MOLECULAR AND CELLULAR BIOLOGY, Oct. 1993, p. 6600–6613
0270-7306/93/106600-14$02.00/0

Vol. 13, No. 10

Initiation and Termination of DNA Replication in Human rRNA Genes

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Received 1 March 1993/Returned for modification 24 March 1993/Accepted 24 July 1993

We have used the multicopy human rRNA genes as a model system to study replication initiation and termination in mammalian chromosomes. Enrichment for replicating molecules was achieved by isolating S-phase enriched populations of cells by centrifugal elutriation, purification of DNA associated with the nuclear matrix, and a chromatographic procedure that enriches for molecules containing single-stranded regions, a characteristic of replication forks. Two-dimensional agarose gel electrophoresis techniques were used to demonstrate that replication appears to initiate at multiple sites throughout most of the 31-kb nontranscribed spacer (NTS) of human ribosomal DNA but not within the 13-kb transcription unit or adjacent regulatory elements. Although initiation events were detected throughout the majority of the NTS, some regions may initiate more frequently than others. Termination of replication, the convergence of opposing replication forks, was found throughout the ribosomal DNA repeat units, and, in some repeats, specifically at the junction of the 3′ end of the transcription unit and the NTS. This site-specific termination of replication is the result of pausing of replication forks near the sites of transcription termination. The naturally occurring multicopy rRNA gene family offers a unique system to study mammalian DNA replication without the use of chemical synchronization agents.

The genetic determinants for initiation of DNA replication in mammalian cells have proven difficult to identify and characterize unequivocally. Recent data suggest that in contrast to the site-specific initiation of replication in bacteria, the yeast *Saccharomyces cerevisiae*, and mammalian viruses, origins of replication in higher eukaryotic chromosomes may be more complex than previously envisaged (for reviews, see references 5, 21, 32, and 56). A great deal of interest has thus focused on determining the sites where replication initiates, with the aim of identifying *cis*-acting regulatory sequences.

Considerable progress has been made in identifying DNA sequences that serve as replication origins in *S. cerevisiae*. A number of sequences that allow plasmids to replicate autonomously in *S. cerevisiae* have been identified (reviewed in references 15 and 27). Two-dimensional (2D) gel electrophoresis replicon mapping techniques have been used to show that only about half of the 10 autonomous-replicating-sequence elements on chromosome III that have been reported function as origins in their normal chromosomal context (reference 22 and references therein). These 2D gel methods are based on the analysis of steady-state levels of replicating molecules and provide information about their conformation (9) and the direction of replication fork movement (59).

The replication of several extrachromosomal mammalian viral genomes has also been studied by 2D gel techniques. Replication of the 7.9-kb bovine papillomavirus genome proceeds bidirectionally from a specific origin mapped to an approximately 60-bp segment (68, 76). Termination of replication occurs 180° opposite the origin in a zone rather than at a specific DNA sequence (64). Evidence has also been presented that replication initiates at only some of the potential origins in oligomeric forms of bovine papillomavirus DNA (64, 76). Replication of the simian virus 40 genome also initiates at a specific site and terminates 180° from the origin (reviewed in reference 16). The origin of replication (*oriP*) of the large 172-kb Epstein-Barr virus (EBV) in its latent cycle functions in a somewhat different manner. The 1.8-kb *oriP* segment contains two essential components, a dyad symmetry element and a family of repeated sequences (FR). From analyses of plasmids containing *oriP*, replication was found to initiate at or near the dyad symmetry element and to proceed bidirectionally until one fork reaches the FR, where it is stalled by a replication fork barrier (28). The other fork progresses through the remainder of the plasmid and pauses when it reaches the opposite side of the FR. Thus, the EBV *oriP* contains both the initiation and the termination sites for DNA replication.

Autonomous replication assays for mammalian origins of replication have not revealed evidence for site-specific initiation. Krysan et al. (46), for example, in efforts to isolate autonomous-replicating-sequence elements from human DNA, have constructed a vector which contains a truncated copy of the EBV *oriP* and the gene encoding the only viral protein required for viral replication, EBV nuclear antigen 1 (EBNA-1). The portion of *oriP* that remains contains the FR which is thought to provide a nuclear retention function. In these experiments, the insertion of almost any human DNA fragment of appreciable size (average sizes about 10 kb) allows these plasmids to replicate autonomously in human cells (36). Furthermore, 2D gel analyses of one of these plasmids indicated that initiation of replication occurred at multiple sites throughout a large portion of the plasmid (45). These data suggest that the genetic elements for initiation of replication in mammalian chromosomes may be much larger or more complex than in the simpler systems described above.

The replication of the Chinese hamster ovary (CHO) dihydrofolate reductase (DHFR) locus has been studied in

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great detail. Determinations of the timing of replication within the region (52), the direction of fork movement (33), the bias in the template strand utilized for leading- or lagging-strand synthesis (13, 14), and the size and abundance of nascent strands across the locus (70) have delineated a relatively small region (0.5 to 2 kb) in which an origin may be located. Two-dimensional replicon mapping techniques, however, have provided evidence that initiation occurs at multiple sites spanning an approximately 55-kb region distal to the DHFR gene, encompassing the region noted above (23). The possibility remains that multiple origins exist in the region but that preferred initiation sites may be utilized under certain conditions.

Recently, the 2D gel techniques have also been used to examine replication in a region of chromosome III of the fission yeast *Schizosaccharomyces pombe* (77). Replication in this locus appears to initiate at multiple sites, although the initiation zone is much smaller (3 to 5 kb) than in the mammalian systems. Replication initiation during amplification of chorion genes in *Drosophila melanogaster* (20, 35) and DNA puff II/9A in *Sciara coprophila* (53) has also been examined by the 2D gel methods. In both cases, initiation occurred at multiple sites within a 6- to 8-kb zone, although a higher incidence of initiation was detected within the central region. In contrast, initiation was detected randomly throughout the tandemly repeated histone genes in *Drosophila* embryos and cultured cells (65, 66).

In an attempt to gain further insight into mammalian chromosomal DNA replication, we have undertaken an analysis of the replication of human rRNA genes. Approximately 400 ribosomal DNA (rDNA) repeats, each 44 kb in length, are clustered in tandem repeats in the nucleolar organizer regions of the five acrocentric autosomes (chromosomes 13, 14, 15, 21, and 22). This naturally occurring multigene family encodes a 13-kb primary transcript which is processed in the nucleolus to yield the mature 18, 5.8, and 28S rRNAs. The remainder of the repeat unit, the 31-kb nontranscribed spacer (NTS), is thought to provide a number of functions including the regulation of transcription by RNA polymerase I, the signals for initiation of DNA replication, signals for recombination, and elements necessary for the assembly and organization of the nucleolus (for a review, see reference 31).

Studies of the replication of rRNA gene repeat units have been carried out for several organisms. Origins of replication have been identified in the NTS of *S. cerevisiae* (27, 54, 62), *Physarum polycephalum* (72), *Tetrahymena thermophila* (67), the sea urchin *Lytechinus variegatus*, (7), the pea *Pisum sativum* (37, 69), and *Xenopus laevis* (8) rDNA by a variety of approaches. In murine rDNA, several short (~400-bp) NTS sequences have been shown to increase the frequency of amplification-dependent transformation of mouse cells (73, 74). Using 2D gel analyses, we provide evidence here for initiation of replication throughout most of the NTS region of human rDNA. Initiation was not detected within the transcription unit, the promoter region, or the transcription termination region. This delocalized pattern of initiation is similar to the initiation zone detected in the DHFR locus in CHO cells (23, 71).

Although extensive work has been done on replication initiation, very little is known about replication termination in eukaryotic chromosomes. Replication termination in the circular *Escherichia coli* and *Bacillus subtilis* chromosomes occurs opposite the origins in a region flanked by terminator sites, thus forming a replication fork trap (for a review, see reference 39). Comparable mechanisms for termination have not been observed in eukaryotic chromosomes which contain multiple origins of replication. In *S. cerevisiae*, termination of replication spanning a 4.3-kb zone between two origins in a region of chromosome III has been observed (78). This is apparently the result of forks converging at random sites within this region rather than termination at specific sequences. However, a polar barrier to replication forks entering the 3′ end of the rRNA transcription units has been observed in *S. cerevisiae* (10, 54) and in *P. sativum* (38). We report here the presence of a replication barrier in a similar location in human rDNA and demonstrate that replication forks are stalled in both directions. In addition, site-specific termination of replication occurs in this region in some rDNA repeat units. This region has previously been studied in detail since it contains binding sites for proteins that play a role in the termination of mouse, rat, and human rRNA transcription (29, 43, 48, 60).

MATERIALS AND METHODS

Cell culture and centrifugal elutriation. The three human cell lines used in these studies were grown in suspension culture in RPMI 1640 supplemented with 10% fetal calf serum unless otherwise noted. The human lymphoblastoid cell lines 721 (41) and Raji (61) are transformed with the EBV. K562, a human erythroleukemia cell line (57), was grown in medium supplemented with 15% Serum Plus (JRH Biosciences) instead of fetal calf serum. Cells were maintained as exponential cultures (i.e., at densities between 2 and 6×10^5 cells per ml) prior to experiments for the analysis of replicative forms. Fractionation of cells according to their position in the cell cycle was done by centrifugal elutriation as described by Brown et al. (12).

DNA probes. All human rDNA clones were kindly supplied by J. Sylvester, Hahnemann University, Philadelphia, Pa. The probes used were as follows: C_{EB}, a 404-bp *Eco*RI-*Bam*HI fragment; C_{HB}, a 470-bp *Hin*dIII-*Bam*HI fragment; C_{PE} a 684-bp *Pst*I-*Eco*RI fragment; B_{ES}, a 1.2-kb *Eco*RI-*Sal*I fragment; A_{BB}, a 1.4-kb *Bam*HI-*Bam*HI fragment; D_{ES}, a 961-bp *Eco*RI-*Sal*I fragment; D_{AS}, a 386-bp *Apa*I-*Sac*I fragment; D_{PB}, a 538-bp *Pvu*II-*Bam*HI fragment; and D_{XX}, a 300-bp *Xba*I-*Xba*I fragment (see Fig. 2 for probe locations). Some of these probes have been described previously (75).

DNA isolation and restriction endonuclease digestion. Nuclear matrix-associated DNA was isolated as described elsewhere (method E in reference 24), with the following modifications. After the cells had been collected by centrifugation at 1,600 rpm for 25 min in a Beckman J6B centrifuge, 1.5×10^8 cells were washed once in 25 ml of cell wash buffer lacking digitonin. After recentrifugation at 1,600 rpm for 15 min, the cell pellet was resuspended in 40 ml of cell wash buffer containing 0.1% digitonin. Isolation of nuclei and matrices was as described, except that the centrifugation conditions for the entire preparation were adapted for the Beckman J6B centrifuge. Most of the DNA preparations utilized the restriction endonuclease *Eco*RI; however, in some experiments, *Hin*dIII was used. RNase T_1 (Worthington Biochemical Corporation) was included at 270 U/ml during the incubation with RNase A (15 μg/ml). The proteinase K concentration was reduced to 250 μg/ml, and the incubation time was reduced to 2 h. For some experiments, matrix-associated DNA was digested with additional restriction endonucleases prior to benzoyl naphthyl DEAE (BND)-cellulose chromatography. Approximately 50 μg of matrix-associated DNA from asynchronous cultures and 10 to 30 μg from S-phase enriched cell populations were used for BND-

cellulose chromatography as described by Dijkwel et al. (24). After precipitation and resuspension, the entire caffeine wash was loaded in a single lane for 2D gel analysis.

High-molecular-weight total genomic DNA was isolated from S phase enriched cell populations as described elsewhere (1), except that the proteinase K treatment was at 50°C instead of 65°C. Special care was taken during all manipulations to minimize shear by mixing gently and transferring DNA solutions with wide-bore pipettes and pipette tips. After the final dialysis, 500 μl of the highly viscous DNA was equilibrated in appropriate restriction endonuclease buffer (final volume, 3 ml) on ice for 1 h with occasional gentle mixing. Restriction endonuclease (3,000 U for each enzyme used; New England Biolabs), RNase T_1 (270 U/ml; Worthington Biochemical Corporation), and RNase A (15 μg/ml) were added and the mixture was incubated at 37°C for 3 h with occasional mixing. An additional 3,000 U of restriction endonuclease was added, and the digest was incubated for an additional 3 h. Sodium chloride was added to 0.3 M, and the DNA was precipitated by the addition of 2 volumes of ethanol. The DNA was collected by centrifugation and dissolved in 0.5 ml of 10 mM Tris-HCl (pH 8)–1 mM EDTA (TE). When the digestion was incomplete, incubation with the restriction endonuclease was repeated with 1,000 U for 2 h. Approximately 150 μg was then adjusted to 0.5 ml with TE containing 0.3 M NaCl prior to BND-cellulose chromatography. After precipitation and resuspension, the DNA from the entire caffeine wash was loaded in a single lane for 2D gel electrophoresis. DNA prepared by this method was used only for the experiment shown in Fig. 3; all other experiments utilized matrix-associated DNA.

Two-dimensional agarose gel electrophoresis. Neutral-neutral 2D gel electrophoresis of fragments between 2 and 5 kb was performed as previously described by Brewer and Fangman (9). For restriction fragments between 5 and 19 kb, we have modified the conditions described by Krysan and Calos (45). DNA restriction fragments were first separated in 0.28% agarose gels (Bio-Rad high-strength analytical grade) in TBE (89 mM Tris-HCl [pH 8], 89 mM boric acid, 2 mM EDTA) at room temperature. The voltage and times were varied for different-sized fragments as follows: fragments of 5 to 9 kb were separated at 0.66 V/cm for 40 to 48 h, fragments of 9 to 12 kb were separated at 0.45 V/cm for 65 to 72 h, and fragments of 12 to 19 kb were separated at 0.34 V/cm for 90 to 96 h. Gels were stained with 0.3 μg of ethidium bromide per ml in TBE for 15 min, and the lane containing molecular weight markers was photographed. The sample lane was excised without exposure to UV light, rotated 90°, and placed at the top of a gel tray, and then a 0.58% agarose gel containing 0.3 μg of ethidium bromide per ml in TBE was poured. Second-dimension conditions for all fragment sizes above 5 kb were 0.89 V/cm for 40 to 48 h at room temperature in TBE containing 0.3 μg of ethidium bromide per ml.

The neutral-alkaline 2D gel conditions were modified from the methods of Nawotka and Huberman (59). The segment analyzed in Fig. 5 was a 12-kb *Eco*RI restriction fragment; conditions described above for 12- to 19-kb fragments in neutral-neutral 2D gel analysis were used in the first dimension. The segment analyzed in Fig. 8 was a 5.8-kb *Eco*RI-*Bam*HI restriction fragment; conditions described above for fragments of 5 to 9 kb were used in the first dimension. The gels were treated as described above, except that 0.8% agarose (see Fig. 5) or 1% agarose (see Fig. 8) in water was used for the second-dimension gel. The solidified gels were placed in an electrophoresis apparatus containing circulating alkaline electrophoresis buffer (40 mM NaOH, 2 mM EDTA) and incubated at room temperature for 1 h. Electrophoresis was at 0.57 V/cm for 48 h (see Fig. 5) or 24 h (see Fig. 8). To visualize the molecular weight markers, gels were stained with 0.5 μg of ethidium bromide per ml in neutralization solution (0.5 M Tris-HCl [pH 7.5], 1.5 M NaCl) for 45 min. After being photographed, gels were rinsed with two to three changes of water for 15 min prior to Southern transfer.

Southern transfer and hybridization. DNA was transferred to GeneScreen Plus membranes (NEN) according to the recommendations of the manufacturer. Gels were treated with 0.25 M HCl for 15 min, then twice for 15 min each in 0.4 N NaOH–0.6 M NaCl, followed by two 15-min treatments in 0.5 M Tris-HCl (pH 7.5)–1.5 M NaCl. The transfer solution was 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). After transfer, the membranes were treated for 45 s in 0.4 N NaOH and then for 5 min in 0.2 M Tris-HCl (pH 7.5)–2× SSC and air dried overnight. Filters were prehybridized at 65°C for at least 2 h in 1 M NaCl–1% sodium dodecyl sulfate (SDS)–10% dextran sulfate. Probes labeled by the random primer method (0.5 to 1 × 10^6 cpm/ml of hybridization solution) were mixed with sonicated salmon sperm DNA (final concentration of 100 μg/ml of hybridization solution), boiled for 10 min, chilled on ice, and added directly to the prehybridization solution. Poly(A)$_n$ (Sigma) was used as carrier with probes A_{BB} and D_{ES} (see Fig. 2) because of probe homology to salmon sperm 28S rDNA sequences. After 24 to 48 h, the membranes were washed twice in 2× SSC for 5 min each at room temperature, twice for 15 min each in 2× SSC–1% SDS at 65°C, and then once in 0.4× SSC–0.1% SDS at room temperature for 5 min. If excessive signal remained, the transfers were washed in 0.4× SSC–0.1% SDS at 65°C for 30 min. Transfers were exposed to X-ray film (Kodak X-Omat AR) with intensifying screens at −70°C. Exposures were from 1 to 21 days. If additional hybridizations were performed, the probe was removed from the membranes by placing them in boiling 0.1× SSC–0.1% SDS and allowing the solution to return to room temperature.

RESULTS

Strategy for the enrichment of replicative intermediates. Because of the relatively low levels of replicating molecules from specific genomic loci in asynchronous populations of mammalian cells, we used a series of enrichment steps prior to analysis of replicative intermediates from human rDNA. First, cells from an exponential culture were fractionated by size by centrifugal elutriation (12). Since cellular DNA content is proportional to size, cells from various positions of the cell cycle can be isolated by this technique. By collecting cells in S phase (i.e., cells actively engaged in DNA synthesis), the percentage of cells in G_1, G_2, and mitosis is reduced and the proportion of replicative intermediates in DNA preparations is thereby increased severalfold. The isolation of cells at specific positions in S phase also permits studies of the temporal order of DNA replication. One advantage in using this technique for obtaining a synchronous cell population is that chemical treatments which may influence normal replicative processes are avoided. The majority of the experiments described here were performed with DNA isolated from early-, middle-, or late-S-phase cells. A second enrichment step involved isolation of DNA associated with the nuclear matrix as described by Dijkwel et al. (24). This procedure enriches approximately 10-fold for replicative intermediates and, importantly, minimizes the

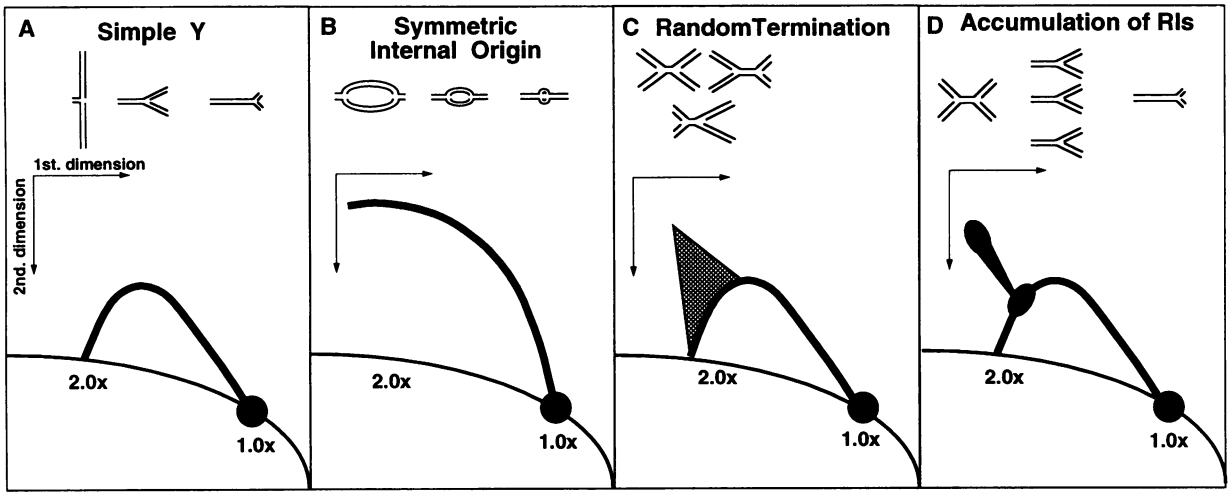

FIG. 1. Patterns generated by branched DNA molecules upon neutral-neutral 2D gel electrophoresis. The first dimension of the 2D gel separates DNA molecules according to their mass, whereas the second dimension is designed to maximize the effect the shape of a DNA molecule has on its migration. Four different types of replication fork movement through a DNA segment are illustrated in panels A through D. At the top of each panel, the branched molecules that are generated as the DNA segment doubles in mass are shown in the relative positions to which they will migrate in the first dimension. The position to which a segment of linear double-stranded DNA will migrate after the second dimension is indicated along the arc at the bottom of each diagram. The positions of linear double-stranded DNA of mass 1× and 2× are indicated. (A) A simple Y pattern is generated by replication forks traversing the segment from one end to the other from an initiation site outside the segment. (B) A bubble pattern is generated from a DNA segment that contains a symmetrically located origin of replication. (C) A diffuse triangular region in which replicative intermediates emanating from the simple Y arc are detected indicates the convergence of opposing replication forks at multiple sites throughout a segment. (D) An accumulation of Y forms at a specific site indicates the presence of a barrier to replication fork movement. The accumulation of double-Y forms indicates replication termination at a specific site. RI, replicative intermediate.

shear and branch migration of replicative intermediates encountered in conventional DNA isolation procedures. A final enrichment step relied on the selective adsorption to BND-cellulose of DNA molecules containing single-stranded regions, a characteristic of replication forks. This method provided an additional 10-fold enrichment. Precautions were taken throughout all manipulations to control shear by gentle handling and to limit the time of restriction endonuclease digestion.

Replication initiation appears to occur at multiple sites in the NTS but is not detected in the transcription unit. In order to determine the structures of replicative intermediates spanning human rDNA repeat units, we utilized the neutral-neutral 2D agarose gel electrophoresis method developed by Brewer and Fangman (9). This technique permits visualization of the steady-state levels of molecules in the process of replication. Three classes of intermediates are observed (Fig. 1): molecules containing a single fork originating from an external origin and progressing from one end of a fragment to the other (simple Y forms), molecules containing a bubble due to an internal initiation site, and molecules in which two replication forks converge (double-Y forms). DNA molecules (including replicative intermediates) are separated primarily by size in the first dimension. As a fragment is replicated, its mass doubles, so that the replicative intermediates derived from that segment are distributed over a portion of the gel lane. The migration of DNA molecules in the second dimension is influenced primarily by shape; branched intermediates containing replication forks thus migrate more slowly than linear molecules. Since each type of replicative form has a different shape, the population from each family (i.e., Y forms, bubbles, and double-Y forms) migrates in a unique arc or pattern (Fig. 1). To facilitate our investigation of the replication of human rDNA, we have modified the 2D gel conditions of Brewer and Fangman (9) and Krysan and Calos (45) so that replicative intermediates of fragments up to about 18 kb can be visualized. Electrophoresis conditions were optimized for each fragment examined in order to avoid distortion of the 2D gel patterns (40, 45).

A series of autoradiographs representing 2D gel analyses of restriction fragments from the human rDNA repeat unit is shown in Fig. 2. A map of human rDNA with relevant restriction sites, segments analyzed, and probe locations is also shown. Patterns consistent with replication initiation (bubble arcs) were detected from all NTS segments tested (for example, patterns 1, 2, 3, 4, 5, 6, and 8 shown in Fig. 2). Bubble arcs were consistently not observed in segments composed solely of transcription unit sequences (Fig. 2, patterns 7 and 9). The ratios of the intensity of bubble arcs to Y arcs are apparently not identical in all regions of the NTS. The strongest bubble arc signals have consistently been observed in the region of the segment in which pattern 3 was obtained (Fig. 2). Slightly weaker bubble patterns were observed for segments in region D (see pattern 8, for example), while the segment in which pattern 1 was obtained produced very weak bubble patterns (Fig. 2).

Additional evidence for the lack of initiation in transcribed sequences is shown by patterns 2 and 3 and 4 and 5 (Fig. 2). In these examples, an *Eco*RI restriction site polymorphism results in the detection of replicative intermediates from two distinct segments. The shorter fragments (Fig. 2, patterns 3 and 5) are composed entirely of NTS sequences and display bubble patterns, whereas the larger fragments (patterns 2 and 4) contain both the same NTS sequences as well as transcription unit sequences and exhibit very weak bubble

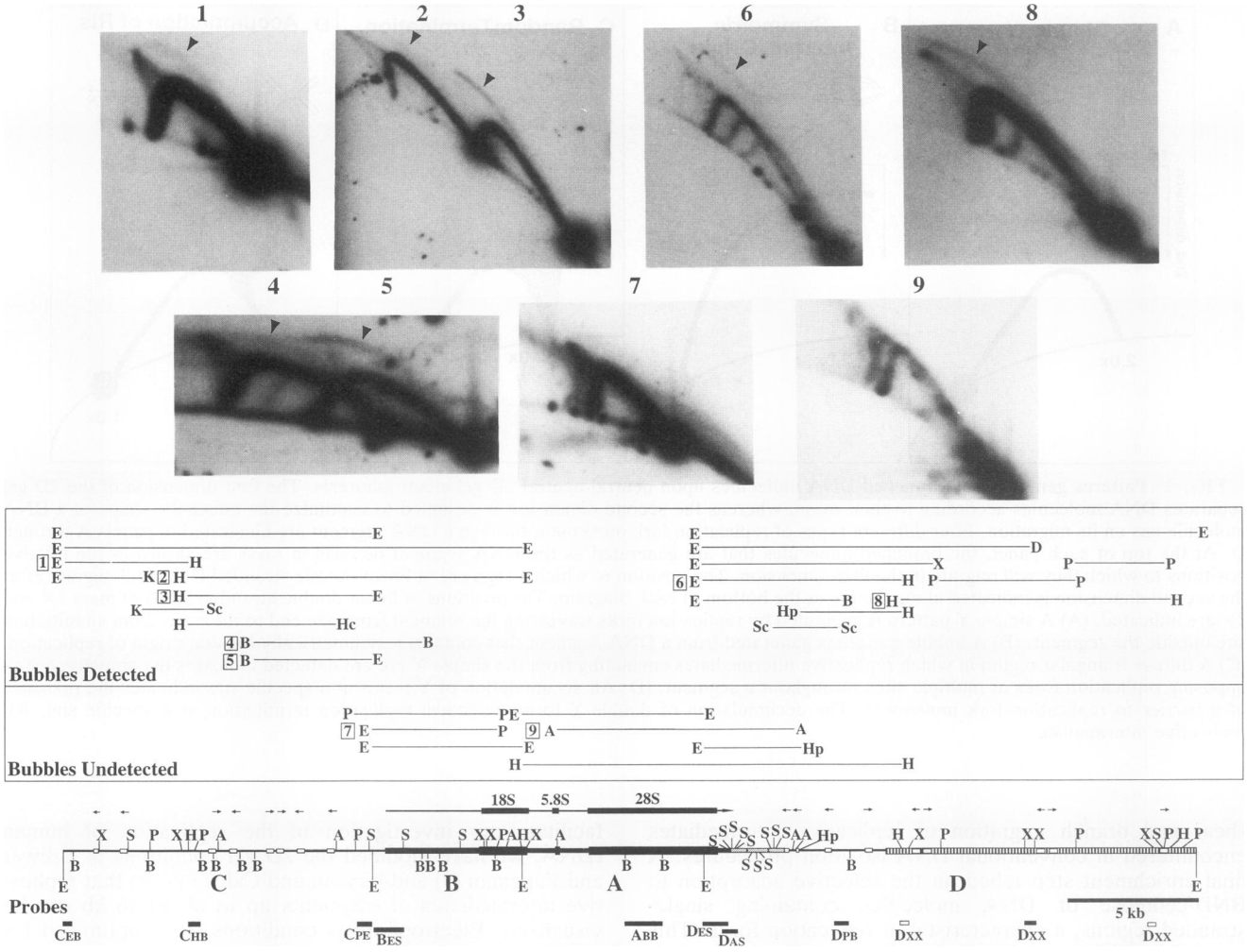

FIG. 2. Replication initiation appears to occur at multiple sites in the NTS of human rDNA. Enrichment for replicating molecules was achieved by isolation of cells in various positions of S phase by centrifugal elutriation, purification of nuclear matrix-associated DNA, and BND-cellulose chromatography. Southern transfers of neutral-neutral 2D agarose gels were hybridized to probes for human rDNA. Each tandemly repeated 44-kb rDNA repeat unit is distinguished by four *Eco*RI restriction fragments (A, B, C, and D). The long arrow above the map corresponds to the transcribed region, with the 18, 5.8, and 28S rRNA-coding regions shown as thicker rectangles. The long striped rectangle on the map represents the region of the rDNA repeat that has not been sequenced. The three stippled rectangles near the 3′ end of the transcription unit identify repeat elements that are present in variable numbers in different rDNA repeats (26, 51). Small open boxes indicate the locations of *Alu* repetitive elements; the small arrows above them denote their relative orientations. The locations of some of the relevant restriction sites are shown (A, *Ase*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; P, *Pst*I; S, *Sal*I; X, *Xba*I). The probes used in hybridizations are shown below the map. The D_{XX} probe cross-hybridizes to two other regions (open boxes) because of a 5-kb tandemly repeated sequence in the NTS (63). Restriction fragments used in 2D gel analyses of replication intermediates are schematized above the map as thin lines. The restriction endonucleases used are shown for each fragment (the restriction endonucleases *Hin*cII [Hc], *Kpn*I [K], and *Sac*I [Sc] are not shown on the map). The numbered panels displayed above the schematic fragments show the results of 2D gel analysis for restriction fragments indicated on the map by boxed numbers. Arrows point to bubble arcs. The two arcs with patterns 2 and 3 and 4 and 5 result from an *Eco*RI restriction site polymorphism. Pattern 1 is from early-S-phase K562 cells, 2 and 3, 4 and 5, and 8 are from late-S-phase Raji cells, 6 and 7 are from late-S-phase K562 cells, and 9 is from asynchronous Raji cells. The *Eco*RI restriction fragments within the transcription unit labeled A and B were each analyzed at least five times. Exposures (more intense than pattern 9) of 2D gels of the A segment contained significant background because of probe homology with the carrier DNA (data not shown). Bubble arcs were not observed in these longer exposures.

patterns. The shorter fragments serve as an internal control to demonstrate that bubble arcs could be detected in the same 2D gel. In order to most reliably detect molecules containing bubbles, the site for initiation should lie in the middle third of the fragment (55). The weaker bubble patterns from the two fragments containing both spacer and transcribed sequences (Fig. 2, patterns 2 and 4) suggest that initiation is not occurring to a significant extent within the central regions of those segments. Interestingly, the promoter elements for rRNA transcription are located there, indicating that replication initiation does not occur in that portion of the NTS. Similarly, we have not detected initiation in the region where transcription termination occurs (60).

In experiments in which initiation was not detected within a particular restriction fragment, we have reprobed those

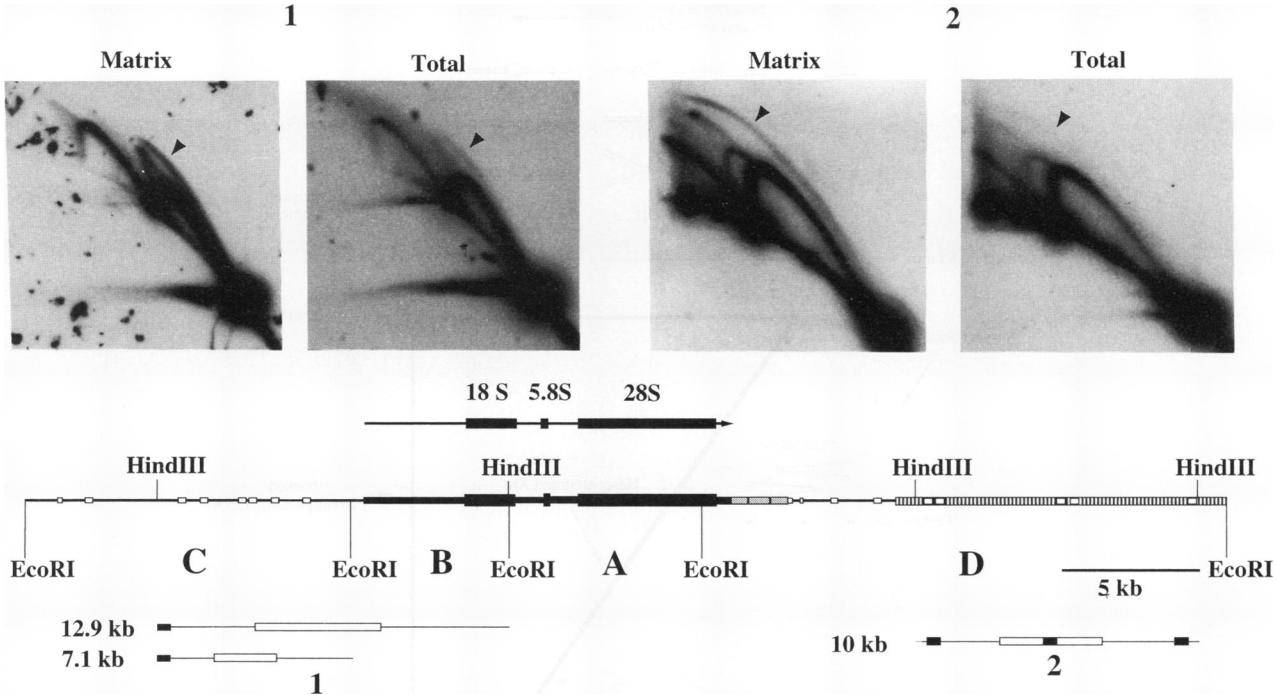

FIG. 3. Initiation patterns observed in DNA associated with the nuclear matrix are also seen in total DNA. A map of human rDNA with the locations of relevant restriction sites is shown at the bottom. Other features on the map are the same as those described in the legend to Fig. 2. The numbered lines below the map correspond to restriction fragments analyzed in 2D gels with total DNA and nuclear matrix-associated DNA. The open rectangles indicate the middle third of the fragment, the region in which bubble patterns are most easily observed. The shaded rectangles show the locations of the probes. The 2D gel patterns designated 1 are from late-S-phase Raji cells, and patterns designated 2 are from late-S-phase K562 cells. Arrows point to bubble arcs. The multiple arcs are due to restriction site polymorphisms and are not the result of partial digestion. Similar results were obtained with the two methods of DNA preparation.

transfers for fragments that have yielded evidence for initiation. A summary of all restriction fragments analyzed is shown in Fig. 2, arranged according to the presence or absence of bubble patterns. The majority of the restriction fragments shown were analyzed at least twice; some have been reproduced in different cell types and during different intervals of S phase with similar results. Most of the patterns obtained from NTS segments are composites of multiple types of replicative intermediates. In addition to weak bubble patterns, a strong complete Y arc was observed in all NTS fragments tested, indicating that some initiation events occurred from origins located outside the fragment in some repeat units.

Two types of replication termination were observed. First, termination appeared to occur throughout the repeat unit, as evidenced by the triangular hybridization pattern emanating from the Y arcs (schematic diagram in Fig. 1 and examples in Fig. 2). We interpret these replication intermediates to be due to the convergence of opposing replication forks at multiple locations across the repeat on the basis of comparable patterns seen for replication termination in the bovine papillomavirus genome (64). Accumulations of replicative intermediates along the Y arcs with patterns 6 and 9 shown in Fig. 2, however, suggested that replication forks were stalled at a specific location within those restriction fragments. A more detailed characterization of replicative intermediates from this region is presented below.

In order to demonstrate that bubble patterns were not an artifact of the nuclear matrix isolation procedure, we prepared total cellular DNA by more conventional techniques. The method we have used includes very gentle lysis conditions and centrifugation through a sucrose step gradient. This DNA isolation procedure was developed specifically to minimize shear and to eliminate exposure to organic solvents prior to the cloning of large fragments of DNA into yeast artificial chromosome vectors (1). Figure 3 shows a comparison of 2D gel patterns from nuclear matrix-associated DNA and total DNA for restriction fragments in the NTS. The two DNA preparation methods yielded similar patterns. Comparisons of 2D gel patterns from transcribed regions with the two types of DNA preparations gave similar results as well, suggesting that the lack of bubble patterns in transcribed regions was not unique to the matrix-associated DNA preparation. We have also performed a control experiment with matrix-associated DNA isolated from circulating leukocytes. Since these cells are primarily in the G_0-G_1 phase of the cell cycle, they should contain very low levels of replicative intermediates. Transfers from 2D gels were hybridized with a probe specific for rDNA and subsequently with total human DNA. Film exposures of up to 3 weeks exhibited very weak signals from replicative intermediates containing a single replication fork but not bubbles (data not shown), indicating that the artifactual generation of replicative intermediates did not occur during the isolation of matrix-associated DNA.

Replication forks proceed in both directions in the NTS. We have also used the neutral-alkaline 2D gel method (59) to analyze the direction of replication fork progression in portions of the rDNA repeat. The first-dimension conditions are identical to those for the neutral-neutral 2D gels. The

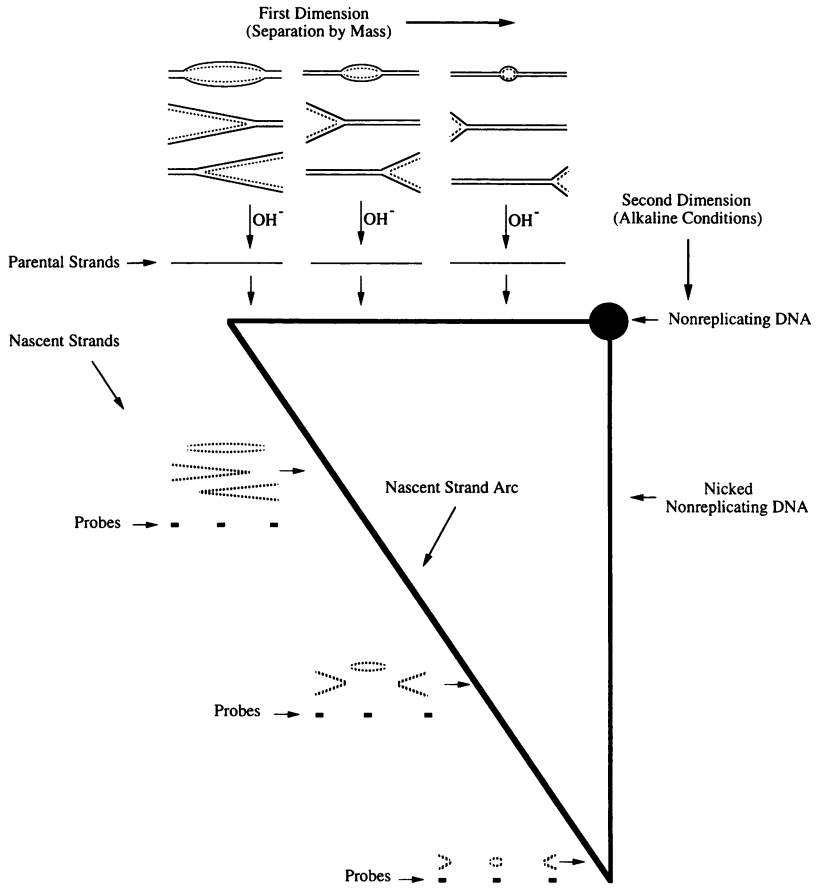

FIG. 4. Neutral-alkaline 2D gel patterns generated by various replicative intermediates. At the top, three populations of replicative intermediates of different sizes, separated by mass, in the first dimension are shown. Within each size class, three types of replicative intermediates are depicted: molecules containing a bubble and molecules containing a fork originating from an origin external to the fragment and entering either the left or the right terminus of the fragment. The second-dimension gel is performed under alkaline conditions such that nascent strands (dashed lines) are separated from parental strands (solid lines). The denatured parental strands are all of the same size and migrate as a horizontal streak in the 2D gel. The liberated nascent strands are of various sizes and migrate as a diagonal arc. The three populations of nascent strands shown at the bottom left are derived from the intact replicative intermediates shown at the top. For clarity, they are reproduced here as shown at the top; however, they are denatured and thus single stranded. Three probes are noted below each of the three populations of nascent strands. Sequential hybridization of a Southern transfer with several probes at different locations within the segment analyzed provides information about the direction of replication fork movement and the location of initiation sites.

conditions for the second dimension, however, differ from those used for the neutral-neutral 2D gels in that electrophoresis is performed under alkaline conditions. Nascent strands are thus separated from parental strands and migrate according to their size (diagram in Fig. 4). Probes are chosen from multiple sites within the fragment analyzed to detect the sizes of nascent strands in various regions of the segment. This technique reveals information regarding the direction of replication fork movement and the location of initiation sites within a particular segment. Figure 5 shows the analysis of a 12-kb *Eco*RI restriction fragment from the NTS of human rDNA. The same transfer membrane was hybridized with probes from both ends and the middle of the restriction fragment. Both terminal probes (Fig. 5; C_{EB}, left pattern, and C_{PE}, right pattern) identify a diagonal nascent strand arc. These nascent strands are shown as dashed lines on the graphic representation of the corresponding replicative intermediates. In both cases, the diagonal arc extends to very small sizes in the second dimension, indicating that some replication forks originating outside the fragment progress through the entire segment from left to right (probe C_{EB}) and right to left (probe C_{PE}). The slight difference in signal intensity between the two terminal probes is probably due to the lack of initiation within the transcription unit and promoter region (which is coincident with the right terminus) as well as the pausing of forks at the 3′ end of the transcription unit. These two factors may result in fewer forks progressing from right to left in this region. The slight variation in intensity along the diagonal nascent strand arcs probably results from differences in the rate of replication fork progression. In contrast to the two terminal probes, the probe derived from the middle of the fragment (Fig. 5; C_{HB}, middle pattern) detects nascent strands from forks that originate from within as well as from outside the segment. Nascent strands from external initiation sites are detected only when their size is greater than the distance from the end of the fragment to the position of the sequence used as probe. Since the probe (C_{HB}) is approximately in the middle of the fragment, these nascent strands detected are from 5 to 6 kb to 12 kb (the size of the full-length fragment). This is

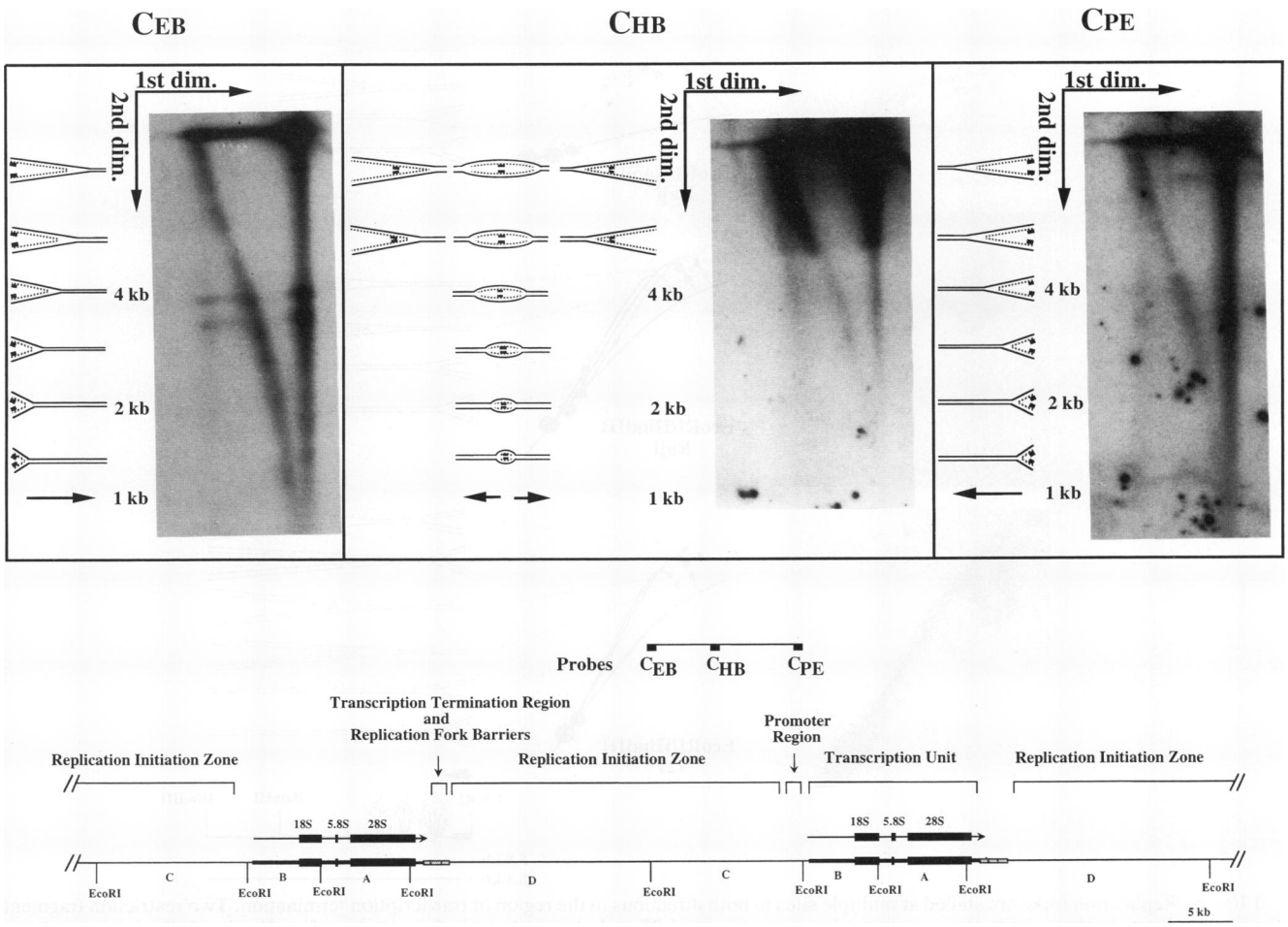

FIG. 5. Replication forks proceed in both directions in the NTS: additional evidence for initiation in the NTS by neutral-alkaline 2D gel electrophoresis. Neutral-alkaline 2D gel electrophoresis was performed on matrix-associated DNA isolated from K562 cells in early S phase. The map at the bottom shows two tandemly repeated rDNA units. The organization of rDNA in most organisms is generally accepted to be clusters of tandem repeats, although it is possible that other types of higher-order organization may exist. Portions of the rDNA repeats noted above the map include the promoter region, the transcription unit, the region where transcription terminates, the replication initiation zone, and the replication barrier region. The line above the map shows the 12-kb *Eco*RI restriction fragment, and the shaded rectangles indicate the positions of the probes used in hybridizations (C_{EB}, C_{HB}, and C_{PE}). The upper portion of the figure shows the results of hybridization of the identical 2D gel transfer with each probe. The left portion of each panel provides an interpretation of the structures of replicative intermediates that contribute to the observed patterns. In the left panel, the probes (shaded rectangles) detect nascent strands (dotted lines) of all sizes, indicating that replication forks are progressing from left to right through this fragment. The right panel demonstrates that forks also progress from right to left. The middle panel, however, shows the results for a probe (C_{HB}) located in the middle of the fragment. This probe gives a very strong signal from nascent strands larger than 5 kb. In addition, a weaker nascent strand signal extending to smaller sizes can be seen, indicative of initiation in the vicinity of the probe sequence. The faint spots and horizontal lines seen near 4 kb with probes C_{EB} and C_{PE} are probably due to site-specific nicks in nascent and parental strands of the restriction fragment. Vertical lines of hybridization are the result of random nicks in the parental strands.

shown in Fig. 5 as an abrupt decrease in the intensity of the arc at 5 to 6 kb. Nascent strands that originate from within the segment should produce a fainter signal that extends to smaller sizes with probe C_{HB}. The presence of these strands in the fragment shown in Fig. 5 indicates that some initiation events occur in the middle of the fragment analyzed.

Replication barriers and termination. Our analysis of the replicative intermediates from human rDNA revealed barriers to replication fork progression near the sequences involved in transcription termination. The transcription termination factor TTF1 has been shown to interact with a repeated sequence element in this region (*Sal* boxes; each binding site contains a *Sal*I restriction site) to promote termination of RNA polymerase I transcription of mouse, rat, and human rDNA (29, 43, 48, 60). In human rDNA, this region exhibits a length polymorphism previously described in detail (26, 51). A 600- to 800-bp fragment can be tandemly repeated up to four times; as a result, several Y arcs are seen for fragments spanning this area in 2D gel analyses. In addition, restriction site polymorphisms occur at multiple sites within the NTS region (2, 26, 47, 51, 63). These variations are observed in the 2D gels as additional spots along the arc of linear molecules and are not the result of partial digestion.

Figure 6 shows a series of 2D gel experiments that localize the barriers in more detail. Two pairs of spots with an

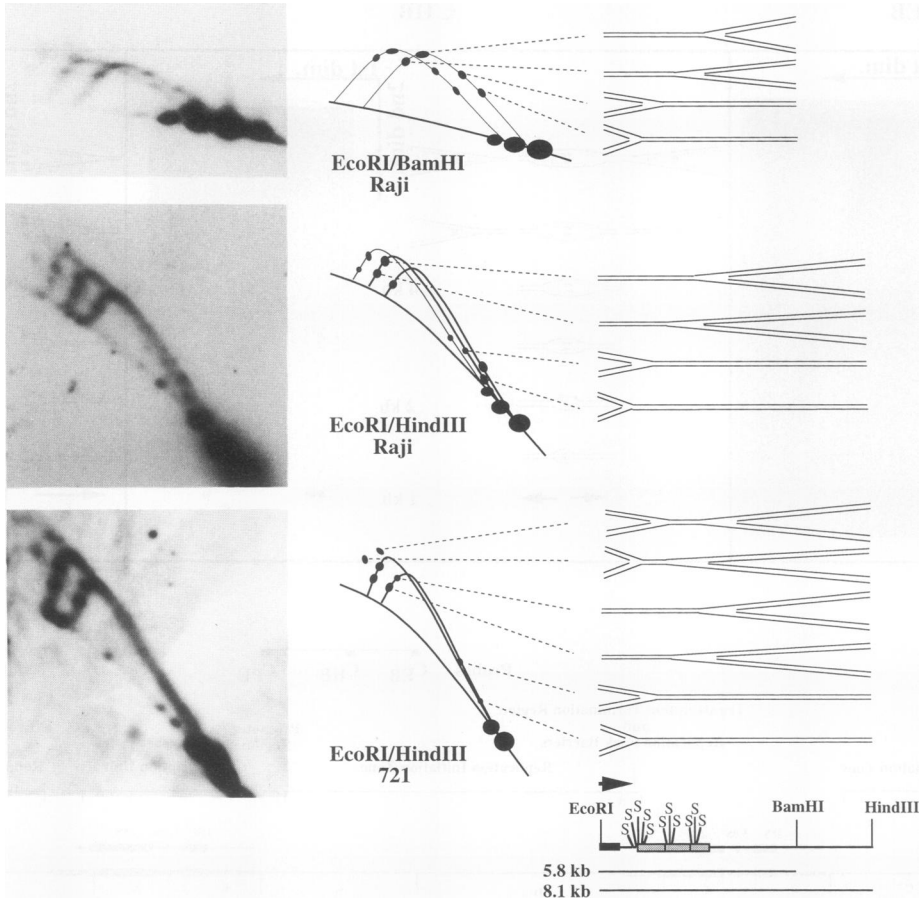

FIG. 6. Replication forks are stalled at multiple sites in both directions in the region of transcription termination. Two restriction fragments (horizontal lines, bottom right) were analyzed by neutral-neutral 2D gel electrophoresis for pausing of replication forks in the region of transcription termination. The map at bottom right shows *Sal*I restriction sites (S) to denote the locations of *Sal* boxes (transcription termination factor binding sites [see text]). The three stippled boxes represent tandemly repeated elements in this region (see text). The arrow shows the direction of transcription and the approximate location of transcription termination. Autoradiographs of 2D gel analyses for the 5.8-kb *Eco*RI-*Bam*HI fragment from late-S-phase Raji cells and the 8.1-kb *Eco*RI-*Hin*dIII fragment from late-S-phase Raji cells and asynchronous 721 cells are shown on the left. The spots of greater intensity along the Y arcs correspond to the accumulations of specific single-Y forms. The illustrations on the right provide an interpretation of the structures of these replicative intermediates. The lower autoradiograph also shows the accumulation of double-Y structures (termination patterns) in the cell line 721 (the complexity of the polymorphisms in the Raji cell line obscures these accumulations).

intensity greater than that of the remainder of the Y arc are shown. One pair is present on the ascending portion of the arc, consistent with the interpretation that forks progressing in the direction of transcription are blocked. The other pair is present on the descending portion of the arc, consistent with the presence of a barrier to forks progressing in the direction opposite to the direction of transcription. This is demonstrated by the relative positions of the spots when eight segments of different sizes are examined (Fig. 7). This analysis permitted us to locate the pause sites in the region involved in transcription termination described above. The relative signals from molecules containing stalled forks are different in the three cell lines studied. The K562 cell line appears to generate weaker signals than either 721 or Raji cells (compare panel 6 in Fig. 2 with Fig. 6). Similar results were obtained for cells in different intervals of S phase. The accumulation of molecules containing forks blocked in the direction opposite to that of transcription appears to be greater than in the direction of transcription. Signals indicative of replication termination due to the convergence of forks moving in opposite directions were observed at multiple sites within the transcribed region. This implies that the barriers may not be absolute and that some leftward-moving forks pass through the barriers and enter the transcribed region (for example, Fig. 2, pattern 7). It is possible, however, that in some repeat units forks are efficiently stalled. In addition to the accumulation of specific Y-shaped molecules, we have also observed accumulations of double-Y forms corresponding to the convergence of two replication forks specifically in the region of the barrier (Fig. 6, lower panel).

In order to study the barriers further and to map their location in greater detail, we performed neutral-alkaline 2D gel electrophoresis of segments in this region. Figure 8 shows a neutral-neutral and the equivalent neutral-alkaline 2D gel for a 5.8-kb *Eco*RI-*Bam*HI fragment from asynchronous 721 cells. The neutral-neutral 2D gel is overexposed to show the double-Y accumulations. Shorter exposures gave

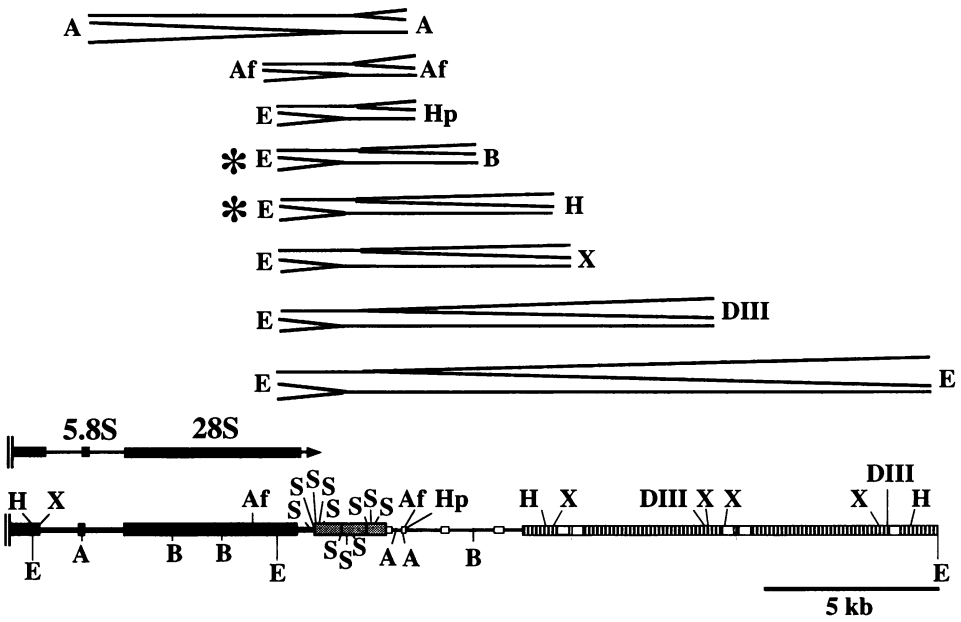

FIG. 7. Summary of restriction fragments containing stalled forks: the barriers are located in the transcription termination region. Eight restriction fragments in which pausing of replication forks was detected by neutral-neutral 2D gel electrophoresis are shown at the top. Replication fork pausing in these fragments was consistently observed in the transcription termination region in both directions. The letters correspond to the restriction endonucleases used, as described in the legend to Fig. 2; DIII, *Dra*III site. The map is as described in the legend to Fig. 2. The two restriction fragments shown in Fig. 6 are indicated by asterisks.

patterns similar to that seen for the same restriction fragment in Raji cells (Fig. 6, top panel). The accumulations of nascent strands in the neutral-alkaline gel were detected with probes from both ends of the fragment. Several spots with a more intense signal were observed along the diagonal nascent strand arc. The sizes of these nascent strand accumulations relative to the two fragment termini are consistent with the barriers being located within the tandem repeats and functioning in both directions. Accumulations corresponding to double Ys in the neutral-neutral 2D gel were also visualized in the neutral-alkaline gel. The nascent strands from these molecules can be seen to the left of the diagonal arc in Fig. 8 and are of the same length as those seen on the diagonal arc. The two probes from either end of the fragment identify accumulations of nascent strands of various sizes which are consistent with strands originating from molecules containing single forks stalled at the replication barriers. Comparable experiments were performed with the Raji cell line with similar results (data not shown).

DISCUSSION

Replication initiation. We have analyzed the replication of human rDNA as a model for the replication of mammalian chromosomes. The replication of this gene family in various organisms has been studied in many laboratories, primarily because it occurs naturally in multiple copies. This feature makes the analysis easier because of the higher levels of replication intermediates. We have found that initiation of replication in human rDNA appears to occur at multiple sites throughout the majority of the NTS region but not in the transcription unit, the promoter region, or the transcription termination region. Most of the segments we examined in the NTS exhibited a composite pattern of replicative intermediates. This indicates that, in a subset of repeat units, a particular segment may contain a replication fork that originates from an external origin which proceeds from one end to the other while the same segment in another set of repeat units may contain initiation sites. In still other copies of this segment in different repeats, two forks may converge at different locations, resulting in termination of replication at multiple sites. These data indicate that different repeat units can utilize different origins of replication. We do not know if a single initiation event occurs for each repeat or whether initiation occurs in a subset of the repeats, as observed in yeast rDNA (10, 54). It is also possible that multiple origins may be used in some of the repeats. Three different cell lines (two EBV-transformed B-cell lines, Raji and 721, and the K562 erythroleukemia cell line) have given similar results. None of the experiments reported here were performed with cultures treated with chemical synchronizing agents.

Several control experiments were carried out to provide evidence that the rDNA molecules containing branches and bubbles we observed in 2D gels were replicative intermediates. First, these molecules were not observed in nonreplicating human B lymphocytes. Second, they were enriched severalfold in DNA prepared from S-phase cells compared with cells from exponential cultures. Third, in preparations of early-replicating DNA we have observed bubbles in only one of two partially overlapping polymorphic restriction fragments (data not shown). Both of these segments contain bubbles when replicated late in S phase. This result indicates that the bubbles are associated with the replication of the DNA rather than being induced during DNA isolation by exposure to extracts from S-phase cells. An additional control in which bubbles were observed in DNA obtained from sucrose gradients indicated that bubbles were not formed as an artifact when DNA was prepared from the nuclear matrix. The size of the large replication bubbles we

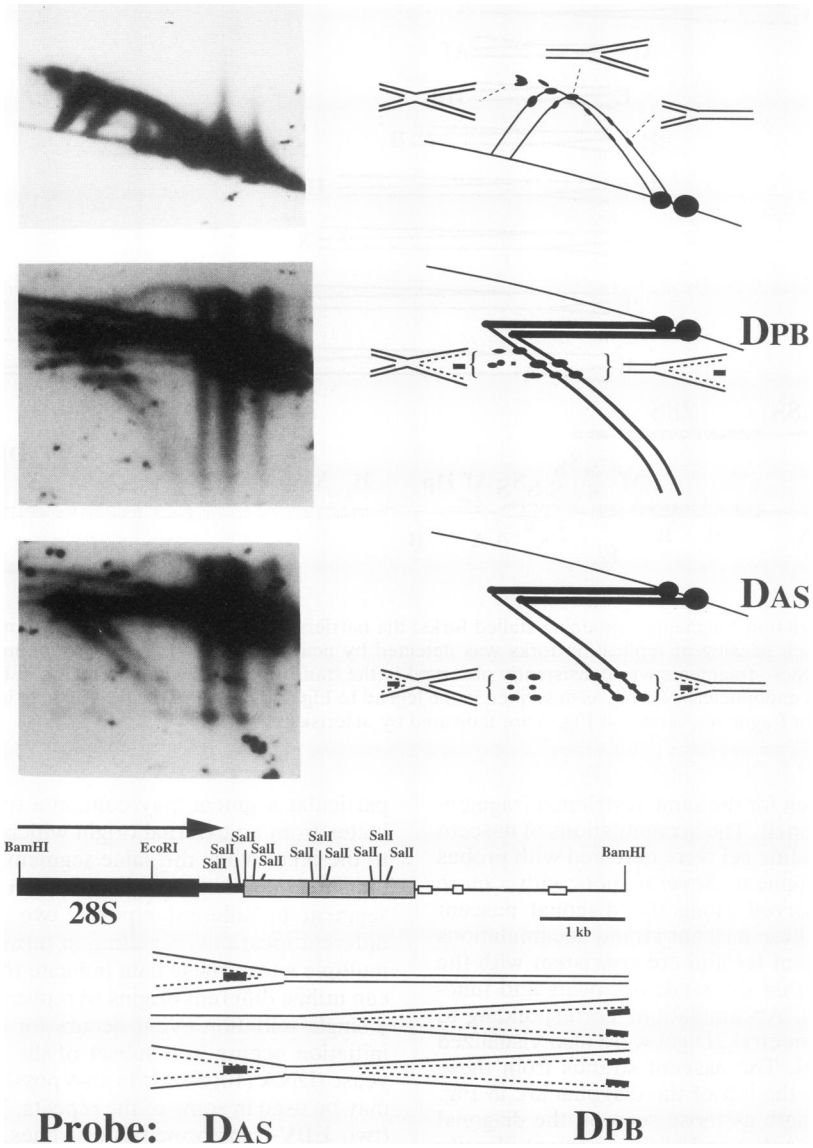

FIG. 8. Termination of replication occurs at the 3′ end of the transcription unit in some rDNA repeats. Neutral-alkaline 2D gel electrophoresis was used to demonstrate the accumulation of double-Y structures in the region where forks are stalled. Matrix-associated DNA samples from asynchronous 721 cells were subjected to identical first-dimension gel conditions. One sample was then used in a neutral second-dimension gel and the other was used in an alkaline second-dimension gel. The top autoradiograph shows the neutral-neutral 2D gel results for the same 5.8-kb *Eco*RI-*Bam*HI fragment analyzed in Fig. 6 for Raji cells. The spots along the Y arc represent the accumulation of simple Y forms because of replication fork pausing. The accumulations in the upper left portion of the autoradiograph represent a population of double-Y forms (terminating molecules). The two lower autoradiographs result from two separate hybridizations of the neutral-alkaline 2D gel with probes from both ends of the fragment (D_{AS} and D_{PB}). The locations of these probes along the fragment are shown as solid rectangles on the replicative intermediates diagrammed at the bottom of the figure. The spots along the diagonal nascent strand arc represent the accumulation of nascent strands of sizes consistent with pausing of forks proceeding in both directions in the transcription termination region. The spots to the left of the diagonal nascent strand arc represent accumulations of nascent strands derived from double-Y structures. Vertical lines of hybridization are the result of nicks in the parental DNA strands. Diagrams of replication intermediates that have accumulated are shown next to the spots; the dashed lines indicate the nascent strands, solid lines indicate parental strands, and the shaded rectangles indicate the locations of the probes.

observe (from fragments up to 19 kb) suggests that they do not represent the result of abortive initiation events.

For particular segments, we have compared the integrated intensity of the signals from molecules containing bubbles with those of molecules containing single forks. These values vary by approximately 10-fold, with the intensity of the bubble arc being highest in the regions flanking the promoter and the transcription termination region. This suggests that there may be a difference in the frequency of origin usage. However, since we cannot determine whether bubbles located within some DNA segments are less stable than those located within other segments under the prepar-

ative conditions we use, it is difficult to quantitate the relative frequency of initiation at different sites.

Why is initiation not detected in transcribed regions or adjacent transcriptional regulatory regions? It is possible that initiation in fact occurs, but at undetectable levels or in a small subset of the repeat units. It is also possible that the rate of replication in these regions is more rapid, resulting in lower steady-state levels of bubble-containing molecules. Alternatively, these intermediates may be preferentially lost or destroyed during isolation of DNA. If we assume, however, that little or no initiation takes place in the transcription unit, one obvious explanation may be that this region has evolved for a different purpose, namely, to code for particular structural RNAs. One would not generally expect coding regions to contain additional genetic information prescribed for origins of replication. Alternatively, the masking of candidate initiation sites by features of the chromatin structure, which in these regions includes the transcription machinery, may influence initiation site selection.

The initiation of DNA replication in the mammalian genome has been studied in the greatest detail for the DHFR locus in the CHO cell line (reviewed in references 21 and 32). The DHFR locus has been studied as part of a 240-kb amplicon by 2D gel electrophoresis (by both the neutral-neutral and neutral-alkaline techniques). Initiation of replication was found to occur throughout the 55-kb intergenic spacer but not in the 3′ exon of the DHFR gene or in a distal transcription unit (2BE2121) that has been identified in this amplicon (23, 24). While a lower frequency of initiation was observed within a few kilobases of the transcribed genes, no major difference in the frequency of initiation over the remainder of the intergenic spacer was reported. Other investigators have determined the site at which there is a switch in the strand serving as a template for Okazaki fragment synthesis (13). This transition defined the location of an origin of bidirectional replication within a 27-kb region. The same region has been identified as the location of an origin of replication by several independent techniques, and several models have been proposed to resolve this apparent paradox (reviewed in references 5, 18, 32, and 56). It is possible that there may be preferred sites of initiation and many secondary nonspecific sites.

Replication termination. Studies of the termination of replication have not received as much attention as the search for origins. While fiber autoradiography has provided evidence for replication termination in mammalian cells (for a review, see reference 25), there have to date been no reports of site-specific replication barriers in the genomes of organisms other than *E. coli* and *B. subtilis* (39), *S. cerevisiae* (10, 11, 44, 54), and pea (38). We have detected two types of replication termination in human rDNA. First, convergence of replication forks occurred at apparently random sites across the entire repeat unit. Second, a portion of the repeats contained specific termination sites at the junction between the 3′ end of the transcription unit and the nontranscribed region. The location of stalled replication forks in human rDNA is similar to the position of the barriers in *S. cerevisiae* rDNA (10, 11, 44, 54) and pea rDNA (38). In human rDNA, replication forks are stalled in both directions, whereas the barriers in *S. cerevisiae* and pea rDNA are polar and arrest forks entering the 3′ end of the transcription unit. The detection of termination patterns within the human rRNA transcription unit indicates that some leftward-moving forks passed through the barriers. This may be the result of forks that are impeded but eventually traverse the barrier region. Alternatively, a subset of repeat units that differ in some manner and that do not stall replication forks in this region may exist.

The replication barriers in human rDNA are located in a region that has previously been shown to be involved in termination of transcription by RNA polymerase I (60). The complexity of the 2D gel patterns in this region is partially the result of a length polymorphism. Each rDNA repeat may contain between one and four copies of a tandemly repeated 600- to 800-bp element; thus, multiple Y arcs and multiple barriers are seen. This polymorphism has been suggested to be the result of unequal homologous recombination (26, 51). The relationship between replication termination and recombination events remains to be determined. This complex region also contains multiple binding sites (*Sal* boxes) for a protein (TTFI) with a role in termination of transcription. Extensive analyses of the comparable region in mouse rDNA have shown that proteins bind DNA sequences flanking the *Sal* boxes as well and can influence the efficiency and accuracy of transcription termination (29). The human locus contains several simple repetitive sequence tracts (51), including poly(T) (up to 29 nucleotides), polypyrimidine (up to ~350 nucleotides with several interspersed purines), and polypurine-polypyrimidine (up to 60 nucleotides). Interestingly, pyrimidine-rich sequences have the potential for forming triplex DNA and are sites where DNA polymerases pause in vitro (4, 50). Studies of the comparable region in *S. cerevisiae* have shown that the barrier is not due to active transcription (11, 44). It is not known whether proteins are involved in barrier formation or whether the DNA sequence itself is responsible.

Initial work in our laboratory on the replication of human rDNA focused on determining the timing of replication of this gene family during S phase (3). Although we found that the sum of all human rDNA repeat units replicated throughout S phase, we have more recently obtained evidence for two distinct classes of genes, one that replicates predominantly in the first half of S phase and another that replicates predominantly in the second half of S phase (unpublished data). This observation was possible because of the linkage of the replication timing differences with a polymorphic *Eco*RI restriction site in the NTS. This polymorphism has been well documented and varies in allele frequency between individuals and cell lines (2, 47). The observation of two forms of rDNA repeats with different replication patterns is not entirely unexpected, since classes of rDNA that differ in various properties have previously been observed. It has been shown, for example, by using psoralen cross-linking, that two distinct chromatin structures of rDNA are present in *S. cerevisiae* (18), *X. laevis* and *Xenopus borealis* (58), and the Friend murine erythroleukemia cell line (17). These studies also suggest that the two chromatin structures observed are associated with different states of transcriptional activity. Additional evidence has shown that only a subset of all rRNA genes are actively transcribed in a variety of vertebrate cells (30). Also, the methylation patterns of mouse, rat, and human rDNA have suggested the presence of hypo- and hyper-methylated subsets of genes (6, 19, 42, 49). Many tissue-specific genes are early replicating when they are transcriptionally active and later replicating when they are inactive (e.g., see reference 34 and references therein). Thus, some or all of the early-replicating rDNA repeats may include transcriptionally repressed units. The barriers to replication we have detected in human rDNA may also be related in some manner to transcriptional status. Human rDNA thus provides a useful model system for examining the potential role of structural features, including

chromatin structure, methylation status, and transcriptional activity in activation and repression of DNA replication origins.

ACKNOWLEDGMENTS

We are grateful to Joel Huberman and Bernardo Schvartzman for helpful discussions throughout the course of these studies and for their comments, as well as those by William Burhans, on the manuscript. We acknowledge helpful comments from Peter Dijkwel during the early stages of this work. We thank James Sylvester for supplying clones and unpublished sequence information. We also appreciate the valuable help provided by Irina Tcherepanova.

This work was supported by the National Institutes of Health grant RO1 GM45751 and by fellowships to R.L. from the American Cancer Society and to T.H.K.P. from the American Heart Association. Support was also provided by Cancer Center core grant NIH/NCI P30-CA13330.

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