THE MOLECULAR REVOLUTION IN BIOLOGY AND ITS INFLUENCE ON VETERINARY SCIENCE

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INTRODUCTION

Developments in molecular biology have received much attention in the popular press during recent years. In 1997 there was Dolly, the cloned sheep, and all the ethical arguments around this first cloning of a mammal (Fig. 1). Then there is the never-ending debate around the issue of genetically engineered foods and, early this year, the announcement of the provisional DNA sequence of the human genome and all its implications, both positive and negative. No wonder several experts have claimed that molecular biology constitutes the "next" scientific revolution, after the present information technology revolution, which will drastically change our lives. It is therefore timely to briefly review the development of molecular biology over the past 50 years in terms of selected highlights and to

examine its influence on veterinary science. To make the review more relevant, I will illustrate it with one example from our own molecular biology programme, i.e. our study of the bluetongue virus over a period of 35 years, and attempt to transmit to you some of the excitement that accompanied this research. The study, which I initiated in 1964, was later continued at the OVI under the outstanding leadership of Prof Henk Huismans, now head of the Genetics Department of the University of Pretoria, and Dr. Albie van Dijk whom I wish to thank for the visual material used in my talk.

In order to understand the essence of the revolution it must be remembered that up to the Second World War the biological sciences were essentially descriptive in nature with the living cell as the smallest unit. With the exception of biochemical reactions, which described life processes in chemical terms, very little was

known about the subcellular and molecular aspects of life. In the post-war years a number of physicists, disillusioned by the destructive image of their discipline, turned to biology as a new field of study and identified genetics as a basic discipline almost completely lacking in knowledge of molecular mechanisms. They developed bacteriophage replication into an experimental system which could approach the precision and reproducibility of results that physicists were used to. Thus a new new scientific discipline, molecular biology, originated from the collaborative efforts of three very different disciplines: physics, genetics and biochemistry.

As a starting point for molecular biology I chose, rather arbitrarily, 1950, the year in which the term was popularized by Astbury, a British X-ray crystallographer in his Harvey lecture. It was also the year in which Chargaff published a paper reporting

strict relationships between the amounts of specific nucleotides in nucleic acids, proving the long-held tetranucleotide model to be untenable. (Fig. 2) I remember distinctly that in 1952, during my second year Biochemistry course at this faculty, we were still taught that all nucleic acids consist of tetrads of the four nucleotides! Chargaff's discovery contributed significantly to the later discovery of the double helix DNA structure.

In 1951 the first amino acid sequence of a polypeptide, that of the phenylalanin chain of insulin, was published. It was the first step towards the elucidation of the molecular structure of proteins, which play an essential role in the molecular revolution.

In 1952, in an experiment that was to become a classic in its field, Hershey & Chase demonstrated that phage DNA alone was infective, destroying the dogma which reigned at the time that infectivity is dependent on a living organism. It also proved without

any doubt that DNA contains all the genetic information for reproduction. A milestone was reached in 1953 when Watson and Crick proposed the double-helix structure for DNA. For the first time a feasible model was proposed for the exact duplication of genetic information during cell division in terms of the controlled duplication of a macromolecule by means of base-pairing. A major technical advance was the development of an *in vitro* system for the synthesis of proteins by Zamechnik & Keller in 1954. Often in the history of science the decisive break-throughs are the development of new techniques which enable researchers to find answers to questions previously unattainable. The *in vitro* system clarified the role of phosphate energy donors in protein synthesis but also provided the means by which the role of nucleic acids in protein synthesis could later be elucidated.

In 1956 the discovery that the RNA of a plant virus, tobacco mosaic virus (TMV), is infective, demonstrated that RNA, in stead of DNA, could act as genetic material in micro-organisms lacking DNA. This publication attracted the attention of many virologists, myself included, and can be regarded as the starting point of molecular virology.

During the following year, 1957, a theoretical paper by Brenner discussed the possibility of nucleotide triplets acting as codes for amino acids during information transfer from DNA to protein. Although he rejected the hypothesis on the grounds of an insufficient number of triplets available, it was later shown to be correct in terms of a so-called degenerate code.

In 1958 two essential discoveries were made: that of sRNA by Hoagland and co-workers and of DNA polymerase by Kornberg et

al. Small soluble RNA molecules were shown to play an intermediate or "adaptor" role between DNA and protein synthesis. Different sRNA (or tRNA) molecules bind specifically to different amino acids at one end and at the other end recognizes a specific codon on the DNA by means of base pairing. This solved the problem of the specific alignment of amino acids according to the genetic information in the DNA, before their linking into proteins. The Kornberg enzyme was later shown to be only one of a number of DNA synthesizing enzymes, but it played a vital role in critical experiments requiring DNA replication. The isolation of a similar enzyme for the polymerization of RNA on a DNA template by Weiss & Gladstone in the following year complemented this work and enabled researchers to develop what came to be called the "central dogma" of molecular biology, i.e. the transfer of genetic information from DNA to RNA to protein.

Another technical advance of major importance was the demonstration by Marmur & Lane and Doty et al in 1960 that the two strands of the DNA helix can be separated by heating and renatured by cooling. In a separate study Rich found that DNA-RNA hybrids could be formed in the same way by exploiting base pairing. This technique, called hybridization, forms the basis of all recombinant work and all experiments involving DNA recognition, such as PCRs.

1961 seemed to be a very productive year with 3 papers being published that changed the scene decisively. The first addressed the problem of how the expression of genes in terms of the 'central dogma' is controlled. The operon theory proposed by Jacob & Monod, which postulated the existence of an 'operator' gene which controls the expression of a group of structural genes, and postulated the existence of an unstable RNA intermediate in

protein synthesis, broke new ground and remained the basis for later work. The discovery of messenger RNA (mRNA) by Brenner et al. and Gros et al. confirmed the hypothesis and further elucidated the mechanism of protein synthesis. This work was complemented by the assignment of the first 2 codons, or coding triplets, i.e. those for phenylalanine and proline (UUU and AAA) by Nirenberg & Matthaei.

However,
In 1970 the 'central dogma' was proven to be incorrect, or rather inadequate, by the simultaneous discovery of reverse transcriptase by two groups respectively led by Baltimore and Temin. This enzyme, found in retroviruses, catalyzes the synthesis of DNA on an RNA template, i.e. reverses the flow of information. You can imagine the excitement this controversial discovery caused! Later the enzyme was also used for the construction of cDNA libraries using mRNA as starting material. During the same period another

set of enzymes called restriction endonucleases were isolated from bacteria. These enzymes, which cleave DNA at very specific sites, was already used by Danna & Nathans for gene mapping the following year. Later it became one of the most important experimental tools for genetic analysis, DNA sequencing and for genetic engineering techniques. It also led to the construction of the first recombinant plasmid by Cohen and co-workers in 1973. Molecular biologists were now able to cut a specific gene or piece of DNA from one organism by using a specific restriction enzyme and to insert it into the DNA of another organism, creating what came to be called a recombinant organism. This so-called 'cloning' of a gene can be regarded as the birth of genetic engineering and remember, it happened only 20 years after Watson & Crick elucidated the structure of DNA.

1975 saw the development of two more experimental techniques that would greatly influence further developments. The first was a blotting technique developed by Southern for the detection of specific DNA sequences after electrophoretic separation in gels, a technique that is still in general use today. The second, the production of monoclonal antibodies in cell cultures of fused cells was developed by Kohler & Millstein and was destined to revolutionize immunology and bring that discipline into the molecular fold too.

Using a combination of the techniques described above, recombinant DNA technology was used to develop molecular diagnostic probes, epitomized by the first probe in 1976 for the prenatal diagnosis of alpha-thalassemia, an inherited disease of humans. This was to be followed by probes for the quick diagnosis of many other diseases of animals and man.

The determination of the sequence of nucleotides in DNA, i.e. reading the genetic 'blueprint' of living organisms, remained a dream for many years. In 1977 two techniques that were developed to achieve this were published by Sanger et al. and by Maxam & Gilbert, respectively. These technologies are still in use today in essentially unaltered from and initiated a new era in genetics.

Another milestone in genetic engineering was reached in 1978 when Chang and co-workers described the expression in E. coli of an eukaryotic gene, coding for a mouse enzyme, again demonstrating the feasibility of gene transfer. In 1981 Brinster et al. achieved the expression of a gene which was micro-injected into the pronucleus of a fertilized mouse egg cell. This

achievement can be regarded as a prelude to the eventual cloning of whole animals.

The development of the Ti plasmid as vector for the cloning of genes in plant cells, as reported by Herrera-Estrella in 1983, greatly facilitated the development of recombinant plants, which gave rise to the highly controversial issue of genetically manipulated food. Even though all transformations to date are aimed at increased yields and decreasing the use of chemical insecticides, opponents claim that long-term risks are undetermined and constitute a possible health hazard.

The next major technical break-through was the development of the PCR or polymerase chain reaction, which was conceived by Mullis in 1983 and first used in 1985 by Saiki et al. in a study of sickle cell anaemia. This technology, which leads to the almost

unlimited amplification of DNA sequences, has already revolutionized the diagnostic industry by immensely increasing the sensitivity of any test depending on the detection of DNA sequences. It also finds application in most areas of recombinant technology.

From a medical point of view one of the most important achievements of the molecular revolution was the development of the first recombinant vaccine against hepatitis B, which was approved in 1986 by the FDA for use in humans. Since the virus causing the disease can not be grown *in vitro*, surface protein used for vaccination could previously only be obtained from human blood which made it prohibitively expensive and also risky. By cloning the gene coding for the immunogenic protein in a bacterium, a safe, effective vaccine could be produced commercially at a reasonable cost. Many similar vaccines have been or are being developed for

both medical and veterinary use. It is certainly one of the most promising and exciting practical applications of molecular biology in the medical field.

Further highlights during the past few years include the first patent for a genetically altered animal, which was awarded to the Harvard university for a mouse highly susceptible to breast cancer; the launching of the human genome project in 1990, the cloning of the first mammal (back to Dolly!) in 1997 and the publication of the first provisional sequence of the human genome this year (2000). It is impossible at this stage to evaluate the significance of the latter event. It can be safely speculated, though, that it marks another turning point in the molecular revolution, and that undreamed of benefits will accrue to mankind from the ability to read and study its own genetic blueprint.

In the second part of my talk I would like to illustrate the impact of the developments reviewed so far on our study of the molecular biology of the orbivirus family which includes the bluetongue and horsesickness viruses. When this project was started in 1964 we knew practically nothing about the structure, function, replication or even classification of the viruses. In order to study these aspects we first had to develop the means to produce and purify significant quantities of the virus. When this was achieved the chemical analysis and electron microscopy of the viral particles could proceed. Our first exciting breakthrough came when we determined that bluetongue virus possesses a double-stranded RNA genome - a revolutionary finding at a point in time when double-stranded DNA was the norm and single-stranded RNA the exception in the case of certain viruses. I remember distinctly how difficult it was to convince the scientific community of the correctness of our results. Eventually it turned out to be a

characteristic of all members of the reoviridae family. To complicate matters even more we found that the RNA genome was not present in the form of one continuous strand as usual, but as ten separate segments. Furthermore, Physico-chemical analysis indicated the presence of two particles of different size and for a considerable time we suspected the presence of a contaminating virus in our viral stock. Eventually electron microscopy and a study of the viral proteins, separated by gel electrophoresis, enabled us to solve the problem by demonstrating that the virus possesses a double layered capsid with 5 proteins forming a core particle surrounded by an outer capsid layer consisting of two proteins. The presence or absence of the outer capsid gives rise to the two sizes of viral particle. These results are summarized in Fig. 3 which also shows the classification of the reoviridae.

A major problem that remained was the functional relationship between the ten genome segments and the 7 viral proteins (VP1 - VP7). This problem was solved by the demonstration that another 3 (later 4) non-structural viral proteins (NS1 - NS3) could be isolated from infected cells. It was then possible to prove that each of the ten genome segments act as a gene coding for one of the viral proteins. The coding assignment for African horsesickness virus (AHSV) is shown in the next figure (Fig. 4). It was later shown that segment 10 in fact codes for 2 variant proteins NS3 and NS4.

By this time various other research groups, mainly in Australia, the UK and the US had become interested in these fascinating viruses, and contributed significantly to further research reported here. Following the allocation of specific structural and biochemical functions to the various proteins, a diagrammatic structure for the

bluetongue virus was compiled, showing the relative positions of the different components (Fig 5). It is important to note here that the same diagramme applies to the horsesickness virus, which is structurally identical to the bluetongue virus. Further refinement by means of X-ray diffraction studies led to the beautiful model shown in the next figure (Fig. 6).

You may have asked yourself by now what the practical use of all this esoteric research is. My reply is that the ultimate aim of the project has always been to improve the diagnostics and the vaccines for these two economically important diseases. Let us therefore have a look at what has been achieved in this respect.

The development of molecular diagnostic probes and the technology required for their use, of cloning techniques and of sequencing technologies, all of which have been discussed, enabled

us to develop sophisticated new approaches to diagnostics. As shown in the next figure (Fig. 7), cloning the VP2 genes of all 9 African horsesickness serotypes made it possible to prepare labeled probes for the specific detection of each serotype in one simple experiment. By adding an NS2 probe, which is group specific, a newly isolated virus can therefore be distinguished from other orbiviruses and its serotype determined in one test the result of which could be available within 24 hours, compared to 2 - 4 weeks using conventional serum neutralization methods. This is of enormous practical benefit.

The amino acid sequencing of VP2 have been used to detect differences between various isolates of the same serotype, and gave the first indication of geographic variation within serotypes as shown by the phylogenetic tree shown in the next figure (Fig. 8). Isolates of serotypes 1 and 3 made in South Africa and Australia

clearly differed, for example. A study of the nucleotide sequences of genome segments of various isolates confirmed and expanded this observation as seen in the next figure (Fig. 9). There is a clear clustering of the various BTV serotypes into two groups: those originating in South Africa and North and Central America in one group and those isolated in Australia and Eastern countries in the other. The practical value of this test is that the broad origin of a new outbreak in say southern Europe can be determined quickly. It is obvious that the molecular diagnostic techniques not only yield faster results but also results that were not obtainable before. Molecular epidemiology has become a new field of study.

The rationale for vaccine development in the case of orbiviruses is the need for a single vaccine to protect against all relevant serotypes, replacing the present combinations of polyvalent vaccines, and, especially in the case of AHS, for a vaccine where the resulting immunity could be distinguished from that resulting

from natural infection. Both these requirements could be met by a subunit vaccine which became a practical possibility when Huismans & van Dijk demonstrated in 1990 that BTV-VP2 on its own is immunogenic and protects sheep against infection. This result confirmed the feasibility of cloning the VP2 genes of relevant serotypes in a suitable vector for the production of a universal recombinant subunit vaccine. In fact, such an experimental vaccine for bluetongue was produced as early as 1990 in collaboration with researchers from the Oxford University using the insect baculovirus as vector. The immunogenicity was enhanced by the co-expression of VP2 and VP5 and by the addition of VP3 and VP7, which forms core-like particles on expression. (Fig.10). Unfortunately low yields and high cost precluded the use of this first recombinant vaccine on a commercial basis. Since that time our attention has shifted to AHSV. Again it was shown that cloned BP2 fully protects animals against infection, and all the relevant genes of all serotypes have already been

cloned but the problems associated with commercial production have not been solved yet.

In conclusion, I have summarized the main areas in which the molecular revolution has contributed to the development of Veterinary Science, or can be expected to do so (Fig.11) The most important area is undoubtedly improved diagnostics in terms of molecular probes, and tests based on PCR and monoclonal antibodies. The development of more efficient, safer vaccines have been slower than anticipated for various reasons, but still holds promise for the future. The production of certain biologicals on a commercial scale using recombinant technology, e.g. growth hormone and insulin, has been more successful in human than in veterinary medicine, but the former has found an application in the stimulation of milk production. The genetic manipulation of rumen organisms holds promise as an approach towards improved nutrition

as well as towards a form of natural detoxification. Nutrition and production can of course also be improved by increasing the nutrient value of foodstuffs by means of genetic manipulation, as has already been done in creating maize with a higher tryptophane content. And this brings us back to where we started: with Dolly. Following her successful cloning much effort has gone into similar attempts with other species, with limited success. I believe it is unlikely that cloning of animals will soon be a practical way of increasing production. However there seems to be a real chance that the cloning of genetically manipulated piglets may provide us in future with "tissue factories' producing compatible organs and tissues for human transplant purposes. The combination of molecular biology and veterinary science may thus in future serve mankind in unexpected ways.

Fig. 2

SOME HIGHLIGHTS OF THE MOLECULAR REVOLUTION

1950 : Nucleotide composition of DNA (Chargaff)
1951 : Amino acid sequence analysis of insulin (Sanger & Tuppy)
1952 : Demonstration of infectivity of phage DNA (Hershey & Chase)
1953 : Double helix structure of DNA (Watson & Crick)
1954 : *In vitro* synthesis of proteins (Zamechnik & Keller)
1956 : Infectivity of TMV – RNA (Gierer & Schramm)
1957 : Triplet genetic code (Brenner)
1958 : Discovery of sRNA (Hoagland et al.)
Discovery of DNA polymerase (Kornberg et al.)
1959 : Discovery of RNA polymerase (Weiss & Gladstone)
1960 : DNA/RNA strand separation & renaturation (Marmur, Doty et al.)
1961 : Control of gene expression (Jacob & Monod)
Discovery of mRNA (Brenner et al. and Gros et al.)
First two codons assigned (Nirenberg & Matthaei)
1970 : Discovery of reverse transcriptase (Baltimore and Temin et al.)
Discovery of restriction endonucleases (Smith & Wilcox)
1971 : DNA mapping with restriction endonucleases (Danna & Nathans)
1973 : Construction of first recombinant plasmid (Cohen et al.)
1975 : Blotting technique for detection of DNA sequences (Southern)
Production of monoclonal antibodies (Kohler & Millstein)
1976 : First diagnostic probe for a human inherited disease (Kan et al.)
1977 : Sequencing of DNA (Sanger et al. and Maxam & Gilbert)
1978 : Expression of eukaryotic genes in bacteria (Chang et al.)
1980 : Restriction enzymes and genetic linkage analysis (Botstein et al.)
1981 : Expression of a gene injected into a mouse egg cell (Brinster +)
1983 : Ti plasmid transformation of plant cells (Herrera-Estrella et al.)
Polymerase chain reaction (PCR) (Kary Mullis)
1986 : First genetically engineered human vaccine against hepatitis B
1988 : First patented genetically altered mouse
1990 : Human genome project launched
1997 : First mammal cloned (Dolly the sheep)
2000 : Provisional sequence of the human genome published

Fig 11

MAIN APPLICATIONS IN VETERINARY SCIENCE

1. New diagnostic techniques : molecular probes, PCR etc.
2. Monoclonal antibodies for diagnostic and therapeutic purposes
3. New and/or improved vaccines
4. Recombinant biologicals e.g. growth hormone, insulin
5. Genetic manipulation of rumen microflora
6. Genetic manipulation of fodder plants
7. Cloning of livestock : improved production; tissues for transplants