40

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New Approaches to the Identification of Parasites and Their Vectors

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Chapter V

The Application of Recombinant DNA Technology to Problems of Parasite and Vector Identification

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ABSTRACT

The application of recombinant DNA technology to problems of parasite and vector identification on an intrinsic level has opened new vistas in this type of analysis. Parasite or vector genes or DNA fragments can be isolated, grown in bacteria and studied at the level of DNA sequences for homologies or evolutionary relatedness. The genes can be from either the nuclear genome or the mitochondrial genome of the eukaryotic parasite or vector and can be compared in terms of general site polymorphisms, specific sequence changes, or sequence conservations. Both species and strain differentiation can be performed in some cases. The technology is simple and powerful and requires little parasite material. In addition to parasite identification, knowledge of the relatedness of parasite and vector genes and specific non-gene regions should lead to a deeper understanding of the molecular evolution of these organisms and perhaps of the host-parasite relationship itself.

KEY WORDS

Recombinant DNA technology; parasite and vector identifica-

tion; genes; DNA sequences; genetic techniques.

The rationale for the study of differences on the DNA level as a means of identifying and classifying parasites and their vectors lies in the fact that living organisms are "living fossils", in that the evolutionary record of each organism is contained within the sequence of nucleotides in its DNA. In fact, the identification of parasites is essentially a by-product of the study of molecular evolution in which one searches for divergence rather than homology.

I shall first briefly discuss the basic concepts of molecular evolution of the DNA of eukaryotes. Most of the DNA in higher eukaryotes is not used for coding of proteins. This noncoding DNA consists of intervening sequences within genes, spacer sequences, and the so-called moderately repetitive and highly repetitive sequences. The study of molecular evolution therefore involves not only the evolution of protein coding genes but also the evolution of these sequences of enigmatic function. The ability to detect and clone single copy genes and specific non-gene regions has revolutionized the study of molecular evolution in recent years. In addition, the study of the evolution of the separate and simpler genetic system in the mitochondrion of the eukaryotic cell has added another dimension to this field.

One of the most useful discoveries in molecular evolution is that the rate of amino acid substitutions, i.e. nucleotide substitutions that lead to changes in amino acids, is characteristic for each polypeptide and appears to be constant over time. This allows the rate to be used as an "evolutionary clock" (Wilson et al., 1977). In the mitochondrial genetic system of animals, the rate of nucleotide substitution is approximately ten times the rate in the nuclear genetic system (Brown et al., 1979), allowing the mitochondrial evolutionary clock to be used for a fine structure analysis of more recent evolutionary changes in maternal lineage (Brown, 1980). Conversely, the rate of silent site nucleotide substitutions is substantially greater than the rate of amino acid site substitutions, and the rate is nonlinear with time (Grunstein et al., 1976; Kafatos, et al., 1977; Efstratiadis et al., 1980; Perler et al., 1980). The evolution of intervening sequences

seems to occur more rapidly than that of coding sequences (Konkel et al., 1979), but in some cases there is a strong evolutionary stability of the position and length of intervening sequences (Leder et al., 1978), and in other cases it is precisely these sequences that have been lost (Nishioka and Leder, 1979).

The existence of mobile transposable elements in eukaryotic nuclear genomes is well established and this adds yet another dimension to molecular evolution. 'Pseudogenes' have been discovered (Lacy and Maniatis, 1980), which represent altered gene sequences, rendering them nonfunctional. And, finally, many copy genes previously presumed to be single have been shown to be members of multigene families, which in some cases have been found to develop jointly by a process of "concerted evolution" (Zimmer et al., 1980).

For the identification of parasites and their vectors, a complete understanding of the evolutionary significance of the DNA sequence divergences observed is at first glance relatively unimportant. However, such an understanding will add a dimension of depth to our knowledge of the parasites and their vectors that could not be obtained in any other way, and may in fact lead to a more basic understanding of the host-parasite relationship, which itself has in many cases a long evolutionary history.

This paper will be concerned with a brief discussion of some representative techniques available for the analysis of the DNA sequences of parasites and their vectors. Several excellent general references include the three-volume set by Williamson (1981), the lab manual by Maniatis et al. (1982), the volume on methods in enzymology edited by Wu (1979), and the book by Schleif and Wensick (1981).

The isolation of high molecular weight DNA from organisms is basically identical to the original method developed by Avery for pneumococcus. It involves cell disruption by detergent lysis in a salt-containing buffer at neutral pH followed by deproteinization either by treatment with nuclease-free protease or by repeated extraction with a strong denaturing agent such as phenol, phenol/chloroform or chloroform. Prior removal of the cell wall by mechanical rupture or enzyme treatment is necessary in some cases. The use of a high concentration of EDTA during the initial lysis inhibits endogenous nucleases, which can damage the DNA. RNA is either

removed by treatment with RNase or by sedimentation in CsCl. DNA is easily precipitated with ethanol and can be purified by a final isopycnic centrifugation in CsCl.

Isopycnic buoyant density centrifugation in gradients of CsCl is a useful method to separate DNA molecules on the basis of % G + C. This buoyant separation can be greatly enhanced by use of the AT-binding fluorescent dye, Hoechst 33258 (Simpson, 1979) (Figure 1). The use of the fluorescent intercalating dye, ethidium bromide, in the CsCl gradient allows the separation of closed circular molecules from open circular and linear molecules (Radloff et al., 1967). This is the basic preparatory method for the isolation of plasmid DNA, circular mitochondrial DNA and other circular molecules.

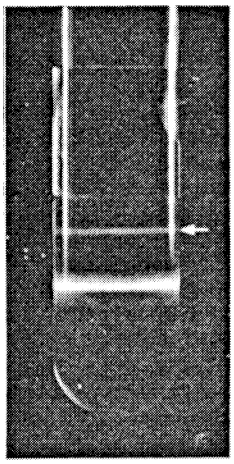

Figure 1. Hoechst 33258-CsCl isopycnic gradient visualized by long wavelength UV illumination. The DNA is Leishmania tarentolae kinetoplast DNA digested with EcoRI. The upper band represents the linearized maxicircle DNA and the lower band undigested network DNA and linearized minicircles containing EcoRI sites. From Simpson (1979) by permission.

The main analytical and preparatory technique used to separate DNA molecules on a size basis is electrophoresis through a supporting matrix of agarose (Aaij and Borst, 1972; Sharp et al., 1973; Southern, 1979) (Figure 2) or polyacrylamide (Maniatis et al., 1975). Agarose is used to separate molecules from 0.1 to 60 kb and acrylamide is used for DNA molecules less than 1 kb in length and can actually resolve fragments differing by a single nucleotide. Acrylamide gradients can also be used to sharpen the bands and extend the limits of resolution (Figure 3). Agarose gel electrophoresis can also be used to separate conformational

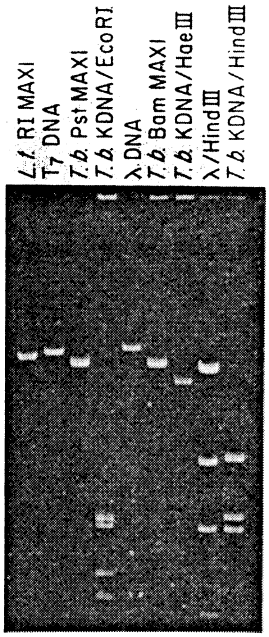

Figure 2. Agarose gel (0.5%) of PstI and BamHI linearized maxicircle DNA from T. brucei strain 366D kDNA and EcoRI linearized maxicircle DNA from L. tarentolae kDNA. T. brucei kDNA samples digested with HindIII, EcoRI and HaeIII are also included. Several molecular weight markers are run as standards. From Simpson and Simpson (1980) by permission.

isomers, such as closed circular, nicked circular and linear DNA molecules (Johnson and Grossman, 1977). Agarose electrophoresis is usually performed in submerged horizontal agarose slabs known as "submarine gels", which minimize thermal distortion of banding patterns (McDonnel et al., 1977) (Figure 4). Minigels or 'blitz' gels can be run on microscope slides in which the total running time can be as short as 10-15 minutes. This enables one to assay the extent of reactions in progress. Visualization of DNA in gels can be done nondestructively by staining with ethidium bromide during the run and transillumination with UV light. The lower limit of resolution is approximately 10 ng per band. Illumination of ethidium-DNA with short wavelength UV light (254 nm) gives good visualization but has the disadvantage of inducing extensive photodamage in the DNA. Long wavelength UV light (366 nm) does not damage but does not allow good visualization. UV light at 300 nm is a good compromise in terms of decreased photodamage and high sensitivity (Brunk and Simpson, 1977) (Figure 5, Table 1). DNA can also be

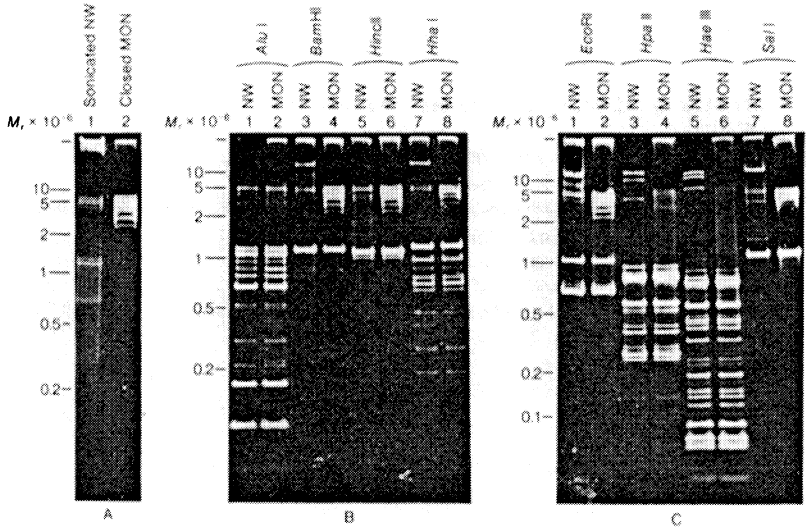

Figure 3. Comparison of restriction enzyme digested monomeric minicircles (MON) and closed network DNA (NW) from L. tarentolae by acrylamide gradient gel electrophoresis. The gel was 3.5-10% acrylamide with a 3% stack. A - Sonicated network DNA and physically isolated monomeric minicircles. Note the distribution of minicircle bands of molecules with different numbers of superhelical turns. B and C - Network DNA and minicircle DNA digested with several enzymes. Note the presence of several minor high molecular weight bands in the network digest lanes derived from maxicircle DNA. Several reference DNAs (not shown) were coelectrophoresed to obtain the molecular weight scales. From Simpson (1979) by permission.

visualized with greater sensitivity by silver staining (Merril et al., 1979) or by autoradiography if the molecules are labelled with ^{32}P.

Recovery of DNA from agarose or acrylamide can be a problem since enzyme-inhibiting contaminants often copurify with the DNA. However, there are several rapid procedures for elution of DNA from agarose and acrylamide by diffusion or electrophoresis that usually yield fragments clean enough to be used for further enzymatic steps (McDonnel et al., 1977; Weislander, 1979; Girvitz et al., 1980; Dretzen et al., 1981) (Table 2). In general, the recovery of DNA fragments decreases with the size of the fragment from almost 100% of fragments less than 1 kb to no more than 20% of fragments

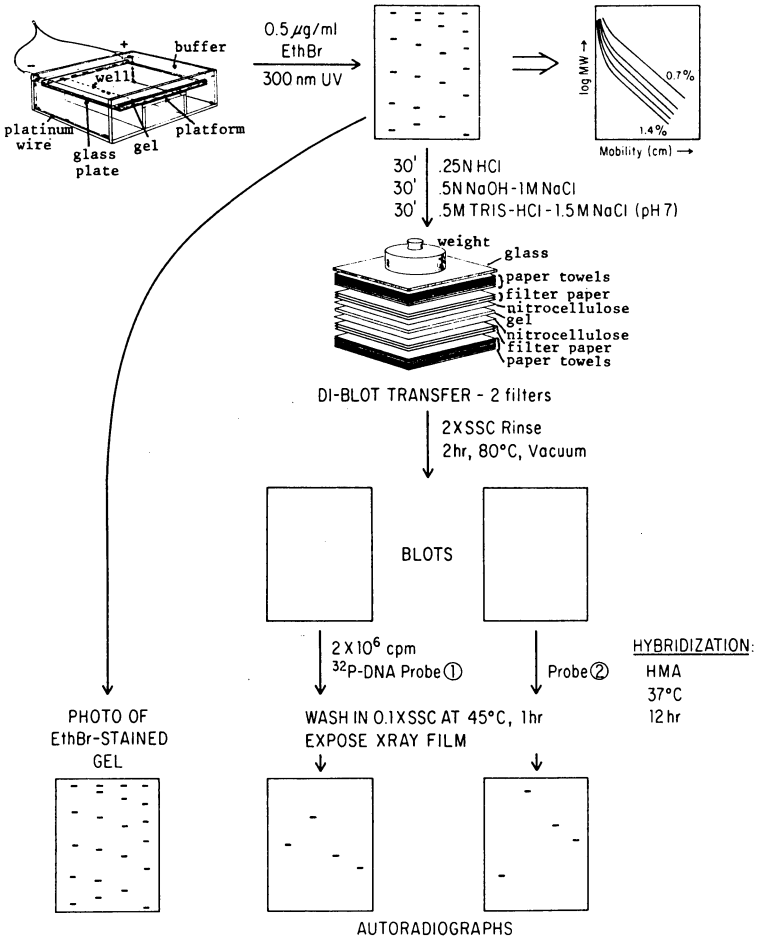

Figure 4. Diagrammatic representation of Southern blot hybridization. DNA samples are electrophoresed in an agarose submarine gel in the presence of ethidium bromide. The banding pattern is photographed by transillumination with 300 nm UV light and the fragment sizes are calculated from the mobilities by reference to standard curves of corun reference DNAs. The gel is blotted onto 2 nitrocellulose filters, which are hybridized with ^{32}P-labelled DNA probes in HMA medium (50% formamide, 0.2% sodium dodecyl sulfate, 5 x SSL, 0.5 mg/ml sonicated denatured salmon sperm DNA, 0.5 mg/ml poly r(A), 0.02% polyvinylpyrollidine, 0.02% Ficoll, 0.02% bovine serum albumin). The filters are washed at a high stringency and autoradiographed. The autoradiograph bands are compared to the ethidium stained bands.

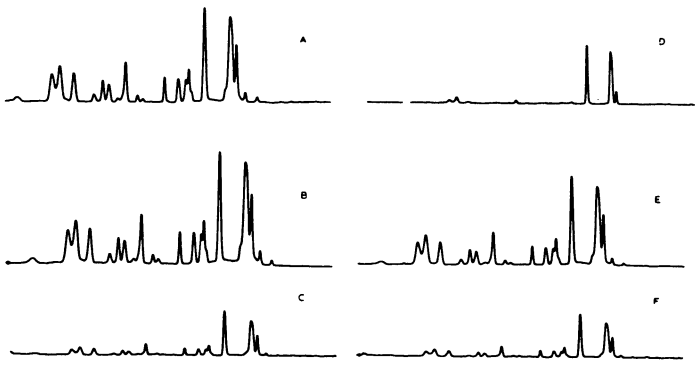

Figure 5. The pattern of fluorescent ethidium bromide-DNA bands on polyacrylamide gels excited with various UV sources for different periods. A - Excitation with a 254 nm source photographed immediately; B - Excitation with a 300 nm source photographed immediately; C - Excitation with a 366 nm source photographed immediately; D - Excitation with a 254 nm source for 20 min. prior to photography; E - Excitation with a 300 nm source for 20 min. prior to photography; F - Excitation with a 366 nm source for 20 min. prior to photography. All negatives were traced with a Joyce-Loebl densitometer. From Brunk and Simpson (1977) by permission.

greater than 20 kb. Both double-stranded and single-stranded DNA molecules can be separated in gels. Gel systems that separate single-stranded molecules include alkaline agarose (McDonnel et al., 1977), agarose in the presence of poly UG (Goldbach et al., 1978), and 7 M urea-acrylamide run at 60°C (Sanger and Coulson, 1975). The latter gels are used to separate the 'ladders' of oligonucleotides produced by the Sanger or the Gilbert-Maxam sequencing methods.

Table 1. Intensities and photodamage parameters for UV sources

Source wavelength (nm)	Output (nE/min. cm^2)	Photonicking D_{37}(nE)	Photodimerization k(%/μ E)
254	390	390	8.40
300	290	5 700	0.10
366	660	28 000	< 0.001

Table 2. Some procedures for recovery of DNA from gels

1. Acrylamide - small DNA fragments: Stained gel bands are minced in 0.5 M NH_4OAc-1 mM EDTA and allowed to diffuse overnight at 37°C. Gel fragments are removed by filtration.

2. Agarose - small or large DNA fragments:

   (1) Stained gel bands are inserted into a dialysis bag with some dilute electrophoresis buffer, and the DNA is electroeluted at 100 V for 2 hrs into the buffer in the bag.

   (2) Troughs are cut in front of DNA bands and the DNA is electrophoresed onto a piece of dialysis membrane in the trough or onto DEAE cellulose or hydroxyapetite in the trough. DNA is recovered from the DEAE cellulose by high salt elution and from the hydroxyapetite by low salt elution.

   (3) Stained gel bands are dissolved in saturated KI and the DNA is recovered by isopycnic centrifugation in a KI gradient.

   (4) Low melting temperature agarose: The stained bands are melted at 65°C in buffer, cooled to 25°C, and the agarose is removed by phenol extraction.

The analysis of DNA sequences is based on simple analytical and preparatory procedures and on the availability of specific enzymes that can perform such remarkable reactions as sequence-specific cleavage, ligation, repair synthesis, phosphorylation and dephosphorylation, methylation and homopolymer 'tailing'. A list of several useful enzymes and their modes of action is presented in Table 3. All of these enzymes are available commercially.

Restriction enzymes usually recognize 4 or 6 base-pair, often palindromic, sequences and produce cleavages in both strands either in a staggered fashion to produce so-called 'sticky' or cohesive ends, or in a non-staggered fashion to produce 'blunt' ends (Roberts, 1982). Several restriction

Table 3. Some enzymes used in DNA research

Enzyme	Use	Activities
1. *E. coli* DNA polymerase I	Nick translation	5'→3' polymerase 5'→3' exonuclease 3'→5' exonuclease
2. Klenow fragment of Pol I	DNA sequencing, filling in recessed 3' ends, cDNA second-strand synthesis	5'→3' polymerase 3'→5' exonuclease
3. T4 DNA polymerase	End labelling of 3' overhangs, blunt-ending molecules	5'→3' polymerase 3'→5' exonuclease (200 x that of Pol I)
4. AMV reverse transcriptase	cDNA copies of mRNA	5'→3' DNA polymerase RNase H activity
5. T4 DNA ligase	Ligation of adjacent 3-OH and 5-P termini of compatible cohesive ends of DNA. Ligation of blunt-ended DNA molecules or synthetic linkers	Ligation. Efficiency of blunt-end ligation is 100 x less than that of cohesive ends
6. Alkaline phosphatase (bacterial or calf)	Dephosphorylation of 5' ends of DNA or RNA prior to labelling with ^{32}P or to prevent self-ligation of DNA	Dephosphorylation
7. T4 DNA kinase	Labelling of 5' ends of DNA or RNA with ^{32}P	Transfer of ^{32}P of ATP to 5'-OH terminus 3-phosphatase
8. S1 nuclease	Digestion of single-stranded regions of DNA. Used to blunt-end DNA molecules and to cleave the hairpin loop in cDNA synthesis	Single-strand specific nuclease DNA or RNA
9. DNase I	Nicking of DNA prior to labelling with Pol I in nick translation. Introduction of single random nick into closed circular DNA molecule in presence of EthBr	Endonuclease. In presence of Mg^{++} produces nicks. In presence of Mn^{++} produces double-strand breaks
10. Terminal transferase	Addition of homopolymer tails to DNA and vector	Adds deoxynucleotide to 3'-OH terminus of DNA. Active on protruding 3'-OH or blunt-ended or recessed 3'-OH

enzymes do not recognize sequences in which the C is methylated (Table 4), thereby providing a valuable tool to measure the extent of methylation in DNA (Roberts, 1982).

Table 4. Use of restriction enzymes to measure extent of methylation of C and A residues in DNA

Enzyme	Site	Activity
Hpa II	C'C G G	+
	C^{M}C G G	-
MspI	C'C G G	+
	C'^{M}C G G	+
Sau3A	'G A T C	+
	'G^{M}A T C	+
MboI	'G A T C	+
	G^{M}A T C	-
DpnI	G A T C	-
	G'^{M} A T C	+

DNA fragments can be labelled *in vitro* with ^{32}P to extremely high specific activities (10^{8} cpm/μg) by several methods. For example, 5' end labelling can be accomplished by use of polynucleotide kinase (Richardson, 1971) and α-^{32}P ATP after removal of the terminal phosphate with bacterial or calf alkaline phosphatase (Chaconas and Van de Sande, 1980), and 3' end labelling by filling in 5' overhangs with reverse transcriptase or Klenow fragment of DNA polymerase (Jacobsen et al., 1974) and α-^{32}P dNTPs. Random labelling of DNA fragments can be accomplished by "nick translation" using DNA polymerase I with DNase I and α-^{32}P dNTPs (Rigby et al., 1977).

Hybridization of single-stranded DNA molecules to produce base-paired, double-stranded molecules is dependent on salt

concentration, temperature, DNA concentration, and on the extent of sequence homology between the reacting species (Bonner et al., 1973). The thermal stability of a heterologous hybrid is a measure of the percentage of mismatched base pairs (Hyman et al., 1973). The hybridization of labelled probes with gel-separated DNA fragments is readily performed by the Southern transfer or blot method (Southern, 1975; Smith and Summers, 1980) (Figure 4). This method involves the transfer of the entire gel banding pattern onto a solid filter support by either capillary action or electrophoresis. The filter, either nitrocellulose membrane or diazotized paper (Alwine et al., 1977), is then hybridized with the labelled probe, washed at the desired stringency and the extent of hybridization visualized by autoradiography. This deceptively simple method is one of the most valuable tools of DNA research and is especially important for the primary detection of sequence divergence between strains and species of parasites (Howley et al., 1979). With the use of high specific-activity labelled probes, even single copy genes can be detected in blots of total genomic DNA separated in agarose (Jeffreys and Flavell, 1977).

Another method to distinguish DNA sequences is to examine restriction site polymorphisms, that is, the appearance and absence of specific restriction sites as determined by the migration of restriction fragments in a gel. Quantitative methods have been elaborated to convert observed polymorphisms into nucleotide sequence divergence (Nei and Li, 1979). This method applied to mitochondrial DNA has proved very useful in the study of the evolution of higher organisms (Brown, 1980) and has also been used to distinguish different strains of pathogenic kinetoplastid protozoa by examination of kinetoplast DNA sequences, both minicircular (Morel et al., 1980) and maxicircular (Borst et al., 1981). (See also Figures 6 and 7).

A more direct method to compare large-scale differences between DNA molecules is heteroduplex analysis in the electron microscope (Davis et al., 1971). The DNAs are denatured and annealed together and spread on an aqueous or formamide hypophase for visualization after staining with uranyl or shadowing with platinum-palladium. Distinction of double-stranded and single-stranded regions as thick strands and thin, kinky strands, respectively, allows the ready detection of regions of sequence homology and divergence. The limit of resolution of denaturation loops is approximately 100 bp.

The most direct method to compare DNA sequences is direct sequence analysis. There are two methods for DNA sequence analysis, the chemical degradation method (Maxam and Gilbert, 1977) and the enzymatic dideoxynucleotide chain termination method (Sanger et al., 1977). The latter method combined with cloning in the single-stranded vector, phage M13, is capable of yielding several thousand nucleotides of sequence data per day (Staden, 1982) and, in practice, is limited by the rate of analysis of the data. Computer analysis is a necessity for handling the large amount of data and for homology searching; there are several programs available for such purposes

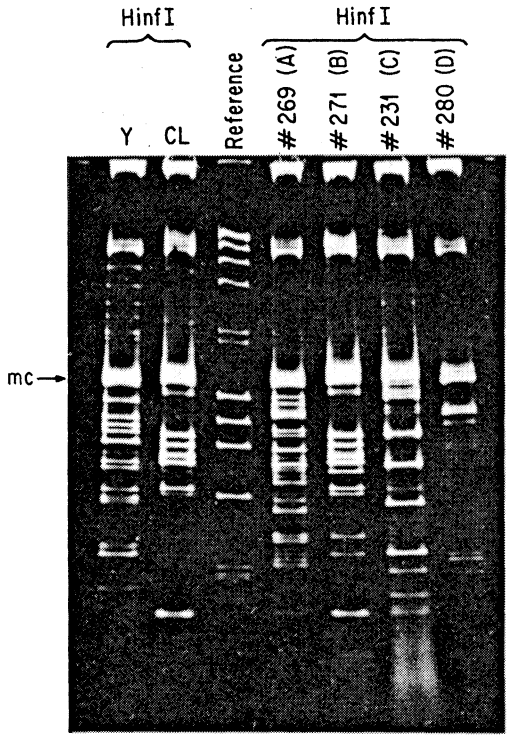

Figure 6. Acrylamide gradient gel electrophoresis comparison of kinetoplast DNA HinfI digests from the Y and CL strains and from four stocks from human patients that represent zymodeme groups A-B. (mc = minicircle regions) From Morel et al. (1980) by permission.

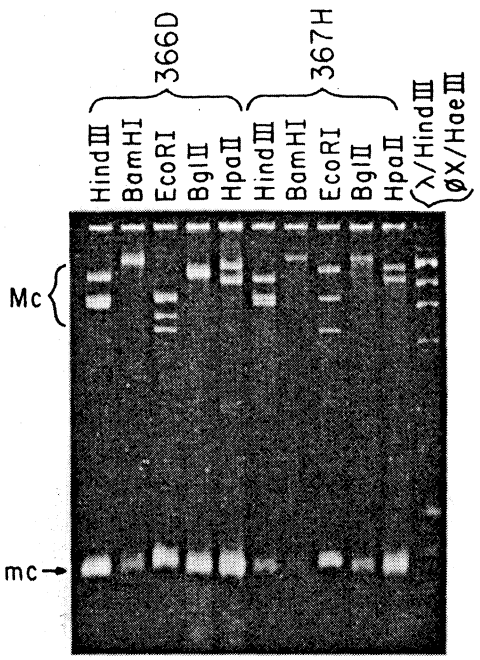

Figure 7. Agarose gel electrophoresis comparison of digests of kinetoplast DNA from two clonal strains of T. brucei, 366D and 367H. Agarose concentration is 0.8%. The minicircle (mc) and maxicircle (Mc) regions are indicated. Note the restriction site polymorphisms for EcoRI and BglII in the maxicircle DNAs. From Simpson and Simpson (1980) by permission.

(see Volume 10, No. 1 of Nucleic Acids Research, 1982), and there are several sequence data banks available for comparison of previously sequenced genes. The generation of both random or 'shotgun' clones (Messing et al., 1981; Anderson, 1981) and nonrandom overlapping clones (Frischauf et al., 1980; Hong, 1982) in M13 phage has been used for rapid sequence analysis of large DNA molecules. Another important aspect of DNA technology is the direct synthesis of DNA molecules (Itakura and Riggs, 1980). The phosphotriester method using charged dimer or trimer added to a resin-linked chain has been used to synthesize up to 40-mers of a defined sequence in a few days (Ito et al., 1982). This has led to the general availability of defined sequence DNA fragments for use as

linkers, adaptors, primers and hybridization probes (Wallace et al., 1981). As an example of the usefulness of synthetic DNA, knowledge of a portion of the amino acid sequence of a protein can be used to synthesize a set of hybridization probes (15 to 16-mers), which could be used to select that particular gene from a genomic library by hybridization (Montgomery et al., 1978).

The cloning of specific DNA fragments in bacterial plasmids, phages (Blattner et al., 1977) or cosmids (Collins and Hohn, 1978) adds yet another dimension to tools that can be used for the identification of parasites. Both cDNA (Williams, 1981) and genomic libraries (Dahl et al., 1981) can be constructed containing essentially all DNA and RNA sequences in the cell. A library consists of a pool of recombinant bacteria or phages containing random fragments of the eukaryotic genome. One can readily calculate the number of recombinants necessary to achieve a 99% probability of having a given DNA sequence represented in a library of genome fragments of a specified size (Clarke and Carbon, 1976). Specific genes can be isolated by several means involving either colony hybridization (Grunstein and Hogness, 1975; Hanahan and Meselson, 1980) (Figure 8), plaque hybridization (Benton and Davis, 1977), expression in the host cell (Broome and Gilbert, 1978; Clarke et al., 1979), or positive (Ricciardi et al., 1979) or negative (Paterson et al., 1977) selection and *in vitro* translation, and these genes could be used for comparison of the parasites. The most easily available genes are those for the ribosomal RNAs. The use of clonal ribosomal genes for species and even strain comparison has already been initiated for both *Plasmodium* (Goman et al., 1982; Hyde et al., 1981; T. McCutchan, personal communication) and *Schistosoma* (Simpson et al., 1982; A. Simpson, personal communication). Other parasite genes or DNA molecules that have been cloned and are available for strain and species differentiation include the tubulin genes of *Leishmania* (N. Agabian, personal communication), the variable surface antigen genes of *Trypanosoma* (Hoeijmakers et al., 1980), several kinetoplast minicircle molecules of *Leishmania* (Simpson et al., 1980; Arnot and Baker, 1981), *Trypanosoma* (Donelson et al., 1979; Morel et al., 1980; Brunel et al., 1980; Simpson and Simpson, 1980) and *Crithidia* (Hoeijmakers and Borst, 1982), and portions of the kinetoplast maxicircle molecules of *Leishmania* (Masuda et al., 1979) and *Trypanosoma* (Brunel et al., 1980; Simpson and Simpson, 1980).

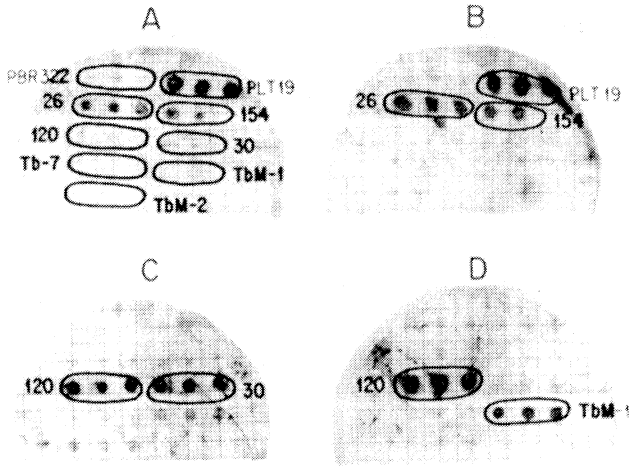

Figure 8. Colony hybridization of recombinant E. coli clones containing L. tarentolae and T. brucei kinetoplast DNA fragments. The clones were spotted in triplicate on each of the four S&S BA 85 grid filters, grown overnight, chloramphenicol-amplified, and processed with NaOH, Tris, and 2 x SSC. The filters were hybridized with the following ^{32}P-labelled probes: A - Total kDNA from L. tarentolae; B - Total monomeric minicircle kDNA from L. tarentolae; C - Total maxicircle kDNA from L. tarentolae; D - Insert DNA from pLt120 plasmid which contains a 6.6 kb fragment of maxicircle DNA of L. tarentolae. The autoradiographs are shown over the actual filters. From Simpson and Simpson (1980) by permission.

The catenated minicircle DNA molecules found in the kinetoplast region of the single mitochondrion of the kinetoplastid protozoa constitute a heterogeneous sequence population in any one strain or species. Restriction fragments are released from minicircle DNA in nonmolar yields and the complexity of the pattern in a high-resolution acrylamide gel represents a unique 'fingerprint', which has been used qualitatively in strain identification in T. cruzi and pathogenic Leishmania on account of the rapid rate of sequence evolution of the minicircle DNA (Morel et al., 1980) (Figure 6). In contrast to minicircle DNA, maxicircle DNA is homogeneous and quantitative analysis of restriction site polymorphisms can be applied (Figure 7). By this method, no distinctive mitochondrial genetic differences could be found between animal and human infective T. brucei-type trypanosomes (Borst et al., 1981) nor between Crithidia fasciculata and C. luciliae

(Hoeijmakers and Borst, 1982). This may imply that the human infective forms of T. brucei represent host range mutants and not separate species and that the Crithidia samples represent two strains of the same species (Borst et al., 1981; Hoeijmakers et al., 1980).

Clearly, the use of DNA technology for the identification of parasites is just beginning but should prove to be a powerful tool for this purpose. In addition, detailed information on sequence divergence of specific genes and specific non-coding regions of the DNA of parasites should lead to a deeper understanding of the molecular evolution of these eukaryotic organisms and perhaps of the host-parasite relationship itself.

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