USE OF MOLECULAR GENETIC ENGINEERING IN THE STUDY OF ANIMAL PARASITES AND THEIR VECTORS

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ABSTRACT

Molecular genetics coupled with advances in immunology and parasite culture has become a powerful tool to study animal parasites and their vectors. Recombinant DNA techniques allow one to identify individual genes of DNA probes, amplify the nucleic acid of interest, and use this material to study: the role of the gene product in the biology of the organism; the evolution of parasites and their hosts; heterogeneity between species and within species; taxonomy and develop refined taxonomic tools; the immunology and biochemistry of host-parasite interactions; identification of specific cells or tissues that produce gene products; cytogenetics and localization of genes on chromosome in the study of animal parasites and their vectors will be presented.

INTRODUCTION

Molecular genetics coupled with advances in immunology and parasite culture has become a powerful tool to study animal parasites and their vectors. Recombinant DNA techniques allow the isolation and amplification of a DNA segment from a complex system like a helminth or protozoan parasite to a simpler more defined system e.g. *Escherichia coli* where it can be manipulated. Once amplified the DNA segment can be identified by obtaining sequence information. If the sequence represents a gene, the amino acid sequence encoded by the gene can be deduced and through homology searches of available data bases using a computer, the polypeptide can be identified. The sequence information will also contribute to an understanding of the structure and organization of the DNA fragment. The DNA fragment can be used as a hybridization probe to: identify organisms, establish relationship between and among organisms, define the distribution of organisms both geographically and within various host species, identify the location of genes on chromosomes, study developmentally regulated gene expression and identify cells that are expressing the gene. The DNA fragment can be engineered into a prokaryotic or an eukaryotic expression system to produce the gene product. Sufficient amounts of the gene product will be available for a number of desired biochemical and immunological studies. Many parasites have complex life cycles in which a single genome that contains all the genetic information for survival moves through different units of environments in space and in time. The expression of parasite genes in any development stage at any point in time is reflected in the

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population of mRNAs of that parasite stage. To study stage- specific genes and gene products mRNA is isolated from the parasite stage of interest (often it is the most abundant stage of the parasite), reverse transcribed into cDNA, made double stranded and inserted into an appropriate cloning vector. Each recombinant clone (vector containing a parasite cDNA) represents a single mRNA. A cDNA library represents a collection of recombinants representing the different genes that were expressed at the time the mRNA was isolated. Immunological or molecular techniques are then used to identify the cDNA clones of interest.

Selected examples of the application of genetic engineering in the study of animal parasites and their vectors follow.

DNA PROBES FOR IDENTIFICATION

The development and use of DNA probes for the identification of parasites and their vectors is base on the premise that within any organism there are unique DNA sequences in the genome which differentiate that organism from closely related organisms. Most genomic DNA differences are not specific to sex or developmental stage. DNA probes are sensitive and specific reagents capable of detecting and identifying target nucleotide sequences. The process, molecular hybridization is the formation of a double helix from two complementary strands. The interaction between the complementary strands is more sensitive and stringent than that between antigen and antibody. The development of a DNA probe requires that a particular DNA sequence be identified, isolated and cloned. Two approaches are commonly employed to identify and isolate DNA probes. One involves identifying species-specific sequences, often by sequential hybridization of genomic DNA of the two or more species being compared to cloned DNA of one species. This approach typically identifies highly repetitive DNA sequences. Alternatively repeated gene families, such as the ribosomal RNA genes, 5S RNA genes, or the mitochondrial genome can be cloned to identify species-specific differences in the variable portions of these DNAs such as spacers, gaps or introns. DNA that is identified by either method is used as a hybridization probe to identify target sequences in genomic DNA from various organisms. In order to detect the hybridization reaction, the probe must be labelled so that it can be visualized. Two labelling methods are in common use: one employs radiolabelled nucleotides that are detected by autoradiography, the other employs biotinylated nucleotides that are detected by an avidin-enzyme conjugate after exposure to a suitable substrate. The molecular structure of DNA is stable in desiccated or alcohol stored specimens. Thus fresh target material is not always necessary. In the preparation of DNA probes, the final test is to compare the sensitivity and accuracy of identification with established benchmark methods. The techniques of DNA isolation, cloning, restriction enzyme analysis, blotting and hybridization have become simplified and standard in many laboratories throughout the world[1].

I. DNA probes for identification

Identification of Leishmania species: The identification of *Leishmania* species is based on a variety of ecological, biological, biochemical and immunological criteria. Each cultured isolate of the parasite has been analyzed by one or more of these characters

and categorized as to species or subspecies. As human leishmaniasis is caused by at least 14 different species and subspecies of *Leishmania* and the clinical manifestations (visceral, cutaneous, or mucocutaneous) of the disease depend in part on the infecting *Leishmania* organism, a sensitive and reliable detection and identification method that was field applicable was needed. DNA probes specific for the various *Leishmania* species were developed to provide a direct diagnosis of patients with leishmaniasis and to eliminate the need for culturing parasites before species identification.[2] The use of DNA probes allows direct diagnosis from lesion material without requiring isolation of the parasite. The DNA probe was derived from the kinetoplast DNA (kDNA), an unusual kind of mitochondrial DNA found in all trypanosomatids, from *Leishmania*. The kDNA is composed of two components: the maxicircle which is 20-40 kilobases in size, present in 10-50 copies per cell, and corresponds to the conventional mitochondrial genomes; and the minicircle which 1 kilobase long, present in 10^4-10^5 copies per cell, and has no known function. The basis for the DNA probes is the minicircle which has a high rate of DNA sequence divergence i.e. changes rapidly over a relatively short evolutionary period as measured by restriction site polymorphism and DNA hybridization in contrast to the more conserved maxicircle. For example, kDNA minicircles isolated from *L. mexicana* do not share sequence homology with those isolated from *L. braziliensis*[3]. This allowed researchers to distinguish between major species complexes. To develop DNA probes with narrower specificities that would distinguish subspecies and even geographic isolates, restriction fragments of kDNA minicircle were cloned. This was possible because within the minicircle population, there are DNA sequences that have undergone rapid sequence divergence even within a single minicircle and thus serve to differentiate closely related organisms and individual isolates[4]. For example, cloned DNA fragments from minicircles that were species specific could differentiate between visceral and cutaneous *Leishmania* causing species were engineered[5].

The above example shows how DNA probes derived from repeated DNA sequences can be used for identification and classification. Another logical use for DNA probes is epidemiological studies. The same DNA probes can be used to study the species range of the host and the vector, and the geographic distribution of the parasite. For example, the epidemiology of leishmaniasis is extremely complex and varies in detail in various geographic areas. A major difficulty in studying leishmanial ecology is that several insect vectors, mammalian reservoirs and species of parasite can be found in the same small region. The animal reservoirs are frequently infected with mixtures of parasites as are the sandfly vectors that feed on them. DNA probes have been used to determine infection rates and identify infecting organisms in field samples collected from vertebrate hosts (tissue from human lesions) and/or vectors (sandflies). As few as 30 organisms can be detected using DNA probes[6].

Species identification of vectors has also been accomplished using DNA probes. For example, a DNA probe method was developed to identify species of individual mosquitoes in the *Anopheles gambiae* complex[7,8]. The *A.gambiae* complex consists of six morphologically indistinguishable sibling species that include the major malaria vectors. The DNA probe was isolated from a repeated gene, the ribosomal RNA encoding

DNA contains an intergenic spacer region that shows variation in an EcoR1 restriction enzyme site. Thus, a DNA fragment that contains the intergenic region was used as a hybridization probe on EcoR1 digested DNA from the 6 members of the *A. gambiae* complex. When the DNA probe method of identification was compared with standard cytogenetic techniques, 97% of cytologically identified mosquitoes were also identified by the DNA probe method.[8]

DNA fragments containing portions of conserved genes such as the ribosomal RNA genes can be used to establish taxonomic and evolutionary relationships. For these types of studies DNA sequence information is obtained for the genes of interest from two or more organisms and the sequence information analyzed usually with the aid of a computer for similarities[9,10]. For example, *Pneumocystis carinii* is the cause of a lethal pneumonia which occurs in immunocompromised hosts like premature babies and AIDS patients. The basic biochemical and genetic characteristics of *Pneumocystis* are poorly understood and its taxonomic classification as a protozoan or fungus is uncertain. To address the taxonomic issue total RNA was isolated from *Pneumocystis*- and parts of the ribosomal RNA (rRNA) were sequenced. This sequence information was compared to corresponding sequences of prganisms from other taxa. The *Pneumocystis* sequences were more similar to those of fungi than those of protozoa[11]. The premise of these studies is that genes are the fundamental units of heredity and thus the base sequence of a gene is a very high resolution marker that is capable of identifying evolutionary relationships and provide another tool along with morphological and biochemical criteria to evaluate taxonomic relationships. Comparison of ribosomal RNA sequences has emerged as the molecular method of choice for these types of studies.

DNA probes have been used in conjunction with cytogenetics to map the location of genes and genetic elements on chromosomes. Chromosomal localization of genetic elements will contribute to our understanding of the genetics of parasites and their vectors. For example, studies with *Schistosoma mansoni* have revealed the location of two repeated DNA elements, SM t-2 and rDNA. SM t-2 is a member of a family of short repetitive DNA elements found throughout the genome of *S.mansoni*. The members are repeated 7,000 to 10,000 times per haploid genome and some are associated with a large restriction fragment of DNA specific to female worms[12]. The rDNA genes are present as a cluster of a 100 tandem repeats[13]. Localization of the DNA repeat elements was by *in situ* hybridization of a biotinylated DNA probe on mitotic and meiotic metaphase chromosomes of *S.mansoni*. The SM t-2 hybridizing sequences were found dispersed throughout the genome, hybridizing to the sex chromosomes and autosomes. The SM t-2 probe showed specific hybridization to the euchromatic gap region within the large heterochromatic block of the short arm of the W chromosome. This specific hybridization coupled with the lack of chiasma formation in this region of the ZW bivalent was used to explain the sex- specific hybridization pattern reported for SM t-2 (see below). The rDNA repeat was localized to the secondary constriction of the short arm of crhomosome 3[14].

DNA probes have been used to identify the sex of a parasitic organism. For example, schistosome parasites, unlike other trematode parasites have separate sexes. Sex is determined by a chromosomal mechanism in which the male is ZZ and the female is ZW. Adult schistosomes exhibit sexual dimor-

phism and can be easily distinguished, however, the sex of the other stages in the life cycle cannot be visually assessed. A cloned DNA probe was identified that could accurately identify the sex of cercariae[15]. This cloned DNA probe, SM t-2 was later shown to hybridize to a euchromatic gap region sandwiched in the heterochromatin of the W chromosome of the female parasite[12,14].

II. Developmentally regulated gene expression

Animal parasites have complex life cycles that often take them through radically different units of environment. Thus a parasite may spend part of its life cycle in a vertebrate host, another part in one or more intermediate vertebrate or invertebrate hosts and during transmission from host to host the parasite may be in a free-living environment. However, it is the same genome of the parasite that moves through the different environments that are discontinuous in space and in time. In order to adapt to the radically different environments presumably the parasite regulates the expression of different genes during development. A measure of gene expression is transcription or the presence of specific mRNA. Thus in studies of gene expression, the steady state level of particular mRNAs obtained from different developmental stages are measured by northern analysis. Briefly, total RNA is isolated from various developmental stages, quantitated, denatured and either dotted onto a membrane or size fractionated by gel electrophoresis and blotted onto a membrane. The RNA bound to the membrane is then hybridized with different labelled DNA probes and the intensity of the hybridization signal between RNAs from different developmental stages is compared. As a control a DNA probe that represents a constitutively expressed gene is used. For example, in the schistosomes where the sexes are separate and female development is dependent on pairing with the male worm, three different female specific genes have been identified[16]. One of these genes encodes an eggshell protein and is represented by a cDNA clone, pSMf61-46[17]. pSM61-46 is able to hybridize to RNA isolated from mature female worms but not to RNA isolated from immature female worms, male worms or the egg stage. Thus the eggshell protein gene represented by pSMf 61-46 is expressed in mature female worms.

From cercariae, a trematode life cycle stage that results from intramolluscan development, genes have been isolated that code for proteinases. For example, an elastase gene from cercariae of *Schistosoma mansoni* has been cloned[18]. The elastase cDNA clone will only hybridize to RNA isolated from the intramolluscan stages of the parasite but not to RNA isolated from mature cercariae or adult worms. Thus the elastase gene whose product seems to be important in penetration is only expressed during cercarial development.

Various enzymes play a role in parasite development and thus show developmentally regulated gene expression. For example, superoxide dismutase (SOD), a metalloenzyme that catalyzes the dismutation of the superoxide radical to molecular oxygen and hydrogen peroxide, is found in all eukaryotic cells. Recently, the gene encoding SOD from *S.mansoni* was isolated and characterized[19]. Studies on the steady state levels of SOD mRNA show that adult worms contain 5-10 times more SOD mRNA than do the egg and cercarial stages. All stages of the parasite contain SOD mRNA because every cell needs this enzyme to survive. However, the adult

worm stage uses more of this enzyme as judged by the higher amount of SOD mRNA detected. Presumably, this is because a significant amount of SOD activity is associated with the tegument of the adult worm and the tegumental associated SOD is thought to play a role in immune evasion.

III. Isolate genes of interest and study their gene products

The power of recombinant DNA technology allows the isolation of DNA or RNA, the construction of gene libraries and with the proper reagents e.g. antibodies, the isolation of specific genes. These genes can then be engineered into an appropriate expression vector to produce quantities of the gene product for study. An example of this type of strategy and study is the isolation of a tropomyosin gene from *S.mansoni*, expression of schistosome tropomyosin in bacteria, purification of the gene product, and demonstration that the purified gene product has potential in immunodiagnosis[20,21]

Polypeptides of three hour schistosomula were identified by separating membrane-containing extracts by non-equilibrium two-dimensional gel electrophoresis (NEPHGE). To identify which of the schistosomula polypeptides were antigens, the separated polypeptides were transferred by western blot to nitrocellulose and the blot probed with sera from humans infected with schistosomiasis mansoni. An acidic group of polypeptides with a molecular size of 40 kilodaltons (pI-4.85) were identified as immunodominant antigens. These polypeptides were removed from silver-stained NEPHGE gels to produce specific antisera in rabbits[20] This antisera raised against the group of 40 kilodalton antigens was used to identify cDNA clones that were expressing one of these antigens. (In this particular example a number of recombinant clones had been identified that contained the cDNA insert of interest. The antisera was used to identify which of these were expression clones). A cDNA clone containing an insert of 1.3 kilobases was identified and DNA sequence information was obtained. A single open reading frame was shown to code for 284 amino acids. Computer analysis of the deduced amino acid sequence homology to amino acid sequences of eighteen different tropomyosins from various species[21]. The cDNA that encoded schistosome tropomyosin was cloned into the plasmid expression vector, pOTSNCO and induced to over express schistosome tropomyosin. The recombinant tropomyosin which was very hydrophilic was purified by ammonium sulphate fractionation and FPLC using a DEAE ion exchange column. The purified schistosome tropomyosin eluted as a single major peak at a salt concentration of 40%. The recombinant tropomyosin was now available for study in large quantities[22]. For example, studies of recombinant schistosome tropomyosin as an immunodiagnostic reagent revealed that only sera from patients with chronic schistosomiasis mansoni but not sera from patients with other parasitic diseases including schistosomiasis haematobia and schistosomiasis japonica reacted with the recombinant protein. Thus immunologically tropomyosin seems to be species-specific[22]

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QUESTIONS AND ANSWERS :

1. Question: What progress has been made on a Plasmodium vivax DNA probe for diagnostic purposes in humans ?
What has been done along the lines of isolating stage-specific DNA genomes ? (i.e. blood stage vs. sporozoite).

   Answer : *Plasmodium vivax* probes have been identified.
I am not sure if they have been field tested.
Genomic DNA is usually the same no matter what stage it is isolated from. The exception to this is sex-specific DNA sequences where the Y chromosome has heterochromatic DNA not present in the X chromosome.