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Replication Initiates at Multiple Dispersed Sites in the Ribosomal DNA Plasmid of the Protozoan Parasite *Entamoeba histolytica*

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In the protozoan parasite *Entamoeba histolytica* (which causes amoebiasis in humans), the rRNA genes (rDNA) in the nucleus are carried on an extrachromosomal circular plasmid. For strain HM-1:IMSS, the size of the rDNA plasmid is 24.5 kb, and 200 copies per genome are present. Each circle contains two rRNA transcription units as inverted repeats separated by upstream and downstream spacers. We have studied the replication of this molecule by neutral/neutral two-dimensional gel electrophoresis and by electron microscopy. All restriction fragments analyzed by two-dimensional gel electrophoresis gave signals corresponding to simple Y's and bubbles. This showed that replication initiated in this plasmid at multiple, dispersed locations spread throughout the plasmid. On the basis of the intensity of the bubble arcs, initiations from the rRNA transcription units seemed to occur more frequently than those from intergenic spacers. Multiple, dispersed initiation sites were also seen in the rDNA plasmid of strain HK-9 when it was analyzed by two-dimensional gel electrophoresis. Electron microscopic visualization of replicating plasmid molecules in strain HM-1:IMSS showed multiple replication bubbles in the same molecule. The location of bubbles on the rDNA circle was mapped by digesting with *Pvu*I or *Bsa*HI, which linearize the molecule, and with *Sac*II, which cuts the circle twice. The distance of the bubbles from one end of the molecule was measured by electron microscopy. The data corroborated those from two-dimensional gels and showed that replication bubbles were distributed throughout the molecule and that they appeared more frequently in rRNA transcription units. The same interpretation was drawn from electron microscopic analysis of the HK-9 plasmid. Direct demonstration of more than one bubble in the same molecule is clear evidence that replication of this plasmid initiates at multiple sites. Potential replication origins are distributed throughout the plasmid. Such a mechanism is not known to operate in any naturally occurring prokaryotic or eukaryotic plasmid.

Plasmids and viruses provide an opportunity to gain insights into the replication mechanisms of the host cell, as they utilize a number of host proteins for their own DNA replication. Extrachromosomal plasmid elements are fairly widespread amongst the parasitic protozoa (25, 28, 52), but detailed analysis of their replication is not available. In fact, amongst eukaryotic plasmids, only the *Saccharomyces cerevisiae* 2μm plasmid (24, 51) and *Dictyostelium* plasmids (30) are well studied. On the other hand, a large number of bacterial plasmids, e.g., ColE1 (47), F (14), R6K (15), and pSC101 (9), have been extensively analyzed. A common feature in all of these molecules is that DNA replication initiates at a relatively fixed site, called the origin of replication (*ori*). The exact mechanism by which this site is recognized for initiating DNA synthesis is unique to each plasmid. However, certain general features are evident, e.g., the presence of short repeats (iterons) in the *ori*, to which a plasmid-encoded replication initiation protein binds, *dnaA* boxes recognized by the host *dnaA* protein, and an AT-rich region in which the DNA is bent. DNA-protein interactions in the *ori* lead to conformational changes which may pave the way for loading the replication machinery, in particular the *dnaB* helicase. Unlike most bacterial plasmids, the yeast 2μm plasmid does not encode an initiator protein of its own. It utilizes host proteins to initiate DNA replication at a unique site called the autonomously replicating sequence (ARS) element. ARS elements each contain an 11-bp core sequence called the ARS consensus sequence (ACS) and an AT-rich region to the 3′ side of the T-rich strand of the ACS. Such ARS elements occur in the yeast genome as well and can function as replication initiation sites when cloned on an artificial plasmid (21). The *Dictyostelium discoideum* plasmids also initiate replication at a specific site (1, 11). In the *D. discoideum* plasmid 2, a 49-bp element present in three copies, is an essential part of the origin.

Studies with bacteriophages and animal cell viruses also led to the conclusion that in these replicons, DNA synthesis initiates within a well-defined locus, *ori*. Proteins which bind to the *ori* locus have been identified, and mutational analysis has confirmed the functional role of several nucleotide sequence elements within *ori*. Existing data have therefore justified the suggestion that simple genomes of prokaryotes, protozoa, metazoan plasmids, viruses, and organellar DNAs have well-defined origins of replication compared with metazoan chromosomes, for which replication may initiate at multiple sites located in broad zones (17). In this study we show that in the protozoan parasite *Entamoeba histolytica* (which causes amoebiasis in humans), the high-copy-number, 24.5-kb nuclear rRNA gene (rDNA) plasmid is quite exceptional, as it initiates DNA replication from multiple sites located throughout the plasmid. This is the first report of multiple, dispersed replication initiations on a naturally occurring plasmid in either prokaryotes or eukaryotes. Multiple initiation events have previously been shown to exist only in artificial plasmids introduced into *Xenopus* eggs and egg extracts (32, 35) and in human cells (33).

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MATERIALS AND METHODS

Strains and cell growth. *E. histolytica* HM-1:IMSS clone 6 and HK-9 were maintained in TYI-S-33 medium at 36°C (19).

DNA isolation and purification. Cells at the end of the log phase of growth were pooled from 50 tubes after chilling in ice-water for 10 min. A cell pellet was obtained by centrifuging at 275 × *g* for 7 min at 4°C. Cells (5×10^7 to 1×10^8) were resuspended in 5 ml of buffer (100 mM NaCl, 10 mM EDTA, 10 mM Tris-Cl [pH 8.0]) and lysed by addition of 0.2% sodium dodecyl sulfate (SDS). The resulting suspension was extracted once with an equal volume of phenol-chloroform-isoamylalcohol (25:24:1). The aqueous layer was passed by gravity flow through Qiagen tips (Qiagen Inc.) which were preequilibrated with 1 ml of QBT buffer (750 mM NaCl, 50 mM MOPS [3-{*N*-morpholino}propanesulfonic acid], 15% ethanol, 0.15% Triton X-100 [pH 7.0]) according to the manufacturer's instructions. The tips were then washed four times with 1 ml of QC buffer (1.5 M NaCl, 50 mM MOPS, 15% ethanol [pH 7.0]). Finally the DNA was eluted with 0.8 ml of QF buffer (1.25 M NaCl, 50 mM Tris-HCl, 15% ethanol [pH 8.5]). DNA was precipitated with 0.7 volume of isopropanol. The pellet collected by centrifugation was washed with 70% ethanol, air dried for 5 min, and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]).

Analysis of replication intermediates by two-dimensional gel electrophoresis. Neutral/neutral two-dimensional gel electrophoresis was carried out by the procedure of Brewer and Fangman (6). DNA samples (5 μg) were digested with appropriate restriction enzymes (20 to 30 U in a 30-μl reaction mixture). Electrophoresis in the first dimension was carried out in 0.4% agarose (FMC) at 1 V/cm for 16 to 18 h. Samples were loaded in duplicate, and one of the lanes was stained with ethidium bromide to locate the DNA bands. The unstained lane was cut out and used for electrophoresis in the second dimension. This was done in 1% agarose at 5 V/cm for 5 to 6 h. After electrophoresis, the gel was incubated briefly (10 min) in 0.25 N HCl and then blotted on a nylon membrane (GeneScreen Plus; NEN Dupont) with an alkaline transfer buffer (0.4 M NaOH, 1 M NaCl) according to the manufacturer's instructions. The DNA probes used were labelled with [^{32}P]dATP by the random-oligonucleotide-priming labelling procedure (23). The hybridization solution contained 1% SDS, 1 M NaCl, and 10% dextran sulfate. Filters were hybridized with 0.15 ml of hybridization solution per cm^2 and 4×10^5 dpm of probe per ml at 65°C for 16 to 20 h. Filters were washed as follows: twice in 2× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate) at room temperature and twice in 2× SSC–1% SDS at 65°C (30 min for each wash) and finally once in 0.1× SSC at room temperature. Autoradiographic exposure (Kodak XAR film) at −70°C was for 4 to 7 days. The membranes were stripped with 0.2 M NaOH for 1 h at room temperature if rehybridization with a second probe was required.

Electron microscopy. DNA molecules were prepared for microscopy by following essentially the method of Davis et al. (16). DNA samples (10 μg/ml in 10 mM Tris-HCl–1 mM EDTA [pH 8.0]) were mixed with cytochrome *c* (0.005%) and 50% formamide and spread on water. This monolayer of DNA molecules was gently picked up onto carbon-coated (400-mesh) grids, stained with uranyl acetate (5 mM stock solution in 50 mM HCl, freshly diluted 100-fold with 95% ethanol), and washed in 95% ethanol. The grids were rotary shadowed with Pt-Pd (80:20) and examined under a Philips EM 410 transmission electron microscope. Photographs at various magnifications were taken. Length was measured with either a map measurer or a marked tape after magnification by projection.

RESULTS

Two-dimensional gel analysis of 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 (3, 29, 43). There is no evidence of any chromosomal copies of these genes. The rDNA plasmid, named EhR1, has been completely sequenced (43). As shown in Fig. 1, it contains two complete rRNA transcription units arranged as inverted repeats. A region labelled Tr, just upstream of the leftward rDNA, is transcribed into a 0.7-kb poly(A)$^+$ RNA which lacks any extensive open reading frames. The function of this transcript is unknown at present, but it could have a regulatory role in the replication of EhR1. Several families of short repeat sequences lie in the regions downstream and upstream of the rRNA transcription units. One of these, the *Dra*I repeat, is 170 bp long and contains three close matches to the yeast ACS. The DNA fragment containing these repeats could confer replicating ability (as measured by yeast transformation efficiency) when cloned into the yeast plasmid vector YIp5, which lacks its own ACS (38). However, it was not known whether these repeats performed a similar function in EhR1 replicating in amoeba cells.

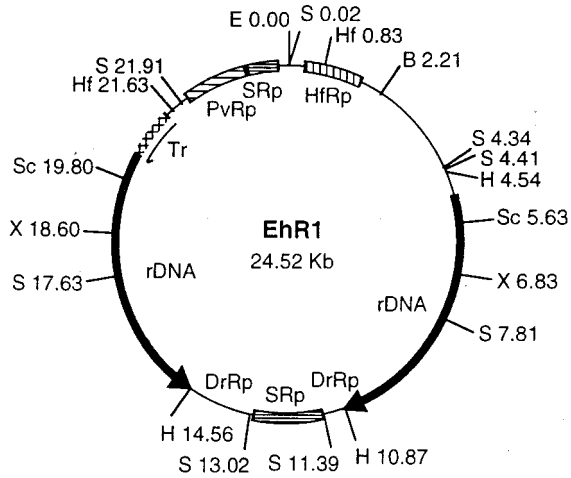

FIG. 1. The rDNA plasmid of *E. histolytica* HM-1:IMSS (43). Bold arrows show the two rDNA inverted repeats and their directions of transcription. Nucleotide positions (in kilobases) are indicated at each restriction enzyme site. Abbreviations for enzyme sites: B, *Bsa*HI; E, *Eco*RI; H, *Hin*dIII; Hf, *Hin*fI; S, *Sca*I; Sc, *Sac*II; and X, *Xho*I. Abbreviations for families of short, tandem repeats in downstream and upstream spacers: DrRp, *Dra*I repeats; SRp, *Sca*I repeats; PvRp, *Pvu*I repeats; and HfRp, *Hin*fI repeats. Individual repeat units within each family have not been marked. To avoid overcrowding the map, not all the sites where *Eco*RI and *Hin*fI cut the plasmid have been indicated; only the *Eco*RI site at the 12 o'clock position and the *Hin*fI sites used in two-dimensional gel analysis are shown. The region Tr in the upstream spacers codes for a 0.7-kb transcript in the direction shown by the thin arrow.

To precisely map the origin of replication of this plasmid, we used neutral/neutral two-dimensional gel electrophoresis as developed by Brewer and Fangman (6). In this method (for details, see reference 6), electrophoretic conditions employed in the second dimension separate DNA molecules on the basis of conformation. Replication intermediates are thus retarded in their migration compared with linear molecules of the same size. The replicon to be studied is digested with restriction enzymes, and the electrophoretic migration of each fragment is determined. A fragment in which replication originates in the middle and proceeds bidirectionally contains complete bubbles and is readily distinguished from those containing Y's (in which replication forks originating from the outside enter the fragment) or X's (in which forks moving in opposite directions meet and terminate). If replication does not originate in the middle of the fragment, the pattern obtained is discontinuous. The arc of a replicating molecule starts as a bubble but turns into a Y as one of the forks traverses the restriction site. However, if the origin is placed very asymmetrically (less than a third of the way down the fragment), the bubble arc is generally indistinguishable from a Y arc. The various patterns generated by replication intermediates are best distinguished in restriction fragments with sizes of 4 to 5 kb. For much larger or smaller fragments, electrophoretic conditions have to be suitably modified. Total *E. histolytica* genomic DNA enriched for the rDNA plasmid EhR1 was digested with *Sca*I, *Xho*I, *Hin*dIII, and *Hin*fI to generate fragments in the 4-kb range, representing the entire circle (Fig. 2a). 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. 2a (filled bars). Restriction enzyme-digested DNA was subjected to two-dimensional gel electrophoresis and blotted on nylon membranes. The various DNA probes used for hybridization are shown as hatched bars in Fig. 2a, and the hybridization patterns obtained are shown in the panels (Fig. 2b). All probes are

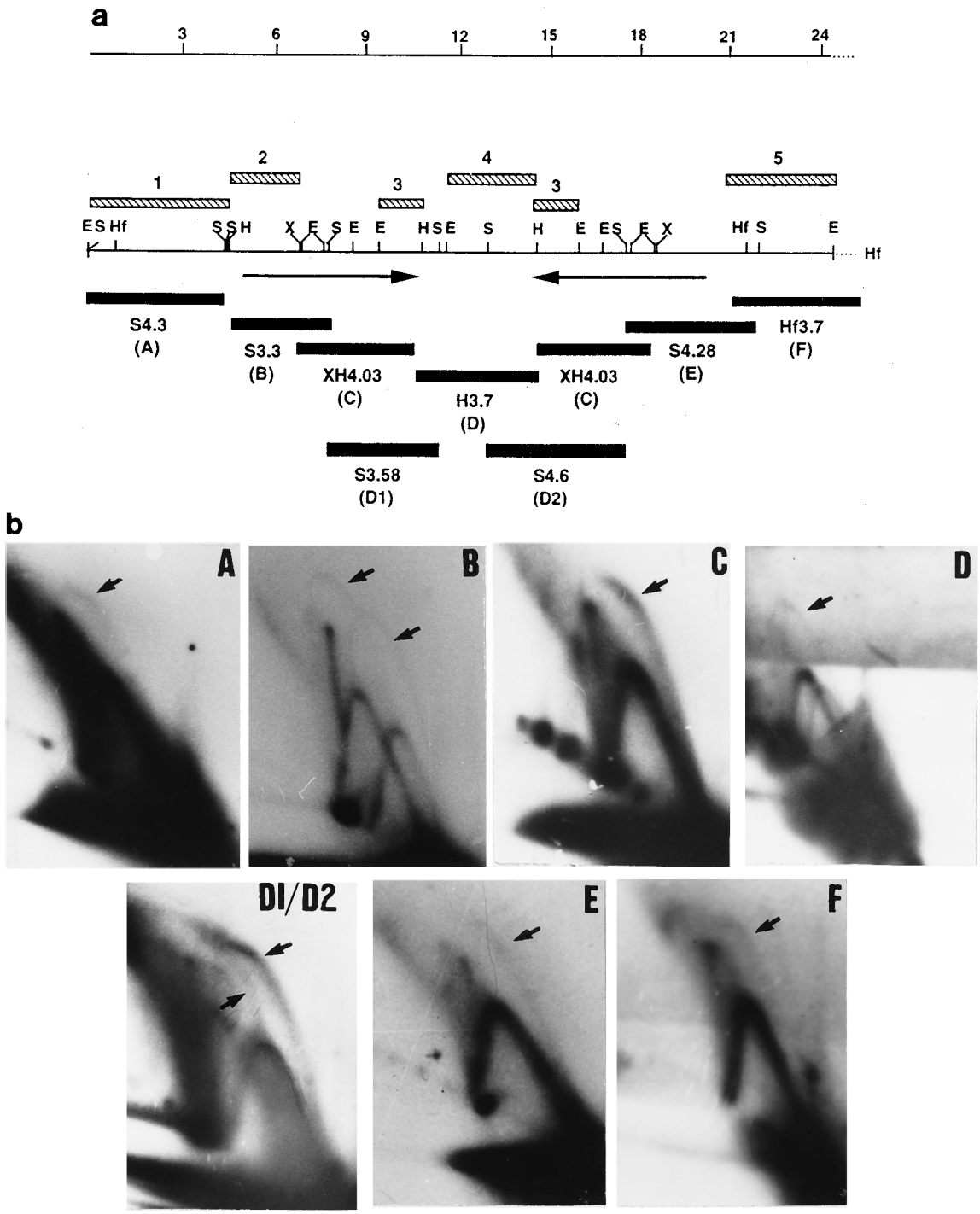

FIG. 2. Two-dimensional gel electrophoresis of replication intermediates in EhR1. (a) A linear map of the 24.5-kb plasmid cut at the 12 o'clock *Eco*RI site (as shown in Fig. 1) is shown. Abbreviations for enzyme sites: E, *Eco*RI; H, *Hin*dIII; Hf, *Hin*fI; S, *Sca*I; X, *Xho*I. The DNA fragments analyzed by two dimensional-gel electrophoresis are shown as filled bars below the line. The restriction enzyme(s) used to generate each fragment and fragment size (in kilobases) 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 Materials and Methods. Southern blots were hybridized with DNA probes specific for each fragment as shown in panel a. Arrows indicate positions of bubble arcs.

specific for the DNA fragments being studied, as confirmed from the nucleotide sequence of the plasmid (43). Probe 2 hybridizes with both fragments S3.3 and S4.28 (Fig. 2b, 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. 2b, panel E). This probe was generated from a 5.9-kb *Eco*RI fragment containing part of the leftward rDNA (as shown in Fig. 1) and the upstream spacer

up to the 12-o'clock *Eco*RI 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 two-dimensional gel electrophoresis showed that all fragments gave signals expected for replication bubbles and simple Y's. 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 bubble arcs was consistently greater in fragments C and D1-D2 (Fig. 2b), which contain the rDNA transcription unit, than in fragments spanning the downstream and upstream spacers. For example, as shown in Fig. 2b, 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 was 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 initiations take place in these regions also. The complete and uniformly labelled 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 *Sca*I fragment containing part of the rDNA and upstream sequence of the leftward rDNA repeat (Fig. 2b, panels B and 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 significance of this pattern are not apparent at this point. It could be worth noting that this pattern coincides with the 3′ end of the Tr transcript (Fig. 1).

Electron microscopic analysis of replicating molecules. In strain HM-1:IMSS, the predominant circular molecule is the 24.5-kb rDNA circle (18). Molecules of this size were viewed with an electron microscope (Fig. 3A to D). Of more than 1,200 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 were 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 (37) 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 (53). The relative abundance of bubbles of various sizes is shown in Fig. 3E. Large bubbles were not seen with the frequency expected on the basis of the intensity of bubble arcs in two-dimensional 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. Thus, replication bubbles seem to be clustered in one half of the plasmid, although their actual locations may vary from molecule to molecule.

To map the locations of replication bubbles in individual molecules, the plasmid was linearized with *Pvu*I or *Bsa*HI (which cut the circle at a single cluster of sites or just once, respectively). The plasmid was also digested with *Sac*II, 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 than did intact circles. The location of each bubble was plotted relative to the 12 o'clock *Eco*RI site. Since it is not possible to orient the molecules, we considered both possible locations of the replication bubble(s) in each molecule (Fig. 4). Large and small bubbles were distributed evenly, with no preferential clustering. The 14.1-kb *Sac*II 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. 4, molecules 1 to 10). In addition, the 10.3-kb *Sac*II 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. 4, molecules 11 to 20). The total number of bubbles seen in the 14.1-kb *Sac*II fragment was 20 (only 10 are plotted in Fig. 4), 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 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 the 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 two-dimensional gel electrophoresis, in which bubble arcs were more prominent in the fragments spanning the rDNA than in the spacers.

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 HM-1:IMSS strain alone, we repeated the experiments described above with the HK-9 strain, in which the rDNA plasmid has a slightly different architecture (43). Each 15-kb rDNA plasmid in this strain contains only one rRNA transcription unit (Fig. 5a). 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. 1). The sequences upstream of rDNA in HK-9, up to the 12 o'clock *Eco*RI site, are exactly identical in the two plasmids (43). The sequence upstream of the leftward unit in EhR1 up to the 12 o'clock *Eco*RI site (containing Tr and *Pvu*I repeats [Fig. 1]) is missing in the HK-9 plasmid. The families of *Dra*I and *Sca*I repeats found in the downstream intergenic spacer of EhR1 are present in the HK-9 plasmid downstream of its rDNA. The HK-9 plasmid was digested with *Eco*RI and *Hin*dIII (Fig. 5a, fragments A and D) and *Sca*I and *Hin*dIII

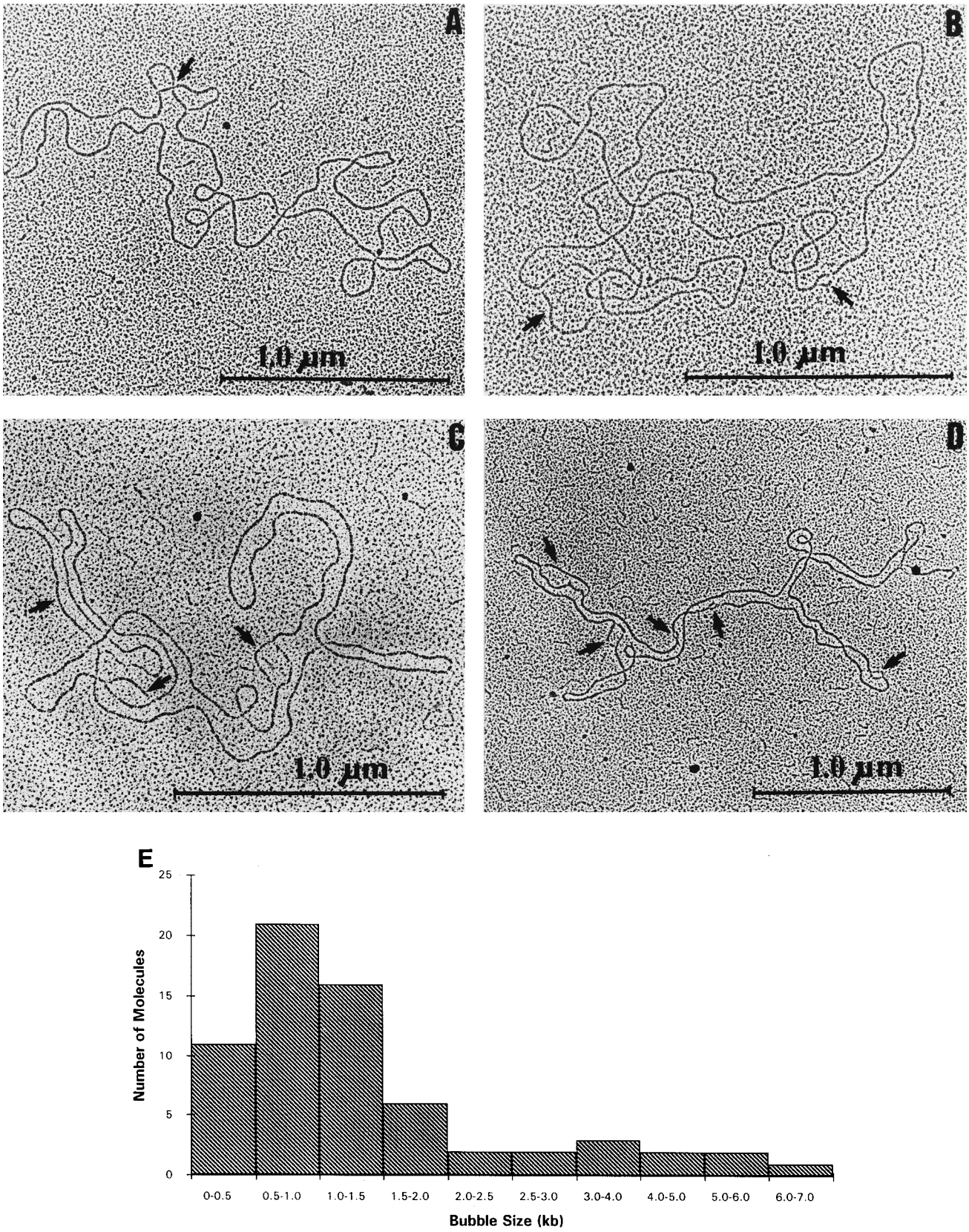

FIG. 3. Electron microscopic analysis of replicating EhR1 molecules. Circular DNA was enriched by being passed through a Qiagen column and was visualized by electron microscopy as described in Materials and Methods. Micrographs of replicating circular molecules (24.5 kb) with one (A), two (B), three (C), and five bubbles (D) are shown. Arrows point towards the middle of each replication bubble. (E) Size distribution of replicating bubbles. The contour lengths of 67 bubbles were measured and plotted to show the relative abundance of each size class.

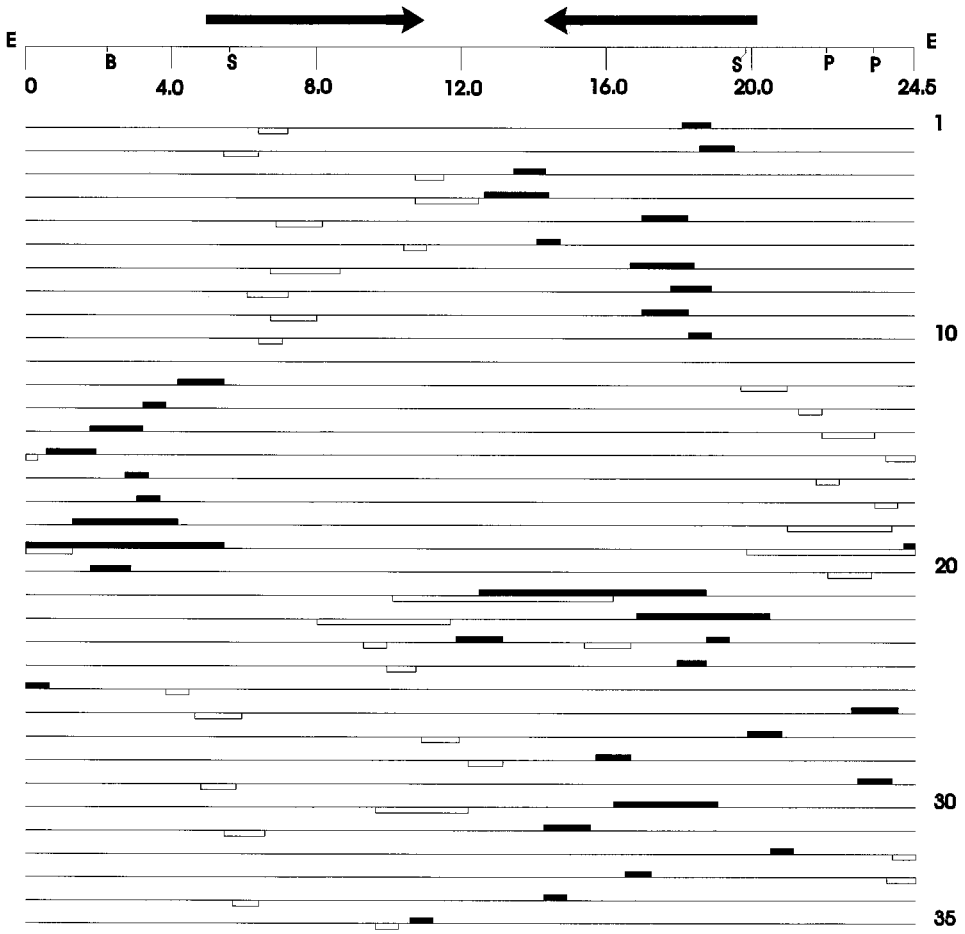

FIG. 4. Electron microscopic mapping of replication bubbles on linearized EhR1 molecules. A linear map of EhR1 oriented clockwise from the 12 o'clock *Eco*RI (E) site (relative to the map in Fig. 1) is shown on the top line. Arrows above the line show the locations of the two rDNA units. The scale below the line is in kilobases. The restriction enzyme sites used to linearize the plasmid are shown below the line: B, *Bsa*HI; S, *Sac*II; and P, *Pvu*I. Data from some of the molecules studied with each enzyme are shown (a total of 35 molecules). The location and 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 *Sac*II fragment which contains both the transcription units. Molecules 11 to 20 show the bubbles in the 10.3-kb *Sac*II fragment which contains the upstream spacer region. Molecules 21 to 30 and 31 to 35 were linearized with *Bsa*HI and *Pvu*I, respectively. Molecule 23 has two bubbles.

(Fig. 5a, fragments B and C) to generate four fragments appropriate for two-dimensional gel analysis. The hybridization probes used were the same as those described above for Fig. 2a. Probes 1, 2, 3, and 4 were used for fragments A, B, C, and D, respectively. The results of the two-dimensional gel analysis are shown in Fig. 5b. All fragments gave signals indicative of simple Y's 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. 2b, panel D). Thus, the mechanisms of replication of the two plasmids appear to be similar, with initiation sites spread in large segments of the molecule.

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. The plasmid was linearized with *Bsa*HI and *Sac*II, both of which have unique sites in the molecule (unlike EhR1, which is cut twice by *Sac*II). A total of 26 replicating molecules were analyzed. The size range of 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 former strain (Fig. 6). On plotting the location of bubbles in linearized molecules, no hot spots of initiation were evident (Fig. 7), 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.

DISCUSSION

The neutral/neutral two-dimensional gel electrophoresis technique of Brewer and Fangman (6) has been successfully used to map origins of replication in a large number of replicons (49). The rDNA plasmid of *E. histolytica* is well suited for analysis by this method since it can be cut into restriction fragments with an optimal size (4 to 5 kb) for differentiating replication intermediates, and it is an abundant class of DNA, constituting as much as 10% of the *E. histolytica* genome. Data

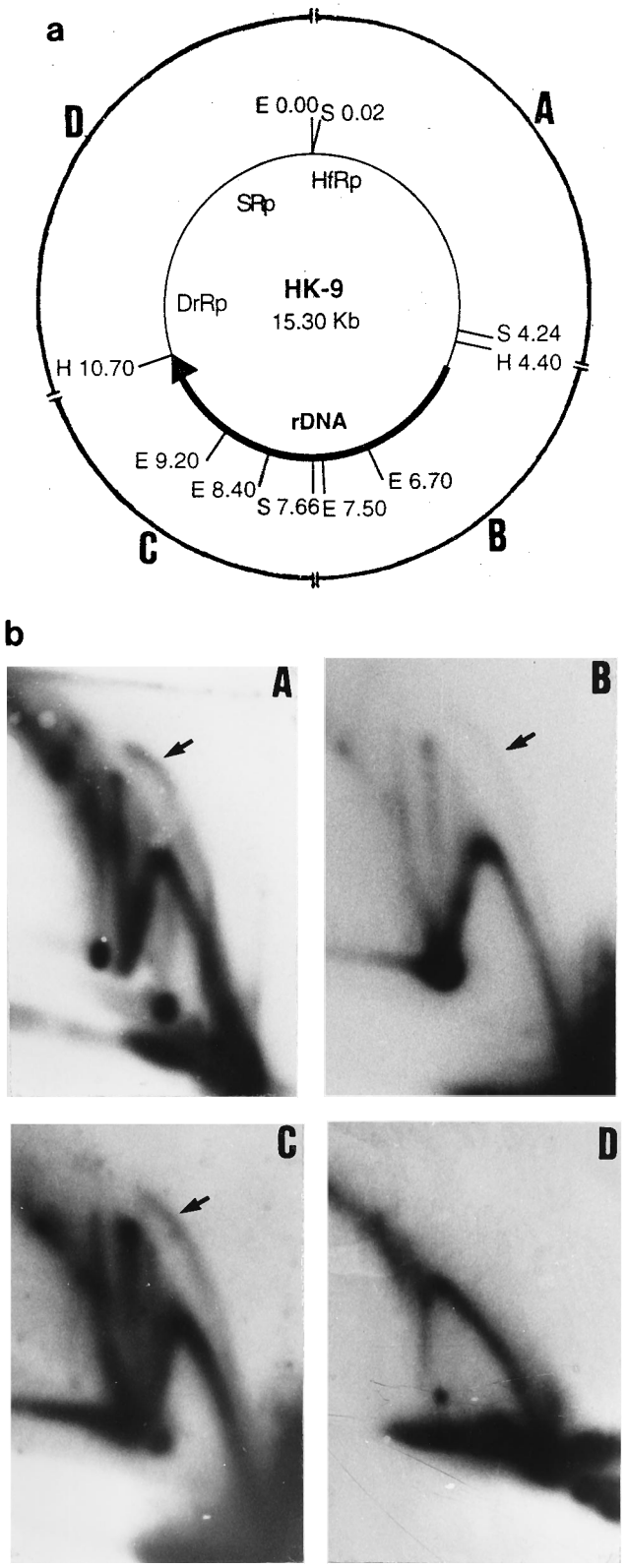

FIG. 5. Two-dimensional gel electrophoretic analysis of rDNA plasmid from strain HK-9. (a) The orientation of the single rDNA transcription unit and locations of *Dra*I repeats (DrRp), *Sca*I repeats (SRp), and *Hin*fI repeats (HfRp) are indicated. Abbreviations for restriction enzyme sites: E, *Eco*RI; H, *Hin*dIII; and S, *Sca*I. The fragments analyzed were generated by digestion with *Eco*RI and *Hin*dIII (for A and D) and *Sca*I and *Hin*dIII (for B and C). (b) Autoradiograms of Southern blots of fragments (panel letters correspond with fragment letters in panel a). Other details are given in the legend to Fig. 2b. The DNA probes used were those described in the legend to Fig. 2 (probes 1, 2, 3, and 4 for A, B, C, and D, respectively). Arrows indicate bubble arcs.

obtained by this method showed that all restriction fragments of the rDNA plasmid in strain HM-1:IMSS (EhR1) gave signals corresponding to complete Y and bubble arcs, and at least four fragments showed triangular smears of double-Y molecules (Fig. 2b). The bubble arcs in all fragments were continuous and uniformly labelled. There was no evidence of replication fork barriers, except perhaps in the 4.28-kb *Sca*I fragment. The simplest interpretation of this result is that replication of EhR1 initiates and terminates at multiple locations spread throughout the plasmid. Complete bubble arcs of uniform intensity can arise only if replication initiates in the middle of a DNA fragment (for bidirectional replication) or from one end (for unidirectional replication). Since all restriction fragments tested gave uniform bubble arcs, it is unlikely that this could result from a single, appropriately placed initiation event in each fragment. It is reasonable to conclude that multiple initiations take place in each fragment in a population of molecules. The existence of multiple bubbles on the same molecule is discussed below.

Direct electron microscopic observation of replicating molecules also led to the conclusion that bubbles indicative of replication origins are distributed throughout the plasmid (Fig. 3 and 4). Although no hot spots could be identified with the available data, the frequency of initiation events was greater in rRNA transcription units than in the upstream and downstream intergenic spacers. This was borne out by both two-dimensional gel analysis and electron microscopic data. However, replication did not originate primarily in the transcription units only. An estimate of the frequency of initiation events occurring in the transcription units compared with that in the upstream spacer was obtained from the two *Sac*II fragments of EhR1 (Fig. 4). Twenty bubbles were scored in the 14.1-kb fragment that contains the transcription units, and 13 bubbles were scored in the 10.3-kb upstream spacer fragment. These data strongly suggest that initiation events occur throughout the plasmid.

It is significant that replication initiated more frequently within the transcription units. This is contrary to observations of rDNAs in other systems; e.g., replication of the yeast rDNA cluster initiates in the intergenic spacer (7, 8, 34, 42) at intervals of about 5 repeat units. The replication origin in sea urchin (4) and *Xenopus laevis* (5) chromosomal rDNA was also mapped to the intergenic spacer. However, rDNA replication during early embryogenesis in *X. laevis* (studied on a plasmid) initiated and terminated at multiple, apparently random locations throughout the rDNA transcription unit (35). Interestingly, initiation of rDNA replication is developmentally regulated in this system. Early embryos in which rDNA is not transcribed can initiate replication throughout the rDNA. Around late-blastula stage, when rRNA transcription resumes, initiations occur primarily in the intergenic spacer, with a specific repression of initiation in transcription units (31). In *Tetrahymena* spp., extrachromosomal rDNA in the macronucleus is arranged as pairs of palindromic repeats. These are replicated from an origin located in the upstream spacer (10). In the yeast rDNA cluster, replication initiates in the intergenic spacer and proceeds bidirectionally. Replication forks moving in the direction opposite to transcription are stalled at a site within the spacer called the replication fork barrier. No such barrier was detected in the upstream and downstream spacers

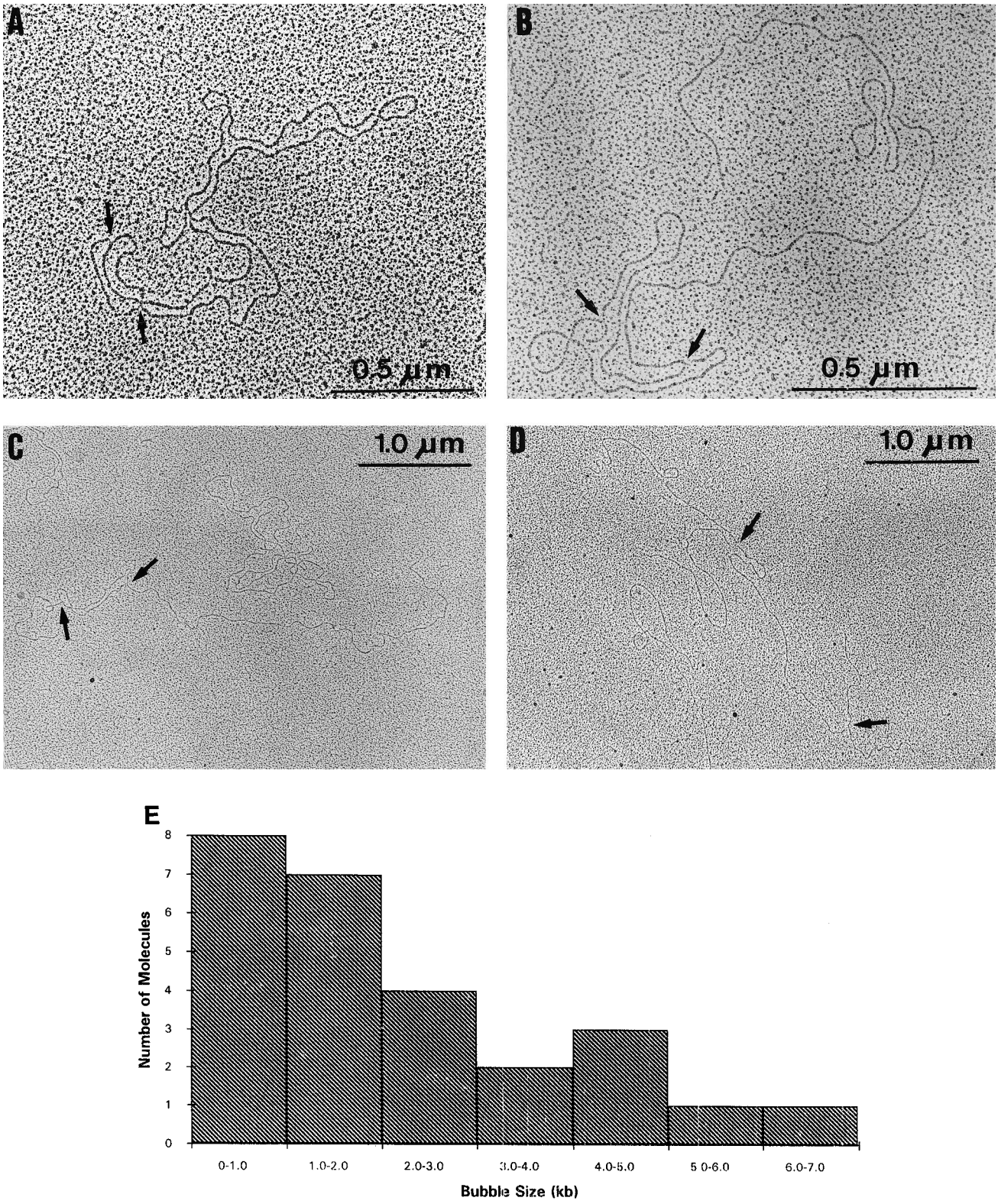

FIG. 6. Electron microscopic analysis of replicating HK-9 plasmids. The DNA was prepared for electron microscopy as described in Materials and Methods. Micrographs of bubbles in an intact 15-kb plasmid (A) and plasmids linearized with *Sac*II (B to D) are shown. Arrows point to the bubble junctions. The molecule in panel D has Y's at the two ends. (E) Size distribution of bubbles (for details, see legend to Fig. 3E). Contour lengths for a total of 26 molecules were measured.

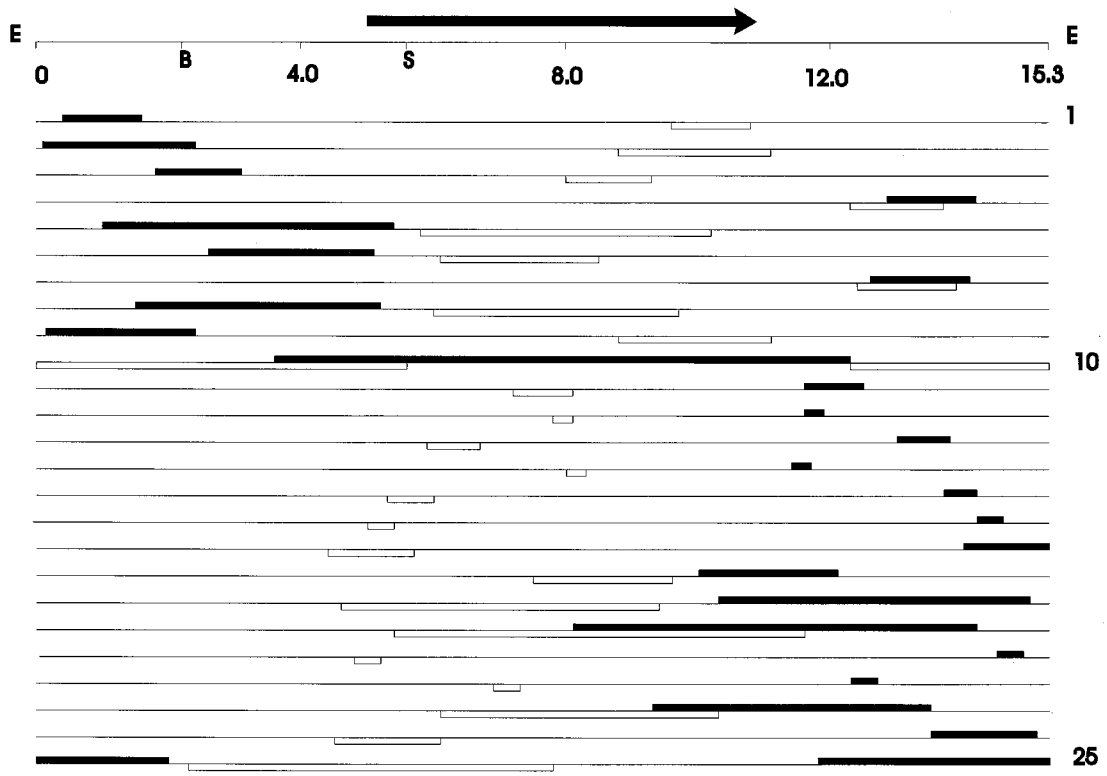

FIG. 7. Electron microscopic mapping of replication bubbles in the HK-9 plasmid. Details are given in the legend to Fig. 4. The plasmid was linearized with *Bsa*HI (B) and *Sac*II (S). The bubbles are depicted with respect to the 12 o'clock *Eco*RI site shown in Fig. 5. Unlike in EhR1, *Sac*II cuts the HK-9 plasmid only once. Molecules 1 to 10 and 11 to 25 were linearized with *Sac*II and *Bsa*HI, respectively.

of EhR1. Since the two rRNA transcription units of EhR1 are arranged in inverted orientation with respect to each other, a replication barrier may be expected in the downstream spacer if forks moving opposite to the direction of transcription were stalled. Since no such barrier was observed, either replication forks in EhR1 are unaffected by rRNA transcription or plasmid molecules engaged in rRNA synthesis are not used as templates for DNA replication.

The key observation in this paper is the absence of a fixed replication origin in EhR1 and the related rDNA plasmid of strain HK-9, which is in contrast to the replication mechanisms employed by other naturally occurring plasmids. Some artificially constructed eukaryotic plasmids, for example those replicating in *Xenopus* eggs and egg extracts (32, 35) and in human cells (33), are similar to EhR1 in that they do not use a defined replication origin. In vivo and in vitro studies with plasmids introduced into the oocyte system led to the conclusion that although replication initiated at multiple, random locations, an individual plasmid molecule initiated replication at a single site only. This inference was drawn by measuring the rate of DNA synthesis and the time required to completely replicate a single molecule (35). However, multiple closely spaced replication bubbles on one molecule could also give rise to a similar result. We have directly visualized replicating molecules by electron microscopy and found evidence of multiple initiation events within a single molecule. Although the available data show that the total number of molecules observed to have multiple bubbles was not very large, we did not find very closely spaced bubbles in any molecule. The shortest distance between two adjacent replication bubbles was 1.8 kb. In our two-dimensional gel electrophoretic analysis, the average size of DNA fragments examined was 4 kb. This size and the low abundance of molecules with multiple bubbles may explain why we did not observe any unusual pattern expected of fragments with more than one bubble by two-dimensional gel electrophoresis. From electron microscopic analysis of molecules with multiple bubbles, there was no evidence of a temporal arrangement; bubbles of different sizes appeared to be in random order. However, interestingly, in no event did bubbles occupy more than half the length of the molecule. If each bubble represents an active replication fork, our data imply that replication initiates at several sites in the rDNA plasmid until about half of the molecule is actively engaged. Following this, no subsequent initiations take place and the molecule is replicated by the already-formed replication forks. Thus, each molecule of EhR1 is replicated by a cluster of origins, whose location varies in individual molecules. Clusters of microbubbles seen by electron microscopy in replicating *Drosophila* (53), sea urchin (2), and *Physarum* (26) DNA have also been thought to represent clusters of replication origins which advance to form macrobubbles. However, while these exist in the context of large chromosomes, the clusters that we observe are in very small replicons. Such a mode of replication has not been observed before for any plasmid or viral genome.

rDNA plasmids have been found in a few other unicellular eukaryotes, for example, *Naegleria gruberi* (12) and *Euglena gracilis* (41). While replication of the *Naegleria* plasmid has not been studied, some information about the *Euglena* plasmid is available. Replicating forms of the latter were viewed by electron microscopy, and rolling-circle intermediates were detected (40). The origin of replication was not mapped. Further data on rDNA plasmids are required before we can understand whether our observation of multiple initiations in EhR1 is a general feature of these plasmids. Replication of the circular

genome of vertebrate mitochondria proceeds by a unique displacement-loop mechanism in which the two DNA strands initiate replication asynchronously (13). However, both strands contain defined sites which are used as origins of replication. In the kinetoplastid protozoa, which include parasites like *Trypanosoma* spp. and *Leishmania* spp., the mitochondrial equivalent is called a kinetoplast. Each kinetoplast contains thousands of 1- to 2-kb circular DNA molecules, called minicircles, whose replication has been investigated in detail (45). Here too, the two DNA strands initiate replication independently, but from defined origins. Among circular genomes studied so far, none seems to replicate by the mechanism operating in the *E. histolytica* rDNA plasmid.

Studies with several metazoan chromosomal loci, e.g., the amplified dihydrofolate reductase domain of Chinese hamster ovary cells (50), the *Drosophila* chorion gene cluster (27, 39), *Drosophila* histone gene repeats and a single-copy locus (44), and the *Schizosaccharomyces pombe ura4* gene (20), showed that all of these are replicated from multiple origins. Although the frequency of initiation at one site compared may vary with respect to that at neighboring sites, it is clear from these studies that several closely spaced sites within a circumscribed zone are deployed for replication. This is quite akin to the situation we encounter with EhR1. How are initiation zones demarcated from neighboring DNA? Is the DNA sequence in this zone more easily amenable to unwinding, as is the case for ARS elements in yeast cells (48)? In the *E. histolytica* rDNA plasmid, replication bubbles appear more frequently in less (62 to 70%) AT-rich regions (rRNA transcription units) than in highly (73 to 85%) AT-rich regions (intergenic spacers). The yeast ACS is obviously not involved in replication initiation in this plasmid, as no correlation between the occurrence of this sequence and initiation frequency was found. When the plasmid sequence was searched for matches with the yeast ACS [(A/T)TTTAT(A/G)TTT(A/T)], three 10-of-11 matches were found in each 170-bp *Dra*I repeat unit located in the downstream intergenic spacer. In contrast, the entire 6-kb transcription unit had only 10 such matches. No complete match with the ACS was found. Further experiments are required to know whether the act of rDNA transcription itself may facilitate the unwinding of DNA for the replication of EhR1, as in bacteriophage lambda (36) and *Escherichia coli* (46) origins of replication, which are known to be activated by the transcription of neighboring DNA.

What defines a replication origin in eukaryotic chromosomes remains elusive. It has been proposed that an origin may consist of discrete *cis*-acting elements which are made accessible only through higher-order chromatin structure (22). The *E. histolytica* rDNA plasmid, being an abundant DNA molecule in the cell, and for which the complete nucleotide sequence is known, offers a relatively simple system for looking for possible *cis*-acting elements from multiple origins of replication in a single replicon. It is hoped that metazoan chromosomes share mechanistic details with this protozoan plasmid.

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