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# Chromatin Structure and Transcriptional Activity around the Replication Forks Arrested at the 3′ End of the Yeast rRNA Genes

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**Replication intermediates containing forks arrested at the replication fork barrier near the 3′ end of the yeast rRNA genes were analyzed at the chromatin level by using in vivo psoralen cross-linking as a probe for chromatin structure. These specific intermediates were purified from preparative two-dimensional agarose gels, and the extent of cross-linking in the different portions of the branched molecules was examined by electron microscopy and by using a psoralen gel retardation assay. The unreplicated section corresponding to the rRNA coding region upstream of the arrested forks appeared mostly heavily cross-linked, characteristic of transcriptionally active rRNA genes devoid of nucleosomes, whereas the replicated daughter strands representing newly synthesized spacer sequences showed a nucleosomal organization typical for bulk chromatin. The failure to detect replication forks arrested at the 3′ end of inactive rRNA gene copies and the fact that most DNA encoding rRNA (rDNA) is replicated in the same direction as transcription suggest that replication forks seldom originate from origins of replication located immediately downstream of inactive genes.**

The development of two-dimensional (2D) gel electrophoretic techniques for the direct analysis of replication intermediates of specific chromosomal regions has contributed substantially to our understanding of the process of DNA replication in eukaryotic organisms (for reviews, see references 5, 17, 22, and 25). Using these techniques, origins of replication have been localized to specific, few-hundred-base-pair-long DNA regions in several systems, including the yeast *Saccharomyces cerevisiae* (3, 14, 27, 28, 31), the slime mold *Physarum polycephalum* (2), and several animal viruses (19, 41, 49). In contrast, the nature of origins of DNA replication in the genomes of higher eukaryotes seems to be more complex and is still under debate (reviewed in references 13, 20, and 32).

The use of 2D gels has also allowed significant insights into the subsequent steps of eukaryotic DNA replication, namely, replication fork progression and replicon fusion. Several studies seem to confirm the general features of the bidirectional replication model, which proposes that pairs of divergent replication forks travel away from their origins until they meet the forks coming in the opposite directions from neighboring replicons (26). In one case, it has been shown that two converging replication forks meet and terminate at nonspecific sites located somewhere halfway between the two origins from which they have originated (50). Nevertheless, results from other specific chromosomal domains seem to indicate many more irregularities in replication fork movement. In this context, one of the extreme cases is represented by the replication fork barrier (RFB) near the 3′ end of the yeast rRNA genes (4, 31). This barrier works in a polar manner in that it only prevents replication forks from entering the 35S rRNA coding region in the opposite direction of transcription, resulting in most of the DNA encoding rRNA (rDNA) being replicated unidirectionally (31). From the location and polarity of this barrier and from the fact that the yeast rDNA locus can be functional at the same time in both replicative and transcriptional processes (39), it has been proposed that transcription elongation per se might be responsible for replication fork blockage (4). However, later studies have shown that DNA sequences near the 3′ end of the yeast ribosomal transcription unit are able to function as a polar RFB in the absence of transcription (6). These a sequences retain their function when inserted into extrachromosomal plasmids replicating in *S. cerevisiae* but not in *Escherichia coli*, indicating the possible involvement of sequence- and species-specific DNA-binding protein(s) in the arrest of replication fork movement (6, 29). The recent discovery of a similar barrier in the rDNA locus of plant cells points to a possible common mechanism that may be utilized by most eukaryotic organisms to accommodate transcription and replication at the same time on the same chromosomal region (24).

To learn more about the mechanisms that coordinate simultaneous transcription and replication at the rDNA locus in *S. cerevisiae*, we decided to analyze the chromatin organization in vivo of the tandemly repeated rRNA genes during the replication process. We have repeatedly shown in several eukaryotic systems that rRNA gene expression is accompanied by distinct changes in chromatin structure which are reflected by the loss or drastic modification of the nucleosomes on the transcribed regions and surrounding regulatory sequences (9, 33, 34, 44). These changes are readily detected in vivo as an increased accessibility of chromatin DNA to the intercalating drug psoralen, which can introduce cross-links into the DNA upon UV irradiation, thereby marking the accessible sites (8, 21). By using this approach, we have recently shown that in exponentially growing yeast cells, transcriptionally active and inactive rRNA genes coexist as nucleosome-free and nucleosome-packaged gene copies, respectively (10). After the in vivo psoralen photoreaction, the structural information is re-

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tained on intact, isolated DNA; therefore, it is possible to study the structure of replicating chromatin by analyzing the cross-linking pattern of specific DNA replication intermediates which have been separated on neutral/neutral 2D agarose gels (3). In this study, by combining in vivo psoralen cross-linking and preparative 2D gel electrophoresis, we focused our attention on the chromatin structure around the replication forks arrested at the RFB and on the distribution of these forks with respect to transcriptionally active and inactive rRNA gene copies in the yeast rDNA locus.

## MATERIALS AND METHODS

**Strains and culture conditions.** *S. cerevisiae* FTY 23 (*MATα ura3-52 his3-1 gal2 gal10 trp1 URA3*/YRp TRURAP) was used for all the analysis with exponentially growing cells (47). Cells were grown in YPD medium (42) at 30°C to a density of about $10^7$ cells per ml. *S. cerevisiae* A1 (*MATa ade2-101 ura3-52 his3Δ200 lys2-801 Δbar1::LYS2*; kindly provided by Markus Aebi) was used for cell synchronization. Cells grown in YPD at 30°C to a density of $5 \times 10^7$ cells per ml were synchronized by blocking with α-factor. All of the following steps were performed at 30°C. Cells were pelleted, washed once with YPD, and resuspended in the same medium containing 1 μM α-factor (Sigma). After 2 h, cells were washed three times with water and allowed to enter S phase by being incubated in fresh YPD medium for a further 40 min.

**Psoralen cross-linking.** About $10^{10}$ cells collected by centrifugation were washed twice in ice-cold water, resuspended in 20 ml of TE buffer (10 mM Tris HCl [pH 7.6], 0.5 mM EDTA), and irradiated on ice in an open plastic dish (100 $cm^2$) with a 500-W high-pressure mercury lamp (model TQ 700; Original Hanau) in the presence of 4,5',8-trimethylpsoralen (Sigma). The lamp was placed at a distance of 7 cm and was filtered by Pyrex glass to remove radiation below 300 nm. Psoralen stock solution in ethanol (0.05 volume of 200 μg/ml) was added five times at intervals of 15 min for a total irradiation time of 75 min.

**rDNA isolation, gel electrophoresis, transfer, and hybridization.** rDNA was purified on CsCl gradients exactly as previously described (10). Neutral/neutral 2D agarose gel electrophoresis was performed as described by Brewer and Fangman (3), with some modifications. The first dimension was 0.4% agarose (type II-medium EEO; Sigma) and was run at 1 V/cm for 20 h. The second dimension was 1% low-gelling-temperature agarose (SeaPlaque; FMC) and was run at 4 V/cm for 9 h. DNA was recovered from the gel by the GELase protocol (Epicentre Technologies). For psoralen gel retardation assay gel electrophoresis, alkaline Southern blotting and hybridizations were done as previously described (34). The probes used are shown in the restriction maps in the respective figures and represent rDNA fragments derived from subclones of plasmids pSZ1 and pSZ26 (46).

**Electron microscopy.** rDNA recovered from low-gelling-temperature 2D agarose gels was digested with *Sph*I, extracted with phenol-chloroform, precipitated with ethanol, and prepared for electron microscopy essentially as described previously (44). DNA contour length measurements were made with a Hewlett-Packard digitizer on photographic prints.

## RESULTS

**Chromatin structure around the replication forks arrested at the RFB.** The psoralen photoreaction has provided detailed information about the structure of transcribing (11, 33, 44) as well as replicating (1, 45) chromatin. Since psoralen cross-links occur in the linker region but not in the nucleosomal DNA, the positions of the nucleosomes remain stably imprinted on the DNA. When the deproteinized DNA is spread for electron microscopy under denaturing conditions, nucleosomal DNA is visualized as a single-stranded bubble of 140 to 160 nucleotides (8, 21). On the other hand, highly cross-linked DNA derived from fully accessible, nonnucleosomal chromatin regions is prevented from denaturing and has a double-stranded appearance (10, 33, 44).

We wished to use the psoralen technique and subsequent electron microscopy to examine the structure of replication forks arrested at the RFB. The prerequisite for an electron microscopic analysis is the purification of the specific DNA fragment under study. For this purpose, by using a CsCl gradient, we first purified rDNA from total DNA isolated from photoreacted S-phase yeast cells. Specific rDNA replication intermediates containing forks arrested at the RFB were then purified from preparative neutral/neutral 2D agarose gels (3). By this technique, DNA restriction fragments are first separated according to their mass in a low-percentage agarose gel. During the second dimension, replication intermediates are separated away from linear DNA under electrophoretic conditions that separate DNA molecules primarily according to their shape. The series of replication intermediates of a given DNA restriction fragment will migrate above the diagonal of linear molecules along a distinct arc starting at the position of the nonreplicating linear fragment. The shape of the arc will depend on how the fragment is replicated, i.e., on the different structures of the replication intermediates (3, 5). Figure 1b shows an ethidium bromide-stained 2D gel of purified rDNA digested with *Bgl*II, which cuts the rDNA repeat unit into two equal-size fragments of 4.5 kb (map in Fig. 1a). On the faint diagonal of linear molecules, the prominent DNA spots correspond to the expected 4.5-kb fragments (L in Fig. 1b) and to some partial rDNA digestion products, indicating that the fraction analyzed contains mainly purified rDNA. To locate the replication intermediates of the *Bgl*II restriction fragment containing the RFB, an aliquot of the same DNA was separated on a similar 2D gel, blotted, and hybridized with a probe from the rDNA spacer region (Fig. 1c). As expected, this probe detects the characteristic simple Y arc. The intense spot near the inflection point represents the accumulation of replication intermediates arrested at the RFB, which correspond to fork 4 in Fig. 1a (4). It should be noted that psoralen cross-links have no direct influence on the migration of the DNA in this 2D gel system. Instead, because cross-links tend to stabilize replication intermediates by preventing branch migration, they contribute to the production of intense and clean arc patterns (15). Using the autoradiogram in Fig. 1c, we could locate on the ethidium bromide-stained gel the position of the arrested replication intermediates, which are actually visible as a faint spot (RFB in Fig. 1b). The replication intermediates migrating in the RFB spot were then recovered from the gel, digested with *Sph*I, and spread for electron microscopy under denaturing conditions. *Sph*I digestion produces distinct asymmetric Y-shaped molecules and enables us to distinguish the arrested forks from the other rDNA replication intermediates

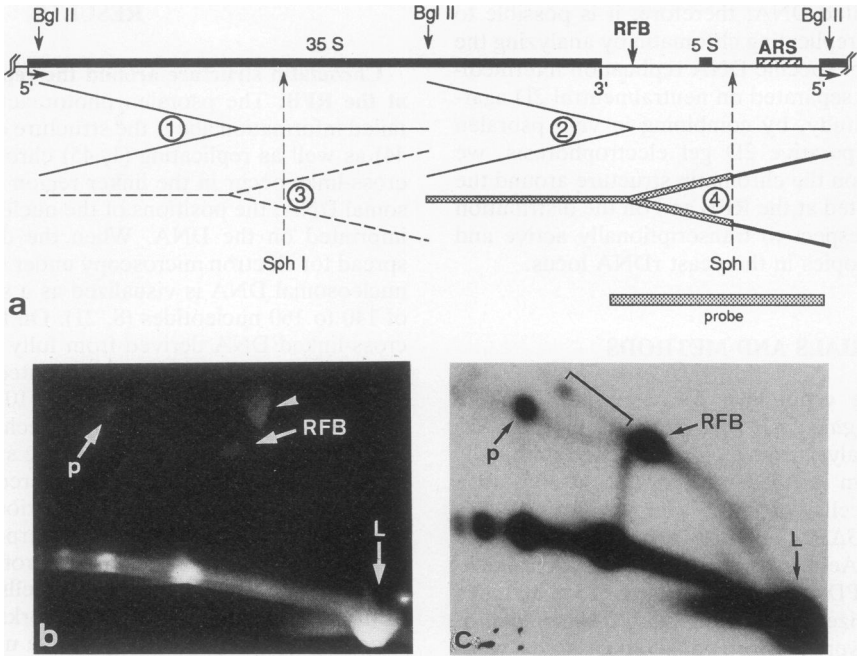


FIG. 1. Purification of replicative intermediates containing replication forks arrested at the RFB. (a) Structural organization and restriction map of the rDNA repeat unit of *S. cerevisiae*. The 35S precursor coding region and the sequences coding for the 5S rRNA are indicated as filled boxes. In the ribosomal intergenic spacer (thin line between 3′ and 5′), the autonomously replicating sequence (ARS) element (hatched box) and the RFB are also indicated. The schematic drawing below the map shows the four possible Y-shaped rDNA replication intermediates migrating in the spot labelled RFB in the 2D gel in panel b. (b) CsCl-purified rDNA from psoralen cross-linked S-phase cells was digested with *Bgl*II, separated on a 2D gel, and stained with ethidium bromide. The first dimension was from left to right; the second was from top to bottom. The spots corresponding to 4.5-kb linear fragments (L) and to the Y-shaped intermediates containing the forks arrested at the RFB are indicated (RFB). The faint spot labelled p represents a partial digest of forks arrested at the RFB. The additional spots along the diagonal of linear fragments are also partial *Bgl*II digestion products. (The arrowhead above the RFB points to some circular 2μm plasmid DNA which copurifies with the rDNA.) The DNA migrating in the RFB spot was then eluted from the gel and analyzed in Fig. 2. (c) An aliquot of the rDNA analyzed in panel b was separated on a similar gel, blotted, and hybridized with the probe indicated in panel a. The bracket indicates fragments with an arrested fork being completed by a second replication fork (see Fig. 6 in reference 4).

of equal size and shape that comigrate in the same position in the 2D gel (forks 1 to 3 in Fig. 1a).

One hundred sixteen arrested replication intermediates were analyzed in this study; all corresponded to the shaded portion of fork 4 in Fig. 1a. The micrographs in Fig. 2a and b show two representative molecules. In all of the molecules, the longer unreplicated section, corresponding to rRNA coding sequences upstream of the arrested fork, appeared fairly continuously cross-linked. This cross-linking pattern is typical for the chromatin structure of transcriptionally active rRNA genes devoid of nucleosomes (10, 33, 44). In contrast, the row of single-stranded bubbles on both newly replicated ribosomal spacer segments is consistent with most of these sequences being packaged in nucleosomes. The *R* value calculated for the newly replicated spacers (i.e., the ratio of the sum of the length of all single-stranded bubbles to the total DNA length [45]), which represents a measure of the nucleosome density, gave an average of 0.68 ± 0.08, which is very similar to that obtained for nonreplicating ribosomal spacer sequences (0.71 ± 0.09 [10]).

In 64 of the molecules analyzed, we observed a single-stranded bubble of 155 ± 25 nucleotides located on the unreplicated DNA just in front of the branch point (arrow in Fig. 2a). By measuring the relative lengths of the different branches of each of these molecules, we calculated that on average 32.8% (±2%) of the *Bgl*II-*Sph*I fragment has replicated. We could therefore map the branch point to a position 376 ± 70 bp downstream of the 3′ end of the 25S rRNA coding region (Fig. 2d). This position is in agreement with results from 2D gel studies, in which it has been shown that replication forks arrest at two closely spaced sites located somewhere in the 129-bp *Hin*dIII-*Hpa*I fragment (6) (map in Fig. 2c). The short stretch of DNA between the branch point and the first nucleosomal bubble on the newly replicated spacer segments is on average 109 ± 56 nucleotides long and appears primarily double stranded in the spread DNA (Fig. 2a).

The average length of the single-stranded bubble in front of the arrested replication forks (155 ± 25 nucleotides) is compatible with the length of the protected DNA in a nucleosome (8, 21). However, when the same DNA region was cross-linked as purified, cloned DNA, a single-stranded bubble of 142 ± 25 nucleotides was detected exactly at the same position, indicating the presence of DNA sequences with a low cross-linkability (data not shown). In fact, inspection of the nucleotide sequence (43) revealed the presence of a 153-bp-long region located between bp 222 and 375 downstream of the 3′ end of the 25S rRNA that does not contain any 5′-TA dinucleotide, which has been shown to represent the most preferred cross-link site (16). Therefore, it was not possible to draw any definitive conclusion about the extent of protection of the DNA by bound proteins in this particular site.

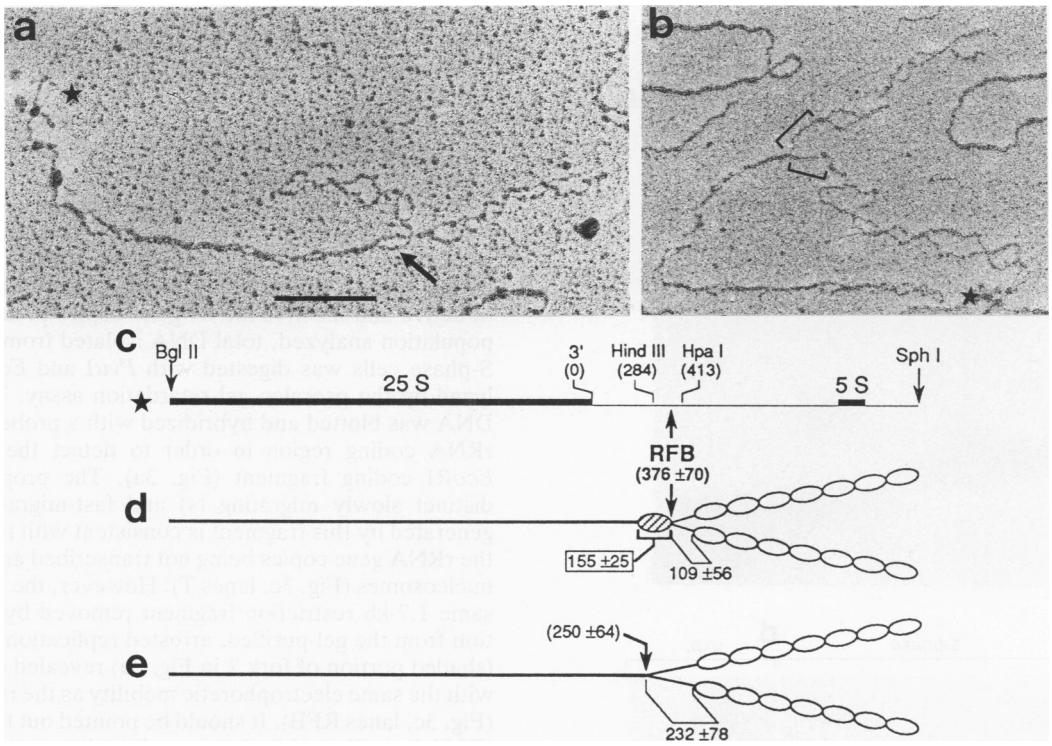


FIG. 2. (a and b) Electron microscopy of psoralen cross-linked rDNA replicative intermediates containing replication forks arrested at the RFB. The rDNA eluted from the RFB spot in the gel in Fig. 1b was digested with *Sph*I (see map in Fig. 1a) and spread for electron microscopy under denaturing conditions. The two Y-shaped molecules in the electron micrographs represent replication intermediates arrested at the RFB and correspond to the shaded portion of fork 4 in Fig. 1a. The star indicates the *Bgl*II site at the longer unreplicated parental strand. The arrow in panel a points to a single-stranded bubble just in front of the replication fork; such bubbles were found in about 50% of the molecules analyzed. The bar represents 0.5 kb. The brackets in panel b indicate single-stranded regions on both replicated arms immediately behind the branch point. The schematic drawings (d and e) summarize the chromatin organization around the arrested forks. In the map of the 3.48-kb *Bgl*II-*Sph*I fragment (c), the *Hin*dIII and *Hpa*I restriction sites are also indicated; the numbers in brackets indicate their positions with respect to the 3′ end of the 25S rRNA coding region (43). The position of the branch point was mapped separately from 64 molecules with (d) and 52 molecules without (e) the single-stranded bubble at the fork. The total length of each molecule (unreplicated portion plus average length of the two replicated branches) was normalized to 3,477 bp. The average length of the bubble at the RFB and the distance between the branch point and the first single-stranded bubble on the daughter strands are also indicated.

In the remaining 52 molecules analyzed, there was no single-stranded bubble adjacent to the unreplicated DNA at the fork, and the branch point was located closer to the 3′ end of the 25S rRNA coding region at a position 250 ± 64 bp downstream (Fig. 2b). Since on these replication intermediates, the DNA behind the fork appeared single stranded on both replicated arms, we conclude that the branch point does not coincide with the site where replication has stopped. From the position of the branch point and from its distance from the first nucleosomal bubble on the newly replicated spacers, it is possible that these molecules represent a fraction of the same replication intermediates as the one in Fig. 2a which simply has not been cross-linked at site 376 ± 70 (compare Fig. 2d and e). Alternatively, the molecules without the bubble in front of the replication fork may represent the fraction of replication intermediates in which the fork has proceeded beyond site 376 ± 70 and has arrested at the second site located slightly to the left in the *Hin*dIII-*Hpa*I fragment as reported by Brewer et al. (6). Since in this DNA region there are no cross-linkable sites, the parental DNA in front of the arrested fork has been denatured under the spreading conditions until the next cross-link located outside the *Hin*dIII-*Hpa*I fragment at position 250 ± 64.

**Distribution of the arrested forks with respect to active and inactive rRNA genes.** All of the arrested replication intermediates examined by electron microscopy showed a fairly continuously cross-linked 25S rRNA coding region characteristic of transcriptionally active rRNA genes. The fact that none of the coding regions analyzed were organized as a row of single-stranded bubbles, indicative of inactive, nucleosomal chromatin, seems to indicate that replication forks never stop at the 3′ end of the inactive gene copies. Since this result is based on a limited number of molecules, we decided to reexamine the extent of cross-linking of the rRNA coding region in front of the arrested forks by using the psoralen gel retardation assay, which permits the analysis of the entire population of a specific restriction fragment in a given DNA sample. By this method, DNA restriction fragments derived from active (nonnucleosomal) and inactive (nucleosomal) rRNA gene copies can be separated on a native agarose gel according to their different extents of cross-linking under conditions in which the more a fragment is cross-linked, the more slowly it migrates (9, 10, 34). When these two different types of rRNA genes coexist in the cell population analyzed, each ribosomal coding fragment is separated into two distinct bands: a slowly migrating band representing highly cross-linked DNA derived from transcriptionally active genes and a fast-migrating band containing slightly cross-

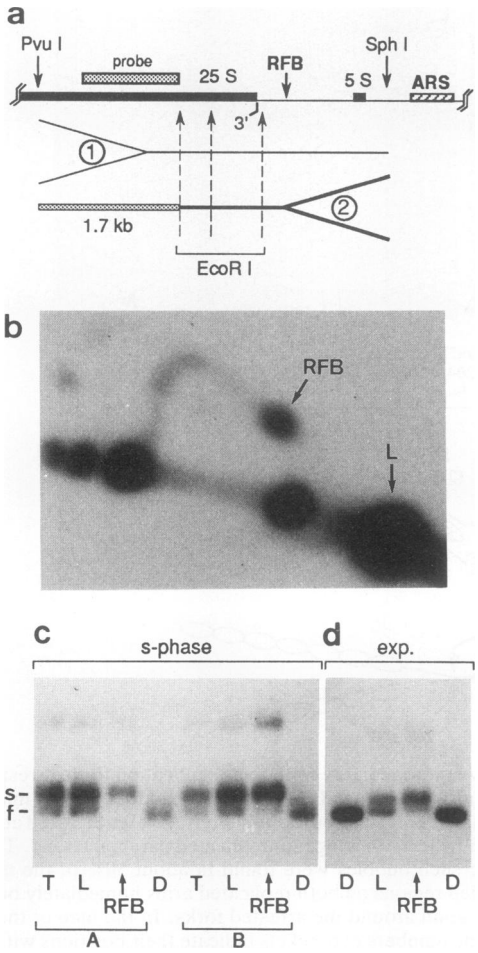


FIG. 3. Psoralen accessibility of rRNA coding segments in front of the replication forks arrested at the RFB. (a) Restriction map of the 4-kb *Pvu*I-*Sph*I rDNA region surrounding the RFB. The Y-shaped structures below the map represent the two replication intermediates migrating in the RFB spot in the 2D gel in panel b. (b) Purified rDNA from psoralen cross-linked exponentially growing cells was digested with *Pvu*I and *Sph*I, separated on a 2D gel, blotted, and hybridized with the probe indicated in panel a. The spots corresponding to the linear 4-kb *Pvu*I-*Sph*I fragment (L) and to the intermediates containing the arrested forks (RFB) are indicated. The additional spots represent linear partial digestion products. (c and d) Psoralen gel retardation assay of the 1.7-kb *Pvu*I-*Eco*RI rDNA coding fragment. The 1.5% agarose gel was blotted and hybridized with the probe indicated in panel a. Lanes: T, total DNA from psoralen cross-linked cells digested with *Pvu*I and *Eco*RI; L and RFB, rDNA eluted from the linear 4-kb spot (L) and from the RFB spot from a 2D gel similar to the one in panel b and digested with *Eco*RI; D, non-cross-linked control DNA digested with *Pvu*I and *Eco*RI. The different DNA samples were from S-phase (c) and exponentially growing (d) cells. Samples in A and B are from two independent experiments.

linked DNA originating from the inactive, nucleosome-packed gene copies. To obtain unambiguous information about the activity state of the genes at which the forks have stopped, arrested replication intermediates can first be purified as before. Then, the unreplicated coding portion of these intermediates can be excised with the appropriate restriction enzyme and analyzed by the psoralen gel retardation assay.

To investigate the distribution of the arrested forks, we have analyzed the replication intermediates of the 4-kb *Pvu*I-*Sph*I fragment containing about twice as much coding as 3′ flanking spacer sequences (map in Fig. 3a). As shown in the 2D gel in Fig. 3b, this restriction fragment produced the expected simple Y arc with an intense hybridization signal at a position consistent with the accumulation of branched molecules that are approximately 30% replicated and which correspond to replication intermediates arrested at the RFB (fork 2 in Fig. 3a). The autoradiogram was used to locate and isolate the arrested intermediates separated on a parallel, preparative 2D gel (not shown). To determine the proportion of active and inactive rRNA gene copies present in the cell population analyzed, total DNA isolated from photoreacted S-phase cells was digested with *Pvu*I and *Eco*RI and analyzed by the psoralen gel retardation assay. The separated DNA was blotted and hybridized with a probe from the 25S rRNA coding region in order to detect the 1.7-kb *Pvu*I-*Eco*RI coding fragment (Fig. 3a). The proportion of the distinct slowly migrating (s) and fast-migrating (f) bands generated by this fragment is consistent with roughly 20% of the rRNA gene copies being not transcribed and packaged in nucleosomes (Fig. 3c, lanes T). However, the analysis of the same 1.7-kb restriction fragment removed by *Eco*RI digestion from the gel-purified, arrested replication intermediates (shaded portion of fork 2 in Fig. 3a) revealed only one band with the same electrophoretic mobility as the retarded s band (Fig. 3c, lanes RFB). It should be pointed out that since most rDNA is replicated in the same direction as transcription (6, 31), the gel-purified fraction containing the arrested replication intermediates will also contain branched molecules of equal size and shape corresponding to fork 1 in Fig. 3a. However, the *Pvu*I-*Eco*RI portion of such molecules has a branched structure which migrates much more slowly in the gel retardation assay and therefore does not interfere with our analysis. Inspection of the mobility of the *Pvu*I-*Eco*RI fragments derived from gel-purified, nonreplicating linear molecules revealed the two distinct s and f bands with the same proportional intensity as in unfractionated total DNA (compare lanes L with lanes T in Fig. 3c), indicating that both highly cross-linked DNA and slightly cross-linked DNA are recovered with the same efficiency from the preparative 2D gel. Taken together, these data confirm the results obtained by electron microscopy indicating that in the population of cells analyzed, most if not all of the arrested forks have stopped at RFBs located at the 3′ end of the transcriptionally active, nonnucleosomal rRNA gene copies.

Since it is known that yeast rDNA replicates throughout the S phase (7), it is possible that at the time the synchronized cells were collected, only the transcriptionally active gene copies were replicating. To avoid this possibility, we performed the same analysis with exponentially growing cells. The result of this experiment revealed basically the same picture as the one obtained with S-phase cells (Fig. 3d). Whereas fragments derived from the bulk of linear, nonreplicating molecules indicate the presence of roughly as many active as inactive rRNA genes (lane L), the electrophoretic pattern of the same 1.7-kb fragment which originated from the purified replication intermediates is consistent with most of these sequences being in an active chromatin state (lane RFB). In fact, only trace amounts of the fragment derived from the arrested forks showed the fast mobility characteristic of slightly cross-linked, nucleosomal DNA. The fact that S-phase cells have more active genes than exponentially growing cells is consistent with the known stimulatory effect on rRNA transcription of the mating pheromone α-factor

used for cell synchronization (48) (compare lanes L in Fig. 3c and d).

Since so far most of the recovered replication intermediates contained highly cross-linked rRNA coding sequences, we were concerned that the less cross-linked intermediates derived from the replicating, inactive gene copies might have been somehow specifically lost during the isolation procedures. In an attempt to detect replication intermediates of the inactive rRNA gene copies, we applied the same strategy to examine the extent of cross-linking of the replicating 4.5-kb *Bgl*II fragment containing only rRNA coding sequences (see map in Fig. 4a). This fragment is replicated by forks moving from one end to the other and generates the characteristic simple Y arc when separated on a 2D gel (Fig. 4b). Since most of the rRNA genes are replicated in the same direction as transcription (6, 31), the majority of the replication intermediates of this coding fragment migrating in regions 1 and 2 of the arc in Fig. 4b are expected to have the same structure and orientation as those of forks 1 and 2 in Fig. 4a, respectively. DNA molecules migrating in these regions were purified from a parallel gel, digested with *Eco*RI (Fig. 4a), and analyzed by the psoralen gel retardation assay. The separated DNA was blotted and hybridized with the probe indicated in Fig. 4a in order to detect the 1.7-kb *Eco*RI-*Bgl*II portion of the replication intermediates under investigation (shaded portions of forks 1 and 2 in Fig. 4a). DNA samples analyzed in this experiment were isolated either from photoreacted S-phase or exponentially growing cells, which gave basically the same results (Fig. 4c and d). As expected, the *Eco*RI-*Bgl*II fragments originating from the forks migrating in region 2 had a branched structure and were detected as a retarded smear, confirming the unidirectional replication of these genes (brackets in Fig. 4c and d, lanes 2). In contrast, the same fragment derived from DNA molecules originating from region 1 was resolved in the two distinct s and f bands characteristic of DNA derived from active and inactive rRNA gene copies, respectively (Fig. 4c and d, lanes 1). In summary, the data presented in Fig. 4 indicate the presence of replication forks entering transcriptionally active and inactive rRNA genes in the same direction as transcription.

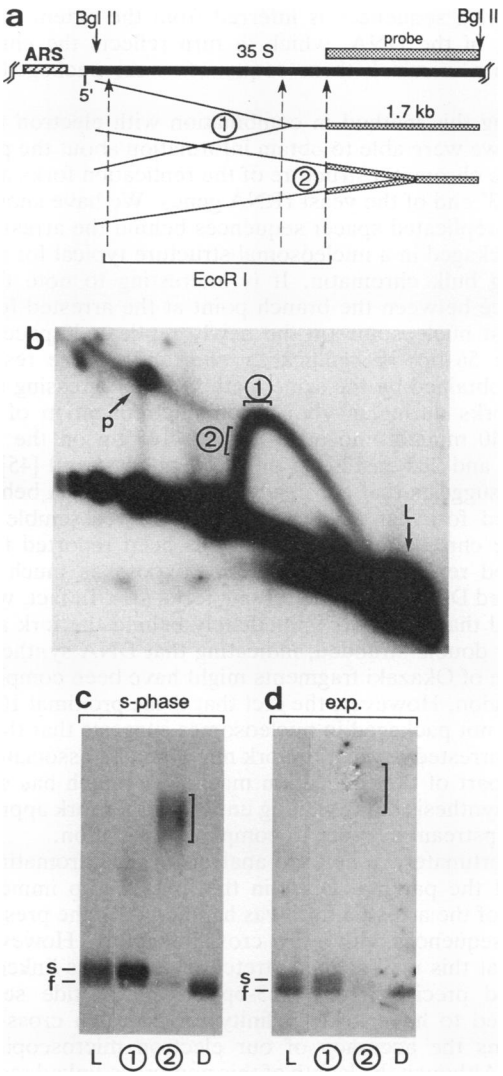


FIG. 4. Psoralen accessibility of replicating rRNA coding regions. (a) rDNA restriction map of the region spanning the 4.5-kb *Bgl*II coding fragment. Forks 1 and 2 represent the replication intermediates migrating in regions 1 and 2 of the Y arc in the 2D gel in panel b, respectively. (b) rDNA from psoralen cross-linked S-phase cells was digested with *Bgl*II, separated on a 2D gel, blotted, and hybridized with the probe indicated in panel a. L points to the position of the 4.5-kb linear fragment; p indicates a partial digest of forks arrested at the RFB (see Fig. 1c). (c and d) Psoralen gel retardation assay of the 1.7-kb *Eco*RI-*Bgl*II fragment. The 1.5% gel was blotted and hybridized with the probe indicated in panel a. Lanes: L, rDNA eluted from the linear 4.5-kb spot from a 2D gel similar to the one in panel b and digested with *Eco*RI; 1 and 2, rDNA eluted from regions 1 and 2 of the same 2D gel and digested with *Eco*RI; D, non-cross-linked control DNA digested with *Bgl*II and *Eco*RI. The slowly migrating material in lanes 2 (brackets) represents Y-shaped replication intermediates of the fragment analyzed and corresponds to the shaded area of fork 2 in panel a. The DNA samples were from S-phase (c) and exponentially growing (d) cells.

## DISCUSSION

Electron microscopic analysis of chromatin prepared by the Miller spreading technique has provided a useful method for visualizing simultaneous transcription and replication of rDNA (18, 35, 39). However, one of the limitations of this approach is the difficulty in obtaining well-spread preparations which permit reliable information about the direction of movement and precise location of the replication forks with respect to the transcribed sequences. Furthermore, since the only reference is represented by the characteristic gradient of nascent rRNA transcripts, positional information is lost when replication forks are approaching the inactive gene copies. On the other hand, the 2D gel mapping techniques yield much more qualitative and quantitative information about replication fork progression than electron microscopy and have contributed significantly to our understanding of the organization of rDNA replication in *S. cerevisiae* (4, 6, 31). However, the information obtained is only at the DNA level, and several important aspects such as chromatin structure and transcriptional activity of the replicating genes are lost. Here we show that the combination of psoralen photoreaction and preparative neutral/neutral 2D gel electrophoresis provides a powerful tool with which to study in vivo the coordination of the processes of transcription and replication in the yeast rDNA. Whereas details about the replication pattern of a specific rDNA intermediate are deduced from its relative abundance and position in the 2D gel, information about the original transcriptional state of the

replicating sequences is inferred from the extent of cross-linking of the DNA, which in turn reflects the chromatin structure in which these sequences were packaged in the cell.

Using this method in combination with electron microscopy, we were able to obtain information about the position and the chromatin structure of the replication forks arrested at the 3′ end of the yeast rRNA genes. We have shown that newly replicated spacer sequences behind the arrested fork are packaged in a nucleosomal structure typical for nonreplicating bulk chromatin. It is interesting to note that the distance between the branch point at the arrested fork and the first nucleosome on the newly replicated spacer DNA (109 ± 56 bp) is significantly shorter than the respective value obtained by the same method for progressing replication forks during in vivo chromatin replication of simian virus 40 minichromosomes (225 ± 145 bp on the leading strand and 285 ± 120 bp on the lagging strand [45]). This result suggests that the newly synthesized DNA behind the arrested fork has had enough time to reassemble into a mature chromatin structure. It has been reported that the arrested replication forks do not expose as much single-stranded DNA as do progressing forks (31). In fact, we have noticed that the DNA immediately behind the fork appears mostly double stranded, indicating that DNA synthesis and ligation of Okazaki fragments might have been completed in this region. However, the fact that these proximal 109 ± 56 bp are not packaged in nucleosomes suggests that the DNA at the arrested replication fork might still be associated with all or part of the replication machinery which has stopped DNA synthesis but is waiting until the other fork approaches from upstream in order to complete replication.

Unfortunately, a detailed analysis of the chromatin structure of the parental DNA in the first 150 bp immediately ahead of the arrested fork was hampered by the presence of DNA sequences with a low cross-linkability. However, the fact that this well-defined stretch of non-cross-linked DNA mapped precisely to a 153-bp-long nucleotide sequence expected to have a low affinity for psoralen cross-linking confirms the accuracy of our electron microscopic mappings. Although the length of this non-cross-linked region fits the expected value for nucleosomal DNA, it is highly improbable that a nucleosome would be located directly in front of the arrested fork. In fact, this DNA region includes most of the sequences that have been shown to function both in vitro and in vivo as an RNA polymerase I enhancer (36, 37, 40).

It is interesting to note that the mean position of the arrested replication forks fell about 20 bp downstream of a site that can stop RNA polymerase I approaching from either direction in an in vitro transcription assay (site III in reference 30). This site is located about 250 bp downstream of the actual termination site and has been interpreted to represent a simple roadblock caused by tightly bound proteins (30). In light of our results, it is quite possible that the protein-DNA interaction at this site represents the one involved in the arrest of replication fork movement.

By analyzing the chromatin structure of the unreplicated coding region of the arrested replication intermediates, we have obtained information about the distribution of the arrested forks with respect to the transcriptionally active and inactive rRNA gene copies. The fact that the arrested forks were found mostly at the 3′ end of the active gene copies suggests a strict correlation between transcriptional activity of the gene and efficiency of the nearby RFB. This result seems to contradict the data obtained with an RNA polymerase I mutant strain that indicated the presence of arrested forks in the rDNA locus in the absence of transcription (6). These two different sets of data can be reconciled as follows. It is generally accepted that one of the first steps in the activation of RNA polymerase I transcription is the binding of one or more sequence-specific proteins at the ribosomal promoter in order to form a stable initiation complex and to turn the gene on (for a review, see reference 38). It has recently been shown that this initial step is greatly stimulated by the presence of the ribosomal enhancer element, which might actually represent the initial binding site for regulatory proteins (37, 40). We can therefore view the active rRNA gene copies as the subset of genes that have their enhancers and promoters occupied by regulatory factors, in contrast to the inactive copies, in which these sequences are packaged in nucleosomes (10, 34). Since the sequences responsible for replication fork blockage are part of the enhancer region (29, 40), it is possible that the same factor(s) responsible for enhancer function is also involved in replication fork blockage. Alternatively, the initial binding of the enhancer factor(s) may facilitate the binding of a distinct RFB factor(s) by opening the chromatin structure in that region. According to this hypothesis, the RFB sequences around the transcriptionally active gene copies would be occupied by the respective factors and would be functional in arresting replication forks, whereas the same sequences at the inactive copies would not. It is reasonable to think that all these protein-DNA interactions might also have occurred at the RFBs in the RNA polymerase I mutant strain in which only the subsequent steps of the transcriptional process have been prevented, namely, RNA polymerase I initiation and elongation (6).

However, there must be an additional explanation to reconcile the unidirectional replication pattern of the yeast rDNA with our failure to detect replication forks arrested at the 3′ end of the inactive rRNA gene copies. In fact, the model presented above suggests that if there were replication forks approaching inactive genes from downstream, they would proceed without impediment through the coding sequences in a 3′-to-5′ direction. Since we have shown that in the cell population analyzed, the relative amount of the inactive gene copies varies from about 20 to 50%, we should expect the same proportion of replication forks proceeding in the opposite direction as transcription. However, results from both neutral/neutral and neutral/alkaline 2D gel techniques have indicated that only less than 10% (6) or even less, 5% (31), of the forks are entering the rRNA coding region from downstream. Furthermore, the results presented in Fig. 4 confirmed this unidirectional replication pattern. Since it has been shown that in the yeast rDNA only a subset of origins are used (17, 31), we can reconcile these data with a replication model in which mainly origins of replication located immediately downstream of transcriptionally active rRNA gene copies are activated. Each leftward moving fork will then stop at the RFB located at the adjacent active gene and will never have the chance to meet inactive gene copies. The rightward moving fork will proceed unimpeded through the downstream genes until it meets the next arrested fork. In this scenario, both active and inactive genes will be replicated in a 5′-to-3′ direction. This prediction is confirmed by the results in Fig. 4, which demonstrate the presence of replication forks which have entered the 5′ ends of both types of genes.

By using the Miller chromatin spreading technique, Saffer and Miller (39) examined the distribution of the activated origins within the transcribing yeast rDNA locus by analyz-

ing the location of the replication bubbles with respect to active and inactive rRNA genes. Although they found that the majority of the replication bubbles were located immediately downstream of active rDNA transcription units, in 9 of 28 examples, the bubbles were located downstream of inactive genes. Since a consecutive row of heavily transcribing rDNA repeats appears highly entangled in the electron microscope, it is difficult to visualize the intergenic spacer in such preparations; therefore, it is possible that in the previous study (39), replication events occurring between active rDNA transcription units were underestimated.

We recently reported that in *S. cerevisiae*, the ribosomal spacer sequences between active genes show an unusual cross-linking pattern, suggesting a peculiar chromatin structure, whereas spacers flanking the inactive copies are packaged in a regular nucleosomal array (10). The results presented here suggest that these structural features may be related to origin function. Recent studies in several systems have indicated that initiation of both transcription and replication might be regulated by common regulatory elements (reviewed in references 12 and 23). Since rRNA synthesis in *S. cerevisiae* is tightly coupled to the proliferation rate of the cell, and since both transcriptional and replicative elements are colocalized in the relative short ribosomal intergenic spacer, it is reasonable to expect a coordinate control of both processes. Experiments are under way to determine directly the distribution of the activated origins with respect to the transcriptionally active and inactive rRNA gene copies in the yeast rDNA locus.

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