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Organization of Replication of Ribosomal DNA in *Saccharomyces cerevisiae*†

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Using recently developed replicon mapping techniques, we have analyzed the replication of the ribosomal DNA in *Saccharomyces cerevisiae*. The results show that (i) the functional origin of replication colocalizes with an autonomously replicating sequence element previously mapped to the nontranscribed spacer region, (ii) only a fraction of the potential origins are utilized in a single S phase, and (iii) the replication forks moving counter to the direction of transcription of the 37S precursor RNA stop at or near the termination site of transcription. Consequently, most ribosomal DNA is replicated unidirectionally by forks moving in the direction of transcription and most replicons are larger than the repeat unit. The significance of this finding for the replication of abundantly transcribed genes is discussed.

Eucaryotic chromosome replication initiates at multiple sites, called origins, and growing evidence suggests that these origins are usually located at specific nucleotide sequences in chromosomal DNA (reviewed in reference 8). However, the nature of these sequences is not yet fully understood.

In *Saccharomyces cerevisiae*, specific sequence elements, called autonomously replicating sequences (ARS), which facilitate the autonomous replication of plasmids have been identified (7, 12, 20, 30). Although ARS elements facilitate the replication of plasmids, it was not known whether they could serve as replication origins until recently, when Brewer and Fangman (6) and Huberman et al. (15) independently developed two-dimensional (2D) gel techniques for mapping functional origins. Both groups used their techniques to demonstrate that the replication origin colocalizes with the ARS element in the yeast 2μm plasmid, and Brewer and Fangman (6) also observed colocalization of the origin with the ARS element in an ARS1-containing plasmid. These reports established that ARS elements could function as replication origins in yeast plasmids. We wished to know whether ARS elements could also serve as replication origins in chromosomal DNA. Here we report the results of our investigation of replication origin usage in yeast ribosomal DNA (rDNA).

The rDNA in *S. cerevisiae* consists of about 120 identical repeat units, arranged head to tail on chromosome XII (26). Each repeat contains a transcription unit for the 5S rRNA and a separate transcription unit for the 37S precursor RNA, which generates the 5.8S, 18S, and 25S rRNAs (1–3, 10, 23, 27, 29, 31). The two transcripts are separated by two nontranscribed spacers (NTS1 and NTS2; see Fig. 2A).

Several aspects of yeast rDNA replication have been studied previously. A weak ARS element was identified in NTS2 (29). The mitotic stability of plasmids containing the rDNA ARS is at least an order of magnitude lower than the stability of plasmids containing ARS1 or ARS2. The rDNA ARS was localized to a 571-base-pair (bp) fragment containing three 10-of-11-bp matches (29) with the *S. cerevisiae* ARS consensus sequence (7). Electron microscopy studies suggest that the origin of replication, defined as the center of observed replication "bubbles," is located in NTS2 (28). The size of replicons is reported to range from 1 to 5 repeat units (28, 32), suggesting that although the units in the repeat are identical (33), not every available origin is used in each S phase.

We have investigated the replication of yeast rDNA using the recently developed replicon mapping techniques (6, 15). Our results suggest that the ARS element and replication origin colocalize, that replication proceeds primarily unidirectionally through the rDNA in the direction of transcription of the 37S precursor, and that the average replicon size is at least several repeat units.

MATERIALS AND METHODS

Cell growth. *S. cerevisiae*, strain 4910-3-3 (provided by Leland Hartwell), was grown in YPD medium (10 g of yeast extract, 20 g of peptone, and 20 g of glucose per liter) at 23°C to a density of 1.5×10^7 cells per ml.

2D neutral-alkaline replicon mapping. DNA isolation, benzoylated naphthoylated DEAE-cellulose (BND-cellulose) fractionation, 2D gel electrophoresis, hybridization, and rehybridization were performed as previously described (15, 24).

2D neutral-neutral replicon mapping. 2D neutral analysis was performed as described by Brewer and Fangman (6), with a few modifications. The first-dimension running buffer contained 0.1 μg of ethidium bromide per ml, and the second-dimension buffer contained 0.5 μg of ethidium bromide per ml. The second dimension was run at 6 V/cm without buffer recirculation at 4°C for 3 h.

Preparation of DNA in agarose beads. We used the procedure of Overhauser and Radic (25), which was based on that of Jackson and Cook (18). Yeast cells (8×10^9) were washed three times in SE (SE is 75 mM NaCl and 25 mM EDTA [pH 8]) and suspended in SE at 2×10^9 cells per ml. A 4-ml portion of the suspension was warmed to 45°C and mixed with 4 ml of 1% low-melting-point agarose (45°C). Light mineral oil (20 ml; 45°C) was added, and the suspension was mixed vigorously to form a uniform emulsion. The emulsion was poured into 100 ml of cold SE with stirring, thus forming beads. After centrifugation ($500 \times g$ for 10 min) the pelleted beads (~5 ml) were washed once in SE and suspended in 9.5 ml of SE; 0.5 ml of 2-mercaptoethanol and 5 mg of Zymoly-

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† Dedicated to Arthur Kornberg, a continuing pioneer in DNA replication, on the occasion of his 70th birthday.

ase-100T (Seikagaku Kogyo Co., Ltd.) were added, and the beads were incubated at 37°C for 2 h. After being washed in SE, the beads were suspended in 20 ml of a solution containing 1% (wt/vol) Sarkosyl (ICN Pharmaceuticals, Inc.), 25 mM EDTA (pH 8), and 50 μg of proteinase K (Boehringer Mannheim Biochemicals) per ml and incubated overnight at 50°C. After beads were washed twice in TE (TE is 10 mM Tris hydrochloride [pH 8] and 1 mM EDTA [pH 8]), 50 μl (~1.2 μg of DNA) of the beads was digested with 5 U of *Bgl*I per μg according to the recommendation of the manufacturer and loaded directly into the well for the first dimension of the neutral-alkaline 2D analysis.

Preparation of probes. A restriction map of the ribosomal repeat was compiled by using available sequence data and restriction maps (1–3, 10, 23, 27, 29, 31) and additional conventional restriction mapping. The total length of the repeat unit is 9,084 bp, and the numbering of base pairs starts at the initiation site of the 37S precursor RNA (see Fig. 2).

Probes were prepared from pBR322 plasmids containing *Eco*RI fragments A through G of the ribosomal repeat (a kind gift of Tom Petes) by the random-oligonucleotide primed labeling procedure (9). Probe G is *Eco*RI(45)-*Eco*RI(262) fragment G, with a size of 217 bp; probe HA is the *Eco*RI(262)-*Hin*dIII(458) fragment, with a size of 196 bp; probe HB is the *Hin*dIII(458)-*Eco*RI(2283) fragment, with a size of 1,825 bp; probe MA is the *Eco*RI(2866)-*Mlu*I(3877) fragment, with a size of 1,011 bp; probe E is *Eco*RI(6076)-*Eco*RI(6667) fragment E, with a size of 591 bp; probe PB is the *Pvu*II(7606)-*Eco*RI(45) fragment, with a size of 1,519 bp; probe SB is the *Sma*I(8875)-*Eco*RI(45) fragment, with a size of 254 bp.

Electroelution and electron microscopy. Agarose blocks containing DNA were placed in a dialysis bag with a minimum of TE buffer. The DNA was electroeluted at 5 V/cm in 0.5× TAE (1× TAE is 40 mM Tris-acetate and 2 mM EDTA). After 3 h, the polarity was reversed for 40 s, and the buffer containing the DNA was recovered from the dialysis bag. The DNA was prepared for electron microscopy as described previously (13). 2μm plasmid was used as a size standard.

RESULTS AND DISCUSSION

Neutral-alkaline 2D agarose gel origin mapping. The technique of 2D neutral-alkaline origin mapping has been described previously (15, 24). In summary, chromosomal DNA is isolated from unsynchronized, exponentially growing cells and digested with a restriction enzyme. After digestion, the DNA is enriched for restriction fragments containing replication forks by BND-cellulose chromatography. Double-stranded DNA is eluted from BND-cellulose with salt and is referred to as the salt wash in this paper. Partially single-stranded DNA, including replication-fork-containing DNA, is eluted with caffeine and is referred to as the caffeine wash.

In subsequent 2D electrophoresis, the first (neutral) dimension separates the double-stranded restriction fragments according to size. In the second (alkaline) dimension, the DNA is denatured so that each DNA strand migrates according to its size. After transfer to a nylon membrane, different probes are used to analyze the blot.

Figure 1 is a cartoon of typical patterns obtained by this technique. When a stretch of DNA bounded by restriction enzyme cut sites, R, is replicated by forks moving from left to right (Fig. 1A), then after digestion with the restriction enzyme and 2D gel electrophoresis, probes 1 and 2 generate the patterns shown in Fig. 1B. The nonreplicating intact

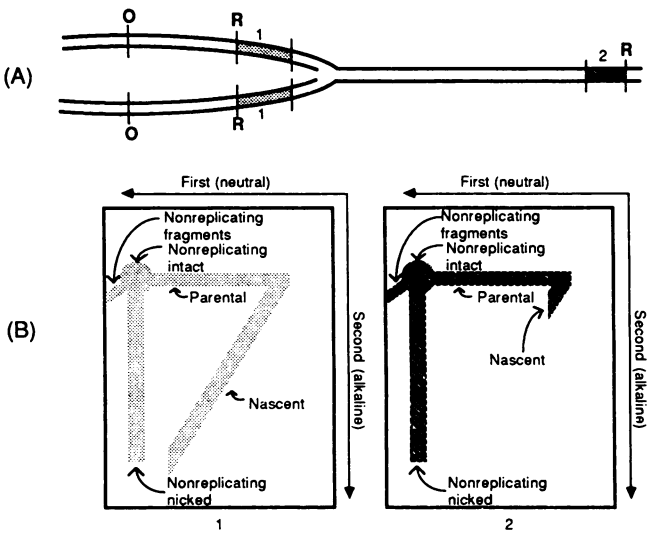

FIG. 1. Schematic diagram of the 2D neutral-alkaline origin mapping technique. See text for detailed explanation. O, Origin; R, restriction enzyme cut sites.

restriction fragments migrate to a position defined in the first dimension by the length of the double-stranded restriction fragment and in the second dimension by the length of the single strand of that fragment.

The vertical smear downwards, called "nonreplicating nicked", is due to nicks in the nonreplicating DNA. In the first dimension, nicked nonreplicating DNA migrates with intact nonreplicating DNA. In the second dimension, however, molecules with one nick generate three DNA strands, one of intact length and two shorter fragments. If the nicks occur randomly along the restriction fragment, a smear is produced (Fig. 1B). A specific nick would generate two spots whose combined length would equal the intact restriction fragment length. Note that detection of the smear or spots depends on choice of probe. A probe located at either end of the restriction fragment would detect a whole size range of single-stranded DNAs generated by nicking (Fig. 1B). However, a probe located in the middle of the restriction fragment would detect only longer nicked strands, and thus the observed smear would be shorter.

The nonreplicating fragments, which form a diagonal line to the left of the "nonreplicating intact" spot, are the result of random double-strand breaks in the chromosomal DNA. If the average size of the prepared chromosomal DNA is 100 kilobase pairs (kb) and the size of the restriction fragment of interest is 10 kb, then 1 in every 10 restriction fragments would contain a double-strand break. These nonreplicating fragments migrate faster in both dimensions than the intact nonreplicating restriction fragments, creating the diagonal shown in Fig. 1B. In some cases (incomplete digestion or partial homology of the probe to larger fragments), a weak signal from this diagonal of nonreplicating fragments may extend to the right of the "nonreplicating intact" spot (see for instance Fig. 3C and D).

The replication-fork-containing restriction fragments migrate more slowly in the first dimension than the nonreplicating restriction fragments, in proportion to their extent of replication. Denaturation in the second dimension generates two parental strands of restriction fragment length and two nascent strands, the size of which ranges from a few nucleotides to full restriction fragment length in proportion to the

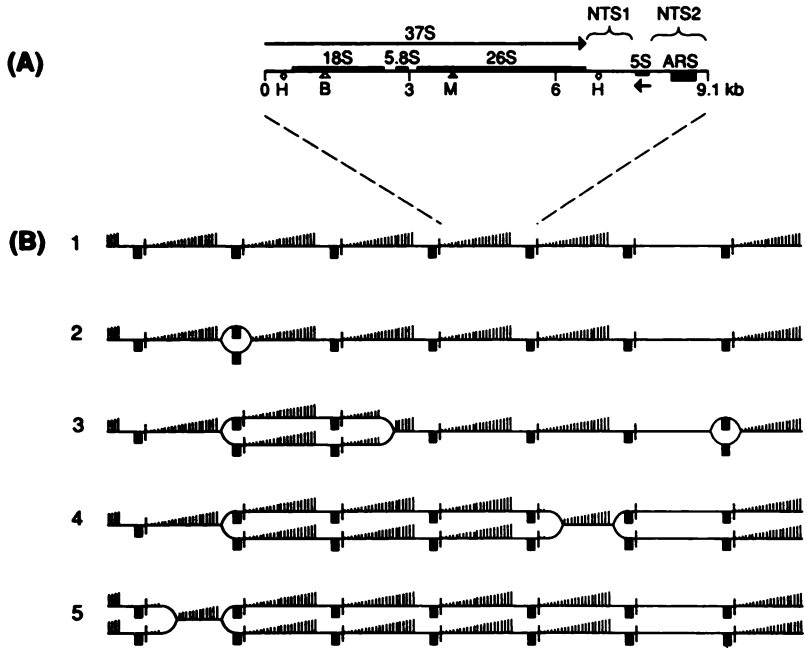

FIG. 2. Model for replication of the rDNA of *S. cerevisiae*. (A) Diagram of a single, 9.1-kb repeat unit. See Materials and Methods for construction of this diagram. Indicated are the directions of transcription of the 37S (0 to 6,570 bp) and 5S (7,715 to 7,835 bp) RNAs (arrows), the two nontranscribed spacer regions (NTS1, 6,569 to 7,714 bp; NTS2, 7,836 to 9,084 bp) and the ARS (black box). The ARS is a *Bsu*I-*Alu*I fragment and is located from 8,357 to 8,928 bp. Restriction sites used in this study are *Hin*dIII (H), sites 458 and 6855 bp; *Bgl*I (B), site 1288 bp; and *Mlu*I (M), site 3877 bp. (B) Schematic diagram of the replication of several repeat units. The ARS elements are indicated by black boxes, and transcription of the 37S precursors is indicated by the vertical lines increasing in length from left to right. See text for further details.

extent of replication, thus generating the nascent line in Fig. 1B. In the example shown, probe 1 detects nascent strands of all sizes, while probe 2 detects only the longest nascent strands.

By using this procedure, the direction(s) of replication fork movement through any restriction fragment can be analyzed. By use of more probes along the restriction fragment, possible internal replication origins can be detected (15, 17, 24).

Model for the replication of the rDNA repeat in *S. cerevisiae*. Yeast rDNA consists of about 120 identical tandemly repeated units. Individual units are 9.1 kb long. The location of the ARS element, the directions of transcription of the 37S precursor RNA and the 5S RNA, and the restriction sites used in this study are shown in Fig. 2A. In this paper, positions within each repeat unit are indicated by their distances in kilobases rightwards from the beginning of the 37S transcript.

The results obtained by 2D origin mapping revealed a much more complex pattern than expected. Careful analysis of all the data suggested a model for the replication of rDNA. To facilitate the explanation of these data, we present the deduced model first. Why this model is favored above other possible models is discussed later.

The data suggested that replication initiates at a position that maps within the ARS element and proceeds bidirectionally (Fig. 2B1 and B2). Replication forks moving leftwards terminate upon arrival at the transcription termination site of the first transcribed 37S RNA region which they encounter (Fig. 2B2 through B4) or at a rightwards-moving replication fork (not shown). Forks moving rightwards continue replication, in the direction of transcription of the 37S RNA, until they meet a fork from the opposite direction, either moving (not shown) or terminated (Fig. 2B4 and B5). At least 95% of the forks moving leftwards terminate at the transcription termination site (see below). Thus, the main direction of replication is in the direction of transcription of the 37S RNA.

Rightwards-moving replication forks frequently proceed through several repeat units before meeting a replication fork from the opposite direction (Fig. 2B). Therefore, not all available origins are used. We estimate that about 1 in 3 to 10 origins is actually used in one round of rDNA replication (see below).

Replication termination at a specific site in the rDNA repeat unit. The results shown in Fig. 3 provide a good illustration of the complex patterns which may be generated during neutral-alkaline 2D origin mapping of yeast rDNA. In these experiments, *Bgl*I-digested DNA was hybridized with a probe covering the ARS element, probe PB (Fig. 3A1). The resulting autoradiograms (Fig. 3C through E) contain many signals not predicted in Fig. 1. The explanation of these extra signals (Fig. 3A and B) supports most of the model proposed in Fig. 2. Conversely, it is easiest to explain the extra signals in terms of that model.

The spots labeled "terminated fork nascent" and "terminated fork parental" (Fig. 3B) are due to the accumulation of strands from the terminated leftwards-moving forks. Terminated-fork-containing restriction fragments (Fig. 3A3) migrate in the first dimension at ~14.5 kb, suggesting an extent of replication of ~5.4 kb in this restriction fragment. However, nonlinear structures migrate in neutral agarose gels more slowly than double-stranded linear fragments of equivalent mass (6). A more accurate estimation of the extent of replication is provided by the size of the nascent strands, ~3.4 kb, detected in the second dimension. The data in Fig.

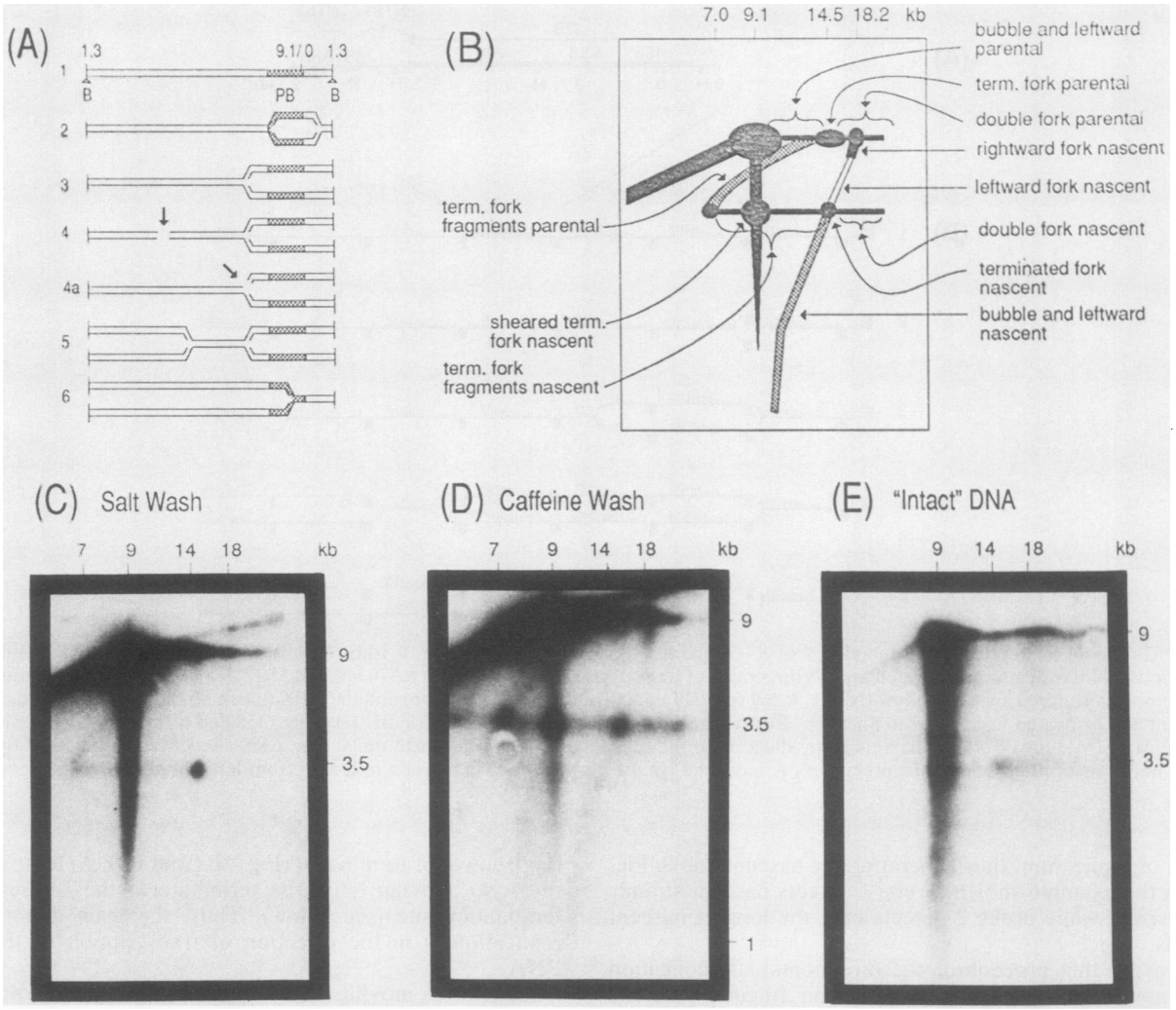

FIG. 3. Neutral-alkaline 2D analysis of *Bgl*I-digested rDNA. (A) Schematic diagram of the possible structures of a *Bgl*I unit during replication. B, *Bgl*I site at 1,288 bp; PB (shaded box), location of probe PB (see Materials and Methods for exact location). (B) Cartoon of panel D. The various indicated lines and spots are discussed in the text. (C) Neutral-alkaline 2D electrophoresis of 1.2 μg of salt wash DNA, hybridized with probe PB. Exposure time, 18 h. (D) Neutral-alkaline 2D electrophoresis of 1.2 μg of caffeine wash DNA, hybridized with probe PB. Exposure time, 3 days. (E) Neutral-alkaline 2D electrophoresis of 1.2 μg of DNA, isolated and digested in agarose beads (18, 25). The agarose beads were loaded directly in the first dimension after digestion. Probe, PB; exposure time, 18 h.

3C and D, combined with the location of the PB probe near the right-hand end of the restriction fragment, suggest that the fork travels from right to left and terminates at about position 7.0 kb in the repeat unit (Fig. 3A1 through A3).

The diagonal arc labeled "terminated fork fragments parental" and the horizontal line labeled "terminated fork fragments nascent" (Fig. 3B) are produced by random double-strand breaks in the nonreplicated part of the restriction fragment, analogous to the nonreplicating fragments in Fig. 1 (Fig. 3A4). In the first dimension these fragments migrated from the size of ~7.0 kb (the fork only) to ~14.5 kb (the full-length terminated-replication-fork-containing restriction fragments). In the second dimension the parental fragments migrated from ~3.4 to 9.1 kb, creating the diagonal arc. The nascent strands of these fragments migrated as a line at ~3.4 kb in the second dimension.

The lines labeled "terminated fork fragments nascent" and "terminated fork fragments parental" and the spot labeled "terminated fork nascent" were detected in both the salt wash (Fig. 3C) and the caffeine wash (Fig. 3D). The abundance of these signals in the salt wash (shorter exposure; Fig. 3C) suggests that terminated replication forks do not expose as much single-stranded DNA as do progressing replication forks. However, a break at the fork itself (a possible "hot spot" for breaks) can remove one of the arms of the fork (Fig. 3A4a). This leads to detection of the spot labeled "sheared terminated fork nascent" (Fig. 3B and D). This spot is detected only in the caffeine wash, possibly because a break at the fork causes exposure of single-stranded DNA. A break at the fork (as in Fig. 3A4a) combined with random breakage in the nonreplicated part of the restriction fragment explains the extension of the line labeled "terminated fork fragments nascent" to the left of the 7-kb mark in Fig. 3D. This extension is absent in the salt wash (Fig. 3C) for the same reason that the spot labeled "sheared terminated fork nascent" is absent.

The lines labeled "double fork nascent" and "double fork parental" (Fig. 3B) are due to replication forks moving in from the left in the terminated-fork-containing restriction fragments, thus creating X structures (Fig. 3A5). In the first

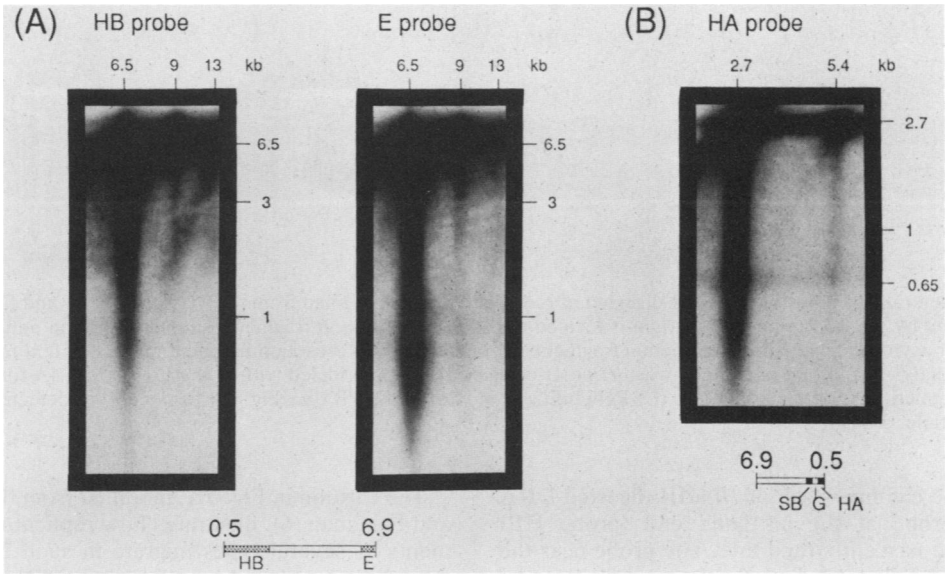

FIG. 4. Neutral-alkaline 2D analysis of *Hin*dIII-digested rDNA. (A) Left panel, Neutral-alkaline 2D analysis of 1.2 μg of caffeine wash *Hin*dIII-digested DNA, hybridized with probe HB; exposure time, 5 h. Right panel, Rehybridization with probe E; exposure time, 18 h. The spot and vertical smear at 9 kb in the first dimension, detected by both probes, are due to incomplete *Hin*dIII digestion of the DNA. The diagram indicates the positions of the probes in the large *Hin*dIII fragment. (B) Rehybridization of the blot from panel A with probe HA; exposure time, 4 days. The diagram indicates the position of the probes HA, G, and SB in the small *Hin*dIII fragment (for exact locations of the probes, see Materials and Methods).

dimension, the X structures migrated more slowly than a linear fragment of ~14.5 kb, in proportion to the progression of the second fork. In the second dimension, the PB probe detected the parental strands at 9.1 kb and the nascent strands of the terminated fork at 3.4 kb. As mentioned above, nonlinear structures migrate more slowly than double-stranded linear fragments of equivalent mass. Therefore, the detected parental and nascent strand lines extend rightwards in the first dimension beyond the size of an almost completely replicated restriction fragment, 18.2 kb.

The caffeine wash shows additional lines labeled "bubble and leftward nascent" and "leftward fork nascent" (Fig. 3B and D) analogous to the line labeled "nascent" in Fig. 1. The detection of nascent strands shorter than 1 kb indicates that the origin of replication is within 0.5 kb (assuming bidirectional replication) of the region covered by the PB probe. The nascent strands are easily detectable up to ~3.4 kb, the size of the nascent strands in the terminated replication forks. Replication to the left beyond the termination point should produce nascent strands longer than 3.4 kb; a very faint signal from longer nascent strands, labeled "leftward fork nascent," is visible in the original autoradiogram of Fig. 3D, suggesting occasional passage of the forks to the left. Densitometric analysis revealed that the intensity of the line labeled "leftward fork nascent" is less than 5% of the intensity of the line labeled "bubble and leftward fork nascent" (data not shown). This suggests that at least 95% of the leftwards-moving forks terminate at ~7.0 kb.

Probe PB detects only the longest nascent strands produced by replication forks travelling from left to right through the restriction fragment (Fig. 3A6), creating the short intense line labeled "rightward fork nascent" (Fig. 3B and D). Comparison of the intensity of this line with the intensity of the line labeled "bubble and leftward nascent" (due to de novo initiation) suggests that replication proceeds largely from left to right through several repeat units. More precise data on the ratio between de novo initiation and rightwards-proceeding replication were obtained by neutral-neutral 2D analysis (see below).

To test whether the lines and spots which we have explained as being the result of random DNA breakage really are due to such breakage, we used DNA that had been prepared without shear after encapsulating the yeast cells in agarose beads (18, 25). This DNA consisted largely of full-size chromosomal molecules, as analyzed by field inversion gel electrophoresis (data not shown). After *Bgl*I digestion, the DNA in the beads was subjected to neutral-alkaline 2D analysis, blotted to a nylon membrane, and then hybridized with probe PB. All the lines and spots attributed to DNA breakage are missing (Fig. 3E), but the spot called "terminated fork nascent" and the line labeled "double fork nascent" are still present (Fig. 3E), strongly supporting the interpretation given above. The absence of nascent-strand diagonals is due to the fact that this DNA could not be enriched for replicating structures by BND-cellulose.

Taken together, these observations indicate the presence of a replication termination site at about position 7.0 kb and the presence of a replication origin close to or at the ARS; the data also suggest mainly unidirectional replication from left to right through the rDNA. This interpretation was further substantiated by use of additional probes and two additional restriction digests, *Mlu*I (data not shown) and *Hin*dIII (see below), as well as by use of a different 2D gel technique and electron microscopy (see below).

Predominantly unidirectional replication through the 37S coding region of rDNA. To further analyze the replication of the ribosomal repeat, chromosomal DNA was digested with *Hin*dIII. This generated two fragments per repeat unit (Fig. 2A; Fig. 4): one 6.4-kb (positions 0.5 to 6.9) and one 2.7-kb (positions 6.9 to 0.5) fragment. The 6.9 position is close to the predicted replication termination site. Therefore, most of the replication forks are expected to move from left (0.5) to right (6.9) in the large *Hin*dIII fragment. To test this prediction, probes at each end of the restriction fragment were

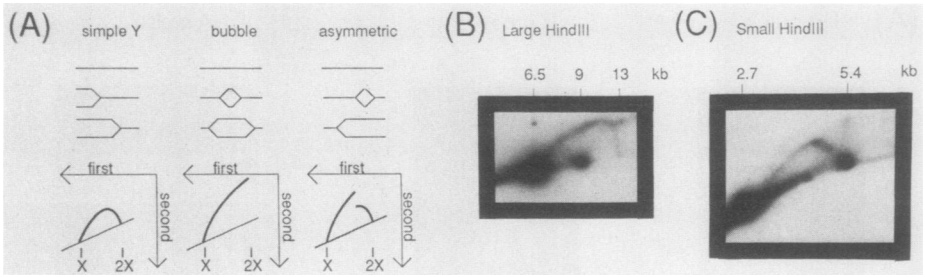

FIG. 5. Neutral-neutral 2D analysis of *Hin*dIII-digested rDNA. (A) Cartoon, modified from the work of Brewer and Fangman (6), showing the patterns generated by this technique if the origin is located outside the restriction fragment (simple Y), in the middle of the restriction fragment (bubble), or asymmetrically in the restriction fragment (asymmetric). X, Restriction fragment mass. See text for further details. (B) Neutral-neutral 2D analysis of 0.3 μg of caffeine wash *Hin*dIII-digested DNA, hybridized with probe E (see Fig. 4A for location), detecting the large *Hin*dIII fragment; exposure time, 15 h. (C) Rehybridization with probe PB (see Fig. 3A for location), detecting the small *Hin*dIII fragment; exposure time, 3 days.

used to analyze the caffeine wash of *Hin*dIII-digested DNA (Fig. 4A). The probe at the left-hand end (probe HB) detected an intense nascent-strand line. The probe near the right-hand end (E) failed to detect any nascent strands. Other results (Fig. 3) suggested occasional (less than 5%) readthrough from right to left. The reason for these different observations is most likely experimental variations. Both results indicate, however, that the main direction of replication is from left to right through the 37S coding region.

The replication origin is at or near the ARS element. The smaller *Hin*dIII fragment contains the ARS element and the putative origin of replication. The position of the origin was estimated by using small probes along this fragment in the analysis of the same blot as in Fig. 4A. The smallest nascent strands detected by probe HA are 0.65 kb long (Fig. 4B), consistent with origin location at position ~0.05 kb. However, probes G and SB (see Fig. 4B and Materials and Methods for location; data not shown) also detected nascent strands of minimum length of ~0.65 kb, consistent with initiation at ~8.7 to 8.9 kb. These inconsistent results may be due to the fact that detection of the shortest strands (less than a few hundred base pairs) is rendered difficult by the shortness of the homologous region and by the diffusion of short strands (17). Alternatively, the events during initiation of replication may be more complex than was assumed in our experimental approach. In any case, the data presented here place the origin of replication at ~8.9 ± 0.3 kb; this position is within the ARS element (see the legend to Fig. 2) but at the right-hand side.

It is interesting to note the horizontal line at 0.65 kb due to a specific nick (Fig. 4B). The nick is located at position 8.9 kb (0.65 kb from the right-hand *Hin*dIII site), inside the ARS fragment, and it coincides with a topoisomerase I consensus site (position 8884 to 8899 bp) identified by Bonven et al. (4).

Neutral-neutral 2D analysis of the ribosomal repeat. Brewer and Fangman (6) developed a 2D neutral-neutral agarose electrophoresis technique for mapping replication origins and characterizing replicating restriction fragments. Their technique exploits the fact that nonlinear DNA molecules migrate more slowly than linear molecules of the same mass, and this retardation is enhanced by high voltage, high agarose concentration, low temperature, and high ethidium bromide concentration. After a first-dimension run, identical to the neutral-alkaline first dimension, the second dimension is run under conditions designed to maximize the retardation of nonlinear molecules. After transfer to a nylon membrane, the blot may be hybridized with any probe recognizing the restriction fragment of interest.

The cartoon in Fig. 5A, modified from the work of Brewer and Fangman (6), illustrates how replicating restriction fragments of several types migrate in such 2D gels. Simple Y structures, produced by unidirectional replication through a restriction fragment, migrate as an arc which rises above and then returns to the line of simple linear fragments (Fig. 5A). Replication intermediates containing bubbles migrate as an arc which originates at the linear line. If the origin is located asymmetrically in the restriction fragment, replication intermediates contain bubbles until one of the forks has reached the end of the restriction fragment. From that point onwards they are simple Ys. This generates a composite signal (Fig. 5A).

Replication intermediates of the large *Hin*dIII fragment are expected to have a simple Y shape. Comparison between the result of probing a neutral-neutral 2D gel of the caffeine wash of a *Hin*dIII digest with probe E (Fig. 5B) and the cartoon (Fig. 5A) shows that the replicating large *Hin*dIII fragment has the predicted shape. The spot at 9 kb and the faint arc originating from it are due to incomplete digestion of the DNA with *Hin*dIII.

Replication intermediates of the small *Hin*dIII fragment are predicted to have either of two shapes. If the replication origin inside the fragment is used, a composite pattern of bubble and Y forms will be generated because of the asymmetric location of the origin. If the internal origin is not used, the fragment will replicate as a simple Y because of a fork coming from the left (>95%) or right (<5%).

Reprobing the gel of Fig. 5B with the PB probe revealed that there is a mixture of both possibilities (Fig. 5C). From 2.7 kb to 4.0 ± 0.3 kb in the first dimension, the arcs due to replication bubbles (clearly visible in original) and Y-shaped intermediates are both detected. Because of abnormal migration of nonlinear structures in the first dimension, the actual size of the replicating fragments at transition from bubble to Y shape is difficult to estimate but is probably slightly less than 4 kb. The maximum possible bubble size at the transition is about 1.3 kb. Therefore, the origin of replication is likely to be located slightly less than 0.65 kb from the right-hand end of the small *Hin*dIII restriction fragment, at about position 8.9 kb or slightly greater. From about 4.0 to 5.4 kb in the first dimension all the replication intermediates are of the Y form. The intensity of this portion of the arc (relatively high in Fig. 5C) is variable in similar experiments.

The intense spot close to the linear line at 5.4 kb in the first dimension is due to detection of accumulated terminated forks within the small *Hin*dIII fragment. The fact that the

spot is detected at nearly the fully replicated size of the small *Hin*dIII fragment suggests that the termination is very close to the left-hand end of this fragment. No equivalent signal due to a terminated fork was detected in Fig. 5B; if present, such a spot would be located at about 12.8 kb in the first dimension. Therefore the termination site must be to the right of position 6854 bp in the rDNA repeat unit, at ~7.0 kb.

The intensity of the arc due to Y structures is about five times the intensity of the line due to bubble structures (by densitometry). This indicates that, in this experiment, on average one of six of the possible origins was used during each S phase. However, some variation has been observed in other experiments (data not shown), with intensity ratios ranging from about 3 to about 10.

Visualization of terminated replication forks. If replication forks terminate at a specific site, restriction fragments containing the terminated forks should accumulate in a neutral-neutral 2D gel as a spot, above the linear line (Fig. 5A). To reduce interference from nonterminated replication intermediates (Fig. 3C and D), the salt wash fractions of *Mlu*I and *Bgl*I restriction digests were run in neutral-neutral 2D agarose gels. After transfer to nylon membranes and hybridization with an rDNA probe, spots of material accumulated at the expected size could be detected in both digests (Fig. 6A). The nature of the additional spots and streaks in both digests is not known.

The autoradiographs were superimposed on identical gels which had not been transferred, and the corresponding spots were cut out of the gel. After electroelution the DNA was visualized with the electron microscope (Fig. 6B).

The forks recovered from *Mlu*I-digested DNA consist, as expected, of two long arms and one short arm (Fig. 6B). *Bgl*I-digested DNA contains forks with one long arm and two short arms (Fig. 6B). Analysis of replication forks from each digest showed that the position of fork termination fell within the range 6.85 ± 0.3 kb (for 21 of 24 observed forks in the *Bgl*I digest and for 16 of 21 observed forks in the *Mlu*I digest).

Replication of the yeast rDNA. Yeast rDNA could theoretically be replicated in different ways. Apart from the model shown in Fig. 2B there are numerous other possibilities. For example, instead of being initiated from specific sequences, replication could start from random sites. Also, replication could proceed bidirectionally, either initiating at each origin or using a subset of available origins. Alternatively, there might be no initiation sites in the rDNA, and the whole locus would then be replicated from outside origins (note, however, that this would require an unusually high fork movement rate to replicate the entire 1,200 kb of the rDNA repeat). For each of these models it is possible to predict the expected 2D gel patterns in the experiments we have performed. However, the patterns detected during our study could not be explained by any of the models mentioned above. The only model we have been able to imagine which satisfactorily explains the experimental data is the model presented in Fig. 2B.

First, both neutral-alkaline (Fig. 3D and 4B) and neutral-neutral (Fig. 5C) 2D gel analyses suggest that a single bidirectional origin is located at 8.9 ± 0.3 kb, at the right-hand end of the ARS-containing restriction fragment identified by Skryabin et al. (29). Second, data obtained by neutral-alkaline 2D gel analysis (Fig. 3), supplemented with data from neutral-neutral analysis (Fig. 5C) and electron microscopy (Fig. 6), suggest that leftwards-moving replication forks terminate at position ~7.0 kb. Third, neutral-alkaline gel analyses (Fig. 3D and 4A) show that replication through the 37S coding region is predominantly in the direction of transcription. Fourth, data obtained with both 2D techniques (Fig. 3D, 4A, and 5C) indicate that only 1 of 3 to 10 possible origins is used in one round of replication. These conclusions together lead to the additional inference that most rDNA is replicated unidirectionally (Fig. 2).

Electron microscopy studies of rDNA replication in *Drosophila melanogaster* (22) and *S. cerevisiae* (28) are in good agreement with the proposed model. In neither study was countertranscriptional entry of replication forks into active large rDNA transcription units observed. Furthermore, Saffer and Miller (28) were able to map the centers of small replication bubbles to a position within NTS2 identical (within experimental error) to the origin mapped by us.

The replicon size reported by Saffer and Miller (28) is 1 to 3 repeat units. Because of the technical limitations of electron microscopy, larger replicons may not have been detected. Using a sucrose gradient method, Walmsley et al. (32) estimated an average replicon size of 5 repeat units. Our data suggest an average size varying from 3 to 10 units, depending on cell strain and growth conditions. The consensus of all three studies is that not all potential origins are used during a single round of replication.

Recently, Hernandez et al. (11) claimed to have detected replication termini in the rDNA of pea root cells by using a neutral-alkaline 2D gel technique. However, the fact that the nicked restriction fragments observed by these investigators were not retarded in the first dimension suggests that they were not due to replication-fork-containing structures. Their relationship to rDNA replication remains to be determined.

Our finding that replication of yeast rDNA is predominantly unidirectional in the direction of the major transcript (37S precursor) is consistent with the prediction by Brewer (5) that long, heavily transcribed regions should be replicated in the direction of transcription. Her prediction is based on a striking correlation between the directions of replication and transcription through the larger genes in *Escherichia coli* and on the likelihood of potential conflict between converging RNA and DNA polymerases in large genes. Smaller genes, such as the 121-bp 5S RNA gene in yeast rDNA, are probably transcribed and replicated rapidly enough that the probability of unresolvable polymerase conflict is low (5). Therefore, the yeast 5S RNA gene should not interfere with replication forks moving rightwards through more than 1 repeat unit (Fig. 2B3 and B4).

As discussed by Brewer (5), termination of leftwards replication at ~7.0 kb (near the 3′ end of the 37S region) could be caused by conflict with RNA polymerases. However, available data do not exclude the possibility of specific (polar) replication terminator sequences at that location. Further work is required to distinguish these possibilities.

Note that although leftwards replication terminates at ~7.0 kb, the 3′ end of the 37S precursor is at ~6.8 kb (19). The ~200-bp interval between the transcription and replication termination sites might be due to accumulation of positive superhelical turns ahead of both polymerases (21), which would prevent a direct protein-protein collision (5).

Our observation of predominantly unidirectional replication in yeast rDNA may seem surprising in view of the current paradigm that eucaryotic chromosomal replication is bidirectional. However, unidirectional replication was noticed previously in fiber autoradiographic studies of mammalian DNA replication (14, 16) and was estimated to occur in 10% of mammalian replicons (16). Because rDNA constitutes less than 1% of mammalian DNA, additional chromo-

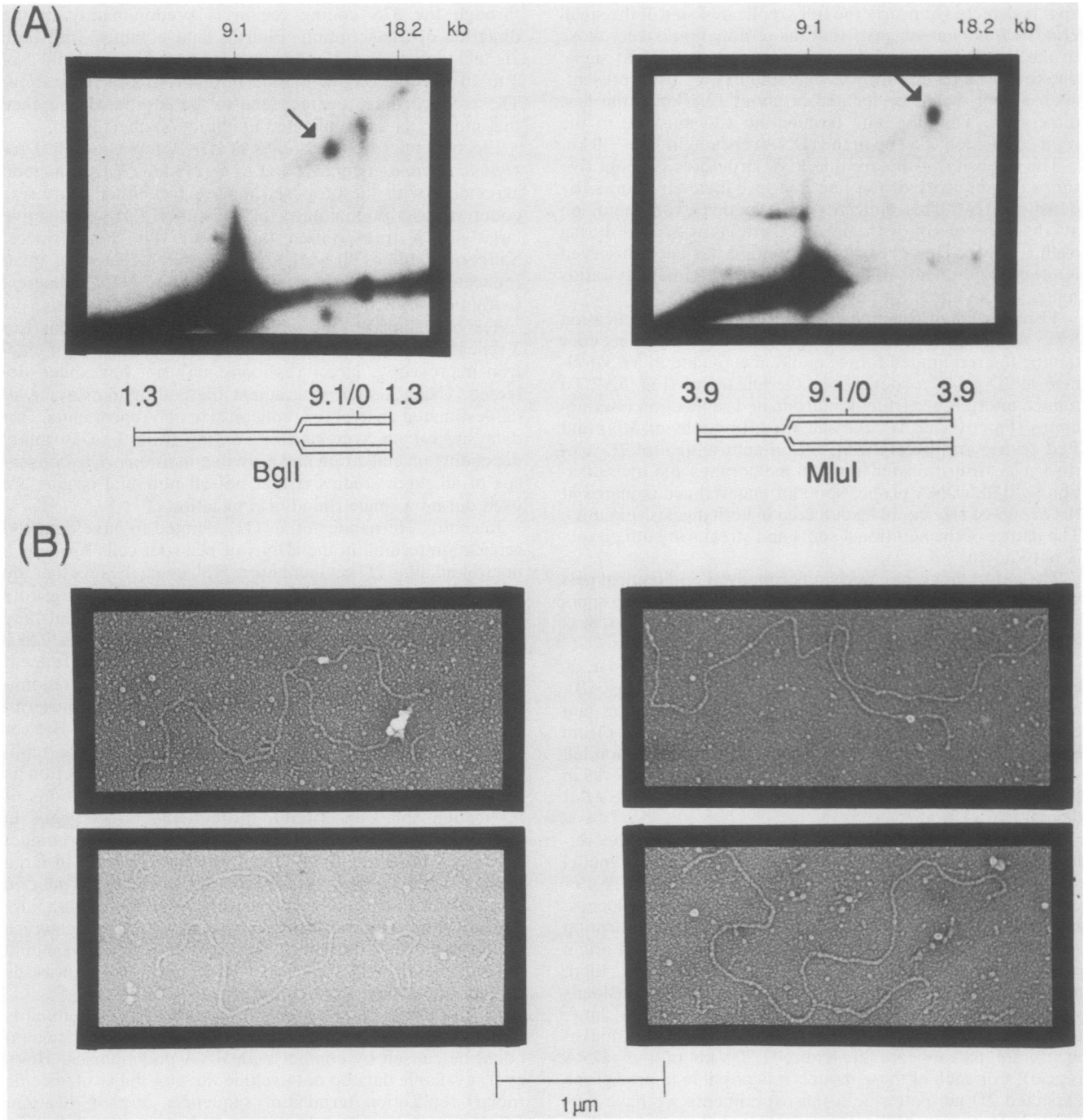

FIG. 6. Electron microscopy visualization of accumulated terminated replication forks. (A) Neutral-neutral 2D analysis of 0.3 μg of salt wash DNA, digested with *Bgl*I (left panel) or *Mlu*I (right panel) and then hybridized with probe MA (see Materials and Methods for exact location); exposure time, 5 h. Arrows indicate the spots due to accumulation of the terminated-fork-containing restriction fragments. Diagrams show the predicted structures of the fragments if termination is at 7.0 kb. (B) Electron microscope pictures of replication forks. After digestion with either *Bgl*I or *Mlu*I, 2 μg of salt wash DNA was run on gels identical to those shown in panel A. The autoradiographs shown in panel A were superimposed on the gels, and the corresponding spots were cut out of the gels. After electroelution from agarose the DNA was prepared for electron microscopy visualization (see Materials and Methods). Left panels, *Bgl*I-digested DNA; right panels, *Mlu*I-digested DNA.

some regions (not just rDNA) must also replicate unidirectionally in mammalian and, presumably, yeast cells.

Our results show that the yeast rDNA replication origin colocalizes with an ARS element. It has recently been demonstrated that a replication origin on yeast chromosome III also colocalizes with an ARS element (17). These observations support the idea that yeast replication origins are ARS elements, but many more origins must be mapped before the extent of correlation between ARS elements and origins can be accurately determined.

Our inability to detect nascent strands shorter than 650 bases near the rDNA origin and the presence of a specific

nick in this region suggest that the initiation events at this origin may be more complex than previously imagined. More accurate mapping of the initiation site(s) in this region is now under way.

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