Mapping the Sites of Initiation of DNA Replication in Rat and Human rRNA Genes*

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To study the organization of DNA replication in mammalian rRNA genes, the sites of initiation of DNA synthesis in rat and human rRNA genes were mapped by two independent techniques. In rat cells the growth of the nascent DNA chains was blocked by Trioxsalen cross-links introduced in vivo. The fraction of "restricted" nascent DNA chains labeled in vivo was isolated, and the abundance in this fraction of cloned ribosomal DNA sequences was determined by hybridization. In the experiments with human cells, the nascent DNA chains were allowed to grow unrestricted for a certain period of time and the movement of the replication forks along the rRNA genes was followed by hybridization of cloned ribosomal DNA sequences to the "unrestricted" nascent DNA fragments fractionated according to size. The results show that in both rRNA genes there are two well defined regions of initiation of DNA synthesis. The first one is located upstream of the transcription units and the second one is located at the 3'-end of the coding regions of the ribosomal DNA repeats.

A number of techniques for mapping sites of initiation of DNA synthesis in vivo have been developed during several recent years. However, their application has led to conflicting results as to whether origins of DNA replication are located at defined positions in mammalian genomes. In the cases when methods that analyze the newly synthesized DNA chains were applied to the dihydrofolate reductase (DHFR)† gene domain in Chinese hamster ovary cells, which is the most studied model for initiation of mammalian DNA replication, the investigators were able to determine the presence of well defined origins of bidirectional replication (1). On the other hand, the analysis of genomic DNA for the presence of replication bubbles and forks by the method of two-dimensional gel electrophoresis failed to indicate the presence of such origins (2). The rRNA genes are repeated tandemly about 400 times in mammalian genomes. This makes them a suitable model for studies of initiation of DNA replication without the need for synchronization of the cells and amplification of the nascent DNA fragments. Three recent papers have analyzed the initiation of DNA replication in the rRNA gene cluster of human cells. In the first one, the authors used two-dimensional gel electrophoresis and concluded that initiation of replication takes place throughout most of the nontranscribed spacer, but not in the transcription unit and the adjacent regulatory elements (3). In the second paper, the authors used a method called "nascent strand abundance analysis," based on a combination of sedimentation and electrophoretic fractionation of DNA. They reached the conclusion that although initiation of DNA replication was more frequent a few kilobase pairs upstream of the transcribed region, it could occur everywhere in the ribosomal DNA repeat, including the transcription unit itself (4). In the third paper, the authors studied the in vitro replication of plasmids containing cloned human ribosomal DNA sequences and showed that replication initiated specifically within two 7-kb DNA fragments located upstream of the promoter and downstream of the 3'-end of the coding region (5). The results of the first two papers imply that initiation of DNA synthesis may be a random process. However, there is evidence indicating that initiation of DNA replication follows a well defined pattern in vivo. This pattern is specific for each cell line and varies during embryogenesis and development. Thus, certain genes or sequences are replicated early in S-phase in some cells, while the same genes and sequences are replicated late in the S-phase of other cells (6). A second argument against the concept of random initiation of DNA replication is that a completely random initiation, regardless of the number of initiation events and the length of the S-phase, would leave a part of DNA unreplicated in each cell cycle.

Since the problem of the organization of initiation of DNA replication in the rRNA gene cluster or elsewhere in the mammalian genome has not been satisfactorily solved so far, in the present article we used two well established biochemical procedures developed by Anachkova and Hamlin (7), and by Vassilev and Johnson (8), to map replication origins in rat and human rRNA genes, respectively. We modified these procedures to avoid certain drawbacks of the original protocols and the results we obtained did not support the hypothesis of random initiation of DNA replication in vivo. We came to the conclusion that in both rat and human rRNA genes, DNA replication most probably initiates at two well defined areas located a few kilobases upstream of the promoter and at the 3'-end of the transcription unit, and that no initiation of DNA synthesis normally occurs outside these zones.

EXPERIMENTAL PROCEDURES

Cell Cultures—Guerin ascites tumor cells were propagated in Swiss male albino rats. At days 7–9 after inoculation (late log phase), the ascites liquid was withdrawn under sterile conditions and the cells were further cultured in minimal essential medium for suspension cultures (Sigma), buffered with 50 mM HEPES. HeLa cells were grown as suspension cultures in minimal essential medium, supplemented with 5% fetal bovine serum (Sigma). Genomic DNA was randomly labeled with 0.025 μCi/ml [3H]dThd (DuPont, 50 mCi/mmol) for 24 h.

Isolation of "Restricted" Nascent DNA Fragments—Cells were spun down at 800 × g and resuspended in fresh medium without serum to
make $5 \times 10^{10}$ cells/mL. DNA was cross-linked by four successive treatments with Trioxsalen and near UV light as described previously (9) to give one Trioxsalen bridge per 1.5 kb on the average. Cross-linked cells were incubated in the presence of 50 $\mu$M BrdUrd (Sigma) and 20 $\mu$Ci/mL [$^{3}H$]dT (70–90 Ci/mmol), or [$^{3}H$]dC, (20–40 Ci/mmol, DuPont) for 1 h to label the nascent DNA fragments synthesized between cross-links. Cells were lysed in 0.5% SDS, 1 mNaCl, 10 mM EDTA, 50 mM Tris-HCl, pH 8, and the proteins were digested with 200 $\mu$g/ml Proteinase K (Merck) at 37°C for 4 h. After deproteinization with phenol/chloroform (1:1) and with chloroform, 1 volume of ethanol was layered and the high molecular weight chromosomal DNA was recovered by spooling on a glass rod. DNA was dissolved in 10 mM Tris-HCl, 1 mM EDTA, pH 7.4, to make approximately 500 $\mu$g/ml, made 0.2 M in NaOH and digested in 5–20% sucrose density gradients prepared in 0.2 M NaOH, 1 mM EDTA, 1 mM NaCl, 0.01 M phosphate buffer, pH 7, containing 0.5% Tween 20 and 100 Ci/ml [3H]dC, or [3H]dT for 10 min. Labeling was terminated by pouring the cell suspension in 10 volumes of ice-cold 0.14 M NaCl, 0.01 M phosphate buffer, pH 7, and DNA was isolated as in the previous paragraph. To size fractionate nascent DNA chains, DNA was dissolved in 10 mM Tris-HCl, 1 mM EDTA, pH 7.4, to make 0.2 $\mu$m in NaOH and applied on top of linear 5–20% sucrose density gradients prepared in 0.2 M NaOH, 1 mM EDTA. Gradients were centrifuged in Beckman SW 27 rotor at 25,000 rpm, 10°C for 18 h. Aliquots were counted, the fractions containing the nascent DNA chains were pooled together, and DNA was immunoprecipitated as described later in the text.

Isolation of “Unrestricted” Nascent DNA Fractions—Exponentially growing HeLa cells were spun down and resuspended in fresh medium without serum to make $5 \times 10^{6}$–$10^{7}$ cells/mL. They were incubated with 50 $\mu$M BrdUrd and 10 $\mu$Ci/ml [$^{3}H$]dC, or [$^{3}H$]dT for 10 min. Labeling was terminated by pelleting the cell suspension in 10 volumes of ice-cold 0.14 M NaCl, 0.01 M phosphate buffer, pH 7, and DNA was isolated as described previously (9). To size fractionate nascent DNA chains, DNA was dissolved in 10 mM Tris-HCl, 1 mM EDTA, pH 7.4, to make 200–500 $\mu$g/ml, made 0.2 $\mu$m in NaOH and applied on top of linear 5–20% sucrose density gradients prepared in 0.2 M NaOH, 1 mM EDTA. Gradients were centrifuged in Beckman SW 27 rotor at 25,000 rpm, 10°C for 18 h. Tubes were unloaded from the bottom and the respective size fractions of DNA were pooled together and centrifuged under the same experimental conditions using Beckman SW 41 rotor.

Immuno-precipitation of the Nascent DNA Strands—The nascent DNA fractions recovered from the gradients were dissolved in 0.14 M NaCl, 0.01 M phosphate buffer, pH 7, containing 0.5% Tween 20 and 100 $\mu$g/ml bovine serum albumin in a final volume of 400 $\mu$l. An equal volume of monoclonal anti-BrdUrd antibody (Beckton and Dickinson) was added, and after 1 h at room temperature the antigen-antibody complex was precipitated with an excess of second antibody (anti-mouse IgG rabbit IgG fraction, Sigma). After another hour at room temperature the samples were kept at 4°C overnight and the precipitate was collected by centrifugation in an Eppendorf microcentrifuge for 10 min. It was washed with 0.14 M NaCl, 0.01 M phosphate buffer, pH 7, resuspended in 200 $\mu$l of 1 M NaCl, 0.05% SDS, 50 mM Tris-HCl, 100 mM NaCl, 0.5% SDS, 50 mM Tris-HCl, 10 mM EDTA, pH 7, and digested with 200 $\mu$g/ml Proteinase K (Merck). After deproteinization with phenol/chloroform (1:1) and chloroform, DNA was precipitated with 2.5 volumes of ethanol.

Hybridization and DNA Probes—For dot-blot hybridization, DNA was loaded onto nitrocellulose membranes (Hybond-C, Amersham) as recommended by the manufacturer using a manifold dot-blotter (Bio-Rad). DNA was transferred carried out using standard procedures (7% SDS, 0.25 M phosphate buffer, 1% bovine serum albumin, at 68°C overnight). The membranes were rinsed with 0.3 M NaCl, 0.03 M sodium citrate, pH 7, at room temperature (twice), washed with 0.1 M NaCl, 0.03 M sodium citrate, pH 7, 0.1% SDS at 68°C for 30 min (twice), and finally rinsed with 0.015 M NaCl, 0.0015 M sodium citrate, pH 7, at room temperature. Areas of the membrane containing individual dots were cut out and counted in Beckman liquid scintillation counter LS1800.

The rat ribosomal DNA probes (10–12) were as follows: RrII was a 5.9-kb EcoRI fragment; Rr133, a 5.9-kb EcoRI fragment; probe 2, DES, a 0.96-kb XbaI fragment; probe 3, CHB, a 0.47-kb EcoRI fragment; and probe 5, CEB, a 0.4-kb XbaI fragment. The mouse ribosomal DNA probes (13) were as follows: probe 1, BES, a 1.2-kb EcoRI/SalI fragment; probe 2, DSS, a 0.96-kb EcoRI/SalI fragment; probe 3, DXX, a 0.3-kb Xbal/Xbal fragment; probe 4, CXX, a 0.4-kb EcoRI/BamHI fragment; and probe 5, CXX, a 0.47-kb HindIII/BamHI fragment (see Fig. 5 for probe locations). 1.2-kb DNA fragment excised from plasmid pL.dIII/RI/III used as a negative control for the DHFR gene, and a 211-base pair DNA fragment excised from plasmid pHJA-IVS-I (13) and containing the 5′-end of human $\beta$-actin gene, were used as controls.

RESULTS

Experimental Approach—The restricted nascent chain growth technique was originally developed in our laboratory (9) and has been successfully used to map “ori-β” in the DHFR domain of Chinese hamster ovary cells (7) and to isolate a number of mouse replication origins (14). The rationale behind this approach is depicted schematically in Fig. 1. Cells whose DNA had been uniformly labeled with [$^{14}C$]dT were treated with Trioxsalen and long wave ultraviolet light to introduce cross-links in DNA in vivo and were incubated with BrdUrd and [$^{3}H$]dT, and the short nascent DNA fragments synthesized at origins of replication located between the cross-links (zone 2) were isolated by alkaline sucrose gradient centrifugation. The low molecular weight weight fraction was purified by immunoprecipitation with anti-BrdUrd antibody and used for hybridization with dot-blotted in excess DNA probes. Hybridization signal was obtained only with probes located at, or close to the origin of replication (zone 2), and not with probes located far from the origin region (zones 1 and 3).

![Fig. 1. Diagram of the restricted nascent chains growth experimental approach.](image)

Following Trioxsalen cross-linking, cells were allowed to synthesize DNA in the presence of BrdUrd and [$^{3}H$]dT, or [$^{3}H$]dC, and the short nascent DNA fragments synthesized at origins of replication located between the cross-links (zone 2) were isolated by alkaline sucrose gradient centrifugation. The low molecular weight weight fraction was purified by immunoprecipitation with anti-BrdUrd antibody and used for hybridization with dot-blotted in excess DNA probes. Hybridization signal was obtained only with probes located at, or close to the origin of replication (zone 2), and not with probes located far from the origin region (zones 1 and 3).

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Mapping Replication Origins in the Rat rRNA Genes—Fig. 3
shows the physical map of the rat ribosomal DNA repeat and
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Rr56, a 5.2-kb DNA sequence located upstream of the promoter sequence, and Rr151, a 3.9-kb sequence located at the 3′-end of the transcription unit, gave good hybridization signals (Fig. 4A).
This showed that there may be two initiation zones in the rat ribosomal DNA repeat. One of them is more pronounced (which would probably mean that it is used more often) and is located upstream of the promoter region, and the second one is

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Mapping Replication Origins in the Human rRNA Genes—Exponentially growing HeLa cells were cross-linked with Trioxsalen and labeled as above. The nascent DNA fraction was isolated and hybridized to five dot-blotted probes that span the length of the human ribosomal DNA repeat. In this case we were able to detect the presence of a replication origin in the nontranscribed spacer 5' of the rRNA gene, but the results were not statistically significant because of the weak hybridization signal. For an unknown reason HeLa cells incorporated labeled precursors less readily than Gueuin cells and the specific radioactivity of the labeled DNA was low. Moreover, both the cloned probes and the nascent DNA fragments were relatively short, which could also lead to the low hybridization signal. To get more conclusive results about the presence and location of replication origins in the human ribosomal DNA repeat we applied the unrestricted nascent chains growth technique in which longer nascent DNA fragments are used. We consider this technique comparable to the restricted nascent chains growth approach since in two different laboratories the positions determined for the DHFR ori-\( \beta \) by the two techniques coincided (7, 18). DNA from exponentially growing HeLa cells, pulse-labeled with \( ^{3}H \)dC and BrdUrd, was size fractionated by two successive alkaline sucrose density gradient centrifugations to obtain five DNA fractions, relatively homogeneous in respect to chain length. 28 S and 18 S rRNA were run as size markers, and it was calculated that the five fractions had average lengths of 30, 15, 8, 4, and 1.5 kb. The nascent DNA fractions were immunoprecipitated with anti-BrdUrd antibody. Five cloned DNA fragments spanning the ribosomal DNA repeat, designated by numbers 1 through 5 (Fig. 5), were dot-blotted in excess and were hybridized with the five size fractions of the in vivo labeled nascent DNA to determine the relative abundance of the cloned fragments in the fractionated nascent DNA. To compare the results obtained with the different size fractions we had to normalize them. This could be done either by using a constant amount of labeled DNA, regardless of the size of the fragments, or by using increasing amounts of DNA with increasing size of the fragments. In the first case we would measure the hybridization capacity in nucleotides per dot, while in the second case it would be expressed in chains per dot. We tried both approaches and found that the first approach gave more reproducible results. Therefore a constant amount of counts of the five size fractions was used for hybridization. A 211-base pair long DNA fragment from the 5'-end of the human \( \beta \)-actin gene was dot-blotted as a negative control and was hybridized with all size fractions. It gave background hybridization with all of them. Probe 1, located at the initiation site for transcription of ribosomal DNA, exhibited high and uniform hybridization with all size fractions of nascent DNA. Probe 2, located at the 3'-end of 28 S RNA, also gave a strong and reproducible hybridization signal with all size fractions, although the absolute strength of the signal was lower than with probe 1. The signal obtained with probe 3, located in the middle of the nontranscribed spacer, was very weak when hybridized with short nascent DNA fragments and approached the strength of the signals obtained with probes 1 and 2 when hybridized with the longest nascent DNA fragments. This was an indication that this probe was not located near an origin of replication. Probe 4 gave a hybridization pattern similar to that of probe 3, which showed that this sequence was also not located near an origin of replication. Probe 5 showed a hybridization pattern similar to that of probe 1 (Table I). It is difficult to express the relationship between fragment lengths, hybridization signals, and probe locations in precise analytical form. Nevertheless, by empirically arranging the nascent DNA fragments on the physical map of the ribosomal DNA repeat, it was possible to obtain an alignment satisfying the data in Table I (Fig. 5). Thus, it could be argued that since both probes 1 and 5, which are located 7 kb apart, hybridize with size fragments 4-kb long, an initiation zone at least 4 kb in length should be located between these two probes. On the other hand, since probe 4, which is located 5 kb upstream of probe 5, does not hybridize with the 8-kb nascent fragments, the length of this initiation zone should not exceed 4 kb. This is an important finding and the fact that the same size fragments hybridized with some probes and did not hybridize with others showed that the initiation zones are well defined and that no initiation occurs outside these zones. Similar arguments lead to the conclusion that a second replication origin is contained within an
Initiation of DNA Replication in rRNA Genes

**Fig. 5.** Mapping the replication origins in the human ribosomal DNA repeat. The diagram represents the physical map of the human rDNA repeat. The positions of the transcription unit (heavy line) and of 18S and 28S RNA (filled boxes) are indicated. EcoRI restriction fragments A (7 kb), B (6 kb), C (11 kb), and D (19 kb), and the positions of the five rDNA probes are shown under the map. The 1.5-, 4-, 8-, and 15-kb nascent DNA fragments are schematically represented by horizontal lines and are arranged on top of the physical map of human rDNA repeat. To satisfy the hybridization results presented in Table I these fragments should have initiated within two different initiation zones located 5' of the transcription unit and at the 3'-end of the transcription unit (open boxes), respectively. For comparison in the figure are included the estimated positions of the human rDNA replication origins obtained in other laboratories (bottom).

| No. | kb | Probe #1 | Probe #2 | Probe #3 | Probe #4 | Probe #5 |
|-----|----|----------|----------|----------|----------|----------|
| 1   | 1.5| 520 ± 73 | 336 ± 66 | 70 ± 35  | 120 ± 50 | 250 ± 73 |
| 2   | 4  | 488 ± 76 | 320 ± 67 | 120 ± 61 | 195 ± 58 | 501 ± 75 |
| 3   | 8  | 490 ± 75 | 370 ± 70 | 150 ± 70 | 280 ± 70 | 426 ± 64 |
| 4   | 15 | 550 ± 60 | 410 ± 61 | 490 ± 83 | 400 ± 81 | 460 ± 89 |
| 5   | 30 | 501 ± 72 | 332 ± 60 | 430 ± 64 | 430 ± 64 | 487 ± 73 |

TABLE I

Hybridization of the five size-fractions of the labeled in vivo nascent DNA to human ribosomal DNA probes

1 μg of each probe were blotted and hybridized with 2 × 10⁶ counts of the respective size fraction. Results are means of three experiments after subtracting the background counts. Standard deviations are shown.

approximately 3 kb long sequence centered at the 3'-end of the transcription unit (Fig. 5).

**DISCUSSION**

To map the sites of initiation of DNA synthesis in the rat and human ribosomal DNA repeats, we applied two biochemical approaches, proven to be adequate for localization of mammalian origins of replication. They have been tested on the yeast ARS1 (14) and the SV40 (8, 24) origins of replication as model systems, and were applied to a number of other mammalian replication origins (7, 14, 19–23). The positions determined by these approaches excellently concur with the positions determined by other methods (1). The application of these two techniques to the rat and human rRNA genes enabled us to map a well defined zone of initiation of bidirectional DNA replication a few kilobases upstream of the coding regions, and also a less well expressed zone of initiation near the 3'-end of the transcribed units. These results are fully consistent with the findings of Coffman et al. (5), that a 1.38-kb sequence, located immediately upstream of the promoter, and to a lesser extent a 7-kb sequence, located at the 3'-end of the 28 S coding region of human ribosomal DNA, serve as efficient substrates in an in vitro replication system involving proteins from human cells (Fig. 5). The location of the upstream initiation zone agrees with the major initiation sites obtained with two-dimensional gel electrophoresis (3) and nascent strand abundance analysis of human rRNA genes (4) (Fig. 5). However, the conclusions that lower frequency initiation sites are distributed throughout most of the nontranscribed spacer (3) and the coding region (4) are not consistent with our results. The reasons for this discrepancy are not clear at present. The two-dimensional gel electrophoresis is the technique of choice for mapping origins of replication in genomes with low complexity and genomes that do not take up DNA precursors readily, since the method does not involve labeling of nascent DNA. When applied to mammalian genomes, it relies on a number of assumptions that are not proven to be always valid (25). For instance, the method involves enrichment for replication bubbles by using their presumed association with the nuclear matrix and assumes that no structural changes or nicks will occur in DNA during the isolation procedure. The reason the nascent strand abundance technique leads to the conclusion that initiation can occur throughout the ribosomal DNA repeat unit could be that the nascent DNA fractions were not well purified from genomic DNA, or that DNA have been fragmented in the course of the purification procedure. In this way random genomic DNA fragments will be present in the nascent DNA fraction, or fragments from longer nascent chains will be present in the population of shorter chains, both cases leading to a certain randomization of the results.

In our experiments the careful purification of the nascent fragments from random genomic DNA was critical for obtaining meaningful results. For this reason we isolated the nascent DNA fragments by immunoprecipitation. In this way we avoided the risk of compromising the results by using nascent DNA fractions not purified from genomic DNA, because immunochemical specificity can be considered almost absolute. In addition, we have followed the hybridization signal of the in vivo labeled nascent DNA fragments, thus eliminating the effect of any incidentally present nonlabeled contaminating DNA. Finally, in our experiments the fractionation of the newly synthesized DNA was done at the very first step of the isolation procedure and thus any possible artifacts from DNA degra-
The numberings of the nucleotides given hereafter are accorded a spacer sequence (30) for such common features (Fig. 6).

The initiation regions are diagrammed schematically, with nucleotide positions according to Financhek et al. (29) and Sylvester et al. (30). The sequence elements are described in the text. The following symbols were used: filled triangle, SAR; open triangle, ARS; filled box, pyrimidine tracts; open box, DNA unwinding elements.

A support for our conclusion that there are specific replication origins in the ribosomal DNA repeat came from the primary structures of the predicted origin regions. Analyses of the primary structure of other known mammalian chromosomal origin regions have revealed the existence of certain sequence and structural elements that are found in zones of initiation of DNA replication more frequently than would be expected by chance (26–28). These include A-T-rich tracts, sequences similar to the yeast ARS and sequences similar to Drosophila SAR, transcription factor binding sites, and DNA unwinding elements. These sequence elements are found at origins of replication in simple eukaryotic genomes such as yeast and Drosophila, and they also occur at nt positions 180, 1180, and 1610, respectively. The first and lowest minimum is located in the 838-base pair EcoRI/EcoRV fragment that showed the strongest hybridization signal with the origin fraction, and is therefore the most likely origin-containing candidate. The human sequence displays two local minima of helical stability, from nt −4822 to −4678 and around nt position −4318. Both are situated within the 138kb BamHI/Smal fragment which in the in vitro replication reaction was evaluated as the most likely origin-containing fragment (5).

The existence of these origin-related sequence elements in the predicted zones of initiation of DNA synthesis strongly supports the conclusion that they function as replication origins in vivo. These data are consistent with the hypothesis that in eukaryotes replication initiates within clusters of redundant modular elements associated with DNA unwinding function (39). We propose that eukaryotic cells have evolved a limited number of short sequence elements that are used as modules to build different control regions, including regions of low helical stability that can serve as origins of DNA replication. Further studies are necessary to elucidate whether all, or only a subset of these sites, are used as origins and how, in the course of development and differentiation, chromatin is organized in such a way that some of the potential initiation sites are blocked, while others are made accessible for the assembly of the replication initiation complex.

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