A practical approach to the molecular biology of kidney diseases: From basic science to bed side

P. Garrido, E. Vélez[1], A. Izquierdo, J. Matthews, Mª. A. Gutiérrez-Tarrés, P. López Luna, J. Bover[2] and R. J. Bosch
Laboratory of Renal Physiology and Experimental Nephrology, Department of Physiology, Alcalá School of Medicine, University of Alcalá, Alcalá de Henares
[1] Department of Nephrology, Fundación Jiménez Díaz, Madrid, Spain
[2] Department of Nephrology, Fundació Puigvert, Barcelona, Spain.

Summary

Nowadays, the term "Molecular Biology" (MB) is generally applied to the biochemical processes that involve genes and the expression of proteins for which specific genes code. In recent years, astonishing advances have occurred in this field. Currently, there are many important powerful techniques allowing scientists to study the molecular mechanisms involved in many human genetic diseases. Furthermore, it is important to underline that the possibilities are not limited to the diagnosis and study of these genetic diseases. Indeed, by studying gene expression, MB also allows the molecular study of many acquired diseases such as viral hepatitis and cancer. Therefore, these major advances in the knowledge of gene biology are facilitating the arrival of a new era of gene therapy.

This article will describe the most important techniques currently used in MB. Firstly, techniques involved in recombinant DNA technology will be discussed and these will include the study of DNA and the possibility of identifying the expression of abnormal genes, e.g. to identify individuals for paternity. Secondly, a description of techniques designed to study the expression of genes and their regulation will follow and they involve the study of RNA. Thirdly, the impact of genetic molecular studies as tools for medical diagnosis will be discussed and analysed. Finally, a discussion concerning the rational basis for gene therapy and its future perspectives is included.

In this article, we have focused on technical or diagnostic aspects ofMolecular Biology. Although Ethics are also an interesting issue to deal with, theseissues are far beyond the scope of this review.

Key words

- Molecular biology
- DNA
- RNA
- Renal diseases

Genes and the methodical basis for their study

The gene is the unit of inheritance and it is made up of DNA (Deoxyribonucleic acid). Each gene is a nucleic acid sequence that carries the information representing a specific polypeptide (1). A gene is a stable entity, but errors may occur during DNA replication causing changes in the nucleic acid sequence. These changes are called mutations and the new form of the gene will be inherited in a stable manner, just like the previous form. The organism carrying the altered gene is called a mutant; the organism carrying the normal (unaltered) gene is called wild type. From a clinical point of view, DNA analysis is focused towards the diagnosis of mutations responsible for hereditary diseases. Many of the changes in nucleic acid sequences (mutations) do not imply the development of a disease, but they are instead the basis for human variability.

The term locus applies to unique positions within a gene and, in addition, the localization of genetic markers along the chromosome (2). On the other hand, an allele refers to one or more alternative forms of genetic information within a locus, coding for the expression of a particular characteristic. Allelism is the consequence of the existence of two (homologous) copies of each inherited chromosome, one from each progeny (or parent) (1,2). The existence of multi-functional genes within a population are known as polymorphisms.

A disease is considered autosomal dominant if it appears in heterozygous subjects. In other words, the affected subject inherited a normal copy of a gene from the healthy parent and a mutated gene copy from the affected parent (3). Autosomal recessive diseases appear when both parents present a mutated gene copy. In most cases, the underlying strategy to study DNA

***Pedro Garrido** is a Biologist. Currently he is studying for his Ph.D. at the Laboratory of Renal Physiology and Experimental Nephrology, Department of Physiology, Alcalá School of Medicine, University of Alcalá, Alcalá de Henares, Spain.*

is based on detecting a certain type of mutation with the best available technique.

Recombinant DNA technology: History

DNA was isolated for the first time by Miescher in 1869; however, it was not until 1944 when Avery demonstrated that the DNA molecules and not the proteins carried the genetic information during bacterial transformation (1,2). In 1953, Watson and Crick discovered the existence of DNA in a double-helix form by using X-rays and this finding made it possible for Marmur and Doty to discover the process of DNA renaturation (4). The renaturation process is generally described as hybridization when nucleic acids from different sources are involved, i.e. when one preparation consists of DNA and the other consists of RNA.

In 1966 Nirenber, Ochoa and Khorana elucidated the genetic code and found that each trinucleotide sequence (codon) coded for an amino-acid. Other important advances were reported by Sanger et al. as well as Maxam and Gilbert who developed rapid DNA sequencing methods (1) and, in addition, the method to amplify specific DNA sequences, known as Polymerase Chain Reaction (PCR) (5). Nowadays, the introduction of automatic DNA sequencing has made the process much easier and currently it is possible to isolate, sequence and analyse DNA quickly and effectively.

Methods and techniques to study DNA

1. Restriction Enzymes

Restriction endonucleases (RE) are enzymes that protect the bacteria whilst degrading the DNA carried into the cells by viruses (4). Each enzyme recognises a specific 4-8 base pairs in the DNA and cleaves at its target sequence, thus creating restriction fragments (1). These fragments may then be used to create a restriction map which enables comparisons to be made between different regions of DNA without knowing their nucleotide sequences. These facts allow the use of restriction maps to represent a linear sequence of the sites at which particular restriction enzymes find their targets (restriction targets). Therefore, this means that different genomic areas can be compared without knowing the sequence of nucleotides that form it (6), a very important fact determining relationships within the human genome.

2. Gel electrophoresis

The negative charge of nucleic acids allows the study of DNA molecules by gel electrophoresis, based on separation according to size. The most common gel used is agarose and it allows the different molecules to pass through, separating them along the way. The addition of ethidium bromide (EtBr) – an intercalating agent – stains the gel and allows gel visualization under ultraviolet light when it is bound to DNA (1). A more sensitive method involves the incorporation of a radioisotope (e.g. 32P) instead of EtBr before electrophoresis. This method relies on the detection of particles by autoradiography.

3. Nucleic acid hybridization

This is the binding of specific DNA or RNA sequences with great specificity along with their homologous or complementary sequences (2,4). This technique allows the identification of specific nucleic acid sequences and it is the basis of Southern (DNA) and Northern (RNA) blot techniques.

4. Southern blot

This technique allows the transferral of DNA from an electrophoresis gel to a nitrocellulose or nylon membrane (1). It allows the identification by hybridization of nucleic acid sequences using a radioactive-labelled probe. Therefore, the DNA probe detects nucleic acid sequences which are complementary to part or all of the probes. Other related techniques are the Northern blot analysis, which detects RNA, and the Western blot, used to detect proteins.

5. Polymerase Chain Reaction (PCR)

The PCR allows amplification of specific DNA segments from any source, using DNA as a template, the four nucleotides from which DNA is made, the DNA polymerase enzyme (natural occuring enzymes that are responsible for DNA synthesis) and two oligonucleotide primers capable of recognizing the beginning and the end of the DNA template in order to copy it (5,6). The in-vitro reaction involves a variable number of repeated cycles of denaturation, annealing and DNA extension. These reactions are performed in a PCR thermocycler.

6. Restriction Fragments Length Polymorphisms (RFLP)

This more recently developed method is based on the detection of those variations in DNA sequences that have a change within their restriction targets (1). The restriction fragments obtained are different depending on the allele. This technique has made it possible to analyse, detect and identify genes associated with certain diseases and which have never been identified by using RFLP markers. It is an example of a genetic linkage map and it enables analysis of large genomes.

7. DNA cloning and genetic engineering

This technique enables the amplification of individual DNA sequences. Cloning a DNA fragment allows the production of indefinite amounts from even a single original molecule (4,6). A clone is defined as a large number of cells or molecules all identical to an original ancestral cell or molecule (1). Once any particular segment of DNA has been cloned, its properties can be characterized.

Cloning technology involves the construction of novel DNA molecules by joining sequences from different sources. The product is often described as recombinant DNA, and the overall techniques as genetic engineering.

8. DNA sequencing

The DNA sequence is virtually always looked on as the prima-

ry objective once a gene has been cloned (6). This method discloses the DNA nucleotide sequences so that it aids in the prediction of the protein amino-acid sequence for which they code.

Techniques used to study gene expression (RNA)

1. Nucleic acid hybridization (RNA) northern blot

As mentioned before, this is the same technique as Southern blot but it uses RNA instead of DNA. It demonstrates both qualitatively and quantitatively the presence of RNA (gene expression) coding for certain proteins (1).

2. cDNA synthesis. Reverse Transcription (RT)

The use of the inverse (reverse) transcriptase retroviral enzyme allows scientists to carry out the synthesis of cDNA. The cDNA is DNA which has been made by using mRNA (messenger RNA) as a template (4). As indicated by its own name, it is the reverse process to transcription.

3. Reverse Transcription and Polymeraese Chain Reaction (RT-PCR).

This is the most powerful, sensitive and versatile technique used at the present time to study gene expression both qualitatively and quantitatively. It can be used to detect either the presence or absence of a gene (6) and it differs from regular PCR that a reverse transcription (RT) of a specific target from either total RNA or mRNA is performed before any PCR amplification.

4. RNAse Protection Assay (RPA).

This technique involves RNA hybridization in solution (4). When the DNA forms a hybrid with its homologous chain, it can not be degraded by the activity of the ribonucleases present in the assay and therefore it becomes possible to study the qualitative and quantitative expression of certain genes.

Molecular basis of certain kidney diseases: Diagnostic implications

From a clinical point of view, the initial objective of molecular studies is to be able to diagnose the genetic implications of diseases in order to give appropiate counselling and treatment to the patient. When the genetic anomaly is totally identified, it may be studied using DNA analysis and biochemical methods (7). Following this sort of analysis, PCR may be used if short repetition markers are present, otherwise RFLP is the preferred method of identification.

A. Infectious illnesses

Almost all organisms contain DNA or RNA. Therefore, they all can be theoretically detected by PCR or RT-PCR, respectively, as far as their sequences are known. These methods are very expensive and only several of them have gained wide clinical use. Thus, MB techniques are routinely used for the detection of several viruses such as HCV, HBV, HIV and CMV. For instance the viral load of the later can even be quantified by the so-called quantitative PCR after renal transplantation. Slowly-growing mycobacterias such as Mycobacterium tuberculosis maybe detected by PCR techniques much faster. Other bacteria, virus, parasites can also be detected by MB techniques (Toxoplasma sp., Plasmodium sp., Bordetella pertussis and so on and so forth).

An important advantage of molecular analysis is that it does not require an immune response (production of antibodies) (8) and thus, a considerable amount of time can be saved. In addition, molecular analysis may be carried out on almost any type of sample obtained from the patient e.g. blood, tissues, hair, etc.

B. Molecular biology applications using tissue biopsies

The widespread use of tissue biopsies over the last few decades has allowed scientists to better describe both diseases and their pathophysiologic pathways. It was not until the advent of PCR that it was possible to apply MB techniques to human biopsies, because it required a quantity of nucleic acids impossible to obtain to run the previously available methods. In recent years, many studies have been carried out involving MB studies on tissue biopsies or blood samples in a wide range of human diseases.

C. Hereditary kidney diseases

A cyst is a cavity lined by epithelium and filled with liquid or semi-solid material (3). Renal cysts may develop from the glomerular capsule along the length of the renal tubule by heritable, developmental or acquired processes. A kidney may contain a single or many cysts. The term "Polycystic kidney disease" includes two genetically distinct outcomes (9), namely, autosomal dominant (ADPKD) and autosomal recessive polycystic kidney disease (ARPKD).

Autosomal dominant polycystic kidney disease (ADPKD)

ADPKD is a common inherited disorder transmitted by a dominant gene and characterized by multiple cyst development and growth in the kidney leading to end-stage renal failure around the fifth-sixth decade of life (3). It affects 1 of every 1000 newly born children. It also affects other organs such as the liver, brain, heart valves, etc. demonstrating the systemic nature of the disease. As any autosomal dominant disease, an individual with ADPKD must have the defective gene on one of a pair of autosomal chromosomes. The PKD-1 gene, that is responsible for the 85% of cases in adults, has recently been identified and it was found to be nearer to the 1-alfa-globin gene and to one of the tuberous sclerosis genes on chromosome 16 p. This PKD-1 gene encodes for a protein known as polycystine 1. The absence of this protein affects cell-cell and cell-matrix interaction and facilitates cyst formation. The PKD-2 gene (the second gene implicated in the disease) is located on chromosome 4, and it encodes for a protein (polycystine 2) which is involved in voltage dependent calcium channels. Both genes also differ in

clinical grounds, and although heterogeneicity and even intrafamiliar variability is common in ADPKD, end-stage renal disease is reached later in patients affected by the PKD-2 mutation.

Autosomal recessive polycystic kidney disease (ARPKD)

This rare, recessive type of inherited polycystic kidney disease is usually discovered in infants; however, young adults may develop the disorder. Thus, it is known as 'infantile' polycystic kidney disease in its classical form ARPKD is a disorder whose genetic defect has been localizated in chromosome 6 (3), although the protein for which encodes is still unknown and current diagnosis is mainly undertaken by ultrasonography.

Hereditary nephritis

The Alport syndrome (one of the most common hereditary nephritis) is a progressive glomerulopathy frequently associated with loss of auditory capacity and crystalline alterations (3,8). The primary change in almost all cases resides in the non-collagen domains (NC1) of type-IV collagen, which is usually located in the X chromosome. Using Southern blot techniques and DNA probes, it is possible to identify several mutations within the syndrome. Nowadays, some probes detecting a single mutation have been developed; however, no routine methods for molecular diagnosis are yet available.

X-linked Nephrogenic Diabetes Insipidus (NDI).

From a clinical point of view, the most frequent form NDI is acquired. Hereditary NDI is a much less frequent disease transmitted by X chromosome, and it is caused by a mutation in the V2 receptor gene (3). Recently, a second hereditary recessive NDI form has been identified. This mutation is located in a kidney cellular water channel known as aquaporine-2, and it obstructs the normal water traffic through the tubular cells (3,9). Currently, it is possible to prenatally diagnose women to detect carriers of the disease in order to administer treatment.

Wilms tumor (Nephroblastoma)

This disease entails an embrionic renal tumor that originates by abnormal differentation of mesenquimal cells (3) and it is common in childhood. It occurs either in the absence or the presence of an abnormal tumor supressor gene.

Molecular biology and therapy: Perspectives

In the last decade, many techniques have been developed within genetic engineering allowing the transfer and incorporation of genes into cells, tissues, organs and even to all the cells of an organism (transgenic animals). Furthermore, genetic engineering has enabled the suppression of gene expression by inhibiting the synthesis of the protein for which the gene codes.

It is possible to find physical and biological methods for gene transfer (3). Physical gene transfer methods are used to transfer genes to cell lines in culture. Basically, cells are incubated with DNA of the gene and then this DNA can be incorporated to the cellular genome using calcium phosphate or electricity, which increases the cellular membrane permeability to DNA (4). Using these techniques it is possible to obtain the expression of the transferred gene (10).

The biological gene transfer methods use viruses, mainly adenovirus and retroviruses. Retroviruses are RNA virus which possess the enzyme inverse transcriptase. This enzyme is also used to carry out the in vitro synthesis of cDNA (1,7). The retrovirus penetrates into the cells through a membrane receptor. Initially, the RNA virus is transcribed into cDNA, which in turn, enters the nucleus and is integrated in the cellular genome, a process directed by the viral enzyme integrase. The viral genome (provirus) is inherited in a Mendellian fashion and passes on into the daughter cells. As the provirus codes for its promoter (region of the gene that regulates its transcription), the infected cells express the viral gene and synthesize viral particles indefinitely, this being the innoculous cycle for the infected cell (11).

In recent years, successful techniques have been developed and they have been used in conjunction with a range of different experimental approaches; one of them involves gene transfer in kidney tissue. Currently, it is possible to transfer gene markers. Cytokine genes have already been transferred in experimental animals as well. Therefore, this area gathers an increasing scientific interest and it is currently an area of continuous expansion.

It is also important to point out that gene therapy not only includes single techniques of gene incorporation but also techniques suppressing the expression of potentially pathogenic genes. To date, two techniques have been developed allowing the suppression of gene expression (1,3). Firstly, the so-called 'antisense' technique, where complementary sequences are employed in an opposite direction to that of the nucleic acids present. This enables hybridization with homologous sequences within the cellular genome, thus obstructing genetic expression. Secondly, enzymes that can degrade specific RNA molecules (called ribozymes) are used. These enzymes facilitate the suppression of the gene product for which they code.

Pathologies which can benefit from techniques which suppress gene expression include several forms of glomerulonephritis. It has recently been proven that cytokines act as mediators of lesions in some experimental forms of glomerulonephritis (3). Border and Cabbage demonstrated that by administering antibodies against the transforming growth factor beta (TGF-b), it was possible to suppress the effects of the platelet growth factor as well as interleukin-1 in other experimental models of glomerulonephritis (3).

Finally, it may be possible to apply these techniques to oncology in the very near future. Immunotherapy is one example. The transfer of the interleukin-2 gene to tumour cells in rats has already been accomplished, and it has demonstrated that this genetic transfer effectively allows the recognition of the tumour cells and ultimately their destruction by the rat's own immunological system. The National Institutes of Health in the

United States has stated that it currently has approximately 60 projects involving research into human genetic therapy, highlighting oncology among them and including Hodgkin's disease, leukaemia, ovarian cancer, and melanoma. Other research areas include cystic fibrosis, familial hypercholesterolemia, bone marrow transplants and immunodeficiency syndromes, to name but a few.

In summary, the current use of MB techniques has a major impact on the administration of treatment and it has also the ability of preventing many of the worldwide fatal diseases before they can affect individuals within the population.

Acknowledgements

This work was partially supported by grants from the Programa Sectorial de Promoción General del Conocimiento from the Spanish Ministerio de Educación y Cultura (PB 98-0700-C02-01), the Consejería de Educación de la Comunidad de Madrid (08.6/0038./2000) and the Spanish Society of Nephrology.

Received: October 2001
Reviewed: November 2001
Revised: January 2002
Accepted: February 2002

Address for correspondence

Esperanza Vélez
Department of Nephrology
Fundación Jiménez Díaz
Avda. Reyes Católicos, 2
28040 Madrid
Spain
email: evelez@mi.madritel.es

References

1. Lewin B. Genes VII. Oxford University Press, 2001.
2. Alberts B, Bra D, Lewis J, Raff M, Roberts K, Watson JD. Molecular Biology of the Cell. 2ª Ed. Garland Publishing Inc, 1999.
3. Fauci AS, Braunwald E, Isselbacher KJ, Wilson JD, Martin JB, Kasper DL, Hauser SL, Longo DL. Harrison. Principios de Medicina Interna. 14th Ed. McGraw-Hill Interamericana, 1998.
4. Luque J. y Herráez A. Texto Ilustrado de Biología Molecular e Ingeniería Genética. Conceptos, Técnicas y Aplicaciones en Ciencias de la Salud. Ed. Harcourt, S.A 2001.
5. Innis MA, Gelfand DH, Sninsky JJ. PCR Strategies. Academica Press, 1995.
6. Brown TA. Essential Molecular Biology. A Practical Approach (Vol II). Irl Press, 1995.
7. Stobo JD, Hellmann DB, Ladenson PW, Petty BG, Traill TA. The Principles and Practice of Medicine. Prentice Hall Int. Inc, 1996.
8. Ganong WF. Review of Medical Physiology. 19th Ed. Prentice Hall Int. Inc, 1999.
9. Schrie W. Diseases of the Kidney. 4th Ed. Gottschalk, C.W. Little, Brown and Company.
10. Woolf AS, Bosch RJ, Fine LG. Gene transfer into the mammalian kidney: First steps towards renal gene therapy. Kidney International 1993; 43(39): S116-119.
11. Bosch RJ. Nefrología y terapia génica: ciencia básica para el clínico. Medicina 1996 (Buenos Aires) 56: 313-318.

Continued from page 76

7. Ware JE. SF-36 Health Survey Manual and Interpretation Guide. Boston: Nimrod Press; 1993.
8. Johnson JP, McCauley CR, Copley JB. The quality of life of hemodialysis and transplant patients. Kidney International 1982; 22: 286-291.
9. Aaronson NK, Acquadro C, Alonso J, Apolone G, Bucquet D, Bullinger M, Bungay K, Fukuhara S, Gandek B, Keller S, Razavi D, Sanson-Fisher R, Sullivan M, Wood-Dauphinee S, Wagner A, Ware JE. International quality of life assessment (IQOLA) project. Quality of Life Research 1992; 1: 349-351.
10. Ware JE, Gandek B. Overview of the SF-36 health survey and the international quality of life assessment (IQOLA) project. Journal of Clinical Epidemiology 1998; 51(11): 903-912.
11. Anderson RT, Aaronson NK, Wilkin D. Critical review of the international assessments of health-related quality of life: generic instruments. In The International Assessment of Health-related Quality of Life: Theory, Translation, Measurement and Analysis, eds S. A. Shumaker & R. A. Berzon, Rapid Communications, Oxford 1995: 11-37.
12. Mingardi G, Cornalba L, Cortinovis E, Ruggiata R, Mosconi P, Apolone G. Health-related quality of life in dialysis patients. a report from an Italian study using the SF-36 health survey. Nephrology, Dialysis, Transplantation 1999; 14: 1503-1510.
13. Fukuhara S, Bito S, Green J, Hsiao A, Kurokawa K. Translation, adaptation, and validation of the SF-36 health survey for use in Japan. Journal of Clinical Epidemiology 1998; 51(11): 1037-1044.
14. Fujisawa M, Ichikawa Y, Yoshida K, Isotani S, Higuchi A, Nagano S, Arakawa S, Hamami G, Matsumoto O, Kamidono S. Assessment of health-related quality of life in renal transplant and hemodialysis patients using the SF-36 health survey. Urology 2000; 56(2): 201-206.