RESTRICTION ENZYME ANALYSIS OF LEISHMANIA TARENTOLAE KINETOPLAST DNA

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INTRODUCTION

The most striking aspect of the kinetoplast DNA (K-DNA) of the kinetoplastid protozoa is the presence of approximately 10^4 catenated covalently closed duplex minicircles, the molecular weight of which is species-specific (1, 2). We have previously reported several methods to isolate closed minicircles from physically disrupted L. tarentolae K-DNA networks (3) and have studied several properties of these molecules (4, 5, 6). We observed an unusual triphasic melting curve of sonicated total K-DNA (1) and open monomeric minicircles (4). Steinert and Van Assel (7) have also reported high resolution melting profiles of K-DNA from several species of hemoflagellates, and in each case a unique multimeric melting pattern was observed. We attempted in the case of L. tarentolae K-DNA to ascertain whether the sequence heterogeneity was intra-molecular or inter-molecular (4). It was early noted that the isolated network minicircles showed a somewhat non-Gaussian distribution of contour lengths, in that there were more small molecules than large ones (1). Most of the molecules, however, did fit a unimodal distribution with a standard deviation expected for a homogeneous population of molecules of that size (0.29 ± 0.02 μm). Several other experimental facts also indicated to us that the observed heterogeneity was mainly intra-molecular: 1) Renaturation kinetic analysis yielded a complexity of 1.34 X the minicircle molecular weight (5); 2) Thermal chromatography on hydroxyapetite of single nicked open minicircles derived from the free or loosely bound class of K-DNA showed a single monophasic melt with no evidence of a minor class of molecules melting at a separate temperature (4); 3) Fingerprint analysis of a ribosubstituted DNA transcript of randomly nicked minicircles implied a total complexity of about 1600 nucleotides, as deduced from the number of unique oligonucleotide spots compared to the pattern obtained with a T1 digest of E. coli 16 S ribosomal RNA (6); 4) Analytical CsCl analysis of the leading and trailing edges of a preparative CsCl equilibrium band of isolated minicircles showed no differences in buoyant density (4). In the case of T. cruzi K-DNA, Brack and Delain (8) also concluded that all minicircles had the same base sequence. This was deduced from a partial denaturation mapping analysis, in which it was shown that all minicircles possessed 4 congruent AT-rich regions.

Recent evidence from gel electrophoresis and from restriction enzyme analysis

Begin Here has also indicated sequence heterogeneity in the K-DNA minicircles of several species and in some cases the evidence has implied an inter-molecular sequence heterogeneity. Both Riou and Yot (9) and Kleisen, Borst and Weijers (10) have reported the resolution of several length classes of open minicircles and once cleaved linears by high resolution gel electrophoresis. However, the maximum difference in molecular weight between C. luciliae minicircles only represents 4% of the monomer molecular weight, a difference which is too small to be resolved in the electron microscope. In addition, the ΔT_m of native and renatured K-DNA duplexes of C. luciliae is only 1°C, which implies a maximum sequence heterogeneity of 2% of the nucleotide pairs.

Further evidence for sequence heterogeneity in K-DNA minicircles has arisen from interpretations of the electrophoretic fragment patterns obtained with DNA digested with several restriction endonucleases. A homogeneous population of circular molecules completely digested with any single restriction enzyme should present a pattern of homogeneous fragment classes in molar yields. This would be visualized on a gel as a series of sharp bands, the intensities of which are linearly proportional to the molecular weights. By this criterion, in the absence of partial digestion products, the K-DNA minicircles of C. luciliae are clearly heterogeneous in base sequence. Furthermore, Kleisen et al. (10, 11) found that the summated molecular weights of the fragments exceeds 12 times the molecular weight of the minicircle. In addition, some restriction enzymes were found to digest only a fraction of the minicircle population. They concluded that there are at least 13 different minicircle sequence classes in C. luciliae K-DNA. Similar results were obtained by Riou and Yot (9) for T. cruzi K-DNA. In an attempt to reconcile the restriction enzyme data with the complexity data and the small change in the ΔT_m of renatured duplexes, the concept of a "micro-heterogeneity" has arisen. The exact relationship between the postulated 13 separate classes of minicircles for C. luciliae, the observed four size classes of open minicircles, and the extensive "microheterogeneity" has not yet been resolved.

Another type of heterogeneity in K-DNA was evidenced by the discovery of Steinert and Van Assel (7) of a minor class of 12 μm "maxicircles" associated with K-DNA networks of C. luciliae. We had previously shown that long DNA molecules were associated with purified K-DNA of L. tarentolae and could be released by DNase II digestion (1). We also demonstrated that these long molecules could be seen in highly purified covalently closed L. tarentolae networks and were therefore not nuclear DNA and were probably closed circular in situ (12). Kleisen et al. (13) have recently succeeded in releasing linear DNA fragments with a maximum molecular weight of 26×10^6 daltons from purified networks of C. luciliae by treatment with S1 nuclease. They also found that this non minicircular species

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could be recovered as a unique class of linear fragments after digestion with any of several different restriction enzymes. They identified this species with the 12 μm "maxi circles" of Steinert and Van Assel (7). However, it is still unclear whether all large circular K-DNA molecules are homogeneous in base sequence. It is possible that some circular molecules represent tandom repeats of minicircles.

In this paper we wish to report several observations on the K-DNA of L. tarentolae.

RESULTS

Gel electrophoresis of purified K-DNA minicircles of L. tarentolae: Closed monomeric minicircles were isolated from sonicated closed networks by a method involving an ethidium bromide CsCl gradient followed by neutral sucrose sedimentation of the lower band. Closed monomers exhibited a multimeric banding pattern in acrylamide-agarose gels. The pattern is heterogeneous and cannot be entirely accounted for by the separation of circles differing in superhelical density (14, 15, 16). Single nicked open monomers (17) ran as a single major band with possibly two minor bands. The major band corresponded to a minor band in the pattern of closed monomers.

We speculate that the heterogeneous pattern of closed monomers is due to a combination of superhelical density differences and small length differences. The minor bands seen in the gel of open monomers may represent minor size classes of monomers.

Renaturation kinetics: In vivo ^{3}H-labeled closed monomers were sonicated to 270 nucleotide fragments which were denatured and annealed at 60°C in 0.12 M Na phosphate buffer. The annealing was measured by hydroxyapetite binding. The Cot curve showed a good single component fit with a complexity of 0.76 X the minicircle molecular weight. However, when the last 15% was selected by Cot fractionation, the DNA renatured at a slower rate (12.7 X the minicircle molecular weight) which corresponded well with the rate shown by the "high Cot component" of total sonicated network DNA. Thermal chromatography of the reannealed "high Cot K-DNA" yielded a broad multiphasic melt from 70°-88°C, which differed strikingly from the monophasic melt of unfractionated network fragments. The low melting temperature of the reannealed "high Cot component" could be due either to the presence of mismatched sequences or to a higher mole fraction of A and T in the DNA. We have no evidence yet to distinguish between these possibilities.

Density evidence for a high AT component in network DNA: We previously reported that the density of purified K-DNA networks was 1.703 g/μ (1), whereas the density of purified monomeric minicircles was 1.705 g/cc (4). These experiments were recently repeated with similar results, and the small difference in

buoyant densities was confirmed by a mixing experiment. The method of isolation of the monomeric minicircles had no effect on the buoyant density obtained.

The increase in buoyant density of the isolated minicircle may imply the presence of a less dense or higher AT component in the network DNA.

Restriction of L. tarentolae K-DNA: Closed K-DNA networks and closed monomeric minicircles were digested extensively with 10 different restriction endonucleases (Eco RI, Eco RII, Hind II, Hind III, Hpa I, Hpa II, Bam HI, Hae II, Hae III, Eco RI*) and the digests separated by electrophoresis on acrylamide gels. We found the same three basic phenomena reported by Kleisen et al. (10, 11, 13):

1. Certain enzymes only digested a portion of the network DNA or the minicircles (Eco RI, Hind II, Hind III, Hae II, Bam HI, Hpa I).
2. Other enzymes produced complete fragmentation of networks and minicircles, but the gel patterns were clearly heterogeneous in that the bands were not in molar yields and the sum of the molecular weights of the bands was greater than the minicircle molecular weight (Hpa II, Hae III).
3. Several high molecular weight bands, composed of linear DNA as shown by electron microscopy, were seen in network digests and were absent in minicircle digests (Hpa II, Hae III).

The fraction of closed networks and closed monomers resistant to digestion with each enzyme was measured by ethidium bromide CsCl equilibrium banding of digested ^{32}P-labeled DNA. The relative amount of DNA in each band was measured by autoradiography of dried gels. It is clear that there are major bands and minor bands with no relationship between band intensity and molecular weight. It is also interesting that there are bands with mobilities implying molecular weights greater than unit minicircle size. Some of these bands were shown to consist of linear DNA molecules, but others may possibly be resistant catenanes or cleavage products of fused dimers or fused oligomers.

CONCLUSIONS

We consider several possible explanations for our minicircle results and those discussed in the Introduction.

1. There is a minor class or several minor classes of minicircles which differ in base sequence from the majority of minicircles in the network.
2. All minicircles possess identical base sequences, but a portion of the minicircles possess blocked or modified restriction sites, giving rise to "partials" which are resistant to further digestion.
3. All minicircles share intramolecular repetitious sequences in addition to regions of sequence heterogeneity.

These hypotheses need not be mutually exclusive. Hypothesis 3 is attractive because the presence of internal repetitious sequences would increase the

probability of recombination between adjacent minicircles, as has been reported by Manning and Wolstenholme (18) for C. acanthocephali.

The existence of several distinct length classes of minicircles implies an inter-molecular heterogeneity, but in no case has an exact correlation between minicircle size and sequence complexity been established. As suggested by Kleisen et al. (10), the observed length heterogeneity and sequence heterogeneity could be due to frequent insertions and deletions. One possible mechanism for the introduction and maintenance of sequence diversity into a homogeneous population of molecules is unequal crossing-over (19) combined with the accumulation of point mutations.

The additional non minicircular bands that we have seen with restricted network DNA confirm the results of Kleisen et al. (13) and most likely arise from the "high Cot" component of the network DNA. However, this must be shown directly by isolation and characterization of this DNA.

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