C. RESULTS
C.1. Studies on replication of rDNA

C.1.1. Two dimensional gel electrophoresis

C.1.1.1. Interpretation of data

The two-dimensional (2-D) gel electrophoresis method (Brewer and Fangman, 1987) is widely used to separate branched DNA molecules, such as replication intermediates from linear DNA. Details of the methodology is discussed in section B.13. Replication of the various model plasmids has been studied by this method, and the hybridization patterns characteristic of defined nonlinear replication intermediates (e.g., Y forms, replication bubbles, asymmetric double -Y forms, etc.) have been established (Brewer et al., 1988). Fig. 6 shows the basic types of replication fork movement through a DNA fragment. Upon 2-D gel electrophoresis the linear molecules migrate along a diagonal arc while the replicating intermediates are retarded in their migration and give the characteristic patterns as indicated.

C.1.1.2. Methodological Problems

Though 2-D gel electrophoresis is a very standard technique these days, it took a long time to standardize the method for *E. histolytica*. DNA preparations from this organism are generally contaminated with a lot of carbohydrates which interfere with restriction enzyme digestion. In addition, it is difficult to grow up large batches of cells and DNA yields are poor. Several methods were tried to get good quality of DNA for successful completion of 2-D gel electrophoresis. Both the methods (for isolating circular DNA molecules and total genomic DNA from *E. histolytica*) have been described in section B.7. Special care was taken to get genomic DNA with well-preserved replicating intermediates (e.g., avoiding pipetting in and out too many times which causes mechanical shearing of DNA).

C.1.2. 2-D gel analysis of circular rDNA molecules

C.1.2.1. 2-D gel analysis of rDNA plasmid present in *E. histolytica* strain HM-1:IMSS (EhR1)

It has been reported earlier that the rRNA genes in *E. histolytica* reside on a nuclear plasmid which, in strain HM-1:IMSS, is a 24.5 kb circle and exists in about 200 copies per genome. There is no evidence of any chromosomal copies of these genes. The rDNA plasmid, named EhR1 (Fig. 2), has been completely sequenced (Sehgal et al., 1994). Organization of nucleotide sequences in EhR1 has already been described in section A.9. The relative positions of different restriction enzymes which have been used for the present study and also for subsequent studies are shown in Fig. 8.
Fig. 8. The rDNA plasmid of *E. histolytica* HM-1:IMSS. Salient features of this plasmid have already been described in Fig. 2. In this figure different enzyme sites used for 2-D gel analysis and EM studies have been shown. To avoid overcrowding, all recognition sites of *Hinf*I, *Sca*I and *EcoRI* have not been marked. Only those relevant to subsequent experiments are shown. Broad arrows show the two rDNA inverted repeats. Various repeats upstream and downstream to the rDNA units are also shown. The number next to each enzyme site refers to the nucleotide position at which the enzyme acts with reference to the 12 o'clock *EcoRI* site. Abbreviations for enzyme sites: B, *Bsa*II; E, *EcoRI*; H, *HindIII*; Hf, *Hinf*I; S, *Sca*I; Sc, *Sacl*II and X, *Xho*I. Abbreviations for repeats: DrRp, *Dra*I repeats; Srp, *Sca*I repeats; PvRp, *Pvul* I repeats; HfRp, *Hinf*I repeats.
Fig. 9. Two-dimensional gel electrophoresis of replication intermediates in EhR1. (a) A linear map of the 24.5 kb plasmid cut at the 12 o'clock EcoRI site (as shown in fig. 8) is shown. Abbreviations for enzyme sites: E, EcoRI; H, HindIII; Hf, HinfI; S, ScaI; X, XhoI. The DNA fragments analyzed by two-dimensional gel electrophoresis are shown as filled bars below the line. The restriction enzyme(s) used to generate each fragment and the fragment size (in kb) are indicated below each bar. The various DNA probes used are shown as hatched bars above the line. The corresponding restriction fragments were cloned in the plasmid vector pTZ18R, and the inserts, purified from gel slices after electrophoresis, were radiolabelled. (b) Autoradiograms of Southern blots of individual DNA fragments (panel letters correspond with fragment letters in panel a). E. histolytica DNA was digested with the indicated enzymes to generate fragments of interest. These were separated by two-dimensional gel electrophoresis as described in the section B 13. Southern blots were hybridized with DNA probes specific for each fragment as shown in panel a. Arrows indicate the position of bubble arcs.
Total E. histolytica genomic DNA enriched for the rDNA plasmid EhR1 was digested with Scal, XhoI, HindIII and HintI to generate fragments in the 4 to 5 kb range, and representing the entire circle (Fig. 8). The exact size of each fragment, the enzyme(s) used to generate the fragment, and the fragment's location on EhR1 are shown in Fig. 9a (filled bars). Restriction enzyme-digested DNA was subjected to 2-D gel electrophoresis and blotted on nylon membranes. The various DNA probes used for hybridization are shown as hatched bars in Fig. 9a, and the hybridization patterns obtained are shown in panels A-F (Fig. 9b). All probes are specific to the DNA fragments being studied, as confirmed from earlier Southern blot data and from the nucleotide sequence of the plasmid (Sehgal et al., 1994). Probe 2 hybridizes with both fragments S3.3 and S4.28 (Fig. 9b, panel B) since it contains the small-subunit rRNA sequence present in both fragments. To look at the pattern in fragment S4.28 alone, probe 5 was used (Fig. 9b, panel E). This probe was generated from a 5.9 kb EcoRI fragment containing part of the leftward rDNA (as shown in Fig. 2) and the upstream spacer up to the 12-o'clock EcoRI site. The rRNA coding sequences were deleted from this fragment by exonuclease III digestion. The upstream spacer region was contained in fragments A and F, and the downstream spacer was contained in fragments D, D1, and D2. The pattern of replication intermediates from each of these fragments after 2-D gel electrophoresis showed that all fragments gave signals expected for replication bubbles and simple Ys. Some of them (A, C, E, and F) also had triangular smears of X-shaped molecules. Although the intensity of bubble arcs was sometimes low, they were nevertheless detectable in all fragments; showing that replication of this plasmid could initiate from any of the restriction fragments tested. From numerous replicates of these data, it is apparent that the relative intensity of the bubble arcs was consistently greater in fragments C and D1-D2 (Fig. 9b), which contain the rDNA transcription unit, than in fragments spanning the downstream and upstream spacers. For example, as shown in Fig. 9b the fork arcs in panels A, C, and E are of comparable intensities, but the bubble arc is much stronger in panel C. Assuming that there is no preferential breakage of bubble structures in spacer regions during DNA isolation, these data suggest that replication initiated more frequently from within the rDNA transcription units. However, the bubble signals in spacer fragments are not trivial, and initiation takes place in these regions also. The complete and uniformly labeled Y arcs in all fragments show that each fragment is traversed by replication forks originating elsewhere. The triangular smear of X-shaped molecules found in multiple fragments shows that replication may terminate at many locations in this plasmid.
The 4.28 kb *ScaI* fragment containing part of the rDNA and upstream sequence of the leftward rDNA repeat (Fig. 9b, panels Band E) shows a prominent pattern emanating from molecules that are more than half replicated and shoots vertically upwards to meet the diagonal of X-shaped molecules. This could be a consequence of branch migration, but the origin and the significance of this pattern are not apparent at this moment. It could be worth noting that this pattern coincides with the 3' end of the Tr unit. (Fig. 2).

C.1.2.2. Two-dimensional gel analysis of rDNA plasmid from strain HK-9

To show that multiple replication initiations in the *E. histolytica* rDNA plasmid were not unique to the HM1:IMSS strain alone, we repeated the experiments described above with the HK-9 strain, in which the rDNA plasmid has a slightly different architecture (Sehgal et al., 1994). Each 15-kb rDNA plasmid in this strain contains only one transcription unit (Fig. 10a). Compared with the HM-1:IMSS plasmid, the single rDNA unit in the HK-9 plasmid is oriented like the rightward unit in EhR1 (Fig. 2). The sequences upstream of rDNA in HK-9, upto the 12 o'clock *EcoRI* site, are exactly identical in the two plasmids (Sehgal et al., 1994). The sequence upstream of the leftward unit in EhR1 upto the 12 o'clock *EcoRI* site (containing Tr and *PvuI* repeats [Fig. 2]) is missing in the HK-9 plasmid. The families of *DraI* and *ScaI* repeats found in the downstream intergenic spacer of EhR1 are present in the same location in the HK-9 plasmid also. The HK-9 plasmid was digested with *EcoRI* and *HindIII* (Fig. 10a, fragments A and D) and *ScaI* and *HindIII* (Fig. 10a, fragments B and C) to generate four fragments appropriate for 2-D gel analysis. The hybridization probes used were the same as those described for Fig. 9a. Probes 1, 2, 3, and 4 were used for fragments A, B, C, and D respectively. The results of the 2-D gel analysis are shown in Fig. 10b. All fragments gave signals indicative of simple Ys and bubble arcs. The bubble arcs in panels A and C are of comparable intensities. The same blot was used for panels B and C by reprobing with two different probes. As is evident, all signals in panel B are fainter than the corresponding ones in panel C. Therefore, the relative intensity of the bubble arc in fragment B is not much weaker than that in fragment C. However, the bubble signal in fragment D was very faint, indicating that replication initiation in this region was very infrequent. A similar result was obtained with the analogous region in EhR1 (Fig. 9b, panel D). Thus the mechanism of replication of the two plasmids appears to be similar, with initiation sites spread in large segments of the molecule.
Fig. 10. Two-dimensional gel electrophoretic analysis of rDNA plasmid from strain HK-9.
(a) The orientation of the single rDNA transcription unit and locations of Dral repeats (DrRp), ScaI repeats (SrRp), HindIII repeats (HfRp) and nucleotide positions of some restriction enzyme sites are indicated. Abbreviations for restriction enzymes: E, EcoRI; H, HindIII; and S, ScaI. The fragments analyzed were generated by digestion with EcoRI and HindIII (for A and D), and ScaI and HindIII (for B and C). (b) Autoradiograms of Southern blots of fragments (panel letters correspond with fragment letters in panel a). The DNA probes used were those described in the legend of Fig. 9 (probes 1,2,3 and 4 for A, B, C and D respectively). Arrows indicate bubble arcs.
C.1.3. Electron microscopic observation of replicating molecules
C.1.3.1. Electron microscopic studies on EhR1
C.1.3.1.1. Direct observation of replicating EhR1 molecules
In strain HM-1:IMSS, the predominant circular molecule is the 24.5-kb rDNA circle (Dhar et al., 1995). Molecules of this size were viewed with an electron microscope (Fig. 11). Of more than 1200 molecules studied, about 5.6% showed "eye" structures in which both arms were double stranded and of equal size. Short single-stranded regions at fork junctions were also visible in many of them. On the basis of these criteria, such structures are considered to be replication bubbles, in contrast with bubbles containing both single-stranded arms, which were encountered occasionally. The latter could arise because of partial denaturation and were excluded from analysis.
Large single-stranded DNA stretches as seen in replicating metazoan chromosomes were not observed. Of the replicating molecules examined, a large proportion (64%) contained a single bubble. However, a significant number of molecules contained two bubbles (14%), three bubbles (16%), and four or more bubbles (6%). The observed size of bubbles varied from 200 bp to 7.0 kb. Thus both microbubbles (<900 bp) and macrobubbles (>900 bp) were observed. The relative abundance of bubbles of various sizes is shown in Fig. 12. Large bubbles were not seen with the frequency expected on the basis of the intensity of bubble arcs in 2-D gels. This was most likely due to the extensive purification of DNA which was found to be necessary to get proper spreading of DNA for electron microscopy. Large bubbles were probably preferentially lost in this process. In molecules with multiple bubbles, the interbubble distance varied from 1.8 to 8.7 kb. In no molecule did bubbles occupy more than half the length of the plasmid, although their actual locations may vary from molecule to molecule.

Direct observation of double-stranded bubbles in circular molecules clearly indicates that these molecules are self-replicating.

C.1.3.1.2. Mapping the location of replication bubbles in EhR1
To map the replication origins in individual molecules, the plasmid was linearised with PvuI or BsaHI (which cut the molecule at a single cluster of sites or just once, respectively). The plasmid was also digested with SacII, which cuts twice to yield two fragments of 10.3 and 14.1 kb. Replication bubbles were visualized under an electron microscope in linear DNA fragments of expected sizes generated by each enzyme. Since restriction enzyme-digested DNA had to be further purified by several phenol-chloroform extractions before it could be spread for electron microscopy, linearized molecules showed fewer larger bubbles and multiple bubbles on the same molecule.
Fig. 11. Electron microscopic analysis of replicating EhR1 molecules. Circular DNA was enriched by passing through a Qiagen column as described in the section B.7.2.2. Micrographs of replicating circular molecules (24.5 kb) with one (A), two (B), three (C) and five bubbles (D) are shown. Arrows point towards the middle of each replicating bubble.
Fig. 12. Size distribution of replicating bubbles in EhR1. The contour lengths of 67 bubbles were measured and plotted to show the relative abundance of each size class.
Fig. 13. Electron microscopic mapping of replication bubbles on linearized EhR1 molecules. A linear map of EhR1 oriented clockwise from the 12 o'clock EcoRI (E) site (relative to the map in Fig. 2) is shown on the top line. Arrows above the line show the location of the two rDNA units. The scale below the line is in kilobases. The restriction enzyme sites used to linearize the plasmid are shown below the line: B, BsaHI; S, SacII; and P, PvuI. Data from some of the molecules studied with each enzyme are shown (a total of 35 molecules). The location and the size of the bubble(s) in each molecule are plotted in both possible orientations from the cut end (filled and open bars). Molecules 1 to 10 (numbers are on the right) correspond to the 14.1 kb SacII fragment which contains both the transcription units. Molecules 11 to 20 show the bubbles in the 10.3 kb SacII fragment which contains the upstream spacer region. Molecules 21 to 30 and 31 to 35 were linearized with BsaHI and PvuI respectively. Molecule 23 has two bubbles.
than did intact circles. The location of each bubble was plotted relative to the 12 o'clock EcoRI site. Since it is not possible to orient the molecules, we considered both possible locations of the replication bubble(s) in each molecule (Fig. 13). Large and small bubbles were distributed evenly, with no preferential clustering. The 14.1 kb SacII fragment contains rRNA transcription units, which are exactly identical, at either end. Replication bubbles in the transcription units in this fragment can therefore be unambiguously mapped (Fig. 13, molecules 1 to 10). In addition, the 10.3 kb SacII fragment contains the upstream spacer region almost exclusively. Any bubbles in this fragment are again unambiguously mapped as not lying in the transcription units (Fig. 13, molecules 11 to 20). The total number of bubbles seen in the 14.1 kb SacII fragment was 20 as opposed to 13 seen in the 10.3 kb fragment. Thus, although the frequency of initiation is greater in the transcription units, a large number of initiations take place in the spacer also. By using three different enzymes to linearize the plasmid, hot spots of replication initiation, if any, should become apparent. However, no such hot spots could be detected, except that replication bubbles appeared somewhat more frequently in the rRNA transcription units. The statistical analysis of these data indicated a very large standard deviation, again pointing to the absence of preferred locations of replication bubbles. The data from all three enzymes taken together show that initiation took place twice as frequently in 12 kb of rRNA transcription units as it did in the 12 kb of intergenic spacers (9 kb of upstream spacer and 3 kb of downstream spacer). This is in good agreement with the data from 2-D gel electrophoresis, in which bubble arcs were more prominent in the fragments spanning the rDNA than in the spacers.

**C.1.3.2. Electron microscopic analysis of the HK-9 plasmid**

Replication bubbles in the HK-9 plasmid were also visualized and mapped by electron microscopy, essentially as described for EhR1 (Fig. 14). The plasmid was linearized with BsaHI and SacII, both of which have unique sites in the molecule (unlike EhR1, which is cut twice by SacII). A total of 26 replicating molecules were analyzed. The size range of the bubbles in the HK-9 plasmid was the same as that for EhR1. However, larger bubbles were seen more frequently, probably because better DNA preparations were consistently obtained with the HK-9 strain (Fig. 15). On plotting the location of the bubbles in linearized molecules, no hot spots of initiation were evident (Fig. 16), nor were larger bubbles found in preferential locations. Thus, the data obtained for the HK-9 plasmid were essentially the same as those for EhR1.
Fig. 14. Electron microscopic analysis of replicating HK-9 plasmid. The DNA was purified through Qiagen column as described in the section B 7.2.2. Micrographs of bubbles in an intact 15-kb plasmid (A) and plasmids linearized with *SacII* (B to D) are shown. Arrows point to the bubble junctions. The molecule in panel D has Y's at the two ends.
Fig. 15. Size distribution of replication bubbles in HK-9. The contour lengths of 26 bubbles were measured and plotted to show the relative abundance of each size class.
Fig. 16. Electron microscopic mapping of replication bubbles in the HK-9 plasmid. A linear map of the plasmid oriented clockwise from the 12 o'clock EcoRI (E) site is shown on the top line. The plasmid was linearized with BsaHI (B) and SacII (S). The bubbles are depicted with respect to the 12 o'clock EcoRI site. The location and the size of the bubble(s) in each molecule are plotted in both possible orientations from the cut end (filled and open bars). Unlike in EhR1, SacII cuts the Hk-9 plasmid only once. Molecules 1 to 10 and 11 to 25 were linearized with SacII and BsaHI, respectively.
C.2. Transfection studies in *E. histolytica*

C.2.1. rDNA fragments stabilize a foreign plasmid in *E. histolytica*

C.2.1.1. Stable Transfection of *E. histolytica* cells by foreign plasmids pTCV1-4

The plasmid vector pTCV1 has been constructed in Dr. William Petri's lab (Vines et al., 1995) to serve as a shuttle vector between *E. coli* and *E. histolytica*. This plasmid is 7247-bp in size (Fig. 7). It has a neomycin resistance gene ([BamHI-SalI fragment](#)) under the control of amebic heavy subunit of galactose-binding lectin gene (*hgl*) flanking sequences. It contains 1 kb of 5' flanking region from *hgl5* as upstream regulatory element and 2.3 kb of 3' flanking region from *hgl1* as downstream regulatory element. Neomycin resistance is used as selectable marker in *E. histolytica* cells transformed with pTCV1. The plasmid has the *amp* gene which is used for selection in *E. coli*. Conditions for successful transfection of *E. histolytica* and selection for pTCV1 transformants have been standardized (Vines et al., 1995). rDNA episomal fragments from EhR1 were cloned in the unique [HindIII](#) site of pTCV1 (Fig. 7) to generate constructs pTCV2 to pTCV5 (discussed in section B.15).

DNA from various constructs was transfected into *E. histolytica* by electroporation as described in materials and methods (section B.16). Stable transfectants were obtained with all the constructs (pTCV2 to pTCV4) except pTCV5. The failure to obtain stable transfectants with pTCV5 was not due to technical reasons because parallel experiments with the other DNAs were repeatedly successful. It may, in fact, be due to an inherent property of the HMg insert in pTCV5. Transfected cells were maintained in medium containing neomycin (24 μg/ml). After one week of selection in this medium, cells were transferred to neomycin-free medium.

C.2.1.2. Plasmids pTCV2-5 are maintained as episomes in transfected cells

To determine whether after transfection all the plasmid constructs are being maintained as episomes, genomic DNA was isolated from transfected cells and electrophoresed in a 0.7% agarose gel. DNA samples digested with [Sacl](#) (which cuts the plasmid pTCV1 once) were also electrophoresed in the same gel (The inserts in constructs pTCV3 and pTCV4 have an additional [Sacl](#) site which is carried over from the multiple cloning site of pTZ18R, the plasmid in which these inserts originally resided). The gel was blotted and hybridized with α-[32P]dATP labeled [BamHI-SalI](#) neomycin resistant gene fragment of pTCV1 (Fig. 7). In each undigested sample (Fig. 17, lanes 1-8), a major band corresponding to supercoiled DNA and several slower migrating bands were seen. These may correspond to the open circle and multimeric forms of the plasmids. All the digested samples (lanes 9-16), rise to single bands, the
Fig. 17. Southern blot analysis of plasmid DNA from transfected cells. Genomic DNA from different transfectants (pTCV1-4) was isolated and 1 µg of each DNA sample was digested using 10 units of SacI which linearizes the plasmids (pTCV1 and pTCV2), or cuts them twice (pTCV3 and pTCV4). Digested samples and a set of untreated DNA samples were electrophoresed in a 0.7% agarose gel at 1V/cm for 16 hours. Gel was blotted and hybridized with neo (BamHI-SalI fragment of pTCV1) probe (Fig.7). Lanes 1-8, undigested samples from cells grown without selection pressure (odd numbers) and with selection pressure (even numbers). Lanes 1,2-pTCV2; 3,4-pTCV3; 5,6-pTCV4 and 7,8-pTCV1. SacI digested samples were loaded in lanes 9-16 (same order as in lanes 1-8). λ/HindIII marker is shown on the left (in kb).
sizes of which matched with those expected for the respective linearized monomeric plasmids. This showed that the transfected DNAs were maintained as independent circular molecules without any detectable rearrangements. (The other part of this experiment namely the relative amounts of each plasmid in cells grown with and without selection, is discussed below.)

C.2.1.3. The copy number of transfected plasmids pTCV1-4 is affected by the nature of rDNA insert

A dot blot experiment was carried out with total genomic DNA from cells transfected with the various plasmids (Fig. 18). Indicated amounts (500 ng, 200 ng, 100 ng, and 10 ng) of DNA from each transfectant was spotted on Hybond N+ membrane. Various amounts (2 ng, 1 ng, 500 pg, 200 pg, 100 pg, and 50 pg) of purified pTCV1 DNA was also spotted as a single-copy control. The blot was hybridized with BamHI-SalI neomycin resistance gene fragment of pTCV1 (Fig 7). This fragment is specific for the transfected DNA and does not hybridize with any E. histolytica sequences. The radioactivity of each spot was quantified by liquid scintillation counting. Values were plotted against amount of DNA spotted, and those falling in the linear range were used to calculate the copy number (Dame and McCutchan, 1983). The total genome size of E. histolytica was taken to be 1.5x10^4 kb (Dvorak et al., 1995). The copy number thus determined for each construct is shown on the right. In the presence of selection pressure ('+' lanes), the copy number of DNA in pTCV2 and pTCV3 was consistently greater than pTCV1 while that in pTCV4 was somewhat lower than the parent plasmid. When cells were grown out of selection for 6 weeks ('-' lanes), copy number declined in all cases. However, compared with pTCV1, pTCV2 and pTCV3 were present in more copies. The difference was especially significant in pTCV2. This result indicates that in the absence of selection pressure, the rDNA fragments in pTCV2 and pTCV3 facilitate the retention of the transfected DNA. Interestingly, the rDNA fragment in pTCV4 had a negative effect on copy number of transfected plasmid.

C.2.2. Two-dimensional gel analysis of the transfected plasmids

The four different rDNA episomal fragments cloned in constructs pTCV2 to 5 affected the outcome of transfection in markedly different ways. To determine any correlation of this result with replication efficiency of the cloned episomal fragments, replication intermediates of the various constructs were studied by 2-D neutral/neutral agarose gel electrophoresis (Fig. 19). The parent plasmid pTCV1 showed distinct bubble signal in the fragment containing amoebic DNA sequences ('a' in bottom panel
Fig. 18. Dot blot analysis and copy number estimation of different transfected constructs (pTCV1-4). Left panel shows the different constructs used for the dot blot experiment. (-) indicates in the absence of selection pressure and (+) indicates in the presence of selection pressure. Various indicated amounts of DNA from each transfectant were spotted on Hybond membrane. The blot was hybridized with neo probe (BamHI-SalI fragment from pTCV1) (Fig. 7). pTCV1 control DNA was also blotted at different concentration indicated in the figure. 'ND' signifies no DNA was blotted. After hybridization and washing, each spot was cut out and quantified using a liquid scintillation counter. Copy number of each transfectant was determined by following the method described by Dame and McCutchan, 1983. Right panel shows the copy numbers of each construct. No significant signal was detected in pTCV4 (-).
Fig. 19. 2-D gel analysis of transfected plasmids (pTCV1-3). DNA was isolated by modified Hirt fractionation protocol (section B 7.2.1) and subsequently enriched for replication intermediates using BND cellulose. After digestion with PvuII, DNA was electrophoresed in the 1st dimension in a 0.45% agarose gel at 1V/cm for 36 hours. Electrophoresis in the 2nd dimension was carried out at 5V/cm for 12 hours (1% agarose gel). The gel was blotted on a Hybond N+ membrane and Southern blots were hybridized with the probes indicated below. The top panel shows a map of pTCV1 (and its derivatives pTCV2 and pTCV3) giving the location of PvuII sites. The rDNA fragments do not have a PvuII site. The middle panel shows a linearized map of the rDNA episome (EhR1) cut at 12 o'clock EcoRI site. The region marked 'c' is the DNA fragment cloned in pTCV2 and includes the entire transcription unit. The region marked 'd' is cloned in pTCV3 and includes the region downstream of the rDNA units. The bottom panel shows autoradiograms of 2-D gel analysis. 

(a) PvuII-digested pTCV1 hybridized with probe containing PvuII-SalI part of neo gene. (b) same as (a) hybridized with probe containing 'Amp' gene. (c) PvuII-digested pTCV2 hybridized with BamHI-PvuII fragment of neo gene to locate the rDNA containing fragment. (d) PvuII-digested pTCV3 hybridized with the probe used in (c).
in Fig. 19), while no bubble signal was detectable in the bacterial sequences ('b' in bottom panel in Fig. 19). The rDNA episomal fragments in pTCV2 and 3 were screened for bubble signals. None could be seen in pTCV2 ('c' in bottom panel in Fig. 19) but a clear bubble pattern was seen in pTCV3 ('d' in bottom panel in Fig. 19). The absence of bubble signal in the rDNA insert of pTCV2 is unexpected in view of the observation that pTCV2 was the most stable plasmid in absence of selection pressure (Fig. 18). It is possible that a bubble signal, in fact, exists but was not detectable in this particular DNA preparation. The experiment would have to be repeated a few times to ensure the validity of the data. However, if a bubble signal truly does not exist, it shows that the ability of pTCV2 to retain the plasmid in absence of selection may not be due to more frequent replication initiation occurring in the rDNA fragment. Rather, the effect may be exerted at the level of copy number control mechanisms or improved partitioning of the plasmid to daughter cells. In contrast, the rDNA fragments in pTCV4 and pTCV5 may prevent plasmid partitioning, thereby resulting in unsuccessful transfection (pTCV5) or rapid loss of plasmid in the absence of selection pressure (pTCV4).

C.3. Replication in cells synchronised by serum-starvation

C.3.1. Establishment of serum-starvation as a method to synchronize amoeba cells

So far no reliable method has been reported to synchronize amoeba cells. Since amoeba needs bovine serum as a component of growth medium, serum deprivation may work as a tool to synchronize these cells. In many other systems (mammalian cell culture), serum deprivation has been used as a means for cell synchronization (Krek and DeCaprio, 1995). Generally, mid-log phase mammalian cell lines are incubated in a growth medium supplemented with a very low serum concentration (0.5%) to bring almost all the cells at the boundary of G1/S in the cell cycle. Cells are then put back into medium containing the normal serum concentration and as a result 70-80% of the total cell population has been reported to be synchronized. This is a routine method to synchronize mammalian cell lines. The same was tried to synchronize amoeba cells. The status of the cells during starvation was observed under microscope using following parameters: (a) shape of the cells, (b) motility, and (c) number of cells.

Mid log-phase amoeba cells were allowed to incubate in medium containing 0.5% serum for several hours and cells were monitored after regular time intervals (2 hr, 4 hr and so on) to find out optimum duration of serum deprivation for synchronization. During serum starvation, cells did not show marked morphological changes till the first 12 hours. Subsequently, cells started getting rounded up and they
became less motile. After 16 hours of serum deprivation, when cells were put back into normal medium, they reverted to normal growth very rapidly (within 30-45 minutes). Whereas, when cells were serum-starved for 24 hours and then put back into normal medium, they took 2-3 hours to revert to their normal morphology. We reasoned that if cells regained normalcy very soon after serum replenishment, it was an indication that all cellular processes had not come to a halt and synchronization may not be achieved. We therefore chose to serum-starve the cells for 24 hours in subsequent experiments.

C.3.2. Tritiated thymidine incorporation in serum-starved cells
Tritiated thymidine incorporation was carried out to estimate the extent of synchronization after serum starvation. Cells undergoing serum-starvation for 24 hours were put back into normal medium, and after each hour they were given a pulse of 30 minutes in the presence of tritiated thymidine. Cells were TCA precipitated and filtered through GF-C filters (as described in B.18). TCA precipitable counts at each time point were plotted (Fig. 20). Till the first 2 hours there was little or no thymidine uptake. Beyond 2 hours the counts increased, reached a peak at 6 hours and then came down at 8 hours. Again at 16 hours there was a peak of incorporation but beyond that the synchrony was lost. Tritiated thymidine uptake studies were not extended beyond 24 hours of serum starvation. This result shows that by serum deprivation, cell synchronization has been achieved to a large extent, with first S phase being at 6 hours and the second around 16 hours.

C.3.3. Determination of copy number of rDNA circles during serum starvation
Total genomic DNA was isolated from cells undergoing serum-starvation for different time periods (from zero hr to 24 hrs). A dot blot experiment was carried out to find out whether there is any change in rDNA copy number during starvation. Indicated amounts of DNA from each time point was spotted on Gene Screen Plus nylon membrane. DNA from normal cells was also spotted as control. Various amounts of purified DNA of a plasmid construct with the EhR1 rDNA fragment cloned into it were also spotted as control for single copy DNA. This DNA was used as the radioactive probe. The blot was hybridized with it and the results obtained are shown in Fig. 21. Each spot of the autoradiogram was quantified with the help of a gel documentation system using Advanced DNA STAR Programme. When the average values of various time points for a particular concentration of DNA were compared, no drastic differences were seen. This shows that the rDNA copy number does not
Fig. 20. Tritiated Thymidine uptake studies in serum-starved cells. Mid log phase trophozoites were serum-starved (as described in section B.17) for 24 hours followed by replenishment with normal medium (zero hour) and incubation for further 24 hours. Cells were given a pulse for 30 minutes in the presence of 5 μCi/ml tritiated thymidine at different time intervals following serum addition. Cell suspension was then TCA precipitated and filtered through GF-C filters. Thymidine incorporation was determined by using a liquid scintillation counter and the values were plotted.
Fig. 21. Dot blot analysis of DNA samples isolated from amoeba cells at different time points during serum-starvation. Mid-log phase trophozoites were serum-starved (0.5% serum) for 24 hours. DNA was isolated at different time points from cells undergoing serum-starvation. Various amounts of DNA from each time point were spotted in duplicate (i and ii) on Gene Screen Plus membrane. DNA from normal cells and different dilutions of the probe DNA were also spotted. The blot was hybridized with rDNA probe (\textit{Hind}III rDNA fragment from EhR1) and on subsequent autoradiography the intensity of each spot was quantified using advanced DNA STAR programme. DNA was loaded in the following order: A, normal cells; B to E: cells serum-starved for, B, 8 hrs; C, 16 hrs; D, 20 hrs; and E, 24 hours. F, probe DNA. Amount of DNA samples loaded in each row (A-E) were: 1, 20 ng; 2, 10 ng; 3, 5 ng; 4, 2 ng and 5, 1 ng. Amount of probe DNA loaded in F was: 2 ng, 1 ng, 500 pg, 200 pg and 100 pg.
vary during serum starvation. Thus the rDNA copies are stable entities and not subject to fluctuations due to perturbations in growth conditions.

C.3.4. Replication of rDNA in serum-starved cells
C.3.4.1. Electron microscopic analysis of replicating rDNA molecules
C.3.4.1.1. Frequency of replication initiation events in serum-starved cells and cells restored to normal serum
The replication status of rDNA molecules in serum-starved cells and after serum replacement was studied by electron microscopy. DNA was isolated from cells which had undergone 24 hours serum starvation followed by incubation in normal medium for indicated time periods (1 hour, 2 hours and 4 hours). Over 200 rDNA circles were examined in each case and the number of molecules with replication bubbles was recorded (Fig. 22). In exponentially-growing cells, replicating molecules were 4% of total rDNA circles. After 24 hours serum starvation ('0 hour' sample) only 1% molecules showed "eye" structure whereas after giving back serum, '1 hour' sample showed 28% molecules having "eye" structure. Likewise '2 hour' and '4 hour' sample showed 15% and 10% molecules having "eye" structure respectively. This result shows that serum deprivation blocks the reinitiation of rDNA replication and upon restoring serum, a wave of new initiations ensues. The lag period of reinitiation in normal medium is less than one hour. The average bubble size in these molecules is 1.5 to 2.0 kb. It seems that not all the rDNA circles initiate replication at the same time as there was no progressive increment of the bubble size with time. However, this could also be an experimental artifact since we do not find too many molecules with large bubbles by electron microscopy, even in normal cells.

C.3.4.1.2. Mapping of replication initiation sites in rDNA after serum starvation
Our main interest in synchronization through serum starvation was to catch the earliest initiation events. If there is any "hot spot" of initiation it should gain prominence under these conditions. DNA was isolated from cells growing in normal serum for 1 hr, 2 hr and 4 hr (after 24 hours serum deprivation) and digested with SacII which cuts the rDNA circle twice, giving two fragments (14.1 kb and 10.3 kb; see Fig. 8 for restriction map). Purified samples were then spread for observation under electron microscope. 30 to 40 replicating molecules were examined in each case. DNA samples from normal cells (no serum starvation) were also studied under the same conditions, as a control. The location of each bubble was plotted relative to 12o'clock EcoRI site on the EhR1 molecule. The number of replicating molecules with respect
Fig. 22. Relative abundance of replication bubbles in EhR1 in serum-starved cells restored to serum. DNA was isolated from cells immediately after 24 hours serum-starvation (0 hr) and also from cells at different time points following serum replenishment (1 hr, 2 hr and 4 hr). A control of exponentially growing cells was also included. DNA samples were processed for electron microscopy and in each case 200 molecules were examined. Total number of replication bubbles in each sample was recorded. A, exponentially growing cells; B-E: serum-starved cells restored to serum for - B, 0 hr; C, 1 hr; D, 2 hr; and E, 4 hr.
Fig. 23. Electron microscopic localisation of EhR1 replication bubbles in serum-starved cells restored to normal medium. Circular DNA molecules were enriched by using Qiagen purification protocol from cells at different time points following serum replenishment (1 hr, 2 hr and 4 hr) after 24 hours serum deprivation. Exponential cells were also taken as control (Normal). DNA samples were digested with SacII and processed for EM. In each case, replication bubbles were mapped in around 35-40 molecules. The positions of the bubbles were plotted both clockwise (filled bars) and anticlockwise (open bars) from 12 o'clock EcoRI site on a linearized map of EhR1 shown below each histogram (detailed map in Fig. 8 and 9). Data from normal cells and '1 hr' sample are being shown in the present page. Please see next page for '2 hr' and '4 hr' sample.
Fig. 23. Continuation of previous page. Data from '2 hr' and '4 hr' sample are shown in the present page.
to their position on the linearised EhR1 map is shown in Fig. 23. In normal cells it is clear that initiations take place all over the molecule with some preference for the transcription unit. In contrast, data from '1 hr' time point shows most initiations within the 3'-half of the transcription unit and in the downstream spacer. Of 32 bubbles, 20 were in the rDNA and 11 in the downstream spacer. Only one replicating molecule was scored in the upstream spacer. Most bubbles were clustered within 3-4 kb of the EhR1 molecule. With increasing time of growth, this preference was progressively lost. In the '2 hr' sample the zone of initiation within the rDNA was broader than in the '1 hr' sample. More molecules initiated from within the upstream spacer. The '4 hour' sample began to look like DNA from exponential cells. Serum starvation has, thus, made it possible to fine map the hot spots of replication initiation within 3-4 kb range in contrast to normal cells where initiations take place all over the molecule.

C.3.4.2. Two-Dimensional gel analysis of DNA samples isolated from serum-starved cells

To extend the results obtained from electron microscopic observations, 2-D gel analysis of DNA samples isolated from serum-starved cells was also performed.

Total *E. histolytica* genomic DNA was isolated from '2 hr' sample (24 hours serum deprivation followed by two hours back in normal medium). The DNA sample was digested with several restriction enzymes to get appropriate size fragments for 2-D gel electrophoresis to cover the entire EhR1 molecule. After blotting and hybridization with specific radiolabelled probes, the patterns corresponding to the different replicating intermediates obtained from various fragments are shown in Fig. 24. Two fragments (X4.5 which is an XbaI 4.5 kb fragment and NSc3.7, a NdeI-SacII 3.7 kb fragment) from the rDNA unit showed strong bubble arcs (marked by arrows). Fragments upstream and downstream of the rDNA units did not show any bubble structure. A fragment S4.2 (ScaI, 4.2 kb) coming from upstream of the rDNA unit showed a clear Y fork arc without a bubble arc. Likewise another fragment N3.2 (NdeI, 3.2 kb) from downstream of the rDNA fragment did not show any bubble arc. However, a clear Y fork arc was present. This data shows that in serum-starved cells put back in normal medium, replication initiation events take place predominantly in the rDNA unit, which is in accordance with the EM mapping (Fig. 23). No detectable initiation events were found downstream and upstream to the rDNA units.

While the absence of replication bubbles in the rDNA upstream fragments is in accordance with EM observations, the downstream fragment did not show the pattern expected from EM results, namely a replication initiation 'hot spot'. Since the 2-D gel experiments have not been repeated a sufficient number of times, the slight chance of
Fig. 24. 2-D gel electrophoresis of replication intermediates in EhR1 from '2 hr' sample (24 hours serum-deprivation followed by two hours in normal medium). (a) A linear map of the 24.5-kb plasmid cut at the 12 o'clock EcoRI site is shown. Abbreviations for enzyme sites: E, EcoRI; S, Scal; Hf, HinfI; N, Ndel; X, XbaI; Sc, Sall; H, HindIII. The DNA fragments analyzed by 2-D gel electrophoresis are shown as filled bars below the line. The restriction enzyme(s) used to generate each fragment and the fragment size (in kb) are indicated below each bar. The various DNA probes used are shown as empty bars above the line. The corresponding restriction fragments were cloned in plasmid vector pTZ18R, and the inserts, purified from gel slices after electrophoresis, were radiolabelled. (b) Autoradiograms of Southern blots of individual DNA fragments (panel letters correspond with fragment letters in panel a). E. histolytica DNA was digested with the indicated enzymes to generate fragments of interest. These were separated by 2-D gel electrophoresis as described in the section B 13. Southern blots were hybridized with DNA probes specific for each fragment as shown in panel a. Arrows indicate the position of bubble arcs.
bubble pattern being missed due to technical problems cannot be entirely ruled out at this stage. If the data is valid, however, it means that the downstream region of one of the rDNAs (in fragment N3.2) is not a replication 'hot spot'. It has to be tested whether the region downstream of the other rDNA may function as a 'hot spot'. Although the sequences on both sides are symmetrical, such a result may be possible if only one of the rDNA units is actively transcribed and this affects replication initiation.

C.4. Multiple dispersed initiation sites in EhR1 with preference for the transcription unit: Correlation with DNA helical stability

An interesting approach would be to identify a DNA unwinding element (DUE)(see section A.13.4), a required sequence that is hypersensitive to single-strand specific nucleases and serves to facilitate origin unwinding. A DUE is a conserved component of DNA replication origins in bacteria, yeast and mammals (Umek and Kowalski, 1990; Kowalski and Eddy, 1989; Borowiec et al., 1990; Natale et al., 1992). To facilitate further characterization of replication origins in *E. histolytica*, it would be useful to identify potential DUEs from DNA sequence information and to quantify their ease of unwinding.

A computer programme (Thermodyn) has been developed (Natale et al., 1992) that makes use of experimentally determined thermodynamic parameters of the DNA sequence (Breslauer et al., 1986) to calculate the free energy difference between the duplex and single-stranded states i.e., DNA helical stability. Calculations using these parameters, accurately reflect the helical stability and melting properties of DNA. This programme uses the thermodynamic data for nearest neighbour dinucleotides to calculate the free energy difference between the duplex and single stranded states for a given window which slides along the DNA sequence. The window size 300 was used to analyze total sequence of EhR1, as it is the maximum permitted window size for a large sequence.

A helical stability (ΔG) plot for EhR1 (Fig. 25) showed seven different zones with very low ΔG values compared to the average ΔG value for the entire molecule. These zones include-(a) *HinF*1 repeats in the upstream spacer which share large stretches of common sequence with *DraI* repeats found downstream of the rDNA unit; (b & g) 361 bp regions of identity immediately upstream of both the rDNAs; (c & f) sequences located near the 5'-end of 16S rRNA; (d) *DraI* repeats immediately downstream of the rightward rDNA unit; (e) S2 spacer and the *DraI* repeats downstream of the leftward rDNA unit.

Among these zones (potential DUEs?), the lowest ΔG was obtained around nucleotide position 12800 [(+/-)150] in zone 'e' which colocalizes with the S2
Fig. 25. DNA helical stability of EhR1. A computer programme (Thermodyn, developed by Natale et al., 1992) has been used to study the helical stability of EhR1. This programme calculates the free energy difference between the duplex and single-stranded states for a given window which slides along the DNA sequence. \( \Delta G \) (kCal/mol) values for each nucleotide in EhR1 have been plotted. Seven different zones within EhR1 have been found with very low \( \Delta G \) values. These zones and their locations in EhR1 (see Fig. 2) are followed: (a) \textit{Hin}I repeats in the upstream spacer which share large stretches of common sequence with \textit{Dra}I repeats found downstream of the rDNA unit; (b & g) 361 bp regions of identity immediately upstream of both the rDNAs; (c & f) sequences located near the 5' end of \textit{lsu} rRNA; (d) \textit{Dra}I repeats immediately downstream of the rightward rDNA unit; (e) S2 spacer and the \textit{Dra}I repeats downstream of the leftward rDNA unit. Thin line below the plot shows linearized EhR1 molecule from 12 o'clock \textit{Eco}RI site. The locations of rRNA transcription units are marked by inverted arrows.
sequence in the downstream spacer. Adjacent Dral repeats also showed low ΔG values over the entire region. Finding potential DUE elements does not in itself signify origin function. Whether potential DUEs found in EhR1 actually serve an origin function or not, needs to be investigated in future studies.

C.5. Small Circular DNAs of *E. histolytica*

C.5.1. A multitude of small circular DNAs exist in the nucleus of *E. histolytica*

Only a few plasmid elements have so far been identified and characterized in eukaryotic cells. The best known examples are the nuclear plasmids in yeasts and *Dictyostelium*. In *E. histolytica* and *E. moshkovskii*, rRNA genes are located on a multicopy nuclear plasmid. Here we show that several distinct classes of small circular DNAs also coexist in the nuclei of *E. histolytica*.

Circular DNA molecules were preferentially enriched from total nuclear DNA by modified Hirt fractionation method. Three strains of *E. histolytica* and one strain of *E. moshkovskii* were analysed. The DNA was visualized by electron microscopy. Circles of various sizes were observed (Fig. 26). In each case, more than 300 molecules were scored. Several discrete classes of circular DNAs were observed in all the strains. The data is compiled in table 6. The size of DNA molecules reported represents the average of about ten measurements. Larger molecules, although occasionally encountered, were not scored due to the relative rarity of their occurrence. A small fraction of molecules of all size classes was present in supercoiled form.

C.5.2. rDNA circles are the most predominant.

From table 6 it is clear that in each strain, rDNA circles constituted 70-80% of the total circular molecules encountered. In HM-1:IMSS (clone 6) the size of the rDNA plasmid is 25 kb and it accounts for 82.2% of the total circles. In 200:NIH (clone 2), 65.3% of the total circles encountered are rDNA molecules and they belong to the size class of 30 kb. Likewise in *E. histolytica* strain HK-9 and *E. moshkovskii* strain laredo, rDNA circles fall in the size class of 15.0 kb and 19.0 kb respectively and they are predominant among the circles present.

C.5.3. Small circles are not related to rDNA circles.

Are the small circles derivatives of the larger and more abundant rDNA molecule? Extensive sequence relatedness of these circles with the rDNA circle appears unlikely from Southern blot data, although further experiments are required to confirm this. As shown in Fig. 27, total DNA of *E. histolytica* strain HM-1:IMSS probed with EcoRI
Fig. 26. Electron micrographs showing circular DNA molecules of various sizes isolated from three different strains of *E. histolytica* (I-III) and one strain of *E. moshkovskii* (IV). DNA was purified by Hirt fractionation (Hirt, 1967). The procedure for electron microscopy has been described in the section B.14. (I) HM-1:IMSS, (A) 25 kb, (B) 12 kb, (C) 6 kb, (D) 4 kb; (II) 200:NIH, (A) 30 kb, (B) 25 kb, (C) 12 kb, (D) 7 kb, (E) 4 kb; (III) HK-9, (A) 33 kb, (B) 15 kb, (C) 6 kb, (D) 4 kb; (IV) laredo, (A) 37 kb, (B) 19 kb, (C) 7 kb, (D) 4 kb, (E) 2 kb.
Table 6: Relative abundance of circular DNA molecules

<table>
<thead>
<tr>
<th>Strain</th>
<th>aSize of circles [(+/−) S.D.], kb (relative abundance, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Strain</td>
</tr>
<tr>
<td>E. histolytica</td>
<td>HM-1:IMSS (clone 6)</td>
</tr>
<tr>
<td>E. histolytica</td>
<td>200:NIH (clone 2)</td>
</tr>
<tr>
<td>E. histolytica</td>
<td>HK-9 (clone 2)</td>
</tr>
<tr>
<td>E. moshkovskii</td>
<td>Laredo</td>
</tr>
</tbody>
</table>

a Size was estimated by measurement from electron micrographs, S.D., standard deviation
Fig. 27. Southern blot of *E. histolytica* HM-1:IMSS DNA probed with *EcoRI* fragment of the rDNA circle (probe HMe) (see Fig. 2). Total genomic DNA was electrophoresed in 0.4% agarose gel at 1V/cm for 16 hrs; and blotted on GeneScreen plus nylon membrane. The blot was hybridized with $^{32}$P-labelled probe. Arrow shows the 23-kb $\lambda$/HindIII marker band.
fragments of the rDNA circle (probes HMd, HMe and HMg; Fig. 2) hybridized with a number of high molecular mass bands. The fastest moving form of the 25 kb rDNA circle migrated close to the 23 kb band of lambda HindIII digest which was used as a molecular mass marker. Under similar electrophoretic conditions, the smaller rDNA circles of strain HK-9 (15 kb) and E. moshkovskii (19 kb) migrated faster than the 23 kb marker and could be readily distinguished from the 25 kb rDNA band. If any of the small circles in strain HM-1:IMSS had homology with rDNA, one would expect to see faster moving bands of low intensity in the Southern blot of Fig 27. Densitometric scanning of overexposed Southern blot showed no detectable signal below the 25 kb rDNA band. It is not possible to comment on circles larger than 25 kb, as they would not be sufficiently resolved under the electrophoretic conditions used.