the sequencing lane). Since common ethanol precipitations or phenol/chloroform extractions may not remove the contaminants, precipitation of the amplified DNA with N-cetyl-N,N,N-trimethyl-ammonium bromide (CTAB) in the presence of ammonium sulphate is recommended (Jost et al, 1989). CTAB is a positively charged detergent often used in cosmetics or for DNA isolation from plants. When applied to precipitations, polysaccharides and certain proteins remain soluble. Combination of CTAB with ammonium sulphate and ethanol precipitation guarantees small, clean DNA pellets which are mixed with sample dye and loaded onto the sequencing gels. It should be noted that CTAB is a cationic detergent; any anionic detergents present will form a complex

with CTAB at the expense of DNA fragments. In such cases, titration with CTAB in the presence of ammonium sulphate will be required.

After linear amplification, the aqueous phase must be transferred into a precooled test tube, during which any contamination from the covering layer of mineral oil must be avoided, since traces of oil may at the end cause an increase in the background within the sequencing ladder. To avoid this, some investigators filter the samples through a membrane (Millipore Ultrafree MC, 0.45 μm) after the linear amplification and before CTAB precipitation. Immediately after purification, the samples are loaded onto a sequencing gel, as previously described (Saluz and Jost, 1989).

3 Using Exponential Amplification for in vitro Footprinting Where the Amount of Target DNA is Limited

A convenient way to increase the amount of DNA for in vitro experiments is by exponential amplification or polymerase chain reaction (PCR) of the target DNA (Fig. 2) with Taq polymerase (Scharf et al., 1986; Wong et al., 1987; Stoflet et al., 1988; Erlich, 1989; Saluz and Jost, 1990). Using PCR, millions of copies can be obtained from very low quantities of the DNA target sequence. This, therefore, opens a completely novel and very powerful way to circumvent tedious cloning work. A prerequisite for such experiments is the high fidelity of the polymerase chain reaction, and artifacts should be avoided by choosing optimal reaction conditions. When using this approach, the following points should be considered: The two synthetic oligonucleotides used for the exponential amplification should have a similar annealing temperature. It is advantageous to use oligonucleotides with a relatively high melting temperature to decrease reannealing of the double-stranded DNA target (see also Linear Amplification above). Detection is achieved by labelling either one of the primers in separate extension reactions. This has the advantage that the DNA can be visualised immediately after footprinting, but has the disadvantage that the amplified DNA must be used within a relatively short time (few days to weeks) . In addition it should be noted that amplification efficiency may fall off rapidly, especially in later cycles, if either the enzyme concentration or the polymerisation time are insufficient, or if the primer- or dNTP concentration is too low. Therefore, the number of cycles should not be too high (approximately 30). Best results were obtained by separating the amplified DNA from the primers and other contaminants after the polymerase chain reaction. This can be done by electrophoresis of the amplification products on an agarose gel, i.e. not low-melting agarose, followed by electro-elution of the target DNA.

The concentration of the exponentially amplified DNA is determined by running a small aliquot of the purified DNA on an agarose gel alongside DNA size markers of known concentrations (see Fig. 3). Comparison of the intensities of the DNA bands of the standards and the sample gives a good estimation of the concentration

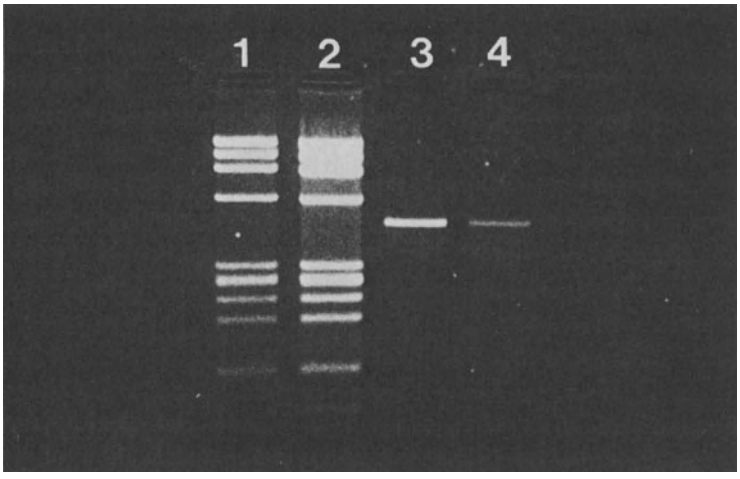


Fig. 3: Concentration of the exponentially amplified and electro-eluted target DNA: A small aliquot of the purified DNA is run on an agarose gel (tracks 3 and 4) alongside DNA size markers of known concentration (tracks 1 and 2). Comparison of the intensities of the DNA bands of the standards (tracks 1,2) and the samples (tracks 3,4; arrow) gives a good estimation of the concentration of the amplified DNA (arrow).

of the amplified target DNA. Problems can arise from contamination due to PCR-product carryover and every effort should be made to avoid this. A list of procedures to minimise this problem has already been published by S. Kwok (1989).

B Practical Procedures for Linear Amplification

1 Materials and Buffers:

Labelling of the Primer

- Purified oligonucleotide (approximately 20-mer) complementary to the target sequence 0.1 µg oligonucleotide/6 µl of water.
- T4 Polynucleotide kinase
- Redistilled water
- Labeled (γ-^{32}P)-ATP (3000 Ci/mmole)
- 10× Kinase buffer: 500 mM Tris-HCl pH 7.6, 100 mM $MgCl_2$, 50 mM dithiothreitol, 1 mM spermidine, 1 mM EDTA

- *E. coli* carrier DNA
- Water bath (37°C)
- Water bath (75°C)
- Floater
- Stop watch
- Microfuge

Linear Amplification and Purification of Amplified DNA
- Labelled primer
- Footprinted target DNA
- 10× Taq reaction buffer: 166 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl pH 8.8 at 25°C, 67 mM $MgCl_2$, 100 mM β-mercaptoethanol, 2 mg BSA/ml of highest purity, for example from BRL
- Taq polymerase (We obtained excellent results with AmpliTaq from Cetus.)
- dNTPs: sequencing grade, Pharmacia; a stock solution containing all four nucleotides each at a concentration of 10 mM
- Mineral oil (Merck; IR-spectroscopy grade)
- DNA thermal cycler
- Microfuge
- Floaters
- Box with ice/water
- Drawn-out microcapillaries
- 1% CTAB solution (N-cetyl-N,N,N-trimethyl-ammonium bromide)
- 0.5 M Ammonium acetate (pH not adjusted)
- 3 M Sodium acetate/5 mM EDTA (pH 5.0)
- Ethanol
- Sorvall centrifuge or equivalent
- SS-34 rotor (Sorvall or equivalent)
- SpeedVac
- 100 mM NaOH, 1 mM EDTA
- 8 M Urea, 0.04% xylenecyanol, 0.04% bromophenol blue (this stock must be stored frozen)
- Millipore Ultrafree MC membranes, 0.45 µm.

2 Step-by-Step Procedure

Labelling of the Primer

> Take a sterile Eppendorf tube and add 2 µl of 10× kinase buffer and 7 µl of the sequence-grade oligonucleotide primer.

> Add 10 µl of (γ-^{32}P)ATP

> Add 1 µl of T4 polynucleotide kinase (5 U/µl).

> Mix, spin briefly in a microfuge and incubate for approximately 30 min at 37°C.

> Inactivate the enzyme by incubating the sample at 65°C for 5 min.

> Add 10 µg of *E. coli* DNA in 80 µl of water.

> Mix, spin briefly and purify the labelled oligonucleotide as indicated in the a Appendix on Sephadex 50 (medium) Spun-column. The oligonucleotide is ready for use.

Linear Amplification and Purification of the Amplified DNA

> To an Eppendorf tube (which must fit your DNA thermal cycler) add consecutively: 69 µl of the footprinted DNA (minimum 200–400 fg), 17 µl of the radioactively labelled primer (approximately 5 ng), 10 µl of 10× Taq reaction buffer.

> Mix gently by tapping the tube and spin for a few seconds in a microfuge.

> Incubate at 95°C for 5 min in a Perkin Elmer- Cetus DNA thermal cycler, programed for denaturing DNA.

> Quick-chill the samples in ice/water (1 min; do not interrupt the denaturing programe).

> Spin the samples for a few seconds in a microfuge.

> Put the samples immediately back on ice/water.

> Add 3 µl of dNTPs (300 µM each in the final mix).

> Add 1 µl of Taq polymerase (diluted to 2–5 units/µl in reaction buffer)

> Mix, centrifuge briefly and put the samples immediately back on ice/water.

> Take a 1-ml plastic pipette and add 100 µl of mineral oil to each sample.

> Put the samples for 1 min at 95°C in the DNA cycler (which is still at 95°C).

> Stop the denaturing programe and start the amplification programe: 30 cycles, each consisting of 1 min denaturation

at 94°C, 2 min annealing (2–4°C below the Tm of the oligonucleotide primer duplex) and 3 min chain elongation at 72°C (this time may be varied if too many background bands occur in the sequencing ladder).

Note:
The times given here are for the effective temperature and do not include heating or cooling time between the steps. If differences between the programed and effective temperatures are observed, the DNA-cycler should be adjusted. This is especially important in the case of improper annealing.

Note:
Oligonucleotides with a high G+C content may have an annealing temperature of over 72°C. In such a situation it is recommended to change the cycling programe as follows: 1 min denaturation at 94°C, 5 min total for annealing and elongation at 2–4°C below the measured melting temperature.

> After the last cycle, immediately put the samples on ice/water and begin the purification.

> Transfer the amplification products (aqueous phase) with drawn-out microcapillaries into a precooled test tube, avoiding contamination from the covering layer of mineral oil (test tubes in a floater on ice/water), and continue with the CTAB precipitation.

Note:
If problems occur due to contamination with oil, transfer the aqueous phase onto a Millipore membrane (Ultrafree MC; 0.45 µm; the filter/tube assembly is precooled in ice) and filter by centrifugation (a few minutes at room temperature, 10000 rpm in an Eppendorf centrifuge) before precipitating with CTAB.

> Add 10 µl of 1% CTAB stock solution (0.1% final concentration), mix by tapping the tube and centrifuge for a few seconds in a microfuge.

> Put the samples for 20 min on ice/water.

> Centrifuge for 15 min at 30000 × g and 4°C (Sorvall centrifuge or equivalent).

> Put the samples on ice/water and remove the supernatant (save the supernatant) with a drawn-out capillary, carefully avoiding transfer of the pellet containing the labelled DNA. Check the efficiency of the precipitation by measuring the

radioactivity in the supernatant and pellets with a hand monitor.

> Add, without drying the pellets, 100–200 µl of 0.5 M ammonium acetate, and dissolve the pellet by firmly tapping the tube and briefly vortexing. The pellets are not very easy to dissolve, so do not worry if there are still some very fine particles visible.

> Add 0.1 volumes of 3 M sodium acetate/5 mM EDTA, pH 5 and 3 volumes of cold ethanol. Mix and incubate at –80°C for 15 min.

> Centrifuge at 30000 × g (Sorvall Centrifuge and SS-34 rotor or equivalents) at 4°C for 15 min.

> Pour out the supernatant very carefully and centrifuge again for a few minutes in a microfuge (test the supernatant with a monitor to be sure that DNA is not lost).

> Remove the residual supernatant with a drawn-out capillary.

> Resuspend the pellet in 150 µl of 0.5 M ammonium acetate, add 15 µl of 3 M sodium acetate/5 mM EDTA, pH 5 and 500 µl of cold ethanol.

> Mix and incubate at –80°C for 15 min.

> Centrifuge at 30000 × g (Sorvall Centrifuge and SS-34 rotor or equivalents) at 4°C for 15 min.

> Pour out the supernatant very carefully as above and centrifuge again for a few minutes in a microfuge.

> Remove the residual supernatant with a drawn-out capillary and dry the pellet for a few minutes in a SpeedVac (the ethanol should evaporate but the pellet should not be completely dry).

> Add 4 µl 100 mM NaOH, 1 mM EDTA and 4 µl 8 M urea, 0.04% bromophenol blue, 0.04% xylenecyanol and redissolve the pellet by tapping the tube and briefly vortexing.

> Centrifuge for a few seconds.

> Immediately prior to loading onto the sequencing gel, heat the samples for approximately 15 s at 94°C (do not chill the sample; load it hot).

C Procedures for Exponential Amplification (Polymerase Chain Reaction)

1 Materials and Buffers

- Two purified, synthetic oligonucleotides (approximately 20-mers) for exponential amplification. (These oligonucleotides should have a similar annealing temperature; concentration: 1 µM oligonucleotide, corresponding to approximately 0.9 µg for 27-mer oligonucleotides).
- Restriction-enzyme digested genomic DNA
- 10× Taq reaction buffer
- Taq polymerase
- dNTPs
- Mineral oil
- DNA thermal cycler
- Microfuge
- Agarose gel containing 1 µg/ml ethidium bromide
- 10× Sample buffer for agarose gels (50% glycerol, 10 mM EDTA pH 8, 0.2% bromophenol blue)
- Ice/water bath

2 Step-by-Step Procedure

> To an Eppendorf tube (which must fit in your DNA cycler) add consecutively 69 µl of the target DNA (fg to pg amounts), 17 µl of a mixture of the two unlabelled, 20-mer amplification primers in water (approximately 0.9 µg each), 10 µl of 10× Taq reaction buffer.

> Mix gently by tapping the tube and spin for a few seconds in a microfuge.

> Incubate at 95°C for 5 min in a DNA thermal cycler, programed for denaturing DNA.

> Quick-chill the samples in ice/water (1 min; do not interrupt the denaturing program).

> Spin the samples for a few seconds in a microfuge.

> Put the samples immediately back on ice/water.

> Add 3 µl of dNTPs (300 µM each in the final mix).

> Add 1 µl of Taq polymerase (diluted to 2–5 units/µl in reaction buffer)

> Mix, centrifuge briefly and put the samples immediately back on ice/water.

> Take a 1-ml pipette and add 100 µl of mineral oil to each sample.

> Incubate the samples in the DNA thermal cycler (which is still at 95°C) for 1 min.

> Stop the denaturing programe and start the amplification programe: 30 cycles, each consisting of 1 minute denaturation at 94°C, 2 minutes annealing (2–4°C below the Tm of the oligonucleotide primer duplexes; usually around 60°C), and 3 minutes chain elongation at 72°C.

Note: The times given here are for the effective temperature and do not include heating or cooling time between the steps.

> Immediately after the last cycle, put the samples on ice/water (if the samples are not to be processed immediately, freeze them at –80°C).

> Transfer the amplification products (aqueous phase) with drawn-out microcapillaries to freshly prepared precooled test tubes, avoiding contamination from the covering layer of mineral oil (test tubes in a floater on ice/water).

> Add 0.1 volumes of 3 M sodium acetate/5 mM EDTA, pH 5 and 3 volumes of cold ethanol. Mix and incubate at –80°C for 15 min.

> Centrifuge at 30000 × g (Sorvall Centrifuge and SS-34 rotor or equivalent) at 4°C for 10–15 min.

> Pour out the supernatant and centrifuge again for a few minutes in a microfuge.

> Remove the residual supernatant with a drawn-out capillary.

> Dry samples (but not to completion) in a SpeedVac.

> Redissolve the pellet in an appropriate volume (10–20 µl) of water, add 0.1 vol of 10× agarose sample dye and load the sample plus size markers on an agarose gel (1 µg ethidium bromide per ml of gel solution).

> Run the bromophenol blue marker to the middle of the gel.

> While visualising the DNA under UV-light (366 nm rather than 254 nm), mark the position of the band by taking a scalpel blade and making a cut 2–3 mm below the band containing the amplified target sequence (to avoid photo-merisation, do not expose the gel for too long to UV-light).

> Switch off the UV-light, put the gel onto a black surface and cut a slot from the gel 2–3 mm below the DNA band of interest. The previous cut marks the upper boundary of the slot, which should be as wide as the band and as deep as the gel.

> Put the gel back into the electrophoresis apparatus and remove buffer until the gel is no longer in direct contact with the buffer. Fix paper wicks to bridge the gel with the buffer and fill the cut out slot(s) with running buffer (1× TBE, without glycerol).

> Re-apply current for 30 s and pipette the buffer from the slot into an Eppendorf tube. Refill the slot with fresh buffer and repeat the above step 6–8 times, pooling all the samples in the same tube. The efficiency of electro-elution can be checked using UV-light.

> Add 0,1 vol. of 3 M sodium acetate/5 mM EDTA, pH 5 and 3 volumes of absolute ethanol.

> Precipitate the DNA on dry ice/ethanol (–80°C) for 15 min and centrifuge at 30000 × g for 10–15 min. Carefully pour off the supernatant, give a short spin in a microfuge and remove all traces of supernatant with a drawn-out capillary.

> Dry the DNA pellet briefly in a SpeedVac and redissolve in 50–100 µl of water.

> Take one-tenth of the DNA solution and run it on a 2% agarose gel alongside size markers of different, known concentrations (for example, phiX-174, HaeIII digests; check the concentrations of the supplied material).

> Take a Polaroid photograph of the gel (see Fig. 3). Estimate the concentration of the amplified DNA by comparison of the band intensity with that of the known standards.

> The DNA is ready for end-labelling. If one of the primers

was labelled for the exponential amplification, the DNA can be used immediately for the footprinting experiment.

D Trouble-shooting Guide

1 Problems Occurring in Linear Amplification with Taq Polymerase

Problem: *Weak signals*

Probable cause and solution:

– Specific radioactivity of the labelled primer was too low. Use only fresh reagent (at least 3000 Ci/mmole). Adjust concentration of the radio-label.
– Not enough DNA was used for the linear amplification. Make sure that the sample has completely dissolved.
– Large variation in the amount of labelled primer, dNTPs or Taq polymerase. Use approximately the same concentrations for all reactions.
CETAB Precipitation:
– After the linear amplification, the aqueous phase must be transferred to a fresh Eppendorf tube. If the sample is contaminated with mineral oil, an increase in the background might be observed. Use only drawn-out capillaries for the transfer or filter the probe through a Millipore membrane (Ultrafree MC).

2 Problems Occurring in Linear Amplification and in Exponential Amplification of DNA with Taq Polymerase:

Problem: *No signals on autoradiography*

Probable cause and solution:

Annealing Temperature:
The temperature chosen for the annealing of the primer(s) was too high. Determine the melting temperature (Tm) of the oligonucleotides (as described in Saluz & Jost, 1990) and calculate the annealing temperature (Ta): Ta = Tm – (2°to 4°C).

Problem: *Weak signals*

Probable cause and solution:

Melting temperature (Tm) lower than annealing temperature (Ta):
– Weak signals may occur if anannealing temperature is chosen lying between the Tm and the maximum value of the sigmoid

melting curve. Decrease the Ta stepwise by 2°C until the signals are stronger but no artifacts occur. Check the Tm of the oligonucleotide primer. If an oligonucleotide is synthesised by a filling-in reaction (Saluz & Jost, 1990), check its final size. Use only Sequenase (version II, USB) and high-quality dNTPs. Avoid the use of deaza-dGTP or dITP for the synthesis of the oligonucleotide primer, since they influence the annealing temperature.
Poor Quality of Taq Polymerase:
– Poor amplification may occur due to a bad batch of Taq polymerase. Store Taq polymerase at –20°C in a non-defrosting freezer. Avoid changes in storage temperature.
Wrong Composition of Taq Polymerase Buffer:
– Poor signals can be caused by a too low Mg^{++} concentration in the buffer. Mg^{++} ions form complexes with dNTPs.
Note that the magnesium concentration is greater than that of the dNTPs, and also that these ions influence the melting temperature of the DNA.

Problem: ***Artifact bands***

Probable cause and solution:

Stringency of Reaction Conditions:
– Intra- and intermolecular structures may cause artifacts. Increase the overall stringency of the amplification procedure, especially the annealing temperature of the primers (usually the lowest temperature). Increase the annealing temperature stepwise. If the results do not improve, decrease the Mg^{++} concentration of the Taq polymerase buffer or choose an oligonucleotide primer with a higher melting temperature (dependent, for example, upon the length of the primer or the G+C content).
Quality or Concentration of dNTPs:
– Incorrect bands can result if one or more of the four dNTPs is limiting; the concentration of dGTP is especially critical. The effective concentration may be too low if an impure dNTP is used. Buy only the best quality. Recheck the final concentrations of the dNTPs.
Quality of the Taq Polymerase:
– Taq polymerase may be contaminated by exonucleases. This is a critical factor, especially for linear amplifications; unspecific nicks occur within the single-stranded target sequence, causing an extraneous band throughout all sequencing lanes. To determine the correct sequence of that region, the polymerase must be changed. Good experience has been had with Taq polymerase produced with recombinant DNA. If the result

does not improve, the correct sequence is determined by sequencing the complementary strand of that region. The appearance of specific single-stranded nicks can be tested by Southern blots; the nick is only revealed on a denaturing gel.

Impure Short DNA Fragments:
– Extraneous bands and irregular spacing can be caused by short fragments of the same length but different mobility due to different base composition. Such bands appear to be a single DNA fragment on a non-denaturing gel. They can be identified by Southern blotting, using denaturing and non-denaturing gels.

Impure Primers:
– If each band in the pattern appears as a doublet or even as a triplet, the primer has a heterogeneous composition. This can be caused by the improper purification of the primer after its synthesis or by autoradiolysis, especially in the case of labelling to a very high specific radioactivity by the filling-in procedure (one break produces two different labelled primers). In the first case, repurify the oligonucleotide primer, and in the second case it is important to use the labelled primer without delay, chosing a stringent annealing temperature so that primer fragments produced by autoradiolysis no longer anneal.

Problem: ***Unspecific band of uneven but high intensity***

Probable cause and solution: Improper annealing of the sequencing primer:
– If the annealing temperature of the oligonucleotide primer is too low, a second (or several) superimposed sequencing ladder(s) may be generated, due to the incorrect hybridisation of the primer with similar complementary sequences. Increase the annealing temperature.

E Bibliography

Bonner, T.I., Brenner, D.J., Neufield, B.R., and Britten, R.J., Reduction in the rate of DNA reassociation by sequence divergence. J. Mol. Biol. 81 (1973) 123–135.

Erlich, H.A., Gelfand, D.H., and Saiki, R.K., Specific DNA amplification. Nature 331 (1988) 461–462.

Huibregtse, J.M., and Engelke, D.R. Direct identification of small sequence changes in chromosomal DNA. Gene 44 (1986) 151–158.

Innis, M.A., Myambo, K.B., Gelfand, D.H., and Brow, M.A., DNA sequencing with *Thermus aquaticus* DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA. Proc. natl. Acad. Sci. USA 85 (1988) 9436–9440.

Jost, J.P., Jiricny, J., and Saluz, H.P., Quantitative precipitation of short oligonucleotides with low concentrations of cetyltrimethylammonium bromide. Nucl. Acids Res. 17 (1989) 2143.

Kwok, S. Procedures to minimize PCR-product carryover. Amplifications 2 (1989) 4.

Oste, Chr., Optimization of magnesium concentration in the PCR reaction. Amplifications 1 (1989) 10–11.

Saiki, R. K., Scharf, S., Faloona, F., Mullis, K.B., Horn,G.T., Erlich, H.A., and Arnheim, N. Enzymatica amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230 (1985) 1350–1354.

Saiki, R. K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn,G.T., Mullis, K.B., and Erlich, H.A. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239 (1988) 487–491.

Saluz, H.P., and Jost, J.P. Genomic sequencing and *in vivo* footprinting. Analyt. Biochemistry *176* (1989) 201–208.

Saluz, H.P., and Jost, J.P., A simple high-resolution procedure to study DNA methylation and in vivo DNA-protein interactions on a single-copy gene level in higher eukaryotes. Proc. natl. Acad. Sci. USA 86 (1989), 2602–2606.

Saluz, H.P., and Jost, J.P. Genomic footprinting with Taq polymerase. Nature 338 (1989) 277.

Saluz, H.P. and Jost, J.P. A laboratory guide for *in vivo* studies of DNA methylation and protein/DNA interactions. Birkhäuser Verlag (1990).

Scharf, S. J., Horn, G.T., and Erlich, H.A. Direct cloning and sequencing analysis of enzymatically amplified genomic sequences. Science 233 (1986) 1076–1078.

Stoflet, E.S., Koeberl, D.D., Sarkar, G., and Sommer, S.S. Genomic amplification with transcript sequencing. Science 239 (1988) 491–494.

Suggs, S.V., Hirose, T., Magake, T., Kawashima, E. H., Johnson, M.J., Itakura, K., and Wallace R.B. in Developmental Biology Using Purified Genes, pp. 683–693. Eds D. Brown and C.F. Fox. Academic Press, New York 1981.

Wong, C., Dowling, C.E., Saiki, R.K., Higuchi, R.G., Erlich, H.A., and Kazazian Jr, H. H. Characterization of β-thalassaemia mutations using direct genomic sequencing of amplified single copy DNA. Nature 330 (1987) 384–386.

XXI Non-radioactive Detection Methods for In Vitro Footprinting.

Andrew Wallace and Hanspeter Saluz

Contents

A Introduction

1 Principles of Non-radioactive Labelling

Whilst the purpose of this chapter is to discuss the use of non-radioactive detection systems, it must be said that radioactive labelling methods offer the most sensitive and robust detection system for biomolecules. Their advantage is that they are inert towards temperature, pH, heavy metals, inhibitors or salts. However, the hazards of radioactive labels have led more and more people to use alternative, non-radioactive

markers in their experiments, particularly for long-term and routine use. Non-radioactive methods may find important applications in recombinant DNA research, large-scale sequencing projects, biomedical, diagnostic, forensic and environmental studies. If one looks at the developmental history of alternative labelling strategies, considerable improvements can be seen in sensitivity, speed and convenience of use over the last two decades, and this trend is likely to continue. Chemiluminescent substrates, such as luminol, have been in widespread use for about 20 years, but such substrates are generally unsuitable for sensitive assays. In 1983 (Whitehead et al., 1983), for example, it was discovered that the signal of luminol could be increased about 1000-fold using additional chemical enhancers. In recent years, several rapid and user-friendly techniques for biomolecule analysis have been developed (Renz and Kurz, 1984). Many of these detection systems rely on the detection of nucleic acids by DNA:DNA or DNA:RNA hybridisation (Carlson et al., 1990). Others can be directly used for end-labelling procedures incorporating rhodamine and fluorescein derivatives which are widely employed in automated DNA sequencing (Cornell et al., 1987). Several kits are commercially available covering various detection strategies. However, many systems described so far, while not yet as sensitive as radioactive detection methods, are usually completely sufficient for the in vitro footprinting methods described in this book.

2 Nature of Non-radioactive Labelling Reagents

The nature and use of non-radioactive labels for sequencing and footprinting projects fit two broad strategies: Firstly, the label can be directly incorporated into the sequence, allowing direct visual detection within the gel or blot (a strategy often used in sequencing). Secondly, the target sequence can be indirectly visualised by blotting onto membranes, followed by hybridisation with a non-radioactively labelled probe, which can be detected with nucleotide hapten analogues incorporated into the DNA or RNA probe, followed by cytochemical colorimetric staining.

An example of the first possibility (direct detection) is the "Oliglow™" strategy from Applied Biosystems. This system offers the advantage that the target molecules are directly detected within the sequencing gel. One must be aware, however, that not all labelling strategies used in sequencing can be applied to in vitro footprinting procedures. Within the scope of this book, therefore, we describe only those staining detection methods which can be used generally. Thus we will only discuss direct detection methods in the context of end-labelled oligonucleotides used as primers for enzymatic copying of the target molecules.

The second possibility will also be described, even though it is probably more tedious, in that it involves electro-blotting and hybridisation steps. Since this is based on indirect end-labelling, one must be aware of the theoretical considerations allowing resolution of the sequence of interest, which are discussed in Volume 1 of

this series (Saluz & Jost, 1987). The length of the probe, therefore, should be not longer than 100–150 nucleotides. One of the advantages of this procedure is, however, that several target sequences can be footprinted at the same time and detected by hybridisation probes specific for each sequence. With these considerations in mind, there are several non-radioactive detection systems available. Most involve conjugation of the probe with an enzyme, such as alkaline phosphatase or horseradish peroxidase, coupled to the probe by antibody/oligonucleotide-hapten recognition or by using biotinylated nucleotide derivatives with a streptavidin-enzyme conjugate. To give an idea of the mechanisms involved, we shall describe some of the most popular systems, which are commercially available in kit form.

3 Commercial Labelling Systems

Properly used, kits can be very helpful. This section does not attempt to describe all the systems available, but only a few of those most widely used.

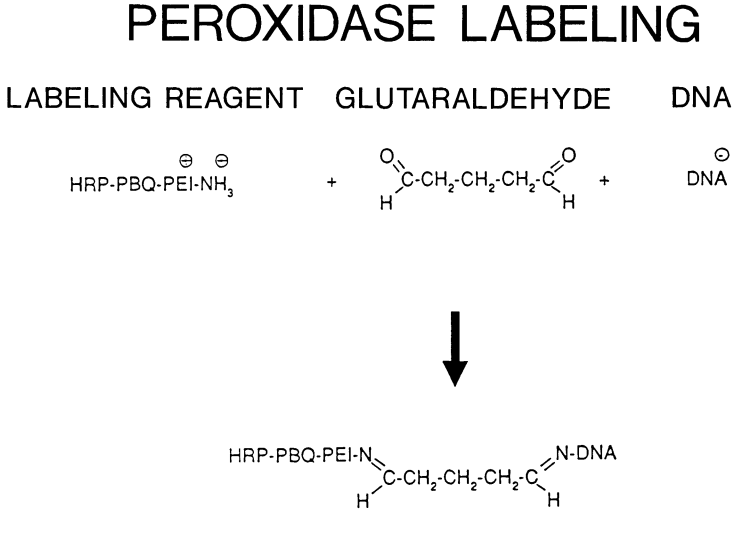


Fig. 1: Peroxidase labelling: The horseradish peroxidase-parabenzoquinone-polyethyleneimine complex is covalently cross-linked to probe DNA with glutaraldehyde. The peroxidase/DNA complex can then be hybridised to immobilised target DNA as described in the text.

a *"Oliglow™" system (Applied Biosystems, part no. 400887):*
This system is based on the use of four dye-labelled primers (one for each sequencing reaction) which are ligated to the specific sequencing probe. In combination with polymerase copying reactions, as described in Chapter XX (Linear and exponential amplification), the primers can be used to label the target DNA.

b *Enhanced Chemiluminescence (Amersham, ECL™ product range):*
This system offers two possibilities: gene detection and oligonucleotide labelling. Here we describe only the former. The principle is to label the nucleic-acid probe with horseradish peroxidase modified with parabenzoquinone and polyethyleneimine to render it positively charged and allow it to bind to the DNA probe. The complex is then covalently cross-linked with glutaraldehyde (Fig. 1). The peroxidase/DNA complex is hybridised to immobilised target DNA. The peroxidase catalyses the breakdown of a peracid salt to give a peroxide ion, which reacts with luminol and an enhancer causing the emission of blue light (Fig. 2). This can be detected with a suitable film. An important advantage of such probes is that they have a long shelf-life (over 6 months) making them well-suited for routine use.

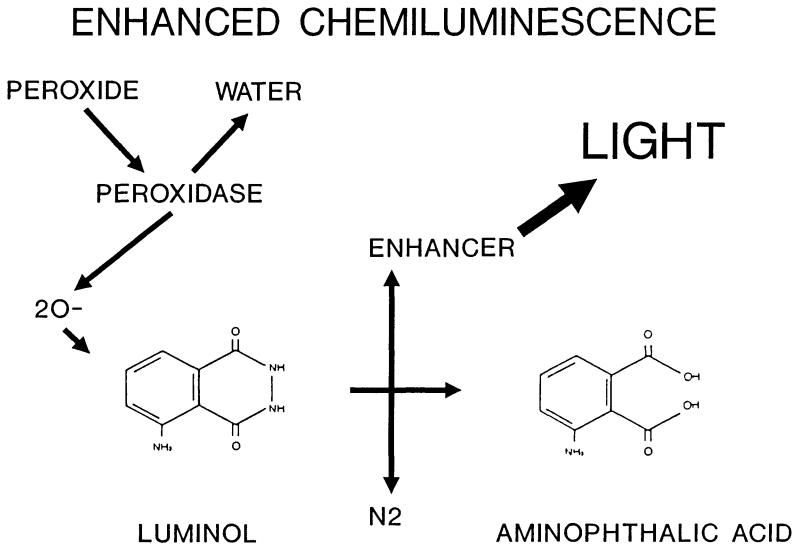


Fig. 2: Enhanced chemiluminescence: The peroxidase catalyses the breakdown of a peracid salt to give a peroxide ion, which reacts with luminol and an enhancer to cause an emission of blue light. This can be detected with a suitable film. An important advantage of such probes is that they have a long shelf-life (over 6 months) making them well-suited for routine use.

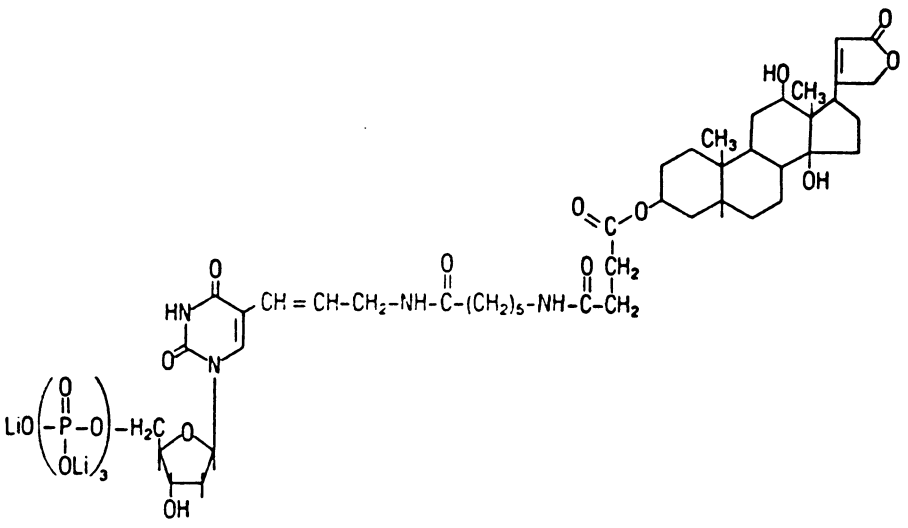


Fig. 3: Digoxigenin-11-dUTP: Structure of the digoxigenin-11-dUTP, used for labelling of probe DNA by incorporation into the DNA with DNA polymerase.

c *"DIG™" DNA labeling and detection kit (Boehringer Mannheim, Cat. No, 1093657):*

This kit is based on the incorporation of the nucleotide analogue digoxigenin-11-dUTP (Fig. 3) into DNA, followed by hybridization of the probe to blotted, immobilised target DNA, and detection of the digoxigenin moeity with an anti-digoxigenin/alkaline phosphatase conjugate. The conjugates are visualised by an enzyme-linked colour reaction (Fig. 4). This system is claimed to detect as little as 0.15 pg of DNA (Application 1 of Applications Manual, Boehringer).

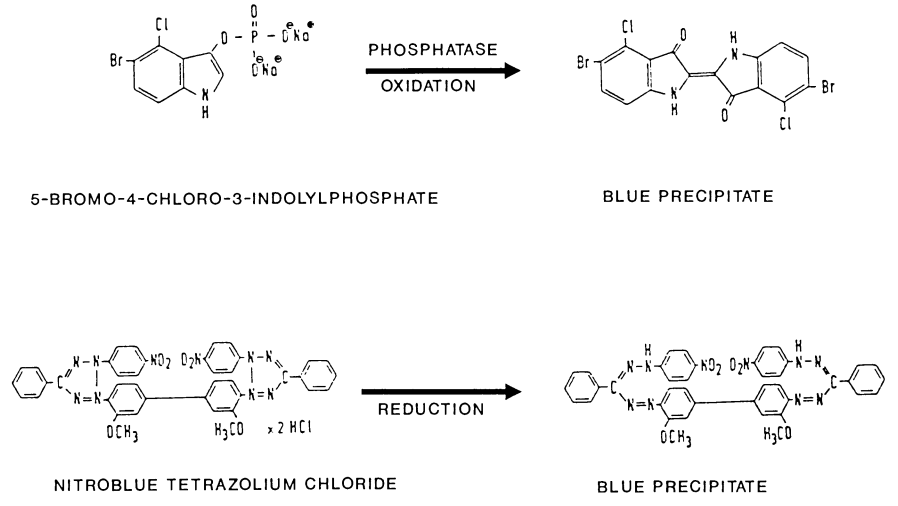


Fig. 4: Enzyme-linked colour reaction: Alkaline phosphatase catalyses a linked oxidation/reduction reaction converting the 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium chloride reagents to produce dyes which form a blue precipitate.

B Practical Applications

Since it is beyond the scope of this chapter to describe electro-blotting systems in detail, we refer the reader to a system used for genomic sequencing (Saluz & Jost, 1986; 1990).

1 Materials and Buffers

"Oliglow™" system (Applied Biosystems, Part No. 400887)

- Probe primer (complimentary to the sequence of interest) with an additional 8-mer oligonucleotide added to the 5' end of the probe. This 8-mer extension is complimentary to part of the "splint" oligo as described below.
- T4 Polynucleotide kinase
- T4 DNA ligase
- Sterile pipette tips

- Sterile microcentrifuge tubes
- 10× Kinase buffer: 250 mM Tris-Cl pH 7.6, containing 100 mM dithiothreitol (DTT) and 100 mM $MgCl_2$
- 10× Ligase buffer: 250 mM Tris-Cl pH 7.8, containing 100 mM $MgCl_2$, 40 mM β-mercaptoethanol and 4 mM ATP
- 10 mM ATP, from which 0.1 mM aliquots are freshly prepared for each experiment

The above items must be prepared in advance. The other materials are supplied in the "Oliglow™" kit.

- "Oliglow™" stock solutions: dissolve the contents of each tube (300 pmol) in 100 µl of TE buffer (final concentration 3 pmol/µl).
- "Splint" oligo stock solutions: dissolve the contents of one tube of splint oligo in 400 µl of TE (final concentration 2 pmol/µl). Store frozen in small aliquots.
- Oligonucleotide Purification Cartridges (Applied Biosystems Part. No. 400771, pack of 10).

Enhanced Chemiluminescence (Amersham, Cat. No. RPN 2113)

- Target DNA immobilised on a membrane
- Hybridisation probe
- Film cassettes

The above must be prepared in advance. All other reagents are available from Amersham.
- ECL™ gene detection system, for use with nitrocellulose membranes: Cat. No. RPN 2100
- Above, for use with nylon membranes: Cat. No. RPN 2110
- DNA labelling and detection reagents: Cat. No. RPN 2101
- Hybridisation buffer: Cat. No. RPN 2102
- Detection reagents: Cat. No. RPN 2105
- Photographic film sensitive to blue light, Hyperfilm-ECL™ (Amersham Cat. No. RPN 2020D) or equivalent

"DIG™" DNA labeling and detection kit (Boehringer Mannheim, Cat. No, 1093657)

- DNA template (complimentary to desired probe sequence)
- Nylon membrane carrying electro-blotted target DNA
- Sterile pipette tips

- Sterile microcentirifuge tubes
- Sterile distilled water
- SpeedVac (Savant or equivalent)
- Small bench-top centrifuge (Eppendorf or equivalent)
- 0.2 M EDTA solution, pH 8.0
- 4 M LiCl
- Pre-chilled absolute ethanol (–20°C)
- Pre-chilled 70% (v/v) ethanol (–20°C)
- 10 mM Tris-HCl pH 8.0, containing 1 mM EDTA (TE buffer)
- 20× SSC: 0.3 M sodium citrate pH 7.0 (at 20°C), containing 3M NaCl
- 5× SSC with formamide: 5× SSC containing 5% (w/v) blocking reagent (supplied in kit), 0.02% (w/v) sodium dodecyl sulphate (SDS), 0.1% (w/v) N-lauryl-sarcosine and 50% (v/v) formamide
- Wash solution 1: 2× SSC containing 0.1% (w/v) SDS
- Wash solution 2: 0.1× SSC containing 0.1% (w/v) SDS

Membrane Staining Solutions:
- Buffer 1: 100 mM Tris-HCl, 150 mM NaCl pH 7.5
- Blocking solution: buffer 1 (100 mM Tris-HCl, 150 mM NaCl pH 7.5) containing 0.5% (w/v) blocking reagent [vial 11 in kit]
- Freshly prepared colour solution: (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5, containing 45 µl NBT solution [vial 9] and 35 µl X-phosphate solution [vial 10] per 10 ml)
- 10 mM Tris-HCl, 1 mM EDTA, pH 8.0

The above materials should be prepared in advance. All other components are supplied in the kit.
- Hexanucleotide random primer mixture
- dNTP labelling mixture
- Klenow enzyme
- NBT solution
- X-phosphate solution

2 Step-by-Step Procedures:

"Oliglow™" system (Applied Biosystems)

> Purification of primer: the primer should be purified as instructed in the cartridge purification kit or simply with NAQ or SEP-PAK cartridges according to the manufacturer's instructions.

> Quantitation of primer: make a dilution (1:50 to 1:100) of an aliquot of the purified primer and measure its absorption at 260 nm using quartz cuvettes. The approximate concentration of the oligo can then be calculated as follows:

$$[\text{Oligomer}]\ (\text{pmol}/\mu\text{l}) = \frac{100 \times A_{260}}{1.54\text{nA} + 0.75\text{nC} + 1.17\text{nG} + 0.92\text{nT}}$$

> Phosphorylation of the primer: add 40 pmol of the dried oligonucleotide to 12 µl water, 2 µl of 10× kinase buffer and 5 µl of 0.1 mM ATP.

> Mix and spin down briefly, then add 1 µl of T4 polynucleotide kinase (5–10 Units).

> Mix and spin down briefly. Incubate for 30 min at 37°C.

> Heat denature the enzyme at 65°C for 5–10 min.

> Use the primer immediately or store it frozen at –20°C.

> Take 6 pmol (2 µl) of one of the four "Oliglow™" primers and add it to 2 µl of the "splint" oligo, 1 µl of phosphorylated primer (2 pmol), 1 µl of 10× ligation buffer plus 3 µl of H_2O.

> Heat the reaction mixture for 5 min at 55°C and place the tubes on ice (annealing of splint to the "Oliglow™" and phosphorylated primer).

> Add 1 µl of freshly prepared ligase/ATP mix (4 µl of 10 mM ATP and 2 µl of T4 DNA ligase [4 Units]).

> Mix, spin down briefly and incubate the reaction mixture for 2–12 h at room temperature.

> The primer (0.2 pmol/µl) is ready for use in either linear or exponential amplification according to the chosen approach for in vitro footprinting (see Chapter XX). *Note*: To visualise the in vitro footprints, the final denaturing gel is placed on a transilluminator bench, exposed to UV light and photographed if necessary.

Enhanced Chemiluminescence (Amersham, Cat. No. RPN 2113)

The Hybridization Probe:

- Excise and purify the probe DNA from the vector sequences.
- Prepare the probe DNA at a final concentration of 10 ng/µl water. Approximately 10–20 ng of probe DNA per millilitre of hybridisation mixture will be sufficient to hybridise 4 cm^2 of a filter membrane.
- Denature the DNA at 95°C for 5 min and then chill on ice/water for a few minutes.
- Spin briefly in a microcentrifuge and add an equal volume of the labelling reagent.
- Mix thoroughly and spin briefly in a microcentrifuge.
- Add an equal volume of glutaraldehyde, mix thoroughly and centrifuge for a few seconds.
- Incubate at 37°C for 10 min and put the sample on ice. The probe is ready for hybridisation. If it is not used within 15 min, store it in 50% glycerol at –20°C (stable for several months).

Hybridisation:

- Clear the hybridisation buffer provided by heating at 65°C for 10 min followed by stirring for approximately 30 min.
- For hybridizations using Hybond-ECL™ or Hybond-N+™ membranes you need approximately 250 µl/cm^2 of hybridisation buffer using small filters, and 125 µl/cm^2 using large filters.
- Dissolve NaCl to 0.5 M in the hybridisation buffer (the salt concentration may need to be adjusted if good results are not obtained).
- Incubate the Hybond-ECL™ filter with agitation for at least 15 min at 42°C (do not use higher temperatures). If Hybond-N+™ membranes are used, 5% (w/v) blocking agent must be added to the hybridisation buffer. The dissolution may take up to 1h.
- Add the labelled probe and continue the incubation with agitation overnight at 42°C (do not use higher temperatures).

Washing:

- Remove the blots, place them in a clean container and add 2 ml/cm^2 of primary wash buffer (360 g urea, 4 g SDS, 25 ml 20× SSC, made up with water to 1000 ml) and incubate with agitation for 20 min at 42°C.
- Repeat the above step.
- Place blots in a fresh container, add 2 ml/cm^2 of secondary wash buffer (2× SSC) and incubate with agitation for 5 min.
- Repeat the above step.

Developing and Exposure:
The following steps must be done as quickly as possible, therefore perform all the following steps in a dark room.
- Add a mixture of detection system 1 and 2 (1:1): 0.125 ml/cm^2 and incubate for precisely 1 min.
- Drain excess buffer and transfer membrane to Saran wrap. Smooth out all air pockets very carefully, place the blots, DNA-side up, in the film cassette and expose them to a suitable film (for example Hyperfilm-ECL™) for 1 min.
- Develop only after exposing the blot to another fresh film.

"DIG™" DNA labelling and detection kit (Boehringer Mannheim, Cat. No., 1093657)

- Add 10 ng–3 µg of the template DNA to a precooled microcentrifuge tube.
- Denature the DNA at 95°C for 10 min.
- Quick-chill the tube in ice/water.
- Add 2 µl of the hexanucleotide random primer mixture and 2 µl of the dNTP labelling mixture.
- Make the volume up to 19 µl with sterile, distilled water.
- Add 2 units (1 µl) of Klenow enzyme.
- Mix and spin down briefly.
- Incubate the reaction mixture at 37°C for at least 1 h, up to a maximum of 20 h.
- Stop the reaction by adding 2 µl of 0.2 M EDTA solution.
- Precipitate the DNA by adding 2.5 µl of 4 M LiCl and 75 µl of pre-chilled (–20°C) absolute ethanol.
- Mix well and incubate at –70°C (dry ice) for 1 h or at –20°C overnight.
- Centrifuge for 10 min at 12000 × g.
- Carefully remove the supernatant and add 40 µl of pre-chilled 70% (v/v) ethanol.
- Centrifuge again as above and dry briefly in the SpeedVac.
- Redissolve the DNA in 50 µl of TE buffer at 37°C for approximately 30 min.

The above procedure generates randomly primed probe DNA, labelled with digoxigenin-11-dUTP, ready for hybridisation and detection.

Hybridisation to Immobilised DNA on Nylon Membranes:
- Bind the blotted DNA to the nylon membranes either by baking for 2 h or by exposure to an appropriate dosage of UV-light (detailed blotting and immobilisation systems are described in Saluz and Jost, 1990).
- Pre-hybridise the membranes with 20 ml/100 cm^2 of the 5× SSC hybridisation solution at 42°C for at least 1 h in a suitable hybridisation device (plastic bags etc.).
- Prepare probe DNA at a concentration of 10 pg/µl with dilution buffer (vial 3 in kit).

- Denature the probe DNA at 95°C for approximately 10 min and chill on ice/water.
- Replace pre-hybridisation solution with 2.5 ml/100 cm^2 of hybridisation solution (5× SSC with formamide) containing the probe DNA at a concentration of 10–50 ng/ml.
- Incubate the filters at 42°C for at least 6 h.
- Discard the hybridisation mix and wash the membranes in 50 ml/100 cm^2 of wash solution 1 for 5 min at room temperature.
- Repeat the above washing step at least once.
- Wash with the same volume of wash solution 2 at 68°C for 15 min.
- Repeat the above step at least once.
- The filters can be used immediately for detection or stored damp in a vacuum-sealed bag at 4°C in the dark.

Detection of Hybridised DNA:
Note: All steps are carried out at room temperature unless otherwise indicated.
- Wash filters in buffer 1 for 1 min.
- Incubate for 30 min with buffer 1 containing 0.5% (w/v) blocking reagent [vial 11 in kit].
- Wash with buffer 1 for 1 min.
- Dilute the antibody conjugate [vial 8] 1:5000 (150 mU/ml) in buffer 1. *Note*: The diluted conjugate is only stable for about 12 h at 4°C.
- Incubate the membranes for 30 min with about 20 ml of diluted antibody conjugate.
- Wash the membranes with 100 ml/100 cm^2 of buffer 1 for 15 min.
- Repeat the above step at least once to remove unbound conjugate.
- Equilibrate the membranes in 20 ml/100 cm^2 of 100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5 for 2 min.
- Incubate the membranes in the dark with 10 ml/100 cm^2 of freshly prepared colour solution for 2 min up to 1 day. Do not shake or mix while the colour is developing.
- When the desired bands appear, stop the reaction by washing the membrane in 50 ml/100 cm^2 of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 for 5 min.
- Record the results by photographing or photocopying the wet membrane. Do not dry the membrane if you wish to reprobe it.
- For permanent storage the membrane can be dried at room temperature or by baking at 80°C, however the colours fade upon drying. *Note*: The colours can be restored by wetting with 10 mM Tris-HCl, 1 mM EDTA, pH 8.0

C Trouble-shooting guide

Note: The following problems and suggested remedies may prove helpful. If all else fails, try using radioactive labels!

Oliglow™ Kit

Problem: ***Insufficient labelling***

Probable cause and solution:

– Low enzyme activity. Low activity of kinase and/or ligase. Replace the enzyme and check carefully the composition of the ligase or kinase buffers. Incubate for appropriate temperature and/or time.
– Poor quality of ATP. Make aliquots of the ATP solution and discard after use.
– Oliglow™ stocks not fully dissolved: Vortex the stock solutions frequently. Complete dissolution may take up to 20 min.

Kits involving hybridisation

Problem: ***High background***

Probable cause and solution:

– Hybridisation. Prepare a dilution series of the probe DNA with dilution buffer in the concentration range of approximately 5 pg/µl to 0.01 pg/µl.
– Too low stringency for hybridisation. Increase hybridisation temperature or decrease salt concentration of hybridisation buffer. Try using non-formamide buffers.

Problem: ***No or low signals***

Probable cause and solution:

– Electro-transfer to nylon membranes. Check that the DNA was transferred completely. If not, increase transfer time. If yes, check immobilisation efficiency.
– Immobilisation. Titrate UV-dose or optimise baking time. If UV-dose was correct, increase amount of DNA to be transferred.
– Quality of hybridisation probe. Check incorporation of peroxidase complex (Amersham kit) or labelled dUTP (Boehringer kit). If insufficient, increase amount of labelling reagent or labelling reaction time. Repurify the probe DNA by phenol/chloroform-extractions and/or ethanol precipitations. Add carrier DNA to improve recovery of labelled DNA. Ensure

that DNA was fully denatured for random priming. Scale up reaction volume several-fold and increase incubation period.
– Hybridisation. Hybridisation temperature was too high and must be optimised. Adjust hybridisation time, probe and/or target DNA concentration.
– Washing. Too stringent washing conditions were used. Decrease washing stringency by increasing salt concentration or by decreasing washing time or temperature.
– Detection system. Increase colour development time up to several days. For "DIG™" (Boehringer) kit, store the anti-digoxigenin-alkaline phosphatase conjugate at 4°C (do not refreeze) or increase conjugate concentration (e.g. 1:500).

D Bibliography

Carlson, D. P., Superko, C., Mackey, J., Gaskill, M. E., and Hansen, P., Chemiluminescent Detection of Nucleic Acid Hybridization. Focus 12, (1990) 9–15.

Cornell, C., Fung, S., Heiner, C., Bridgham, J., Chakerian, V., Heron, E., Jones, B., Menchen, S., Mordan, W., Raff, M., Recknor, M., Smith, L., Springer, J., Woo, S., and Hunkapiller, M., Automated DNA Sequence Analysis. BioTechniques 5, (1987) 342–348.

Renz, M., and Kurz, C., A Colorimetric Method for DNA Hybridization. Nucl. Acids Res. 12, (1984) 3435–3444.

Saluz, H. P., and Jost, J. P., Optimized Genomic Sequencing as a Tool for the Study of Cytosine Methylation in the Regulatory Region of the Chicken Vitellogenin II Gene. Gene 42, (1986) 151–157.

Saluz, H. P., and Jost, J. P., A Laboratory Guide for In Vivo Studies of DNA Methylation and Protein/DNA Interactions (1990) Birkhäuser Verlag, Basel, Boston.

Whitehead, T. P., Thorpe, G. H. G., Carter, T. J. N., Groucutt, C., and Kricka, L. J., Enhanced Luminescence Procedure for Sensitive Determination of Peroxidase-labelled Conjugates in Immunoassay. Nature 305, (1983) 158–159.

XXII Quantitative Determination of Proteins in Crude Extracts

Jan Hofsteenge

Content

A Introduction

The determination of the exact protein concentration in crude extracts of tissues or cells is not a trivial undertaking. Unfortunately, very few methods are available that allow the determination on a routine basis of the *absolute* amount of protein in mixtures (c.f. Peterson, 1983). As a result, a variety of, methods mostly colorimetric, have been developed that allow the determination of the amount of protein *relative* to that of a standard protein (for reviews see Peterson, 1983; Darbre, 1986). How closely these relative values approximate to the absolute amount of protein will depend on the standard chosen and on the type of extract under study. Two procedures that allow the quantitation of protein in the µg-range have gained wide popularity: the Folin phenol method (Lowry et al., 1951) and the Coomassie Blue dye-binding method (Bradford, 1976).

Note: The reaction of *o*-phtaldehyde with primary amines, which yields fluorescent products, provides a method for protein determination with a sensitivity in the nanogramme range (see Peterson, 1983).

It has been stressed that a large number of common laboratory reagents interfere with the Folin phenol method and that a less susceptible method is required. Despite this fact, the method has been widely used, and reviews on its applicability (Peterson, 1979), compilations of interfering substances, and good protocols have been published (Peterson, 1979; Peterson, 1983).

The Coomassie Blue dye-binding method has been reported to be simple, sensitive, and less susceptible to interfering substances (Bradford, 1977; Spector, 1978). In solution, the dye can exist in three ionisation states that differ in spectral properties (Compton & Jones, 1985):

	OH^-		OH^-	
Anion		Neutral		Cation
	H^+		H^+	
595 nm		650 nm		470 nm

Proteins bind the anionic form of the dye, thereby displacing the equilibrium between the different species. In the strongly acidic medium in which the assay is performed, the concentration of the anionic species is very low, and a large increase in absorbance at 595 nm is observed in the presence of protein. A linear response depends on the presence of an excess of free cation over protein. It should be borne in mind that any substance affecting the above equilibrium, and thereby the concentration of free cation, may influence the outcome of the assay (Compton and Jones, 1985).

B Materials and Method

1 Dye Reagent

Reagent of Read & Northcote (1981):
- Dissolve 100 mg Coomassie Blue G (Serva, Heidelberg, FRG) in 100 ml 16 M phosphoric acid (careful, etching !) and 46.7 ml absolute ethanol. The solution is diluted to 1 l with distilled water.
- Filter the dye solution through Whatman No.1 paper to remove insoluble dye and store in a brown bottle.

Note: It has been reported that the content of soluble dye in preparations of Coomassie Blue G-250 powder is variable. If a dye other than Coomassie Blue G from Serva is used, the amount of dye powder added may have to be increased to obtain a final dye concentration of 0.01% (w/v).

Commercially Available Reagent
A commercial stock solution of Coomassie Blue in phosphoric acid/methanol is available from Bio-Rad (Bio-Rad, 1979). The final concentration of the dye in the working solution is approximately 0.005% (w/v), which may be too low for some applications.

2 Protein Standard

- Prepare a solution of bovine serum albumin (BSA; approximately 2 mg/ml) in Tris-buffered saline (TBS).
- Determine the exact protein concentration by measuring, in duplo, the A_{280} of a suitably diluted (5-fold) sample. A concentration of 1 mg/ml will give an A_{280} = 0.660 (Cohn, 1976).
- Based on the above measurement, dilute the protein to exactly 1.0 mg/ml and store the protein solution at –20°C in 0.6 ml aliquots. Before use, rapidly thaw the solution at 37°C.

3 Step-by-Step Procedure

Calibration Curve

> Prepare a working solution of BSA by diluting 500 µl of the 1.0 mg/ml BSA stock solution with 4500 µl of TBS.

Make further dilutions as indicated:

µg in assay	1	2	3	4	5	6	7	8
µl BSA dilution	50	100	150	200	250	300	350	400
µl TBS	450	400	350	300	250	200	150	100

> Discard the dilutions after use.

> Pipette, in duplo, 100 µl of the BSA dilutions in disposable glass tubes and add 1 ml of the dye reagent.

> Mix gently by vortexing for 3 s. For good reproducibility, mark the speed setting on the mixer.

> Incubate 10 min at room temperature.

> Read the absorbance at 595 nm, using a polystyrene cuvette (path-length 1 cm) against a blank that consists of 1 ml reagent containing 100 µl TBS. It is important to pipette gently, to avoid producing air bubbles, and to protect the tip of the pasture pipette with a piece of tygon tubing, to prevent the formation of plastic shavings.

> Plot the mean measured values against the amount of protein (Fig.1).

Measurement of the Sample

> Dilute the sample with TBS so that the protein concentration is in the range of the standard curve.

> Prepare a blank by diluting the sample buffer in the same way as the sample itself (if the protein has been dialysed or concentrated using ultrafiltration, the dialysate or filtrate should be used for this).

> Add 1 ml of dye reagent to 100 µl of the samples and blank, incubate for 10 min, and measure the A_{595} against the blank as described above.

> Calculate the amount of protein from the standard curve.

C Remarks

Although the method is straightforward and easy to perform, a number of points are worth mentioning:

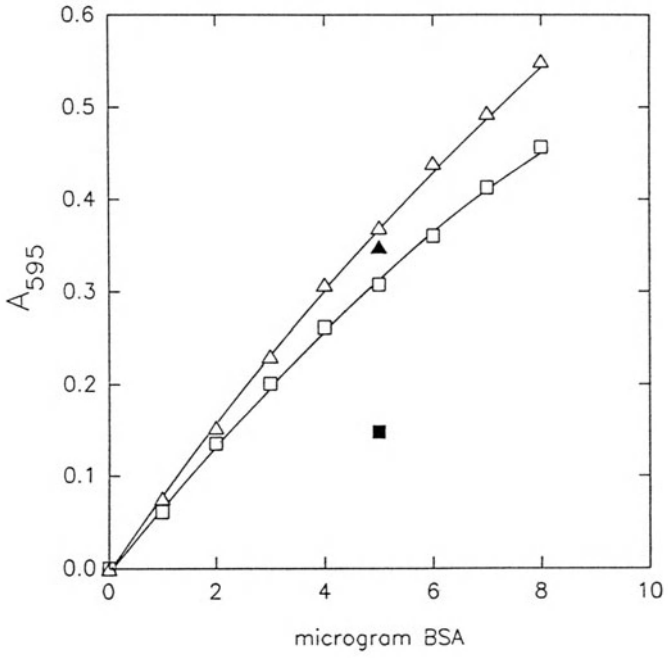


Fig. 1: Calibration-curve for the Coomassie Blue G dye-binding method: Bovine serum albumin was used as the standard. A sample of 100 µl containing the indicated amount of protein was mixed with 1.0 ml of the dye reagent (□ Bio-Rad protein determination kit; △ reagent solution according to Northcote and Read, 1981). The absorbance at 595 nm was determined after 10 min using a polystyrene cuvette. The values of A_{595} obtained with 5µg of lysozyme using the two reagents have also been indicated (■ and ▲).

1 Reagent

It has been pointed out that the response of this method varies considerably with different proteins (Pierce and Suelter, 1977; Van Kley and Hale, 1977). This is certainly true for the commercial dye solution (Bio-Rad, 1979). Although this problem may not be serious when assaying mixtures of proteins in crude extracts, Read and Northcote (1981) have shown that raising the concentration of Coomassie Blue G greatly diminishes this variation with different proteins. It is recommended to test the dye solution by measuring the colour produced by 5 µg lysozyme (prepare a stock solution as described above for BSA; a solution of 1mg of lysozyme/ml will give an $A_{280} = 2.64$). The colour yield should be 80–90% of that of 5µg BSA (see Fig.1). Alternatively, one may determine the concentration of dye by measuring the absorbance at 550 nm (Read and Northcote, 1981).

Since the concentration of dye is close to saturation, a precipitate may form with time. The solution should, therefore, be filtered and standardised weekly. Some protocols recommend adding concentrated dye stocks to a large volume of sample.

Although this undoubtly improves sensitivity, it may affect the accuracy since these concentrated solutions are not easy to pipette precisely. The use of a dispenser (e.g. the Distrivar 5000 from Gilson) and a diluted dye solution are recommended. For a microscale version of the assay see Brogdon and Dickinson, 1983.

2 Standard

We have observed that slightly higher (10%) values are obtained for the readings of the BSA standard when the protein is diluted in TBS rather than in water. Moreover, a solution of this protein in water is difficult to pipette accurately.

The dose-response curve (Fig.1) is non-linear, requiring an adequate number of data points. This non-linearity also precludes the use of a fixed number to correlate A_{595} and the amount of protein.

The procedure described yields *relative* amounts of protein. It has been suggested that standards other than BSA (ovalbumin, IgG) would give results closer to the *absolute* amount. However, this will certainly depend on the extract studied. If the determination of the absolute amount of protein is required, a conversion factor should be determined for the particular extract by an independent method (e.g. total quantitative amino-acid analysis, preferably using post-column derivatisation). This makes the choice of standard irrelevant.

3 Interfering Substances

Interfering substances may affect the standard curve in different ways: They may add a constant background value, shifting the dose-response curve in a parallel fashion. They may interfere with colour formation and alter the slope of the curve. They may change the linearity of the curve. It is, therefore, good practice to include an interfering substance in the dilutions of the standard protein at the same concentration as it occurs in the sample and the blank.

The number of substances that interfere with the method is relatively small. Notably, detergents (both ionic and non-ionic), guanidine-HCl, NaOH, and ampholines are known to interfere (Bradford, 1976; Spector, 1978; Bearden, 1978; Bio-Rad, 1979). Only proteins in solution can be determined and membrane bound proteins must be solubilised. Fanger (1987) has shown that the detergent hexyl-β-glucopyranoside does not significantly affect the reaction.

Note: The method employing Cu^{2+} and bicinchonic acid (Smith et al., 1985) may be used in cases where the presence of detergents is needed (however, not Triton X-100). With this method the same sensitivity as with the Coomassie Blue dye-binding method can be attained. It is affected by a number of other substances (thiol compounds, reducing sugars, chelating agents, Tris).

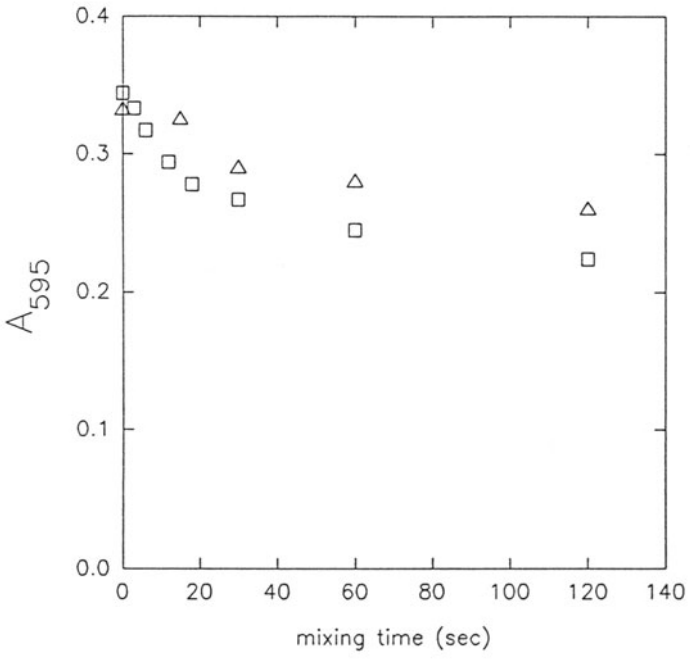


Fig. 2: Effect of Mixing: A 100-µl sample of protein solution (5 µg of BSA △, or 5 µg porcine liver cytosol protein □) was mixed with 1.0 ml of dye reagent by vortexing for the indicated amount of time. A sample that was mixed by gentle inversion was taken as the zero-time point.

4 Mixing Sample and Reagent

It has been recognised that excessive foaming reduces the colour yield (Bio-Rad, 1979). This has a number of consequences: 1) As can be seen in Fig.2, the value for A_{595} is dependent on the time of vortexing. This effect was observed for both BSA as well as a mixture of proteins in a liver cytosol preparation. Thus, it is necessary to standardise the way and time of mixing. 2) Pipetting of the reaction mixture into the cuvette should be done gently (the deleterious effect of bubbles is easily demonstrated by leading air through the solution with a pasture pipette and remeasuring A_{595}). 3) Since removal of the sample from the cuvette inevitably creates some foam, rereading of a sample always results in a lower value for A_{595}.

E Bibliography

Bearden, J.C., Quantitation of submicrogram quantities of protein by an improved protein dye-binding assay. Biochim. Biophys. Acta 533 (1978) 525–529.

Bio-Rad, Protein Assay, Technical Bulletin No. 1069 EG, Bio-Rad Laboratories, Richmond, CA 1979.

Bradford, M.M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt. Biochemistry 72 (1976) 248–254.

Brogdon, W.G., and Dickinson, C., A microassay system for measuring esterase activity and protein concentration in small samples in high-pressure liquid chromatography eluate fractions. Analyt. Biochemistry 131 (1983) 499–503.

Compton, S.J., and Jones, C.G. Mechanism of the dye response and interference in the Bradford assay. Analyt. Biochemistry 151 (1985) 369–374.

Cohn, W.E. in CRC Handbook of Biochemistry and Molecular Biology, 3rd edition, Vol.II, pp.383–545. Ed. G.D.Fasman. CRC Press, Cleveland 1976.

Darbre, A. in Practical Protein Chemistry, pp. 227–335. Ed. A.Darbre. John Wiley and Sons, New York 1986.

Fanger, B.O., Adaptation of the Bradford protein assay to membrane-bound proteins by solubilizing in glucopyranoside detergents. Analyt. Biochem. 162 (1987) 11–17.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. Protein measurement with the Folin phenol reagent. J. Biol. Chemistry 193 (1951) 265–275.

Peterson, G.L., Review of the Folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall. Analyt. Biochemistry 100 (1979) 201–220.

Peterson, G.L., Determination of total protein, in Methods in Enzymology 91 pp.95–119. Eds. C.H.W. Hirs and S.N.Timasheff. Academic Press, New York 1983.

Pierce, J., and Suelter, C.H., An evaluation of the Coomassie Brilliant Blue G-250 dye-binding method for the quantitative protein determination. Analyt. Biochemistry 81 (1977) 478–480.

Read, S.M., and Northcote, D.H., Minimization of variation in the response to different proteins of the Coomassie Blue G dye-binding assay for protein. Analyt. Biochemistry 116 (1981) 53–64.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. Measurement of protein using bicinchonic acid. Analyt. Biochemistry 150 (1985) 76–85.

Spector, T., Refinement of the Coomassie Blue method of protein quantitation. Analyt. Biochemistry 86 (1978) 142–146.

Van Kley, H., and Hale, S.M. Assay for protein by dye binding. Analyt. Biochemistry 81 (1977) 485–487.

Appendix A

DNA Isolation from *Escherichia coli*

J.P. Jost

DNA from *E. coli* is often used as carrier for precipitations, for chemical reactions and for many other purposes in molecular biology. The *E. coli* DNA we used for some procedures described in this book has been prepared as described below.

Materials and Buffers

- Luria broth (L-broth) for bacterial cultures
- Sorvall centrifuge with GSA rotor or equivalents
- Sonicator
- 25% Sucrose, 0.05 M Tris-HCl (pH 8)
- Lysozyme
- 0.5 M EDTA, pH 8.5
- 5 M Sodium perchlorate
- Phenol: chloroform: isoamylalcohol (25 : 24 : 1)
- 3 M Sodium acetate, pH 5

Step-by-Step Procecure

> Grow any *E. coli* strain (for example, HB 101) in 250 ml of Luria broth (L-broth) in a 1-l Erlenmeyer flask to stationary phase.

> Centrifuge the bacteria in a GSA rotor (or equivalent) at 6000 rpm for 10 min at 0°C.

> Suspend the pellet of bacteria in 4 ml of 25% sucrose buffer.

> Add 1.3 ml of lysozyme/water solution (10 mg/ml).

> Mix and incubate on ice for 5 min.

> Add 1.25 ml of 0.5 M EDTA pH 8.5, mix and incubate on ice for 5 min.

> Add 0.5 ml of 25% SDS and mix.

> Heat at 60°C for 5 min.

> Sonicate at room temperature until the solution is no longer viscous (this step must only be performed if the DNA is to be sheared).

> Add 5 M sodium perchlorate to a final concentration of 1 M.

> Mix with an equal volume of phenol/chloroform and extract for 5 min at room temperature.

> Separate the phases by centrifugation and repeat the extraction five times as described above.

> Dialyse the DNA solution at room temperature against 10 l of 5 mM EDTA for 24 h.

> Add 0,1 volumes of 3 M sodium acetate, pH 5 and precipitate the DNA with 2.5 volumes of ethanol at –20°C overnight.

> Centrifuge and dissolve the pellet at appropriate concentration in water, store in aliquots at –20°C.

Appendix B

The Spun Column Procedure

This procedure can be used to either desalt a DNA or RNA preparation or to separate the labelled nucleic acid from the non-incorporated nucleotides (see Fig. 1).

> Make a tight plug of silicone-treated, sterilised glass wool. Push it to the bottom of a 1-ml disposable syringe by means of the syringe plunger (cut off the rubber seal of the plunger).

> Using a Pasteur pipette, fill up the syringe with a slurry of Sephadex G-50 medium which has been pre-swollen in water and kept at 4°C in the presence of 0.05% NaN_3.

> To pack the Sephadex, put the syringe in a 15-ml Corex tube and centrifuge for a few minutes at 3000 rpm in a clinical centrifuge.

> Add Sephadex and spin again until the syringe contains about 0.9 ml of Sephadex.

> Rinse the Sephadex column three times with water by centrifuging at 3000 rpm for a few minutes.

If the column is not used immediately, prevent its desiccation by adding drops of water until the column is saturated. Seal the bottom and top of the column with Parafilm and store at 4°C.

> Before using the column, remove the water by centrifugation for 10 min at 3000 rpm. In order to avoid the collapse of the column to the bottom of the Corex tube, assemble the filtration unit as outlined in Fig. 1. Remove the water from the Eppendorf tube and reassemble the column.

> Immediately load 50–100 µl of the reaction mixture onto the column and centrifuge for 10 to 15 min at 3000 to 4000 rpm in a clinical centrifuge.

Notes : If the column is used for a change of buffer, equilibrate the Sephadex G-50 with the new buffer by washing the column six times by centrifugation. In cases where large DNA fragments are used (over 400 bp), it is advantageous to replace

Sephadex G-50 by Sephacryl S-300. This allows separation of the labelled DNA from oligonucleotides, free nucleotides, phenol and residual proteins. To remove any DNA by-product smaller than 400 bp, one can use Sephacryl S-400. Pharmacia recommends centrifugation of Sephacryl S-300 and 400 at a maximal centrifugal force of 400 × g during both equilibration and purification procedures. To calculate the speed (in rpm) needed to achieve this force, measure the radius (r) in millimetres from the centre of the spindle to the bottom of the rotor bucket and use the equation rpm = (1000) × (357/r) (see Pharmacia Data file on cDNA spun column).

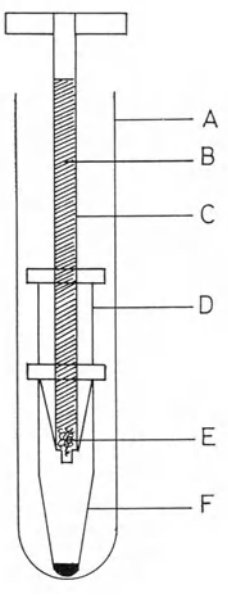


Fig. 1: The spun column:
A: 15 ml Corex tube
B: Sephadex or Sephacryl
C: 1 ml tuberculin syringe
D: Adapter Eppendorf tube with bottom removed
E: Glass wool
F: Eppendorf Tube

Appendix C

Purification of Oligonucleotides

J. Jiricny

How to Get Rid of Ammonia

> Take 50 µl of crude mixture in ammonia and dry down in an Eppendorf tube (a large volume should be first frozen or left open at room temperature to get rid of most of the ammonia gas.)
or

> Make a small (5–10 ml) column of Sephadex G-25 in water. Apply 100–300 µl of the crude oligo mixture (still in ammonia solution) and allow to run in. Elute with water. The oligos come with the void volume (approximately one-third of the column volume). Collect small fractions (5–10 drops) and measure OD_{260}. Keep only the first two or three fractions. If you do not wish to measure the OD, just pool all fractions that do not smell of ammonia. These fractions can be used for PCR.
or

> Add three volumes of cold ethanol to the ammonia solution and precipitate at –80°C. Resuspend in water. (This method is very crude and results in a great loss of oligo).
or

> Extract ammonia into n-butanol (3 volumes). Dry down in a SpeedVac concentrator and resuspend in water.

Purification by Polyacrylamide Gel Electrophoresis

> Transfer approximately 10 OD into an Eppendorf tube and dry down in a SpeedVac concentrator. Resuspend in 10 µl of 90 or 95% formamide (no dye) and apply in a 1-cm-wide

slot of a 0.5-mm-thick, 20 × 20 cm, denaturing polyacrylamide gel. (Acrylamide:bisacrylamide, 29:1, percentage according to oligo length: 20% up to 30-mer, 16% 30-50-mer, 12% 50-80-mer.) Load marker dyes in an adjacent slot and run quite hot.

> Remove the top glass plate from the gel assembly and cover gel with Saran wrap. Mark lanes with a felt-tip pen to avoid errors. Turn the sandwich upside down and remove the gel from the bottom glass. Lay the gel (Saran-wrap-side down) onto a 20 × 20 cm TLC plate coated with an adsorbent containing fluorescent (254 nm) indicator dye and place under a UV-lamp. Cut out the dark band corresponding to the longest product and transfer into an Eppendorf tube.

> Add 0.4 ml elution buffer (0.5 M ammonium acetate pH 6, 10 mM magnesium acetate) to the gel slice and shake vigorously in an Eppendorf shaker or equivalent (at 37°C if possible). Short oligos (up to 20-mer) elute within 2 h. Incubation with shaking overnight is advisable for longer sequences. Remove the buffer containing the eluted oligo. Elution efficiency can be measured by taking out the gel slice, laying it on a piece of Saran wrap and visualising at 254 nm over a TLC plate.

> (I) Precipitate the oligo by adding three volumes of cold ethanol. This is the quickest method, but great losses of short oligos are to be expected. Alternatively
(II) Wash a C_{18} Sep-Pak cartridge with 10 ml methanol followed by 10 ml water. Apply the eluted oligo slowly and wash with 10–12 ml water. Elute the oligo into an Eppendorf tube with 1 ml methanol. Dry down in SpeedVac concentrator and resuspend in water.
or
(III) Concentrate in a SpeedVac concentrator. Apply onto a Sephadex G-50 column (or equivalent) and collect excluded fraction. Measure the OD_{260} off the purified oligo. By rule of thumb, a solution of pure 25-mer with an $OD_{260} = 0.25$ contains 1 pmole/µl; a 90-mer solution of 1 pmole/µl should have an OD reading of 0.9.
or
Measure the absorption of a dilution (1:50 to 1:100) of the purified oligonucleotide at 260 nm. The following equation is used to determine oligonucleotide concentration (and to

account for the effect of base composition on extinction coefficients):

$$\text{Oligomer Concentration (pmol/}\mu\text{l)} = \frac{100 \cdot A_{260}}{1.54n_A + 0.75n_C + 1.17n_G + 0.92n_T}$$

Where: A_{260} = stock solution absorbance at 260 nm.
n_X = number of residues of base X in the oligonucleotide.

Appendix D

Migration of DNA on Native and Denaturating Gels of Different Polyacrylamide Concentration

Tracking dyes migrate through denaturating and non-denaturating gels at the following approximate positions equivalent to nucleotide migration.

On denaturating gels

% polyacrylamide gel	Xylene cyanole nucleotides	Bromophenol blue nucleotides
4	155	30
6	110	25
8	75	20
10	55	10
20	28	8

On non-denaturating gels

% polyacrylamide gel	Xylene cyanole nuleotides	Bromophenol blue nuleotides
3.5	460	100
5.0	260	65
8.0	160	45
12.0	70	20
20.0	45	12

(Maniatis et al, 1982; Sambrook et al, 1989)

Appendix E

Preparation of a Nuclear Lysate from Chicken Liver

J.P. Jost

This method for preparing nuclear lysates is not suitable for in vitro transcription, for which it is necessary to purify the nuclei through two discontinuous sucrose gradients before the lysis with ammonium sulfate. However, these lysates are suitable for further purification of factors binding to DNA.

Buffers

- 2 M Sucrose buffer: 2 M sucrose, 10% glycerol, 10 mM Hepes pH 7.6, 25 mM KCl, 1 mM EDTA
  Before use add 1 ml/100 ml of 100× polyamine mixture, 100 µl/100 ml of 1 M DTT and 0.5 ml/100 ml of 0.1 M PMSF.
- Dithiothreitol (DTT) 1 M in water, stored frozen at –20°C
- 100× Polyamine mixture: spermidine 50 mM, spermine 15 mM, in water, frozen in aliquots at –20°C
  The presence of polyamines prevents extensive degradation of the DNA during the lysis of the nuclei and gives a much more compact chromatin pellet upon centrifugation.
- PMSF stock solution (phenylmethylsulphonyl fluoride) 0.1 M in ethanol, kept at –20°C
- Lysis buffer: 10 mM Hepes pH 7.6, 10 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol
  Just before use add 0.01 volumes of polyamine mixture, 0.01 volumes of 0.1 M PMSF and 0.001 volumes of 1 M DTT.
- For the lysis of nuclei, use 4 M ammonium sulphate buffered with 50 mM Hepes pH 7.5.
- Dialysis buffer: 25 mM Hepes pH 7.6, 0.1 mM EDTA, 40 mM KCl, 1 mM DTT, 10% glycerol, 2 mM benzamidine, 20 mM β-glycero-phosphate Benzamidine is a protease inhibitor and β-glycerophosphate acts as a competing substrate for the protein phosphatases.)

Step-by-Step Procedure

> Perfuse two livers with ice-cold 0.15 M sodium chloride.

> Put the livers (50–60 g) in 100 ml of 2 M sucrose buffer in a Waring blender.

> Homogenise at low speed for a few seconds until there are no large pieces of liver.

> Homogenise with a glass-teflon homogeniser, 5 strokes at 2000 rpm.

> Make up the total volume of the homogenate to 220 ml with ice–cold 2 M sucrose buffer and mix well.

> Fill up six centrifuge tubes (SW-28 Beckman) and centrifuge at 0°C for 1 h at 25000 rpm. The crude nuclei will sediment through the sucrose while cell debris and membranes will float on top of the sucrose.

> Decant the tubes, leave them upside down to drain off the residual sucrose and wipe the side of the tube clean with a tissue.

> Resuspend the nuclei in 60 ml lysis buffer with a glass-teflon homogeniser (two strokes at 500 rpm).

> Put the homogenous nuclei suspension in a beaker on ice.

> Add 0,1 volumes of 4 M ammonium sulphate-buffer slowly while stirring with a magnet.

> Stir the lysed nuclei very slowly on ice for 40 min.

> Put the lysed nuclei into six tubes of SW-40 (Beckman) using a 60-ml plastic syringe fitted with a wide-gauge canula. Take out the plunger of the syringe, pour in the viscous mass of chromatin, and put back the plunger. Push the chromatin slowly to the bottom of the tubes.

> Centrifuge the chromatin at 0°C for 1 h at 35000 rpm.

> Decant the supernatant into a graduated cylinder and discard the pellet.

> Measure the volume of the supernatant and precipitate proteins in the cold with solid ammonium sulphate (65% saturation, use 0.365 g of solid ammonium sulphate per

millilitre of solution). The ammonium sulphate crystals should be first ground and then added slowly over 15 min.

> Stand the solution at 0°C for 30 min.

> Spin down the precipitate in a SW-40 Beckman rotor for 30 min at 30000 rpm at 0°C.

> Decant the supernatant, keep the tubes upside down and wipe the inside walls clean.

> Dissolve the pellets in 3–5 ml of the dialysis buffer.

> Dialyse the solution for 2 × 2 h against two changes of 500 ml dialysis buffer.

> Put the dialysed protein into a Corex tube and centrifuge the insoluble proteins at 10000 rpm for 10 minutes in a Sorvall centrifuge (rotor HB-4). The insoluble protein can be solubilised in the same buffer plus 100 mM KCl and 1% Nonidet NP 40.

> Store the clear supernatant in aliquots at –70°C or in liquid nitrogen. The preparation should have a protein concentration of 15–20 µg/µl and is ready for further purification steps.

Appendix F

Preparation of a Cell Lysate from Tissue-Culture Cells

It is possible to isolate transcription factors and other DNA-binding proteins directly from cell lysates of cultured cells. The procedure described below is derived from the original protocol of Manley et al. (1980).

Materials and Buffers

- **Phosphate-buffered saline**
  Solution 1: in 800 ml water dissolve consecutively 8 g of NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4 \cdot 2\ H_2O$, 0.2 g KH_2PO_4. Solution 2: in 100 ml water dissolve 0.175 g $CaCl_2 \cdot 2\ H_2O$. Solution 3: in 100 ml water dissolve 0.1 g $MgCl_2 \cdot 6\ H_2O$. Autoclave the three solutions separately for 20 min at 120°C. Let them cool to room temperature and mix in a sterile vessel.
- 100× Polyamine mixture: Spermidine 50 mM, spermine 15 mM in water, frozen at –20°C in aliquots The presence of polyamines in the cell lysate prevents extensive degradation of DNA and gives a much more compact chromatin pellet upon centrifugation.
- Sucrose-glycerol buffer. 25% sucrose, 50% glycerol, 50 mM Tris pH 8, 10 mM $MgCl_2$, 2 mM DTT, kept at –20°C
- Lysis buffer: 10 mM Tris pH 7.5–8, 1 mM EDTA, 5 mM DTT, 1× polyamines (diluted from a 100× stock solution, see above) DTT and polyamines are added just before use.
- 4 M Ammonium sulpfate buffered with 50 mM Hepes pH 7.5
- PMSF (phenylmethylsulphonyl fluoride): 100 mM stock solution in ethanol, kept at –20°C
- Dialysis buffer: 20 mM Hepes pH 7.5, 100 mM KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT, 17% glycerol.
- Glass-glass Dounce homogeniser, 15 ml S type from B. Braun, Melsungen, Postfach 110 & 120, D–3508 Melsungen, FRG.

Step-by-Step Procedure

> Scrape cells from Petri dishes after having removed the tissue culture medium.

> Rinse cells twice in cold PBS (in certain cases it is better to leave out Ca^{++} and Mg^{++}). Resuspend the cells by vortexing and centrifuge for 3 min at 3000 rpm in a clinical centrifuge.

> Estimate the packed cell volume and add 4 volumes of the lysis buffer.

> Leave the cell suspension in ice for 20 min.

> Homogenise by hand with 20 strokes in a Dounce homogeniser, keeping the homogeniser in ice.

> Add an equal volume of sucrose-glycerol solution to the lysed cells.

> Mix well and put the lysed cell suspension into a beaker on a magnetic stirrer. Measure the exact volume of the suspension.

> Slowly add 0,1 volumes of 4 M ammonium sulphate buffer. The nuclei will lyse and give a very viscous mass.

> Stir very slowly on ice for 30 min.

> Put the viscous mass of cells in approximately sized centrifuge tubes as indicated for the preparation of lysed nuclei in the previous chapter.

> Centrifuge, for example, for 3.5 h at 50000 rpm in a SW 56 rotor at 0°C.

> Take out the supernatant carefully, measure the volume and slowly add over 15 min 0.33 g of finely powdered ammonium sulphate.

> Leave on ice for at least 30 min.

> Centrifuge the protein precipitate for 30 min at $100000 \times g$.

> Decant the supernatant and dissolve the pellet in dialysis buffer, usually 1/25 the volume of the post-chromatin supernatant.

> Dialyse for 2×2 h against one change of 500 ml of dialysis buffer.

> Centrifuge the dialysed proteins in an Eppendorf tube for 5 min.

> Store the supernatant solution at –70°C or in liquid nitrogen. The concentration of proteins should be 10–20 µg/µl.

Appendix G

Preparation of Nuclear Extracts from Cells in Tissue Culture

This method was developed by Shapiro et al. (1988) for HeLa cells and for the in vitro transcription, is about 20 times more efficient than the standard procedure described by Manley et al. (1983).

Buffers

- Hypotonic buffer: 10 mM Hepes pH 7.9, 0.75 mM EGTA, 1 mM DTT, 10 mM KCl
- 10× Salts: 500 mM Hepes pH 7.9, 7.5 mM spermidine, 1.5 mM spermine, 100 mM KCl, 2 mM EDTA, 10 mM DTT.
  Polyamines are instable so add just before use from a stock solution kept frozen at –20°C, or keep the complete 10× salt solution frozen at –20°C.
- Sucrose restore buffer is prepared by adding 9 volumes of 75% RNase free sucrose (w/v) to 1 volume of 10× salts.

Note: It is not necessary to purchase an expensive RNase free sucrose. Just take any sucrose and destroy the contaminating ribonucleases by adding a few drops of diethylpyrocarbonate to the solution. Diethylpyrocarbonate is highly unstable in aqueous solution and a treatment for 30 min at 50°–80°C will decompose all residual DEPC.

- Nuclear resuspension buffer: 20 mM Hepes pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM EDTA, 2 mM EGTA, 2 mM DTT, 25% glycerol. The final buffer is prepared by adding 9 volumes of nuclear resuspension solution to 1 volume of saturated (at 4°C) ammonium sulphate.

Note: It is also possible to add 1 volume of 4 M ammonium sulphate buffered with 50 mM Hepes pH 7.9.

– Nuclei dialysis buffer: 20 mM Hepes pH 7.9, 20% glycerol (v/v) 100 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA and 2 mM DTT

Step-by-Step Procedure

> Grow cells to a density of approximately 5×10^5 cells/ml.

> Collect cells by centrifugation at 170 × g for 10 min at 4°C.

> Resuspend the pellet in approximately 50 volumes of cold phosphate-buffered saline (10 mM phosphate buffer pH 7.4, 150 mM NaCl).

> Sediment cells at 170 × g for 10 min at 4°C.

> Transfer the washed cells to a 15-ml graduated centrifuge tube by resuspending in a small volume of phosphate-buffered saline.

> Sediment cells at 300 × g for 10 min at 4°C.

> Measure the approximate packed cell volume (PCV) and resuspend cells in 5 PCV of hypotonic buffer.

> Allow the cells to swell for 10 min in ice.

> Sediment the cells by centrifugation at 300 × g for 10 min at 4°C.

> Pour off the buffer and replace with twice the original PCV of hypotonic buffer.

> Homogenise cells with three strokes (by hand) with a tight (glass-glass) Dounce homogeniser (Kontes Glass Co.). This will result in over 95% cell breakage.

> Add immediately 0.1 volumes of ice-cold sucrose restore buffer and gently mix the homogenate with two strokes of a loose-fitting glass/teflon homogeniser.

> Centrifuge the homogenate immediately for 30 s at 10000 rpm in a HB-4 Sorvall rotor (16000 × g) at 4°C with the brake on. (The total centrifugation time should be approximately 2.5 min).

> Carefully pour off the supernatant.

> Resuspend the viscous nuclear pellet in 3 ml of nuclear

resuspension buffer per 10^9 cells and transfer to a straight-wall polycarbonate or polyallomer centrifuge tube.

> Rock the extract on a "Lab Quake" (Lab Industries) or equivalent apparatus for 30 min at 4°C.

> Sediment by centrifugation at 2°C for 90 min at 150000 × g (40000 rpm, Beckman rotor Ti 80).

> Carefully remove the supernatant and put into a small beaker with a magnetic stirring bar.

> Slowly add ammonium sulphate (0.33 g/ml) and stand the solution in ice for 30 min while stirring very slowly.

Note: The ammonium sulphate should be of enzyme grade and should be finely ground before use.

> Centrifuge for 30 min at 100000 × g and 0°C.

> Dissolve the pellet in up to 1 ml of nuclear dialysis buffer per 10^9 cells.

> Dialyse twice for 90 min against two changes of 500 ml dialysis buffer.

> Store the preparation at –80°C or in liquid N_2 in aliquots. The protein concentration should be approximately 10 µg/µl.

Bibliography

Manley, J.L., Fire, A., Samuels, M. and Sharp, P.A. *In vitro* transcription: whole cell extract. Methods Enzymology 10 (1983) 568–582.

Shapiro, D.L., Sharp P.A., Wahli, W. W. and Keller, M.J. A high efficiency HeLa cell nuclear transcription extract. DNA 7, (1988) 47–55.

Appendix H

Inhibitors of Proteases[a]

E. Shaw

Inhibitor	Action	Note	Source
DFP (Diisopropylfluorophosphate)	Ser	b	1,3
PMSF (Phenylmethylsulphonyl fluoride)	Ser	b	1,2,3,4
3,4-Dichloroisocoumarin	Ser	b	1,4
L-1-Chloro-3-Cbz-amido-4-phenyl 2-butanone (ZPCK)	Ser	b	1,2
Benzamidine	Ser	c	1,3
Leupeptin (or antipain)	Ser, Cys	c	1,2,4
Iodoacetamide	Cys	d	1,3
Cbz-Phe-AlaCHN$_2$	Cys	d	2
EDTA, EGTA	Metallo	e	3
1,10-phenanthroline	Metallo	e	1,3
8-Hydroxyquinoline	Metallo	e	3
Pepstatin	Asp	f	1,2,4

Notes:

a) Inhibitors are listed which have a relatively broad action. A great number of more specific inhibitors is available but these are less suitable for obtaining general protection from proteases.

b) These are time dependent when they act and lead to an irreversible inhibition. DFP is neurotoxic but sometimes is anti-proteolytic when PMSF, although less hazardous, is not. 3,4-Dichloroisocoumarin is a recent arrival and apparently more rapid. ZPCK is specific for neutral proteases.

c) Very soluble reversible inhibitors for the trypsin subclass of serine proteases.

d) Irreversible.

e) EDTA and EGTA bind calcium commonly, but this metal may have a structural, not catalytic role. The other inhibitors listed bind zinc, which is often a key metal in this class of proteases.

f) The effect is quite pH dependent; more effective near pH 5 than at neutrality.

Sources

1 Sigma Chem. Co., P.O. Box 14508, St. Louis, Mo., USA 63178-9916
2 Bachem, Hauptstrasse 144, CH–4416 Bubendorf, Switzerland
3 Aldrich-Chemie Gmbh + Co. Postfach 1120, Riedstrasse 2, D–7924, Steinheim, Germany
4 Boehringer Mannheim Biochemica, Sandhoferstr. 116, D–6800, Mannheim 31, P.O. Box 310120, Germany

Appendix I

The Most Common Protein Kinase and Phosphatase Inhibitors

B. Hemmings

Protein kinase inhibitors

Inhibitor	Target protein kinase	Note	*Source*
EDTA	All protein kinases	Chelates Mg^{2+}	–
W5 W7 W12 W13	CAM-PK	Calmodulin antagonist	(a)
H7 H8 H9	PKC, cGMP-PK PKA, MLCK	ATP-binding inhibitor	(a)
Heparin	CKII	Potent inhibitor of purified protein	(a)
Staurosporine (+analogs)	PKC, PKA, MLCK CKI, CKII, S6K	Potent inhibitor of most protein kinases	(a)
Calmidazolium	CAM-PK, MLCK	Calmodulin antagonist	(a)
PKI peptide	PKA	Specific inhibitor of peptide-pseudo-phosphorylation site	(b)
PKC pseudo-substrate peptide	PKC	Specific inhibitor of peptide-pseudo-phosphorylation site	(b)

Source
(a) Calbioche
(b) Peninsula
Both companies supply references for their compounds

Abbreviations
W5, N- (6-aminohexyl)-1-naphthalene-sulphonamide
W7, N- (6-aminohexyl)-5-chloro-1-naphthalene-sulphonamide
W12,N- (4-aminobutyl)-2-naphthalene-sulphonamide
H7, 1- (5 isoquinolinesulphomyl)-2-methyl-piperazine;

Protein phosphatase inhibitors

"Classical" Inhibitors	*Target protein phosphatase*	*Source*
Sodium fluoride Sodium pyrophosphate p-Nitrophenolphosphate β-Glycerophosphate	P-Ser/Thr Types 1, 2A, 2B and 2C	(a)
EGTA	P-Ser/Thr Type 2C	(a)
Vanadate Molybdate Zn^{2+}	P-Tyr Placental PTP-1B CD 45	(a) (a)
Heparin	P-Tyr Placental PTP-1B	(b) (c)
"New" Inhibitors Okadaic Acid Microcystin	P-Ser/Thr Type 1 Type 2A & 2C	(c)
"Peptide" Inhibitors Inhibitor 1 Inhibitor 2	P-Ser/Thr Type 1	(d)

Sources:
(a) Commonly available
(b) Sigma
(c) Calbiochem
(d) Not commercially available but can be easily purified – both are TCA and heat stable proteins

Abbreviations:
Nomenclature of serine/threonine protein phosphatases according to Ingebritsen and Cohen; see Cohen, Ann. Rev. Biochem. 58 (1989) 453–508.
Nomenclature for tyrosine specific protein phosphatases from Tonks and Charbonneau (TIBS 14 (1989) 487–501).
H8, N- [2-(methylamino)-ethyl]-5-isoquinoline-sulphonamide;
H9, N- [2-(aminoethyl)-5-isoquinoline-sulphonamide;
PKA, cAMP-dependent protein kinase;
PKC, Protein kinase C;
cGMP-PK, cGMP-dependent protein kinase;
CAM-PK, Calmodulin-dependent protein kinase;
MLCK, myosin light chain kinase;
CKI, Casein kinase I;
CKII, Casein kinase II;
PKI, cAMP-PK inhibitor protein
S6K, Mitogen-stimulated S6 protein kinase

Appendix J

Tables on the Precipitation of Proteins with Ammonium Sulphate.

Initial concentration of ammonium sulphate, % saturation	*Final concentration of ammonium sulphate, % saturation*																
	10	20	25	30	33	35	40	45	50	55	60	65	70	75	80	90	100
	Grammes solid ammonium sulphate to be added to 1 l of solution																
0	56	114	144	176	196	209	243	277	313	351	390	430	472	516	561	662	767
10		57	86	118	137	150	183	216	251	288	326	365	406	449	494	592	694
20			29	59	78	91	123	155	189	225	262	300	340	382	424	520	619
25				30	49	61	93	125	158	193	230	267	307	348	390	485	583
30					19	30	62	94	127	162	198	235	273	314	356	419	546
33						12	43	74	107	142	177	214	252	292	333	426	522
35							31	63	94	120	161	200	238	278	319	411	506
40								31	63	97	132	168	205	245	285	375	469
45									32	65	99	134	171	210	250	339	431
50										33	66	101	137	176	214	302	392
55											33	67	103	141	179	264	353
60												34	69	105	143	227	314
65													34	70	107	190	275
70														35	72	153	237
75															36	115	198
80																77	157
90																	79

Precipitation with solid ammonium sulphate

From Green, A.A. and Hughes W.L. Protein fractionation on the basis of solubility in aqueous solutions of salts and organic solvents. Methods in Enzymology, volume 1 (1955) p.76, with permission of Adademic Press, copyright 1990.

% Initial ammonium sulphate saturation	% Final Ammonium Sulphate Saturation																			
	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95
0	0	6	11	18	25	34	43	54	67	82	100	122	150	186	234	300	400	600	900	1900
5			6	12	19	27	36	46	58	73	90	111	138	172	217	280	375	533	850	1800
10				6	13	20	29	39	50	64	80	100	125	157	200	260	350	500	800	1700
15					7	14	22	31	42	55	70	89	113	143	183	240	325	467	750	1600
20						7	15	23	34	46	60	78	100	129	167	220	300	433	700	1500
25							7	16	25	37	50	67	88	114	150	200	275	400	650	1400
30								8	17	28	40	56	75	100	133	180	250	367	600	1300
35									9	18	30	45	63	86	117	160	225	333	550	1200
40										9	20	34	50	71	100	140	200	300	500	1100
45											10	22	38	57	83	120	175	267	450	1000
50												11	25	43	67	100	150	233	400	900
55													13	29	50	80	125	200	350	800
60														15	34	60	100	167	300	700
65															17	40	75	133	250	600
70																20	50	100	200	500
75																	25	67	150	400
80																		34	100	300
85																			50	200
90																				100

Millilitres of saturated ammonium sulphate solution at room temperature to be added per 100 ml solution of known initial ammonium sulphate saturation.

XXIII Index

A Laboratory Guide for Cellular and Molecular Plant Biology

Edited by
I. Negrutiu
G. Gharti-Chhetri
Inst. Molecular Biology, Free Univ. of Brussels, Belgium

1991. 392 pages. Hardcover. ISBN 3-7643-2542-9 (BioMethods 4)

Cellular techniques and gene transfer procedures are presented as simplified protocols making them easy to handle for all those entering the field. Hardliners of cell culture will also find new ideas in improving media composition or evaluating plating efficiency. Cellular biologists will have access to molecular techniques through two elaborate chapters on the matter. Finally, in the cytology chapter straightforward techniques are presented which should allow many of us to perform molecular cytology experiments properly and obtain maximum benefit from flow-cytometry. Many of the procedures are described in compact, rapid flowing essays. Overall, illustrations and examples cover various developmental problems, such as functional analysis of genomes and genes and basic aspects of reproduction biology.

Sections from the Contents:

Please order through your bookseller or directly from:
Birkhäuser Verlag AG, P.O. Box 133, CH-4010 Basel / Switzerland
Orders from the USA or Canada should be sent to:
Birkhäuser Boston Inc., c/o Springer Verlag New York Inc.,
44 Hartz Way, Secaucus, NJ 07096-2491 / USA

Birkhäuser
Birkhäuser Verlag AG
Basel · Boston · Berlin