5038–5046 Nucleic Acids Research, 1994, Vol. 22, No. 23 

# Replication fork barriers in the *Xenopus* rDNA

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Received July 26, 1994; Revised and Accepted September 2, 1994

## ABSTRACT

**To investigate replication fork progression along the tandemly repeated rRNA genes of *Xenopus laevis* and *Xenopus borealis*, rDNA replication intermediates from dividing tissue culture cells were analyzed by two-dimensional gel electrophoresis. Analysis of the direction of replication in the rRNA coding regions revealed replication forks moving in both directions. However, in both frog species, polar replication fork barriers (RFB) arresting forks approaching the rRNA transcription units from downstream were identified. Whereas in *X.borealis* the RFB maps to a defined site close to the transcription terminator, in *X.laevis* the arrest of fork movement can occur at multiple positions throughout a 3′ flanking repetitive spacer region. A short DNA element located near the respective RFB sites is shared between these two related frog species, suggesting its possible involvement in the arrest of replication fork movement. In a subset of rDNA repeats, these barriers cause an absolute block to replication fork progression, defining the sites where replicon fusion occurs, whereas in the remainder repeats, most probably in the non-transcribed gene copies, the replication machinery can pass the RFB sequences and replicate the rRNA transcription unit in a 3′-to-5′ direction.**

## INTRODUCTION

In the genome of most eukaryotes, the repeated rRNA genes are organized in tandem arrays in which transcription units coding for the large rRNA precursor are separated by intergenic spacer regions of variable length (for review see 1). It has long been recognized that the rRNA genes are among the most actively transcribed genes of the cell. This very efficient expression is deduced from electron microscopic preparations of nucleolar chromatin in which transcriptionally active rRNA genes appear fully loaded with RNA polymerases and nascent transcripts (2). However, in a given cell, not all the repeated rRNA gene copies are necessarily activated; in fact, by taking advantage of their different chromatin structures, it has been shown that active and inactive rRNA genes coexist in a variety of different eukaryotic cells (3–6).

In the last few years, detailed functional analyses of the rRNA intergenic spacer allowed the identification of several DNA elements that regulate transcription of these genes. The type and arrangement of these regulatory elements appear to be conserved among many different organisms (for review see 7–9). In contrast, much less is known about the sequence elements involved in the replication of the rDNA. Studies directed at identifying the origin of replication in the rDNA of various species seem to confirm the two distinct patterns characteristic of chromosomal replication of simple and complex genomes (reviewed in 10–12). In contrast to the site-specific initiation of replication in the rRNA gene spacer of the lower eukaryotes *Saccharomyces cerevisiae* (13,14), *Physarum polycephalum* (15) and *Tetrahymena thermophila* (16), replication origins in the rDNA of animal cells are distributed over broad regions (17,18).

In contrast to the considerable differences in the selection of the sites where replication initiation takes place among different species, recent studies on replication fork progression in three disparate eucaryotic organisms seem to indicate a common feature of rDNA replication, namely the presence of a replication fork barrier (RFB) near the 3′ end of the rRNA gene (13,17,19,20).

Since its discovery in yeast, studies aimed at understanding the nature of the RFB and its relationship to rRNA gene transcription have been undertaken. From the fact that both transcription and replication can occur simultaneously on the same rDNA region (21,22), it is reasonable to expect that the RFB might somehow be related to the transcriptional process. The initial hypothesis proposing that in yeast the arrest of fork movement may be directly caused by repeated head-on collisions between the massive flow of transcribing RNA polymerases and the replication complex has recently been contradicted by the finding that sequences at the 3′ end of the yeast rRNA transcription unit can act as an RFB when inserted into an extrachromosomal plasmid in the absence of elongating RNA polymerases (23,24). In this organisms, the RFB has been mapped to two closely spaced sites located about 200 bp downstream of the site of transcription termination and has been interpreted as specific protein–DNA complexes that are able to arrest a replication fork coming from downstream, thereby preventing collisions between the transcription and the replication machineries moving in opposite directions (23). However, the fact that in the native chromosomal context the arrested replication

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forks were found mostly at the 3′ end of the transcriptionally active rRNA gene copies suggests a correlation between expression of the gene and efficiency of the nearby RFB (25).

In this study, we focused our attention on replication fork progression along the rRNA gene spacer sequences flanking the 3′ end of the rRNA transcription units in *Xenopus laevis* and *Xenopus borealis*. A peculiar difference between these two related frog species resides in the function of the transcriptional elements at the 3′ end of the rRNA coding region. In contrast to *X.borealis*, the *X.laevis* rRNA transcription unit seems to lack an efficient transcription terminator at its 3′ end (26–28). In the light of these results, we wished to use the 2D gel electrophoretic technique to compare how in these two related organisms rDNA transcription and replication are coordinated. So far, studies on *X.laevis* early embryos or on rDNA plasmids replicated in *X.laevis* eggs and egg extracts failed to detect an RFB (18,29,30). These results might correlate with the absence of rRNA transcription in these particular systems (31).

## MATERIALS AND METHODS

### Culture and synchronization of cells

Xenopus laevis A6 cells and *Xenopus borealis* Xb 693 cells were grown at room temperature (5% $CO_2$/95% air) in 70% Dulbecco's modified Eagle's medium (pH 7.2, Amimed), supplemented with 4.5 g/l glucose, 1% glutamine, and 10% fetal calf serum (Biological Industries). The cells were synchronized at the G1/S phase boundary by first collecting them in the G0 phase by isoleucine starvation followed by release into complete medium containing aphidicolin, essentially as described (32).

### DNA isolation and gel electrophoresis

In order to stabilize the replication intermediates, the DNA was first cross-linked *in vivo* with psoralen. In a typical experiment, about $5\times10^8$ cells collected by trypsinization were resuspended in 20 ml ice cold cell culture medium and irradiated on ice in an open plastic dish with a high-pressure mercury lamp in the presence of 4,5′,8-trimethylpsoralen as previously described (25).

The DNA was gently extracted by two phenol and one chloroform treatment, ethanol precipitated and resuspended in TE (10 mM Tris–HCl pH 7.6, 1 mM EDTA) containing 50 μg/ml RNAse A (Böhringer) at 37°C for 30 min. Ribosomal DNA was purified on CsCl–actinomycin D gradients exactly as previously described (6). Neutral/neutral two-dimensional agarose gel electrophoresis was performed as described (25), except that the first dimension was run for 24 h and the second dimension for 12 h. Alkaline Southern blotting and hybridizations were done as previously described (4). The probes used are shown in the restriction maps of the respective figures and are described in reference (4).

## RESULTS

### Identification of rDNA replication intermediates

Replication fork progression through the rDNA locus of *X.laevis* and *X.borealis* tissue culture cells was studied by analyzing steady-state levels of replication intermediates of specific rDNA restriction fragments by using the neutral/neutral 2D agarose gel electrophoretic technique developed by Brewer and Fangman (33,34). To increase the fraction of the molecules under study, we have purified rDNA on CsCl gradients by taking advantage of its markedly higher GC content than the bulk of the genome. Furthermore, to prevent damage or loss of replication intermediates due to branch migration occuring during the isolation procedure, the DNA was cross-linked *in-vivo* with psoralen. Psoralen cross-links stabilize replication forks and have no influence on the migration of the replication intermediates in this 2D gel system (25,35). In some experiments, the cells were synchronized and harvested in the S phase in order to further increase the ratio of rDNA replication intermediates relative to linear, nonreplicating DNA.

Figure 1b shows an ethidium bromide-stained 2D gel of *X.laevis* rDNA purified from early S phase cells and digested with *Eco*RI, which cuts twice in the rDNA repeat unit (see map in Figure 1a). The two expected rDNA fragments are clearly visible as two prominent spots on the faint diagonal of linear molecules indicating that the DNA fraction analyzed is highly enriched in rDNA sequences. The two less intense spots correspond to fragments containing rRNA gene spacers with different sizes. A Southern blot of this 2D gel, hybridized with a probe specific for the 4.8 kb *Eco*RI coding fragment is shown in Figure 1c. The autoradiograph reveals a classical simple Y arc originating from the intense spot of nonreplicating 4.8 kb linear molecules, consistent with this fragment being replicated from a single fork progressing from one end to the other and generating a series of Y-shaped replication intermediates (33). The nearly vertical line starting from the diagonal of linears at the end of the simple Y-arc corresponds to X-shaped recombination intermediates (19; see also schematic drawing in panel d). The relative intensity of this signal varied between different experiments: since this phenomenon was not relevant to our work it was not further investigated.

### Polar replication fork barriers in the 3′ flanking ribosomal spacer region of *X.laevis*

The arrest of replication fork progression at a specific site within a DNA fragment is reflected by the accumulation of long-lived, Y-shaped replication intermediates of a specific size which generate an intense spot at a defined position along the simple Y arc on a 2D gel (19). By taking advantage of this feature, we looked for the presence of RFBs in the *X.laevis* rDNA. For this purpose, we analyzed the replication intermediates of the 3 kb *Pvu*II–*Bam*HI fragment containing a portion of the 28s rRNA coding region as well as 3′ flanking spacer sequences up to the first spacer promoter (see map in Figure 2a). In the 2D gel, these intermediates trace a complete simple Y pattern indicative of replication that occurs by fork movement through this fragment from one end to the other (Figure 2a). However, a conspicuous region along the ascending portion of the Y-arc (indicated by a bracket in the autoradiograph) shows an intense hybridization signal, suggesting the presence of arrested replication forks. This area corresponds to a series of accumulated Y-shaped intermediates that are approximately from 35 to 50% replicated, somehow indicating the presence of a series of barriers at which fork arrest can occur. In a shorter autoradiographic exposure of the same filter (figure 2a′), the nonreplicating *Pvu*II–*Bam*HI linear fragment is resolved as a single, well defined spot, confirming that the elongated shape of the signal corresponding to the accumulated intermediates is not due to length heterogeneity within this particular rDNA restriction fragment, but rather reflects the presence of a population of molecules of equal length that have been replicated to different extents.

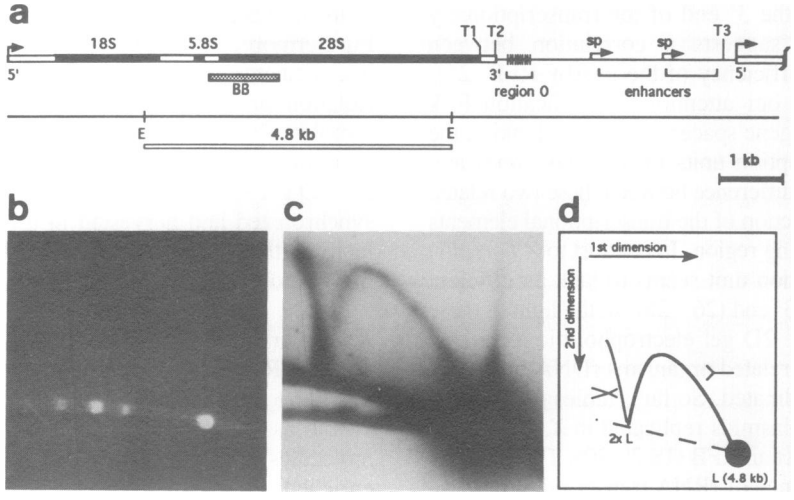


**Figure 1.** Replicative intermediates of the *X.laevis* rDNA coding region. (**a**) Structural organization and *Eco*RI (E) restriction map of the rDNA repeat unit of *X.laevis*. The 40S precursor coding region is indicated as a box; its 5′ (arrow) and 3′ ends (T2) are indicated, and the filled boxes represent the sequences coding for the different rRNA species (T1 is the 3′ end of the 28s rRNA). The ribosomal intergenic spacer is indicated as a line; some relevant regions like spacer promoters (sp), enhancers, repetitive region 0 and the upstream terminator (T3) are indicated. The location of the analyzed 4.8 kb *Eco*RI coding fragment is also shown. (**b**) CsCl-purified rDNA isolated from synchronized cells harvested 1.5 h after release into S phase was digested with *Eco*RI, separated on a 2D gel, and stained with ethidium-bromide. (**c**) The 2D gel in panel b was blotted and hybridized with probe BB (stippled box in panel a). (**d**) Interpretation of the autoradiograph in panel c. The diagonal of linears is shown as a dashed line. A simple-Y arc is rising from the spot of the 4.8 kb linear fragment (L) and returning back to the diagonal of linears at a position of 9.6 kb (2xL). The X-shaped molecules migrating along a straight line starting from position 2xL represent recombination intermediates. The directions of the two electrophoreses are also indicated.

Since a neutral/neutral 2D gel does not provide information about direction of fork movement, the accumulated replication intermediates migrating in the elongated spot in Figure 2a might consist of two types, namely intermediates containing forks that were moving to the left or to the right before their arrest (see schematic drawing in Figure 2a). To determine the polarity and the sites of fork arrest, we examined the replication intermediates of the 4.7 kb *Bam*HI fragment (see map in Figure 2b). This fragment comprises the same 3 kb region as the fragment analyzed previously and includes additional 1.7 kb of coding sequences to the left. We reasoned that if the stalled replication forks in Figure 2a were directed to the left, the addition of sequences to the left would produce branched molecules with a longer unreplicated portion that would migrate more towards the spot of the linear fragments. In contrast, the addition of the same sequences to replication intermediates containing stalled forks directed to the right would produce intermediates with longer replicated branches (i.e., molecules replicated to a higher extent) that would migrate on the simple Y arc further away from the linear fragments. From Figure 2b, it is apparent that the position of the accumulated intermediates (see bracket in Figure 2b) originating from the 4.7 kb *Bam*HI fragment was shifted towards the spot of linear molecules compared to the 3 kb *Pvu*II−*Bam*HI fragment, which is consistent with the presence of arrested replication forks directed to the left (compare bracketed areas in the autoradiographs in Figure 2a and b). Additional irregularities in signal intensity along the simple Y arc in Figure 2b are most probably due to local differences in blotting efficiencies that we occasionally observe (see Figure 6a and b for a similar experiment). Taken together, the results presented in Figure 2 demonstrate the presence of polar replication fork barriers that can arrest replication forks progressing toward the rRNA transcription unit. The size range of the accumulated intermediates is consistent with a series of barriers spread over an about 450 bp long spacer region flanking the 3′ end of the rRNA gene (see bracket labelled RFB in the map in Figure 2a). The fact that the intensity of the hybridization signal of the accumulated intermediates is stronger towards the middle might indicate that there are sites in the middle of the 450 bp region where forks stop at higher frequency.

## A polar replication fork barrier near the 3′ end of the *X.borealis* rRNA transcription unit

In contrast to *X.laevis*, the 3′ end of the rRNA transcription unit of the related species *X.borealis* is characterized by the presence of two closely spaced transcription termination sites which appear to completely prevent RNA polymerase from proceeding into the downstream intergenic spacer (sites T2 in Fig 3a; 28). To investigate replication fork progression around the 3′ end of the *X.borealis* rRNA genes, we examined replication intermediates of the ~4.9 kb *Sst*I fragment containing about 1.2 kb of coding and 3.7 kb of 3′ flanking spacer sequences (see map in Figure 3a). Due to length heterogeneities in the 3′ flanking spacer region, this fragment shows three different size classes which are reflected by the presence of three closely spaced spots on the diagonal of linear molecules on the 2D gel (see short exposure in Figure 3b′). Because of these spacer length heterogeneities, replication intermediates of this fragment reproducibly traced a simple Y pattern with a fuzzy appearance, most probably reflecting the combination of three closely spaced arcs of different intensities intersecting each other at a position close to their inflection points (Figure 3b, for interpretation see Figure 3c). However, distinct, intense spots are visible at a defined position along the descending portion of the arc, consistent with the presence of accumulated intermediates of distinct size classes representing arrested forks that are approximately 70% replicated (arrows in Figure 3b; note

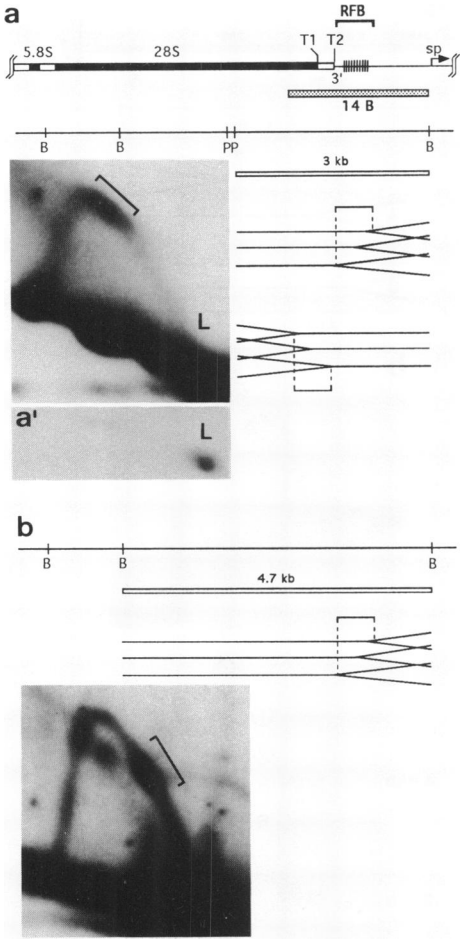


**Figure 2.** Replication fork barriers in the 3′ downstream spacer region of *X.laevis* rRNA genes. (**a**) Purified rDNA isolated from exponentially growing *X.laevis* cells was digested with *Pvu*II and *Bam*HI, separated on a 2D gel, blotted and hybridized with probe 14B in order to detect the 3 kb *Pvu*II−*Bam*HI fragment containing the 3′ end of the rRNA coding region. The locations of the analyzed fragment, the probe (stippled box), the *Pvu*II (P) and the *Bam*HI (B) sites are shown on the map on top of the panel. The bracket on the autoradiograph marks the ellipsoid spot on the Y-arc where arrested replication forks have accumulated. The possible structures and orientations of the replication forks migrating in this spot are indicated to the right. (**a′**) A short exposure of the lower portion of the same filter as the one in panel a shows the defined spot of the 3kb linear fragment (L). (**b**) Replication intermediates of the 4.7 kb *Bam*HI fragment (see restriction map on top of the panel). The 2D gel analyses was as in panel a, except that the rDNA was purified from synchronized *X.laevis* cells, harvested 1.5 h after release into S phase. The structures of the arrested replication forks accumulated in the ellipsoid spot along the Y-arc (bracket on the autoradiograph) are shown below the restriction map. The location of the broad region where these forks are arrested is indicated in the rDNA map in panel a (bracket labelled RFB). The additional spot under the Y-arc in panel b is an artifact of this particular Southern transfer.

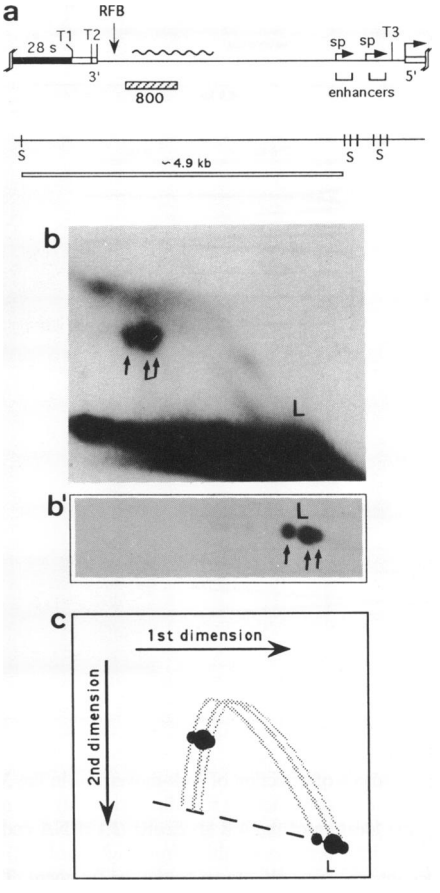


**Figure 3.** Replication fork barrier close to the 3′end of the *X.borealis* rRNA coding region (**a**) Structural organization and *Sst*I (S) restriction map of the rRNA gene spacer of *X.borealis*. The wavy line indicates spacer regions showing length heterogeneitiy. (**b**) Purified rDNA isolated from exponentially growing *X.borealis* cells was digested with *Sst*I, separated on a 2D gel, blotted and hybridized with probe 800 in order to detect the ~4.9 kb rDNA fragment containing the 3′ end of the coding region (see map in panel a). The arrows in the autoradiograph point to the presence of defined spots corresponding to accumulated forks about 70% replicated. (**b′**) A short exposure of the lower portion of the same filter shows three spots corresponding to three different size classes of the analyzed fragment (L). (**c**) Interpretation of the 2D gel in panel b.

that the signal representing the smallest size class is not resolved, most probably because it is too close to the signal of the most abundant size class). By analyzing a shorter fragment from the same rDNA region, we confirmed that the accumulated replication intermediates, as in the case of *X.laevis*, contain arrested forks directed to the left (data not shown). However, in contrast to the related frog species, the discrete sizes of the arrested replication intermediates suggest the presence of a polar replication fork barrier localized in a defined region close to the 3′ end of the rRNA transcription unit (arrow labelled RFB in the rDNA map in Figure 3a).

## Efficiency of replication fork arrest

In the yeast *Saccharomyces cerevisiae*, replication forks moving towards the 3′ end of the rRNA transcription unit are efficiently blocked at the replication fork barrier, resulting in more than 90% of the rRNA genes being replicated in the same direction as transcription (13, 23). To determine the direction of replication fork movement in the *X.laevis* rRNA coding region, we analyzed replication intermediates of various coding fragments by using the modified 2D gel procedure described by Fangman and Brewer (14), which includes an *in situ* digest after the first dimension.

In a first experiment, *Eco*RI digested rDNA separated in the first dimension was redigested in the gel with *Nco*I in order to remove 2 kb from the right hand side of the 4.8 kb rRNA coding fragment (Figure 4a). The *in situ* digested DNA was subsequently separated in the second dimension, blotted and hybridized with probe BB, which detects the 2.8 kb *Eco*RI−*Nco*I portion. The

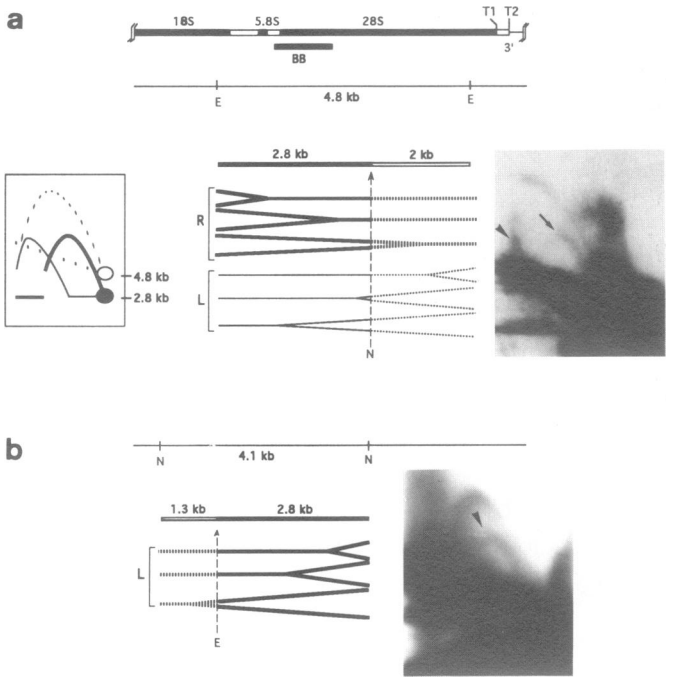


**Figure 4.** 2D gel analysis of direction of fork-movement in the *X.laevis* rRNA coding region: *in situ* digestion after the first dimension. (**a**) The restriction map at the top shows the location of the 4.8 kb *Eco*RI (E) rRNA coding fragment. Y-shaped replication intermediates resulting from rightwards (R) and leftwards (L) moving forks are schematically drawn below the fragment. The position of the *Nco*I site (N) is indicated by the dashed vertical line. *Eco*RI digested, purified rDNA isolated from S phase cells was separated in the first dimension, digested *in situ* with *Nco*I, and subjected to the second dimension. The gel was blotted and hybridized with probe BB (see map at the top). The cartoon on the left represents the expected 2D gel pattern (adapted from reference 14). Replication intermediates of the 2.8 kb *Eco*RI−*Nco*I fragment resulting from rightwards and leftwards moving forks are separated along the thick and the thin arc, respectively, whereas the dashed arc represents replication intermediates of the undigested 4.8 kb *Eco*RI fragment. The positions of the 2.8 and 4.8 kb linear fragments are also indicated. The actual result is shown on the autoradiograph to the right. Portions of the arcs indicative of the presence of both right (arrow) and left (arrowhead) moving forks are indicated. (**b**) The same experiment as the one in panel a was performed with *Nco*I-digested rDNA which was subsequently digested *in situ* with *Eco*RI. The arrowhead in the autoradiograph points to a portion of the arc representing *Eco*RI−*Nco*I fragments being replicated by leftwards moving forks.

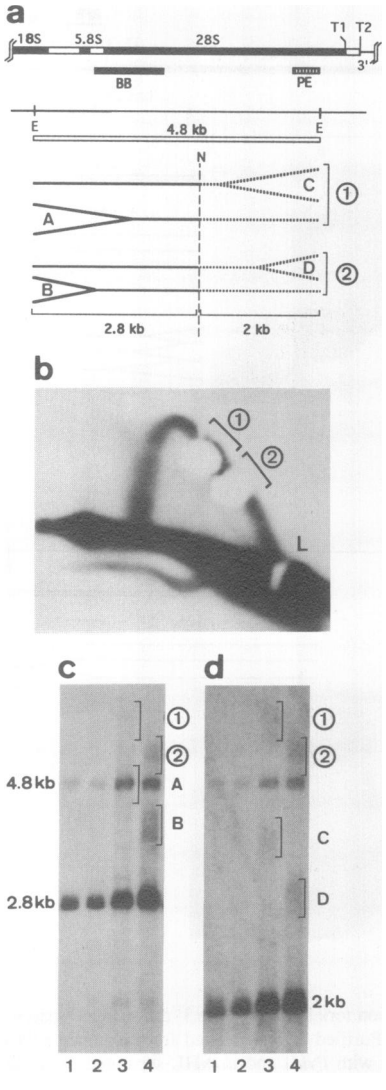


**Figure 5.** Direction of fork-movement in the *X.laevis* rRNA coding region: analyses of 2D gel-purified replication intermediates. (**a**) rDNA map spanning the 4.8 kb *Eco*RI coding fragment. The scheme below the map illustrates the four possible replication forks isolated from region 1 and 2 of the 2D gel in panel b. *Nco*I digestion of these replication intermediates (dashed vertical line) releases 2.8 and 2 kb long linear fragments as well as the four forks labelled A through D. (**b**) Purified rDNA from exponentially growing *X.laevis* cells was digested with *Eco*RI, and separated on a 2D gel. Small gel plugs containing the 4.8 kb linear fragment (L) and replication intermediates migrating along the ascending portion of the Y-arc (region 1 and 2) were removed, and the gel was blotted and hybridized with probe BB (see map). (**c**) DNA eluted from the gel plugs was digested with *Nco*I, separated on a 1.5% agarose gel, blotted and hybridized with probe BB. Lane 1: *X.laevis* total DNA digested with *Eco*RI and *Nco*I. Lanes 2, 3 and 4: DNA from region L, 1 and 2, respectively. The brackets labelled 1 and 2 points to undigested replication intermediates of the 4.8 kb *Eco*RI fragment. The brackets labelled A and B indicate the positions of the rightwards moving forks A and B drawn in panel a. (**d**) The same transfer shown in panel c was hybridized with probe PE specific for the 2 kb *Nco*I−*Eco*RI fragment (see map). The signals labelled C and D represent the leftwards moving forks C and D shown in panel a.

position of the small simple Y arc traced by these shortened replication intermediates will give information about the direction of replication fork movement according to the following rationale. Removal of sequences from the right hand side of replication intermediates containing rightwards moving forks will convert the Y's with long replicated arms to linear molecules, whereas the molecules with short newly replicated arms will retain their branched structures and will trace a small simple Y arc starting at the spot of linear fragments (thick arc in the interpretive diagram in Figure 4a; see also schematic drawing in the center of the Figure). On the other hand, removal of the same sequences from replication intermediates containing leftwards moving forks will convert the less replicated fragments to linear molecules, and only the more replicated, slowest migrating fragments will contribute to a small simple Y arc shifted to the left with respect to the spot of linears (thin arc in Figure 4a). The result of this experiment is shown in the autoradiograph in Figure 4a. The faint, bigger arc corresponds to replication intermediates of the 4.8 kb *Eco*RI coding fragment derived from incomplete cutting by *Nco*I during the *in situ* digest, whereas the smaller arc

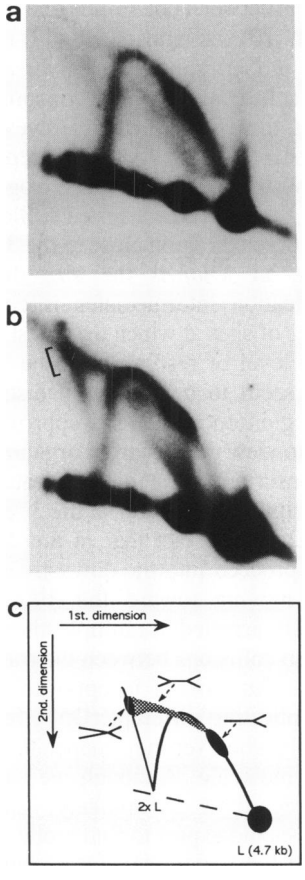


**Figure 6.** Replicon fusion at the arrested replication forks. Purified, *Bam*HI digested rDNA derived from synchronized *X.laevis* cells harvested 2 h (**a**) or 4 h (**b**) after release into S phase was separated on 2D gels, blotted and hybridized with probe 14B specific for the 4.7 kb rDNA fragment containing the 3′ end of the rRNA coding region (see maps in Figure 2 for location of the probe and the fragment analyzed). (**c**) Interpretation of the autoradiograph in panel b. The dotted area starting from the accumulated replication forks and ending at an ellipsoid spot on the arc of the X-shaped intermediates represents double-Y structures corresponding to fragments with an arrested fork being completed by a second replication fork (For details see reference 36).

indicated by the arrow clearly demonstrates the presence of fragments replicated from rightwards moving forks. Unfortunately, high background signal from linear, partial digestion products covers most of the gel area where the small simple Y arc indicative of leftwards moving forks migrates (the signal indicated by the arrowhead most probably represents the apex of this small arc).

To unambiguously prove the existance of replication forks moving through the coding region in a 3′-to-5′ direction, we performed a similar 2D gel analysis in which rDNA was first digested by *Nco*I and then redigested *in situ* by *Eco*RI (see schematic drawing in Figure 4b). In this case, we have removed sequences from the left hand side of the fragment under study, and, therefore, 2.8 kb *Eco*RI−*Nco*I fragments replicated by leftwards moving forks are expected to migrate along a small simple Y arc starting at the spot of linear fragments. The appearance of this type of arc in the autoradiograph in Figure 4b (arrowhead), confirms the presence of replication forks moving through the rRNA transcription unit in the opposite direction as transcription. However, the failure to detect both types of arcs in the same 2D gel, precluded a direct estimation of the proportion of the forks moving in either direction.

To gain more quantitative information about the direction of fork movement through the rRNA coding region, we determined the fraction of rightwards and leftwards moving forks among specific, early Y-shaped replication intermediates that were recovered from preparative neutral/neutral 2D gels (25). The strategy and the actual data of this approach are shown in Figure 5. Ribosomal DNA from exponentially growing *X.laevis* cells was digested with *Eco*RI and separated on a 2D gel. Small gel plugs expected to contain early replication intermediates of the 4.8 kb *Eco*RI coding fragment were removed from the gel and the DNA was recovered. The remainder of the 2D gel, after blotting and hybridization with a probe specific for this fragment, reveals the exact positions along the simple Y arc where these specific intermediates were taken from (regions 1 and 2 in the autoradiograph in Figure 5b; the expected structures and orientations of the recovered intermediates are indicated schematically in panel a). The gel-purified replication intermediates were then redigested with *Nco*I, separated on a normal agarose gel, blotted and hybridized sequentially with probe BB and PE specific for the left 2.8 kb and the right 2 kb portion of the *Eco*RI coding fragment, respectively (Figure 5c and d, respectively; see also panel a for the location of the probes and the *Nco*I site). As it can be seen from the schematic drawing in Figure 5a, *Nco*I digestion of early replication intermediates containing rightwards moving forks produces forks A and B that are detected by the probe to the left as retarded smears above the 2.8 kb linear fragments (brackets labelled A and B in Figure 5c, lanes 3 and 4, respectively; note that forks A partially comigrate with the 4.8 kb linear fragment). On the other hand, replication intermediates containing left moving forks produce forks C and D detected only by the probe to the right (see respective brackets in Figure 5d). The more retarded smears labelled 1 and 2 in both autoradiographs represent large Y-shaped molecules that resulted from the failure of *Nco*I to cleave some of the *Eco*RI intermediates recovered from the 2D gel. A considerable portion of these branched molecules appear to be converted to linear, 4.8 kb long fragments by single-strand breaks at the replication forks, most probably as a result of the action of single-strand nucleases which could have been present during the agarose digestion (i.e., during the recovery of the DNA from the gel plugs) or during the subsequent *Nco*I digestion (see 4.8 kb bands in lanes 3 and 4 of Figure 5c and d). Similarly, a considerable fraction of the 2.8 and 2 kb linear fragments detected by the two probes may also represents breakage of forks A−B and C−D, respectively. However, by making the reasonable assumption that forks moving in either direction have the same probability of breakage, the ratio of the branched molecules detected by the left and the right probe represents the ratio of righwards and leftwards moving forks present in the analyzed population of gel-purified replication intermediates. By taking the signal of the 4.8 kb band as an internal control for hybridization efficiency, it is apparent that both probes detect roughly the same amount of DNA in the regions where forks B and D migrate (compare the respective signals in lane 4 of Figure 5c and d). Since probe BB detects parental DNA of fork B, whereas probe PE detects duplicated DNA of fork D (see Figure 5a), we can conclude that among the early Y-shaped replication intermediates migrating in region 2 of the 2D gel in Figure 5b there are about twice as much rightwards as leftwards moving forks.

A direct estimation of the fraction of rightwards and leftwards moving forks among the replication intermediates of region 1 was precluded by the fact that forks A partially comigrated with the 4.8 kb linear fragment (Figure 5c, lane 3). Since we have found no evidence for initiation of DNA replication either in the 3′ portion of the rRNA coding region nor in the 3′ flanking spacer sequences (see Figure 2), it is apparent that most of the replication forks moving through the 4.8 kb *Eco*RI coding fragment in a 3′-to-5′ direction must have originated from regions further downstream. In summary, these results demonstrate the presence of a considerable fraction of replication forks that have entered the transcription unit from downstream after having passed the replication fork barriers.

### Replicon fusion at the replication fork barriers

The fact that a considerable fraction of the *X.laevis* rRNA transcription units are replicated in a 3′-to-5′ direction implies that either the RFBs represent only pause sites, or that leftwards moving forks are permanently blocked only in a subset of rDNA repeats, whereas in the remainder fraction they can pass the barriers without impediment. The second case predicts that the site where a leftwards moving fork has completely stopped should coincide with the site where replicon fusion occurs. In this scenario replication intermediates containing an arrested replication fork would be resolved by a second fork coming from the upstream replicon, resulting in the formation of double Y-shaped terminating molecules migrating along a characteristic arc in a 2D gel (19,36). To search for the occurrence of replicon fusion at the *X.laevis* replication fork barriers, we analyzed rDNA replication intermediates isolated from synchronized cells harvested at different times after release into S phase. Two hours after release, replication intermediates of the 4.7 kb *Bam*HI fragment trace the characteristic simple Y pattern with the expected accumulation of stalled forks in the ascending portion of the arc (Figure 6a, see Figure 2b for the location of the fragment). However, a 2D gel analyses of the same fragment derived from cells harvested four hours after release into the S phase shows an additional form of intermediates migrating along a broad diagonal line leaving the apex of the simple Y arc and ending at a defined region along the line of recombinants (Figure 6b). This pattern is the one generated by fragments with a stalled fork being completed by a second fork entering from the other side (19,36; see also schematic drawing in Figure 6c). The shape and position of the broad, intense signal almost at the end of the line of recombinants (bracket in Figure 6b) is consistent with an accumulation of a series of X-shaped molecules containing forks meeting at the different replication fork barriers. These results point to the presence of absolute barriers to replication fork movement representing the sites where replicon fusion occurs.

## DISCUSSION

We show that stabilization of replication forks *in vivo* by psoralen cross-linking and subsequent purification of total rDNA by cesium chloride gradient fractionation represents a valid isolation procedure which permits 2D gel analysis of rDNA replication intermediates from asynchronous populations of slowly dividing tissue culture cells.

Analysis of replication fork progression along the 3′ flanking rRNA gene spacer sequences in *X.laevis* and *X.borealis* demonstrates the presence of polar RFBs preventing replication forks from entering the 3′ ends of the rRNA transcription units. The strong parallels between our results and those obtained with yeast (13,19), plant (20) and human cells (17) reinforce the notion that the RFB may in fact represent a universal mechanism with which eukaryotic cells can coordinate concomitant transcription and replication at the rDNA locus. However, a remarkable difference in the location of the sites where replication fork arrest takes place distinguishes these two related frog species from each other. Whereas in *X.borealis* the arrested replication forks appear to be arrested at a defined region close to the 3′ end of the rRNA transcription unit, in *X.laevis* the size distribution of the accumulated replication intermediates is consistent with the presence of a series of sites at which the arrest of fork movement can occur. At the level of resolution of the 2D gel technique, these arrest sites seem to be randomly distributed over a 3′ flanking spacer region encompassing approximately 450 bp.

It is interesting to view this different organization of the RFBs with regard to transcriptional studies indicating the absence of an efficient transcription terminator at the 3′ end of the *X.laevis* rRNA genes (26,27). The fact that in this frog species RNA polymerases may proceed into the downstream spacer and that replication forks moving toward the 3′ end of the rRNA transcription unit are arrested at multiple sites may suggest that the RFBs are due to collisions between the transcription and the replication machineries moving in opposite directions. In this situation, forks replicating different rDNA repeats will stop at different sites simply wherever they happen to meet a converging RNA polymerase molecule. Although we cannot exclude this possibility, we regard it as unlikely, since under these circumstances one should expect to find replication forks arrested throughout most of the intergenic spacer, rather than confined to a portion of the spacer flanking the 3′ end of the transcription unit. From the analysis of the DNA sequence organization of the 3′ flanking spacer region in this *X.laevis* cell line, it is apparent that the arrested forks map to a repetitive region consisting of ten copies of a tandemly repeated 35 bp unit (data not shown; see 'region 0' in Figure 1 and reference 37). This result rather favours a model in which this 35 bp repeat unit might contain a specific DNA element capable of directing replication fork arrest (it should be noted that the 2D gel technique does not have sufficient resolution to distinguish specific arrest sites separated from each other by only 35 bp). The finding that a 13 bp element contained in this 35 bp repeat unit is also present near the RFB at the 3′ end of the *X.borealis* rRNA transcription unit (28) considerably strengthens the notion of a possible involvement of this common DNA element in the arrest of replication fork progression.

Our results about replication fork arrest in the *X.laevis* rDNA are reminiscent of those obtained with the Epstein–Barr virus. In this virus, replication forks are arrested throughout a repeated region consisting of 20 copies of a tandemly repeated 30 bp sequence (38). Each repeat unit contains a binding site for the viral protein EBNA-1, which has been shown to be directly involved in replication fork arrest (39). In analogy to these results and to those obtained with the yeast rDNA (23,24), it is reasonable to expect that fork arrest in the *Xenopus* rDNA might also be mediated by a *trans*-acting factor(s) binding to DNA. The fact that in *X.laevis* early embryos replication forks traverse the rDNA without impediment (18) rules out the possibility that the barriers detected in the tissue culture cells are merely caused by a peculiar DNA structure adopted by the 3′ flanking spacer sequences. The failure to detect an RFB in early embryonic cells may result from the absence of rRNA transcription or the absence

of the putative RFB binding factor(s) at this particular developmental stage (18,31).

Since transcriptional run-on experiments with *X.laevis* nuclei have detected elongating RNA polymerases throughout almost the entire rRNA intergenic spacer (28), one could ask whether RNA polymerases transcribing spacer sequences downstream of the RFBs may encounter a replication fork moving in the opposite direction. If such collisions occur, they might be resolved in favour of replication as has been found for the *E.coli* replication machinery entering the rRNA operon in the opposite direction of transcription (40). Alternatively, transcription elongation *in vivo* through the *X.laevis* rRNA gene spacer might be either very low or restricted to other periods of the cell cycle other than S phase, and, therefore, direct interactions between transcription and replication might be very unlikely. In this context, it is worth mentioning recent results from our laboratory on the chromatin structure along the rDNA in *Xenopus* tissue culture cells. Unlike in *X.borealis*, where most of the spacer regions flanking the 3′ end of the rRNA transcription unit have been found uniformly packaged in an inactive chromatin structure organized in nucleosomes, in *X.laevis*, a subset of rDNA repeats showed 3′ flanking spacer sequences with a heterogeneous chromatin structure indicating the presence of a disturbed nucleosomal organization (4). The peculiar chromatin structure of the *X.laevis* spacers has been interpreted as the result of RNA polymerase I molecules transcribing through the inefficient terminator and entering the intergenic spacer, thereby disrupting nucleosomes. However, in contrast to the heavily transcribed rRNA coding regions which appear completely devoid of nucleosomes (4), the heterogeneous structures at the *X.laevis* 3′ flanking spacer sequences could be an indication that only few RNA polymerases traverse these spacer regions, allowing the presence of residual nucleosomal structures. It is very possible that, once formed, the putative protein−DNA complex at the RFB responsible for the arrest of replication fork movement might also be able to prevent transcribing RNA polymerases from proceeding beyond the fork barriers and disrupting chromatin structures further downstream. Supporting this conclusion, a recent *in vitro* transcriptional study has shown the presence of a roadblock for RNA polymerase I transcription located very close to the RFB site in the yeast rDNA (41). This situation would definitely prevent readthrough transcription entering the *X.laevis* spacer from colliding with replication forks moving in the opposite direction.

By analyzing the direction of fork movement in the *X.laevis* rDNA, we showed that a considerable fraction of the rRNA coding regions is replicated in a 3′-to-5′ direction. One can basically visualize two models explaining this fraction of replication forks moving in the opposite direction of transcription. One possibility is that the barriers represent only pause sites at which the replication machinery pauses for only a fraction of the S phase before continuing into the transcription unit. The other possibility is that in a subset of rDNA repeats the barriers cause an absolute block to replication fork progression, whereas in other repeats, leftwards moving forks are able to pass the barriers without impediment. Since our 2D gel analysis revealed the presence of stalled replication intermediates being completed by a second fork entering from the other side, it is apparent that in a subset of spacers the RFBs represent absolute blocks to left moving forks and define the sites where replicon fusion occurs. These results support the second model proposing two different types of rDNA repeats with distinct replication patterns. It should be noted, however, that it is not possible to distinguish whether the forks entering the 3′ end of the transcription units pause at the RFBs or replicate through these sequences completely unimpeded.

Since we have shown that active and inactive rRNA genes coexist in these *X.laevis* cells (data not shown, see also reference 4), it is reasonable to propose a correlation between these two different replication pattern and the transcriptional state of the genes. In this model, the transcriptionally active rRNA gene copies have a functional barrier at their adjacent 3′ flanking spacers sequences and can be replicated only in the same direction as transcription, whereas in the inactive gene copies replication forks can pass through the RFB sequences and replicate the coding regions in a 3′-to-5′ direction. However, depending where the active replication origins are located with respect to the transcriptionally active and inactive gene copies, inactive genes might also be replicated in a 5′-to-3′ direction (25). These conclusions are supported by recent results obtained with yeast cells in which analysis of the chromatin structure of the stalled rDNA replication intermediates was consistent with most of the replication forks being arrested at the 3′ end of the transcriptionally active gene copies (25). We are currently testing this model by analyzing the chromatin structure of *X.laevis* rDNA replication intermediates, for active and inactive rRNA genes can easily be recognized by their nonnucleosomal and nucleosomal organization, respectively (3,4,25). How could only active genes specifically manage to have a functional RFB at sequences located as far as 500 bp downstream of their 3′ ends? Since in *X.laevis* the sequences flanking the 3′ end of the inactive rRNA gene copies appears regularly packaged in nucleosomes (4), it is very possible that one of the roles of readthrough transcription into the downstream intergenic spacer might be to open up the chromatin structure in order to allow binding of the putative RFB factor and establishment of a functional barrier at the 3′ end of the active gene copies.

## ACKNOWLEDGEMENTS

We are grateful to Duri Rungger for providing the *X.laevis* cell line. We thank Ron Reeder for the generous gifts of the *X.borealis* cell line and the rDNA plasmids and H.Mayer-Rosa for excellent technical assistance. This work was supported by Schweizerischer Nationalfonds zur Förderung der Wissenschaftlichen Forschung (31-310 68.91).

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