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MOLECULAR CARDIOLOGY FOR THE CARDIOLOGISTS.

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PREFACE.

For people who are currently doing basic research in medicine since 20-30 years the last ten years were quite an exciting period during which it was practically possible to attend a logarithmic burst both in the new tools available for research and in our understanding of physiopathology. New biology is essentially different from what 50-60 year old scientists had learnt during their professional education and is based on the knowledge of the DNA structure and above all on the capacity that we now have to resynthesize DNA or RNA fragments and to use them for making proteins or detecting rapid, subtle changes in the cardiac or vascular structure.

Such an explosion in biological sciences reached cardiology rather late as compared to other fields of medicine for several reasons including the fact that cardiology is one of the medical branch which is the most intimately linked to organ physiology, and also because the heart is not an easily accessible tissue. Nevertheless, it is now impossible to follow the recent progress in cardiology without, at the least, knowing the new language which has been created by the practicians of the new biology, because indeed new biology, the so- called "Molecular Biology", as every new domain in science, had invented its own mode of expression. This new language does not only include new words which qualifies new compounds ("intron*", "exon*".....), excessive and needless synonyms ("locus*" for "place", "segregate*" for "transmit"), but also a new syntaxe (a gene "encode" a protein). New words have been labelled " * " and explained in a Glossary at the end of this book. No doubts, in addition, that this new language more or less hides a wish from the high priests of the new religion to sacralise their field of investigation and to maintain the source of political and economical power which it represents.

We had tried to summarize in the most concise way possible the keys which may allow a general practician or a student who want to become internist to read most of the papers published upon this matter and to realize where the developments are the most promising from a practical and clinical point of view. There are indeed several talented books [Darnell 1986, Kaplan et al 1993, Robert 1993] which may introduce a physician to new biology, nevertheless, for most of them, the size may be discouradging.

The intent of this book is firstly to provide a short, easily readible, summary of what the new biology may bring to cardiology in 1995. With this respect, special efforts were made to mutiply comprehensive schemes and drawings, as well as teaching tables. Some of them had already been published.... and experienced in various teaching articles [Swynghedauw et al 1993], and also during a rather long carreer as both a researcher and a teacher in basic cardiology. Practical application are provided as exemples to illustrate the clinical interest of such a new approach, nevertheless these are only exemples, and the general purpose of the book was not to cover every chapter of the cardiovascular pathology.

The book had been divided into 5 parts. The first part is a general introduction to the new linguistic and was essentially made from the two teaching articles that we had previously published in Cardiovascular Research [Swynghedauw et al 1993]. This part contains numerous schemes and a rather long chapter on biotechnology which was at the origin of the new progress and also of the new language.

The second part is the most important and devoted to the normal structure of the heart and vessels. This area has been covered in relation to electrophysiology, mechanics and the physiology of vasomotricity, and special care was made to try to establish bridges between new biology and the classical physiology.

Parts 3 and 4 dealt with Physiopathology. One of the important contributions of molecular biology to cardiology is a better understanding of

the general process of adaptation of the heart and vessels to a permanent mechanical overloading. Such a process is essentially different from the mechanism which is responsible for genetical disorders and results from the put into play of coordinate changes in the expression of the genes. Thus, heart failure, and the hypertensive macroangiopathy, appear as true diseases of biological adaptation. It is now possible to provide a biological explanation for most of the myocardial dysfunctions.

By opposition, the genetic diseases result from a modification in the gene structure itself. Genetics is in rapid progress and very close to have clinical applications, but its language is particularily hermetic for those who are not familiar with this rather new, or renewed, science. This part had been introduced by a linguistic initiation, which does include numerous teaching schemes. Exemples of cardiovascular genetic diseases are given in proportion to the amount of knowledges that we have for each particular disease, which is not, obviously, commensurate to their relative incidence. Consequently, there were, for example, more developments on the hypertrophic cardiomyopathy and the xanthomatose hypercholesterolemia than on the atherosclerotic process or on arterial hypertension which are, for the moment, poorly documented in terms of genetics.

The last part of the book will define the new therapeutic avenues which had been opened by new biology. This includes firstly the reapprisal of drug design, which has now to take into account the fact that we now know that both the vascular and the myocardial phenotypes are modified by the diseased process itself. Consequently, assuming that a drug is a key, drug design has to plan new drugs taking in account that the lock is modified by the disease. The new glycosides and the sodium pump are good examples of this concept. Gene therapy is not really immediately appliable to cardiology. Nevertheless, the physician has now to understand the concept because several applications are likely to occur within the next decade, specially in familial hypercholesterolemia or to prevent restenosis. Another area of interest is the utilization of transgene technology to make

up new strains of animals which may be of great help in the understanding of either a disease or particular physiological mechanisms.

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THE NEW LANGUAGE OF BIOLOGY.

FROM CHROMOSOME TO GENE.

The first part of this chapter will describe the deoxyribonucleic (DNA) and ribonucleic (RNA) acids molecules, their organization within the cell, their synthesis commonly refered to as DNA replication and RNA transcription and the regulation of mRNA transcription.

The chemical basis of heredity is **Figure 1** summarizes the concept that every biologist keeps in mind. I resemble my father and also every human being because the biochemical molecules from which I am made are very similar those from which my parents or other humans are made. In addition this similarity has been transmitted, geneticians would say segregated*, during conception. Both the morphology of a given human being and the main physiological functions are expressions of its chemical structure in terms of proteins. The main physiological functions depend upon various enzymes and receptors whose activity is, in turn, a consequence of the spatial structure of a protein. Proteins are made from more than 20 amino acids which all have different structures and electric charges. There final spatial arrangement is entirely determined by these structures and their charges, i.e. by the amino acids composition which constitutes the primary structure of the protein*.

There are two different levels of spatial arrangement of a protein : the secondary structure which is the spatial arrangement of the amino acid chain itself, i.e. its helicity, and the tertiary structure which is formed by the spatial arrangement of the chain which in turn results in the formation of

the different pockets where ligands (for the receptors) or substrates (for the enzymes) bind. The quaternary structure of a protein is the spatial arrangement of its different subunits and represents the final structure, it also reflects the amino acid composition. The amino acid composition is itself determined and transmitted as a genetic code* and the genetic code is provided by a biochemical molecule, DNA. Such a dogma is, for the moment, based on a rather limited number of examples. We know the primary structure of most of the proteins and also the composition of nearly all of their corresponding genes. Nevertheless, at the present time the quaternary structure and structure-function relationships has been fully explored for only a few proteins. Such an investigation needs the use of several several biophysical techniques including crystallography and also the use of expression vectors, transgenic technology and even genetic epidemiology and organ physiology in order to establish the correlations between a given part of the molecule and the physiological functions. Strictly speaking the above dogma is in fact a working hypothesis which has never been contradicted.

The genotype* is the genetic code and it is present and the same in every nuclei, of a given individual. The genotype can be identified in any cell of a given individual, but it can be transmitted to the progeny* only by the germinal cells*. The phenotype* is the apparent manifestation of the genotype, nevertheless phenotype and genotype are different in the same person. For example, the genotype of a cardiac cell and of a leucocyte are identical in the same individual, but the myocardial phenotype is different from that of the liver, since albumin is present in the liver, but not in the heart. It is therefore possible to identify in the leucocytes a genetic abnormality responsible for a cardiac disease. There is an intermediary step between the genotype and the phenotype which is by RNA, and there is one molecule of special interest, the messenger RNA, mRNA,* which

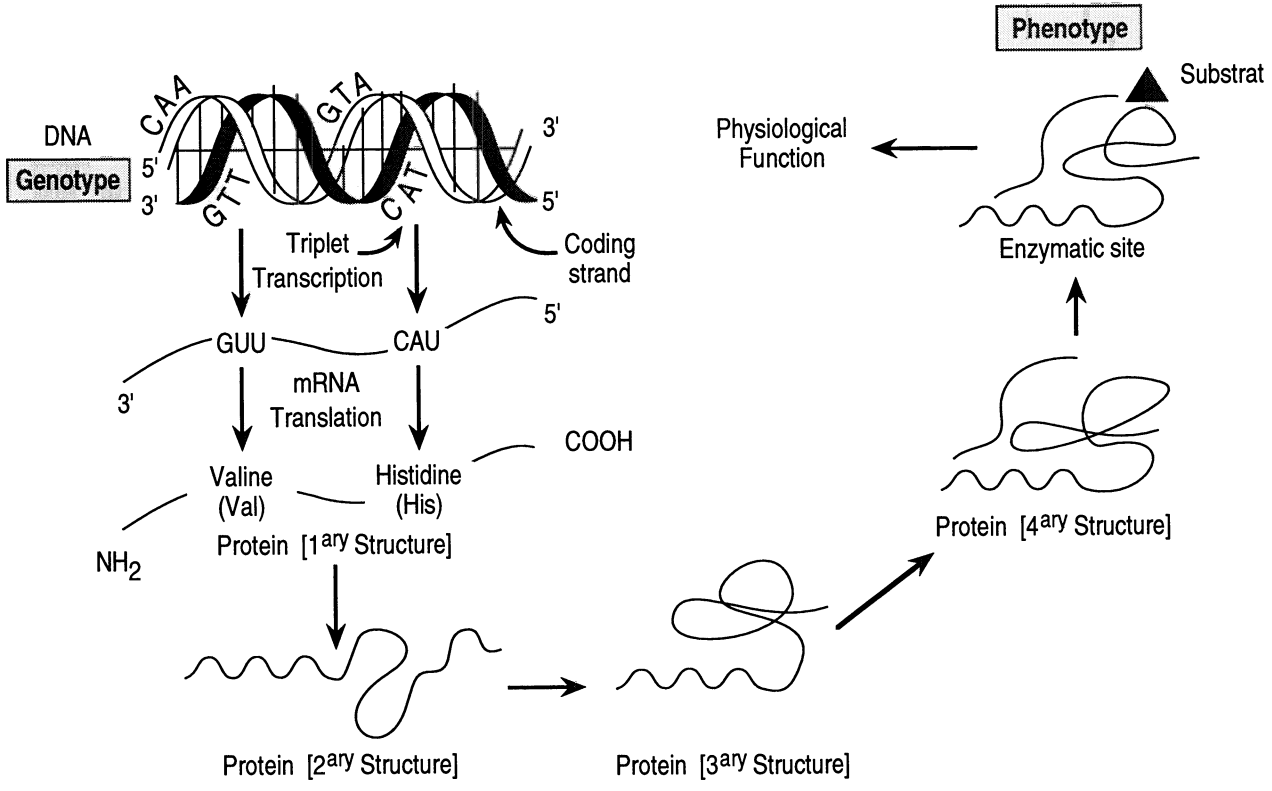

Fig. 1 From Phenotype to Genotype

transcribes* the genetic code into a language which allows the cellular machine to translate* the code into a protein.

Chromosome packaging.

The genes are the molecules which contain all of the information which allows the transcription of a mRNA, the genes represent a rather minor portion of the DNA molecule and most of the molecule is said to be anonymous* because it is not used to make the phenotype. DNA, proteins responsible for DNA replication and transcription and the first products of the expression of genes are located in highly organized structures present in the cell at mitosis and called chromosomes* (**Figure 2**). Chromosomes are large structures measured in nmeters, visible by light microscopy and easily identified by histological techniques in a cell providing the cell is dividing. Diploid* cells contains an even number of chromosomes, 2 x 23 chromosomes in man, which includes 22 pairs of autosomal chromosomes, so-called because they play no role is sex determination, and the 2 sex-determining chromosomes, XX in the female and XY in the male, diploïd cells are called somatic* cells. The germinal cells* (or gametes* and their precursors) are haploïd* and contain only one chromosome from each pair.

Chromosomes can be easily recognized in a chromosomal spread. Each chromosome concists of a pair of chromatids joined by a constricted central region termed the centromere. Chromatids are rigorously identical structures which contain exactly the same genetic material and are morphologically identified by having two arms of unequal length. The morphology of chromosomes can be studied using several histological techniques which allows the detection of a series of distinct bands and enables one to divide the chromosomes into distinct regions. For any animal species, the banding pattern of a given chromosome is reproducible for each staining and is called for example Q-banding pattern when the stain is Quinacrine (**Figure 3**). It allows the identification of the chromosome

(Chromosome 1, 2, 3.....10, 11..). Chromosome bands are named by describing the short arm of the chromosome as p and the long arm as q (Figure 3, see examples Chapter 4). Each arm is in turn divided into numbered regions of various size, and these regions are then further divided into bands and interbands.

Chromosomes are located within the cell nuclei. When the cell is not dividing, i.e. when there are no mitosis or meïosis the chromosomal material is compactly folded into chromatin* which duplicates before it organizes into chromosomes. After chromatin duplication and condensation, the chromosomes containing the same genetic information segregate so that each dividing cell receives exactly the same amount of genetic material. After cell division, the chromosome structure is no longer visible and the chromatin becomes amorphous.

Chromatin, or chromosome, is composed of approximately a one meter-long, 0.2 nanometer-thick string of DNA wound around a core of proteins made of histones, the nucleosomes*. The DNA is also covered with other proteins : structural acidic proteins, transcription factors and various regulatory factors, including the nuclear receptors. The size of each nucleosome is of the order of x nmeters. By electron microscopy, nucleosomes appear as beads on a thin string. The beads consist of 2 copies of small basic proteins called histones, around which is wound a constant length of DNA and are separated by DNA bound to a fifth type of histone. Histones are highly conserved between species. The human genome consists of approximately 30,000,000 nucleosomes. Nucleosomes are the cell's mean of packaging a long string of DNA into a very compact structure.

Cell Division.

Prokaryotic* and eukaryotic* cells are both able to duplicate their genome and to transmit identical copies of the initial genetic material to daughter cells. Mammalian cells are eukaryotic and divide during their cell cycle*.

Autosomal Cells.

Cells from the cardiovascular system are autosomal this includes non muscular cells such as endothelial cells, fibroblasts, macrophages, and muscle cells such as vascular smooth muscle cells and cardiac myocytes, also called cardiocytes. All these cells divide during development, and, at least for the non muscular cells, during the adulthood. The division occurs during a cell cycle which is made of several sequential phases : (i) the most variable period is the first Gap period, G1, during this phase the DNA is compactly folded as chromatin and the chromosomes cannot be seen ; (ii) the S (S for Synthesis) phase (7hours), during this phase the double-helical DNA is replicated into two identical daughter DNA molecules, and histones and various proteins responsible for the activity of the chromosome bind rapidly to the new DNA ; (iii) a second gap period, called G2 (3h), (iv) then chromosomes appears as thin threads into the nucleus, and they attached to microtubules which have been radiated from the centrioles. Mitosis can then proceed for one hour into four substages, prophase, metaphase, anaphase, telophase. At the end of telophase, cytokinesis occurs, the cell divides into two new cells, each of them having the same chromosomes and also chromosomes identical to the chromosomes of the initial cell (**Figure 4**). Adult cardiocytes do not divide, as neuronal cells, and are quiescent cells which remain in phase G0.

An important issue of the cell cycle of autosomal cells is that the genetic material is copied once. At the begining, each of the 22

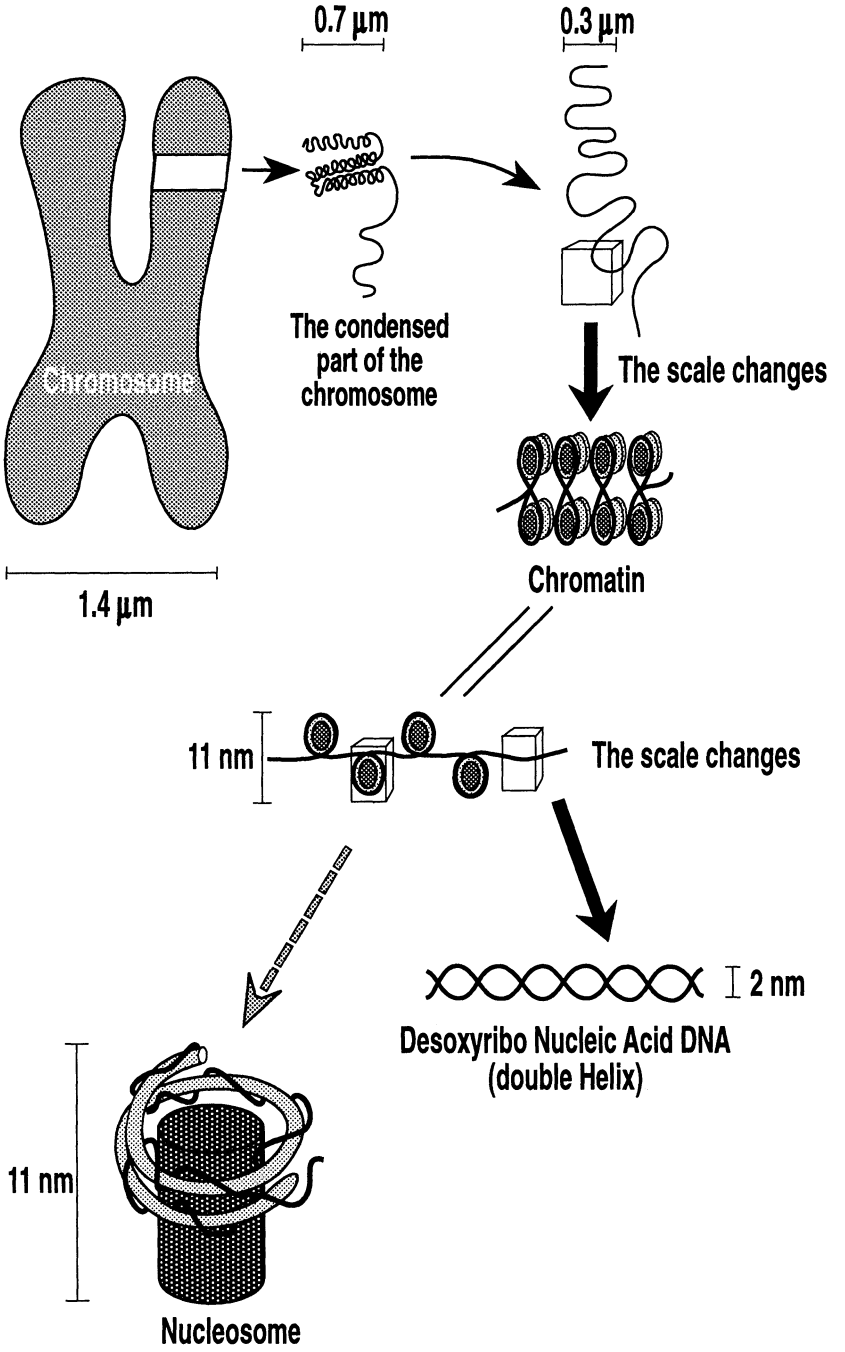

Fig. 2 . From Chromosomes to DNA [Reproduced from Swynguedauw et al, Cardiovasc. Res. 1993, 27, 1414, with permission].

chromosomes exists as a pair, and the cell is 2n. At the end of telophase, immediately before the cell division, each pair of chromosome is doubled and the cell became 4n. After cytokinesis the daughter cells are again 2n and contain exactly the same material as the initial cell, i.e. a pair of the father's chromosomes and a pair of the mother's chromosome. In addition there are no crossing-over events in autosomal cells and the daughter cells are absolutely identical to each other.

Germinal cell.

The germinal cells have a different cycle, at least at the end of the cycle, and mitosis in these cells is called meïosis (**Figure 5**). During meïosis* three important events occurs which all contribute to genetic heterogeneity and may play a role in the genesis of inherited cardiovascular diseases. (i) During the first meïotic division, the chromosomes segregate into the daughter cells randomly with regards to the parental origin. Thus, at least four different types of cells can be generated as shown Figure 5. (ii) During the first prophase homologous chromosomes align with each other lengthwise and makes up a sort of ribbon, called synapsis* so that each member of the pair consists of homologous chromatids. Subsequently, there are several cuts and ligations which result in an exchange, or crossing-over* of genetic material between homologous chromosomes. The point of attachment of the chromatids which participate in the crossing-over is the chiasma*. This is an important source of genetic variation. In addition, the size of the chromatids segments which have been exchanged determines the genetic distance* (see Chapter 4). The new chromosomes after the crossing-over are recombinants*. (ii) The second particularity of meïosis is that it includes two divisions, the first results in two cells which are again 2n. The second division is different and results into two haploïd (1n) cells.

Initially the pregametic cells contain paired chromosomes, one being from the father, and the other from the mother, and each chromosome is

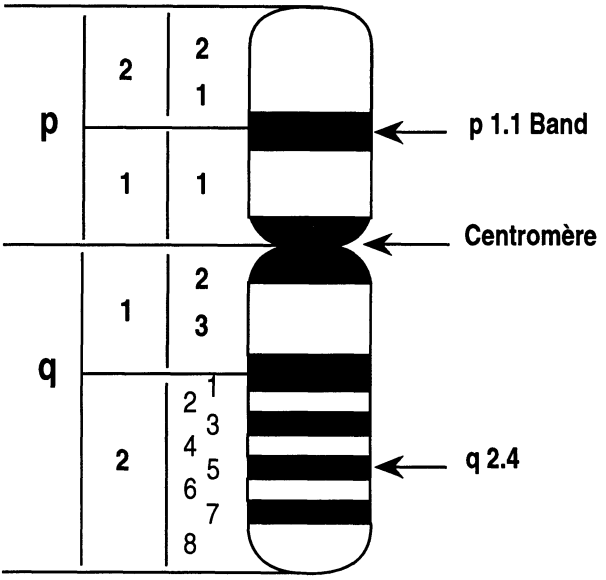

Fig. 3 Banding Pattern of a Chromosome

composed by two identical chromatids. Meïosis results in a mixture of genetic information coming from the two parents. The new cells obtained at the end of a meïosis differ from the initial cells, because during the first meïotic division there are both a random mixing of genetic material during the crossing-over and a random distribution of the parental chromosomes. Finally meïosis involves two separate cell divisions and yields four haploïd (1n) cells from one single diploïd (2n) cell. Thence we need conception and fusion of one male gamete, spermatozoïde, and one female gamete, ovule, to regenerate a diploïd cell, called a zygote, which is an autosomal cells ready for the development.

DNA Molecule.

Biochemistry.

The DNA molecule (**Figure 6**) is a dimer composed of a double strand arranged in α helix, the Watson's helix. Figure 6 shows 4 turns of the helix. It also shows the elementary unit of each strand, the nucleotide, a phosphosugar to which is attached a basic ring. The single strand is a chain of nucleotides linked together by covalent bonds between the phosphate groups and the sugars, the sugar-phosphate backbone. Four different bases are found in each DNA strand : 2 purines, Adenine, A, and Guanine, G, and 2 pyrimidines, Cytosine, C, and Thymine, T. Each base has a specific affinity for only one other base : G recognizes C and A recognizes T. The two strands are held together by non-covalent hydogen bonds between the bases on each strand. Since G forms hydrogen bonds only with C and A only with T, the nucleotide sequence within one strand can be predicted from the other strand which is its mirror image ; the 2 strands are complementary*.

Nomenclature.

The length of a DNA fragment has to be quantified. The unit of length of the DNA molecule is the nucleotide. DNA is double-stranded and the unit is the number of pairs of bases which compose the nucleotides : we say, for example, that we want to clone a DNA fragment of 56 base pairs*, or 56 bp, the number of pairs of nucleotides involved in hydrogen bonding within the fragment. When single-stranded DNA is used, the unit becomes the nucleotide : for example, if the above DNA fragment is dissociated by heat treatment for experimental purpose, and becomes single-stranded, its length becomes 56 nucleotides*. The human genome contains more than 2×10^9 bp.

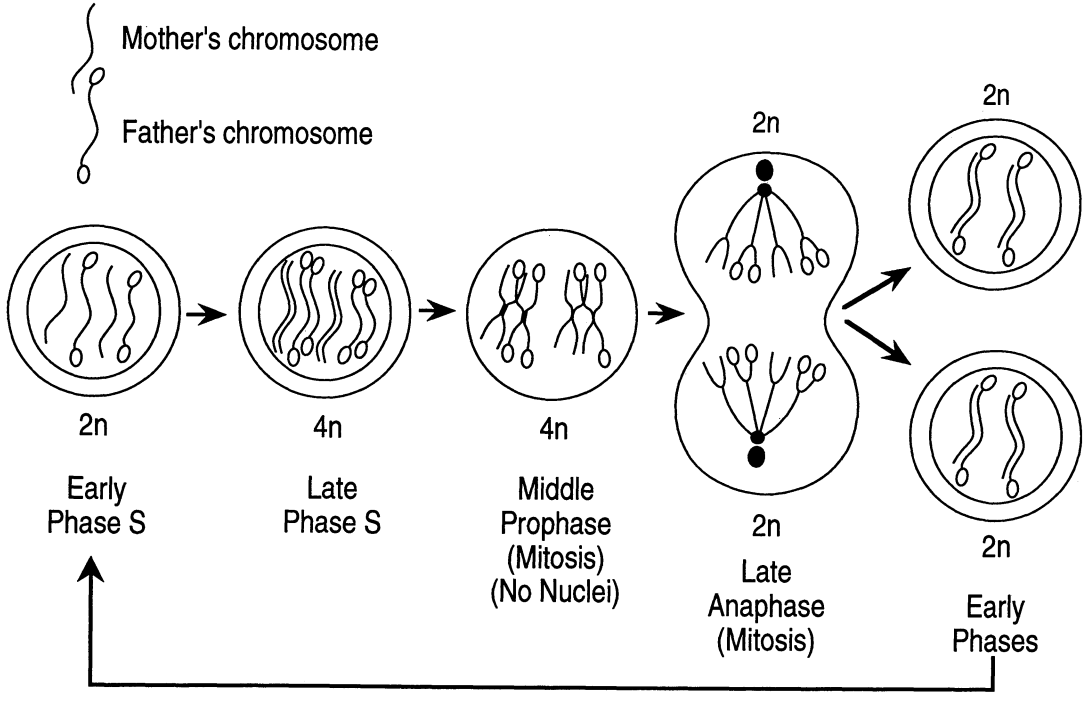

Fig. 4 Cell Cycle in Autosomal Cells : Mitosis.

The DNA strands are oriented and two frequently utilized symbols are 5' and 3'* which is quite a puzzling convention. Within the DNA chain, two hydroxyl positions on the sugar, 5' and 3', form phosphoester bonds with phosphate (Fig. 6, bottom, see also Figure 9), but at each end of the chain, only one of these 2 positions is free of phosphoric acid, the 5' or the 3'. The building block of DNA is a 5'-linked triphosphonucleotide molecule. Therefore the 5' phosphoester building blocks, the nucleotide triphosphates, are added to the 3' hydroxyl end of DNA to synthesize a new DNA molecule. The arrow on the DNA strands in the top of Figure 6 indicates the direction of nucleotide addition, from the phosphate-bound 5' to the phosphate-free 3'. Since nucleotides can be added only in one direction, the synthesis of the two strands occurs in opposite directions and the 5' end of one strand faces the 3' end of the other. Consequently, the two strands are antiparallel*. When describing genes, it is common to say, for example, that within Chromosome 4, gene A is located in 5' relative to gene B, or upstream of gene B, which conventionnally means on Figure 6 that gene A is on the left, in 5' on the sense* strand.

DNA duplication.

Duplication* (**Figure 7**) occurs during the S phase of the cell cycle. Such a process doubles the genetic information. Duplication is far from being perfect and is also very sensitive to chemical and physical aggressions, which means that duplication is intimately linked to the process of repair*. DNA replication is routinely mimicked *in vitro* by using a technique called Polymerase Chain Reaction, PCR* (see further "Current Technologies").

Duplication begins by a separation of the two DNA strands which have to be copied. This is an energy-consuming process in order to break the hydrogen bonds which bind the two strands together, and is made by a helicase. The process results in the formation of a replication fork. The two

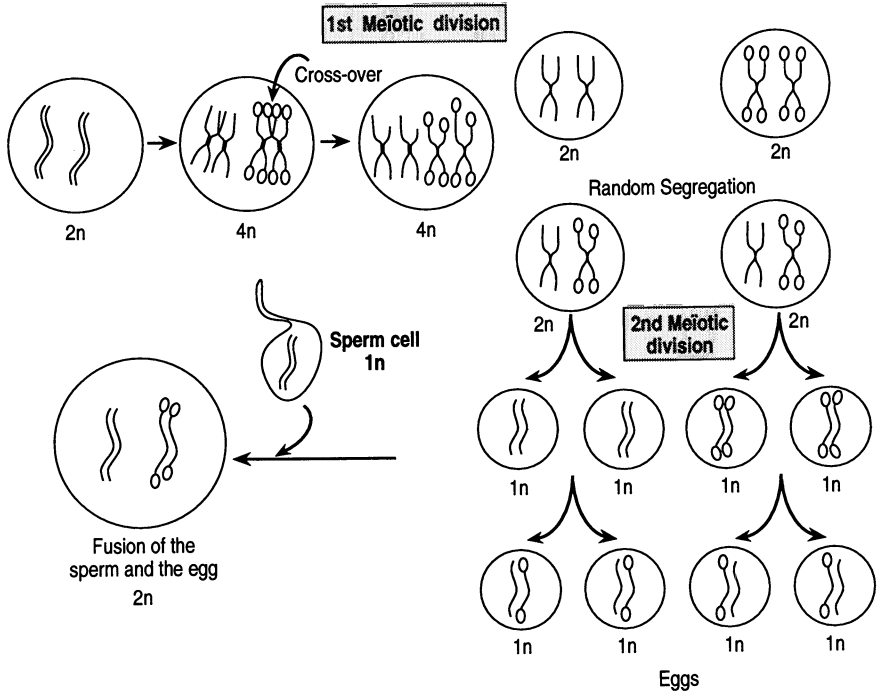

Fig. 5 Cell Cycle in Germinal Cells : Meïosis.

strands then have to be stabilized by Single Strand Binding proteins which forms a muff around the two strands. Replication needs to be started by a primer*, which is a small RNA sequence synthesized by a group of proteins called primosome which includes an RNA polymerase. The synthesis of the primer uses the preexisting DNA as a template and is complementary to the DNA sequence to which it binds, and is 3'5' to bind a 5'3' DNA sequence, or 5'3' to bind a 3'5' DNA sequence. The synthesis of the whole molecule of DNA is a continuous process on the non coding strand from 3' to 5'. On the other strand, from 5' to 3', the process is discontinuous and result in several segments called Okasaki's fragments. Reparative processes will correct and bind together the Okasaki's fragments. At the end of duplication a specfic RNAase will destroy the RNA primers.

Genes and the genetic code.

Gene structure.

The most important parts of the DNA molecule are the portions of the molecule which contribute to the synthesis of proteins and which are called genes*. Gene includes not only the sequences which code for the protein, the coding DNA, but also a large, and frequently unknown, amount of sequences which regulate the synthesis of proteins. Not all DNA codes for mRNA. The cell needs RNA molecules for other purposes, such as making ribosomes. Even DNA which codes for mRNA contains large segments which never appear in the cytoplasmic mRNA and finally most of the DNA never serves for RNA synthesis and is called silent DNA. A gene is composed of two segments : the regulatory and the coding DNA. In prokaryotes the coding DNA is unique and uninterrupted. In eukaryotes, the coding DNA is generally, although there are exceptions, located on several fragments of the gene called exons* (because they are exported to the cytoplasm) ; the DNA fragments located between the exons are termed

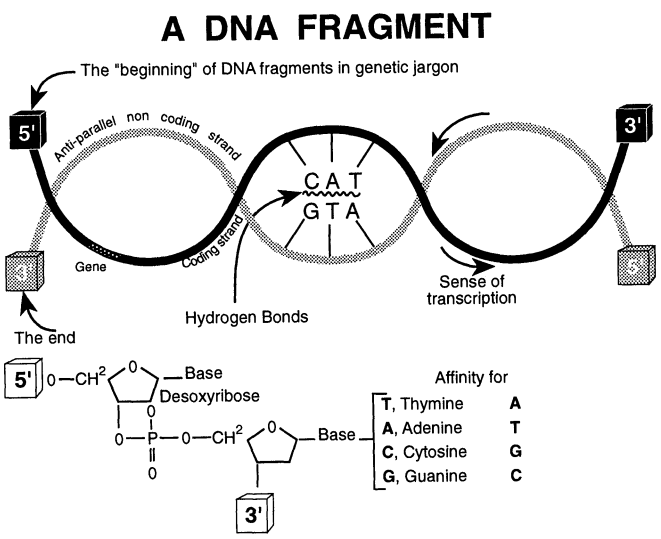

Fig. 6 From DNA to Nucleotide [Reproduced from Cardiovasc. Res. 1993, 27, 1414, with permission].

introns (because they stay inside the nucleus) (**Fig. 8**). Exons are in the same order as the amino acids in the proteins, nevertheless, as a rule, they do not correspond to a particular functional segment of the protein.

The attachment point of the RNA polymerase is a short DNA sequence called the promotor* which is located upstream of the transcription start site but is not sufficient to permit RNA synthesis. Other DNA sequences which recognize protein factors, the enhancers, are also needed.

Main types of genes.

During evolution meïotic divisions has frequently resulted in the multiplication of identical copies of a given gene, sometimes with a few differences which do not modify gene function, but also sometimes with major modifications which suppress the coding function of the gene and results in pseudogenes*. The actin gene for example exists as 50-70 copies with identical, or similar sequences or pseudogenes. The degree of homology* of two sequences indicates the % of nucleotides which are exactly at the same place on the two sequences. Nucleotide sequences are

said to be highly homologous when the degree of homology is between 75-100%.

In mammals, most of the genes are composed by exons of different length, which are separated from each other by introns, which also may have extremely different lengths. The number of exons is variable from one gene to another : the human gene coding for the Myosin Heavy Chain, MHC, isoform β (Molecular Weight, MW : 200,000) has 40 exons, by contrast the gene of the fast isoform of Troponin I, TNI, has only 8 exons. These two proteins are also good examples to show that exons and functional sites are not superimposable : on the β MHC the actin binding site is spread over 2 full exons (14 and 15) and a portion of two others (13 and 16), on TNI the binding site for Troponin C is spread over two exons (4 and 5), whereas the actin binding site, on the same molecule is entirely located on exon 7. Nevertheless there are also intronless genes, which are supposed to be genes of bacterial origin which have been incorporated into the mammalian genome during evolution and maintained as such because of an evolutionary advantage. This group of genes includes histones, and the genes encoding the adrenergic and muscarinic receptors.

It is now common to try to rearrange genes in to families or superfamilies, based upon both genetic structure and functional phenotype. A good example are the genes coding for nuclear hormonal receptors, i.e. progesterone, oestrogenes, gluco and mineralocorticoïdes and thyroxine receptors which have in common a specific N terminal sequence, DNA binding and ligand binding domains, and of course the fact that they are hormonal receptors. The so-called R7G receptor family includes the adrenergic and muscarinic genes receptors as well as the receptors for Angiotensin II. These genes are usually intronless, and possess 7 transmembrane hydrophobic domains and a G protein subunit binding site. They encode hormonal membrane receptors (see Ch. 2).

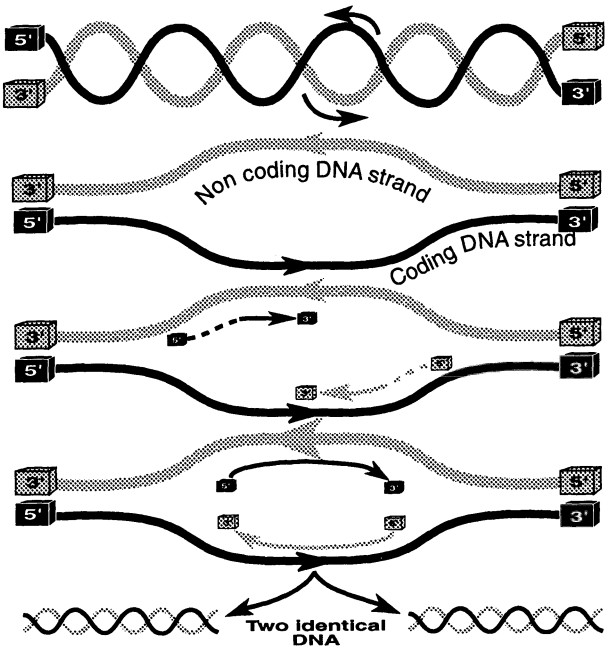

Fig. 7 DNA Replication
[Reproduced from Cardiovasc. Res. 1993, 27, 1414,with permission].

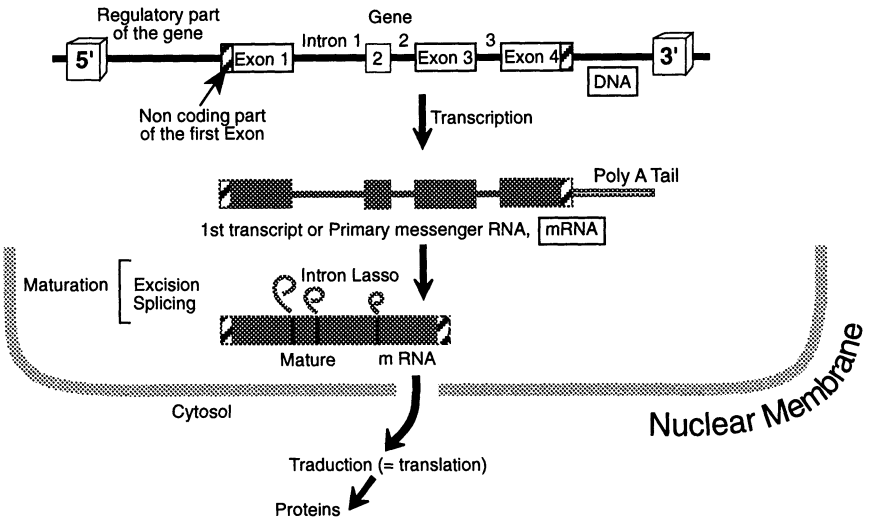

Fig. 8 From Gene to mRNA. RNA Maturation
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Isogenes.

Genes and proteins are not superimposable notions, and moreover there are genes which can encode several proteins. Isoforms are different proteins with the same function, isoenzymes are enzymes with the same function and slight differences in their activity and structure. Nevertheless the genes coding for a group of isoforms can be unique or multiple. Multigenic families include different genes, called isogenes, sometimes located in different chromosomes, coding for isoforms or isoenzymes.

In cardiovascular research a good example of this are the genes coding for the cardiac myosin heavy chain isoforms α and β. These genes are distinct and reflect differences both in the amino acid composition of the protein and the enzymatic activity. They are located on the same chromosome in an antithetic manner. The skeletal muscle embryonic, neonatal and fast isoforms are also coded by a series of genes located on a different chromosome. By contrast, there are genes such as those which encode for tropomyosin or the myosin light chainswhich are able to code for several different isoforms by the process of alternative splicing*. In this case one exon of the gene is common to all isoforms, and alternative splicing allows the gene to express only one or several exons specific for the given isoform.

Genetic Code.

In the coding DNA, a group of three consecutive nucleotides, the codon* or triplet*, corresponds to one amino acid ; for example the triplet CAU corresponds to histidine (**Table 1**). This is the well-known genetic code. There are 4 nucleotides, A, G, C and T, therefore there are 4^3 possible codons. There are 64 codons, but since there are only 20 amino acids, some amino acids, the most abundant, are specified by more than one codon.

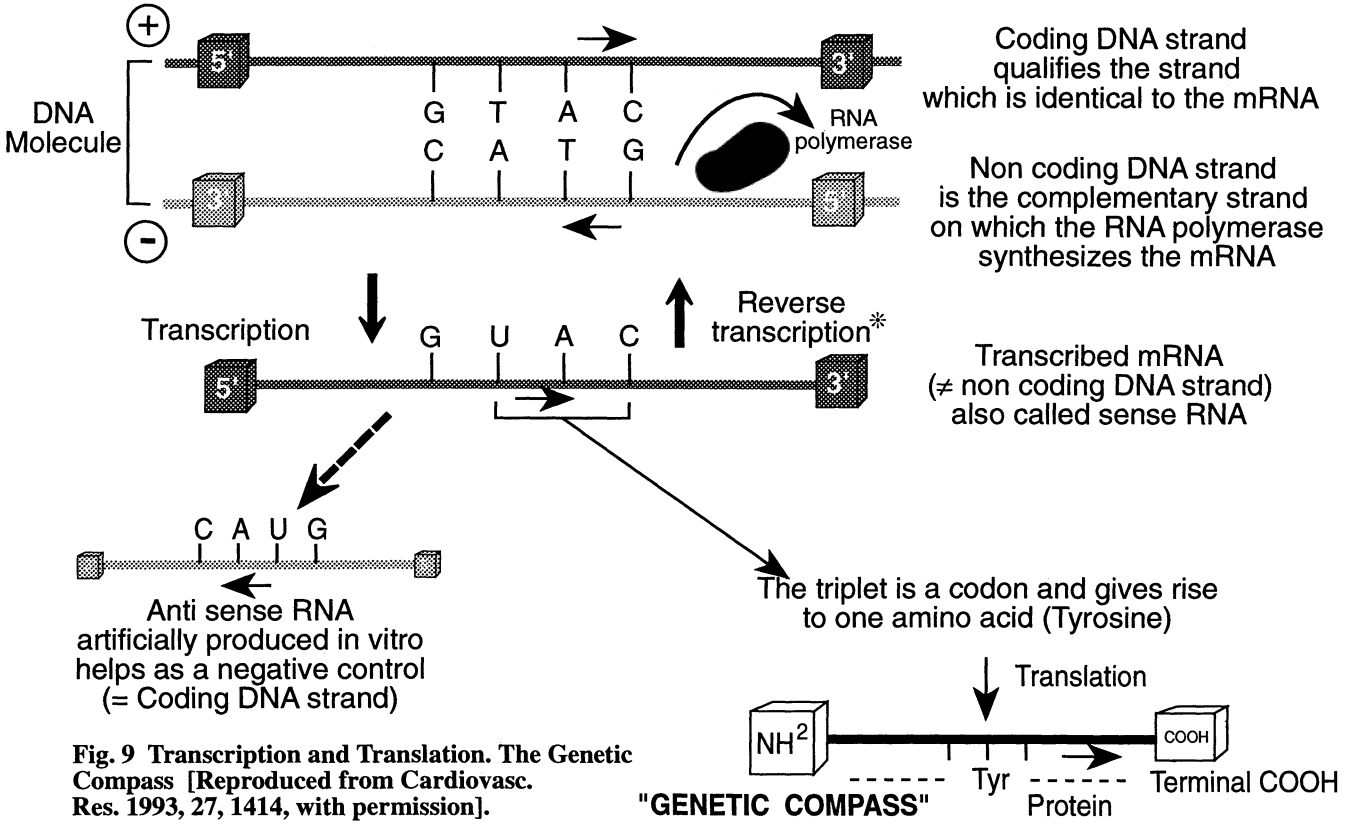


Fig. 9 Transcription and Translation. The Genetic Compass [Reproduced from Cardiovasc. Res. 1993, 27, 1414, with permission].

TABLE 1. The Genetic Code (Bases are in RNA bases, and U is read instead of T. The genetic code is lodated on DNA, and stricto sensu it would be preferable to use T, which is on the DNA).

Amino Acids.	**Corresponding Triplets.**
Methionine	AUG [Start Codon]
Tryptophane	UGG
Phenylalanine	UUU UUC
Histidine	CAU CAC
Glutamine	CAA CAG
Asparagine	AAU AAC
Lysine	AAA AAG
Aspartique Acid	GAU GAC
Glutamic Acid	GAA GAG
Cysteine	UGU UGC
Tyrosine	UAU UAC
Isoleucine	AUU AUC AUA
Valine	GUU GUA GUC GUG
Proline	CCU CCA CCC CCG
Threonine	ACU ACA ACC ACG
Alanine	GCU GCA GCC GCG
Glycine	GGU GGA GGC GGG
Serine	UCU UCA UCC UCG AGU AGC
Leucine	CUU CUA CUC CUG UUA UUG
Arginine	CGU CGA CGC CGG AGA AGG
[Codons Stops]:	UAA UAG UGA

Starting points and termination points are also specified by specific codons. The initiation triplet encodes for Methionine. Stop codons are silentious. The string of nucleotides in the DNA coding strand or in the mRNA determines the final aminoacid sequence in the protein.

Because the arrangement of the bases specifies the aminoacid arrangement, the sequence of the bases in the coding DNA strand is identical to the mRNA and predetermines or codes* for, the arrangement of the aminoacids in the protein, the coding DNA sequence being read from the 5' to the 3' direction, 5' specifies for the amino end of the protein and 3' for its carboxyl end (**Figure 9**). The other strand of DNA which is the mirror image of the coding strand is the non-coding or antisense strand. The RNA polymerase attaches at the 3' end of the non-coding strand of DNA, the antisense strand. It brings in and links together the nucleotides complementary to the strand to which it is attached, e.g. a C when G is on the DNA, an A when T is on the DNA. The new RNA molecule is transcribed from the non coding antisense DNA strand, but only complementary to this strand. It is identical to the coding DNA strand. In figure 9, the RNA polymerase has finished to transcribe and is consequently located on the 5' end of the non coding DNA strand.

In molecular biology, parallel or antiparallel either are verbs, or qualify a strand relative to another : mRNA parallels the coding DNA strand, the two DNA strands are antiparallel. DNA strands are named using several synonymous : the coding strand, is also called 5'-3', or sense strand, or antiparallel (to the other).

Transcription.

Transcription occurs on the non coding strand of DNA and is the synthesis of an RNA molecule which is identical to the coding strand of DNA. Transcription is just a copy. Reverse transcription is also a copy, and occurs in vivo, in cells infested with retrovirus. It is also a useful research tool,

TABLE 2. General and Ubiquitous Transcription Factors.

General Transcription Factors of RNA Polymerases II :

regulate Polymerase activity,

TFIIA, TFIIB, TFIIC.

Ubiquitous DNA-binding Transcription Factors :

- TATA Box-Binding Factor, TFIID, binds the TATA box which is a consensus DNA sequence,

- CCAAT-Binding Factors (CBF, CDP, YB-1, CP2 ....), bind the TATA box which is a consensus DNA sequence,

- GC box-Binding Factors, Sp1 and 2, bind the GGCGGG boxs (in the promotor region, closed to the TATA sequences).

---

since it allows one to synthesize routinely in the test tube DNA, called complementary DNA, cDNA*, from mRNA (Figure 9). Reverse transcription is routinely used to prepare DNA from mRNAs isolated from a given tissue in order to clone the gene of a tissue-specific protein. A good example of this was the cloning of the the gene coding for the Angiotensin I Converting Enzyme which was isolated from the mRNA of endothelial cells.

Transcription is a complex process and involves numerous steps : (i) binding of DNA-dependent RNA polymerases to the DNA and recognition of relevant sequences ; (ii) opening of the double stranded DNA ; (iii) binding of the substrates, i.e. the ribonucleotides ; (iv) initiation which consists of the formation of the first two phosphodiester bonds ; (v) elongation (both initiation and elongation also exist during translation into

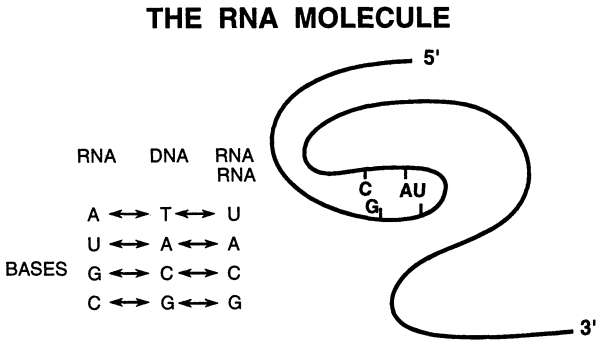


Fig. 10 RNA Molecule [Reproduced from Cardiovasc. Res. 1993, 27, 1414, with permission].

proteins) by further polymerization with ribonucleotides and movement along the DNA template which is the non coding strand of the double helix; (vi) release of the RNA chain. The activity of the RNA polymerases themselves depends on a group of transcription factors which are proteins (**Table 2**). Polymerases and transcription factors unwind the DNA molecule, maintain the transcription bubble constant during the making of RNA and separate the new nascent RNA molecule from the DNA. Other enzymes play a role including DNA topoisomerases which help to release the torsional stress imposed on the DNA strands, kinases, transferases and phosphorylases which are involved in rendering the chromatin accessible to transcription or to regulate transcription.

Biotechnology also one allows to produce in vitro the mirror image of the mRNA, the complementary RNA* or cRNA (Figure 9), also called the antisense RNA which is identical to the antisense DNA strand. Antisense RNA gene is a tool which has many uses, including transgenic technology, therapy, and, it has been proposed as a method to preventrestenosis after PTCA by transfecting the coronary endothelial cells with antisense mRNA specific for growth signals or growth factors.

RNA Molecule.

Structure.

The cell synthesizes three different types of RNA ! mRNA, messenger RNA*, which translates the genetic code into proteins, this is the least abundant rRNA, ribosomal* RNA, which is the most abundant, and tRNA, transfer RNA, which is the RNA which transports the amino acids to the site of the translation. A specific RNA polymerase corresponds to each of these different types of this RNA, is called Polymerase I for rRNA, II for mRNA and III for tRNA. RNA is synthesized in a 5' to 3' direction from one strand of DNA called template* DNA, RNA and its DNA template are antiparallel and have complementary nucleotides, but RNA and the DNA strand complementary to the template are parallel. In other words RNA has the same 5' to 3' orientation and the same nucleotide sequence as the 5'-3' antisense DNA strand (with one exception T does not exist in RNA and is replaced by U).

Figure 10 shows schematically the structure of a mRNA molecule. The sequence of nucleotides present in the transcribed RNA is almost identical to the DNA coding strand : U in the RNA molecule forms hydrogen bonds with A, and A with T, G with C, and C with G. Naturally occuring RNA is a single strand. But if, within the single strand, there are strings of bases which are complementary, the single-stranded RNA will loop on itself to form short length double stranded stems. These stems have important physiological consequences for the different RNA species present in the cell and may also represent a difficult problem when mRNA is manipulated, because the RNA strand can hybridize to itself.

Synthesis.

Figure 8 had shown the different steps involved in synthesis and maturation of mRNA. The coding strand of a gene is composed of exons and introns, introns are transcribed, but they are not coding, however some not all exons are coding. A portion of the first and last exons are non coding and their

ends are modified. The 5' end of the first exon is blocked by a 5' to 5' sugar-phosphate covalent bond called the RNA cap. To the last exon is attached a string of repetitive A, AAAAA, called the poly(A) tail, hence the other name of mRNA, poly(A)-rich RNA. This property is used both to quantitate and to isolate the total mRNAs. The poly(A) tail is thought to increase the efficiency at which mRNA is translated into proteins ; the mRNA cap is necessary for the initiation of protein synthesis.

By definition, introns do not code for a protein, and are not included in cytoplasmic mRNA. Nevertheless introns are transcribed into premRNA, also called nuclear mRNA. They need to be eliminated by excision*. In order to be excised, the introns form loops called lariats* which are degraded in the nucleus. The exons are then joined together by ligation. The two process of excision of introns and successive ligation of exons are called splicing*. Transcription into premRNA, splicing and attachment of the poly(A) tail represents the different steps of the mRNA maturation and constitute an important step of regulation control.

mRNA is finally transported to the cytoplasm where it is translated into protein on the ribosomal machinery via aminoacyl tRNAs.

Regulation of gene expression.

Gene expression [Wingender 1993] is regulated at the level of the regulatory part of the gene located upstream of the coding sequence of a gene (in 5'). This DNA segment is of variable length and the nucleotides are negatively named from the start codon, -240 bp means that we are located 240 bp upstream from the start codon. The regulatory part of the gene includes two different segments : one which is usually close to the first exon, and called the promotor*, and the other which is called the enhancer* and is upstream of the promotor. Nevertheless the spatial configuration of the gene is such that the enhancer can be close to the promotor and have functional connexions with it.

In **Figure 11**, two DNA consensus sequences are shown in the promotor region, the TATA box and the CAAT box which binds RNA polymerase through specific transcription factors. Both contain a consensus DNA sequence with invariant and variable nucleotides. The enhancers also have consensus sequences which are different for the different regulatory proteins called transcriptional factors : Glucocorticoid Responsive Element, GRE, which binds glucocorticoïd receptors, CRE, cAMP Responsive Element or TRE, Triiodothyronine responsive element, etc...

The regulation of gene transcription proceeds in two steps : the firts step is called transregulation* and consists in the synthesis of a protein, a transcriptional factor, usually on a different chromosome, but at the least by genes different from the regulated gene. Transregulation occurs when the transcriptional factor binds to the enhancer. Transregulation is the result of a protein/DNA interaction. Cis-regulation occurs on DNA, when the regulator is contiguous to the gene (**Figure 12**). Enhancers and the promotor are cis regulators.

Figure 13 summarizes what we know about the regulation of gene expression by hormones. There are two main classes of hormone receptors : transmembrane and nuclear receptors. When hormones such isoproterenol and insulin, bind to their transmembrane receptors, they generate a signal which is transduced into a second intracellular signal. The signal is cAMP for isoproterenol which binds the regulatory subunit of Protein Kinase A, this in turn phosphorylates a transcriptional factor which binds DNA at the

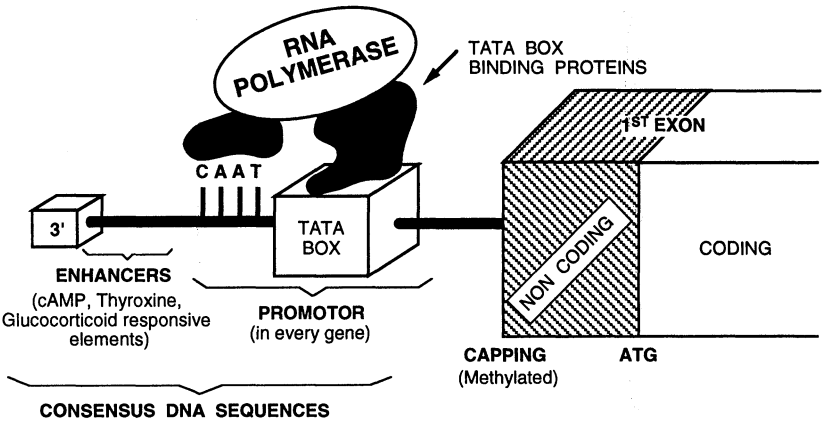
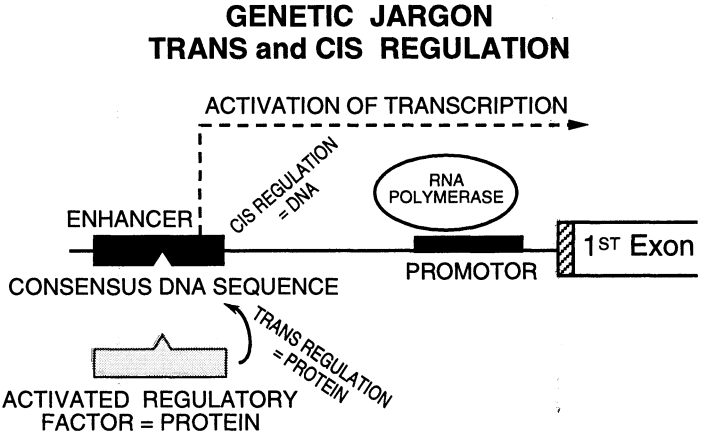


Fig. 11 . Schematic Representation of the Regulatory Part of a Gene [Reproduced from Cardiovasc. Res. 1993, 27, 1414, with permission].



The factor can be activated by a hormone, by phosphorylation, by cAMP, by dimerization, or by dissociation from an inhibitory protein.

Fig. 12 . Cis and Transregulation [Reproduced from Cardiovasc. Res. 1993, 27, 1414, with permission].

level of the cAMP Responsive Element. The signal is the activation of a cascade of kinases for insulin. The net result in both examples is the phosphorylation of enhancer binding proteins by specific kinases into the nucleus. These phosphorylated transacting factors bound to their respective responsible element are now able to activate the RNA polymerase already attached to the promotor. In either case the hormone transported into the cytoplasm forms a complex with its specific receptor and the complex bound to its responsive element in the nucleus, once again activates the promotor-bound polymerase.

The receptors which belong to the superfamily of the nuclear hormonal receptors bind to hydrophilic hormones such as thyroxine or aldosterone, and are directly activated by the hormone. Activated receptors can bind the corresponding DNA consensus sequence without any intermediary proteins. It is important to note that the knowledge of gene structure and particularly of the structure of the enhancer of a gene, can open new areas of investigations. Obviously the existence of a given consensus sequence, lets say a GRE, in the regulatory portion of a gene is not sufficient to attribute such a type of regulation to the gene expression, but is to stimulate physiological investigations in order to have the true demonstration of this type of activation.

An important issue in cardiovascular research is the study of both cardiac and vascular hypertrophy in arterial hypertension, the so-called myocardial remodeling. This problem has two different facets. Several plasma hormones, including aldosterone, angiotensin II, catecholamines have a trophic effect and indeed are likely to play a regulatory role during transcription. All these hormones have a correspondance at the DNA level. Conversely stretch, perhaps mechanical activity, activates growth, but, for the moment, there is no evidence for a stretch responsive element, and research had focused on intermediary steps, such as the phosphoinositol cycle.

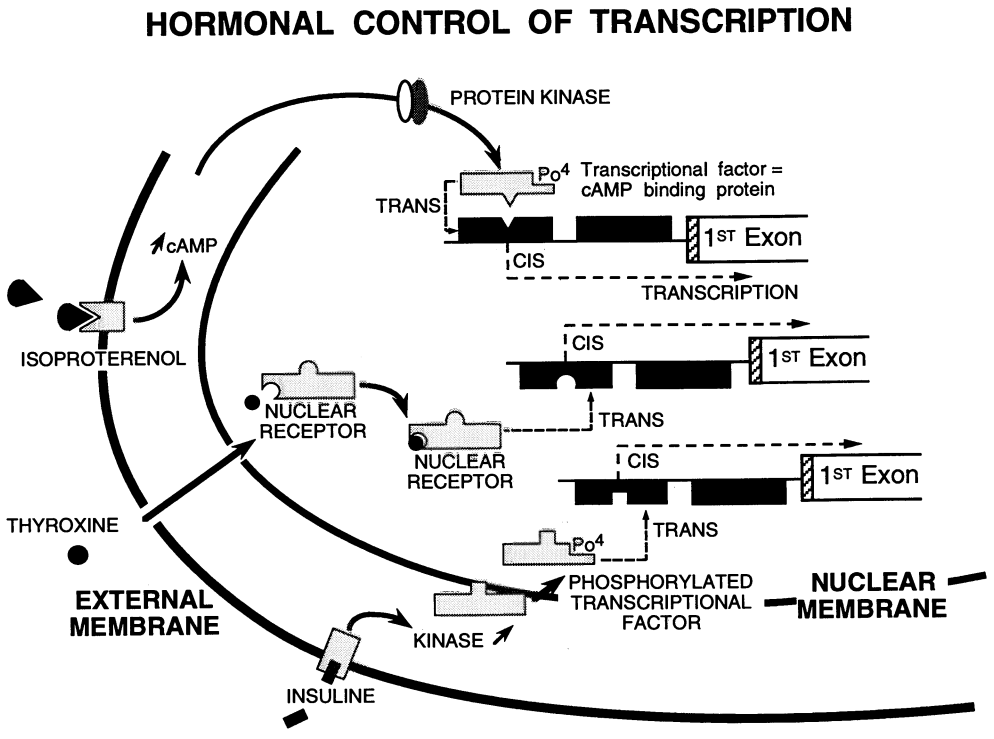


Fig. 13 The Three Modes of Control of Transcription by Hormones [Reproduced from Cardiovasc. Res. 1993, 27, 1414, with permission].

CURRENT TECHNOLOGIES.

Molecular biology offers a broad range of techniques which are largely reponsible for several misunderstandings occuring during the cross-talk between clinicians. The goal of this part of the chapter is to explain the general principles of a few techniques which we have found particularly useful. It is obviously impossible to provide an exhaustive list of all available methodology or to describe at great length the selected techniques. The reader may refer to several excellent laboratory manuals to find technical details related to this chapter [Sambrook et al 1989, Kriegler 1990].

Probes and Stringency.

Probes* (**Figure 14**) in molecular biology are nucleotide sequences, radioactively or non radioactively labelled, which are used for identification, mostly after specific hybridation with a complementary sequence. The most commonly used probes are cDNA probes, genomic probes and oligonucleotides. (i) cDNA represents only that part of DNA which is transcribed in a specific tissue. cDNA originates from reverse transcription of mature mRNAs (Figure 9). cDNA, as mRNA, varies from one tissue to another, during development or in disease states. cDNA is a copy of the exons which are mostly coding, but does also contain, at the beginning of the first exon and at the end of the last one, a non-coding sequence (Figure 8). (ii) In contrast, genomic DNA is isolated directly from the DNA of nuclei and is the same in every cell of an individual. The genomic DNA of a gene contains exons, introns, and the regulatory part of the gene located upstream the first exon. It contains all the hereditary information which makes this individual unique. (iii) Oligonucleotides* have been commercially synthesized. Their length is around 20-40

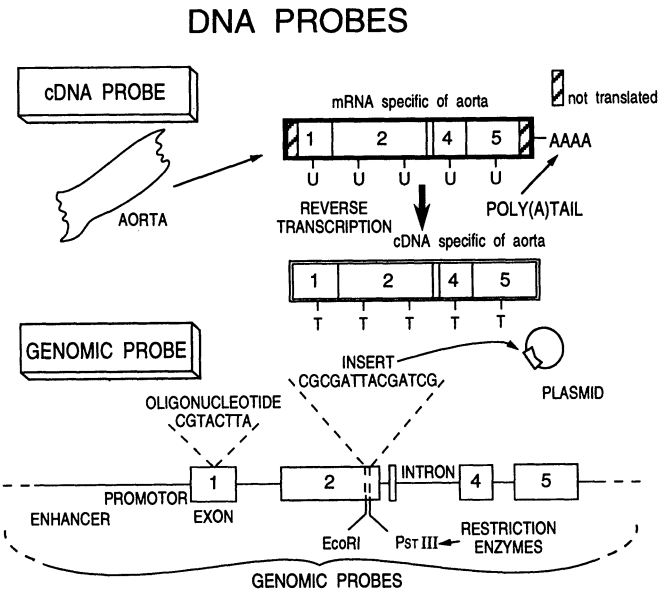


Fig. 14 DNA Probes in Molecular Biology

nucleotides. Usually they are complementary to a small portion of mRNA or DNA. The most commonly utilised primers are oligonucleotides.

Synthesis of radioactive probes.

There are now several techniques to synthesize radioactive cDNA probes. Commercial kits are available for all of them. The principles of some of these techniques will be given because it allows the reader to become more familiar with the language.

The nick translation technique consists of first digesting away a few nucleotides with a DNase (which destroys DNA), then replacing the missing nucleotides with their radioactive homologs in the presence of DNA polymerase (which synthesizes DNA) and the radiolabelled DNA building blocks. The result is a radioactive double-stranded cDNA probe in fragments. There is no net DNA synthesis and the amount of DNA present in the test tube is the same at the beginning and at the end of the reaction. This mode of preparation is still the method of choice when the DNA

fragment of interest used to make the probe is large because the small fragments obtained at the end can diffuse more rapidly to hybridize to mRNA. Nevertheless the nick-translated probe is not very radioactive and good results can only be obtained with abundant RNA (or DNA).

Random priming consists of randomly annealing small DNA primers to denaturated strands. Then DNA polymerase elongates the primers by copying the template in the presence of radioactive nucleotides. There is net DNA synthesis and the amount of DNA present in the test tube at the end of the reaction is twice that present at the beginning. The probe is much more radioactive than after nick-translation.

Both previous techniques yield radioactive coding and non-coding strands, and it is necessary to select conditions which favour RNA-DNA hybridization over DNA-DNA reannealing to get a stable RNA-DNA hybrid. Another technique avoids this problem and consists of initiating DNA synthesis by a specific antisense primer using the antisense DNA as a template (see Fig. 9). At the end, one has only one antisense strand which is radiolabeled. Single-stranded probes are required for the quantitation of mRNA by the S1 protection assay. Radioactive oligonucleotides are prepared using specific enzymes as the polynucleotide kinase or the terminal transferase will add a few deoxynucleotides at the 3' end.

Stringency.

A large part of biotechnologies consists of making DNA-DNA or DNA-RNA hybrids. The unknown DNA or RNA sequence is subsequently identified thanks to the radioactive DNA (or sometimes RNA) probe. The two hybrids are bund by a Hydrogen bond which is stable in given conditions of temperature and ionic strength. For example, the two DNA strands can be separated by heating, and this is a well-known tool to prepare

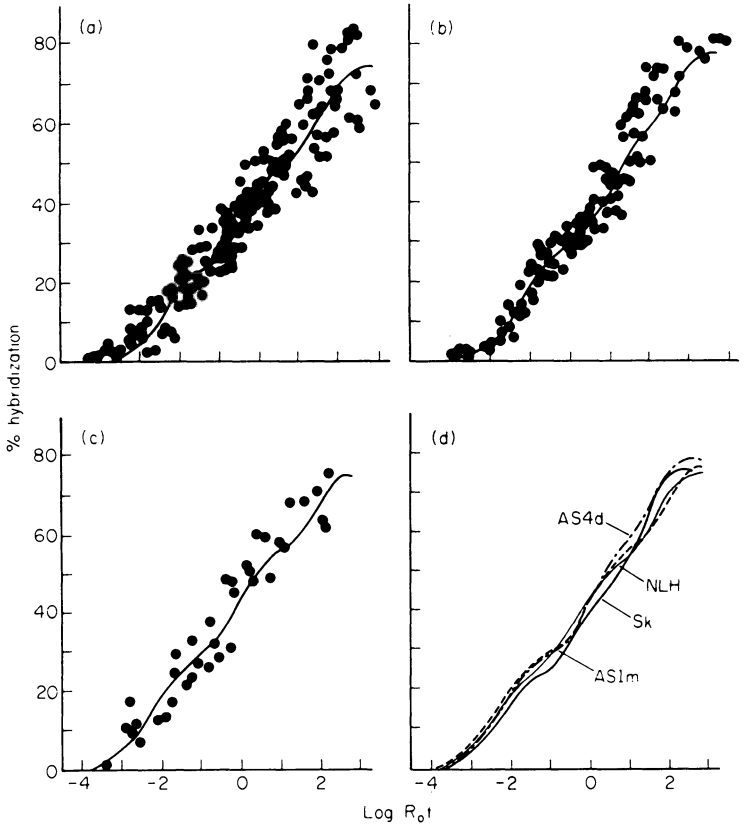


Figure 15. Curves for the hybridization of cDNA Hybridizations from normal rat ventricle to various poly(A)-containing RNAs (Rot1/2): normal (a, NLH) and overloaded rat heart 4 days (b, AS4d) and 1month (c, AS1m) after aortic banding [from de la Bastie et al. 1987, with permission].

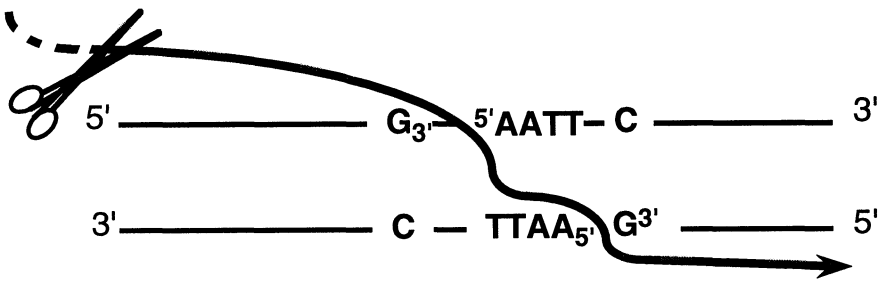
single stranded DNA. The two DNA strands are fully separated at a given temperature, and the intermediary temperature is called Tm, which is a reproducible physical characteristics of a given DNA molecule. The lower the ionic strength, the lower the Tm. Stringency is the product of temperature and ionic strength, and a solution with high stringency is a solution with a low ionic strength and/or high temperature. Every probe hybridizes to its complementary sequences in given stringency conditions, and the determination of the most appropriate conditions of stringency is the first step to allow the quantification of a nucleotide sequence. The determination is quite empirical and may need time.

Hybridization also depends on the time and the concentration of the two molecules to be hybridized, and during the analysis of hybridization in solution it is common to consider these two parameters together using the Concentration, Co, x Time, t1/2,
product, called Cot1/2 when we are dealing with a DNA/DNA hybrid and Rot1/2 when the hybrid is made from RNA and DNA.

An interesting application of this technique was made years ago in cardiovascular research and is illustrated **Figure 15**. In these rather ancient experiments, an attempt was made, unsuccessfully [Swynghedauw et al. 1984, de la Bastie et al. 1987], to detect heterogeneity between three populations of mRNA isolated respectively from normal rat heart and from hearts after an abdominal aortic stenosis. In d the three hybridization curves were superimposed together with a curve obtained with mRNA from skeletal muscle. Computer analysis of such a curve showed that the data could be described by division into three components corresponding to abundant (in the negative range of Log Rot1/2), medium and rare mRNAs. The curves were obviously superimposable, and, at that time, the conclusion was that there were no real differences in mRNA complexity between normal and hypertrophied heart. Latter it was shown that there are in fact differences but

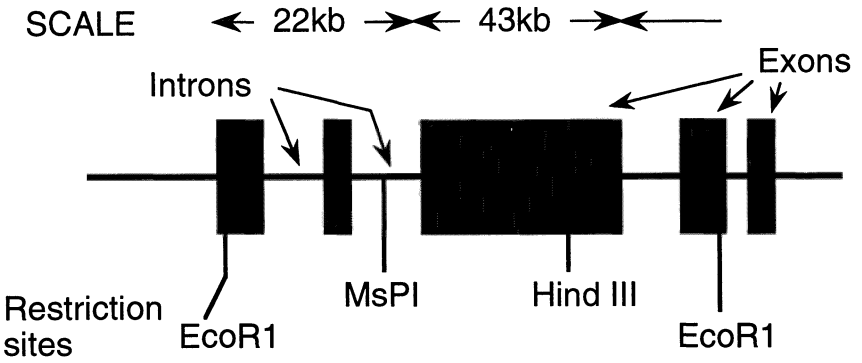
RESTRICTION ENZYME

Eco R1 = 1ST RESTRICTION
ENZYME FOUND IN ESCHERICHIA COLI Ry13



Cut palindromic sequences on the two DNA strands

RESTRICTION SITES



Conventional representation of a gene

Fig. 16 Restriction Enzymes and Restriction sites
[Reproduced from Swynghedauw B., Cardiovasc. Res. 1993, 27, 1566].

only at the level of isoenzyme distribution which cannot be detected by this rather crude technique (see Ch. 3).

Main Enzymes Used in Molecular Biology.

Restriction enzymes

Restriction enzymes* are endonucleases (**Table 3**) which were discovered in bacteria by a Noble prize winner and have the property to cleave sequences present in the double-stranded DNA (**Figure16**).

Table 3. Main Enzymes utilised in Molecular Biology.

Enzymes.	**Biological activity & current utilisation.**
Restriction Enzymes :	endonuclease, to fractionate DNA.
Reverse Transcriptase :	transcriptase & RNAase H, cDNA bank.
DNA Polymerase I :	polymerase & exonuclease, probe labeling.
Taq Polymerases (T7) :	polymerase, to synthesize a whole RNA
Ligases :	DNA or RNA Ligases, to link 3'-5' ends.
S1 Nuclease :	single strand nuclease, to quantitate mRNA.
RNAase A :	thermoresistant, to digest single stranded RNA.

Both strands are cut, sometimes in a staggered manner to give "sticky ends", sometimes at the same nucleotide on both strands to give "blunt ends", as for Alu I. Most of the restriction enzymes cleaves palindromic* sequences on the two DNA strands at a very specific site in terms of nucleotides, such as Eco R1 on Figure 18, nevertheless there are also restriction enzymes capable of cleaving sequences which are only partially defined, such as Nsp II. The DNA sequences which can be cleaved by a restriction enzyme are called restriction sites. There are several hundreds of these enzymes and their nomenclature is based on the bacteria

from which the enzyme has been isolated ; Eco R1 was isolated from the **R**Y13 strain of **E**scherichia **co**li. Most of the restriction enzymes are specific for a given sequence, so they are tools to identify DNA sequences and to provide a rapid idea of the nucleotide sequence. Figure 18 shows the conventional representation of a nucleotide sequence such as a gene that is seen in every article describing a new gene. It includes a scale in base pairs, the length and position of both exons (always represented as boxes) and introns (represented as thin lines) and the position of the restriction sites. Obviously the more numerous the sites the more precise the definition, and it has empirically been shown that a gene sequence can be identified with enough accuracy when 2-3 restriction sites are indicated.

Restriction maps.

Restriction maps* (**Figure 17**) result from the electrophoresis of the fragments obtained after digesting of a given DNA sequence with one or several restriction enzymes. Following digestion, the fragments are separated by electrophoresis according to their molecular weights and can be identified provided we can "stain" them. Non specific staining using BET, for example, a fluorescent (and toxic) chemical which binds every DNA fragments whatever their composition in nucleotides, may show a broad smear if applied, for example, to a complex mixture of genomic DNA and is useless. To obtain a real map, we need a second component, which is usually a radioactive single-straded probe, specific for a given region of the DNA molecule, for example a known, well-identified, portion of a gene. Then the probe allows a geographical localisation of the DNA fragments, by specific hybridization to a given fragment which has previously been separated by electrophoresis. It then becomes possible to analyze the fragments generated by the restriction enzymes and obtain a sort of finger print of the gene. Restriction mapping is, for example, routinely used to control probes or clones* and to avoid errors of identification. As shown in Chapter 4 it is also a very effective way to identify DNA polymorphism*.

Polymerase Chain Reaction (PCR).

This technique is based upon the principle of DNA duplication (Figure 7). As stated previously, DNA replication needs a complete dissociation of the two DNA strands as a prerequisite and also the synthesis of two primers which hybridize specifically to a given segment of the two strands and limit the portion of DNA to be duplicated. *In vivo* there are enzymes which denature the DNA and synthesize the primers. Once a primer is provided, DNA polymerase may synthetize a coding DNA strand by using the DNA non coding strand as a guideline, and vice-versa. The result is two identical molecules of DNA.

It is possible to reproduce this process *in vitro* by using heat to dissociate the two DNA strands and by synthesizing oligonucleotides (around 20 nucleotide length) to make primers complementary to the two extremities of the DNA sequence of interest [Becker-André 1989, Wang et al. 1989]. It is therefore possible to repeat such a duplication process nearly indefinitely until we have amplified the sequence in such quantities that we can see the newly made DNA by electrophoresis. We have to add to the mixture the appropriate nucleotides and DNA polymerase. Nevertheless the repetition of such an amplification needs several cycles of heating which destroys the DNA polymerase, and the amplification procedure was impossible until the discovery of Thermophilus **aq**uaticus, **Taq,** and its heat-resistant DNA polymerase. The Taq polymerase is resistant to the repeated heating which is necessary to redissociate the DNA strands. The PCR can usually repeat the operation 20-30 times and then amplify a DNA, or RNA strand 100, 1000 and even 10^6 times.

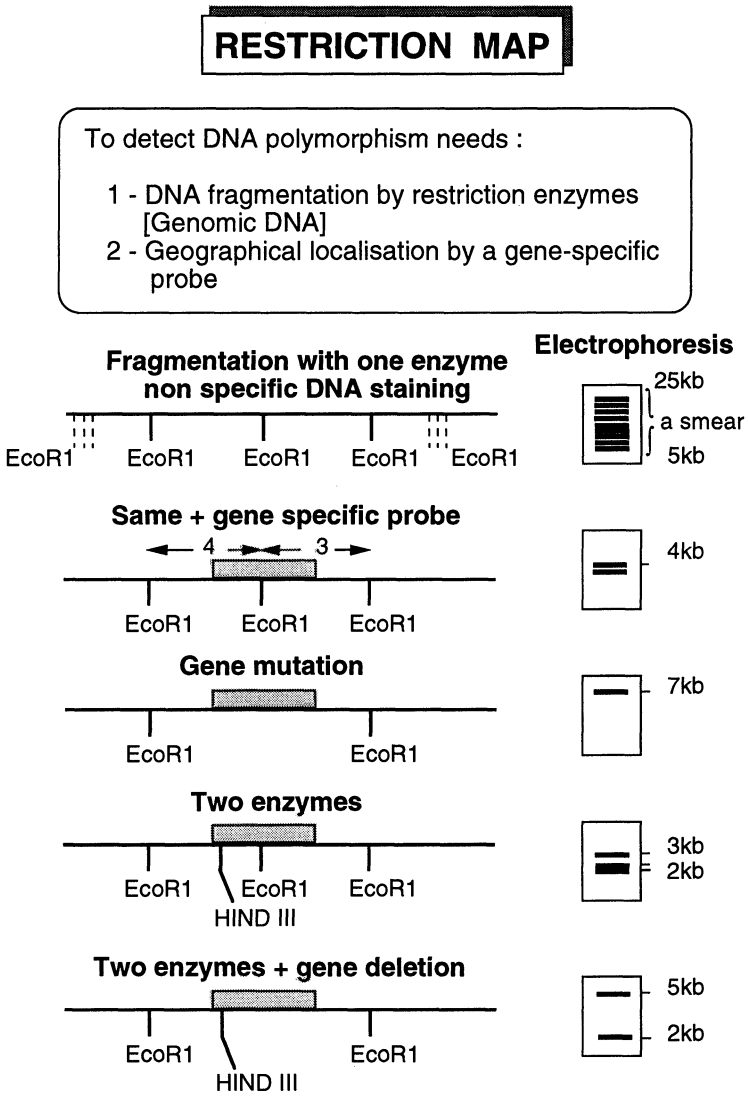


Fig. 17 Restriction Maps [Reproduced from Cardiovasc. Res. 1993, 27, 1566].

PCR is performed in a small inexpensive apparatus and can be programmed to produce cycles of temperature which can be repeated numerous times : 95°C which dissociates the two DNA strands, 55°C which allows the primers to hybridize to each DNA strand, and finally 72°C, the temperature at which the polymerase synthesizes DNA, then back to the beginning of the cycle (**Figure 18**).

When mRNA is to be amplified, it has to be transformed into a single stranded DNA by using reverse transcriptase (Figure 9).

The primers are either 5'-3' and will anneal to the 3' end of the non-coding strand, or **3'-5'**. Only the portion of the sequence which is contained between the two primers will be amplified. During the first cycle, the synthesis of the DNA strands is not limited in length and will go as far as the polymerase chooses to go on the DNA template (Figure 18). However in subsequent cycles, the length of the new strands is limited to the region of DNA recognized by the primers, since after the first cycle strands will stop where the primer has started in the previous cycle.

The amplification procedure will produce a large amount of a single DNA fragment which can be quantified on a gel. One of the problems to obtain quantitative results is that the number of amplication cycles is always unknown, mainly because some cycles remain unfinished. Therefore it is necessary to coamplify an internal probes to really obtain reproducible and quantitative results.

This technique is now a major tool for routine molecular biology and is at the origin of many of the major recent progresses which have been made in genetics. It has many applications including mRNA quantification, even on histological slices, detection of repetitive DNA sequences, synthesis of various DNA fragments in detectable quantities, etc.... PCR has been extensively applied in cardiovascular research. For example, it is now possible to extract total RNA from 3-5 mg samples of human heart

and to reverse-transcribe this RNA into cDNA [Feldman et al 1991]. The internal standard control RNA is produced from a synthetic DNA that contains two pairs of primers complementary to the those used to amplify the two cDNAs of interest. By using appropriate primers, it is possible to quantify the relative levels of mRNAs present in the human heart biopsy samples. Normal human ventricle does not contain the Atrial Natriuretic Factor mRNA, whereas in the failing ventricle this message is abundant, as a compensatory mechanism. The failing human heart contains more Phospholamban (a cofactor of the Ca^{2+}-ATPase of the Sarcoplasmic Reticulum, that plays an important role during relaxation) than normal hearts ($7.5 \times 10^8 \pm 1$ and $1.1 \times 10^8 \pm 0.4$ molecules of mRNA/mg total RNA, $p<0.0001$).

Working with RNA

Quantifying mRNA in cardiology.

Obviously mRNAs, also called transcripts*, are not protein, they don't possess any functional activity except making proteins,
nevertheless their quantification provides information of crucial importance both in cardiovascular physiology and pharmacology.

(i) The techniques used to quantitate mRNAs, including slot blot analysis and quantitative PCR, are often easy and rapid, and it is possible to quantify several messengers at the same time. This has to be compared with the classical biochemistry, or kinetic analyses, which are time consuming and need weeks or months of pilot and/or preliminary experiments before being efficient. A good example comes from studies on the Na^+, K^+ ATPase, the sodium pump which maintains cell depolarization and binds ouabain. During mechanical overload of the heart, the expression of the gene coding for the a subunit of this enzyme is modified, resulting in modifications of the enzymatic activity and pharmacological properties. To

demonstrate that the enzymatic function and sensitivity to ouabain were changed required 4 years [Lelièvre et al. 1986, Chevalier et al. 1989], while the molecular biological results were completed within 6 months [Charlemagne et al 1994] (**Fig. 19**).

(ii) During physiological steady states, such as senescence [Besse et al 1993] or after chronic administration of a drug, such as a Converting Enzyme Inhibitor [Callens-El Amrani 1989], mRNA levels are generally correlate with the corresponding protein concentration, so that RNA quantitation may be equated with protein content. However one should be aware that such a relationships is far from being the rule. There are a lot of exceptions, for example collagen which is certainly not fully transcriptionnally regulated in the heart [Besse et al. 1993].

(iii) Rapid biological transitions, such as those obtained after acute mechanical overload [Bauters et al.1988, Delcayre et al. 1992] or hormonal injection, or acute administration of a drug, represent a major concern in cardiology. mRNA levels may be altered long before there is any detectable change in the corresponding proteins, and it is important to know that transcripts may change without any translation into proteins [Snoeckx et al. 1991].

(iv) The routine strategy to identify the level of regulation of a given modification in protein level (Figure 8), includes the determination of the corresponding mRNA level. Nevertheless it is important to know that, by so doing, we are not measuring transcription. An increased mRNA level is frequently the result of increased gene transcription, but it may also result from modifications occuring at any steps of mRNA maturation in the nucleus. For example, during chronic cardiac overload in rats a shift occurs in the different myosin isoforms [Lompré et al. 1979, see Ch. 3]. It has been shown that this shift is preceeded by a change in the corresponding mRNAs, the change in mRNAs occurs roughly 2-3 days before that of the

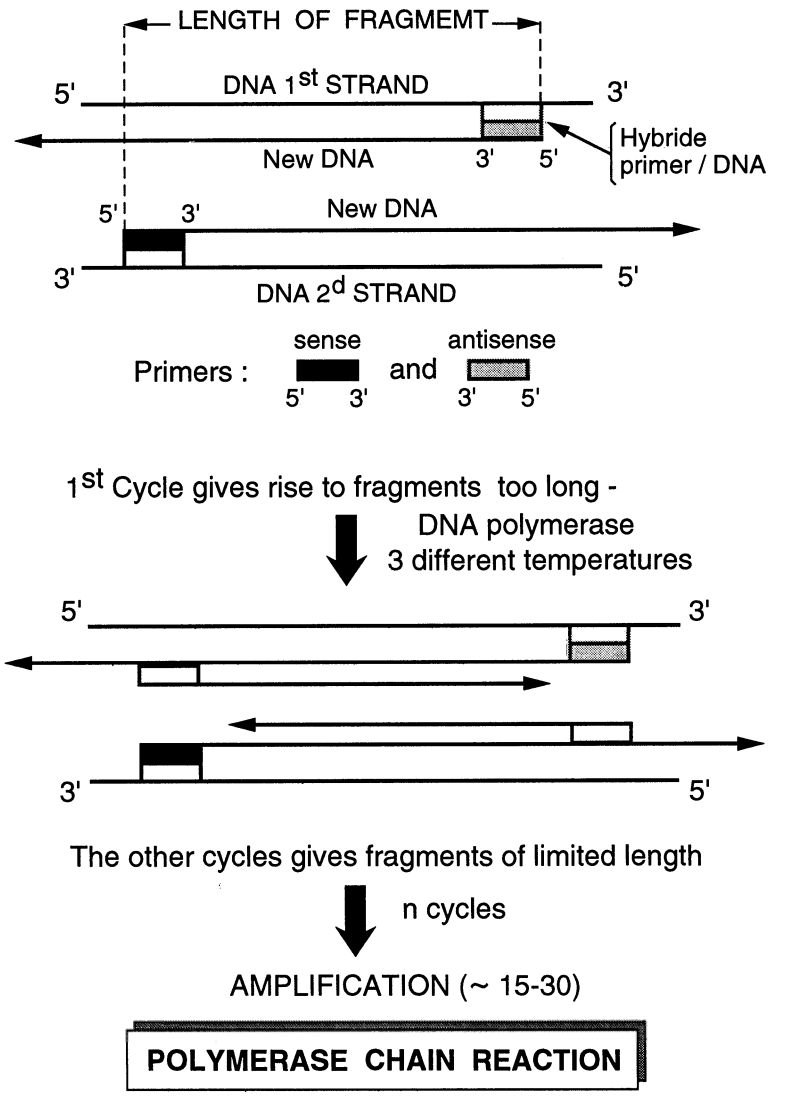
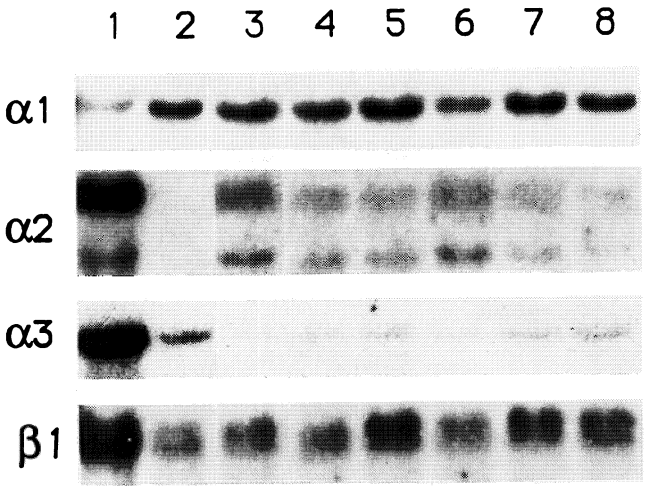


Fig. 18 Polymerase Chain Reaction (PCR) [Reproduced from Cardiovasc. Res., 1993, 27, 1566, with permission].



19. Northern Blot analysis of Na, K-ATPase α isoforms and β1 subunit mRNAs in hypertrophied rat heart. 1-8 indicates lanes. After electrophoresis the different isosubunits have been identified by hybridation with a specific radioactive probe using autoradiography. Each spot has been cut and rearranged to show the various isoforms on the same picture. Lanes 1 & 2: neonatal brain and heart. 3 & 6 : Left ventricle of a normal rat. 4, 5, 7 & 8 : hypertrophied LV (aortic stenosis) [reproduced from Charlemagne et al. 1994, with permission].

proteins [Lompré et al. 1979] which allows one to conclude that the regulation is pretranslational and that the chicken is the mRNA and the egg is the protein. By contrast, the myocardial collagen mRNA concentration decreases while the protein content increases [Besse et al. 1994].

The role of transcription can only be determined by using other techniques such as transcriptional run-on which consists of studying transcription directly on isolated nuclei, and allows one to know if the

increased mRNA level is also accompanied by a parallel augmentation of premRNAs (those that contain transcribed introns, Figure 8). By so doing, it has, for example, been demonstrated that the cardiac myosin level is mainly regulated at the transcriptional level [Boheler et al 1992].

How to prepare and handle RNA.

A major problem in working with RNA is RNase contamination. Most RNases are heat-stable and are present everywhere including on the fingers of investigators. In the cardiovascular system, RNAases start to destroy tissue RNA soon after death, and renders post-mortem tissue determination of mRNA impossible. This is the reason why biopsy samples taken during open-heart surgery, transplantation or catheterism have to be rapidly stored in liquid nitrogen. Nevertheless, working on RNA is possible in nearly any cardiovascular laboratory providing a minimum of care (disposable gloves, heat-baked glassware, etc...) is taken.

mRNA needs to be quantified total RNA that is protein- and DNA-free. This is done on fresh or deep-frozen tissues. One of the most popular techniques in cardiovascular research is based on rapid homogenization in a chaotropic agent to inactivate RNases, followed by an extraction in acidic phenol/chloroform to remove proteins and DNA, precipitation of the RNA by alcohol, solubilization of the remaining double-stranded nucleic acids but not single-stranded ribosomal and mRNA in high salt solutions and several cycles of solubilization-reprecipitation in ethanol [Chomczynski et al 1987].

It may be necessary to isolate pure mRNAs, for example to quantify rare transcripts. Commercial kits are available. They are based on the principle of affinity chromatography and exploit a structural property specific to mRNAs, i.e. a poly(A) tail at the 3' end of mRNA (Figure 8). Such a tail can hybridize to complementary bases, Uridine or Thymidine, in high ionic strength, and dehybridize at low ionic strength. A restriction to this technique is that there are mRNAs which have no poly(A) tail.

The yield of total RNA is approximately 1 mg of total RNA per mg of fresh cardiac tissue. It is less in elastic arteries which contain much more connective tissue and fewer cells. Affinity chromatography allows the preparation of mRNA that is 95% pure.

Quantification of abundant mRNAs.

The quantitation of mRNAs is based on the hybridization of a single mRNA species (there are about 10,000 different mRNA species and 10^6 RNA molecules in any given cell) to a specific radioactive probe, and then the identification and quantification of the hybrid by electrophoresis followed by autoradiography.

The most commonly used techniques to quantitate nucleic acids, including DNA and RNA, are called Southern* and Northern* blot analysis. Southern blot was historically the first technique and was developed by P. Southern to analyse DNA cut into fragments of manageable size. "Northern" was intended as a pun and now refers specifically to mRNA analysis. DNA fragments or mRNAs are fractionated by electrophoresis, transfered to a nitrocellulose or nylon membrane by capillarity and then exposed to a specific radioactive probe. The probe will hybridize to the homologous DNA fragment or mRNA ; unhybridized probe is then removed by washing with buffers of increasing stringencies (see above); finally the radioactive hybrid is identified by autoradiography and quantified by scanning (Fig. 19). When the appropriate conditions of stringency are well determined, it is possible to apply total RNA directly onto a membrane without electrophoresis. This so-called slot or dot blot analysis is rapid and allows an absolute quantitation of a given mRNA by depositing known amounts of sense RNA as a standard directly to the membrane. The sense RNA is prepared by transcribing the sequence of interest previously inserted into a special plasmid which contains two different promotors one on each side of the cDNA insert. It thus permits the transcription of both antisense RNA which is a negative control and sense RNA (see Figure 9).

RNA has to be quantified relative to something, and results have usually been expressed as a relative amount of a given mRNA to either a fraction of ribosomal RNA which has been probed using a radioactive probe specific for, for example, the 18 S ribosomal RNA, or the total poly(A) containing sequences which represent the total mRNAs, or another mRNA which is known to be unaffected by the experimental procedure.

To study shifts in isogene expression or to analyse the expression of isogenes or genes that belong to the same family, needs special care since these nucleotide sequences are highly conserved and homologous. When it is impossible to obtain a specific probe for each isoform, one needs to use other techniques such as S1 nuclease or RNase protection assays (see below), or PCR. In the latter case it is necessary to know the sequence of the various isoforms, and necessary to ask to a special computer programme where are the sequences specific for the isoform and where are the sequences that the various isoforms have in common.

Quantification of rare mRNAs.

Membrane proteins, receptors, regulatory peptides all belong to this group of mRNAs. There are several methods which can been used to study these mRNAs. The mRNA concentration can be increased, as previously explained. It is also possible to enhance the specific radioactivity of the probes by using several different radioactive nucleotides or by using more tissue. PCR again may be used if we are dealing with very small samples and if it is possible to make a reference probe. Another frequently used technique is RNase protection.

S1 Nuclease assay and the RNase protection assay consists of making a DNA-RNA hybrid for the first technique or an RNA-RNA hybrid for the RNase protection and to hydrolyze the single strands of DNA or RNA with S1 nuclease or RNases. One uses large quantities of total RNA (100-200 mg which represent 100-200 mg of myocardium for example) in a dilute solution. The S1 nuclease or RNases will digest all mismatched

hybrids which have single-stranded regions. The probe used for S1 nuclease is a complementary cDNA probe, the same than that used for a Northern blot. RNases protection utilizes an antisense-RNA transcribed in the test-tube, as above. These techniques can be recommended for detection of isoforms, or homologous sequences, since both S1 nuclease and the RNases are very sensitive tools and will digest mismatched hybrids even if the single strand non hybridized regions is one nucleotide length. One must be aware however that these two techniques are rather time consuming.

Working with DNA.

Working with DNA is much easier since environmental DNases are less abundant and active than RNases.

cDNA amplification

This is certainly the first technique that should be learnt by people who want to start molecular biology even at an embryonic level. The technique uses bacteria as a tool (**Figure 20**). DNA amplification is an absolute must, and is the technique required to make the probe of interest in a sufficient amount for making experiments on a large scale. It is so simple that any physiologist will learn it quickly.

The fragment of interest that has to be amplified is introduced into a plasmid and is called an insert. The plasmid into which an insert has been introduced is now called a recombinant plasmid*. The plasmid is commercially available and is a piece of bacterial DNA which behaves like an independent minichromosome. It contains a gene confering resistance to

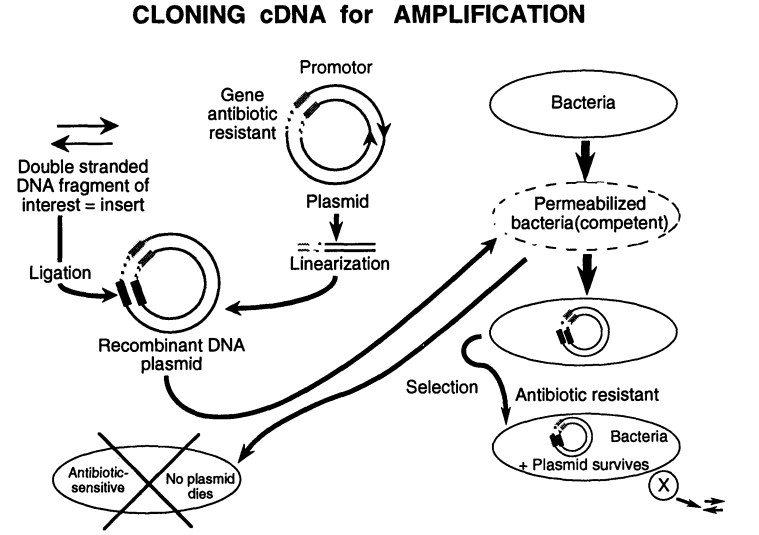


Fig. 20 Cloning and Amplification [Reproduced from Cardiovasc. Res. 1993, 27, 1566].

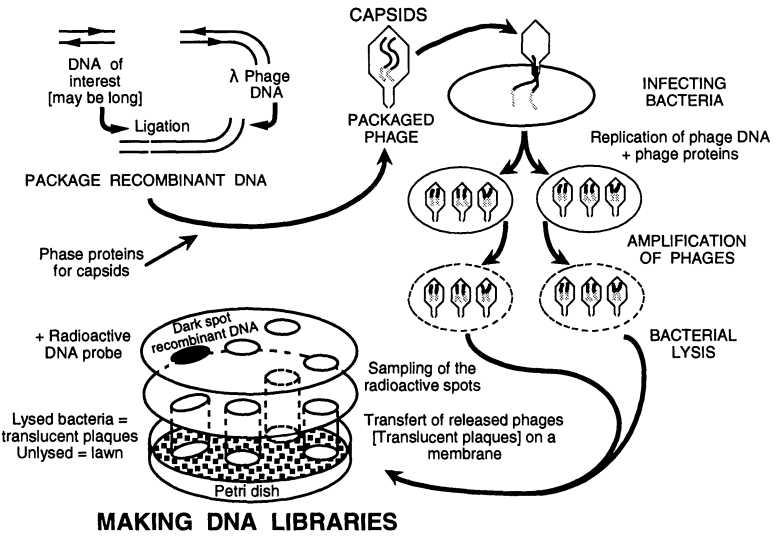


Fig. 21 DNA library using 1 Phage [Reproduced from Cardiovasc. Res. 1993, 27, 1566].

an antibiotic, such as amplicilline or tetracycline. The plasmid is introduced into permeabilized bacteria, replicates by itself and the gene conferring resistance to the antibiotic is transcribed and translated. Only the bacteria containing the recombinant plasmids are resistant to the antibiotic added to the medium and survive. They all contain the same recombinant plasmids. It is possible to multiply bacteria until we obtain enough material. Techniques to purify plasmids from bacterial DNA are easily available. Then belong to the routine techniques which do not require specialists or well-trained microbiologists.

Making a DNA library .

This technique is more difficult (**Figure 21**) and is to be handled by more experienced workers. A DNA library is so called because it produces different DNA fragments available and ready to be isolated. Figure 8 shows how the DNA of interest is selected. Let us follow the protocol used by F. Soubrier [1988] to isolate the gene encoding the rat angiotensine I converting enzyme, which is a protease responsible for the making of angiotensine II, a major determinant of arterial pressure. They firstly partially sequenced the protein, and, by using the genetic code (Table 1), they synthesized the corresponding oligonucleotides. A cDNA library was then constructed from mRNAs isolated from endothelium which is the tissue that contains the converting enzyme. The oligonucleotides are so specific that they can only hybridize to the DNA fragments of the library that contain the corresponding gene products. It is then possible to isolate the spot that contains the hybrid, and then to reintroduce this spot into bacteria for amplification.

One of the technique used to make the library is described in Figure 21. The starting material is a mixture of cDNA fragments copied from all the mRNAs present in the heart which expresses, among other genes, the

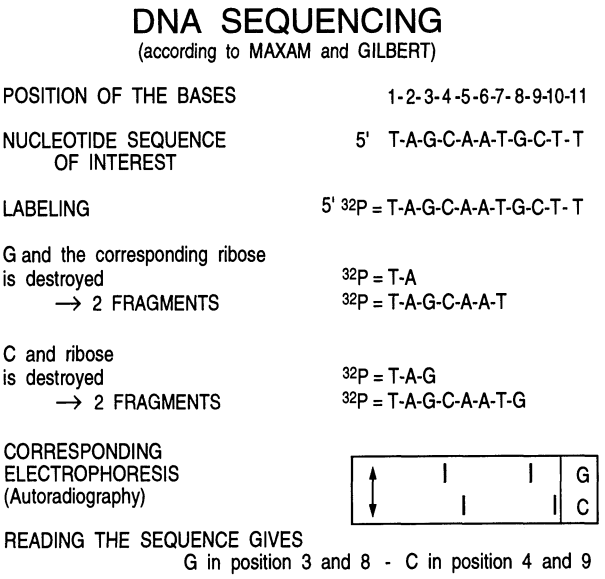


Fig. 22 DNA Sequencing using the chemical technique of Maxam and Gilbert

converting enzyme. The angiotensin converting enzyme is a rather rare protein and its mRNA is rather rare. In order to increase the chances to have a rat clone, the cDNA fragment is firstly introduced into bacteria by using phage transfection which is more effective than plasmid transformation. The cDNA molecules are first ligated to a l phage* DNA to make a recombinant DNA. Then the recombinant DNA is packaged into capsids to give infectious phages. Capsids have the properties to attach to bacteria and to inject their DNA into the bugs where it replicates and destroys the bacteria. The lysed bacteria release more phages which infect more bacteria and so on. On the Petri dish, lysed bacteria appear as translucent plaques, and unlysed bacteria appears as a "lawn". The released material is then transfered to a nitrocellulose membrane and DNA is hybridized to the radioactive oligonucleotide probe. The radioactive spot is then aligned with the corresponding plaque still containing many phages, all with the same insert.

Another approach consists in making radioactive antibodies against the corresponding peptide. It is then necessary to use phages capable of

inducing the expression of the DNA that was incorporated. The cDNAs are ligated after a promotor in the l phage, and the transfected bacteria can express the recombinant proteins. The plaques can now be screened with an antibody against the peptide of interest. The unique recombinant phage from a single plaque is reintroduced into bacteria so that we can collect enough l DNA to subclone the insert in plasmids. New phages have been developed which also infect bacteria, but it is possible to recover plasmids directly from the bacteria.

DNA sequencing.

The chemical method of Maxam and Gilbert is the most popular and automatic sequencers using this technique are now available (**Figure 22**). The fragment of DNA is labeled on one end either using ^{32}P or fluorescent tags and then subjected to a set of four partial, but base-specific, cleavages. The resulting subfragments are separated by size by electrophoresis and the labeled fragments are detected by autoradiography or detection of fluorescence. The sequence can be read off the autoradiogram from the ladder of each of the base-specific tracks, starting from the left on Figure 26. Such a technique is incredibly rapid as compared to amino acid sequencing and allows the sequencing of most of the genes, and consequently of the proteins during a short period of time.

2

NORMAL HEART AND VESSELS.
Molecular structure in relation with physiology.

Our current understanding of the molecular structure of heart and vessels has considerably increased over the last ten years. Most, nearly all, of the genes coding for cardiovascular proteins have been cloned and sequenced and, in addition, the amino acid composition of the cardiovascular proteins that had not previously been sequenced, is now known. Informations has also been obtained from genomic sequences that concern the regulatory parts of the genes, and subsequently their potential physiological regulation. Obviously such a chapter may constitute an entire book. A selection has therefore been made amongst an enormous mass of information in order to retain the data which has a potential interest in terms of physiological and pharmacological properties. This is the reason why the chapter has been presented in terms of structure/function relationships.

MEMBRANE PROTEINS AND ELECTRICAL ACTIVITY

Both the heart and the vessels possess external membranes, endoplasmic (called sarcoplasmic in the muscle) reticulum membranes and nuclear membranes, the last two are internal membranes and, in part, responsible for intracellular compartimentation. The role of the external membrane is to maintain the polarity of the cell and transmit information coming from the

Table 4. Main families or superfamilies of membrane proteins.

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1 The R7G receptor family :

- 7 transmembrane domains, G protein binding site ;
- adrenergic, muscarinic, dopaminergic, Angio II, bradykinin, endothelin receptors.

2. The voltage-gated plasma membrane ionic channels :

- several transmembrane domains containing 6 transmembrane helices, one helix (S4) is a voltage sensor, inactivation is controlled by a lid ;
- Na^+, K^+, Ca^{2+} channels.

3. The P-type class of ATPases :

- catalytic cycle that involves a phosphorylated protein intermediate, 10 transmembrane domains (still controversial) ;
- Na^+, K^+ ATPase, Plasma Membrane Calcium ATPase, Calcium ATPase of the Sarcoplasmic Reticulum.

4. The thyroid and steroid nuclear receptor superfamily :

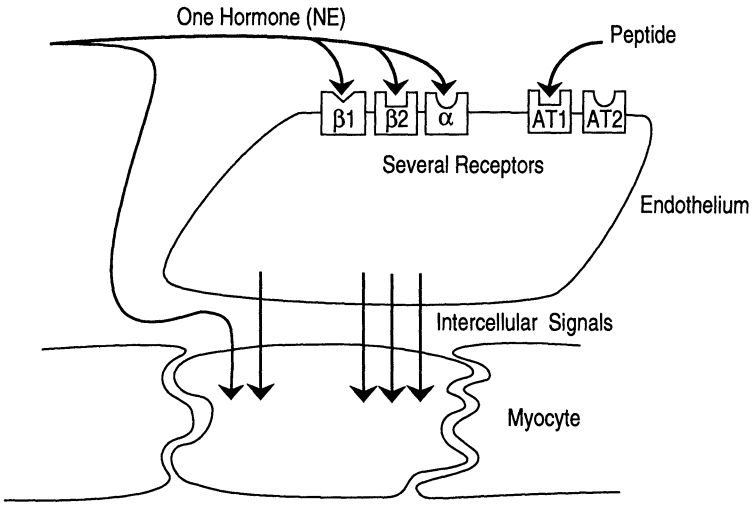
- ligand & DNA binding domain with Zn finger dependent structure ;
- mineralo & corticosteroid, estrogen, progesterone, thyroxine receptors.

5. Receptors with Tyrosine Kinase activity :

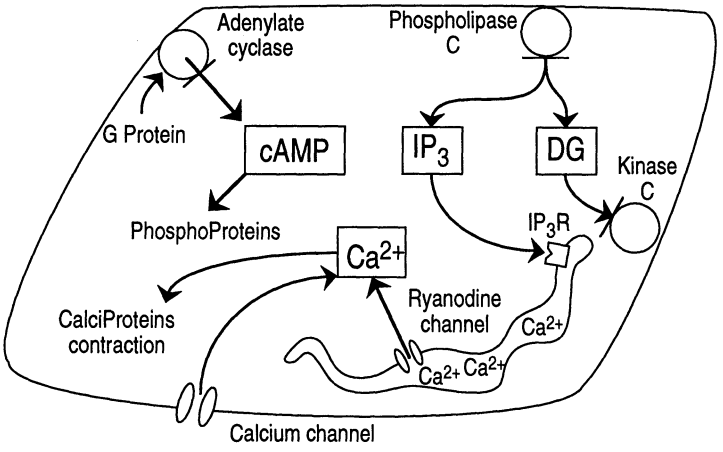
- extracellular ligand binding domain, cytosolic tyrosine kinase activity, dimers or monomers active after dimerisation ;
- PDGF, EGF, FGF, insulin receptors.

6. Exchangers :

- 12 transmembrane domains, use energy indirectly through the sodium gradient ;
- Na^+/H^+ and Na^+/Ca^{2+} exchangers.



Intercellular Cross-Talk



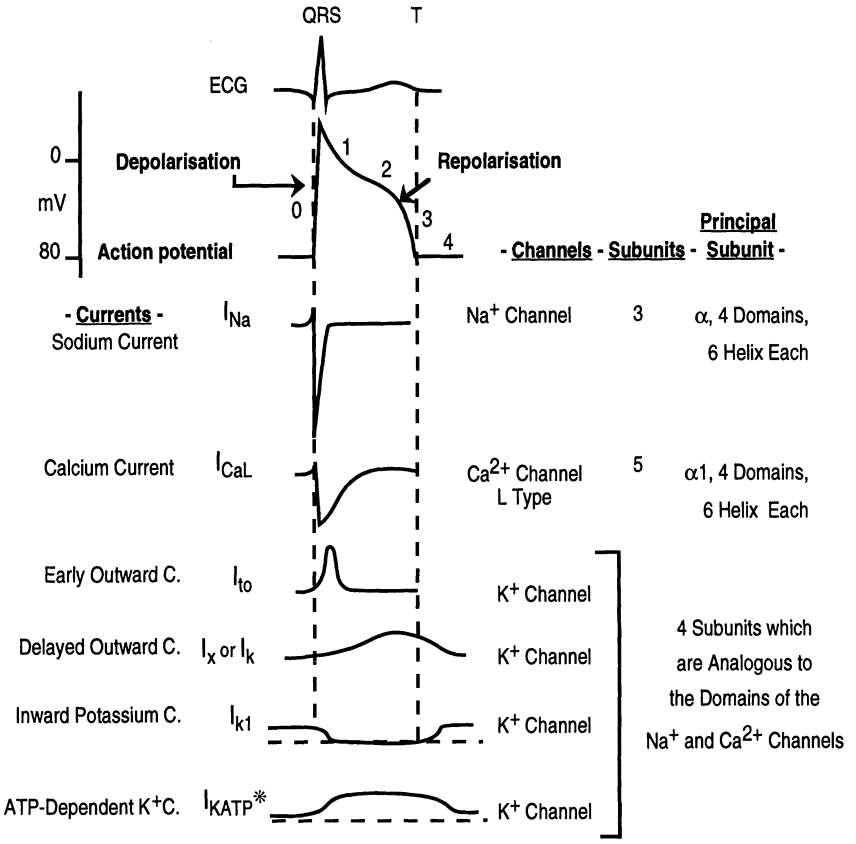
Intracellular Messengers

Fig. 23 Inter and intracellular cross-talk

environment. The transmission procedure is also called transduction*. The endoplasmic reticulum has to store rapidly available calcium within the cell. The role of the nuclear membrane is to isolate the genetic information from the cytoplasm, but there are numerous types of cells which do not have a nuclear membrane, and even in the heart the nuclear membrane is porous. Progress in molecular biology has simplified our current understanding of membrane proteins and allows a classification of membrane proteins that is based upon structural/function relationships (**Table 4**).

External membrane.

The external membrane, or sarcolemma in the myocardium, is a phospholipid hydrophobic bilayer covered by glycoproteins (or glycocalyx) in which three groups of proteins are inserted : ionic channels, receptors and a few enzymes. Such a bilayer electrically isolates the cell from the extracellular space. This is the place where the cell receives external signals from other cells (**Figure 23**). These signals are numerous and include hormones, ions, electrical currents, stretch and growth factors. In addition, each signal acts through different targets, e.g. norepinephrine binds to four different receptors. The multiplicity of intercellular signals is in contrast with the paucity of intracellular messengers that do not include more than 3-4 components including calcium, (the most important messenger in muscles), cAMP, IP_3 and DiacylGlycerol. Membrane proteins not only transduce the external signals into intracellular messenger, but also sequentially amplify this information. In addition intracellular messengers have diverse functions. For example, calcium not only couples mechanical contraction to excitation, but it also simultaneously activates calmodulin, and in turn at least 20 different enzymes (Figure 23).



* Only active in diseased states

Fig. 24 Molecular basis of ECG

Biological determinants of the Action Potential.

Cells are polarised and the intracellular space, as compared to neutral, has a potential of -80 mV. Polarisation is maintained by this ionic gradient. The intracellular K^+ concentration is higher, and the Na^+ or Ca^{2+} lower than in the extracellular space, conversely energy production (directly or indirectly) is required to release Na^+ or Ca^{2+} out of the cell, or to allow K^+ to penetrate into the intracellular space. These ionic movements create electrical currents whose intensity can be predicted from the ionic gradient according to Nernst's equation [Coraboeuf 1994]. Currents are expressed in Amperes and are the product of voltage, i.e. the difference between the intra and extracellular potentials, conductance times (or permeability, the reverse of resistance). The driving potential of an ion is responsible for the movements of this ion and represents the difference between membrane potential E_m and the equilibrium potential for that ion, E_{ion}, which is calculated from Nernst's equation. For example, the resting potential, - 80 mV, is close to E_K (- 90 mV), but not identical, because there is still a certain degree of permeability for the Na^+ and Ca^{2+} ions at rest. Resistances are in fact transmembrane channels which are holes in the membrane whose gating is commanded by voltage or hormones. The structure of these ionic channels is now known and structural/function relationships are begining to be established.

Action Potential, AP, (**Figure 24**) is a passive event. It depends on an inward current due to the gating of a sodium channel and the passive influx of sodium. The opening of the channel itself is voltage-dependent and is triggered by a wave of depolarisation that originates from the sinusal node. The sodium current will in turn trigger the subsequent gating of a calcium current, which is also a passive event. The calcium channel is also a voltage-dependent ionic channel. The cell is now fully polarised and even slightly hyperpolarised and needs to lose positive charges in order to recover its normal negative resting potential. These are again passive events due to

the gating of a rather complex group of channels, potassium channels. The cell will then lose as many positive charges as were introduced by the sodium current and at the end of the AP the cell will have recovered a potential of - 80 mV. Nevertheless, it contains too much Na^+ and not enough K^+. The Na^+, K^+-ATPase, also called the sodium pump, provides energy to reestablish the normal gradients and release sodium in exchange for potassium, and ATP consumption. Calcium has also to be released, this is the task of the Ca^{2+} ATPase of the Endoplasmic Reticulum and Na^+/Ca^{2+} exchange and Ca^{2+}ATPase of the external membrane (see further Fig. 38).

In current practice, ECG monitors the first derivative of the sum of all the AP of either the ventricles or the atria and may provide information concerning AP and ionic channels (Figure 24). In addition to the well-known modifications due to hypertrophy, ECG allows the measurement of the QT interval, which is of major pronostic value and represents the duration of the AP. Ischemia-induced elevation of the ST segment for example, results from both a shortening of the AP and depolarisation due to the loss of ATP during ischemia opens the ATP-dependent potassium channel. Depolarization is a consequence of both the intracellular loss of potassium and the accumulation of potassium in the extracellular space.

Figure 24 tries to establish a bridge between what is currently known in clinical practice and both cellular physiology and molecular biology. The ECG corresponds to AP, and T wave indicates the repolarisation process. On this figure, below AP, were schematically indicated currents, I, as obtained by potential steps triggering currents during voltage-clamp experiments. The currents are in Amperes, on the scheme inward currents are downward and outward currents upward. The addition of these currents gives rise to the AP. The right side of the figure shows the corresponding ionic channels and some features concerning their structure. The sodium current is due to the gating of the principal subunit of the sodium channel, the same is true for the calcium channels L (the most important) and T

(which is specific for the conduction system). Potassium channels are different since most of the potassium channels principal subunits all contribute to the formation of the ionic pore as explained below.

In the cardiovascular system there are several potassium currents which are rapidly going to become a major target for pharmacology, these currents result from the various assembly of different homologous subunits.

(i) The three main voltage-dependent potassium currents (Escande et al. 1993) are :

- the transient outward potassium current, I_{to}, which occurs at the beginning of the action potential and is one of the main determinant of its duration ; when I_{to} is less active the action potential duration is increased.

- There are two I_K currents, one is rapid, I_{Kr}, the other is slow, I_{Ks}. These currents are outward delayed rectifier currents and occurs latter, and are one of the main responsible of repolarization.

- I_{K1} is an inward background (rectifying) current occuring during the rest period and which slightly corrects the resting potential.

(ii) Other currents are activated by ligands as AcetylCholine (in the conduction system), or inactivated by ATP (during ischemia) or sensitive to both potassium and calcium or sodium.

Ionic channels and currents.

Channels are holes in the membranes, but holes whose gating is regulated (**Table 5**). The ionic movement is indeed commanded both by the ionic gradient and the degree of opening of the channel. Ionic channels are complex proteins encoded by multiple genes which have been already cloned. Our understanding of the structure/function relationship in this field is based on molecular cloning and isolation of the gene, gene structure, and mutagenesis experiments (see Ch. 1).

Table 5. Main ionic channels of the cardiovascular system.

1. Voltage-gated cationic channels : L or T types calcium channels, sodium channel, potassium channels.

2. Anionic channels*: CLC voltage-gated chloride channels, Cystic Fibrosis Transmembrane conductance Regulator and phospholemman.

3. Ligand-regulated cationic channels : AcetylCholine-dependent, GABA and amino acid-dependent channels*.

4. Channels regulated by a second intracellular messenger : cAMP or cGMP-gated, calcium-dependent potassium channels, ATP-dependent potassium channels.

5. Gap junctions (connexins).

6. Stretch channels, the existence of these channels is still questionable.

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* Still poorly studied in the heart and vessels.

Voltage-gated channels. Recent studies have identified a superfamily of voltage-gated plasma-membrane cation channels [reviewed in Catterall 1994]. These proteins are composed of several subunits: sodium and calcium channels are made from one principal and several auxiliary subunits (α, $\beta 1$, MW 260 and 38 kd, and $\beta 2$ for the sodium channel; $\alpha 1$, $\alpha 2$, β, γ and δ, MW 175, 143, 54, 30 and 27 kd, for the L type calcium channel) ; potassium channels are slightly different and have four principal and equivalent subunits and no auxiliary subunits. The primary structure of the principal subunit of the sodium or calcium channels and that of the principal subunits of the potassium channels is based on the same motif, or domain (**Figure 25**). Structural studies have revealed strong analogies between the different motifs from which the ionic channels are made.

In sodium and calcium channels the main subunit consists of four homologous transmembrane domains that surround a central pore (Figure 25). Each of the four domains contains six transmembrane hydrophobic α-helices called S1, S2, S3, S4, S5 and S6. In most of potassium channels,

these domains are very similar to those of the Na and Ca channels, nevertheless each domain is a separate peptide encoded by a separate gene and the functional subunit of the channel is in fact a heterotetramer and the α–helices are separated peptides (Escande et al. 1993). In other words each potassium channel is composed of four quarters of a sodium channel, and the possibility of potassium channel subunits assembling into heteromultimeric channels provides a structural basis for the generation of a rich variety of functioning channel proteins. The exact correspondence between molecular data and potassium currents has not yet been achieved, but there are strong suggestions that both I_{kr} (the rapid delayed rectifier) and I_{to} belong to this group of heterotetramers. The inward rectifier background channel, responsible for I_{k1}, has only recently been cloned and expressed. I_{ks} is another type of K channel responsible for delayed rectification and a major determinant of the duration of the action potential. Nevertheless the structure of the corresponding channel, called *minK*, is an exception and is different since the channel protein predicted by the cDNA sequence contains 130 amino acids residues and only one transmembrane spanning region (instead of six). *MinK* gene is a good candidate for the congenital long QTsyndrome.

In those channels whose gating depends upon voltage changes, the S4 transmembrane segment is responsible for the activation of the channel and acts as a voltage-sensor, i.e. a protein fragment whose spatial structure is sensitive to changes in voltage and is therefore able to transmit a cellular message to the whole protein (Figure 3). The S4 fragment is helicoïdal and is made from repeated groups of three amino acids composed by a positively

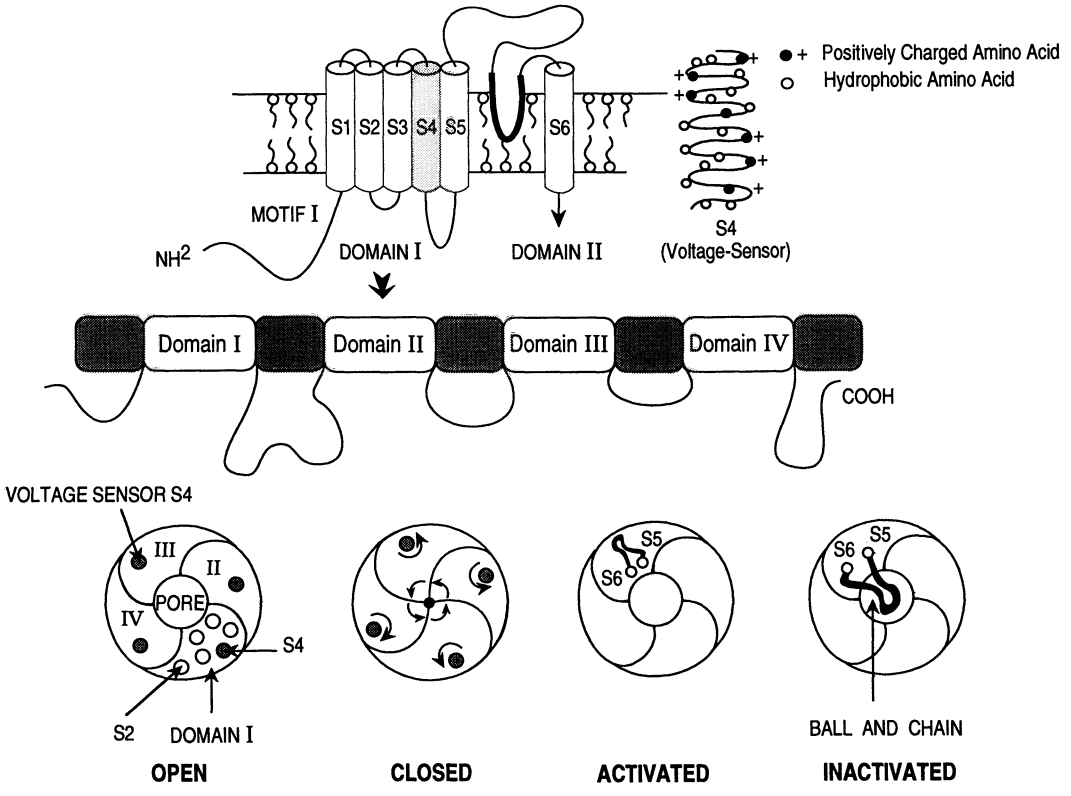


Fig. 25 Molecular structure of ionic channels

charged amino acid followed by two hydrophobic residues (residue is commonly used as a synonymous of amino acid). For example, neutralization of several positively charged residues can be obtained by mutagenesis. It results in substantial shifts of voltage dependence. Inactivation is a process which differs from one type of channel to an other. From a structural point of view, the intracellular loop connecting domain III to domain IV forms a hinged lid which can alternatively occlude the intracellular mouth of the pore. In potassium channels the structural basis of inactivation is very similar, but the lid is more alike a ball and chain and the portion of the peptide that is responsible for the occlusion of the pore is located at the amino terminal of the peptide.

Both the sodium and the calcium channels have phosphorylatable sites and are regulated by phosphorylation through the catecholamines-G protein-adenylate cyclase-protein kinase A cascade. Phosphorylation of the calcium channels activates the gating of the channel and by so doing enhances the inward calcium current and the duration of the action potential. Sodium channels are also phosphorylatable by cAMP, phosphorylation has a different effect depending on the membrane potential.

Ligand (or transmitter)-gated channels. In another group of ionic channels, as such the calcium-dependent potassium channels, sequence analysis has revealed high-affinity binding sites for calcium. In cyclic nucleotide-gated channels, such as cAMP- or cGMP- (in the vessels) gated channels, gating depends upon binding of the ligand on a consensus sequence for binding the cyclic nucleotide that resembles that of the cyclic nucleotide-dependent protein kinases. The AcetylCholine nicotinic receptor is a cationic channel that is regulated by AcetylCholine and mediates the transduction in the autonomous system at the ganglionic leve. It is made up of five subunits which form a channel. Each subunit is made from four transmembrane spanning domains and an intracellular loop between domain 3 and 4 containing an amphipatic helix which interacts with a cytoskeleton-

bridging protein. There is much of evidence to suggest that domain 2 lines the aqueous ion channel.

Gap-junctions are the largest ionic channels of the cardiovascular system. They establish communications between the myocardial myocytes and are made from polymorphic proteins called connexins. The channel has a structure very similar to that of the voltage-gated channels described-above, nevertheless the hole is bigger and has 6 connexin domains or subunits instead of four.

Plasma membrane receptors and transduction systems.

Stricto sensu, receptors are proteins that receive and transduce information, AngioTensin II receptor subtype1, AT1, binds Angiotensin II, Angio II, and by so doing knows that the vasculature needs to be vasoconstricted. Then, it activates the PI cycle and produces intracellular messenger such as IP_3 that, in turn, may bind an IP_3 receptor located on the endoplasmic reticulum inside the cell which, in turn, will release calcium from the internal stores. Therefore the role of Angiotensin II Receptor is to transform a signal that is carried by a peptide, Angio II, into another signal, chemically different and that has a broader and less specific field of activity.

Receptors not only transduce signals, but they also amplify the information. The β1-Adrenergic Receptor, β1-AR, binds epinephrine, and by so doing knows that contractility has to be activated by exercising. Then the receptor binds and dissociates several G protein complexes that, in turn, liberate the $G_{\alpha s}$ subunits which in turn activate several molecules of adenylate cyclase which, produces several molecules of cAMP.

Classification of receptor families is actually based upon structure/function relationships. Not all the families play a significant role in the cardiovascular system, and this chapter will only deals with those that really have a physiological effect in either myocardial or vascular physiology (Table 4). Nuclear hormonal receptors and tyrosine kinase

receptors will be described in detailed latter on, in relation with their respective physiological activities.

The R7 G [Strosberg 1987] family includes receptors that binds G proteins and possess seven hydrophobic spanning regions in their molecular structure. In addition most are encoded by intronless genes. Most of the plasma membrane receptors of the cardiovascular system which have important physiological roles belong to this family.

The β-Adrenergic system consists of three elements, including a receptor, a coupling protein binding guanine nucleotides (G protein), and the adenylate cyclase. The phosphodiesterases that catalyse the hydrolysis of cAMP, are not in a strict sense a part of the system, but play a major role in the regulation of the physiological effects. The β2-AR (glycoprotein, 64 kD) is well-characterized. The corresponding gene is intronless. Most important for the physiologist is the presence of several consensus DNA sequences that play a regulatory role. These sequences include Glucocorticoid, cAMP and Thyroxine Responsive Elements (GRE, CRE, TRE) all of which suggest hormonal control in the expression of the gene (**Fig. 26**). Every endocrinologist is indeed aware of the fact that, for example, thyrotoxicosis is accompanied by an increased sensitivity to catecholamines, which results from an overexpression of β-AR genes. The protein structure includes an extracellular N-terminal tail that binds glycoproteins, seven membrane-spanning regions that are arranged as a tunnel in which the agonist (or the β-agonist) binds, and a phosphorylatable C-terminal end that binds G protein.

The β2-AR receptor, as every receptor of the R7G family, is fully activated when it binds the corresponding hormone and then becomes able to interact with the heterotrimeric G protein complex, leading to an exchange of the GDP previously bound to the Gα-subunit with GTP (Fig. 26). The binding of GTP decreases the affinity of the receptor for the G protein and

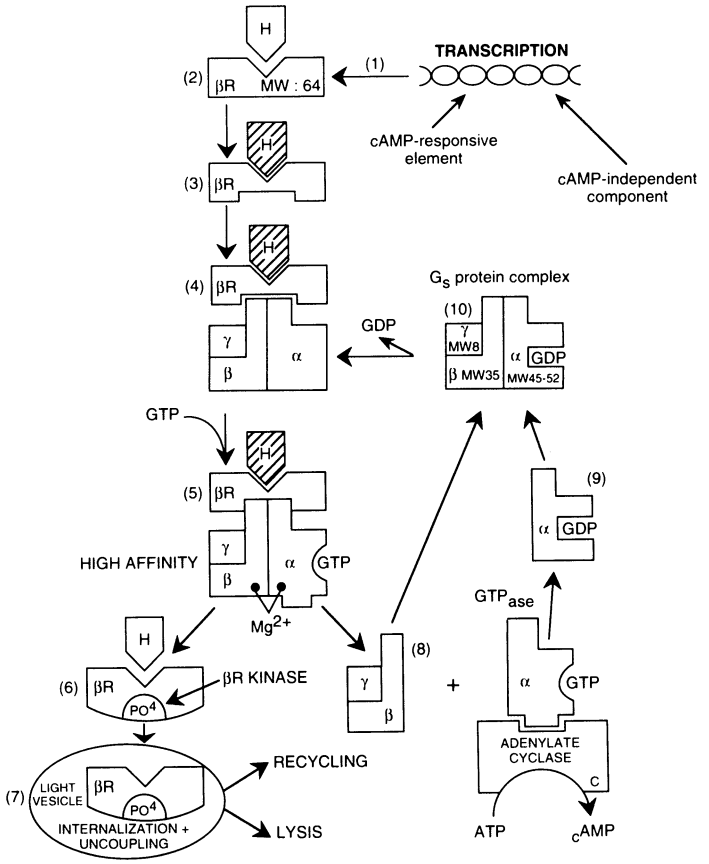


Fig. 26. The transduction system of the β-adrenergic receptors, β-R. From the β-R gene (1) to the internalized vesicles. Right : process of activation of the adenylate cyclase. H : Hormone. α, β, γ : G protein subunits. (From Swynghedauw, 1990, with permission).

dissociates the G protein complex into α and β–γ subunits. The α–GTP subunit then has the capacity to activate Adenylate Cyclase, or other effectors such as ionic channels. The α-subunit is also a GTPase, and the G protein complex needs to be reconstituted by GTP hydrolysis.

The cAMP concentration also depend upon a membrane polymorphic enzyme, phosphodiesterase, that transforms the active nucleotide into AMP. The enzyme exists as several isoforms whose repartition varies in the heart and vessels, and is inhibited by several compounds.

External receptors that belongs to this family can be internalized (Figure 26) and inactivated, as a protection when the plasma concentration of the corresponding agonist is increased. This so-called homologous down-regulation is an extremely complex phenomena that may involve either a simple internalization of the receptor which then becomes uncoupled from the transduction system, or an internalization followed by phosphorylation (by a specific β-Adrenergic Receptor Kinase, Fig. 26) and destruction, or even inhibition of transcription by a negative feed-back that utilizes the cAMP Responsive Element located on the gene [Lalli et al. 1994]. Conversely prolonged treatment with a β-blocker upregulates the receptor and usually results in an increased receptor density.

The transgenic technology has been applied to adrenergic receptor physiology. By using the promotor of Atrial Natriuretic Factor, Bertin et al. [1992] were able to target in the atria an 8 x fold increase in the β1-adrenergic receptor density. Such a genetic manipulation suppressed the normal variability in heart rate clearly showing that this important non invasive quantification of the autonomous system depends on the balance between the adrenergic and the muscarinic components of the system, even locally. More recently [Milano et al 1993], a 200 fold increase in the β2-AR was obtained in the ventricle by using the myosin promotor. Surprisingly such an enormous augmentation in the receptor density has a rather modest effect on ventricular contractility emphasizing the fact that most of the

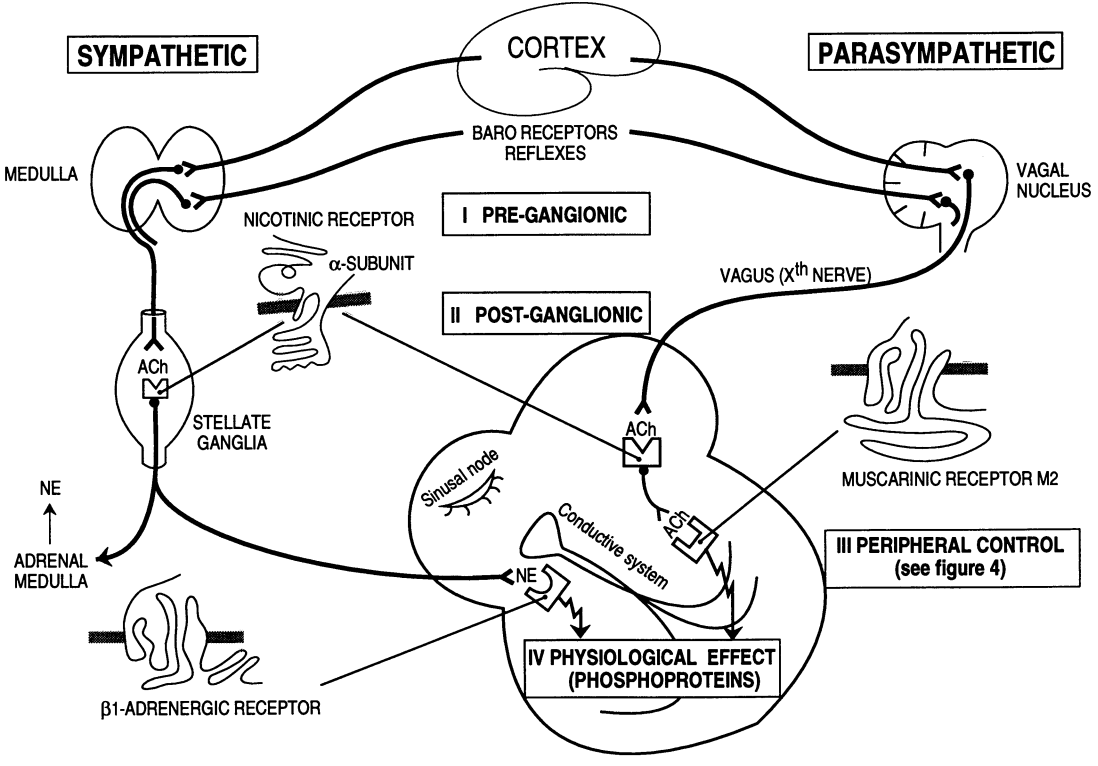


Fig. 27. Receptors of the cardiovascular autonomous nervous system.

receptors are in fact non functional and that consequently the plasma membrane receptor density (or either in functional channels) is too high.

The adrenergic system comprises several receptors including β1 and 2, α1 and 2 and their subtypes. The system is a component of the Autonomous Nervous System, ANS, and, as such, is coupled with the muscarinic receptors. For the physiologist, the pathologist and the pharmacologist it is important to note that nearly every component of this system are polymorphic, inluding the receptors, α–subunits of the G proteins, adenylate cyclase (there are 7 isoforms of the cyclase and at least two are present in the heart) and phosphodiesterases (3-4 isoforms in the cardiovascular apparatus), resulting in infinite possibilities of regulation.

Figure 27 is an attempt to reintegrate what is known about the ANS, from classical physiology and what we have recently learnt from molecular biology. Both the sympathetic and the parasympathetic systems have a preganglionic step, a postganglionic step, a peripheral control and finally physiological effects.

(I)- In both cases AcetylCholine and the corresponding AcetylCholine nicotinic receptor mediates the transduction at the ganglionic level, nevertheless this level is located in the stellate ganglions in the sympathetic cascade. By contrast, the ganglionic level of the parasympathetic system is located in the organ itself, i.e. around the sinusal node and the auriculo-ventricular node. The nicotinic receptor has a five-subunit stoïchiometry, and acts as an ionic channel (see above "Ionic channels").

(II)- The postganglionic neurones do not contain myeline, and release from their terminal varicosities either NorEpinephrine, NE, for the sympathetic system, or AcetylCholine for the parasympathetic system. These two neurotransmitters in turn act on specific receptors which are in

the myocardium itself and belong to the R7G family, respectively the β1-adrenergic receptor and the muscarinic receptor subtype M2.

(III)- The receptors themselves are connected to a transduction system which includes the G proteins (Gs for the sympathetic and Gi for the parasympathetic systems) which are membrane proteins, the Adenylate Cyclases isoforms (5 and 6 in the heart) and the synthesis of cAMP. cAMP, in turn, binds cooperatively to two sites on the Regulatory subunit of Protein Kinase A, releasing the active catalytic subunit (Figure 4 and 5).

(IV)- Finally the catalytic subunit of protein kinase A is translocated from its cytoplasmic and Golgi complex anchoring sites and triggers physiological activity by phosphorylating various phosphoproteins (on a Serine in the context X-Arg-Arg-X-**Ser**-X), including troponin I, phospholamban, several ionic channels, but also transcriptional factors (see below cardiac and vascular growth). Other receptors of the R7G family include dopaminergic receptors, angiotensin II, bradykinin, endothelin receptors (see below).

G proteins.

The G proteins are polymorphic membrane proteins that play a major role in controling signal transduction. G proteins were discovered by the last two winners of the Nobel price, Rodbell and Gilman [Gilman 1987] and are composed of three different polymorphic subunits, a, b, g, (MWs 45-52, 35 and 8 kd).

Molecular cloning has identified at least 20 different α-subunits. The αs-subunit isoforms are sensitive to cholera toxin and are encoded from splicing products of a single gene. They mediate the activation of adenylate cyclase and L-type calcium channels through the activation of β1-adrenergic, histamine, prostaglandin E2, 5-HydroxyTyramine receptors. The αi-subunits 1, 2 and 3, but only αi2 and 3 are expressed in the heart, are sensitive to pertussis toxin and mediate adenylate cyclase inhibition and, in the sinusal node, activation of the potassium channels. G proteins also

mediate the regulation of the phosphoinositol pathway and the activation of phospholipase A2 through the activation of the other adrenergic recptors and also the Angio II receptors.

Enzymes.

Na^+, K^+-ATPase is directly responsible for intracellular sodium and potassium homeostasis and indirectly responsible for that of calcium, because the enzyme is coupled with the Na^+/Ca^{2+} exchange. Hydrolysis of one molecule of ATP permits the release of three Na^+ ions and the uptake of two K^+ ions, each against the electrochemical gradients. The enzyme creates a sodium gradient which is used by the cell to drive numerous transport process, including the Na^+/Ca^{2+} exchange and the Na^+/H^+ exchange. As a consequence, the sodium pump is electrogenic and takes part in the repolarization of the membrane with each excitation-contraction cycle. The ATPase belongs to the P-type class of ATPases, which also includes the Plasma Membrane Calcium ATPase and the Calcium ATPase of the Sarcoplasmic Reticulum (Table 4). These enzymes share a similar catalytic cycle that involves a phosphorylated protein intermediate. An important, and frequently forgotten, notion is that the enzyme consumes approximately 20% of the ATP at rest in the whole organism, which renders Na^+/K^+ homeostasis an extremely expensive procedure.

Na^+, K^+ ATPase is also the specific receptor for digitalis. Inhibition of the enzyme by cardiac glycosides provokes an increase in intracellular sodium, and subsequently of calcium via the Na^+/Ca^{2+} exchange. This increase in calcium will, in turn, cause the inotropic effect (the mechanism of which is not as simple since the inotropic effect is only systolic, and not diastolic, as expected).

The protein is a dimer comprising one α–subunit (MW 112 kd) carrying all the known receptor sites of the enzyme and one glycosylated β-

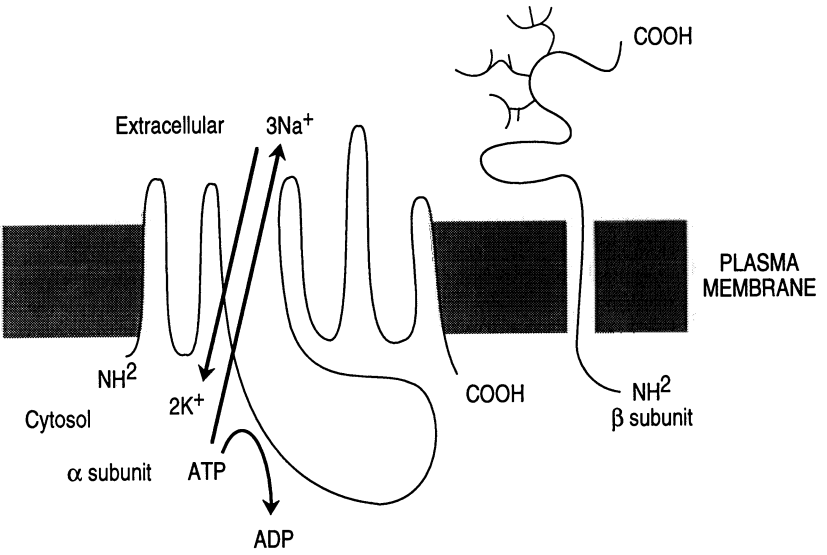


Fig. 28 Model of the structural domains of Na^+, K^+ ATPase

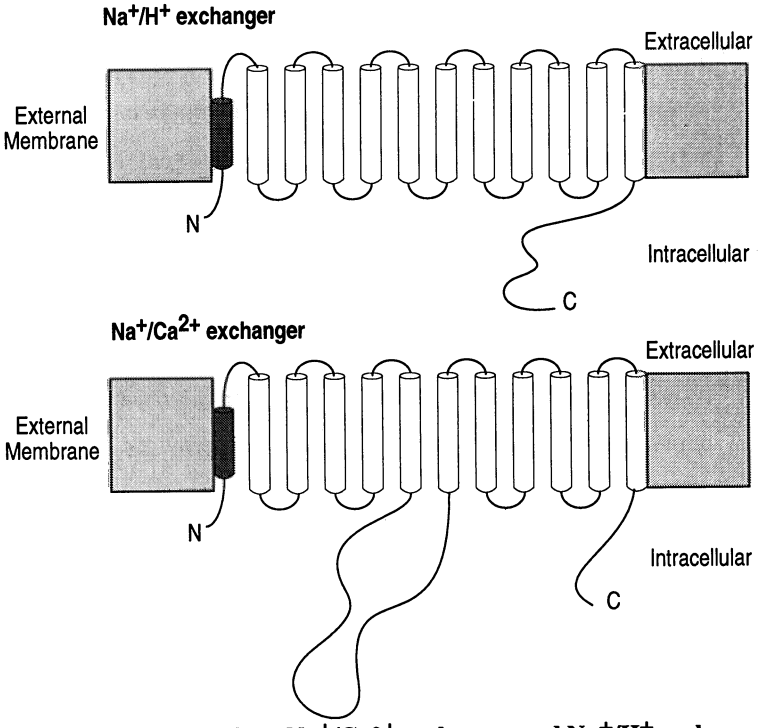


Fig. 29 Structure of the $Na^+/Ca2^+$ exchanger and Na^+/H^+ exchanger [redrawn from Reithmeier 1994].

subunit which is indispensible for the activity (**Figure 28**) [Lingrel 1994]. Nevertheless whether the enzyme functions in higher orders, for example as $(\alpha\beta)_2$, in cells is presently unknown. The most up-to-date "working" model of membrane topology includes ten transmembrane domains (called H1.... H10), divided into two groups, a group of four on the N-terminal side and a group of six on the C-terminal side. Multiple extracellular and transmembrane regions are involved in determining ouabain sensitivity, this area of research is still in process. Ouabain binds on the external side and the hydrolytic site is located on the intracellular side.

Both subunits are polymorphic. Three isoforms of the α-subunits are presently known. In the rat α1 is an ubiquitous form present in every tissues and has a low affinity to ouabain and high affinity for sodium, α2 which has a high affinity both for ouabain (1,000-fold higher than α1) and for sodium and is found in only a few organs such as the ventricles and the aorta, α3 which is embryonic and posses the same high affinity as α2 for ouabain, but has a low affinity for sodium. In human α1 is different and has a very high affinity for ouabain, and α3 is not specfic for embryo and is found during adulthood. Such species differences in normal conditions may explain the species differences observed in cardiac hypertrophy. These three isoforms are encoded by three different genes. In the heart, the enzyme has both a low and a high affinity site for ouabain, the first being roughly responsible for toxicity, and the second for inotropic effect. These sites correspond to the α-subunits isoforms which provides a structural basis to search for new inotropes with less toxicity. The three isoforms are encoded by three different isogenes that are both developmentally and hormonally regulated.

Recent studies have demonstrated the existence of a functionally active calmodulin-dependent Plasma Membrane Calcium ATPase which may play a major role in releasing calcium both in the heart and in the smooth

muscle of the vessels. This ATPase is encoded by four different genes, each alternatively spliced at several different positions.

Exchangers.

Exchangers and cotransporters are membrane proteins which have evolved to fulfill general functions such as the maintance of calcium homeostasis and pH. They have a similar structure and span the membrane about 12 times (instead of seven for the R7G). The most important components of this family in the cardiovascular system are, for the moment, the Na^+/Ca^{2+} exchanger and the Na^+/H^+ exchangers (or antiporters) [**Figure 29**].

The Na^+/Ca^{2+} exchanger is highly expressed in the myocardium where it is the major pathway for the extrusion of calcium out of the cell [Reithmeier 1994]. The exchanger is electrogenic and exchanges one calcium for three sodiums. It can function in the two directions, nevertheless, because of the normal stoïchiometry of the two cations, it normally releases calcium out of the cell and acts as a major determinant of both diastole and vasodilation. The protein has 970 amino acids which are arranged into 12 putative transmembrane segments with a large cytosolic domain located between segments 5 and 6. This cytosolic domain contains the regulatory elements sensitive to calcium and to a specific inhibitory peptide. In human, the protein is encoded by one gene that contains 6 central exons encoding the cytosolic loop, and which, by alternative splicing, can make 32 different transcripts specific for different tissues (cardiac, renal and brain.... isoforms have been identified).

The Na^+/H^+ exchanger and intracellular control of pH. Cells, possess an intrinsec buffering capacity due to the intracellular proteins, phosphates, and above all the CO_2/HCO_3^- content. In addtion the cell can release H^+ when their production exceeds the buffering capacity of the cell. Three mechanisms can play this role, including the passive release of lactic acid, the Na^+/H^+ exchanger and the Na^+-HCO_3 symporter. The Na^+/H^+ exchanger uses the electrochemical gradient as a source of energy to release

one proton and to take up in exchange one sodium molecule. The exchanger does not work when the pH is too alkaline and is above 7.3. It is specifically inhibited by amiloride. There are at least four isoforms (the isoform 1 is found in the heart) and the general structure of the antiporter is similar to that of the Na^+/Ca^{2+} exchanger with 12 transmembrane segments and a C-terminal cytosolic loop. The antiporter plays a major physiopathologic role during ischemia-reperfusion.

Endo (or Sarco) plasmic Reticulum.

Both in the heart and vascular smooth muscle cells, the Sarcoplasmic Reticulum, SR, consists of tubules (10-50 nm in diameter) that anastomose and divide in all directions, and form a lace-like network that wrap around myofibrils and spread across the Z-lines discs. A specialized part of the SR is called junctional and lies in proximity to the T tubules of the plasma membrane and forms triads. In skeletal muscle, triads are structures connected one to each other through a protein called foot. In the heart the connection between Sr and SL is only functional. The main function of the SR is to store and release calcium, and to couple contraction with electrical activity. **Figure 30** shows the main proteins that play a functional role in the SR :

- uptake of calcium is against the gradient, needs energy and is controled by an ATPase,

- calcium storage depends on a calciprotein (see below), calsequestrine, which binds a considerable amount of calcium per mole (about 40),

- calcium release differs from one type of muscle to another and depends upon rather complex calcium channels, termed ryanodine receptor in

the heart, and IP3 receptor in the vessels. Ryanodine and IP3 receptor are very homologous proteins, but encoded by different genes.

Calcium ATPase of SR and phospholamban.

The ATPase was first isolated from fast skeletal muscle where it is the most abundant. The enzyme is able to transport two calcium molecules per molecule of ATP hydrolysed. The ATPase belongs to the P-type class of ATPases (Table 4). The model shown in Figure 30 includes 10 helical hydrophobic segments connected to three cytosolic hydrophilic loops which contains both the ATP binding site and the phosphorylation site. Several isoforms are now well-characterized (the cardiac isoform is termed SERCA 2a, MW 105-115 kD). These enzyme are encoded by at least three genes, all of which may be alternatively spliced to generate different protein isoforms. The cardiac and vascular isoforms have the particularity to be regulated by a protein, acting as a cofactor, phospholamban that is present in a molar ratio of 1:1. The density of the Ca ATPase in SR is approximately 6,000 higher than that of the Ca channels of SL.

Phospholamban is a pentamer made up of five subunits (MW 6,000 D) that form a sort of tunnel. Each subunit is phosphorylatable and posses two different phosphorylation sites which correspond to two different protein kinases, one of which is the cAMP-dependent protein kinase protein kinase C, the other is calmodulin-dependent. The phosphorylation of phospholamban induces transconformational changes of the calcium ATPase whose pumping ability is, in turn, activated. The increased rate of relaxation (also called the lusinotropic effect) which is observed after β-agonist stimulation is due to an enhanced uptake of calcium by SR, which, in turn, corresponds to the phosphorylation of phospholamban by cAMP and the activation of the Calcium ATPase.

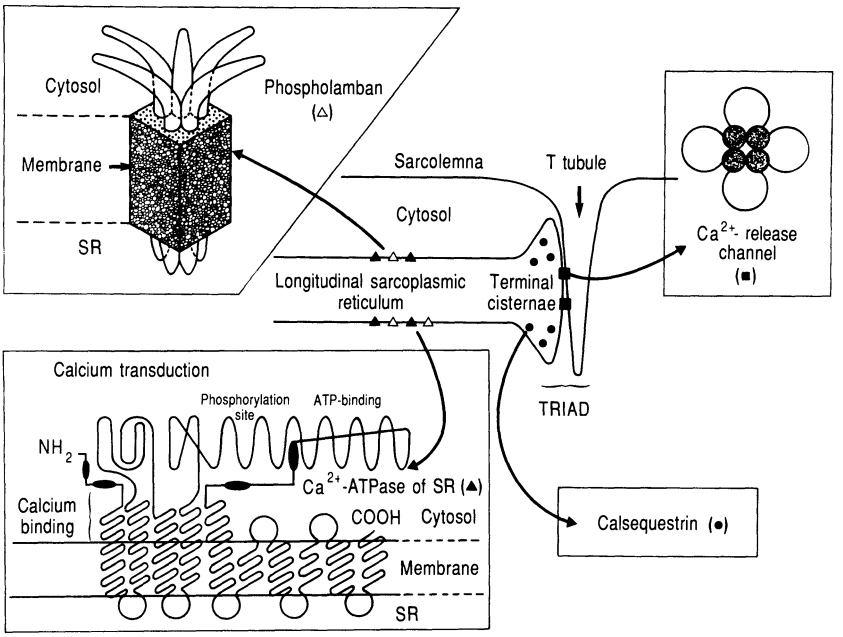
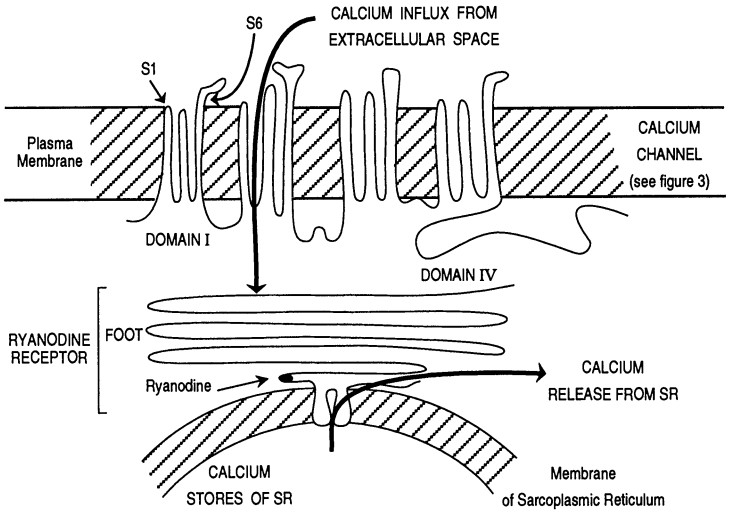


Fig. 30. Proteins of the Sarcoplasmic Reticulum. Middle : Triad. Periphery : proteins. (From Swynghedauw, 1990, with permission).

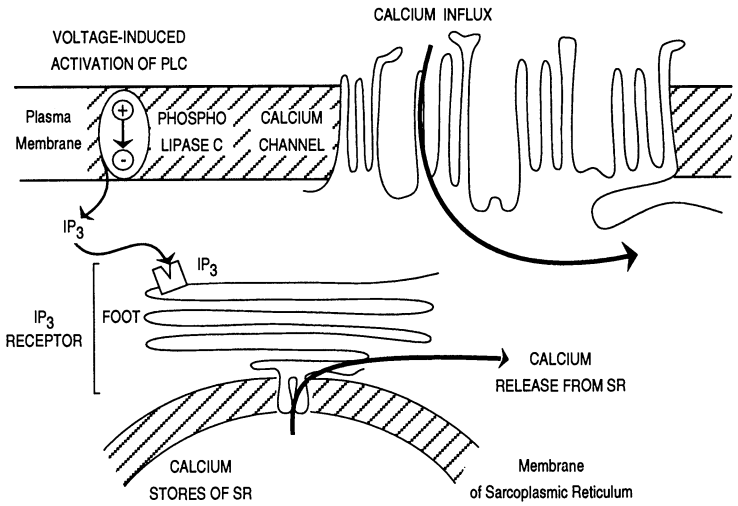
Ryanodine and IP3 receptors.

The calcium release from SR is directly or indirectly voltage-dependent, nevertheless the mechanism of the calcium release from SR varies from one muscle to the other. In the fast skeletal muscle the release depends upon a spanning protein which directly transmits the voltage change to the calcium channel of the SR. In the heart (**Figure 31**) the release is autocatalytic and triggered by the calcium inward current which transmits the signal to the



CALCIUM-INDUCED CALCIUM-RELEASE IN THE HEART

Fig. 31 Structural basis for the cardiac calcium-induced calcium-relase in the SR. The figure shows only one subunit of the ryanodine receptor.



IP3-INDUCED CALCIUM-RELEASE IN THE VESSELS

Fig. 32 Structural basis for the vascular IP3-induced calcium-release in the SR.

ryanodine receptor (ryanodine is a synthetic marker that does not exist in vivo). In the vessels the release is commanded by voltage-dependent IP3 production from the phosphoinositol cycle (**Figure 32**) and the signal, i.e. IP3, acts on an IP3 receptor which is also the calcium channel of SR.

In the SR, there are, at least, three calcium channels isoforms [Coronado et al 1994] (Figures 31 & 32). These channels are different from the plasma membrane dihydropyridine-sensitive calcium channels described above. The protein is a tetramer and comprises four subunits (MW around 500 kD) which forms a channel. Each subunit is composed by four hydrophobic transmembrane segments (called M1, 2, 3, and 4) which anchor this bulky protein to the SR membrane, and a hydrophilic cytoplasmic domain termed foot.

Membrane depolarization, as neurotransmitters or hormones, evokes a release of calcium from SR stores through these calcium channels, nevertheless, from a pure physiological point of view, the important point is that the calcium release from SR is not permanent. The release is pulsatory and triggered by the voltage changes.

Nuclear receptors.

Thyroxine, glucocorticoid, mineralocorticoid, progesterone and, estrogen receptors are all part of a large family of intracellular hormone receptors which share a common general structure, including a central highly conserved DNA binding site of about 70 amino acids, a C-terminal steroid recognition region, and a finger-like structure that is activated in the presence of an atom of Zinc (Table 4). These receptors are in fact true transcriptional factors which bind to the corresponding consensus DNA sequence located upstream from gene. The thyroxine receptor is also an oncogene, called c-erb-A, and has a particular interest in cardiology since there are positive and negative Thyroxine Responsive Elements in the

regulatory region of genes coding for adrenergic receptors, cardiac isoforms of myosin heavy chains, phospholamban etc....

The mineralo (or type I aldosterone receptor) and glucocorticoid (type II) receptors demonstrate a poor selectivity between glucocorticoids and aldosterone. Aldosterone in fact only interacts with the mineraloreceptor type I because, in the aldosterone target tissue, this class of receptor is associated with 11-β–Hydroxy-Steroid Dehydrogenase, an enzymatic complex which transforms cortisol into an inactive form unable to bind to the receptor. Both types of receptors, and also the 11bHSD, are present in the myocytes of the myocardium [Lombes et al 1992]. Aldosterone activates several plasma membrane channels, exchangers or enzymes, and also has an effect on the transcription of these components. Aldosterone could have a determinant role during fibrogenesis [Brilla et al 1990, Robert et al 1994].

Particularities of the vascular cell membrane proteins.

Vascular contraction is tonic, as compared to myocardial contraction, and is frequently maintained for a long period of time. In parallel, membrane resting and action potential are different since the resting potential in smooth muscle is - 40 to - 55 mV instead of - 80 mV in the heart. The external membrane lacks sodium channels, and the inward current is carried by voltage-dependent and receptor-operated L-type (sensitive to calcium blockers) calcium channels (**Table 6**) [Tedgui et al 1994, Hathaway et al 1991]. Several K^+ channels have been identified as a major target for vasodilators, including calcium-activated K^+ channel, ATP-sensitive K^+ channel and delayed rectifyer potassium channel. Stretch activates cation channels and may depolarize the cell. The smooth muscle SR plays a major role since blocking calcium release from SR by ryanodine stops smooth muscle contraction. Smooth muscle has a specific Ca ATPase isoform and also possess an active phospholamban that is able to control calcium uptake and produces relaxation exactly as in the heart.

R7-G receptors are present, especially the α1 and β2-adrenergic, M3 and M2 muscarinic, angiotensine II, serotonin receptors. These receptors are

Table 6. Molecular basis of contractility. Cardiac versus vascular smooth muscle. Membrane proteins.

	Heart.	Smooth muscle.
Sarcoplasmic Reticulum.		
Calcium ATPase isoforms :	cardiac	smooth
Phospholamban :	+	+
Calcium channels of SR :	Ca induced	IP3 induced
External Membrane. Ionic Channels.		
Na channel	+	0
Ca channels	L, T-type	L, T-type like
K channels	I_{to}, I_K, I_{K1}	I_K ? ?
	ACh, ATP, Ca-induced	ATP,Ca-induced
Na, K ATPase	α1 & α2	α1 & α2
Na/Ca exchange	+	+
Na/H exchange	specific isoform	specific isoform?
Gap junction & connexin	Connexon 43	Connexon 43
External Membrane. Receptors.		
Adrenergic	β1 & 2, α 1	β2, α1 & 2
Muscarinic	M2	M3 & 2 Angio II
	rare	AT1 & 2

coupled to several G proteins, including Gs and Gp. Coupling operates through cAMP, but also cGMP, and the PI cycle. As explained below, most of the vasoconstricting agents which activate the PI cycle, including

Angio II, rapidly trigger contraction through the liberation of IP3 and consequently the release of calcium from SR. A second step which proceeds more slowly, follows and is the DiacylGlyderol-induced activation of protein kinase C and the corresponding phosphorylations.

CONTRACTILE PROTEINS AND CONTRACTILITY

Systolic ejection is the ultimate goal of myocardial function, and results from the harmonious and simultaneous cyclic contraction of cardiac myocytes. Relaxation of the same cells allows the myocardial filling procedure. In the vessels, contraction is not rythmic, and relaxation is the way by which the vascular tone is maintained and numerous regulatory peptides regulate this process. In both cardiac and vascular muscles contraction is triggered by the electrical events, the intracellular messenger is calcium, but the origin of the calcium varies according to the species and the type of muscle. Contraction itself results from the sliding of two groups of proteins, called contractile proteins. The sliding process is regulated at the level of the thin filament in the heart (through troponin), and at that of the thick filament in the vessels (through the phosphorylated light chain of myosin).

Smooth muscle is not only smooth, i.e. apparently disorganized, as compared to myocardium which is striated, i.e. highly and geometrically organized, but also the structure of the sarcomere is different, and smooth muscle contractile protein structures and genes differ both from the myocardium and fast skeletal muscles in such a way that antibodies raised against smooth muscle proteins do not cross-react with those raised against striated muscle.

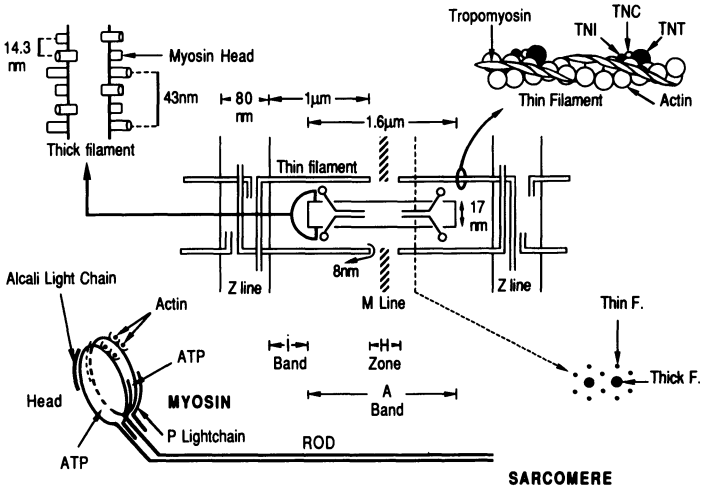


Fig. 33. Striated muscle sarcomer. Middle : structure of a sarcomere. Upper right : thin filament. Upper left : thick filament. Lower : myosin (From Swynghedauw, 1992, with permission).

Cardiac sarcomere.

Electron micrographs of cardiac fibers show a striated appearance made up of regular alternating dark and light bands (A and I-bands, **Figure 33**) [Swynghedauw 1986]. In the centre of the A-band is an H-zone and an M-line. The sarcomere is the basic contractile unit, i.e. the smallest component of the muscle to shorten. It is delimited by the two Z lines. At higher magnification, the sarcomere shows interpenetration of thick (1.5 mm long) and thin filaments (I mm long, start at the Z line). A cross section at this point shows that each thick filament is surrounded by 6 thin filaments. Thick and thin filaments are interconnected through the myosin bridges projecting from the thick filaments.

Thick filaments proteins.

The thick filament contains 400 molecules of myosin (MW 500 kD) which is a fibrous protein composed of two Heavy Chains (MW 200 kD) and two pairs of Light Chains (one is phosphorylated, the other is called alkali). Myosin plays an important role in the mechanical events involved in muscle contraction. The structure of the thick filament is bipolar and the the myosin molecules are arranged in an antiparallel manner. Myosin bridges are uniformly spaced every 14.3 nm (Figure 11) forming a helix with a coil of 43 nm.

The myosin molecule is made of a long helicoidal rod fragment that contributes to the formation of the filament itself, and a head which is the bridge connecting the two filaments to each other. The head, also called S1, posses the sites both for the ATPase activity of the molecule and the binding of actin. There are two heads per myosin molecule, and consequently each molecule of myosin can hydrolyzes two molecules of ATP. The active site of the myosin ATPase is located on the myosin heavy chains and the main activator of the enzymatic activity is actin.

Cardiac myosin heavy chain isoforms in man and in rat are the products of two highly homologous genes with 40 exons. These gene called α and β, and located in a head-to-tail arrangement about 4.2 kb apart on chromosome 14. In rat, these two different cardiac myosin heavy chains interact to product three isomyosins, called V_1, V_2, and V_3, which are $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$ myosin heavy chain isoforms respectively. The cardiac myosin heavy chains are developmentally and tissue regulated and distinct patterns are observed in ventricles and atria of the same species, as well as in human and rat ventricles. These is however always a predominancy of $\alpha\alpha$ in the atria of all species, including man, and in the rat ventricle. Thyroxine activates the expression of the $\alpha\alpha$ isoform. The specific ATPase activity of the α myosin heavy chain isoform is 3-4 times higher than that of β and correlates with the maximum shortening velocity of an unloaded cardiac or smooth muscle. The human ventricle has a slower shortening velocity when compared to the rat ventricle, and consequently it has, a higher content of

the ββ myosin heavy chain isoforms (in contrast rat ventricle myosin heavy chains are entirely αα) [reviewed in Swynghedauw 1986].

Cardiac myosin light chain isoforms are also heterogeneous. They belongs to a monogenic family, and are encoded differentially by a process of alternative splicing. Phosphorylated and non phosphorylated light chains are different in the ventricles and in the atria. The non phosphorylatable atrial light chain is also skeletal embryonic and the corresponding gene is located on chromosome 11. The same light chain in the ventricle is also the slow skeletal muscle light chain and the gene is on chromosome 9 (in mice).

Thin filament proteins.

The thin filaments consist of two globular chains of actin 4 nm in diameter twisted into a double helix which contains 13.5 actin molecules per turn (Fig. 33). A coiled-coiled and coiled dimer of tropomyosin, occupies the actin-actin cleft. Every 385 nm there is a regulatory complex which is composed of the three different TropoNins, TN, subtypes called TNI, TNC and TNT. The TN complex does not interact directly with myosin, it does so through tropomyosin and actin.

Actin (MW 41,785) isoforms are numerous and encoded by different genes which are located on different chromosomes, and nearly 60 of them, including pseudogenes, have been reported. The striated muscle acti isoforms are α-skeletal (on chromosome 3) and α-cardiac (on chromosome 17) actins which are highly homologous proteins. Both are expressed into the heart. Smooth muscle isoactins are β and γ isoforms and are different from the striated muscle isoforms. Troponin is absent from smooth muscle, but tropomyosin is present (**Table 7**). Cardiac TNC and TNI are specific for the myocardium and are encoded by separate genes that are also different from the genes coding for the fast skeletal muscle isoforms. Cardiac TNC (MW 18,459) binds calcium and, by so doing, activates contraction, and this is the last step in excitation-contraction coupling.

Nevertheless it has only three calcium binding sites, instead of four in the skeletal TNC (MW 17,965). Cardiac TNI (MW 23,550) inhibits contraction, and is slightly longer than the fast skeletal isoform (MW 20,864). Cardiac TNI is unique because it contains an amino acid that can be phosphorylated by cAMP through a Protein Kinase C.

Troponin T links TNC and TNI to Tropomyosin, and is extremely polymorphic in the heart and the various isoforms are developmentally regulated. TNT (MW 38,000) isoforms are encoded by alternative splicing by the same gene which covers 18 exons. Tropomyosin is a dimer forming a super coiled double helix 4 nm long which sits in the two grooves formed by the twisting of 7 molecules of actin. Isotropomyosins are homo or heterodimers encoded by by two different genes α and β, that are, in turn, able to encode by alternative splicing several isoforms.

Contraction movement.

Contraction is due to the relative sliding of thin and thick filaments due to the movement of the myosin head. **Figure 34** summarizes the different steps of contraction in the heart. An important point of this scheme is the dual role of ATP which is not only the source of energy, as everybody knows, but also the most basic determinant of relaxation. The outer circle shows the enzymatic reaction, and the inner circle is a scheme of the accompanying mechanical movement (M : Myosin head. A : Actin. TN-TM : troponin-Tropomyosin complex). From left to right:

- relaxation which is characterized by an inhibition (or a weakening) of the actin-myosin link due to the high concentrations of ATP (the ATPase is no longer active whereas both the mitochondrial and anaerobic synthesis are continue).

- The initial burst is the first extremely rapid phase of the ATPase reaction, during this step ATP binds to myosin, whose spatial structure changes, it is then hydrolyzed to produce mechanical energy.

- Simultaneously, due to the decrease in ATP concentration, actin binds to myosin, and consequently the tropomyosin-troponin complex is indirectly connected to myosin. The actin-myosin link is still weak because the intracellular calcium concentration is low and TNI hinders the interaction between the two proteins.

- The increase in the intracellular calcium concentration enhances the affinity of TNC for TNI and instigates a steric and stoichiometric displacement of tropomyosin which leaves the actin grooves and allows the actin-myosin relationships to be reinforced. Actin can then activate the myosin ATPase, the ATP hydrolysis is completed and the hydrolytic products are released.

Relationships with physiological properties.

The Tension/Length curve is steeper in the heart than in the striated muscle (**Figure 35**). On a cardiac or skeletal fiber, the length is initial length, or preload, and is a bell shape curve, and the top of the bell corresponds to the point where there is a maximum number of actin/myosin bridges formed. Tension in Figure 35, is active tension, and when the Tension/Length curve is drawn using membrane-free (skinned fibers) samples, it is possible to add ATP and measure ATPase at various lengths. It has been shown that the ATPase/Length curve has the same bell shaped appearance as the curve

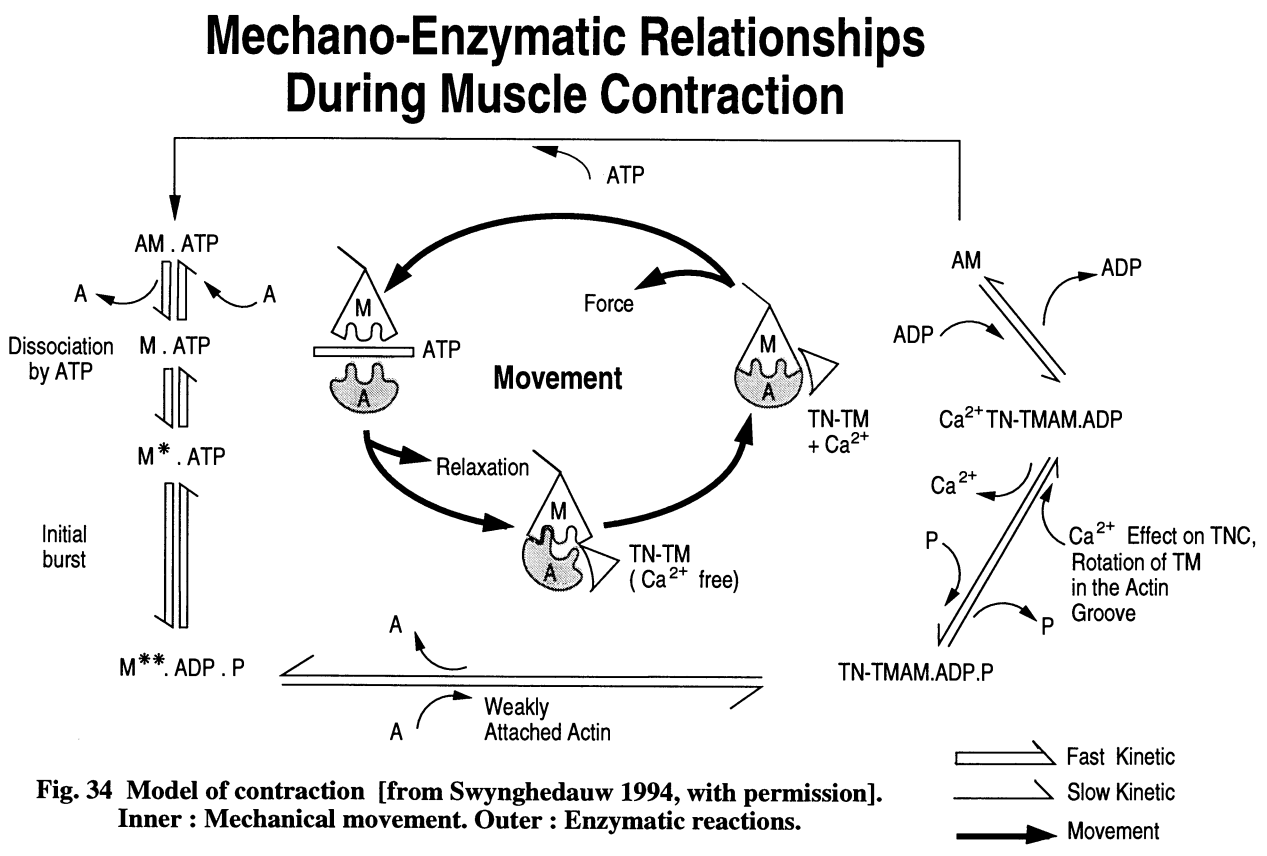


Fig. 34 Model of contraction [from Swynghedauw 1994, with permission]. Inner : Mechanical movement. Outer : Enzymatic reactions.

obtained with fresh tissue. In vivo the Length corresponds to the end-diastolic volume and active tension to the pressure developed.

The Velocity/After-Load curve is drawn at a constant preload (or initial length) and Vmax is the initial shortening velocity for unloaded muscle. Vmax depends on the maximum myosin ATPase activity which, in turn, is determined by the genetic characteristics of the molecule, the number of actin molecules bound to myosin (i.e. the load), and also, with some restrictions, the intracellular calcium concentration. The myosin ATPase enzymatic site is located on the heads of the heavy chains, and, from a genetic point of view, depend on the isoform which is present. In phylogeny, in various muscles from different animal species, myosin ATPase, the biochemical parameter, is positively correlated with Vmax, the physiological parameter. Such a phylogenetic relationships is also found in cardiac diseases.

The vascular smooth muscle sarcomere.

Smooth muscle cells are located in the media of the arterial wall. The vascular smooth muscle sarcomere is arranged differently (**Figure 36**). As opposed to striated muscle (**Table 7**), the structure of the smooth muscle sarcomere is unipolar and myosin molecules are parallel. In addition, the smooth muscle contains less myosin and more actin per g of tissue than does the cardiac tissue (the Actin/Myosin ratio is indeed 4 in the striated muscle and around 30-35 in the arterial smooth muscle), which gives the sarcomere a very particular shape (Fig. 14). Smooth muscle contains only 30% of the myosin found in myocardium, nevertheless they develop the same range of force because the force produced per myosin head is 3 fold higher in smooth muscle than in striated muscles. In addition the smooth muscle myosin is much more efficient than the striated muscle and uses less ATP per cross-bridge. Myosin subunits of the smooth muscle are also

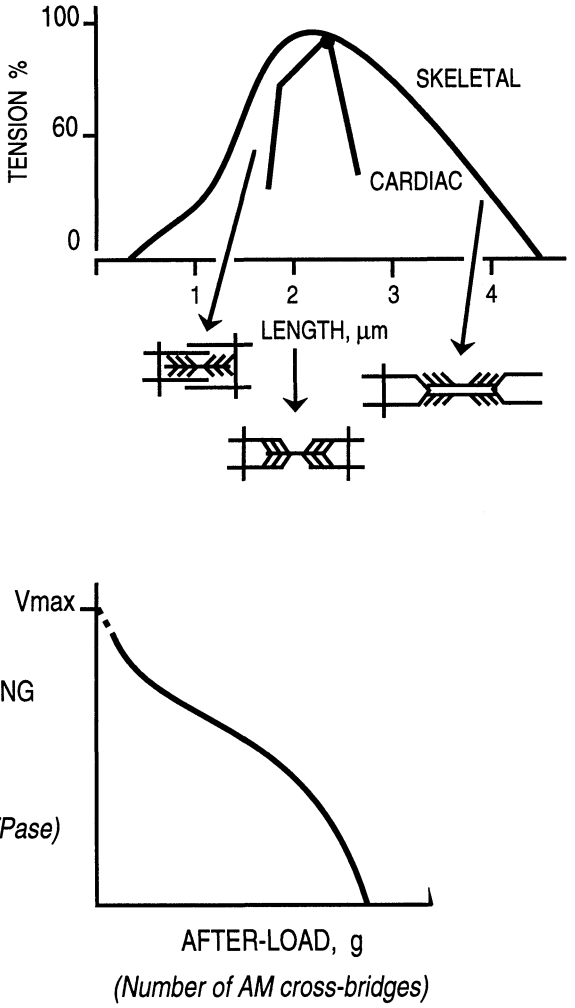


Fig. 35 Physiological-Molecular correspondences.
Upper : Active Tension/Length curve. Lower : Velocity/Length curve.

Table 7. Molecular basis of contractility. Cardiac versus vascular smooth muscle.

	Heart.	**Smooth muscle.**
Contraction.		
Type	Phasic	Tonic
Refractory period	+	0
Contractile proteins.		
Myosin Heavy Chains	α and β	1 and 2
P Myosin LC2	2_V and 2_A	20kD, 2 isoforms
Alkali Myosin LC2	1_V and 1_A	17kD, 2 isoforms
Actin	α-card/skel	α–smooth
Tropomyosin	card α2, β	smooth α 3, β
Troponins C and I	cardiac specific	0
Troponin T	several isoforms	0
Myosin LC Kinase activity	poor	important
Calponin	0	+
Caldesmon	0	+

polymorphic, but the physiological significance of this polymorphism has not yet been explored in detail. The contractile activity of the smooth muscle is also regulated by calcium, nevertheless the mechanism of the regulation is different since there is no troponin in the smooth muscle.

In smooth muscle the regulation of contraction depends upon the state of phosphorylation of the phosphorylatable myosin light chain.

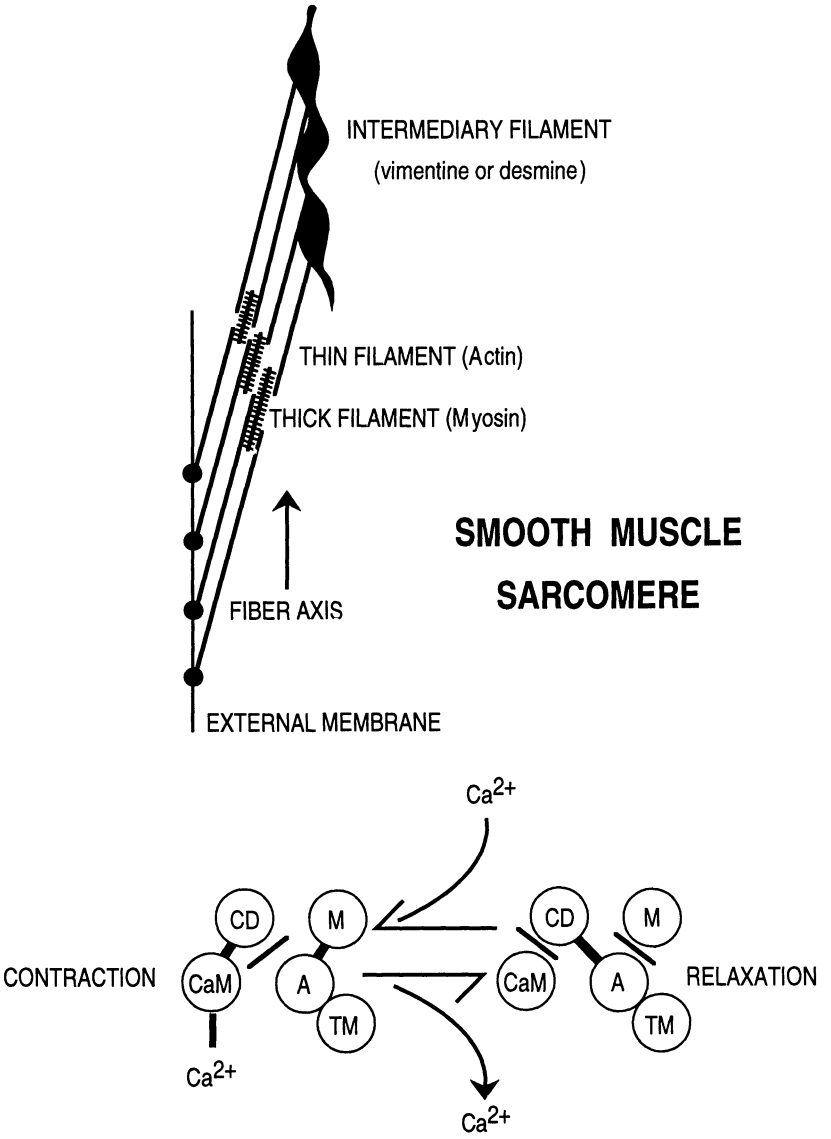


Fig. 36 Structure of the smooth muscle.
Upper : sarcomere. Lower : regulation of contraction

Phosphorylation of this subunit is the signal that increases the rate constant of the rate-limiting step in the actomyosin ATPase cycle (see Fig. 34), activates the cycling of the cross-bridges and initiates the mechanical event. The light chains are phosphorylated by a specific kinase (MW 160 kD) which is activated by calcium through the calcium-binding protein, calmodulin. Myosin light chain phosphorylation is correlated both with the intracellular calcium concentration and the velocity of muscle shortening. The affinity of the myosin light chain kinase for calmodulin decreases when the kinase is itself phosphorylated by protein kinase C. This mechanism explains the vasodilatory effects of adrenergic agonists. A characteristic feature of smooth muscle is that during the contraction cycle, muscle tension increases more slowly than shortening and is maintained at a reduced ATP consumption while the calcium concentration decreases. This so-called "latch state" reflects a very specific regulation of contraction and may be related to other proteins. Caldesmon, CD (Figure 36), is one of these, it is a phosphorylatable calmodulin-dependent protein which competes with myosin on actin. When calcium binds calmodulin, CaM, caldesmon can no longer bind actin and contraction occurs.

To summarize, the cardiac smooth muscle is made up of specific contractile proteins different from those of the myocardium. Its contents less myosin, and more actin than the striated muscle. The regulation of the smooth muscle contraction is located on the thick filament, because smooth muscle does not contain troponin, and involves the cAMP and calcium-dependent phosphorylation of a specific myosin light chain.

CALCIPROTEINS AND CALCIUM MOVEMENTS

Calcium is a major intracellular messenger which not only plays a role in muscles, but also in nearly all other cells. In cardiac and vascular smooth muscle, the function of calcium is to transmit the electrical signal to the sarcomere in order to trigger the mechanical event. In all cells, including myocytes, calcium has two origins: the extracellular space and the endoplasmic reticulum. The importance of these two sources varies from one type of muscle to an other, but also for a given type of muscle, from one animal species to the other. For example, the fast skeletal muscle of a rabbit is able to contract in a calcium-free medium because all the calcium used for contraction comes from the Sarcoplasmic Reticulum, SR. On the other hand, in the frog ventricle that possesses very little SR contraction is

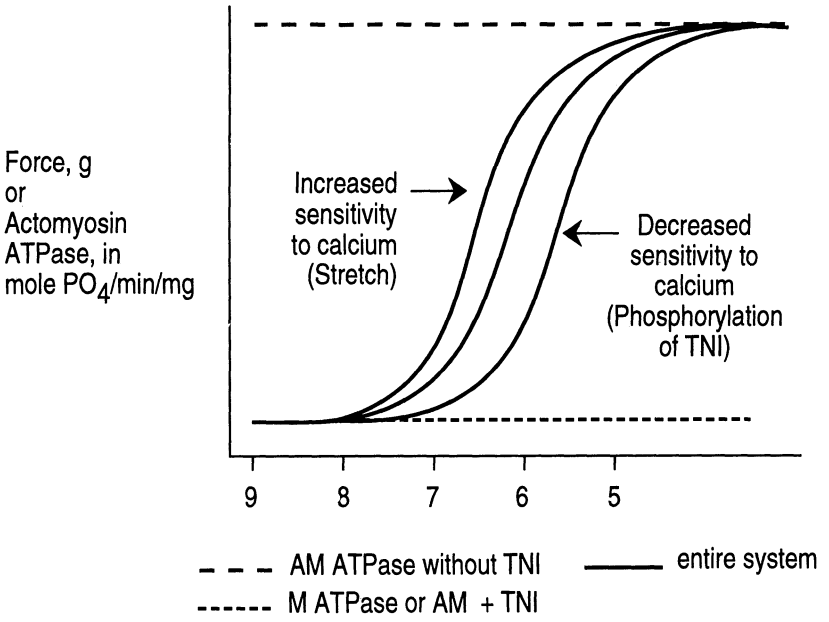


Fig. 37 Calcium transient

triggered only by external calcium. The rat ventricle is close to the skeletal model, nevertheless external calcium plays a role and the muscle can't

contract in a calcium-free medium. The human ventricle also uses both sources of calcium.

Such phylogenic differences explain why the biological process of adaptation to mechanical overload is not the same in every species.

Intracellular calcium homeostasis.

The intracellular concentration of calcium is maintained between 10nM and 10µM. The so called physiological calcium transient is located between these two limits (**Figure 37**). Figure 37 shows simultaneously the effects of a variation in calcium concentration on force in a membrane-free (skinned) preparation, and also on actomyosin ATPase activity, the two curves superimpose. The upper limit is imposed by the intracellular concentration of free phosphate which is around 10µM. Such a concentration is high enough to precipitate calcium as calcium phosphate when the intracellular concentration of free calcium reaches10µM. Calcium homeostasis is obtained as long as the free intracellular concentration of calcium remains below such a threshold. During acute ischemia, the external membrane becomes permeable to calcium and the intracellular concentration of calcium exceeds the threshold of 10µM, this results in the formation of calcium phosphate which precipitates as calcifictions around the infarcted zone.

The calcium transient is an increase in intracellular in calcium concentration that is triggered either by a change in voltage or by chemical mediators. It originates from both the extracellular space and the intracellular stores (**Figure 38**). Calcium concentration in all cells peaks around 1µM, and then binds calciproteins (**Figure 37 & 38**) to activate contraction and

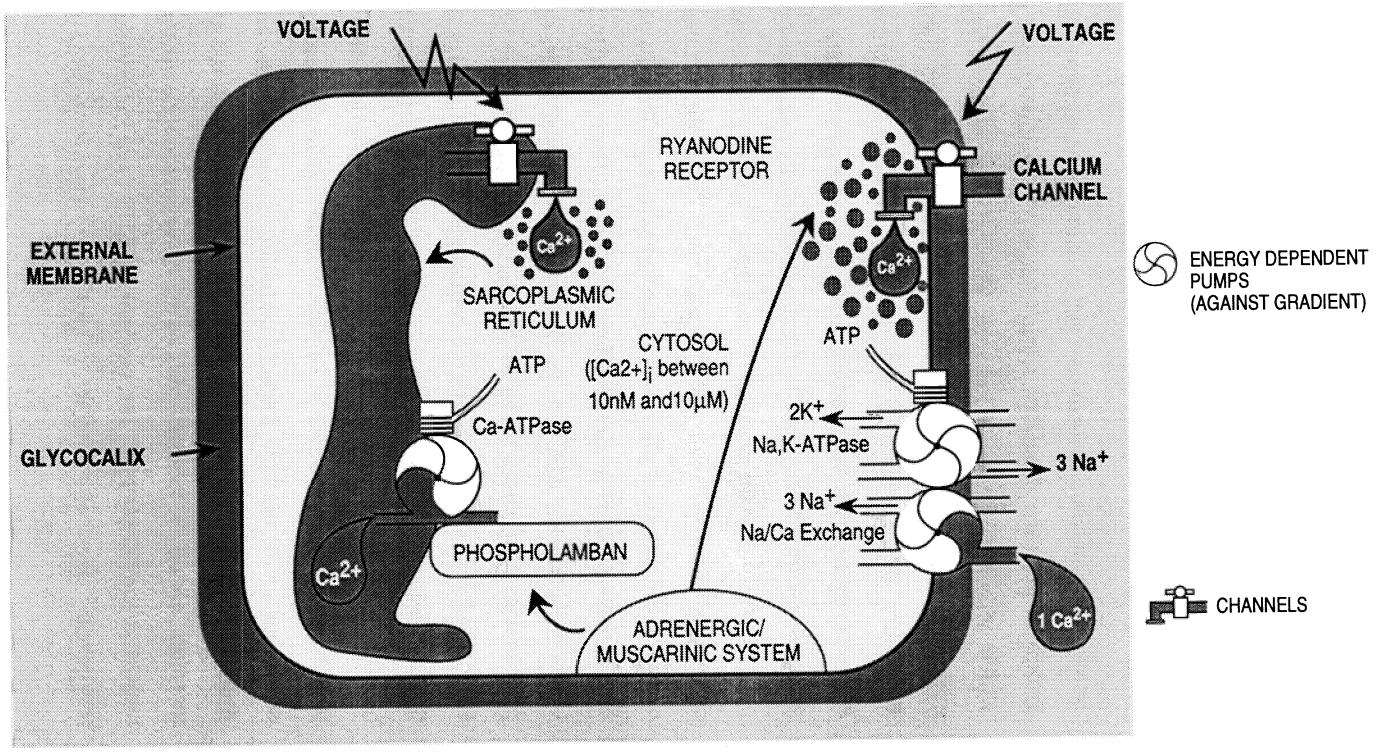
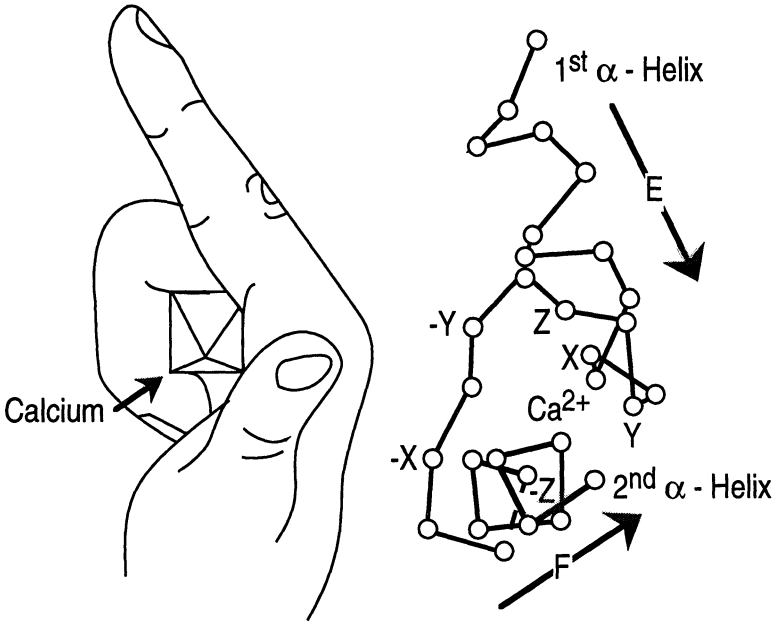


Fig. 38 Main Determinants of the intra-cellular calcium homeostasis

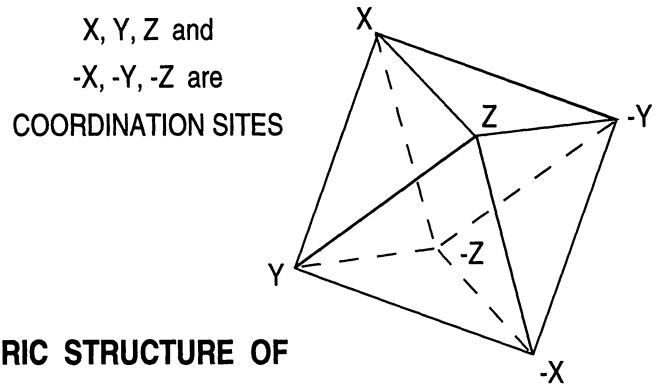
metabolism. Consequently, the calcium concentration returns rapidly to low levels, around 0.1μM, owing to active mechanisms located either on the external membrane or on the SR.

Determinants of calcium movement.

In any tissue, calcium homeostasis and calcium transient depends upon two groups of proteins: those that are in charge of calcium uptake, and those which activates release of calcium. The calcium gradient between the cytosol and either the extracellular compartment or the Sarcoplasmic Reticulum, SR, is around 20,000 nM which means that calcium does not need energy to enter the cytosol, it is only necessary to gate a channel, wherease calcium release needs directly or indirectly a lot of energy to pull out calcium against the gradient (Figure 38). The two gates responsible for calcium uptake are calcium channels located either on the Sarcolemma, SL, or on the SR, the latter are called ryanodine receptors. The Ca channels of SL are voltage-gated in all cells, but, in addition, there are also Receptor-Operated Channels, ROC, in the blood vessels. In the heart, the sinusal pace-maker transmits a wave of depolarization through the conduction system to every Ca channels in the cardiocytes. The voltage change gates the channels and allows calcium to enter the cell and triggers contraction both directly and through the ryanodine receptors (Ca-induced Ca-release phenomena). The contraction of smooth muscle cells in the vessels depends of various mediators (see further) including norepinephrine, endothelin, Angio II... Most of the corresponding receptors, such as adrenergic receptors, are on the terminal ends of the nerves and only exist on the cells located at the periphery of the vascular wall. In these cells contraction is triggered by the ROC, and is then propagated to



E-F HAND STRUCTURE
OF A CALCIUM BINDING SITE



OCTAEDRIC STRUCTURE OF
CALCIUM COORDINATION SITES

Fig. 39 Calciproteins [redrawn from Kretsinger et al 1975].

voltage-operated Ca channels of other cells which are located more deeply in the vascular wall. Vascular ryanodine receptors are also activated by various mediators and are sensitive to IP3.

Ca^{2+} release also depends on both SL and SR. At the level of the SL, there are two mechanisms (Fig. 38) : the Na^{+}/Ca^{2+} exchange that is able to both release and take up calcium, but is usually working as a Ca^{2+} release system, and a Plasma Membrane Ca^{2+} ATPase, PMCA. The exchanger indirectly uses the energy of the Na^{+}, K^{+}-ATPase, the sodium pump, since it needs a low internal sodium concentration to be allowed to do its job. Digitalis, by blocking the Na^{+}, K^{+}-ATPase, increases the calcium concentration through the Na^{+}/Ca^{2+} exchanger. PMCA is rather poorly active in the heart, but the smooth muscle isoform is very active. Calcium uptake from the SR depends upon a Ca^{2+}ATPase which is, in both the smooth and the cardiac muscles, regulated by phospholamban, a cAMP dependent phosphoprotein.

Calciproteins.

Phospholipids or DNA bind divalent cations, nevertheless, because of the high intracellular Mg^{2+} concentration (1 mM), proteins are the only molecules that bind calcium with a pKd (Ca) of 5-7. Calcium-binding proteins, or calciproteins, have either a high capacity and low affinity for Ca^{2+}, or low capacity but high affinity. Calcium modifies their properties and/or stability. Finally, both the function and interactions of the protein depends on calcium binding.

It has been demonstrated that a large number of calciproteins possess one or several calcium binding site which have a common consensus amino acid sequence, termed the E-F hand structure (**Figure 39**), and includes : two turns of α-helix, a 12 residue loop containing six calcium-coordinating ligands that maintain calcium in a pocket, and again two turns of α-helix. A model has been proposed, based on the structure of

parvalbumin, a calciprotein with very high affinity for calcium. It is generally admitted that the affinity for calcium of a given binding site is higher when the structure is close to the model.

Calciproteins, include parvalbumin (a Ca^{2+} transporter in certain muscles), troponin C, the non phosphorylated myosin light chain that has incomplete E-F hand regions, suggesting that calcium affinity is low, calsequestrin, and, very likely, the ATPase of SR. Calmodulin has 4 homologous divalent cationic binding sites and is active as (3 Ca^{2+}-1 Mg^{2+}). It is the cofactor of a large number of enzymes, including myosin light chain kinase (predominant in the vessels), phosphodiesterase, adenylate cyclase, glycogen synthetase and phosphorylase, phospholamban, and the protein responsible for microtubular disassembly.

The E-F hand structure is not the only structure able to bind calcium. Other consensus structures for a calcium-binding site have been reported, including that discovered by Geisov [Nature, 320, 636, 1986] which consists of 17 aminoacids which form a loop followed by an α-helix and is found in calcimedin, endonexin, calelectrin, calpactin and lipocortin.

COLLAGEN, EXTRACELLULAR MATRIX AND COMPLIANCE.

Extracellular Matrix. Role. Composition.

Cardiac myocytes and capillaries are surrounded by an extensive connective tissue lattice work, or endoskeleton, which is mainly composed of collagen, but also contains glycosaminoglycans, elastin and fibronectin. Extracellular matrix is certainly much more dynamic than previously believed and is not only a support for myocytes and an extracellular skeleton, but also controls the morphology and phenotype of cells, specially durin development. Fibronectin is particularly known to affect cell adhesion, migartion and

cytodifferentiation and is implicated in organogenesis. Three levels of myocardial connective tissue have be described (**Figure 40**): epimysium which envelops the entire cardiac muscle, perimysium which associates groups of cells and endomysium that supports and interconnects individual myocytes [Brilla et al. 1990, Weber et al. 1989]. Each myocyte is linked to adjacent cells by struts of collagen of 150 nm diameter which run perpendicularly to the basement membranes and prevent excessive elongation or shortening of the cardiocytes. They also synchronize myocyte contraction by uniformly disseminating tension so that cells are equally elongated during contraction and store energy during the cardiac cycle. Therefore collagen plays a major physiological role during contraction and relaxation by coupling the

Table 8. Functions of the myocardial and vascular collagen network [From Weber et al. 1993 and Tedgui et al. 1994].

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1. Connections between myocytes to maintain cardiovascular architecture.

2. Collagen forms a rigid network around the cardiac myocytes and the vascular wall and prevent excessive stretch.

3. Transmission of force generated by contractile elements to the chamber.

4. Storage of energy during diastole in the heart and during diastole in the aorta. In the aorta, during diastole energy is used to deliver blood to the periphery through the elastic fibers.

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mechanical activity of the different contractile units. Each capillary is equally interconnected with surrounding myocytes by another type of struts (Figure 40) whose role is to maintain the capillary flow during systole. The

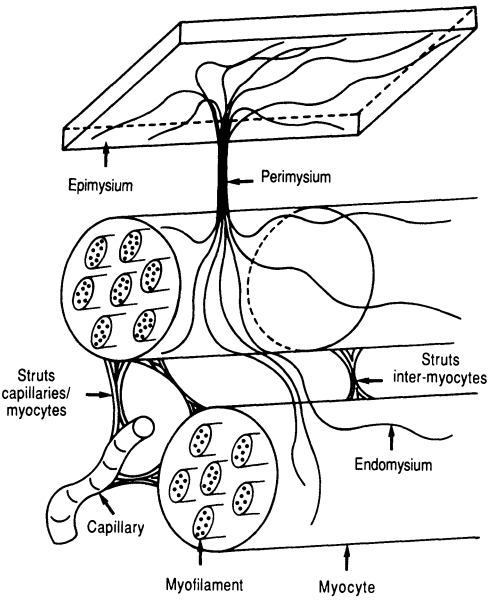

Fig. 40 Structure of the collagen network (From Swynghedauw, 1990, with permission).

presence of a normal collagen matrix is essential during the development, and blocking collagen synthesis by treating new-born rats with β-aminopropionitril results in multiple areas of necrosis and aneurism of the ventricle.

Collagen represents 3% of the myocardial mass, as compare to 50% for the muscle itself. Myocardial collagen is routinely quantitated by quantitative morphology or by measuring the amount of hydroxyproline, an amino acid specific for this protein. The myocardial collagen matrix consists primarily of type I collagen (85% which aggregates into thick fibers), and type III collagen which forms thinner fibers and represents 11% of the total collagen content. Valve leaflets contain 20% type III collagen. Type IV collagen is aminor component and is included into arterial wall intima and also basal membrane of cardiomyocytes. Arterial walls contain type IV collagen in intima and cardiomyocytes posses. The media contains lamellar units which are composed of muscle cells surrounded by elastic

fibers made of elastin, and maintained in order by collagen fibrils. As in the heart, the collagen is mainly type I and III.

Collagen I has the tensile strength of steel [Weber et al 1993]. Collagen plays a major physiological role as detailed in **Table 8.** Collagen is a determinant of both ventricular and arterial stiffness, and the collagen content of the LV is correlated with the tissue compliance (which is different from chamber compliance and is calculated from the stress/strain relationships). Cardiac fibrosis is due to an increased collagen concentration and occurs in three different conditions : senescence, after ischemia or under the influence of hormones (Angio II, catecholamines and aldosterone) [Weber et al. 1989]. It renders the myocardial tissue less compliant and favors arrhythmias by both increasing tissular heterogeneity and fragmenting the passage of ventricular excitation. Fibronectins are another important component of the extracellular matrix both of the heart and the blood vessels. Fibronectin is a 500 kDa dimeric glycoprotein which originates either from exsudation of soluble plasma fibronectin, pFN, or from the local synthesis and secretion of a cellular form, cFN. Both forms are synthesized from a single gene and are composed of three types of repeats, types I, II and III. pFN and cFN differ in the number of their altenatively spliced sequences, the IIIA and B repeats being specific for cFN. The expression of these different form of fibronectin are developmentally regulated and specifically reexpressed in adult in hypertrophied heart. FN has specific domains of binding to collagen and to cell receptors, namely integerins which are firmly attached to cytoskeleton.

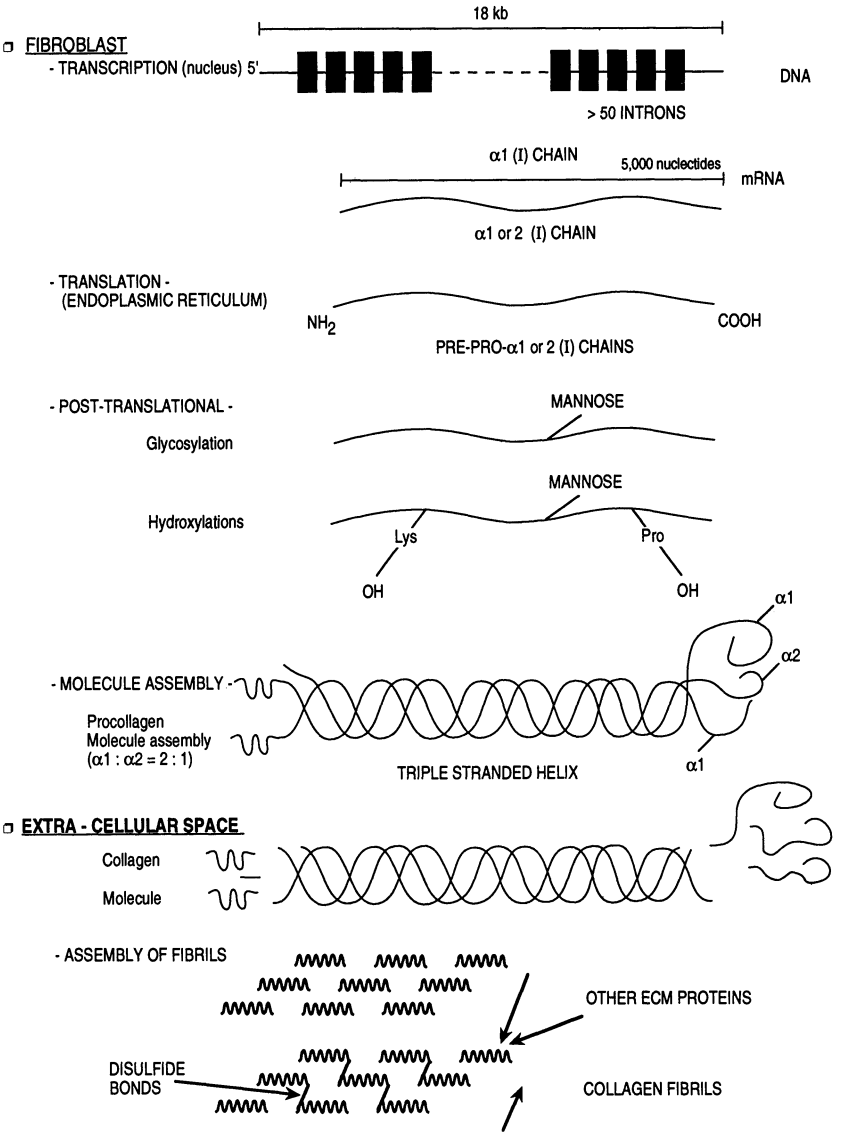

Fig. 41 Type I Collagen Synthesis
[redraw from a scheme from Valérie Robert with permission]

Collagen genes. Collagen synthesis and degradation.

Type I collagen is a long (300nm) and bulky heteropolymer, consisting of three chains (or subunits), 2 α1(I) and one α2(I), which form a triple-helical domain. Each chain is made up of more than a thousand amino acid residues. The genes encoding the α1 and α2 chains are different, but similar in structure since they both contain more than 50 exons. In man, the locus for α1 is on chromosome 17 and consists of approximately 18 kb, the gene of α2 is on chromosome 7 and consists of 38 kb of sequence.

Collagen is both synthesized and degraded by fibroblasts, and attempts to demonstrate collagen synthesis in myocytes have been unsuccessful. The biosynthesis of collagen is a rather complex phenomena, and, at least in the cardiovascular system, consists of a poorly explored cascade of events (**Figure 41**). Collagen half-life is unique with respect to its length, which is approximatly 100 days, as compared to that of myosin for example which is around 7 days. In some tissues the half-life can even be much longer. This is due to both a slow rate of synthesis (0.56% per day in the ventricle) and degradation.

The initial translational products, prepro-α1 (I) or 2 (I) chain, are translocated in the endoplasmic reticulum of the fibroblasts, cleaved into pro-α1 (I) or 2 (I) chain and then several post-translational modifications take place, including hydroxylations and glycosylation. These modifications are a necessary prerequisite for stabilisation of the structures and assembly of the chains into a molecule which is then secreted into the extracellular space to constitute fibrils in the extracellular matrix (Figure 41).

The packing of collagen molecules into fibrils is associated with the formation of covalent crosslinks between the molecules (Figure 41). The molecules are arranged in parallel and in register with respect to one another. They are separated by regular gaps and assembled into a matrix that contains several other extracellular matrix molecules.

PEPTIDES REGULATING CARDIAC OR VASCULAR FUNCTION.

There is, for the moment a huge number of factors, which are mostly peptides, that play a role in the regulation of cardiac and vascular function, obviously some are still unknown and the list is far from being complete.

Renin-Angiotensin System, RAS.

This system is the most popular because of the discovery of two class of drugs that interact with one of its components. Numerous symposia had also rendered the RAS very familar to clinicians. This area is becoming exciting both because it is a major target for pharmacological research and a major goal for detecting genetic abnormalities in essential hypertension. In addition the recent discovery of various receptor subtypes and the trophic role of angiotensin II open new ways for exploring the physiopathology of various cardiac diseases including heart failure, myocardial infarction and hypertensive macroangiopathy.

The RAS is a proteolytic pathway that transforms an inactive peptide, Angiotensinogene, Angne, into an active peptide, Angiotensin II, Angio II. Angio II is a potent vasoconstrictor playing a major role in the physiological regulation of blood pressure (**Figure 42**). There is an intermediary step which consists in the transformation of Angne by a protease, renin, into a smaller peptide, Angiotensin I, Angio I. Angio I is in turn degraded by a second metallopeptidase, Angio I Converting Enzyme.

Structure of RAS Components.

Angiotensinogene. The protein is a rather bulky globular protein (MW 55-65 kd) found in the plasma, and mainly synthesized in the pericentral zone

of liver globules, but also in the myocardium (mainly in the atria) and arterial walls. The gene is unique and composed of 5 exons and encompasses 13 kb. The 5' flanking region of the gene contains three Glucocorticoid Responsive Elements, a Thyroid and an Estrogen Responsive Element. These structural data were confirmed by physiological studies that demonstrated an increased plasma level of Angne following stimulation by the above hormones. The liver content in Angne mRNA increases after bilateral nephrectomy or when the plasma level of Angio II is enhanced, nevertheless, as a rule for the different components of the RAS, Angne mRNA is differentially regulated in various tissues.

Renin is a single chain 37-40 kb glycoprotein enzyme, found in plasma (routine quantification includes either immunological quantification of the protein, or enzymatic detection of Plasma Renin Activity) mainly synthesized in the juxtaglomerular apparatus in the afferent arterioles of the kidney. Renin is specific for Angne and the unique enzyme responsible for transforming Angne into Angio I. The gene is unique in human (but in rats there are two genes) and is composed of 10 exons and encompasses 12.5 kb. During the first step of translation the renin mRNA firstly yields a preprorenin, and then a prorenin that appears in the circulation where it is further transformed into renin. The 5' flanking region of the gene contains DNA consensus sequences specific for cAMP, glucocorticoid, estrogen and progesterone, and the plasma level of renin is consequently hormonally regulated. The Angne renin mRNA is differentially regulated in various tissues.

Angiotensin Converting Enzyme, ACE. ACE is a dipeptidyl metallo (that binds Zinc to be activated) cardoxypeptidase, in contrast with renin, ACE is a rather non specific protease that not only transforms Angio I into

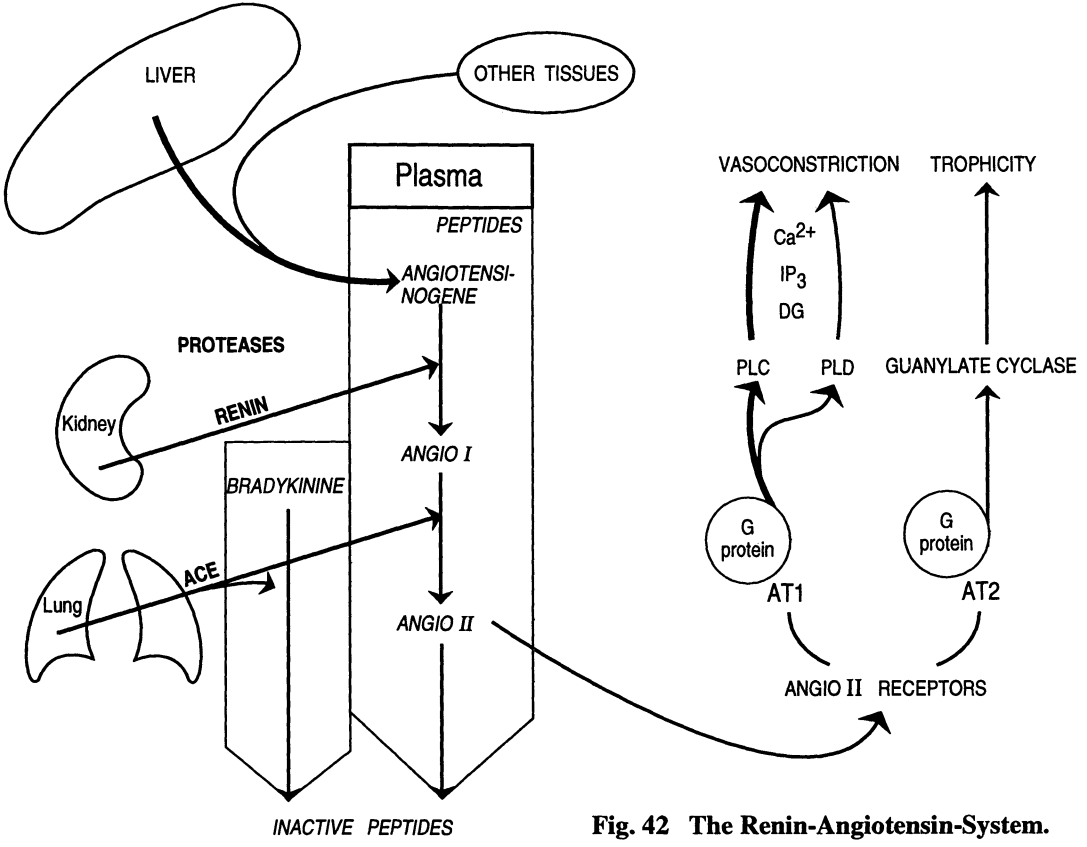

Fig. 42 The Renin-Angiotensin-System.

Angio II, but also can degrade bradykinin into an inactive form. Consequently ACE inhibition not only results in a lower plasma level of Angio II, but also in an increased concentration in bradykinin which is a potent vasodilator. ACE exists as two different isoforms, the endothelial form (MW 140-160 kd) and the testicular form (MW 90-100 kd), which are encoded by two different isomRNAs (4.5-5 kb and 2.6 kb) that are transcribed by alternative splicing (and initiation) of a single gene. The gene is composed of at least 6 exons. The ACE gene has consensus DNA sequences specific for glucocorticoid and cAMP, and indeed physiological studies have demonstrated that the expression of the gene is hormonally regulated.

AngioTensin II, AT, receptors. AT receptors are going to become a major target for therapy since several specific inhibitors have now been proposed. The availability of these compounds has provided definitive evidence for the heterogeneity of AT receptors: the AngioTensin II receptor subtype 1, AT1, is specifically blocked by Losartan and subdivided into AT1A and B (both are coded by two different genes located on chromosomes 17 and 2 respectively), while AT2 is inhibited by PD 123177, PD 123319 and CGP 42112A (the latter also has an agonist effect). Both belong to the R7G family. (i) AT1 mediates vasoconstriction, aldosterone secretion, Angio II-induced water drinking, tachycardia and plays the major role in controlling blood pressure. Activation of AT1 receptors result in a biphasic increase of the calcium transient that corresponds to an initial release of calcium from the calcium stores and a furthera ctivation of the inward current. AT1s are coupled to Phospholipase C through a Gq protein and by so doing activate the Phosphoinositol cycle and hydrolyze phosphatidylcholine which in turn mobilizes intracellular calcium concentration and reduces intracellular pH. they are also coupled to phospholipase A2 and D. (ii) AT2 binding sites are present in adult tissues, but are more abundant in embryonic and neonatal tissues and it has been suggested that such a receptor subtype mediates growth and should be

responsible for the well-documented trophic effects of Angio II. The AT2 receptors are possibly negatively coupled to guanylate cyclase, but this is still debatable, AT2 receptors have been identified in both the heart and the aorta. The role and mechanism of action of AT2 are still debatable.

Circulating and Tissular RAS.

There are now several evidences for a vascular and a myocardial RAS. All of the components of the RAS have been demonstrated by molecular biological techniques to be present in the arterial wall and myocardium, including renin, Angne and ACE. In addition, converting enzyme inhibition is still capable of lowering blood pressure after blocking circulating Angio II with appropriate antibodies. The functional role of the tissular RAS is likely to be of minor importance in normal conditions, nevertheless this system may play a more significant role under pharmacological influences or in certain conditions such as ageing.

Atrial Natriuretic Factor, ANF.

The first demonstration of the regulation of the atrial system by modifications in sodium balance or volume belongs to PY Hatt's group [Marie et al. 1976] who demonstrated that sodium restriction results in an increased granular appearance in the atria whereas high sodium diet, desoxycorticosterone, or chronic volume overload decreases granulation density. The ANF was further identified and the corresponding gene cloned. ANF gene is located on chromosome 1 in man, and is translated into a preproANF and a proANF. In man active ANF is in a 28 aminoacid peptide which is stored in granulations as a propeptide. In adult mammals, the ANF mRNA is quite abundant in both right and left atria, and rare in other tissues. ANF production is developmentally regulated, and the ventricular level of ANF mRNA during embryogenesis is comparable to that of atria in adults. ANF production is activated by atrial distension, but also by several

hormones such as glucocorticoids and thyroid hormone. Mechanical volume overload in atria, and pressure onverload in ventricles induce the production of ANF mRNA and secretion of the peptide by the myocardium. The putative regulatory region was located 2.5 kb upstream of the coding part of the gene.

ANF is a potent diuretic which enhances natriuresis by decreasing the proximal tubule reabsorption of sodium, and inhibitng aldosterone production. In addition ANF has a vasodilator activity. Thus it opposes nearly every effect of Angio II. ANF exerts its effects through specific receptors and the cGMP pathway.

Endothelium-Derived Vasoactive Factors.

It is now well-established that a number of vasoactive factors indirectly induced vasodilatation or vasoconstriction through the release of substances produced by the vascular endothelium. Endothelium-dependent relaxation plays a major physiological role in maintaining vascular tone. It is dominated by the effects of short-lived substance(s) produced as Nitric Oxide, NO, released by several vasoactive factors by the endothelial cells and acting on smooth muscle guanylate cyclase. Acetylcholine, for example, indirectly induces vasodilatation, but requires the presence of an intact endothelium. Prostacyclin formation (PGI_2) also plays a role in vasorelaxation, but this a minor role as compare to NO.

Endothelium-dependent contraction is much more complex and includes the production of a variety of different compounds including endothelin and locally synthesized Angio II, but also prostaglandin H_2 and thromboxan A_2, which all are acting in a second step, but by different mechanisms, on smooth muscle cells. It may play a physiological role

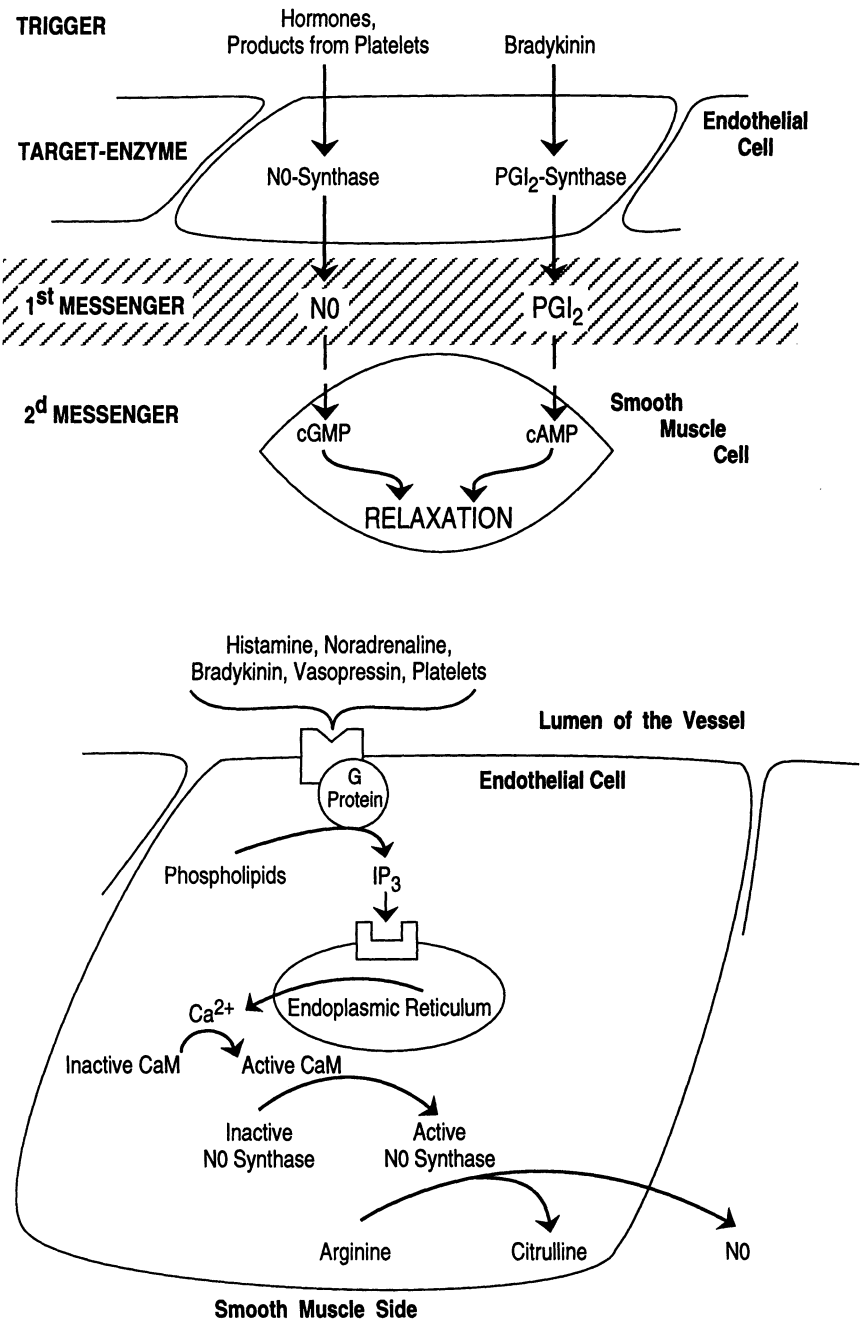

Fig. 43 Endothelial-Dependent Factors causing Vasodilatation.
Upper : endothelial cascade. Lower : NO Production

during autoregulation of regional flow, but is mainly involved in pathological states.

Endothelium-dependent relaxation.

Endothelial cells produce at least three relaxing factors, including EDRF/NO (Endothelium Dependent Relaxing Factor/Nitric Oxide), which is the most important, prostacyclin PGI_2 (**Figure 43**) and a hyperpolarizing factor (EDHF). A clinician has to be aware of the recent developments of research in the field of NO, because there are several drugs which act through such a mechanism, as Molsidomine for example which is a NO donor.

EDRF/NO is the major physiological regulator of systemic resistance vessels tone since inhibition of the NO production decreases local blood flow. Concordant observations suggest indeed that arterial blood pressure is continuously regulated by NO which is formed and released from the arterial endothelium. In cardiovascular pharmacology NO is the compound directly responsible for coronary-dilatation induced by nitroprusside or indirectly responsible for vasorelaxation after treatment by nitroglycerine. NO has a potent vasodilatory effect, but is also a trophic factor which inhibits cell proliferation. NO, is a regulator molecule for endothelial cells of blood vessels (and also leucocytes and neurons) and is formed on demand by the action of NO Synthase on Arginine or directly by the shear stress [Dinerman et al. 1993, Shepherd et al. 1991]. The most important target of NO is the soluble guanylate cyclase in smooth muscle cells. NO stimulates this cyclase as a consequence of the reaction of NO with the haem prosthetic group of the cyclase to form nitrosohaem. The result is an over 50-fold greater rate of cGMP synthesis than that catalysed by unnitrosylated cyclase. The resulting rise in cGMP is responsible for relaxation.

In endothelial cells, NO Synthase is activated by various compounds, including acetylcholine, bradykinine, histamine, arginine, vasopressine,

norepinephrine and several substances released by platelets, which all enhance intracellular calcium concentration through a cascade of events which involves the phosphoinositol cycle and the IP_3 production (**Figure 43-44**). IP_3 binds to an IP_3 receptor on the endoplasmic reticulum and then releases calcium from endogenous stores as explained above (this important mechanism is reviewed in detail by Williamson et al 1991). Calcium activates NO Synthase through calmodulin which is a calciprotein (see above). NO Synthase has been molecularly cloned and is a polymorphic molecule which can exist as three isoforms, each being specific for different tissues. The gene structure displays several binding sites : for oxidative factors as NADPH, Flavin Adenonucleotide and sites for heme and calmodulin. NO synthase is a phosphorylatable enzyme and can be phosphorylated by a cAMP-dependent protein kinase C which attenuates the enzyme activity. NO is generated in the endothelial cells and released into the extracellular space. It generates cGMP in the smooth muscle cells. cGMP elicites muscle relaxation through its influence on calcium release from the endoplasmic reticulum and calcium influx through receptor-operated calcium channels.

Prostacyclin PGI_2 is another less important short-lived relaxing factor released by the endothelium, it is the major product of the metabolism of arachidonic acid by cyclooxygenase and its endothelial production is also activated by various factors including thrombin, bradykinin or shear stress (**Figure 44**). The physiological effects of prostacyclin are mediated by cAMP. Nevertheless inhibition of prostacyclin production, unlike inhibition of NO production, does not have any substantial effect on blood flow.

Differences exist between arteries and veins and even from one region to another. For example, thrombin relaxes large epicardial arteries in dogs by releasing EDRF, while it constricts the deep coronary resistance vessels and contracts veins.

Endothelium-dependent contraction.

Endothelial cells produce and release several vasoconstricting substances that act through, at least, four different pathways: the Renin-Angiotensin System, Cyclooxygenase-dependent endothelium-derived contracting factors, endothelin (**Figure 45**) and pO_2.

Preproendothelin is encoded by a gene located on chromosome 6 in man and is sequentially transformed to proendothelin and then to endothelin by an endothelin converting enzyme [Lotersztajn 1993]. This is again a major target for pharmacological research in cardiology and as such has to be known by physicians. Endothelin is a 21 amino acid peptide and its production is transcriptionally regulated and activated by thrombine, cytokines, epinephrine, vasopressine, Angio II, endotoxine and shear stress forces. ANF and relaxing factors such as NO and prostacyclin reduce its production. There are three different isoendothelins, called 1, 2 and 3, that are encoded by three different isogenes. Endothelin is different from Angio II in terms of its vasoconstrictor effects since a single injection of endothelin results in a rapid and short episode of vasodilatation followed by a long-lasting period of hypertension for 2-3 hours. The receptors are G protein-dependent receptors containing seven spanning domains and belong to the R7G family described above. The ET_A receptor is located on smooth muscles cell and is responsible for endothelin-induced vasoconstriction. By contrast, the ET_B receptor is on endothelial cells and activates NO synthase or IGI_2 formation, and is responsible for the short episode of vasodilation. The second messenger pathway is rather complex since endothelin and the corresponding endothelin receptors may act through nearly all the known pathways including cAMP production, PI cycle and arachidonic acid (Figure 44). This complicated issue is poorly informative in terms of clinical

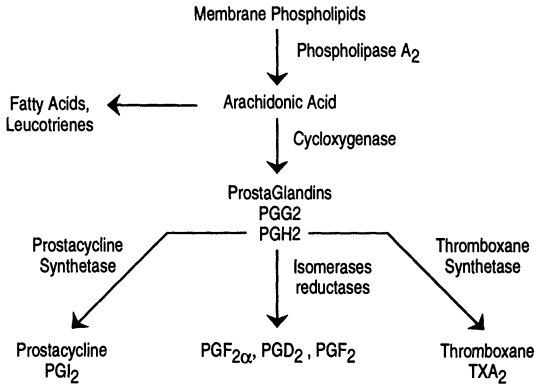

Fig. 44 Metabolism of Arachidonic Acid and Prostaglandins.

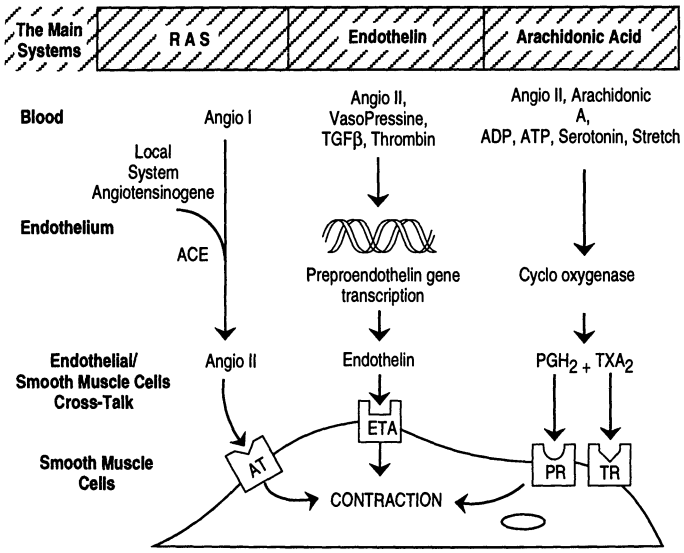

Fig. 45 Endothelial-Dependent Factors causing Vasoconstriction.

practice, nevertheless it suggests that this topic become a future domain in terms of pharmacological research. It is very unlikely that endothelin plays a role in maintaining blood pressure since inhibition of proendothelin production has no effect on blood pressure, but does certainly play a role in pathological conditions.

Inhibitors of cyclooxygenase, such as indomethacin, can block endothelium-dependent contractions. Conversely endothelial-dependent contractions can be induced by arachidonic acid, stretch, serotonine, or even by acetylcholine (which is normally a vasodilator) in arteries from Spontaneously Hypertensive Rats.

Endothelial cells/Cardiocytes cross-talk.

This new area of research has received recently considerable attention. Pionneer works (reviewed in Brutsaert et al 1992) have clearly demonstrated that the selesctive destruction of the endocardial endocardium induced abbreviation of isometric twitch duration with isometric force declining sooner during relaxation. Both a release of endocardial substances with inotropic properties and a trans-endocardial endothelial physicochemical control are postulated as possible underlying mechanisms. Endocardial and coronary endothelii are also likely to play an important role in the regulation of cardiac hypertrophy.

The kinin-kallicrein system.

The system is very similar to the Renin-Agiotensin System (Fig. 42) and includes an active peptide called bradykinin which has a potent endothelium-dependent vasodilatory effect. Circulating bradykinin is inactivated by ACE, therefore converting enzyme inhibitors can inhibit bradykinin degradation and also produce vasodilatation through this additional mechanism. The precursor of bradykinin is kallicrein which is synthesized in the kidney, this

organ is the only one to produce and degrade kinins. The kinin-kallicrein system is likely to exist as a tissue system, as the RAS.

CARDIAC AND VASCULAR GROWTH.

Cardiac hypertrophy is a clinical problem of crucial importance that will be described in detail in the next chapter, vascular hypertrophy is however also important and it is now well-documented that the vascular wall thickens in response to chronic arterial hypertension which constitutes both an important risk factor and a major target for drug therapy.

Cardiac and vascular (mainly arterial and arteriolar) hypertrophy of pathological origin results from the abnormal triggering of the normal growth process. It is therefore important to know the main avenues used by the cardiovascular system for growth and development. The topic is becoming popular and the amount of informations relative to growth signals in the heart and in the arteries is increasing every month.

A major problem in this field of investigation comes from the complexity of the situation. Arterial hypertension, and also the physiological regulation of blood pressure, for example, are complex situations resulting from the simultaneous action of several hormonal and mechanical factors. Both pure mechanics and hormones or peptides may have a trophic effect, and it is sometimes impossible to know what is really determinant in the growth process. The question is far from being academic since the two factors are accessible to therapeutic intervention.

Growth of the cells.

Cardiac myocytes are terminally differentiated cells incapable of division in adults. In man, cardiac hypertrophy induced by mechanical overload is due to an increase in the size of the cardiocytes with an unchanged number of the

cells, in this condition non-muscular cells (endothelial, fibroblasts, cells of blood origin..) increase both in number and size. Adult cardiocytes never divide, at least by mitosis, whereas non-muscle cells are still able to produce mitotic figures. Growth during the foetal period is different and results from both hyperplasia and hypertrophy of all cells, including the cardiocytes.

Vascular endothelial cells are able to replicate in the adult. Smooth muscle cells of the small arteries usually become hyperplasic when submitted to mechanical stress, by contrast the same cells in the aorta are unable to divide and, the response to mechanical overload is hypertrophy without cell division. Nevertheless, such a phenotypic response depends on the experimental conditions and there are conditions during which smooth muscle cells of the aorta can also proliferate [Tedgui et al. 1994].

Protein Synthesis.

It is usual to describe the process of growth in three steps: the triggers, the growth pathways, and the targets (**Figure 46**) [Swynghedauw 1986, van Bilsen et al 1991, Tedgui et al 1994]. These steps are remarkably similar both in the heart and the vessels. Obviously there are also process which are posttranscriptionally regulated, however for the moment these have only been poorly documented, in addition the expression of the genes coding for translational factors has to be regulated.

Triggers and pathways.

By trigger, we mean every event able to initiate the process such as stretch or mechanical activity (termed mechanotransduction), hormones, or peptides. Growth factors are not stricto sensu triggers since their expression is itself triggered by various transmitters.

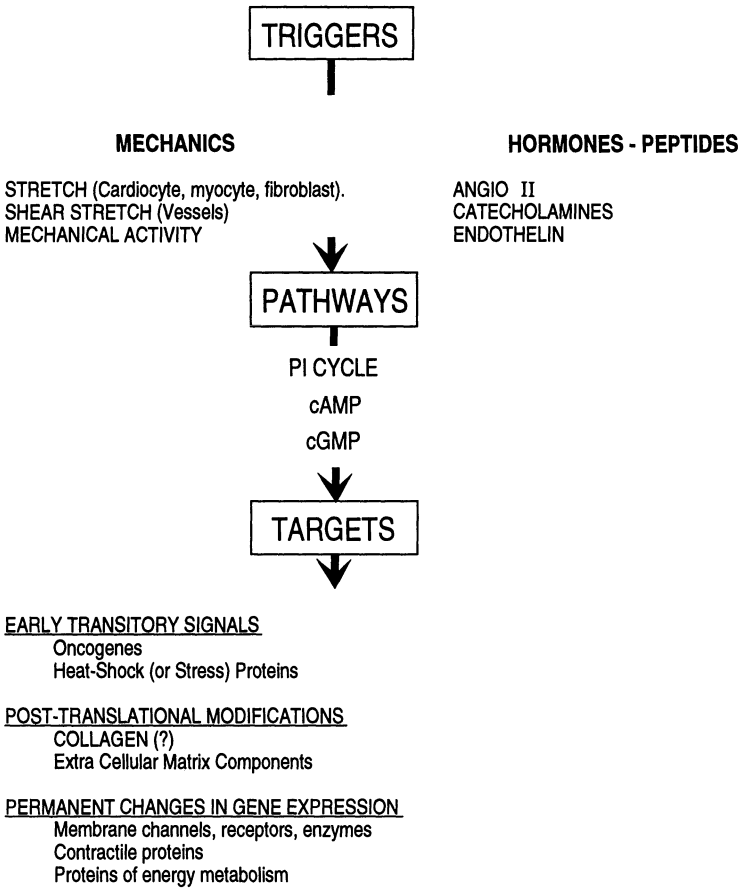

Fig. 46 The biological cascade at the origin of cardiac or vascular growth

Mechanical factors. In many biological systems mechanical forces regulate genetic expression : in bacteria changes in turgor pressure induce osmoregulatory genes, in plants both gravity and wind regulate growth, in mammals stretching an axon, a fibroblast or a myocyte activates growth. The mechanogenetic regulation of transcription is far from being a simple mechanism and is likely to involve different pathways, including mechanosensory channels, stretch receptors, the direct effect of stretch on membrane phospholipases, and the cytoskeleton itself [Erdos et al 1991].

Mechanical forces obviously are specific triggers for muscle growth, and indeed play a role in myometrium, vessels and myocardium. For example, in skeletal muscle, passive stretch such as that obtained by bilateral removal of the insertion of the tendon of the distal portion of the synergistic gastrocnemius muscle results in an activation of protein synthesis and a differential modification of the expression of the gene coding for myosin isoforms in the controlateral muscles. Passive stretch of the heart activates both protein synthesis and modulates myocardial gene expression in isolated adult and neonatal cardiocytes, papillary muscle, and also on coronary perfused heart (in the latter, stretch is obtained through the Gregg's effect, i.e. sarcomere stretching due to an excessive coronary distension).

The endothelium plays a particular role in this process. Endothelium is located at the interface between blood flow and the vascular or cardiac wall and functions as a mechanical sensor which is permanently exposed to pressure and stretch forces as welle as high fluid shear stress [Davies et al 1993]. Nevertheless, endothelium is not unique in responding to mechanical forces, and nearly every cell accomodates to such a trigger. Blood flow regulates the internal diameter of vessels both acutely by vasomotricity and chronically, by the reorganization of vascular wall cellular and extracellular components. The forces are transmitted and distributed throughout the cell via the cytoskeleton and between cells by the extracellular matrix :

- pressure stretch can activate several ionic channels,

- shear stretch may acts on nuclear transcription through K channels, the adenylate cyclase and also the phosphoinositol pathway,

- recent evidence suggests that integrins which anchor the cytoskeleton to the plasma membrane, may elicit tyrosine kinase phosphorylation,

- the stretch-induced increased expression of c-fos in isolated cardiocytes is accompanied by a parallel augmentation of the intracellular level of IP3. Similar data were also reported following the influence of shear stress in smooth muscle cells.

Hormones. Several hormones and peptides possess a trophic effect in addition to their known physiological function :

- Stimulation of α1-adrenergic receptors by norepinephrine is coupled to the transcription (measured in vitro by nuclear run-on assay) of genes encoding early developmental isoforms of contractile proteins (β-myosin heavy chain and skeletal α-actin) and c-myc in the cardiocytes. The intracellular pathway connecting the α1-receptor to the genome probably involves the PhosphoLipase C-dependent phosphorylation of a specific transcription factor [Simpson PC et al 1990].

- β1-adrenergic stimulation has also, in certain experimental conditions, a specific trophic effect and stimulates noncontractile protein synthesis in isolated adult cardiocytes [Dubus et al 1990].

- The trophic effect of thyroid hormones has been well-documented and includes both hypertrophy and a differentiel activation of several isogenes. Thyroxine activates the expression of several genes, including α-myosin heavy chain, β-adrenergic receptor.

- Angio II induces the growth response and stimulates cellular proliferation in smooth muscle cells grown in serum, suggesting that such a proliferative effect of Angio II requires the presence of other factors, and indeed Angio II amplifies the proliferative response to EGF or PDGFB. It has mainly a direct hypertrophic effect through the well-known enhancer

element, AP1. The activation of this enhancer depends on Protein Kinase C pathway. Angiotensin II induces the expression of c-myc, c-fos, PDGF A and TGF β. The determinants of the trophic effects of Angio II are quite complex, since Angio II can activate both a proliferative and an antiproliferative pathway. Converting Enzyme Inhibition is known to prevent both vascular fibrosis and hypertrophy and to have an effect on cardiac hypertrophy which is, in part, independant from the effect on blood pressure.

The DNA target. Trans-acting DNA binding factors.

As explained above (Ch.1, "Genes and the genetic code"), transcription is regulated by nuclear proteins, also called transcription factors, that recognize and bind specific consensus DNA sequences usually located within the gene promotor. These proteins as well as being involved in differentiation play a major role in transmiting hormonal and mechanical signals. It is, for the moment, impossible to ascribe a specific transcriptional factor that could be involved either in cardiovascular differentiation or mechanotransduction. We know that the transcriptional factor termed MyoD is absent from the heart during development, but whether MyoD-like proteins are present or another group or complex of transcriptional factors are involved is presently unknown and under active research. Several transcriptional factors specific for hormonal transduction, including cAMP Responsive Elements (Fig. 13, Ch.1) have also been described and several amino acid sequences able to bind DNA have been reported. We know, for most of them, the corresponding nucleotide sequence. The amino acid sequences include :

- a DNA binding dimerization motif termed Helix-Loop-Helix, HLH, which is linked to a basic region, bHLH. This motif is found in MyoD1, Myogenin, E12, E47. When introduced into non-muscle cells, MyoD1 is able to activate the endogenous skeletal muscle programme and, for example, to trigger the expression of myosin in fibroblasts.

- The Leucine Zipper, bZIP, that binds DNA to protooncogenes such as c-myc, c-jun and c-fos, but also to the cAMP-Responsive Element Binding protein (which is the factor connected to the adrenergic system), CERB (see below).

- The Helix-Turn-Helix, HTH, motif is found in homeoprotein and SOX.

- Activator Protein 2, AP2, and the Serum Responsive Factor are also transcriptional factors which play an important role in the cardiovascular system.

- Zinc fingers motifs which consist of 60-100 amino acids and are found in the members of the superfamily of nuclear hormone receptors (thyroid, steroid receptors, see above) and also in Sp1 transcription factor.

Activated Protein Kinase modulates the function of transcriptional factors which may bind specific consensus sequences (TGACGTCA, cAMP Responsive Elements or CRE) in the regulatory region of genes sensitive to cAMP. The CREB belongs to the basic region/Leucine Zipper transcription factor class that is characterized by a DNA-binding domain composed of 30 basic amino acids. A heptad leucine repeat close to this domain can form amphiphatic α-helices with leucine residues aligned along one ridge associating in a coiled-coil conformation (Leucine Zipper), forming finally a dimer. The dimer juxtaposes the basic region and forms a particular Y-shaped structure that has the property to wrap around the major groove of the DNA Watson's helix. Interestingly, the expression of the gene encoding the above-described transcriptional factor is likely to be autoregulated by a sort of feed-back mechanism by the Inducible cAMP Early Repressor that is involved in circadian rhythms [reviewed in Lalli et al 1994].

DNA target. Early transitory responses.

Both mechanical stress and hormonal signals elicit an early transitory increase in theexpression of several groups of genes including protooncogenes, stress proteins and cytokines.

Table 9. Major protooncogenes. (Chr 5... indicates chromosomal localisation in man).

Cytosolic c-onc :

- with tyrosine Kinase activity :

  c-erb-B : EGF receptor (see Fig. 24, Chr 7),

  c-fms : CSF1 receptor (Chr 5q34),

  c-src : inhibits differentiation (MW 60,000, Chr 20q12).

- transducers (GTPases) :

  c-Ki-ras2, c-Ha-ras1 (Chr 11) (MW 21,000) : activate phospholipase C.

- others :

  c-erb-A : thyroid hormone receptor (Chr 17),

  c-sis : PDGF B (MW 30,000, Chr 22).

Nuclear c-onc :

c-fos (MW 55,000, Chr 14) and c-jun,

c-myc (MW 62,000, Chr 8q24).

Protooncogenes, c-onc. Initially the term oncogene, onc, was used to qualify genes able to induce cancer in experimental conditions in eucaryotes (**Table 9**). The first oncogenes to be discovered were viral and termed v-onc. Later, it was demonstrated that onc exists in normal conditions and encodes numerous proteins playing a role in cell growth, and are termed

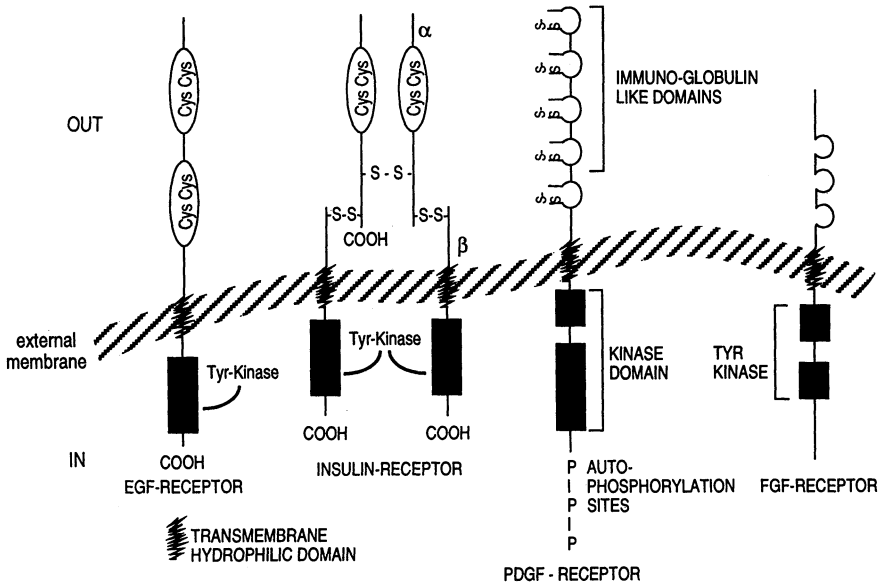

Fig. 47 Tyrosine Kinase receptors

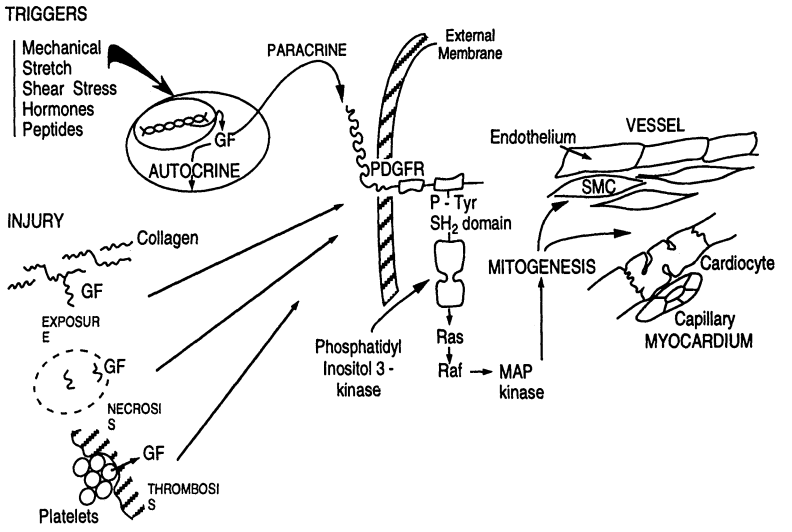

Fig. 48 Mechanisms of action of Growth factors (GF)

proto or cellular oncogenes. The definition of onc is vague and in fact includes proteins which have diverse functions, but may become transforming when modified. Nuclear c-onc mRNAs, including the c-fos/c-jun or c-fos/AP1 nucleoprotein complex and c-myc are transiently expressed when protein kinase C is activated or intracellular calcium or cAMP concentrations are enhanced both during embryonic development, and also after an acute mechanical overload or hormonal stimulation, in the heart and vessels. Whether or nor these transcripts are also translated into oncoproteins, at least during mechanical overload, is still controversial. These oncoproteins bind DNA and confer competence to the cell to express a new genetic programme.

Cytokines. Cytokines are a family of glycoproteins non immunoglobulin in nature acting non-enzymatically in pico to nanomolar concentration by interacting with specific receptors, which mostly belongs to the Tyrosine Kinase receptor family (**Fig. 47**). Cytokines mostly exert an effect on cell proliferation and biosynthesis and for this reason are frequently considered as synonymous of Growth Factors, GF [Cummins 1992]. Nevertheless, cytokines have functions other than growth. In addition distinct cytokines often exhibit similar biological activities.

GF are a polymorphic group of polypeptides that includes genuine growth factors such as EGF, TGF etc..., but also some hormones for example insulin, which has a trophic effect in addition to its known effect on glycemia, and vasoactive peptides such as endothelin (**Table 10**). As explained above, Angio II can also be considered as a GF. These polypeptides can be either induced by mechanical stretch, shear stress, hormones or peptides (there are also GFs which induce the synthesis of other GFs), or released from blood cells as platelets or neutrophils, or exposed during injury (FGF for example is linked to the extracellular matrix), or released by necrotic cells (**Fig. 48**).

Table 10. Main families of growth factors (see Fig. 25 for receptor structure).

Epithelial Growth Factor, EGF :

mitogenic, stimulate angiogenesis, endothelial cell proliferation and migration ;

EGF, Transforming Growth Factor-α, TGF-α (MW 6,000)* ;

receptor : with cytoplasmic Tyrosine Kinase activity ;

distribution : wide.

Platelet-Derived Growth Factor, PDGF :

mitogenic, facilitates capillary formation ;

heterodimer (AA, AB, BB, MW 14 & 17,000), Vascular Endothelial Growth Factor, VEGF;

receptor : with Tyrosine Kinase activity ;

distribution : wide, endothelial, PDGF is also a chemoattractant for neutrophils.

Fibroblast (or Heparin-Binding) Growth Factor, FGF :

mitogen for fibroblasts and vascular endothelium, promotes angiogenesis (proliferation, migration and organization of endothelial cells) ;

acidic and basic FGF, aFGF & bFGF (MW 15 to 17,000) ;

tissue distribution : very wide for bFGF, limited for a FGF,

bFGF binds heparan sulphate glycosaminoglycans in extracellular matrix and is stored there, FGF 3 to 7.

Transforming Growth Factor-β, TGF-β :

can promote, but usually inhibits growth, development and angiogenesis ; stimulates extracellular matrix formation.

TGF-β 1, 2, 3 (homodimer, MW 25,000)*;

receptors : transmembrane proteins with Serine/Threonine Kinase;

tissue distribution : universal.

Others :

- Insulin-like Growth Factor, IGF : R : with Tyrosine Kinase activity; Insulin, IGF-I, IGF-II.
- Interleukins, IL : R : IL receptors; IL 1 to 8.
- Colony Stimulatory Factor, CSF : R : Interleukin receptors or R with Tyrosine Kinase.
- Tumor Necrosis factor, TNF : TNF α & β.
- Neuropeptides : R : R7G; vasopressin, endothelin, gastrin-releasing peptide.

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*TGF-β and TGF-α are unrelated molecules.

GF are then allowed to bind specific receptors that are tyrosine kinases (Fig. 47). When activated by the ligand, the receptors phosphorylate themselves (autophosphorylation) at specific intracellular sites consisting of short tyrosine-containing peptides and bind specifically several signalling proteins, including phosphatidylinositol 3-kinase which activate mitogenesis, phospholipase Cγ that activates phosphatidylinositol hydrolysis and consequently increases the intracellular calcium concentration, GTPase-activated factor (GAP) and an oncogene c-src. The region of signalling proteins which binds the phosphotyrosine-containing peptide is characterized by a SH2 domain [Pazin et al 1992].

Stress Proteins or Heat-Shock Proteins, HSP, were initially described as proteins expressed during heat shock and whose expression protects the cellular components against further agression. HSP may bind mRNAs and prevent RNA lysis. The group includes several members of various MW. HSP 70 (MW 70,000) is induced by heating and ischemia in both the aorta and the myocardium, and by mechanical overload in the heart. The group also includes ubiquitin (MW 7,600) which is cofactor for proteases and plays a role in the normal process of proteolysis.

DNA target. Permanent responses.

The biological cascade describes above can occur during pathological events, such as arterial hypertension, or in more physiological conditions, including embryonic development, normal growth, aging or training. It has now become more and more evident that in every case the cell utilizes the same genetic programme which is the foetal one. The foetal programme includes the general process of activation of every genes implicated in cardiac or arterial wall hypertrophy, and also more specific changes as shifts in isogene expression (isomyosins, isoforms of Na^+, K^+-ATPase, lactate deshydrogenase, creatine kinase), or even the non-induction of the expression of certain genes resulting in a dilution of the corresponding protein and mRNA in the hypertrophied tissue (Ca^{2+}ATPase of SR, ryanodine receptor, β1-adrenergic and muscarinic receptors in certain conditions). Such a general process is not appliable to every biological component of the cardiovascular system, and is different, at least for fibrogenesis and also angiogenesis.

Fibrogenesis is a major problem in cardiology both in the heart and vessels. It results from an accumulation of collagen which can be due to an enhanced synthesis or a decreased degradation, both of these phenomena occur in fibroblasts. Connective tissue infiltration by inflamatory cells play a central role at the beginning of the fibrotic process, for example after ischemia. The activity of these cells in inducing fibrosis is mediated by several growth factors that activate either fibroblast proliferation or collagen synthesis and/or degradation [Maquart 1994]:

- EGF, PDGF, Insulin Growth Factor-I, Interleukin-1, FGF, and Endothelin-1 can all activate fibroblast proliferation and are secreted by inflamatory cells.

- Collagen synthesis can be activated by IGF-1, several Interleukins, and endothelin, but the best known activator is TGF-β, a 25 kD homodimeric polymorphic protein which can be synthesized by nearly all the cells. TGF-β both activates the transcription of collagen and fibronectin genes and inhibits matrix degradation.

- Conversely there are several cytokines, e.g. Tumor Necrosis Factor-α and relaxin, which inhibit collagen or fibronectin synthesis.

Angiogenesis. A commmonly accepted scheme is the following [Schott et al. 1994]:

- GF are induced or exposed by injury (after a myocardial infarction), or released from various cells,

- bFGF, PDGF and EGF bind to specific receptors and the corresponding signal is then transduced via tyrosine kinase activity,

- endothelial cells are then allowed to divide and proliferate and sprout of neovessels appear.

3

DISEASES OF ADAPTATION

Cardiac hypertrophy is not *per se* a disease, but the physiological adaptation of the heart to a disease, and the causal disease is usually arterial, as essential arterial hypertension and/or coronary insufficiency. Cardiac failure indicates the limits of the mechanical adaptation, and is aggravated by an additional factor, namely fibrosis. The senescent heart, in the absence of any cardiovascular disease is mainly, while not solely, an overloaded heart. The overload results from the enhanced impedance of the large vessels that is the basic characteristic of the senescent vessels.

Ventricular adaptation, and dysadaptation, isa species-specific process because the regulation of contraction differs from one animal species to the other, and also because the causes of cardiac hypertrophy could differ according to the species (for example, the rat is very resistant to atherosclerosis, dogs have a very particular valvular pathology and cardiac failure is mostly secondary to hypertension and/or coronary insufficiency). In this chapter, we would successively describe the general process of adaptation, the biological factors responsible for cardiac and vascular hypertrophy, cardiac failure and finally what we know on the senescent heart. The model used for such a description is the rat, except when indicated, because the species has been the most widely studied, but finally we would report what we know concerning the biology of the human failing heart.

BIOLOGICAL ADAPTATION.

Thanks to the development of molecular and cellular biology, we have learnt that, in response to mechanical stimuli, both the myocardium and the vascular walls adapt to increased workloads through several changes in gene expression. The expression of the gene, i.e. the phenotype both in terms of protein and mRNA, and physiological function, is modified. The structure of the gene (see Fig. 1, Ch. 1) is only changed in genetic diseases (see Ch. 4). Nature has not created infinite possibilities of new genetic programmes, and they are all already available in the oncogenetic story. In other words, to know, in a given animal species, what are the genetic programmes that could be reexpressed in a given condition (pathological or physiological). The best strategy consists of exploring simultaneously the gene expression during development and in the pathological state. During cardiac hypertrophy due to mechanical overload, only the foetal isomyosin is reexpressed, simply because there are no other possibilities. In skeletal muscle hypertrophy due to intensive training or during muscle regeneration, several programmes are expressed because there are both several programmes during develpoment and several different isoforms [Swynghedauw 1986].

Nevertheless there are obviously differences between an embryo and a patient suffering from arterial hypertension in terms of genetic expression. In the first case, the changes in genetic expression are part of a sequential programme which is not only entirely known, but which includes several signals for growth using well-defined biological pathways and resulting from a complex interaction between hormones, neurotransmitters and cellular interactions. In the second case, the major, if not ultimately unique, trigger is hemodynamic, i.e. mechanical, and the possible receptors or pathways are, for the moment, largely unexplored.

The general process of biological adaptation.

Biological adaptation is a general process by which an organ or organism changes its genetic expression and tries to express a new genetic programme in response to new environmental requirments that unbalance its thermodynamic status. This is a necessity and if the modifications do not result in a new and improved thermodynamic status, the organ or organism simply does not survive and there is consequently no adaptation. There is no finalism in this basic process, the finalism is in fact defined *a posteriori.*

The main characteristic of biological adaptation to cardiac overload is that the thermodynamic status of the heart becomes adapted to new environmental requirements. Mechanical overload on the skeletal muscle immediately reduces the instantaneous shortening velocity by simply bringing into play the mechanical properties of the myofiber. The load is greater, therefore the fiber contracts more slowly which results in an immediate decrease in the economy of the system (i.e. the amount of developed tension per mole of ATP burnt), since the muscle fiber, as a car, is adapted to have an optimal economy for a given velocity. The process is not finalist and proceeds by trial and error, using the preexisting genetic programme, which in the heart is the foetal programme. By definition there are modifications in gene expression which are beneficial, whereas others are either detrimental or even useless, and finally the modifications in the genetic expression are not limited to one gene but concerns all the cell.

The change in genetic expression will allow the heart to obtain a lower Vmax and to recover a normal economy. By comparison, for a car, when we are permanently obliged to go slower and maintain a normal economy, we have to change the motor. The change in gene expression has not been directed toward this finality by a magic finger, in fact, facing this new situation the genome simply uses another programme. There are not so many available and the programme used in the heart is most likely to be the foetal one.

The foetal genetic programme during cardiac overload.

Hemodynamic overload, whatever the origin, induces several quantitative and qualitative modifications in the cardiac expression of the genome that are well-documented in the rat model (Moalic et al. 1993) and include : (i) an overall augmentation of the synthesis of RNAs and proteins (Meerson et al. 1978); (ii) transitory changes in the expression of genes encoding proteins normally expressed during foetal life, as oncoproteins (Bauters et al. 1988, Izumo et al. 1988, Snoeckx 1991), Heat-Shock Proteins (Delcayre et al. 1988), α–skeletal isoactin (Schwartz et al. 1986) ; (ii) shifts in isogene expression resulting in the reexpression of foetal isoforms of myosin heavy and light chains (Lompré et al. 1979, Schwartz et al. 1981), fibronectin (Samuel et al. 1991), the α-subunit of the Na^+, K^+ ATPase (Charlemagne et al. 1994), the B-subunit of the Creatine Kinase (Ingwall et al. 1985), the M-subunits of the Lactate Deshydrogenase ; (iv) several genes may likely not to be activated, as during the foetal life, and consequently the products of these genes, namely the corresponding mRNAs and proteins, are diluted in the hypertrophied myocyte (Ca^{2+} ATPase of Sarcoplasmic Reticulum, ryanodine channels, β1-adrenergic and muscarinic receptors, Chevalier et al. 1991, Bastie et al. 1990, Naudin et al. 1991).

Another characteristic of biological adaptation is that the process is likely to be the same for different situations. In response to an environmental change the same tissue always, or nearly so, utilizes the same programme. The changes in cardiac gene expression are very similar in a variety of conditions, including foetal development, mechanical volume and pressure-overload, mechanical overload due to myocardial scar, senescence and hypothyroidism. This coïncidence is not fortuitous. Since it is obvious that in all these conditions the genome has been obliged to change its expression due to an external event and the same programme is always used simply because this is the only developmental programme available.

The cascade of events which goes from the initial trigger to the enhanced gene expression is remarkably monomorphous in various types of biological adaptation and has been described in Ch. 2 (Fig. 46).

THE HYPERTROPHIED AND FAILING HEART.

Prolonged mechanical overloading induces several changes in genetic expression both in the heart and vasculature which results in biological adaptation, at least for a long period of time. Failure finally occurs and indicates the limits of the biological process of adaptation. Starling's biophysical approach to heart work regulation studied short-term myocardial adaptation to physiological and pharmacological agents. Phenoconversion, i.e. changes in myocyte phenotype in response to changes in cardiac work, now allows us to establish a correlation between shifts in cardiac gene expression (including sarcomeric proteins, membrane proteins and several pathways involved in transduction process or energetic metabolism) and the well-known physiological modifications which characterize an hypertrophied chronically overloaded heart (**Table 11**).

Energy metabolism in compensatory hypertrophy.

Heat production.

The energy present in the structure of the ATP molecule is transformed into mechanical energy either in aerobic conditions in mitochondria, or, in the absence of oxygen, during glycolysis. Such a transformation is accompanied by heat dissipation. Economy is the ratio between Force and Energy Flux or

Table 11. Permanent changes in genetic expression in the hypertrophied rat ventricle (Moalic et al. 1993, Swynghedauw et al. 1994).

Sarcomere :

- Myosin ATPase specific activity ⇓.
- Myosin Heavy Chains become ββ.
- Atrial (foetal) Myosin Light Chain expression.
- No changes in Troponins, except (?) for TNT 2.

External membrane proteins :

- α-subunit of Na^+, K^+-ATPase : α3 ⇑, α2 ⇓.
- Calcium channels (DHP receptors) : unchanged in density.
- Na^+/Ca^{2+} exchanger ⇓.
- β1–αdrenergic and muscarinic (M2) receptors ⇓.
- Angiotensin II receptors 1_A⇓, 1_B⇑.

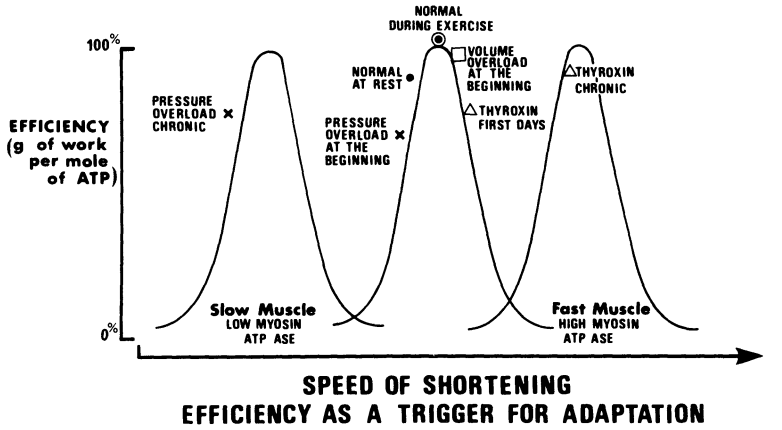
Sarcoplasmic Reticulum proteins:

- Ryanodine receptors ⇓
- Ca^{2+} ATPase ⇓.
- Phospholamban ⇓.

Energy Metabolism.

- Mitochondrial proteins ⇑ (DNA loops).
- Shifts to Creatine Kinase B & Lactate Deshydrogenase MM.

Heat production. Biological adaptation has to maintain or even, as in cardiac mechanics, to improve the economy and also, in vivo, the efficiency of the system. The economy of a given muscle is optimal for a given velocity of shortening. In other words the Economy/Velocity curve has a bell shape



49. Mechanotransduction in cardiac hypertrophy.

(**Fig. 49**). Another finding coming from phylogenic studies is that slow muscles have a better economy than the fast ones.

When a muscle, or a car, are suddenly mechanically overloaded, they instantaneously contract, or run, at a speed slower than normal (in Fig. 35, on a Velocity/Force curve, an increased after-load result in a depressed instantaneous velocity). The immediate consequence is a diminution in the economy of the system, with the muscle using more ATP (or oxygen) per g of developed tension than normal., or the car using more petrol per km than usual. A normal heart during exercising contracts at a velocity that corresponds to an optimum economy, at the top of the bell-shape curve. At rest, contraction is already less economic, and during overload, the velocity is even slower and is responsible for a drop in economy, i.e. to a diminished energy utilisation/ energy production ratio.

There are more qualitative changes in the biological structure of the myocardium in such a way that the myocardial fiber can now contract more slowly, but with a normal efficiency, by having a lower V_{max} and by using a different Force/Velocity curve. In other words, if we want to continue to drive our vehicle economically with an increased load, we have to gradually change its motor. In addition, according to the Laplace's law, the first beneficial effects of a modification in the expression of the myocardial genome which characterize hypertrophy is the normalisation of the wall stress.

Table 12. Economy of the hypertrophied heart. Data are recalculated from Alpert N. et al. (1982, 1992) and expressed in % control. *means that the difference is significant.

Degree of hypertrophy :	45%*
Resulting increase in force :	17%*
Heat produced during rest by synthesis, ionic movements :	unchanged
Heat produced by mechanical activity :	- 58%*
Heat produced by mitochondria :	unchanged
Economy of the system (energy utilized/energy produced) :	102%

To quantitate Economy, it is necessary to measure the mechanical performances as force, or the force/time integral, or work (Economy is then termed Efficiency) and the corresponding energy flux. Energy flux can be quantitated by measuring ATP or oxygen consumption or heat production (or petrol consumption). A major technical progress has been the utilisation of the microthermopile which allowed N. Alpert's group (1992) to measure heat production on a rabbit papillary muscle on a beat-to-beat basis.

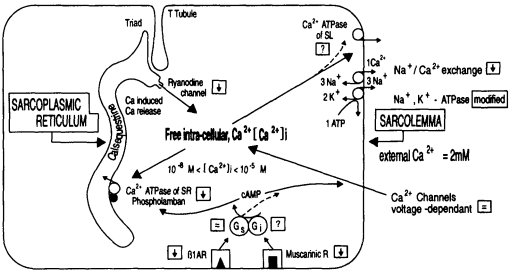
The heart, as any other muscle, is using energy for several purposes (**Table 12**) : (i) for the processes responsible for the survival of the tissue,

such as protein synthesis and ionic movements (even on an arrested heart) ; (ii) contraction, including ATP hydrolysis for cross-bridge cycling, calcium movements and excitation-contraction coupling and (iii) resynthesis of the high-energy phosphate stores which mainly occurs in mitochondria, but can depend on the anaerobic glycolytic pathway during ischemia.

The hypertrophied heart produces more tension at a slower velocity (dP/dt_{max} or V_{max}) and more economically since the heat produced during mechanical activity is reduced when it is normalized per g of tension. Special experimental protocols allow one to partition heat produced by the sliding process, from that produced by the movements of calcium and the activity of the different calcium pumps. Both systems are used during contraction and both function more economically in experimentally induced cardiac hypertrophy (Alpert et al 1982, 1992). This indirect measurement suggests that the genetic determinants of the adaptational process are located both at the level of the contractile apparatus as well as in the various components of the membrane which play a role in calcium movements (see below).

Biological basis of compensatory hypertrophy.

The improved heat production during compensatory hypertrophy depends on the sliding process and calcium movements both of which are determinants of V_{max}.



The 2 components of intracellular Ca homeostasis in the hypertrophied cardiocyte

Fig. 50 (from Moalic et al. 1992, with permission).

The two mechanisms are both species and tissue-specific, and depend on the "plasticity" of myocardial gene expression during cardiac overload. They are both involved in the ventricles of some species (rat) and in the atria of all mammalians, including man. By contrast, the membrane changes are the only determinant of the adaptational changes which occur in the ventricles of man, cat, dog, guinea pig and ferret. Such a species-specificity also exists in normal animals in terms of calcium metabolism (see Ch. 2, Calciproteins).

These differences are revealed by mechanical studies of isolated skinned fibers (ventricular fibers without any membrane structure) from pressure overloaded rats or guinea pigs. These experimenrs demonstrated a slowing of the turn over of cross-bridge cycling in the papillary muscle of the rat, which was not present in the guinea pig (Clapier-Ventura et al. 1988). In the rat ventricle and in human atria, molecular biological studies have shown the existence of a shift in the expression of the two isogenes coding for the cardiac MHCs (Lompré et al. 1979). The shift from the isomyosin V1 to V3 is correlated with the improved economy of contraction. Such a change does not exist in either the human or guinea pig ventricles which are already 100% V3.

In compensatory hypertrophy, both in humans and in the ferrets, the intracellular calcium transient peaks normally and is prolonged, (Gwathmey et al. 1987), which may account for the slower shortening velocity. Calcium transient is determined by a large number of membrane proteins all of which

are modified by the adaptational process (see below, and **Fig. 50**). Nevertheless, as explained below, the slowing of the transient can also have an arrhythmogenic effect.

Changes in gene expression in relation to myocardial function.

Changes in myocardial function that preceeds the stage of heart failure, and congestive cardiac failure obviously indicates the limits and imperfections of the adaptational process and depends upon both myocardial and peripheral factors. Recent advances in the biology of cardiac hypertrophy and failure allows one to clearly separate two groups of factors which act nearly independently, namely the detrimental aspects of mechanical adaptation of cardiocytes (**Fig. 51**) and fibrosis (**Fig. 52**). In addition, another factor of major interest to clinicians that is vascular hypertrophy due to arterial hypertension.

Adaptational changes in gene expression in relation to systolic dysfunction.
Clinically detectable alteration in systolic function occurs later in vivo that in vitro, as detected on a papillary muscle or by biochemical or molecular biological methods. The alterations in systolic function are initially adaptational, but also they simultaneously indicate the beginning of failure (Fig. 51). The cardiac output can be maintained by several compensatory mechanisms including the Starling's law, peripheral adaptation and the sympathetic drive. Chronic pressure overload of the heart decreases V_{max} and

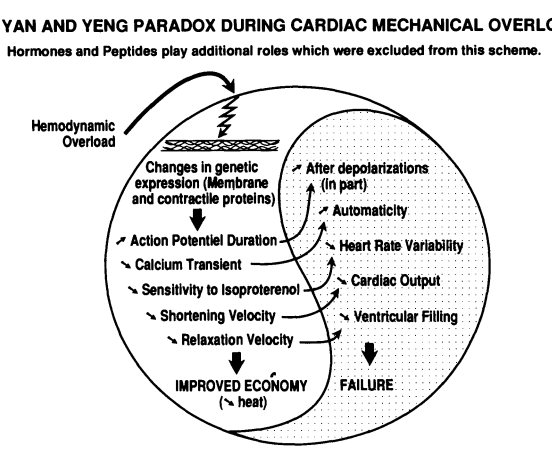


Fig. 51.

changes the expression of genes coding for membrane or contractile proteins early before the appearance of any sign of heart failure.

Atrial contraction. When passive compliance is altered by fibrosis, atrial contraction plays a more important role in compensating for the deficiency in ventricular filling as shown by echoDoppler (E/A ratio). Atrial contractility is mainly under the control of the contractile proteins and above all depends upon the ATPase activity and the type of isomyosin present in the atria. In the normal human heart, as in most of the mammals, the atria contains almost 100% of the V1 isoform that is the fast isomyosin and is composed by the αα–myosin heavy chain isoform. During hemodynamic overload, the composition of the atria changes from 100% V1 to %V3 isoform, and this shift is correlated with the size of the atria as determined by echocardiography (Mercadier et al. 1987).

In rat ventricles, the same shift in isomyosin occurs and is correlated with the shortening velocity (Lompré et al. 1979, Schwartz et al. 1981). In other species, such as man where the ventricles are nearly 100% V3, adaptation is more likely to occur by a slowing of the calcium transient, which, in turn, is determined by changes in the expression of genes encoding membrane proteins, or to other more debatable sarcomeric mechanisms.

Adaptational changes in gene expression in relation to diastolic dysfunction.
For the clinician, diastole includes active relaxation, early rapid filling, diastasis and the very last small increase in diastolic volume due to atrial contraction (Brutsaert et al. 1989). Diastolic dysfunction in cardiac hypertrophy results from alterations in active relaxation, atrial contraction and passive compliance of the left ventricular chamber. Active relaxation is really the only component which depends upon the biological processes of adaptation, whereas atrial contarction and passive compliance are both linked together and are consequences of fibrosis.

During cardiac hypertrophy, the alterations in both active relaxation and shortening velocity are linked together. In all models of cardiac hypertrophy active relaxation was impaired. Molecular biology helps us to understand the mechanism of the impaired relaxation in the hypertrophy. Active relaxation depends on the release of calcium from the cytosol to either SR or the extracellular space (Fig. 38). At least three different studies, based on animal or human myocardium, have demonstrated that overload is associated with a quantitative change in the Ca ATPase of the SR (Bastie et al. 1990). The calcium uptake is impaired and the number of functionally active calcium ATPase pump is decreased suggesting that the gene encoding this enzyme is not sensitive to the general process of adaptation which is activated during cardiac overload, and that consequently the product of the gene, namely the protein, is diluted in the hypertrophied tissue. The other, although less important, biological component of active relaxation, namely the Na^{+}/Ca^{2+} exchanger is also impaired in rats (Hanf et al. 1988) (Fig. 50).

Adaptational changes in gene expression in relation with calcium movements, arrhythmias and changes in heart rate or QT variability.
Clinical studies, as the Framingham study, have shown that the incidence of arrythmias increases with age and with the degree of hypertrophy (Levy et al. 1987). The increase of arrythmias in the hypertrophied heart have different

origins, including fibrosis (see below) and necrosis, changes in action potential duration, the decreased capacity of the myocyte to maintain calcium homeostasis and the diminution of heart rate variability.

(i) Necrosis and fibrosis which are present in the hypertrophied myocardium create an electrical heterogeneity in the tissue. The current flow runs faster through the damaged cells which provides a basis for reentry currents.

(ii) The prolongation of the action potential has been demonstrated in numerous models of experimental cardiac hypertrophy (Swynghedauw et al. 1994) and explains the inversion of the T-wave and the increase of the QT interval. Both of these modifications of the ventricular repolarisation are observed during cardiac hypertrophy in man. The prolongation of the action potential could be one of the major factors that initiates ventricular arrhythmias in hypertrophy. Both clinical studies and experiments on hypertrophied isolated myocytes have demonstrated that the lengthening of the QT-interval, which is the ECG equivalent of the duration of the action potential, has a pronostical significance. The action potential duration itself depends on several ionic channels (**Table 13**) :

Table 13. Ionic currents in cardiac hypertrophy in animal models (from E. Coraboeuf in Swynghedauw et al. 1994).

Name (role).	**Density in hypertrophy.**
Inward currents :	
- Na current, I_{Na}, (rapid depolarisation).	⇑
- Ca current, I_{CaL} (slow inward).	Maintained.
Na^+/Ca^{2+} exchange, $I_{Na\text{-}Ca}$.	Prolonged.
Outward Potassium currents :	
- Transient outward current, I_{to}, (AP duration).	⇓

- Delayed rectifier, I_k, (delayed repolarization).	Slightly reduced.
- Inward rectifier, I_{k1}, ("Background" current).	⇑

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represents the total channels) is unchanged in cardiac hypertrophy (Scamps et al. 1990).

- The potassium currents are outward currents which accelerate the repolarisation. As explained in Ch. 2, there are three main voltage dependant potassium channels. The delayed rectifier outward current, I_k, is reduced in feline models of hypertrophy while the background inward rectifier current, I_{k1}, is likely to be increased. Nevertheless the major defect which has been reported in this condition is a depression of the early transient outward current, I_{to}, in cardiac hypertrophy in rats which is likely to constitute the main explanation for the prolongation of the action potential at least in the rat (Coulombe et al. 1994).

- The voltage sensitive calcium channels that are responsible for the slow inward current, I_{CaL}, contribute to the plateau phase and could be involved in lengthening the action potential. They are however probably not involved, because both the density of the current (that represents the functional channels) and that of the dihydropyridine binding sites (which

- The sodium-calcium exchange creates an inward depolarisation current. Its activation could create a long lasting slow inward current and participate to the lengthening of the action potential. This particular point is still debatable.

(iii) In cardiac hypertrophy, calcium homeostasis reaches a new steady state which is likely to be unstable, even in the compensatory stage. A good example of such an instability comes from the experimental studies performed in vitro on isolated coronary perfused hearts. In normal hearts, a short period of anoxia is known to depress ventricular compliance

and to render the myocardium stiffer. This phenomena is exaggerated in hypertrophy, and such an exaggeration is likely to reflect a difficulty of the hypertrophied heart to buffer any sudden changes in intracellular calcium concentration (Callens-El Amrani et al. 1992).

Recent advances in molecular biological techniques have provided an overall picture of the calcium movements in the hypertrophied myocyte in rat (Fig. 50). The density of both the dihydropyridine receptors and the functional calcium channels remains unchanged and their number increases proportionnaly with the degree of hypertrophy. The sodium-calcium exchanger, the main sarcolemmal determinant for calcium extrusion becomes less active. The Na^+, K^+-ATPase is coupled to the exchanger from a functional point of view and is modified, but the changes are species-specific. In the rat there is a shift from the $\alpha 2$-subunit isoform having a low affinity for ouabain and a high affinity to sodium, to $\alpha 3$, which is the foetal isoform with a high affinity for ouabain and a low affinity for sodium (Chevalier et al. 1989, Lelièvre et al. 1986, Charlemagne et al. 1986, 1994). In the Sarcoplasmic Reticulum, in the rat, the density of both the calcium ATPase and ryanodine receptors decrease in parallel and calcium homeostasis is preserved in the reticulum, neverthelesss this situation is also particular to rats (Bastie et al. 1990, Naudin et al. 1991).

In the rat model, the overall calcium homeostasis is therefore maintained with a new fragile equilibrium and it is easy to hypothesize that every agent that would increase calcium input into the cell would be able to induce alterations of the calcium concentrations, because the buffering capacity of the hypertrophied myocyte are limited. As a consequence the intracellular calcium content of the myocyte increases, and alterations of the diastolic properties of the myocardium or arrhythmias may occur due to the premature release of the calcium contained in the Sarcoplasmic Reticulum.

(iv) The variability of the heart rate is a well-recognized index of the activity of the autonomous system and is strongly attenuated both in the hypertrophied or failing heart and during senescence. It is considered to

be a better risk factor than the ejection fraction and could predict severe arrythmias. The loss of heart rate variability results from either changes in baroreflex control, or an imbalance between the various components of the sympathetic or parasympathetic myocardial pathways.

During the compensatory phase of cardiac hypertrophy in rats, both the β1 adrenergic and muscarinic receptors were parallely down-regulated and their density on the cell membrane is decreased while the concentration of mRNAs coding for both Gαs and Gαi remains unchanged (Narayanan et al. 1982, Chevalier et al. 1991, Moalic et al. 1993). The balance between the two systems is therefore unchanged and the heart rate variability is only slighty modified (Carré et al. 1994).

Fibrosis.

The passive compliance of the left ventricular chamber depends on both chamber dilatation and intrinsic myocardial stiffnes (as assessed by the diastolic Stress-Strain relationship) (Gaasch et al. 1985). The main determinant of both myocardial (and arterial) stiffness and arrhythmias is fibrosis, i.e. collagen concentration (Villari et al. 1993).

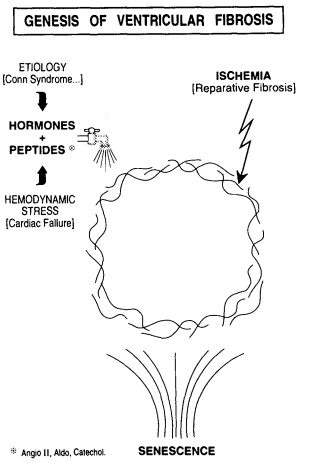

Fig. 52.

Fibrosis is not inevitabily linked to mechanical overload, and there are several experimental and clinical models of mechanically overloaded hearts with unchanged myocardial stiffness and collagen content including volume overload, infra-renal aortic constriction... In chronic cardiac overload the left ventricle collagen concentration is likely to be a factor which is independent of mechanics and whose determinants are senescence (see below), myocardial ischemia and, as recently demonstrated (Weber et al. 1991, Robert et al. 1994) Angio II, catecholamines and aldosterone plasma levels. These three hormonal factors play a role independently, and, for example, aldosterone alone creates fibrosis without the intervention of Angio II. Volume overload is not associated to fibrosis, but with changes in collagen cross-linking (Iimoto et al. 1985, Apstein et al. 1987).

Hypertrophy of the arterial wall.

Chronic arterial hypertension is one of the major causes of cardiac failure. The arterial wall responds to high blood pressure in the same way as the myocardium, and hypertrophies (Tedgui et al. 1994) in order to normalize the wall stress. Both endothelial and smooth muscle cells participate in this process, endothelial cells replicate, while smooth muscle cells either

hypertrophy in the large arteries, or become hyperplasic, in the smaller vessels. Simultaneously the extracellular matrix mass is modified, the elastin content remains unchanged, but collagen and fibronectin concentrations are increased. Both mechanical stress (the increased pressure and shear stress) and vasoactive peptides, such as Angio II, play a major role in these phenotypic modifications.

Species-specificity of the adaptational process. Human heart.

The calcium metabolism in normal ventricles is highly species specific, and it is well-known that, for example, in the rat the SR plays a more important role as a determinant of the calcium transient than the calcium current, while the role of SR is less important in human or rabbit (Fabiato et al. 1983). Therefore, it is easy to predict that the biological process of adaptation may vary between animal species.

In man the problem is complicated by the fact that, for evident ethical reasons, human samples usually come from transplanted hearts, i.e. from patients with advanced congestive cardiac failure, controls are donors which is rare, or are patients with a compensatory cardiac hypertrophy or an ischemic cardiopathy during by-pass surgery and therefore these samples cannot be considered to be normal. By contrast, it is difficult to obtain chronic congestive cardiac failure experimentally, and most of the studies in animals have been performed on compensatory hypertrophy. An excellent book has been published on the cellular and molecular alterations in the failing heart by G. Hasenfuss et al. (1992).

Cellular aspects.

Cardiac failure in humans is a disease of senescence and is very frequently caused by myocardial ischemia, which means that fibrosis is a major problem that complicates everything, including the genesis of arrhythmias and that of the diastolic dysfunction. In humans, the fibrosis is correlated

with the degree of failure (Schaper et al. 1992). Another particularity of the hypertrophied human heart is the existence of highly polyploide cardiocytes, the degree of polyploidy being roughly proportional to the cardiac mass (Adler 1975)

Energetics.

It is now possible to quantitate economy on isolated fibers from human hearts using microthermopiles as in the rabbit (Alpert et al 1982, 1992, Hasenfuss et al. 1992). These experiments were made possible in humans by using cardioplegic solutions, such as BDM (2,3-Butanedione Monoxime), to store the myocardial strips for hours and protect them from dissection injury. The cardioplegic effect of BDM is reversible and preparations have high peak tension.

Using this technique, it has been possible to demonstrate that, in isolated myocardial fibers, peak isometric tension and maximum tension rise are both reduced in man as in animal models, which, for the first time, clearly indicates the existence, in humans, of a deficit at the level of the myofiber. The contractile deficit is highly dependent on the stimulation frequency : in normal human myocardium twitch tension increases by 107% and peaks at 174 min^{-1}, in cardiac failure tension peaks at 81 min^{-1} and then declines (Mulieri et al. 1992). This point has been controversial for years. The major origin of the controversy lies into technical considerations, including the use of appropriate cardioplegia and that of a complete Force-Frequency curve, and of various external calcium concentrations.

The second important conclusion was that the general process of adaptation previously described in animal models also applies to human, and that, from a thermodynamical point of view, the cardiac fiber from a hypertrophied, and even failing human myocardium, contracts more economically. It is possible to quantitate the heat produced during one cardiac contraction, and then to partition this heat into a tension-dependent and a tension-independent heat, as in the rabbit. During cardiac failure, the

peak isometric tension decreases by 46%, while the heat produced at the same moment is reduced by 61% (Hasenfus et al. 1992), showing that the contraction is indeed more economical. It is also possible to calculate the average force-time integral of the individual myosin crossbridges that is roughly the average force-time integral measured by a force gauge on the muscle fiber divided by the number of crossbridges. The number of crossbridges really active during the contraction is calculated from the heat liberated by assuming that during each crossbrige cycle only one high-energy bond is hydrolyzed and produces heat (Alpert et al. 1992). The force-time integral of the myosin crossbridges is increased by 30-40% in human dilated cardiomyopathy as it is in the pressure overloaded rabbit heart which means that crossbridges cycle more economically.

These authors have also asked in humans the same question as we had in the rat model, i.e. what are the biological determinants of such a basic phenomena. Is the basic phenomena located at the level of the myosin isozymes, as it is in the rat, or is it due to other changes in the myofibers, or is it only a consequence of phenotypic modifications at the level of the membrane proteins, as in the guinea pig (Clapier-Ventura et al. 1988) ?

Myocardial phenotypic changes.

The phenotypic modifications observed in the failing heart in humans are different from those reported in the rat model. For the moment, it is difficult to say if these differences reflect differences in the stage of the disease, or are basically related to an ontogenic situation which is not the same, especially in terms of calcium homeostasis.

Sarcomere. The normal human ventricle contracts three times more slowly than the rat ventricle and is nearly exclusively composed of the V3 slow myosin isoform (the ββ myosin heavy chain isoform) which is also predominant during foetal development. The V3 isoform remains the predominant isoform in the ventricle during mechanical overload. In contrast, as described previously the normal atria that contracts faster than

the ventricles, contains nearly 100% of the fast isomyosin V1, and during atrial overload (when the early filling phase of the ventricle is impaired) there is a shift to V3 that parallels the atrial size as measured by echocardiography (Mercadier et al. 1987). There is no doubt that in the human overloaded ventricle there are no shift in isomyosins as in the rat, nevertheless the pronounced enhancement of the force-time integral of the myosin crossbridges, that has been reported in man both during the compensatory and in the failing stages, suggests a deficit at the level of the contractile proteins. There are several reports which have shown a diminution of the myofibrillar ATPase (Leclercq et al. 1976, Swynghedauw 1986) in this condition, and it was suggested that either the myosin light chains (Hirzel et al. 1985), or the thin filament proteins, such as TNT, may be modified and that these modifications would explain both the drop in myofibrillar ATPase and the physiological changes.

Membranes. In humans, the problem of the adrenergic receptors is complicated by the fact that we are usually dealing with advanced cardiac failure with a high plasma catecholamines concentrations, which can *per se* down-regulate the β1-adrenergic receptors. It is therefore possible that the pronounced down-regulation of these receptors which was observed in man reflects both the influence of the circulating hormones and a non-regulation, which participates in the process of adaptation, as in rats. The β2-receptors remain unchanged, but there is a pronounced increase in Giα2 which reflects a process of transregulation in response to the enhanced plasma catecholamines (Eschenagen et al.1992). Such a phenomena may also explain the reduced adenylate cyclase activity.

The Na^+, K^+-ATPase is basically different in humans since the α3-subunit has a high affinity for sodium (the same subunit in rats has a low affinity for sodium), and the situation in chronic hypertrophy is, for the moment, controversial. However it is clear that the concentration and activity of the calcium ATPase of the SR are reduced in human as in animal models, in agreement with the finding of a reduced Force-Frequency curve

(and both correlate, see Hasenfuss et al. 1992). Therefore, adaptation to mechanical overload, in any animal species does not involve the gene encoding this important enzyme. The state of the calcium-release channel is also for the moment a rather controversial issue since it was found that the release of calcium from isolated SR is not altered (Hasenfuss et al. 1992) and whereas the density of the ryanodine channels is modified (unpublished data from our laboratory). The peak calcium-current density is unmodified in humans and rats, nevertheless there is a major change in the Na^{+}/Ca^{2+}

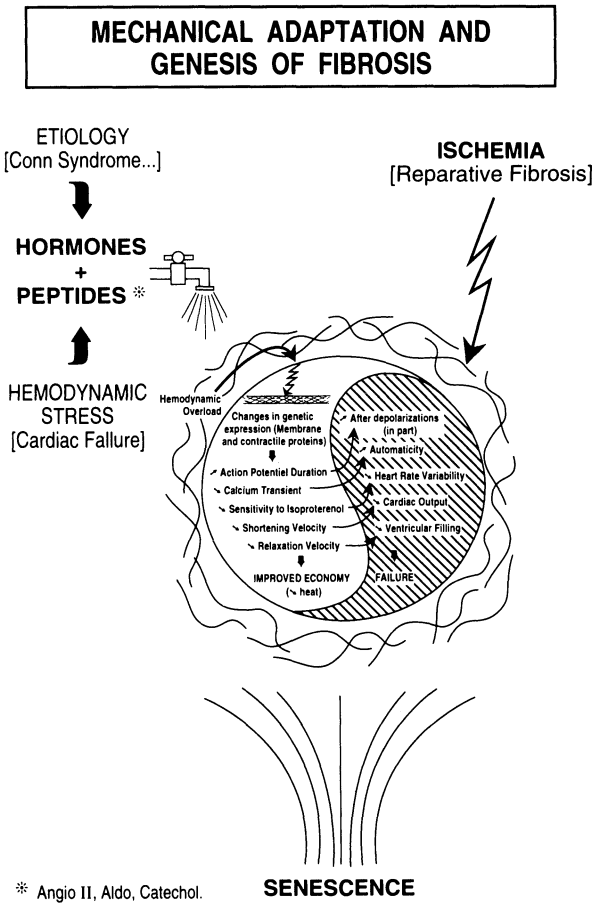

Fig. 53.

exchanger whose concentration increased both in terms of protein and mRNA (Studer et al. 1994). The result is a calcium transient which is slightly modified with a second peak, termed L2, whose origin is still unknown, the calcium transient is barely unmodified providing the measurement is made at physiological calcium concentration and frequency stimulation (Gwathmey et al. 1987, see also discussion in Hasenfuss et al. 1992).

Conclusion.

Cardiac failure can be considered to be a disease of adaptation which involves three different mechanisms (**Fig. 53**) : (i) the limits and imperfections of the general process of myocardial adaptation to the mechanical stress (Fig. 51) ; (ii) the limits and imperfections of the adaptational process at the peripheral level which allows the entire organism to adapt to the low cardiac output ; (iii) fibrosis which is not a direct consequence of mechanical overload, but depends on aging, myocardial ischemia or hormonal changes, including Angio II, aldosterone and catecholamines (Fig. 52).

We need, for the moment, additional findings to reconcile what has been reported in humans and in different animal species. In the rat, the major determinant for an improvement in economy is a slowing in Vmax, which is in turn determined by both an isoenzymic shift in myosin and a slowing of the calcium transient. The hypertrophied rat cardiocyte is likely to be in a rather fragile equilibrium in terms of calcium homeostasis because of an inbalance at the level of the external membrane proteins. The human heart is more fibrotic, and more often failing and it is, for the moment, impossible to decide if these two conditions may or may not explain the numerous differences which exist between rat and man in the changes in genetic expression (at the level of the Na^+/Ca^{2+}exchanger, the Na^+, K^+-ATPase, G protein subunits and also calcium-release channel), the puzzle is still

incomplete (for example, we ignore how are the potassium currents in human).

THE SENESCENT HEART AND VESSELS.

As explained below aged heart is not only enlarged, but also quite different in terms of receptor densities, a major target for drug design, and most of the observed modifications are likely to adapt the heart to the new aortic impedance.

Studies on cardiovascular senescence are far from being academic since epidemiological studies have shown that diseases of the heart are one of the major cause of mortality amongst the overall population and the main cause in people over 65 years of age (Brock et al. 1990). Therefore it is of major importance to determine the state of the heart in undiseased elderly subjects and to assess the limits of normality in aged persons. The clinical problem is far from being simple because obviously the fundamental biological process of senescence is intimately associated with an increased incidence of arterial hypertension, atherosclerosis, and also with modifications in physical activity and nutritional status which all may seriously modify the myocardial structure. From a purely economic point of view, it is quite important to know if aging myocardium is similar to the myocardium of younger adults, and if we can prescribe the same drugs to both groups of people. It is also important to decide if the initial screening of new therapies needs to be done in senescent animal models.

The pionner studies in this field have been carried out by the group of E. Lakatta in Baltimore who recently published an important review

mostly focused on the physiological aspects of this problem (1993). Several other review articles have also recently been published (Besse et al. 1994, Folkow et al. 1993, Kirkland 1992).

Anatomical and cellular data.

In man, anatomical studies have shown that the weight of the heart regularly increases with age by approximately 1 g per year in man and 1.5 g per year in woman (Linzbach 1973) . In spite of the fact that the aging heart is bigger that a young one, this is not really a clinical hypertrophy since the Heart Weight/Body Weight ratio remains unchanged, except in very old people (over 90) in whom a slight decrease in the ratio is observed.

Clinical and experimental studies had revealed that in the aging heart, a loss of myocytes and fibrosis are associated with myocyte hypertrophy which is more significant in the left ventricle (60%) than in the right ventricle (10% to 20%). It has commonly been admitted that, after birth, the cardiac cells have lost their capacity to undergo DNA synthesis, cytokinesis and mitosis (Anversa et al. 1990). A study from Anversa et al. using 3 and 21 month old male Sprague Dawley rats showed a pronounced loss in the total number of cardiocyte located mostly in the endocardium of the left ventricle. Such a loss was only partly compensated by an enhanced myocyte volume. Cardiocyte hypertrophy is a constant finding during senescence and is much more pronounced in the left ventricle than in the right ventricle (**Table 14**).

In most mammalian species the majority of cardiac myocytes are binucleated (Rakusan 1994). However in man binucleation is observed only in 10% of cardiocytes In fact, the human cardiocyte only becomes progressively poplyploïd with age (Adler 1975) since there is a progressive

increase in both the degree of polyploïd cells and the number of highly (16 n) polyploïd nuclei, and number of nucleoli. This process is accelerated by the addition of mechanical overload which can result in an increased number of octoploïd nuclei up to 70%, instead of 15% in controls.

Table 14. Effects of aging on cardiovascular structure and function in healthy subjects (Reviewed in Besse et al. 1994, Fleg et al. 1982, 1987, Folkow 1993, Lakatta 1993).

Functional data.

- Normal cardiac output at rest and during exercise.
- Increased characteristic aortic impedance and arterial stiffness.
- Decreased ventricular early diastolic filling rate, increased atrial contribution to the ventricular filling, but the global ventricular filling is normal.
- Adaptation to exercise implicating more Frank-Starling mechanism than tachycardia, but the exercise-induced increase in cardiac output is normal.
- Decreased cardiovascular response to catecholamines.
- Arrhythmias and attenuation of the Heart Rate Variability.

Anatomical data.

- Increased left ventricular mass, normal heart weight/body weight ratio.
- Dilatation of the aortic orifice and the great vessels.
- Dilatation of arterioles, diminished capillary density.
- Myocyte hypertrophy.
- Fibrosis (interstitial and perivascular).

Ancient pathological studies on human hearts had revealed myocardial fibrosis and an increased collagen concentration in aged hearts and vessels. In addition the collagen structure is also modified with an increased collagen cross-linking. Histochemical studies have shown an abnormal accumulation of fibrillar collagen, both around the vessels and within the interstitium. The concentration of the two main components of fibrosis, namely collagen I and fibronectin, increases during aging. By contrast, both type I and type III procollagen mRNA levels were reduced in the senescent rat heart showing that changes in myocardial collagen mRNA and protein were not synchronous which suggests that during senescence collagen concentration is not transcriptionally regulated (Besse et al. 1994).

Biological determinants of myocardial function at rest.

Systolic function.

The Baltimore study which used only subjects with normal ECG during exercise, has clearly demonstrated that cardiac output, at rest, is not altered with age. The afterload on the left ventricle is represented by aortic impedance which includes not only peripheral resistances, inertia of the blood column, compliance of the large vessels but also velocity and morphology of the return waves. Aortic impedance is greatly increased with aging mainly because of an increased aortic stiffness and diameter (Tedgui et al. 1994). During senescence the enhanced characteristic impedance of the aorta is responsible for a left ventricular overload which in turn activates the expression of several specific genes as detailed below.

Action potential and the myoplasmic calcium transient are also prolonged. Studies on the contractility of papillary muscle were complicated by the fact that the senescent papillary muscle is both thicker and stiffer than the young muscles. Nevertheless it has been shown that isometric as well as isotonic contraction is prolonged in every species studied and that both the

maximum velocity of shortening and the maximum unloaded shortening velocity are depressed (**Table 15**). Relaxation is also impaired. Active force per surface area is correlated with the biochemical parameters and more particularly with the isomyosin shift which suggests that this parameter

Table 15. Senescent Heart versus overloaded heart. Fibrosis, Arrhythmias and Mechanics (from Besse et al. 1992, 1993).

	Overload.	**Senescence.**
Fibrosis :	+ or 0	+++ *
Arrhythmias :	+	+++ *
Mechanics of papillary muscle :		
Max Shortening Velocity	⇓	⇓
Max Relengthening Vel.	⇓	⇓
Active Force per mm^2	⇓	⇓ or =
Energetics:		
Curvature G of F/V curve	⇑	⇑
Contractile and Membrane Proteins:		
Action Potential duration	⇑	⇑
Calcium Transient	⇑	⇑
Isomyosin shift to V3	+	+
Ca^{2+} ATPase of SR	⇓	⇓
Na^{+}/Ca^{2+} exchanger	⇓	⇓
α3 Na^{+}, K^{+} ATPase	+	+

⇓ : decreased. ⇑ : increased. = : unchanged. + : pronounced. +++ : extremely important. * Indicates significant differences between Senescence and Overload.

reflects, in part, the state of contractility. All these modifications were similar to those observed during pressure-overload. Moreover, the skinned myofilament response to calcium was unaltered suggesting that the changes in the velocity of contraction which have been observed depend on contractile proteins, and/or the different determinants of intracellular calcium movements. The slowing of the contraction allows the senescent muscle to maintain the active tension within the normal limits, but to the detriment of the velocity at which this tension is developped. Both physiological and biochemical changes have therefore a beneficial effect in terms of muscle energetics. Contraction energetics of the papillary muscle was calculated from mechanical parameters which had indicated that each contraction was more economical (Table 14) (Besse et al. 1993). An interesting finding, made in man (Hasenfuss et al. 1992), is the fact that the force-time integral of the individual myosin crossbridge cycle (see above) correlates with the age of patients wtih nonfailing myocardium, suggesting that, even in man, ageing is associated with an improvement of cantraction economy.

In the rat ventricle, the isomyosin content shifts progressively with age from V1 to V3, so that by 24 months the rat ventricle contains 80% V3, the ββ isomyosin heavy chain homodimer. This shift of the isomyosins which also occurs during cardiac overload results in a decreased ATPase activity which parallels the decline in the unloaded shortening velocity and, at least in rats, is likely to constitute the main determinant of the adaptational process. Two actin isoforms (α-skeletal and α-cardiac) coexist in newborn rat heart. A study from this laboratory has reported that α-skeletal actin which represents 40% of the mRNA coding for sarcomeric actin at birth is almost absent at 2 months and does not reappear in the senescent heart. This is in opposition to the myosin heavy chain expression (Carrier et al. 1992).

The modifications of the membrane proteins occur at several different levels including the Ca^{2+} ATPase of SR (Maciel et al. 1990), adrenergic and vagal receptors, calcium channels, the Na^{+}/Ca^{2+} exchange

(Heyliger et al. 1988) and the Na^+, K^+-ATPase (Unpublished data from our laboratory). This phenoconversion of the membrane proteins is directly responsible for the slowing of the intracellular calcium transient (Orchard et al.1985) and is very similar to what has been reported to occur during mechanical overload (Fig. 50).

Diastolic function.

During senescence the global diastolic function remains unchanged in spite of a pronounced impairment in the early ventricular filling because the contribution of atrial systole to ventricular filling is increased (Fleg 1987, Lakatta et al. 1993). The impairment in early ventricular filling and the diminution of the E wave shown by EchoDoppler results from both a prolonged active isovolumic relaxation and an increased myocardial stiffness. Thus, a 50% reduction in the rate of left ventricular filling during early diastole is observed between 30 to 80 years.

Active relaxation is modified during senescence. The activity of the Na^+/Ca^{2+} exchanger is attenuated in the aged myocardium as in cardiac hypertrophy of mechanical origin. *In vitro*, the activity of exchanger is progressively reduced from 4 to 27 months in the senescent rat ventricle as is the capacity of the SR to pump calcium (heyliger et al. 1988).

As explained above, myocardial fibrosis is an important component of the senescent heart and is responsible for the major changes in passive compliance in both heart and vessels. The origin of fibrosis in the senescent heart is debatable, and the most likely hypothesis is that it is replacement fibrosis due to myocyte loss, but this important finding is far from being demonstrated (Eghbali et al; 1980, Mamuyama et al. 1992, Besse et al. 1993).

During senescence atrial hypertrophy and a slowing of atrial contraction compensates for the impairment in the early diastolic filling. For ethical reasons, this particular point has never been explored in elderly

people, however it is easy to hypothesize that the same adaptational mechanism exists in the atria during senescence as after overload.

The cardiovascular response to exercise.

In elderly people, during exercise the maximal cardiac output is maintained by an an increased ejection fraction and an increased utilization of the Frank-Starling mechanism with only slight tachycardia, or no increase in the heart rate at all. There are also evidences that the responsiveness to catecholamines is attenuated.

In the senescent Wistar rat heart, the density of both the total number of β–adrenergic and muscarinic receptors is reduced, however the decrease in muscarinic receptor density is greater than that in total β–adrenergic receptors. A similar decrease in the number of muscarinic receptors has also been reported in the cerebral cortex, striatum and hippocampus suggesting that, for unknown reasons, aging has a rather specific and pronounced effect on the muscarinic system (Danner et al. 1990). A diminution of the Gαs mRNA was also observed and the adenylate cyclase is modified. The genetic regulation is probably specific, since the levels of mRNAs encoding these myocardial components of the autonomous system are different. Aging, as mechanical overload, is accompanied by several modifications of the autonomous myocardial system which are likely to be located "down the road", as initially suggested by E. Lakatta (**Table 16**).

Arrhythmias.

Clinical and experimental studies which have examined cardiac rhythm in a healthy geriatric population, carefully evaluated to exclude cardiac disease, demonstrate a prevalence of supraventricular and ventricular ectopic beats

(Fleg et al. 1982). Time domain analysis has also shown that the standard deviation of the R-R interval decreases with age. Fast Fourier

Table 16. Senescent Heart versus overloaded heart. Autonomous nervous and Renin-Angiotensin systems, Atrial Natriuretic Factor.

	Overload.	Senescence.
Autonomous Nervous System :		
β-adrenergic receptors	⇓	= or ⇓
mRNA	⇓	⇓⇓
Muscarinic receptors	⇓	⇓⇓*
mRNA	⇓	⇑
Gαs	=	⇓
Gαi	=	=
Adenylate cyclase activity	⇓	⇓
Renin-Angiotensin System :		
Circulating	⇑	⇓⇓*
Myocardial	⇑	⇑⇑*
Atrial Natriuretic Factor :		
Plasma levels	⇑	⇑
LV mRNA	⇑	⇑

⇓ : decreased. ⇑ : increased. = : unchanged.* Indicates significant differences between Senescence and Overload.

Transformations of the R-R interval show that both High (respiratory oscillations) and Low frequency content were reduced with age although there is no change in High to Low content ratio (Levy et al. 1987, Schwartz et al. 1991).

In the senescent heart, arrhythmias result from both fibrosis and changes in the membrane phenotype. Fibrosis is undoubtedly a major factor of reentry and also auriculoventricular block. Moreover it may create centers of automaticity. The intracellular calcium transient peaks normally, but is prolonged and the duration of the action potential is increased. These two factors are potentially arrhythmogenic. Studies on the L-type calcium current are still rare in senescent myocytes. Only a small increase in the peak current density associated with a reduced inactivation has been demonstrated and, as yet, there is no information concerning the ryanodine receptors. The $\alpha 3$ subunit of the Na^+, K^+ ATPase is reexpressed both in cardiac overload and during senescence and this shift may result in a slight and permanent increase in the intracellular subsarcolemmal sodium concentration and a consecutive slowing of the Na^+/Ca^{2+} exchange, and lengthening of the calcium transient.

Cardiac hormones.

The heart is also an endocrine gland and is able to produce at least one hormone, the Atrial Natriuretic Factor, ANF, which is specifically secreted by the atria in response to a mechanical stress such as an enhanced plasma volume. This small peptide has a strong natriuretic effect which may compensate for a volume overload, and plays an important physiological role. Plasma ANF levels are elevated in healthy elderly people as compared with young individuals, but the factors responsible for these differences are still poorly known. Old rats also have higher plasma ANF levels (Table 16) than young rats. In vitro the basal ANF secretory rate was greater in atria from aged animals, and also the secretory response to phenylephrine, but not to stretch, suggesting that an increased secretory response to adrenergic stimulation may contribute to the enhanced ANF plasma levels. Molecular biological determination of the relative levels of mRNA coding for ANF

showed a strong activation in the left, but not in the right ventricle (Heymes et al. 1994).

Aging in man, and rat (Table 16), is associated with low plasma levels of Angio I, Renin activity and Angiotensinogen. The latter is likely to reflect a diminution in the hepatic level of angiotensinogen mRNA. In contrast the myocardial mRNA levels for both Angiotensinogen and Angiotensin Converting Enzyme were both upregulated. This upregulation is restricted to the left ventricle and does not exist in the right ventricle (Corman et al. 1986, Heymes et al. 1994).

Both angiotensinogen and its mRNA are rather abundant in the atria, but nearly absent in the ventricles of young animals. In young rats ACE is present and active, but rather poorly expressed. During mechanical overload, plasma ANF increases, and the ANF mRNA is activated in the overloaded ventricle. The myocardial Renin-Angiotensin-System, including Angiotensinogen and the Angiotensin Receptors, is also activated after a prolonged mechanical stress. During senescence, the specific left ventricular activation of normally poorly expressed genes such as the genes coding for ANF, Angiotensinogene and ACE suggests that the trigger for activation is common to the two systems and is in fact a left ventricular mechanical overload.

Senescence is known to be associated with several other changes in the hormonal status. (i) The plasma level in free T4 was depressed, but not that of T3, which is the active thyroid hormone. Such a finding allows one to eliminate the role of hypothyroidism in the genesis of the molecular changes in isomyosin content. (ii) The plasma cortisol levels were increased, but aldosterone remained unchanged in spite of a pronounced down-regulation of the Renin-Angiotensin-System, both findings have been fully documented in humans. These findings allow one to rule out a role of these two hormones in senescent fibrogenesis.

Cardiac failure is mainly a disease of the elderly and the average age of the patients of most of the clinical trials, including the CONSENSUS

study on Angiotensin Converting Enzyme Inhibitor, ACEI, is around 65 years. Obviously most of the patients under ACEI have a very low level of plasma Angio I and the question is how ACEI works in elderly ? The answer is most likely to be found in the various tissue Renin-Angiotensin-Systems, at least the one located in the myocardium.

Protein turn-over. Capacity of the aged heart to hypertrophy.

Several reports have suggested that during aging the rate of both protein synthesis and degradation decrease in parallel in the heart as in others tissues, but the myocardial total RNA content remains unchanged Danner et al. 1990). The recent availability of molecular biology techniques allows the quantitation of both the concentration of total polyA containing mRNA (i.e. mRNAs) and specific mRNA. Data from this laboratory has shown that both the yield of total RNA and the total amount of cardiac mRNAs (or polyA containing RNAs) relative to ribosomal RNA remain unchanged.

The main problem, in the heart as in other tissues, is the relative quantification of a given mRNA. There are now data which suggest that cardiac senescence is accompanied by several changes in genetic expression which are not uniform and vary from one gene to another. In brief: (i) there are shifts in the expression of isogenes, such as those coding for α and β Myosin Heavy Chains (α-myosin heavy chain mRNA disappears and is replaced by the isomRNA β). (ii) The relative concentration (in mg per mg) in other mRNAs, such as that encoding the Ca^{2+} ATPase of the Sarcoplasmic Reticulum, SR, decreases suggesting that these genes are rather inactive during senescence and that, consequently, the corresponding protein and mRNA are diluted in a heart whose mass is increased. The total cardiac content (in g per heart), in protein (or mRNA) belonging to this family of genes remains unchanged. (iii) Nevertheless there are also genes such as collagen and the β1-Adrenergic Receptor (Table 15) whose expression is likely to be decreased during senescence. In this family both

the concentration and the total amount of mRNA per heart are decreased, but there is additional evidence of a post-transcriptional regulation. (iv) Finally, as explained below, there are, in the myocardium and very likely in the cardiac myocytes, at least three genes which are activated during senescence, namely the genes encoding the Atrial Natriuretic Factor, Angen and ACE.

The modifications in both protein synthesis and degradation (Crie et al. 1981) and mRNA content which occurs during aging would suggest that the senescent heart may also be unable to adapt to an increased load and would be unable to further hypertrophy in response to a mechanical stress. This question has more than an academic interest since both arterial hypertension and coronary insufficiency, the two main causes of cardiac hypertrophy and failure are indeed much more frequent after 65 years than before. Several experimental investigators have tried to answer this question (Capasso et al. 1986, Isuyama et al. 1987), although the results were somewhat contradictory, the general opinion is that the senescent heart responds more slowly to mechanical overload than the young hearts, but that the final result is the same in terms of myocardial mass (Besse et al. 1993).

The senescent heart, a diseased heart.

As explained previously, careful and detailed clinical investigations have demonstrated that the senescent heart has normal myocardial performances at rest and during exercising because it uses several compensatory mechanisms to maintain a normal output. Experimental investigations have confirmed these findings and shown several modifications both at the cellular and molecular levels in the aging rat heart, which are very similar to those observed during experimental pressure-overload. These modifications include : a slowing of the action potential, the calcium transient and contraction associated with an isomyosin shift to the V3 isoform, a diminished density or activity of the calcium ATPase of SR and Na^{+}/Ca^{2+} exchange and a reduced activity of the adrenergic system.

Fibrosis is also a common feature found during both overload and in senescence. Nevertheless although fibrosis is a constant finding in the aged heart, there are several clinical and experimental examples of mechanically overloaded hearts in which the collagen concentration remains normal and it has recently been suggested that fibrosis in these particular conditions is under hormonal control. Arrhythmias and a loss in heart rate variability are a common feature found in both conditions in human and in animals, nevertheless the types of arrhythmias, their frequency and prognostic value are extremely different. The β–adrenergic/muscarinic receptor ratio remains unchanged during cardiac hypertrophy, while the same ratio is reduced during senescence in the rat heart due to a much more pronounced decrease in muscarinic compared to β-adrenergic receptor density and molecular biological data suggests that the regulation is different.

The disease is multifactorial.

Several alterations, but not all, observed in the hearts of elderly people result from the triggering of a general process of adaptation of any muscle to mechanical overload. The most important of these modifications is thermodynamic and consists in an improved economy of the muscular system. It results from several changes in gene expression, including isogene shifts for both myosin and the sodium pump, non-induction of several genes coding for membrane proteins and down-regulation of others. In the case of senescence, mechanical overload results from an increased characteristic impedance and stiffness of the aorta and a dilatation of all the major arteries which is commonly found in senescent vessels. In other words, the senescent heart is firstly an overloaded heart and reflects the vascular effects of aging.

Nevertheless, there are several changes occuring in the aged myocardium which cannot be explained by a simple mechanical overload, including myocyte loss, a pronounced fibrosis and its clinical consequences,

permanent, or nearly permanent, arrhythmias and a pronounced down-regulation of the muscarinic system.

The senescent heart, even in the absence of arterial hypertension or coronary insufficiency, is potentially a diseased heart. Myocardial function in the elderly is still normal because of several cellular or tissular adaptational mechanisms, including a slowering of calcium transient and action potential, the triggering of Starling's law during exercise, and an increased contribution of the atria to ventricular filling. As such it resembles compensatory hypertrophy occuring before NYHA stage I during the course of an arterial hypertension.

GENETICS FOR NON GENETICIANS.

DNA POLYMORPHISM.

Definitions.

Slight variations in the DNA occur in approximately one of every 3,000 bp, in humans this roughly represents 1,000,0000 differences between two unrelated individuals. DNA polymorphism determines genotypic polymorphism which may be translated into phenotypic polymorphism (Fig. 1), but not necessarily so. DNA polymorphism may indeed be silent and will not cause any phenotypic change. Such changes would either occur in an irrelevant part of the gene or would not change the properties of the encoding protein (remember, for example, that several triplets may code for the same aminoacid). Such a polymorphism is widely used for linkage analysis and polymorphic sites are used as markers. Conventionally "polymorphism" is restricted to allelic variations occuring with a frequency >1% ; rare variants or mutations qualify allelic variations occuring at a frequency <1%. Polymorphism can also occur within the gene, either in the coding part of the gene, or in the regulatory part, upstream of the first exon and is the cause of a genetic disease, if the mutation gives rise to a defective protein or even inhibits gene expression. Nevertheless and fortunately for us, DNA polymorphism rarely gives rise to a disease because most of the mutations do not result in a phenotypic change.

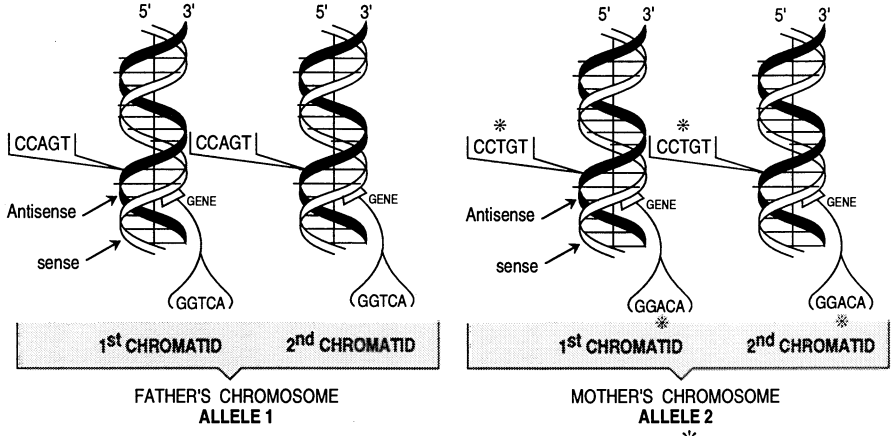

Fig. 54 Allelic Polymorphism During Cell Division (* Mutation)

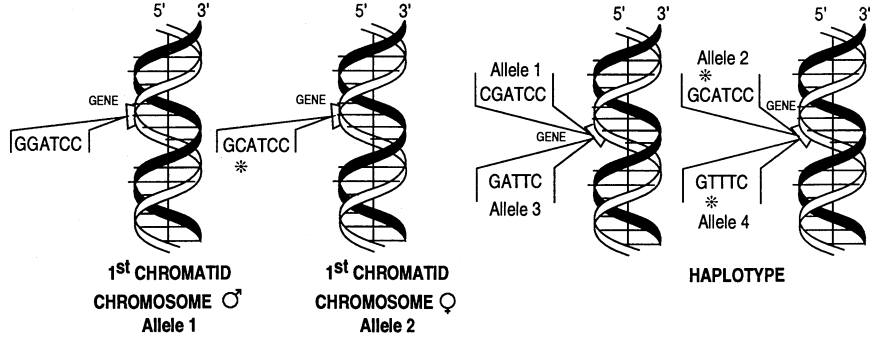

Fig. 55 Left : Restriction Polymorphism on Bam H1 - Right : Haplotype (Allele 1 + 3 or 2 + 4)

DNA polymorphism can be punctual, and then is identifiable after DNA sequencing which is a rather long job. Punctual polymorphism can be located on a restriction site. It then modifies a restriction site and the corresponding restriction map (Fig. 19) and generates restriction fragments of different lengths, and is termed Restriction Fragment Length Polymorphism, RFLP.

Allele has usually been used to qualify DNA polymorphism in the coding part of a gene, the word is now more widely used and qualify polymorphism located either in a gene or in the anonymous part of the genome. As illustrated **Fig. 54**, during cell division there are two copies of Allele 1, one on each chromatid of the same father's chromosome, there are also two copies of Allele 2 on the mother's chromosome. A diseased gene is also the allelic variant of a normal gene. Variants located at a

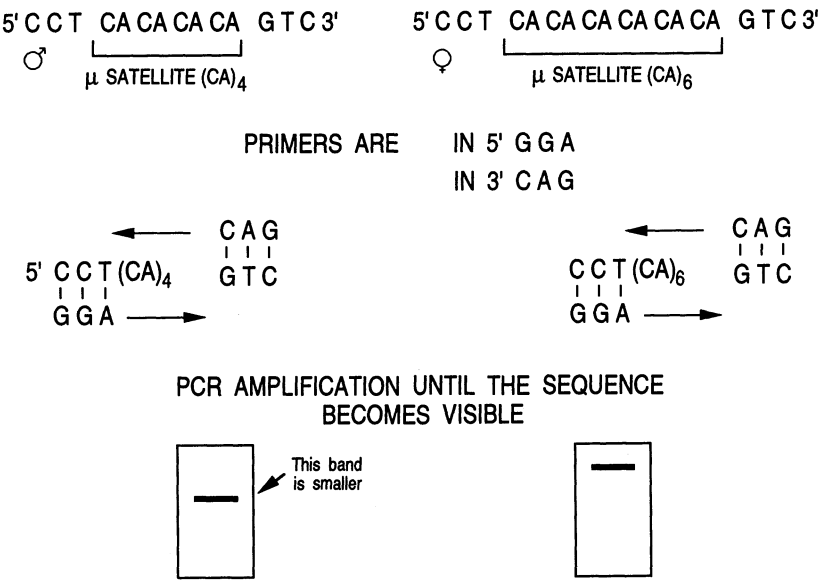

Fig. 56 Short Tandem Repeats identification by PCR (repetition polymorphism or micro satellites).

restriction site reflect Restriction Polymorphism (**Fig. 55**). Alleles can be closed one to each aother as Allele 1/2 and Allele 3/4 on Fig. 55, they are

termed Haplotype, and then the alleles cosegregate. Haplotypes are not randomly inherited, their are linked and the linkage is therefore in disequilibrium.

Repetitive polymorphism is different. It has no physiological consequences, but represents a major tool to explore the genome. There are indeed individual differences in repeated di- and tri nucleotides termed Short Tandem Repeats, STR, or microsatellites. This polymorphism is easily detectable by PCR (**Fig. 56**) by using two primers flanking the repeated units to sequentially amplify the STR. The resulting amplification products have different sizes and can be separated by gel electrophoresis. Fig. 56 shows two alleles, one with 4 repeats on the father's chromosome, the other with 6 repeats on the mother's chromosome. This difference in size can be used to track the inheritance of each chromosome and the degree of linkage of these alleles with a diseased gene.

What causes DNA polymorphism.

Tchernobyl had certainly caused mutations, and, presently, the impredicitible evolution of humanity can unfortunately allow us to predict that genetics has chances to become a major field for future researches. Nevertheless, for the moment, most of mutations do not result from physical or chemical injuries, but are consequences of the normal mixing of the genetic material during the meiosis, and more specifically during the crossing-over (**Fig. 57**). The genetic consequences of meiosis in terms of mixing genetic material are the following :

- after the first meiotic division the homologous chromosomes segregate into the daughter cells randomly with regard to parental origin ;

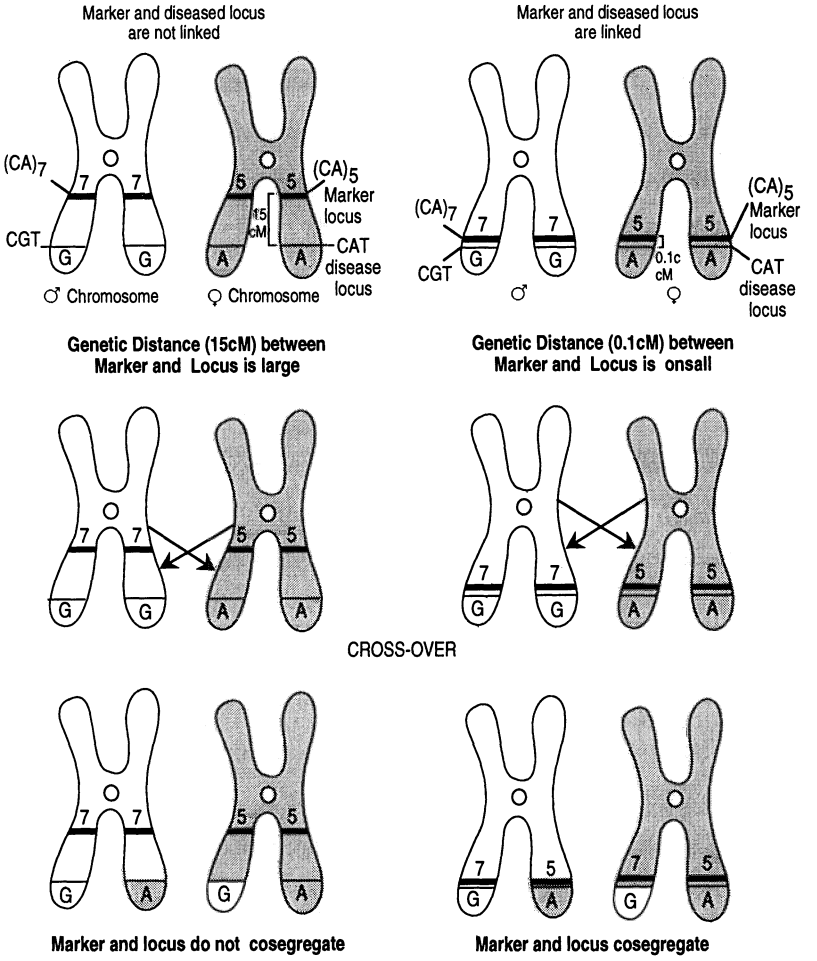

Fig. 57 Physical basis of genetic linkage during meiosis

- during middle prophase I homologous chromosomes synapse and crossing-over between chromatids of homologous chromosomes occurs, synapses do not necessarily coincide (Fig. 59).

- Chromatids assort randomly into the daughter cells.

- In mammals, in the male, each of the four haploid cells produced by meiosis give rise to a sperm cell whereas in the female only one of the haploid cells survive as an egg nucleus. The fusion of an egg and a sperm gives rise to a new diploid cell.

Any two genes, or loci *, that are on the same chromosome are also by definition on the same DNA molecule. They are both therefore, to a certain extent, linked. Nevertheless, as illustrated on Fig. 58, if two loci (the locus of a polymorphic marker, namely a tandem repeat, and that of the diseased gene) are located far apart on the chromosome, recombination events have more chances to occur than if they are close together. The genetic distance is the distance that separates the two loci from each other. It is expressed in cMorgans*, cM. cMs are statistical units, 1 cM is equal to 1% recombination during crossing-over. The likehood to have a crossing-over that separates two loci whose genetic distance is 1cM is therefor one chance in a 100. Strictly speaking genetic distance is not a physical unit, nevertheless 1 cM roughly corresponds to 1,000,000 bp. In Fig. 58, the two loci which are 15 cM apart are poorly linked and the marker provides very little informations concerning the place where the diseased locus is located, the marker is then said poorly informative. In contrast, if the distance is 0.1 cM which means that the marker is close to the diseased gene (it may, for example, be located in the intron of the diseased gene as in Cambien et al. 1992), it would be an informative marker.

Fig. 58 shows one example of how a genetic variant of tandem repeats can be created. During synapsis, the hybridation of the tandem repeats is slightly shifted downward, and the resulting new chromosomes

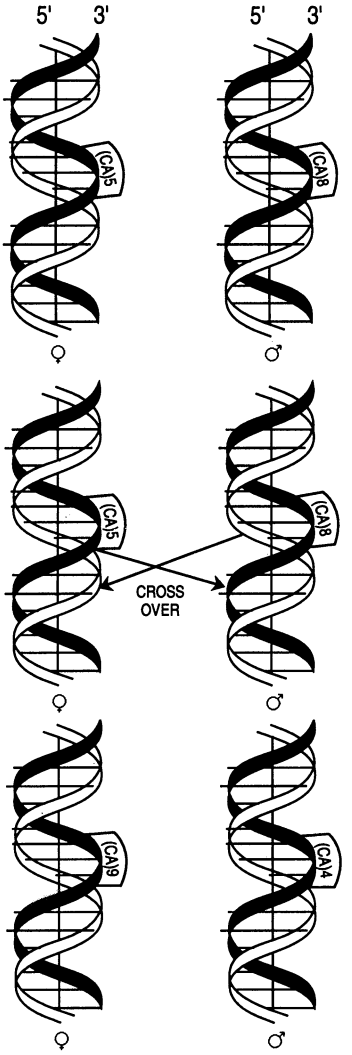

Fig. 58 Unequal Recombination

obtained after the crossing-over have different repeats : $(CA)_9$ for the father's chromosome instead of $(CA)_5$, and $(CA)_4$ for the mother's instead of $(CA)_8$.

Genetic conversion is another example of recombination which results from the triggering of the reparative process (**Fig. 59**). Random assignment of alleles after crossing-over may result in mismatches*, or mispairing*, which means that a given pair of bases will no longer be complementary on

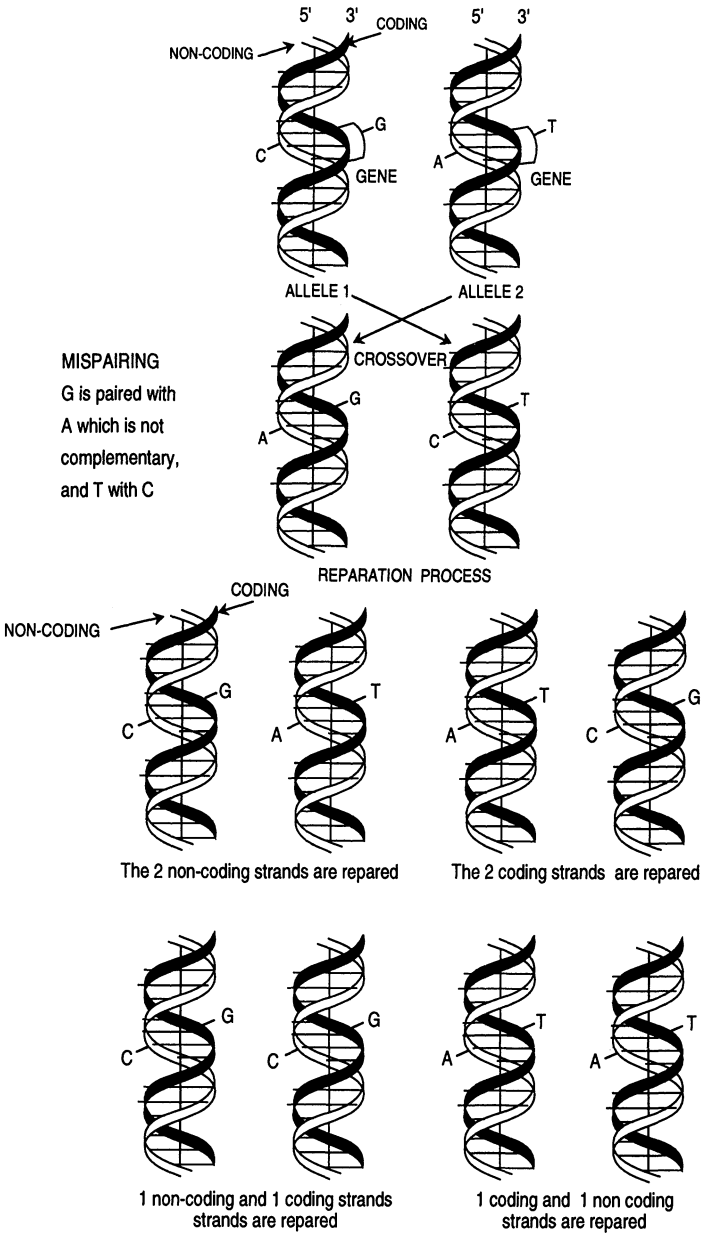

Fig. 59 Genetic conversion

the DNA strands. Spontaneously, a reparative process starts within the nucleus, that uses the complementary strand as a guide, but the process is

random and may use, as a guide, either DNA strand. The result is a rather complex mixture of new variants.

What are the consequences of DNA polymorphism.

DNA polymorphism may create genetic diseases, but is mainly beneficial in terms of evolution, since it produces diversity. Several of the processes above-described that create polymorphism are also mechanisms responsible for biological regulations such as alternative splicing which is also used to modulate the expression of isoforms, or unequal recombinations (Fig. 58) which is likely to be at the origin of the superfamilies of membrane proteins (Table 4).

INHERITED DISEASES.

Strategies to isolate a mutation.

Classical genetics.

Classical genetics starts from a candidate protein which is supposed to be the probable diseased protein. Familial anemia, for example, was thought to be due to mutations within hemoglobin. The gene encoding the hemoglobin subunits was cloned and the mutation responsible for sickle cell anemia was discovered. Unfortunately, in most cases the working hypothesis is too vague or broad and another approach, termed positional cloning or reverse genetics, is necessary (**Fig. 60**).

Reverse genetics.

Reverse genetics is based solely on the knowledge that the disease is monogenic and heritable and that there is a familial approach. Search for the

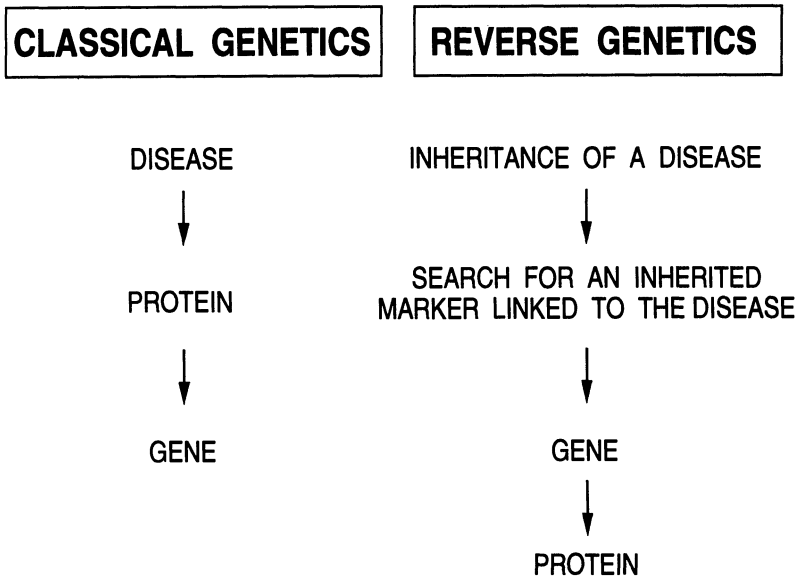

Fig. 60 Genetics - The two strategies.

genetic location of the disease consists in testing many randomly chosen DNA markers until one is found that segregates with the disease, which means that the two DNA segments, the marker and the diseased gene, are linked, i.e. physically located close to each other on the same chromosome arm. The challenge then is to detect a mutation, which is a unique base substitution, in the genome that consists of roughly 200,000 genes and is approximately 3,000,000,000 bp long, and then to localize the mutation within a chromosome that is, on average, 120,000,000 bp long. Finding the mutation can be compared to looking for a needle in a huge haystack ! Such a scale explains, for example, why the final identification of the mutation responsible for cystic fibrosis was so long and needed so many people, so much fundings and effort.

Reverse genetics consists in evaluating the genetic distance which separate the diseased gene from informative markers. There are, for the moment, certainly more than a thousand markers of known chromosomal location, and the number is increasing day by day. In addition, the mapping

of the human genome is progressing rapidly. Markers are important, but the availability of well-characterized families with numerous affected members is also determinant. In addition, a major issue, specially in cardiovascular research, is the relevance of clinical data. The clinical phenotype has to be extremely well-defined *a priori*, and not *a posteriori* : criteria for a normal blood pressure, a myocardial infarction, eclusion criteria need to be rigorously defined long before starting the genetic study.

Statistical analysis is then utilised to establish whether a given marker is linked to the disease, or if the linkage is only occasional. The recombination frequency, θ, between two loci is equal to the percentage of recombinants obtained divided by the total number of meiosis that have been examined and is expressed in cM. $\theta = 0$ means that the marker and the diseased locus are closely linked and may be identical. $\theta = 0.50$, means that the two loci are not linked and can only cosegregate randomly, i.e. in 50% of the cases according to the Mendel's laws. They are located on different chromosomes, the genetic distance is above 50 cM and linkage is unlikely. The statistical analysis consists in calculating the likehood that the two loci are linked at given recombination frequencies $\theta = 0.1$ .... 0.3 .... 0.4 ... 0.5. The result is expressed in log as LOD score Z and a curve is drawn by plotting θ = as a function of Z. The recombination fraction giving the highest LOD score is thus the relation with the highest probability to be the true value. By convention, a LOD score >3 is considered as significant and a LOD score >3 for $\theta = 0.13$ means that the hypothesis that the two loci are linked at a genetical distance of $\theta = 13$ cM is 10^3 (1,000 times) times more likely that the opposite hypothesis. In contrast, the LOD score analysis also allows one to exclude a linkage if the LOD score <- 2 .

The next step is to ascertain the physical distance between the diseased locus and the different markers, and finally to isolate and clone the gene and the mutation. The latter may require years of work, and needs precise strategy which is out of the scope of this book Interestingly there are numerous examples of genetic diseases which has resulted in the

discovery of new genes and new proteins. Two good examples are Cystic Fibrosis which allowed the discovery of a chloride channel, and the Duchenne Muscular Dystrophy that is due to mutations in dystrophine (Kaplan et al. 1993).

Populational approach (candidate gene).

In multifactorial diseases, the phenotype results from the expression of multiple genes which renders segregation studies as described above difficult to interpret. In the populational approach, it is mandatory to have a good candidate gene. DNA markers located on the responsible gene itself or within close proximity are then used, and the frequency of the marker in the diseased population is then compared to that of a control population (Soubrier et al. 1993). Several candidate genes have already been selected for such a purpose, as angiotensinogen or Angiotensine I Converting Enzyme to study arterial hypertension, or apolipoproteins for hyperlipidemia.

Main types of hereditary diseases.

Hereditary diseases are diseases due to a mutation occuring on one or several genes. **Figure 61** summarizes the main categories of genetic diseases.

(i) Monogenic diseases with a founder effect are due to a single mutation that results in the absence of one amino acid and both a defective protein and function. The mutation may be historically unique, the most well-known example is sickle cell anemia which is due to a mutation in codon 6 of the β-globin gene. This mutation results in the substitution of a negative amino acid, glutamic acid, by a neutral residue, valine. Individuals bearing such a mutation also possess the same genetic markers of the locus

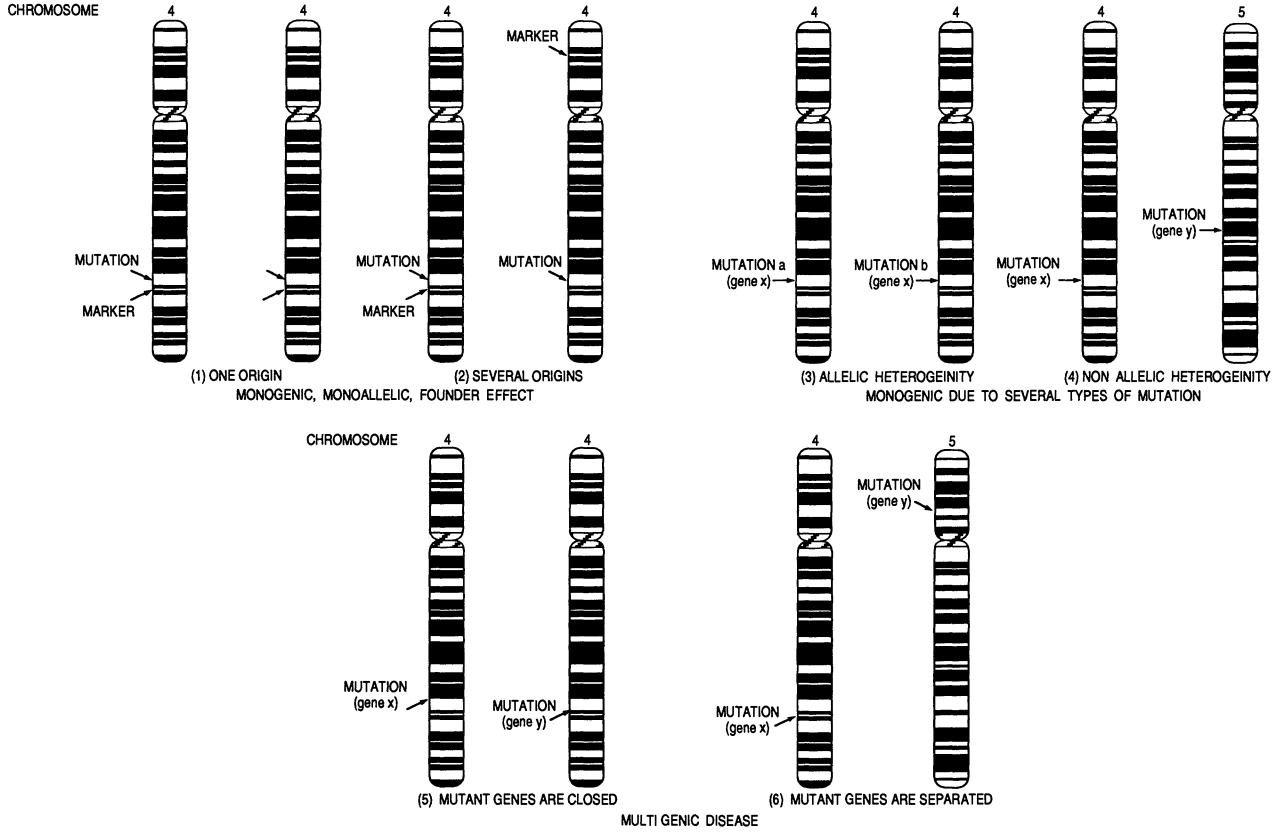

Fig. 61 Mono and Multigenic diseases.

which allows one to say that the accident had only occured once in one founder. The mutation creates a disease, anemia, but it also protects these people against malaria, which explains why such a unique event has finally been disseminated throughout Africa. There are also monogenic monoallelic diseases with a founder effect which are due to the same mutation, nevertheless, because the mutation is not always linked to the same markers, it is possible to conclude that the disease originated from several identical accidents occuring in different places at different moments (Fig. 62). In these conditions both phenotype and genotype are unique.

(ii) Most of the monogenic diseases are however due to several different mutations, the phenotype is identical, but the genotypes are different. It is possible to have different mutations on the same gene, resulting in the same clinical syndrome, this situation is termed allelic heterogeineity. Familial Hypercholesterolemia, for example, may result from approximately 200 different mutations on the lipoprotein receptor gene. There are also non allelic heterogeineity, i.e. mutations occuring on different genes, on different chromosomes (Fig. 61), but again giving rise to the same phenotype. A good example is Familial Hypertrophic Cardiomyopathy that may be due to mutations, on, at the least, five different chromosomes.

(iii) Multigenic diseases are due to several mutations which can be genetically linked, or, by contrast, located on different chromosomes. Arterial hypertension, hyperlipidemias, diabetes belong to this category. Atherosclerosis is determined by the combination of the above diseases, plus additional genetic factors, plus environmental factors (Sing et al. 1990). Atherosclerosis is then both multigenic and multifactorial. Such an extreme complexity has imposed an entirely different strategy to geneticians that want to start research in the cardiovascular field.

To reduce the problem to manageable dimensions, genetic studies have focused on the study of intermediate traits* which were previously

identified as risk factors in terms of clinical manifestations of atherosclerosis as Coronary Artery Disease, CAD. Nevertheless such traits, such as arterial hypertension, diseases of hemostasis, hyperlipidemias or diabetes, are still extremely complex. There are, for example, at least 200 genes which are involved in lipid metabolism and and in which mutations may be responsible for hyperlipidemias. The "genetic architecture" (Sing et al. 1990) responsible for the distribution of a trait in a large population is defined by the number of genes and alleles reponsible for the expression of that trait, but it also includes the impact of alleles on the level of the trait and its relationship with other risk factors. The effort spent on trying to present a complete genetic architecture of inherited risk factors are great, especially in hyperlipidemias, but for the moment an overall picture is still lacking even for one identifiable trait.

INHERITED CARDIOVASCULAR DISEASES AND RISK FACTORS.

Cardiology has been for a long time more closely linked to environmental problems than to genetics. Sudden death was more often attributed to diet or smoking, than to heredity. Genetic epidemiology is a rather new branch of cardiology and data comparing heritability to environmental factors are still rare, although concordant. At present, although it is possible to provide sufficient data to convince cardiologists that genetics will soon become a major tool to prevent, diagnose and perhaps treat cardiovascular diseases, it is impossible to write an extensive review on the subject.

The goal of this chapter is therefore to illustrate the new ways that have been recently suggested by a few pioneer publications. For the moment, an extensive review on genetics in cardiology is likely to be an impossible task. The reader interested to have more complete information in

this field should read the following reviews (Breslow 1993, Chan 1990, Sing et al. 1990, Soubrier et al. 1993, Corvol et al. 1993).

There are, as yet, rather few cardiovascular diseases which are monogenic, such as Familial Cardiomyopathy, FCM, and there isonly one that is both monogenic and monoallelic, the Familial Defective Apo B-100. The strategy which has been used to explore the genetic factors in cardiovascular diseases is different from that commonly applied to problems such as cystic fibrosis or hemoglobinopathies. For similar reasons, this approach is also used for cancer and asthma.

In this chapter, an attempt has been made to start with rather simple, although rare, problems, those arose from monogenic diseases, and then to establish a few bases for the study of more complex problems.

Monogenic cardiovascular diseases.

Familial Defective Apo B-100.

This is a frequent autosomal dominant monogenic, and, for the moment, the only monoallelic (as drepanocytose) familial cardiovascular disease which has been unambiguously identified. Whether the mutation originates from one or several places in the world is unknown.

Hyperlipidemia (type IIa) is caused by an increased level ofLow Density Lipoproteins, LDL, cholesterol level and is associated with premature atheroclerosis. Such a type of hyperlipidemia is due to a defective clearance in LDL, and Familial Defective Apo B-100 is one of the forms of type IIa Hyperlipidemia.

The disease is frequent (1 : 500) and is caused by a mutation in the coding sequence of the apo-B gene (on chromosome 2) that changes an arginine codon at amino acid 3,500 to glutamine (Tybjaerg-Hansen et al.

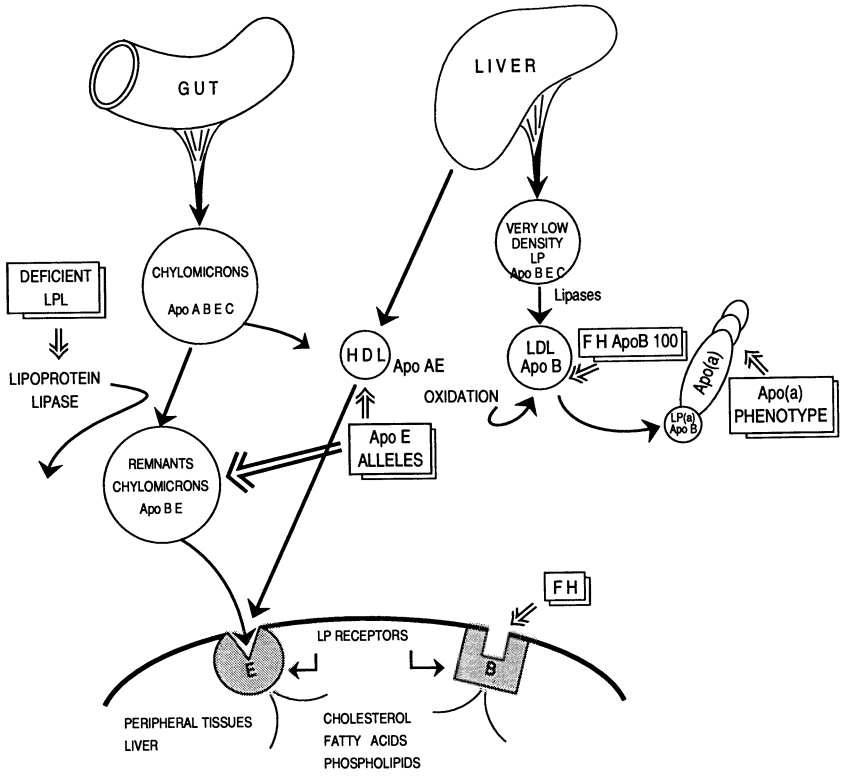

Fig. 62 PATHWAYS FOR LIPID TRANSPORT-MAIN MECHANISMS FOR HYPERLIPIDEMIAS (in Boxes) - LP : Lipoproteins - Apo : ApoLp-HDL,LDL : High, Low Density LP.FH : Familial Hypercholesterolemia

1992). The mutated codon is located in the LDL receptor-binding region of apo-B and results in a protein with a very low affinity for the receptor. LDL are lipoproteins that contain apo-B100 (the hepatic form of apo-B) and originate from the liver, after lipolysis of Very Low Density Lipoproteins. LDL deliver cholesterol to the liver and peripheral tissues (**Fig. 62**). Their cellular uptake is mediated by the LDL receptors which recognize apo-B100. The disease is monoallelic and therefore easily detectable by routine analysis on spotted whole blood using PCR amplification (Hansen et al. 1991). The technique used by Peter Hansen is an interesting clinical application of PCR and consists of introducing a cleavage site for the restriction enzyme MspI (C/CGG) in normal alleles but not in mutant alleles (**Fig. 63**).

Familial Hypercholesterolemia.

This disease is frequent (1 : 500 for heterozygotes) autosomal dominant monogenic, but multiallelic. The phenotype is the same as described previously, i.e. hypercholesterolemia with premature atherosclerosis, and is associated with the occurence of planar xantoma in the homozygous forms. This is a type IIa Hyperlipidemia with a defective clearance of LDL, nevertheless in this case the structure of apoliproteine B is unchanged and the mutation is located on the LDL receptors. The human LDL receptor gene is on chromosome 19q13 and spans 45 Kb and has 18 exons (**Fig. 64**). The first mutations were reported by two Noble prize winners (Brown and Goldstein 1986). Nearly 200 different mutations have now been reported. The mutations are located all along the gene and can result in the total absence of receptor, or a deficiency in lipoprotein transport or fixation, or even in a deficiency in the process of receptor internalization. Such a multiallelism renders the routine detection of the diseased mutation tedious and necessitates the alternate approach of linkage analysis.

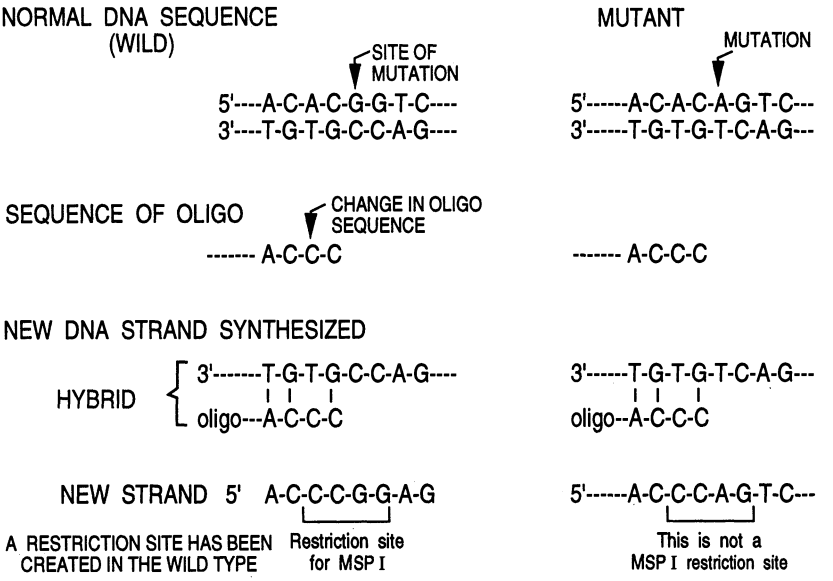

Fig. 63 Detection of the apo B - 3,500 mutation by PCR on blood spots (DRAWN from Hansen et al. 1991).

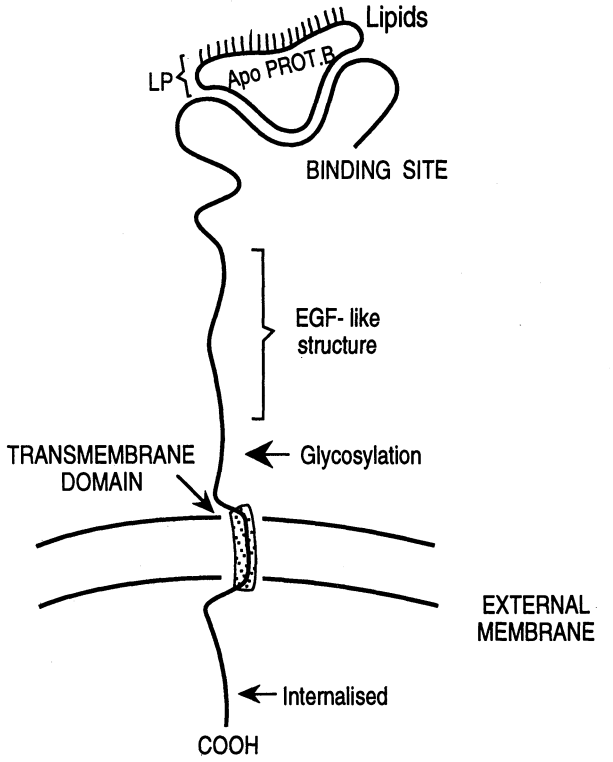

Fig. 64 Lipoprotein (LP) Receptor (redrawn from Brown et al. 1986).

Familial Hypertrophic Cardiomyopathy.

Hypertrophic Cardiomyopathy is a cardiomyopathy that is familial in half of the cases and sporadic in the other half. Familial Hypertrophic Cardiomyopathy, FHC, usually has an autosomal dominant* pattern of inheritance (a few cases of autosomal recessive or non autosomal, X-linked and recessive patterns of inheritance have been reported). The incidence of the disease is around 2.5 per 100,000 inhabitants per year. The penetrance* of FHC is incomplete in young individuals and increases with age. The disease is severe and the annual rate of mortality is around 2 to 4%.

FHC is characterized by a primary myocardial hypertrophy which is most frequently asymetric and usually predominates on the interventricular septum. Systolic ejection is hampered in nearly 60% of the cases and creates an intraventricular gradient. Two major specific features characterize the disease : (i) a high incidence of spontaneous or induced ventricular tachycardia and sudden death (the disease is one of the major causes of sudden death in athletes) ; (ii) a normal systolic function with an accelerated ejection velocity and pronounced diastolic dysfunction. Post-mortem examination, or endomyocardial biopsy reveals an increased myocardial mass, and myocytic and myofilaments disarray with structurally intact sarcomeres on electron micrographs. The severity of the prognosis is variable, and is possibly related to the genotype, there are benign forms, and, conversely, children who die suddenly, the prognosis depending mainly on the severity of the accompanying arrhythmias.

Molecular basis. The overall strategy followed in the study of this disease is an excellent and representative example of the strategy used for positional cloning. It has only been recently shown that the disease is linked to various mutations located either at different places on the same gene (or locus) (allelic heterogeinity), or on different genes (or locus) in different chromosomes (non allelic heterogeineity). In other words different genotypes yield the same, or nearly the same, phenotype. The first locus

was identified by the group of Christine Seidman in 1989 [Jarcho et al 1989, Geisterfer-Lowrance et al 1990], by using genetic-linkage analyses with polymorphic DNA, on chromosome 14 at band q11, close to the gene encoding the α chain of the T-cell receptor. Furthermore, it was demonstrated that, in these kindred, the mutation was a missense $403^{Arg\text{-}Glu}$ on exon 13 on the head of the β–isoform of the cardiac myosin heavy chain on the 50 K segment which is close to the actin binding site. Since this pioneer observation, several distinct missense mutations have been reported on the same gene (at least 17-20, **Figure 65**).

A 2.4 kb deletion (representing 10% of the gene!) involving part of the intron 39, exon 40, the 3'-untranslated region, and part of the β–α myosin heavy chain intergenic region has also been reported in a small family [Marian et al 1992]. An unequal crossover event occuring during meiosis and resulting in an α/β cardiac myosin hybrid gene, flanked by the β and the α isogenes, is also associated with phenotype characteristic of FHC [Tanigawa et al 1990] (**Figure 66**). Similar hybrids were found in hemoglobinopathy due to hemoglobin P (due to a β/δ hemoglobin hybrid gene). Finally other mutations occuring on different chromosomes including chromosome 11p13-q13 [Carrier et al 1993], chromosome 1q3 [Watkins et al 1993], chromosome 15q2 [Thierfelder et al 1993], and very likely a 5th locus, has also been reported.

In normal conditions, the β-isomyosin heavy chain gene encodes a protein which is expressed both in the myocardium and in the slow skeletal muscle fibers. In humans, skeletal muscle in general contains a mixture of fast and slow isoforms, and the myosin mutations have also been found in the skeletal muscle [Cuda et al 1993] in spite of the fact that clinical signs of skeletal myopathy are rare.

The relationship with the clinical situation is far from being simple : clinical symptoms such as sudden death or ischemia may result from different mechanisms, mutations responsible for the disease occur in various part of the genome. It is now clear that several different mutations,

including mutations in non contractile proteins, can result in the same clinical symptoms. The mutation $606^{Val\text{-}Met}$, which results in a neutral charge substitution, is malignant in some kindreds whereas it is associated with near normal survival in others. Conversely sudden death occurs in only 2% of the cases with the mutation $256^{Gly\text{-}Glu}$ which results in a charge change. The amino acid 403 is normally an Arginine, several different mutations, at least 3, were reported at this particular locus and are associated with a severe prognosis, suggesting that this amino acid is a "hot spot". Studies of large kindred is necessary to be certain that a mutation is benign and not accompanied by a high risk of sudden death, to date, only the $908^{Leu\text{-}Val}$ and the $256^{Gly\text{-}Glu}$ mutations satisfy this requirement [Fananapazir et al 1994].

The pathogenesis of the disease is still unknown. The most commonly accepted hypothesis is to consider the abnormalities of the contractile proteins, including the various mutations occuring in the myosin head, as causal and hypertrophy as compensatory. Mutations occur mainly in the head of the myosin molecule, in the specific region where both ATP is hydrolyzed and actin is bound. Mutations on myosin, or others components of the contractile apparatus, could poison assembly of many myosin molecules [Geisterfer-Lowrance 1990] and result in abnormal contractility, and to compensate for such an abnormal contractile state, the heart, as usual, hypertrophies. The skeletal muscle does not hypertrophy, because it does not permanently contract, and, in addition, it possesses a fast myosin isoform which may play a compensatory role.

This hypothesis is supported by the elegant experiments of Cuda et al [1993] which showed that purified mutant β–myosin ($908^{Leu\text{-}Val}$ and $403^{Arg\text{-}Gln}$) had abnormal functions in an *in vitro* assay in which actin filaments are translocated by myosin bound to a coverslip surface.

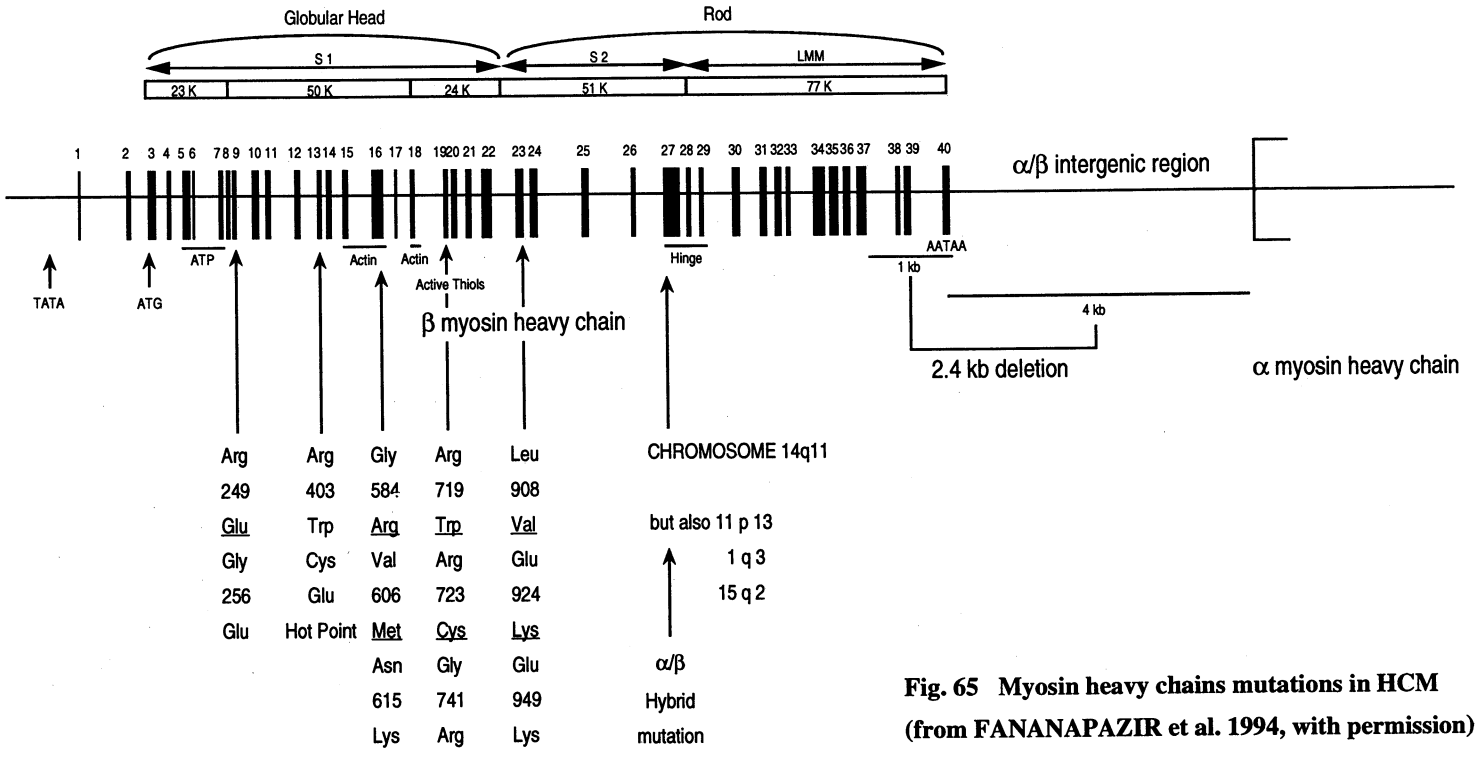

Fig. 65 Myosin heavy chains mutations in HCM (from FANANAPAZIR et al. 1994, with permission)

Nevertheless such a hypothesis did not take into account other findings : (i) if hypertrophy compensates for an altered myosin function, it is surprising to see that *in vivo* systolic ejection is normal, and that there is no compensation at the level of the skeletal muscle. (ii) The compensatory hypothesis does not explain the incidence of sudden death, nor the asymetry of the hypertrophy. (iii) In addition, FHC can be associated with mutations occuring in chromosome 11p13-q13, in a region where there are no contractile proteins genes [L. Carrier et al. 1993]. (iv) Another intriguing clinical finding is the existence of patients who are proned to sudden death and have cardiac arrhythmias and carry the disease gene but who have no signs of hypertrophy. In other words the electrical and the morphological forms of the disease can be dissociated [Epstein et al 1990].

Alternatively one could say that the FHC phenotype needs to be expressed either some particular environmental conditions such as intensive training, or the expression of an associated facilitator gene.

An important issue of the problem is prevention. Could routine genotyping be performed in children or athletes at risk for FHC ? The general opinion at present is to reserve routine genotyping to members of a family when a mutation is discovered, and not to apply this technique in routine practice. The main reason is our poor knowledge on genotype-phenotype correlations, particularly concerning the relations between mutation and the mechanisms of sudden death which can result from ventricular tachycardia, hypotension due to atrial fibrillation, bradyarrhythmias, acute myocardial ischemia, and left ventricular outflow obstruction, it is certainly more important to evaluate individuals for their propensity to develop such events, than to base a prognosis on a single rather complex genotype determination [Fananapazir et al 1994]. Attempts to prevent ischemia with beta-blockers or calcium-blockers in individuals with known mutations have been more successfull, in spite of the fact that

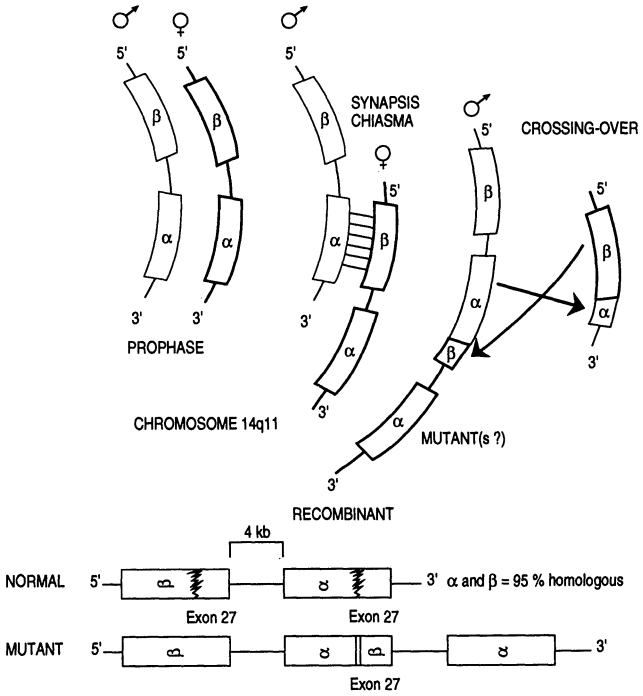

Fig. 66 Familial Hypertrophic Cardiomyopathy.
α/β myosin heavy chain hybrid (Drawn from Tanigawa et al. 1990)

ischemia in FHC has multiple origins, including an inbalance between coronary flow and myocardial mass, endothelial hyperplasia and episodes of atrial or ventricular tachycardia.

Long QT syndrome.

The long QT syndrome is a rather rare familial disorder which is characterized by a prolongation of the QT interval (QTc interval > 0.44 sec $^{1/2}$) and a propensity to ventricular arrhythmias and sudden death especially during youth (Moss et al. 1991). In 1991, 245 different markers were tested and it was shown that a DNA marker defining a restriction site polymorphism at the H-ras-1 locus, pTBB-2, was tightly linked to the disease with a maximum LOD score of 16 at a recombination fraction of zero. A second probe located on the same locus, pUC EJ 6.6, gave the same result (Keating et al. 1991).

Multigenic cardiovascular diseases and risk factors.

Monogenic diseases are well-charcterized and delimited topics. Inherited risks factors are of crucial importance in cardiology, nevertheless the topic is far from being well-defined and it is, for the moment, only possible to provide several pathways and give examples in a field of investigation where knowledge is increasing day and day. Epidemiological studies have isolated several risk factors including hyperlipidemias, hypertension, diabetes and also unknown factors.

Hyperlipidemias.

This topic cannot be reviewed in detail in such a book. However Fig. 63 summarizes both the main pathways for lipid transport and metabolism and the main genetic mechanisms responsible for hyperlipidemias.

Lipids are insoluble in the plasma, by definition, and circulate in the blood stream as Lipoproteins, Lp, which are in a complex of various lipids associated to Apoproteins, Apo (the genetic structure of Apo has been nicely reviewed in Chan et al. 1990). Dietary lipids are packaged in the intestinal cells and secreted in the the lymph as large particles termed chylomicrons and are lipolyzed as smaller remnant chylomicrons (Fig. 62). Endogenous synthesis in the liver results in the formation of Very Low density Lps, VLDL, and LDL. LDL are important particles in hyperlidemias and include Apo B-100 which is an Apo specifically synthesized in the liver. Lp (a) is a recently discovered Lp which is derived from LDL and includes a specific Apo(a) component whose physiological role is unknown, but is polymorphic and likely to play an important role in atherogenesis.

Lps deliver lipids to peripheral tissues and cellular uptake is mediated by receptors that bind the Apo component of Lps. For the moment, several types of Lp receptors (Fig. 64) have been described (at least four), such as

the Lp receptors discovered by Brown and Goldstein (1986) which bind LDL through ApoB, and Lp receptors specific for ApoE.

Genetics of hyperlipidemias includes two different clinical problems. (i) Monogenic hyperlipidemias which have been described above, however there are several other rare diseases due to mutations in other Apo, such as Apo E (type II Hyperlipidemias), or various lipases or components responsible for the reverse cholesterol transport (**Table 17**). For example, several defects in the structure of the gene encoding lipoprotein lipase have been reported, including various types of gene rearrangements and point mutations which cause familial chylomicrominemias (Benlian et al. 1995). (ii) Family associations, including complex segregation analysis and path analysis have also demonstrated that 50% of the normal interindividual variability in total cholesterol is associated with polygenic differences (Lusis 1988). One of the difficulties for appreciating the genetic factor is that the trait, namely plasma cholesterol or Apo content is highly dependant on environmental factors such as fat or alcohol consumption, exercising and even smoking habits (which can influence the Apo E plasma level).

Of particular interest were the family studies which finally evidenced associations between normolipidemic variations and Apo alleles. Three common alleles of Apo E designated E2, E3 and E4, have been identified and occur at frequencies of 7, 78 and 15% respectively (Chan et al. 1990). The two most frequently paired alleles are E3/E3 that is shown in 57% of the population and E4/E3 which is observed in 22% of the population. E2 is associated with lower plasma cholesterol level than E4, and, for example, the mean plasma cholesterol level of the E2/E2 homozygotes is 140 mg/dL, as compared to 197 mg/dL for E4/E4. Such a difference is likely to reflect a

Table 17. Genetics of Hyperlipidemias. (rearranged from Breslow 1993). •Frequent or ••very frequent. A : autosomal. D : dominant. Lp : lipoprotein. Apo : apoLp. 1 : 500 : frequency in the population.

Increased Low Density Lp.

- Apo E phenotype with alleles E4/E3 (population study)••.
- Familial Defective Apo B-100 : AD ; $3,500^{Arg\ Glu}$; 1 : 500•.
- Familial Hypercholesterolemia (LDL receptor deficiency) : AD ; >200 mutations ; 1 : 500•.

Increased Very Low Density Lp and decreased HDL.

- Mutation on Apo A-I gene (defective HDL).
- Defective lipoprotein processing : Hepatic lipase, CETP, Lp Lipase ; 1 : 10^6(familial chylomicrominemias).
- Defective genes controlling HDL catabolism.
- Apo C-III overexpression (?).

Increased Chylomicrons Remnant and Intermediary Density Lp.

- Dysbetalipoproteinemia (or type III Hyperlipidemia) ; polygenic ; Apo E E2/E2 phenotype (1 : 100), needs environmental factor to be expressed•.

Increased Lp (a) level.

- Lp(a) plasma level is genetically determined and may cause hyperlipidemia••.

faster rate of clearance of the E2 alleles than does the E4 form which is attributable to a diminished clearance of Apo E4-bearing lipoproteins by the Apo E receptor (Lusi 1988).

Similar studies were performed with Apo (a) which is a large glycoprotein associated to Apo B-100 in an LDL-like particle termed Lp (a). Apo (a) has a structure very similar to that of plasminogen and, both the protein and cDNA, contain 15-40 copies of a kringle IV-like region, a kringle V-like region, and a variant-protease domain (Fig. 64). There are approximately 40 different isoforms of Apo (a) depending of the number of

kringles which has been expressed. The length of the protein depends upon the number of the kringles, and constitutes an inheritable trait. It varies from one individual to another, and from one population to another. In addition, the shorter the protein the higher the plasma concentration of Lp (a) and the higher the risk of atherosclerosis.

Essential arterial hypertension.

Studies of twins or larger populations have clearly demonstrated that blood pressure aggregates and suggests an approximate 30% degree heritability for blood pressure. Blood pressure is continuously distributed in the population, the genetic basis of the disease is polygenic and it is very unilkely that inheritance follows the classical mendelian laws. The trait is quantitative and has been empirically determined as the level of blood pressure (160/95 mmHg) which is accompanied by cardiovascular complications. Taking into account the dimensions of the problem, the genetics of essentiel arterial hypertension is still a largely unexplored field of investigation.

The candidate gene approach has also been used in the study of hypertension, although on a smaller scale, and the first candidates were the genes coding for the Renin-Angiotensin System, RAS (Soubrier et al. 1993). Linkage, association and sib-pair studies failed to demonstrate any association or linkage between the renin gene and hypertension (Jeunemaître et al. 1992 a). The same negative association or linkage were found for the Atrial Natriuretic Peptide and the Na^+/H^+ antiporter (reviewed in Samani et al. 1994).

By contrast, there is evidence to suggest that such a linkage exists with the Angiotensinogen, Angne, gene : (i) the plasma level of Angne is correlated with blood pressure in large epidemiologic studies, especially in young adults. (ii) Linkage studies have been performed using a highly polymorphic and informative (CA) dinucleotide repeat isolated on the Angne gene. One of the studies was performed both in Paris and Salt Lake City on 215 pairs of sibs affected by arterial hypertension and another study comes

from London and was carried out on 63 mutiplex families. Both demonstrated an excess of concordance for these microsatellite alleles, as compared with the expected concordance if both the disease and the marker segregate independently and conclude that arterial hypertension and the Angne are linked and associated (Jeunemaître et al. 1992 b, Caulfield et al. 1994). (iii) Several mutations have been detected on the Angne gene, and two of them on codons 235 and 174 on exons 2 and 3, gave rise to two pairs of alleles. In one of the above studies (Jeunemaître et al. 1992 b) the distribution of the paired alleles was different in hypertensive subjcts than in controls and the plasma concentration in Angne was correlated with the genotype. Hypertension was not associated with the allelic distribution in the other study (Caulfield et al. 1994).

Significant familial correlations have been found between genetically related individuals and the plasma level of Angiotensin Converting Enzyme, ACE. Once the ACE gene had been cloned, it was shown that this phenotype was associated with an Insertion/Deletion, I/D, polymorphism located in intron 16 of the gene and that the mean plasma level of DD subjects was about twice that of II subjects. Nevertheless, it was impossible to demonstrate, by sib-pair analysis, any linkage between hypertension and a highly polymorphic marker located close to the ACE gene (Soubrier et al. 1993).

One of the most promising features reported in this domain during the last decades, was the finding of a linkage between ACE/ID polymorphism and myocardial infarction (Cambien et al. 1992). The DD genotype was indeed significantly more frequent in 610 patients with myocardial infarction than in the 733 controls, especially among patients with a low body-mass index and low plasma levels of ApoB (or cholesterol, or cholesterol in LDL), i.e. in patients usually considered to be at low risk (with an odd ratio above 3). The DD genotype was not associated with myocardial infarction when risk factors such as smoking habits, plasma levels of ApoA1, Lp(a) or fibrinogen were considered. In this particular

protocol many patients received drugs affecting blood pressure and therefore the conclusions of this article do not apply *stricto sensu* to arterial hypertension. This work has a potential interest for drug trial to see if the genotype is related to sensitivity to Angiotensin Coverting Enzyme inhibitors both in terms of blood pressure and survival.

Diabetes.

Type I diabetes is an autoimmune disease caused by several different mutations on different genes encoding the various components of the immune system, including the HLA system at locus HLA-DRB1 on chromosome 6, genes responsible for the maturation and translocation of antigenes, the insulin gene itself, genes coding for the receptors of antigens such as immunoglobulin receptors or lymphocyte T receptors. There are polymorphic markers linked to the disease and located in the vicinity of the insulin gene. The disease is atherogenic and a major cause of cardiovascular complications (reviewed in Todd et al. 1992 and in P. Froguel et al. in Corvol et al. 1993).

Type II diabetes is not insulin-dependent it is an extremely frequent disease (4-7% of the population) which is also associated with a high incidence of atherosclerosis, but is not autoimmune. Clinically this type of diabetes is a major responsible for blindness, renal insufficiency and amputations. The phenotype is characterized by a decreased utiliation of glucose and an enhanced hepatic production. The insulin response to glucose is altered, and insulin needs to be secreted a higher plasma glucose concentration than normal.

The disease is also highly multifactorial and to be expressed the phenotype needs both genetic and environmental factors as obesity. The genetic approach has been similar to that of arterial hypertension : several rather rare monogenic diseases were firstly identified as deficiencies in glucokinase, and further the techniques used for multigenic diseases were applied to search new genes or candidate genes.

Maturity Onset Diabetes of the Youth, termed MODY,is an autosomal dominant heterogeneous type of diabetes due to several different mutations mostly located in the gene encoding glucokinase. Untill now there are at least 40 different mutations which have been identified on this particular gene, the most frequent mutation is located on exon 7. 0.5-1% of the type II diabetes are due to mutations in the mitochondrial DNA. Mitochondrial DNA is located in ovules and both the disease and the DNA are transmitted by the mother.

The candidate gene approach is actively developed in this area, but other approaches are used such as the cDNA display.. Good candidates are the genes related to glucose metabolism (at least 40) and mutations have been found in the genes encoding insulin and glucagon and their receptors, glycogene synthase, the Fatty Acid Binding Protein...

5

GENE TRANSFER.

Gene transfer is the possibility to incorporate new genetic material into a cell where it can be expressed either as extrachromosomal material, or after being incorporated into chromosomal DNA. The process occurs naturally during a viral infection by a retrovirus. It is also possible to produce gene transfer experimentally by injecting genetic material into isolated cells, or even into adult tissues (Haber 1993). Transgenic technology is a third possibility and consists in transfering genes into gametes to produce strains expressing the new DNA sequences.

The clinical applications are, of course, those which are of interest for the cardiologist. They are, for the moment, with a few exceptions, mostly promising areas, and the time when DNA may become a drug in cardiology is still unpredictible.

GENE TRANSFER DURING VIRAL INFECTION.

Virus are living elements without nuclei and nuclear membrane and whose genetic material consists of either RNA or DNA. Retroviruses are RNA viruses whose genetic material is incorporated into the host's genome during infection (**Fig. 67**). The retrovirus cycle includes a step during which the RNA is retrotranscribed into DNA by the specific viral reverse transcriptase in order to be included into the genome, subsequently the viral material is transcribed and new virus particles are produced. The reverse transcriptase comes from the virus, and it is also able to synthesize non specific

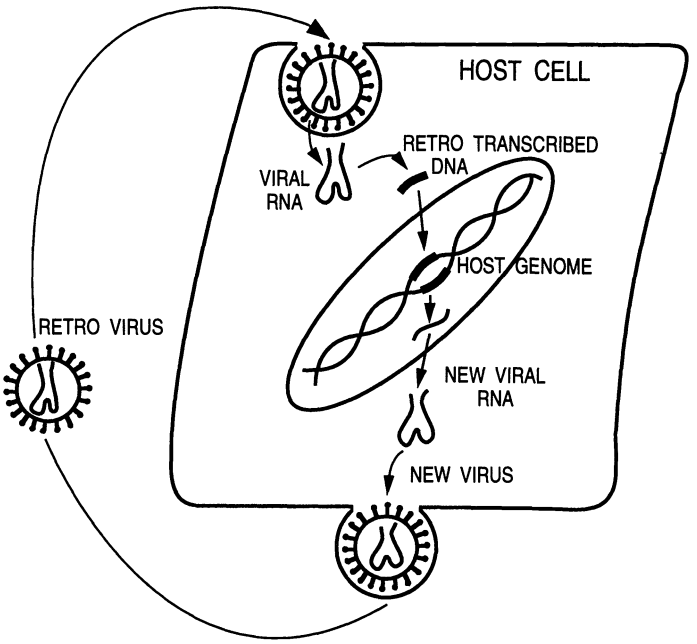

Fig. 67 Retro virus cycle

promotor sequences which are able to activate the expression of adjacent genes. During these steps, both the host's genome, and the viral genome may acquire new DNA sequences, that, in a sense, perverts the transfected genome which, in turn, can become transformant. Such a concept is one of the basis of the oncogenic theory of cancer. This family of retroviruses includes HIV which is the virus responsible for AIDS. It is also very likely that the process of retrovirus infection may have played a role during evolution and that, for example, we had acquired some viral sequences of interest by using this procedure, but of course we shall never have the absolute proof of this. Good candidates are, for example, the intronless genes encoding membrane receptors since the absence of introns is one of the characteristic features of procaryotic genes.

TRANSGENIC ANIMALS AND REVERSE PHYSIOLOGY.

Transgenic animals are becoming a new tool accessible to physiologists and pharmacologists because there are now a number of transgenic animals commercially available as, for example, the transgenic strains of hypertensive rats (**Table 18**). The ability to introduce new genes into the mammalian fertilized ovum was first described in 1980 and the most commonly used technique relies on direct injection of the DNA of interest into the male pronucleus of fertilized eggs from mice. The injected embryos are then reimplanted in a pseudopregnant foster mother and allowed to develop to term (**Fig. 68**). The resulting pups are screened to detect those animals which have incorporated the injected DNA in their genome. DNA is prepared from the tail or ear of the transgenic mice and analyzed by Southern blot. In experienced hands, the injected DNA is usually found in 20 % of the pups. To be useful for physiological or pharmacological studies, the foreign gene must be expressed as a mRNA, but also necessarily as a protein in the relevant organ. In terms of altered functional activity, the final yield may be extremely low and... the financial cost very high.

The microinjected DNA can be an entire gene, including its own promotor, but usually it is a chimeric construct consisting of a tissue-specific promotor and the coding (or non coding, if we want to specifically block, geneticists say to knock out*, gene expression) part of a gene not usually controlled by the selected promotor.

Three different aspects of transgenic technology have been applied to cardiovascular research.

(i) One of the goals is to target gene expression to a specific part of the cardiovascular system. By so doing, we want to overexpress, or

Table 18. Some examples of transgenic strains which have been used in cardiovascular research (reviewed in Field 1993¥. P : Promotor. C : Coding sequence. ANF : Atrial natriuretic Factor. β1 or 2-AR : β1 or 2 Adrenergic Receptor. MHC : myosin heavy chain. LDL : Low Density Lipoprotein. = unchanged.

Genetic construct.	**Phenotype.**
Heart.	
- P : ANF. C : SV40 T Ag oncogene (Field 1988).	Atrial tumor, arrhythmias.
- P : metallothionein. C : c-myc oncogene (Jackson 1990¥).	Hyperplasic cardiocytes.
- P : β-globin. C : c-fps oncogene al. 1991¥)	Cardiac Hypertrophy + (Chow et Fibrosis.
- P : ANF. C : human β1-AR (atria) al. 1993).	⇓Heart Rate Variability (Bertin et + ⇓Sensitivity to Iso.
- P : α-MHC. C : human β2-AR (Milano et al. 1994).	⇑basal ventricular contractility.
Vessels.	
- Mouse renin gene in rats et al. 1990¥).	Arterial Hypertension + (Mullins Plasma renin =.
- Rat angiotensinogene gene (Kimura et al. 1992).	Arterial Hypertension.
- Deficiency in endothelin-1 (embryonic (Kurihara et al 1994).	Arterial Hypertension + stem) Craniofacial abnormalities.
- Overexpression of LDL receptors (Hofmann et al.¥, Brown 1986)	Prevent hyperlipidemia.

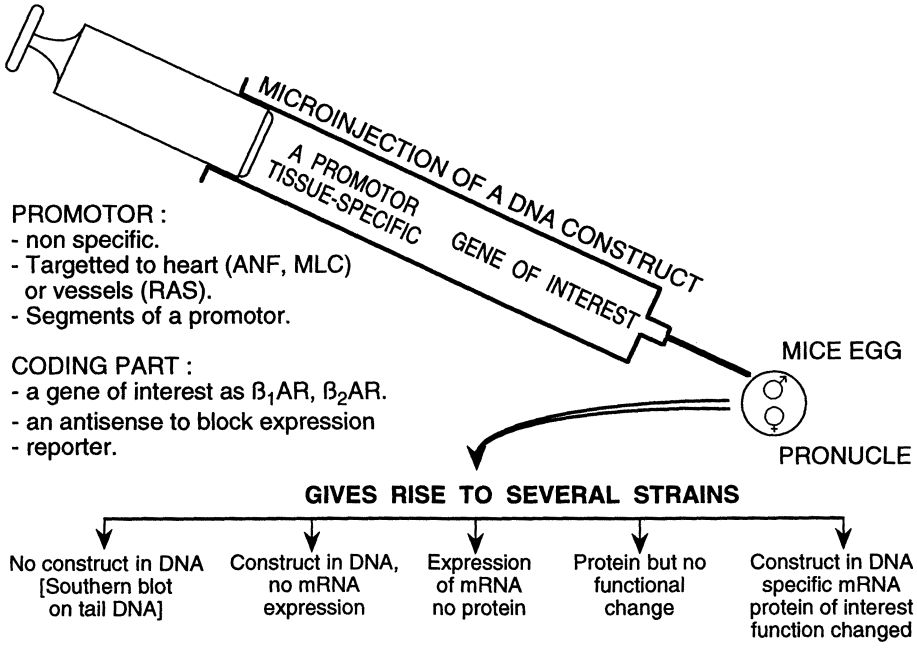

Fig. 68 Transgenic technology

to suppress the expression of a gene, with the hope of altering the corresponding cardiac or vascular function and practicing what we call reverse physiology. To attain such a goal, the choice of the promotor is of crucial importance, and it is now possible, for example, to target expression specifically to the myocardium as opposed to the coronary vessels. For example, the promotor controlling the transcription of the Atrial Natriuretic Factor gene is constitutively active only in the atrium (Ch. 2) and can be used to target the expression of any coding sequence to this part of the heart (Field 1988, Bertin et al 1993). The promotor of the ventricular Myosin Light Chain 2 or α-myosin heavy chain are active only in the ventricles, that of vasopressin directs gene expression in the anterior pituitary and the pancreas while that of neurofilament F is specific for the pituitary. Hence any coding DNA controlled by these promotors will be transcribed only in the tissue in which the promotor is functional. It is also possible to use non-specific promotors, such as the metallothionein promotor and, by chance, get specific cardiac expression (Table 18).

Our main point of interest here is the gene product itself. It could be the coding part of the selected gene controlled by its own promotor if the entire genomic sequence is injected. But then its expression will be regulated as is the endogeneous gene and there will be no advantage in using transgenic mice. Usually the goal is to take advantage of a tissue-specific promotor to overexpress or to inhibit the expression of a chosen protein. One good example is the pioneer work of L. Field (1993) who obtained an arrhythmogenic atrial tumor in transgenic mice by injecting a chimeric gene consisting of the ANF promotor and the coding region of an oncoprotein. In our laboratory we have developed a new transgenic model of atrial β1-adrenoceptor overexpression (Bertin et al. 1993, **Figure 69**). In this model, the expression of the adrenergic receptors has been targeted to the atria by using the ANF promotor sequence, instead of the naturally occuring promotor of the receptor gene, and the purpose of the experiment was to modify the normal β1-adrenergic/muscarinic receptor ratio of atria, and more

particularly of the cells of the sinusal node, to modify the normal regulation of the heart rate and to change heart rate variability, a parameter of crucial importance for predicting sudden death in clinical cardiology. The result of this reverse physiology was a disappearance of heart rate variability in transgenic strains, which demonstrates that the atrial phenotype, in terms of autonomous system components, is as important as baroreflexs or central nervous system influences.

Another interesting approach consists of injecting an antisense mRNA to block the expression of the normally occuring mRNA. For example, Pepin et al. (1992) impaired the glucocorticoid feedback loop of ACTH synthesis and secretion by injecting into the fertilized egg a construct consisting of the neurofilament F promotor controlling the transcription of an RNA complementary to a portion of the 3' non-coding region of the rat glucocorticoid receptor cDNA. This antisense RNA has minimal sequence homology with other members of the steroid hormone receptor gene superfamily, but it hybridizes specifically to the glucocorticoid receptor type mRNA and inhibits its expression. Consequently the transgenic mice becomes obese and exhibits very high plasma levels of ACTH and corticosterone while the glucocorticoid maximal binding capacity of the pituitary was greatly diminished.

(ii) An entirely different strategy consists of analyzing the promotor. A construct is made with the promotor of interest linked to a reporter*, which is a gene encoding an easily scorable protein to monitor the activity of the promotor. Most of the reporter genes code for enzymes not expressed in the tissue where the promotor is active, such as the bacterial enzyme Chloramphenicol AcetylTransferase, never present in mammalian organs, or β–galactosidase normally present only in the gut. Even more elegant is the use of a reporter gene coding for a secreted protein such as human growth hormone which can be directly measured in the blood of transgenic mice by radioimmunoassay.

This very common use of chimeric promotor-reporter gene constructs has been used to identify the DNA sequences which regulate the activity of a promotor after hormonal treatment, during differentiation or after mechanical stretch for example. It has also been used in cardiology to try to identify the regulatory regions sensitive to mechanical overload (Chien 1990).

(iii) Animal species. The mouse is the commonly utilized animal species for very practical reasons : it is inexpensive ; it is very fertile, the gestation is very short and it matures at an early age (3 weeks) so that one can obtain transgenic mice rapidly. ECG and hemodynamic studies are now possible in mice which beats at 500 bpm and has a sympathetic tonus. Transgenic models of hypertension have also been developed in rats which facilitates the measurement of blood pressure since the tail-cuff method has not been yet adapted to mice. Another approach is the use of transgenic pigs to make up xenografts (and also human blood), i.e. to use animals organs which have been rendered tolerant to human immune system by transgenic technology for transplantations.

Gene transfer using transgenic technology is a random process and there is evidence to suggest that the resulting phenotype may also depend upon the location of the newly transfered constructs into the overall genome. Such an inconvenience could be overcome by the use of gene targeting in mouse embryonic stem cells. This is a new and sophisticated technique which provides the means to generate mice of any desired genotype (Capecchi 1989, Kurihara et al. 1994). For the moment, transgenic technology is probably more useful as a tool for experimental research or pharmacology, mainly because it allows one to perform REVERSE PHYSIOLOGY, i.e. to study the physiological properties of a species or organ in which known changes in genetic expression have been induced.

GENE TRANSFERS IN AUTOSOMAL CELLS.

Pionner studies have been carried out by JA Wolff (1990) on skeletal muscle and by E Nabel (1989) on blood vessels. Transfer of a genetic construct was obtained by injecting pure RNA or DNA directly into the quadriceps muscles of mice. A significant protein expression of the reporter genes was then obtained *in situ*, and maintained for at least 2 months (Wolff et al 1990). Porcine endothelial cells expressing recombinant β–galactosidase, a protein encoded by a reporter gene, from a retroviral vector, were introduced with a double balloon catheter into a denuded ilio-femoral artery, and, 2-4 weeks later, it was shown that the β–galactosidase was reexpressed throughout the entire arterial endothelium indicating that the DNA has been successfully transfected into the vessel wall (Nabel et al 1989).

Striated muscles, such as myocardium, have a particular ability to efficiently express injected genetic constructs as compared to other tissues. Genetic constructs, including a reporter gene (e.g. CAT or luciferase) and a promotor such as the Rous sarcoma virus promotor or the α-cardiac myosin heavy chain promotor, were directly injected into the myocardium after thoracotomy in dogs and in rats (Harsdorf et al. 1993, Buttrick et al. 1992). The activity of the reporter genes was detectable in a 1-2 mm area around the injection site, and was still detectable in 30% of the animals one month after injection. The viral promotor was more active than the cardiac-specific sequences. This technique is useful to study the regulation of expression of a promotor, but the geographic extension of the infection is rather limited. Nevertheless this approach has been proposed to treat skeletal muscle myopathies by multiple injections of a material containing the non defective gene.

For the cardiovascular system, we need a more systemic technique, and it has been proposed to intravenously inject the DNA construct by

utilizing a vector such as a adenovirus (Stratford-Perricaudet et al. 1992) or an expression plasmid : cationic liposomes (Zhu et al. 1993). Dose-response curves were obtained with efficient transfection even 9 weeks after injection in all the endothelial cells of the cardiovascular system and extravascular parenchymal cells with no treatment-related toxicity. The adenoviral construct is probably more efficient than other vectors including retroviruses and it has been demonstrated that the viral DNA remains extrachromosomal.

These approaches are obviously preliminary experiments which preceed gene therapy, and, for the moment, there practical utility is mainly to permit study of the mechanisms of regulation of genetic expression in vivo in adults.

GENE THERAPY.

Gene therapy is both a promising and fascinating topic as well as a dangerous game by which mankind is trying to more or less substitute God. For the moment DNA is not a therapy applicable to cardiovascular disease and it is difficult to predict when such an approach could have a practical output. The domain which is certainly the most promising is that of prevention of restenosis after angioplasty. The purpose of this book is not to extensively review (see Barr et al. 1994) a field that is expanding every month, but only to provide the language, as above, that permits a full understanding of what is presently on the pot, and to give a few examples.

In the future it is most unilkely that gene therapy in cardiology will consist of replacing a diseased gene by a normal sequence, mainly because most of the inherited cardiovascular diseases are multigenic, and, for the moment the main targets of this new pharmacology are vessels, the major aim being to try to introduce and then induce the permanent expression of genes able to deliver proteins that would prevent restenosis or thrombosis.

Most of the papers recently published are in fact attempts to optimize gene transfer in the vessels using reporter genes and various vectors or modes of injection.

Liposomal reagents are mixtures of cationic or neutral lipids forming micellar structures in the plasma, they form complexes with DNA, and because of their lipidic structure may fuse to the plasma membrane and then deliver the genetic material to the cells. Liposomes are a priori safer than any viral vector since they are entirely artificial structures. Several groups have attempted to transfer reporter proteins into vascular endothelial and this mode of transfection is for the moment poorly efficient (Barr et al. 1994, Zhu et al. 1993).

Adenovirus is a DNA virus responsible for either flues or benign ailments. Several groups have tried to transfer genes into the cardiovascular sytem using such a vector. The yield of the transfection is for the moment the highest ever obtained (Barr et al. 1994, Steg et al. in press). Nevertheless the longevity of the transfection seems to be rather limited, and such a vector may create an immune response which has not yet been fully appreciated.

Most of these investigations have been centered around the yield of the transfection using reporter genes. Few of them have, for the moment, used genes of real therapeutic interest. A group of DNA sequences has however a particular interest. Antisense sequences or antisense oligonucleotides do indeed have the ability to combine with their complementary (sense) sequences and, consequently, to block gene expression. These sequences have to be combined with a vector to be delivered to the tissue of interest, and, for example, it is now possible to deliver anti growth factors or anti oncogenes oligonucleotides to inhibit the proliferation process responsible for coronary restenosis after PTCA. Antisense c-myb oligonucleotides were for example targeted to smooth muscle cells and by so doing inhibits the synthesis of the corresponding oncoprotein with a subsequent effect on the cellular process (Simons et al.

1992). Obviously such attempts need parallel progress in the understanding of the biological process responsible for the disease, i.e. for the latter problem, to understand how the cascade of events responsible for such an anarchic growth is fonctioning (see Fig. 46). It is, for example, necessary to obtain a greater understanding of the development of the proliferation process after intimal denudation, in the rabbit (Bauters et al. 1994), to more efficiently target arterial gene transfer of DNA sequences.

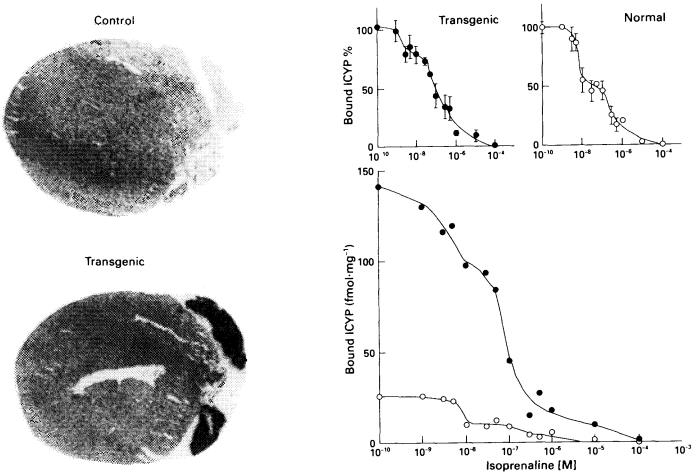

Fig. 69 Overexpression of G protein-coupled human β_1-adrenergic receptor intransgenic mice. Left: autoradiography on mice heart slices using radioactive Cyanopindolol. Right: binding competition curves- ●Transgenic - ○ Normal. (from Bertin et al. 1993, with permission).

TO CONCLUDE

To write such a book alone was an interesting challenge. I had to summarize both 30 years of research and teaching in the cardiovascular field, and the main tendencies of the contemporary research in the domain. In addition, the book was especially adressed to my colleagues clinicians, which are usually overloaded with an enormous amount of informations, usually written for specialists, not for general practitioners. Thanks to this challenge I learnt a lot, particularly in fields such as genetics where I was primarily rather ignorant. Nevertheless, the difficulties that I had, for example, to understand the genetic jargon help me to try to explain the same jargon to clinicians. I was also obliged to make choices, and, for example, to abandon, at least for the moment, several important sections, such as the energy metabolism, the techniques for transfection, the biology of the fibroblasts or the genetics of the diseases of collagen or of obesity.

It is perhaps rather pretentious to try to draw some lines of conclusion in such a moving field, nevertheless I should try at least to summarize my feeling into three points :

(i) A striking point is the fact that our amount of knowledges had really blowded up very recently. I had personnally learnt more in this field the last 5 years that during the 25 preceding years. I remember, for example, that when I was passing my PhD the fact that ionic channels are proteins was nearly a working hypothesis ! Nevertheless, and curiously these progresses have simplified teaching, it is for example more easy to teach electrophysiology, at the least in a Faculty of Medicine, by starting with the molecular structure of the ionic channels than by the currents.

(ii) The second point is that, in our domain, the complexity is growing at an unbelievable rapidity. To understand how a given patient becomes hyperlipidemic, we should have soon to integrate an enormous

mass of knowledge, including multiallelism (200 alleles for the lipoprotein receptor, and 200 genes responsible for the lipid metabolism !) and the environmental biological factors, that it makes us feel dizzy ! It is easy to predict that such a complexity will soon require special methods of analyses capable to integrate the overall data. The future of the modern biology will certainly require the development of non linear methods of analysis, such as the methods based on the chaos theory (Mansier et al. soumis).

(iii) The third remark concerns the new therapeutic approaches. The future of the gene therapy will certainly concern more therapy with a gene such as the prevention of restenosis with antioncogenes, than a substitutive therapy in an inherited disease. In addition a better understanding of the biological mechanisms responsible for arrhythmias, cardiac failure or coronary insufficiency will permit a better targetting of the drug design. This was recently illustrated with the story of NO.

POUR CONCLURE

J'ai eu beaucoup de plaisir à écrire ce livre. Seul, c'était un challenge intéressant. Il fallait à la fois essayer de résumer 30 ans de carriére dans la recherche cardiovasculaire, aussi bien comme chercheur que comme enseignant, et faire le point sur les évolutions essentielles dans le domaine, et se faire comprendre par mes confrères cliniciens submergés par une littérature à la fois pléthorique et peu didactique. Ce challenge m'a permis de désobscurcir les nombreuses insuffisance de ma culture scientifique en ce domaine, en particulier en matiére de génétique. Il m'a aussi obligé à faire des choix, j'ai, par exemple, délibérement laissé de coté un certain nombre de chapitres, comme le métabolisme énergétique, les techniques de transfection ou de mutagénèse, la biologie des fibroblastes, la génétique de l'obésité ou celle des maladies du collagéne. On ne peut pas tout faire, tout au moins la premiére fois.

Il est peut-être prétentieux de faire un bilan des grandes tendances de la biologie cardiovasculaire contemporaine, mais on ne peut à la fois prétendre écrire ce type de livre et refuser d'en tirer des enseignements prospectifs.

(i) Ce qui est d'abord frappant c'est l'aspect explosif des connaissances dans ce domaine, j'ai personnellement plus appris ces 5 derniéres années que pendant les 25 ans qui ont précédés. La biologie moléculaire a mis du temps à envahir le cardiologie, mais la somme de connaissance accumulées grâce à elle était proprement inimaginable il y a 20 ans. Quand je pense qu'au moment où je rédigeais ma thése on se demandait encore si les canaux ioniques étaient bien des proteines ! Mais curieusement ces progrés loin de compliquer l'enseignement, le simplifient, il est plus facile d'enseigner en Faculté de Médecine l'électrophysiologie en commencant par la structure des canaux ioniques que de le faire, comme autrefois, en débutant par les courants.

(ii) La seconde remarque est que dans notre domaine particulier, la complexité va croissant, la conjonction facteurs biologiques dépendant de l'environnement + multiallélisme (il y a dans la xantomatose hypercholesterolémique presqu'une mutation par famille!) donne un peu le vertige. On voit venir le temps où il sera nécessaire d'acquérir des outils sophistiqués capables d'intégrer toutes ces données. En ce domaine on peut prédire, sans craindre de se tromper, que l'avenir sera bientôt dans les méthodes d'analyse sophistiquées capables de définir ce type de relations complexes non linéaires, comme la méthode basée sur la théorie du chaos (Mansier et al. soumis). On peut aussi penser que, paradoxalement, cette biologie risque à terme de redonner une nouvelle jouvence à la relation personne à personne du médecin et de son malade. Les multiples alléles responsables de cette même hypercholestérolémie ayant été découverts, en même temps que les déterminants diététiques ou métaboliques de la lipidémie, l'utilisation de ces données au lit du malade deviendra tellement complexe qu'il faudra que le biologiste fournisse au praticien une vraie synthése au moyen d'outils nouveaux, comme ceux précités. De son coté le praticien, épaulé par l'apport biologique, devra revenir à une approche prioritairement individuelle et plus intuitive.

(iii) La troisiéme remarque porte sur les perspectives thérapeutiques, non tant en matiére de thérapie génique substitutive dans les maladies héréditaires, que thérapie par le géne, comme pour prévenir les resténoses, et aussi "drug design" de plus en plus ciblé au fur et à mesure que progresse notre connaissance des mécanismes, l'histoire du NO par exemple est sur ce point exemplaire.

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GLOSSARY

5'-3'. Indicates the DNA or RNA polarity. A nucleotide consists in a combination of a sugar, a base and a phosphate group. The bases are attached to the 1' carbon position of the sugar and face the interior of the molecule, while the phosphate group forms the backbone of the molecule and binds to the 5' and 3' carbon position of the sugar. The convention is that the order of the nucleotides is read from 5' to 3', DNA or RNA is synthesized in the 5'-3' direction (Fig. 9).

Adenovirus. DNA virus naturally infecting man, frequently used as a vector in gene therapy.

Allele. One of several alternative forms of a gene that occupies a given locus on the same chromosome. By extension, alternative forms of non coding (anonymous polymorphism) DNA sequences located on the same locus.

Anonymous. Anonymous DNA is the portion of DNA that does not encode RNAs. It constitutes the majority of DNA sequences, and includes polymorphic markers.

Antiparallel. See antisense.

Antisense. An antisense RNA is the mirror copy of a mRNA. In an antisense RNA the nucleotides are arranged in the same order as in the non coding, antiparallel, DNA strand on which the RNA polymerase synthesizes the mRNA (Fig. 9).

Autosomal. Mode of inheritance which is not sex linked.

Base pairs, bp. A partnership of nucleotide bases, such as A with T or C with G, in a DNA double strand. A single stranded sequence of 30 nucleotides, is also a double strand of 30 bp.

Cell cycle. The sequence of events that separates two mitotic or (for gametes) meiotic division in eucaryotes (Fig. 4 & 5).

Chiasma. Chromosomal site where crossing-over occurs (see Fig. 4).

Chromatin. Highly organized structure in which DNA is packaged with proteins (histones). Heterochromatin is permanent dark-staining areas of condensed chromatin in which most of the DNA is never transcribed.

Chromosomes. Morphologically distinctive nuclear structures, species-specific in number and shape. Assemblies of transcription units made up from DNA, RNA and proteins, which are precisely duplicated during cell division (Fig. 2 & 3).

Cis/Transregulation. When transcription is indirectly activated through a protein factor which binds to a DNA Responsive Element, this is a Transregulation ; when the activated DNA Responsive Element enhances transcription, this is a cisregulation (Fig. 12-13).

Clone. Genetically identical cells descending from a single common ancestral cell.

Coding. The genetic code is a special arrangement of bases which allows DNA, which is made from nucleotides, to code for a specific arrangement of amino acids, i.e. amino acids. A given gene or mRNA code for a given protein, but stricto sensu a given DNA sequence does not code for a given RNA sequence.

Codon or triplet : a group of three nucleotides that encodes for an amino acid, initiation signal or termination signal.

complementary DNA, cDNA. DNA synthesized from a mRNA copy obtained by reverse transcription from mRNAs, tissue-specific by definition. Does not contain introns (Fig. 14).

complementary RNA, cRNA. Mirror image of mRNA, also termed antisense RNA and is identical to the antisense DNA strand. Tool made by biotechnology. Exists in vivo, but its physiological significance is still unknown (Fig. 9).

Consensus. Sequence which is located in the regulatory part of the gene, binds specifically to a given transcriptional factor and is conserved in various biological species (Fig. 11-13).

Conversion (genetic). Reparation of non complementary DNA strand by using the other strand as a guideline (Fig. 59).

Cross-over. Exchange of genetic material between haploid chromosomes that pair during meiosis.

Diploid. A diploid (2n) cell contains two copies of each chromosome. In mammals, as in every eukaryotic organism all the cells, or nearly all the cells, are diploïd (Fig. 4).

Dominant. Inheritance is dominant when the expected phenotype is expressed in the heterozygous state.

Duplication. See Replication (Fig. 7).

Enhancer. Component of the regulatory part of the gene that includes the consensus sequences (Fig. 11-12).

Eukaryote. Organism that has nuclei, nuclear membranes and mitosis.

Excision. Step of the maturation of mRNAs during which introns are excised, this step is followed by a ligation of the exons (Fig. 8).

Exon. DNA sequence transcribed and found in mature RNA (Fig. 8).

Gene. All nucleic acid sequences which are necessary to produce a peptide or an RNA. Includes the coding sequences, but also the regulatory sequences (Fig. 8, 11 & 12).

Genetic code. The set of correspondences between codons and amino acids (Table 1).

Genetic distance. The distance, in centiMorgans, between linked nucleotidic sequences (Fig. 57).

Genomic DNA. Genuine DNA directly extracted from nuclei, is the same in every cell of a given individual (Fig. 14).

Genotype. The genetic constitution of an individual in terms of DNA sequences (Fig. 1).

Germinal cells. Gametes (Fig. 4).

Haploid. Haploïd cells (1n) contain only one copy of each chromosome (Fig. 3 & 4).

Hereditary disease. A disease caused by a mutant gene.

Homology. Homologous sequences means sequences sharing many nucleotide sequences in common.

Informative. A polymorphic marker is informative when it allows one to identify the father's chromosome from the mother's one, and more particularly when it allows the localisation of a locus on one of these chromosome.

Intron. A DNA sequence present in premRNA and excluded from mature RNAs, the introns are intercalated between two exons (Fig. 8).

Kindred. Relative.

Knock-out. Gene transfer technique that consists in blocking the expression of a gene.

Lariat. After excision the introns appear in the nuclear area as lassos or lariats (Fig. 8).

Linkage (genetic). Cosegregation of several alleles due to their physical proximity. Linkage analysis is a method of analysis of inheritance based on the search of a diseased locus using markers (Figs 57 & 60).

Locus. Location, place.

LOD score or log odds ratio. Expresses the likehood that two loci are linked at a given recombination frquency. A LOD score above 3 is considered to be significant .

Meiosis. Germinal cell division (Fig. 4).

Messenger RNA. Transcript from which protein can be translated (Fig. 8).

Mismatch or maipairing. Random assignation after crossing-over may give rise to double DNA strands in which one or several bases are not complementary (Fig. 59).

Missense mutations. Mutation in which the codon is mutated to direct the incorporation of a different amino acid.

Mutation. Change in a DNA sequence, mostly often used to qualify a change in the sequence of a gene.

Northern blot. A technique used to examine and quantify mRNAs and that is based on electrophoretic separation and hybridization with specific probes (Fig. 19).

Nucleosome. Basic subunit of chromatin (Fig. 2).

Offsprings. Progeny, lineage.

Oligonucleotide. Synthetic single-stranded nucleotidic sequence (Fig. 14).

Palindrome. The two DNA strands are complementary and termed palindromic because the two sequences are identical if read from left to right for one strand and right to left for the other. "Esope reste ici et se repose" is a classical palindromic sentence (Fig. 1 & 6).

PCR. Polymerase Chain Reaction. Qualifies both the technique and the commercially available apparatus used for this technique. The method is an in vitro reproduction of DNA replication (Fig. 18).

Penetrance. The percentage of people having a specified genotype which shows the expected phenotype. Penetrance usually increases with age and is complete when all individuals carrying a given genotype have the expected phenotype.

Phage. Or bactriophage, is a virus that infects bacteria. Used as a vector (Fig. 21).

Phenotype. Observable characteristics of an organism resulting from genomic expression. Morphological feature, physiological property, clinical syndrome or protein (Fig. 1).

Plasmid. Extrachromosomal, circular, autonomously replicating DNA segment. Commercially available as a well defined nucleotidic sequence. Vector.

Poly(A) tail. Non-coding repetitive sequence of Adenine, A, which is synthesized during the maturation of a mRNA, in the nucleus.

Polymorphism. Genomes showing allelic variations. Restriction polymorphism means that the variations modify a restriction site.

Primers. Single-stranded DNA sequence which is paired with a DNA or RNA strand, and provides a free3' end at which DNA polymerase starts the synthesis of a longer DNA sequence. Exists in vivo, but is usually synthesized for the purpose of PCR (Fig. 7 & 18).

Probe. Biochemical radioactively labeled or tagged for ease of identification (Fig. 14).

Procaryote. Microorgaisms that lack a membrane-bound nucleus.

Promotor. A region of the regulatory part of a gene to which RNA polymerase binds (indirectly) (Fig. 11).

Protein structure. 1^{ary} : amino acid sequence. 2^{ary} : helical configuration. 3^{ary} : spatial configuration of each chain; 4^{ary} : subunit interaction.

Pseudogenes. Resemble a known functional gene, but rendered nonfunctional by structural changes at crucial points.

Recessive. Mutation which modifies the phenotype only in homozygotes.

Recombinant. (i) In vivo, at the end of crossing-over recombinants are chromosomes resulting from the reassembly of genes during meiosis (Fig. 5). (ii) In vitro, a recombinant is a composite DNA sequence created by joining foreign DNA with a vector.

Recombination frequency. Number of recombinants divided by the total number of progeny.

Reparation. Process by which the cell is able to restaure a normal DNA molecule after injury or recombination, using the other DNA strand as a guideline.

Replication. DNA synthesis from DNA. The new DNA strands are complementary copies of the DNA templates (Fig. 7).

Reporter. Gene used in a construct to indicate the activity of a promotor of inetrest. The gene usually expresses only proteins which are not normally present in the tissue, such as CAT.

Responsive Element. Consensus nucleotide sequence specific for a given activated transcriptional factor, which is a protein. The element is said to be responsive because it responds to the signal which is transported by the

factor. The signal can be hormonal, but also mechanical, or a signal for differentiation (Fig. 11-13).

Restriction enzymes. Endonucleases that cleave unique, specific sequences of duplex DNA. Class II restriction enzymes cleave palindromic sequences (Fig. 16).

Restriction maps. A linear array of sites on DNA cleaved by restriction enzymes. (Fig. 17). Commonly utilized to rapidly identify a DNA probe.

Retrovirus. RNA viruses. These virus are termed *retro*viruses because they use their own reverse transcriptase activity during the cycle when they are transcribed into DNA and integrated into the genome of the host cell. AIDS is caused by a retrovirus HIV. Oncogenic retrovirus are transformants and have the capacity to transform the host cell into a cancerous cell.

rRNA. Ribosomal RNA.

Segregate. Transmit.

Sense. Sense RNA is mRNA. Sense DNA strand, or non-coding strand is the strand on which the polymerase synthesizes mRNA. See antisense and Fig. 9.

Sib-pair analysis. Method of analysis of genetic transmission based on paired siblings.

Southern blot. The same technique as Northern blot, but using DNA fragments instead of RNA. Historically the first, has been described by EM Southern in 1975.

Splicing. Removal of introns followed by ligation of exons to produce mature translatable mRNAs. Splicing = excision + ligation. Splicing is a mode of regulation of the expression of isoforms (Fig. 8).

Synapsis. Allows crossing-over.

Template. DNA sequence serving as a model to synthesize RNA or DNA sequence.

Trait. Dominant phenotype expression.

Transcript. mRNA.

Transcription. RNA, synthesis from DNA.

Translation. Protein synthesis from mRNA. Translation of a nucleotide sequence into an amino acid sequence.

Transregulation. See Cis/Trans regulation.

Triplet. See codon.

Upstream. A nucleotide sequence is upstream from the coding part of a gene when it is located upstream the initial site of transcription, i.e. upstream from the 5' end. The regulatory sequences are located upstream from the coding part of the gene.

Western blot. Same as Northern or Southern blot, but using antibodies to identify proteins.

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