MOLECULAR AND CELLULAR BIOLOGY, Apr. 1986, p. 976–984
0270-7306/86/040976-09$02.00/0
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Vol. 6, No. 4

Polarity of DNA Replication through the Avian Alpha-Globin Locus

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Received 5 August 1985/Accepted 17 December 1985

Toward understanding the controls affecting eucaryotic chromosome replication, we used a runoff replication assay to investigate whether the activity of a gene is related to its use of an upstream or downstream replication origin. When in vivo-initiated DNA polymerases are allowed to complete replication in vitro in the presence of bromodeoxyuridine triphosphate the density label is preferentially incorporated into origin-distal regions of DNA. Isopycnic centrifugation and blot hybridization analysis of the relative bromodeoxyuridine triphosphate incorporation into fragments spanning the chicken alpha-globin locus indicate that this region is replicated from an upstream origin both in chicken lymphocytes and in erythrocytes. Thus the replication polarity of these genes does not change as a function of transcriptional activity, consistent with earlier suggestions that DNA replication in the transcriptional direction may be a necessary but not sufficient condition for gene expression.

The ordered nature of eucaryotic chromosome replication is evident in the replication of particular chromosome domains during discrete intervals of the S phase and in the synchronous initiation of replicon clusters whose size and number vary in a tissue-specific and developmentally regulated manner (reviewed in references 16, 21, and 47). These observations are consistent with recent data showing that DNA sequences complementary to probes for individual transcribed genes preferentially replicate early in the S phase and that chromosome position influences the timing of gene replication (6, 11, 17, 20). In *Saccharomyces cerevisiae*, electron microscopic and biochemical data imply that DNA synthesis initiates at specific loci (5, 7, 18, 46, 50); the clearest evidence for the existence of discrete origins in higher eucaryotes comes from studies of the amplification of dihydrofolate reductase genes in CHO cells (22, 23) and chorion genes in *Drosophila* follicle cells (36, 44), where the extent of amplification decreases bidirectionally from a central domain.

The apparent nonrandom selection of sites for replication initiation and the asymmetry of chromatin replication at the level of DNA synthesis (16, 35) and protein deposition (28) have contributed to the proposal that replication origin selection may regulate gene activity (42, 49). As an initial step toward testing this hypothesis we have used a runoff replication assay to determine the direction of replication of the chicken alpha-globin genes in cells where these sequences are expressed (erythrocytes) or quiescent (lymphocytes). Following the logic used to locate the simian virus 40 origin of replication (12, 34), isolated nuclei were incubated in a replication cocktail containing bromodeoxyuridine (BrdU) triphosphate (BrdUTP) and allowed to complete the synthesis of DNA chains initiated in vivo. The direction of replication was deduced based on the relative incorporation of BrdU into DNA fragments encompassing the globin locus; those fragments farthest from the origin of replication incorporated the greatest amount of density label. Using this system we find that the alpha-globin genes are preferentially replicated from upstream origins, irrespective of their transcriptional state.

MATERIALS AND METHODS

Isolation of nuclei and cytosol. MSB-1 cells were grown as described previously (27). Erythrocytes were isolated from 5-day White Leghorn chicken embryos by vein puncture. Nuclei and cytosol were prepared by the method of Muller et al. (32). Cells were harvested by low-speed centrifugation, washed once with cold 10 mM Tris chloride [pH 7.8], 4 mM $MgCl_2$, 1 mM disodium EDTA and suspended in the same cold buffer at a cell density of 7×10^7/ml. The cells were homogenized with a Teflon pestle, and the nuclei were pelleted by low-speed centrifugation. The crude cytoplasmic supernatant was removed and centrifuged at $100{,}000 \times g$ for 2 h at 4°C in a Beckman SW60 rotor, and the postribosomal supernatant was saved. Nuclei were washed once and suspended in cold Tris chloride-$MgCl_2$-EDTA. Nuclei and cytosol used for in vitro replication reactions were prepared separately, but from the same cell type.

In vitro replication. Isolated nuclei were incubated in a standard reaction mixture containing 33 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pH 7.8), 50 mM NaCl, 6.7 mM $MgCl_2$, 5 mM ATP, 100 μM dGTP, 100 μM dCTP, 100 μM dATP, 100 μM BrdUTP or TTP, and 50% (vol/vol) cytoplasmic fraction for 45 to 60 min at 37°C. Nonradioactive nucleotides were purchased from Sigma Chemical Co. or Pharmacia Fine Chemicals. Restriction enzymes (Bethesda Research Laboratories) were used in reactions at a ratio of 10 to 100 U/μg of DNA. Preincubations of nuclei with restriction enzyme were at 37°C for 15 to 40 min. Micrococcal nuclease (Boehringer Mannheim Biochemicals) was used at 0.05 μg/ml under the same conditions with the addition of 1 mM $CaCl_2$ to the reaction cocktail.

For measurement of incorporation of radioisotope (New England Nuclear Corp.), reactions were carried out in the presence of 9 μM dTTP and 1 μM [^{3}H]dTTP or 20 μM dCTP

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and 0.02 μM [α-^{32}P]dCTP. Reactions were stopped by spotting samples on Whatman GF/C filters saturated with 10% trichloroacetic acid–0.01 M sodium pyrophosphate. Filters were rinsed with 5% trichloroacetic acid–0.01 M sodium pyrophosphate and then with 95% ethanol. Filters were dried and counted in Redy-Solv MP (Beckman Instruments, Inc.) in a liquid scintillation spectrometer.

Reactions containing samples which were to be analyzed by blot hybridization were terminated by adding EDTA to 20 mM. These samples were incubated with RNase A (50 μg/ml) at 37°C for 1 h followed by overnight incubation with proteinase K (200 μg/ml)–0.5% sodium dodecyl sulfate at 37°C. Samples were deproteinized by phenol-chloroform-isoamyl alcohol (24:24:2) extraction. After dialysis in 10 mM Tris chloride (pH 8.0), 1 mM disodium EDTA, samples were digested to completion with additional restriction enzyme(s) under conditions recommended by the manufacturer (Bethesda Research Laboratories).

Isopycnic centrifugation. BrdU-substituted DNA was resolved on isopycnic CsCl gradients as described by Epner et al. (17). DNA was centrifuged in 15 mM NaCl–15 mM disodium EDTA (pH 7.5)–5.9 M CsCl (refractive index, 1.402) at 39,000 rpm for 60 to 72 h in a Beckman 75 Ti or 50 Ti rotor at 20°C. Gradients were fractionated from above with a Buchler Densi-Flow collecting apparatus. DNA recovered from the gradient was pooled, dialyzed against 10 mM Tris chloride–1 mM disodium EDTA (pH 8.0), concentrated with butanol, and ethanol precipitated. Samples of DNA from each pool were run on a 0.5% agarose minigel and stained with ethidium bromide for quantitation. Approximately 2 to 5 μg of DNA from each pool was run on 1.2% agarose gels in TAE buffer (30) for transfer to nitrocellulose.

Filter hybridization. DNA was transferred from agarose gels to BA85 nitrocellulose filters (Schleicher & Schuell Co.) (43). Filters were hybridized at 65°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0)–0.5% sodium dodecyl sulfate–5× Denhardt solution (1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin)–0.01 M EDTA–10% dextran sulfate–100 μg of salmon sperm DNA per ml to probes labeled to high specific activity (2×10^9 dpm/μg) with [α-^{32}P]dCTP by replacement synthesis by the method of James and Leffak (25). The filters were washed in 0.5× SSC–0.5% sodium dodecyl sulfate at 65°C and exposed to Kodak XR-5 film at −80°C with intensifying screens for 2 to 7 days.

Densitometry. Autoradiograms were scanned with a computer-assisted (Apple IIe) LKB 2202 UltroScan soft laser densitometer. Quantitation of band intensities was performed with the LKB 2190 Gelscan program, which employs a least-squares method to compute the height, width, and area of the Gaussian peak which most accurately fits the measured peak. The data shown are representative of repeated scans across the full width of each gel lane. Most films were scanned on a second Ultroscan densitometer (courtesy of M. T. Muller, Ohio State University), and on a Gelman ACD-18. Each in vitro runoff replication (IVR) experiment was performed in its entirety at least twice. Although there was limited variability in the quantitative ratio of signals between two bands in repeated scans (less than 15%), in no case did the normalized ratio of band signals change from less than unity to greater than unity (or vice versa) within or between experiments. The traces of Fig. 7 through 10 were scaled to equalize the response from the light and dense 0.6-kilobase (kb) *Bam*HI-*Eco*RI fragments to facilitate direct comparison of the signals from the 1.0- and 0.4-kb fragments.

RESULTS

Rationale. Consider a length of DNA containing sites for cleavage by restriction endonucleases RE-1 (site A) and RE-2 (sites B and C) which is undergoing replication in vivo (Fig. 1). In the presence of RE-1 and BrdUTP in vitro IVR through the A-B-C locus will result in preferential density labeling of fragment B-C. Isolation of DNA after IVR followed by digestion with RE-2 and isopycnic centrifugation will enrich for fragment B-C in the dense fractions of the gradient. Because the amount of DNA replicated in vitro is comparatively small (<2% of the input DNA), the unreplicated DNA in the reaction mixture provides an internal standard for the relative signals from fragment A-B versus B-C; thus the enrichment for B-C relative to A-B in dense DNA can be assayed after gel electrophoresis and blot hybridization by normalizing the signal ratio of fragments A-B/B-C in dense DNA to the A-B/B-C signal ratio in light DNA.

This discussion assumes that there is no replication origin within fragment A-B-C. It is unlikely that an origin will occur at random in the alpha-globin locus, because the average avian replicon is some 20- to 50-fold larger than the alpha-globin domain (16). However, this possibility can be tested directly by comparing the normalized A-B/B-C ratios when IVR is performed in the presence and absence of RE-1. If the A-B-C locus is replicated in vivo from an origin greater than approximately 3 kb away, during IVR in the absence of RE-1 the differential labeling of fragment B-C will be masked by forks which label the A-B-C domain uniformly. Alternatively, if replication initiates close to the RE-1 cutting site within the A-B-C region, the addition of RE-1 to the IVR reaction will have little effect on the normalized A-B/B-C ratio (see below).

Reaction kinetics. Nuclei were incubated as described by Muller et al. (32) (Materials and Methods). Inclusion of a buffered postribosomal cytosol fraction in this in vitro replication system enhances ligation of Okazaki fragments and extension of in vivo-initiated DNA chains for more than 10 to 20 kb in the absence of in vitro initiations (32; see below). (We have confirmed that replication does not initiate in vitro at a significant level based on the density of IVR incorporated DNA label after an in vivo BrdU pulse [39; see below] and by the UV light-induced release of this label [32]; results not shown.) Under the prescribed conditions labeled TTP or BrdUTP is incorporated with biphasic kinetics, characteristic of similar in vitro replication systems (32, 39, 40, 48) (Fig.

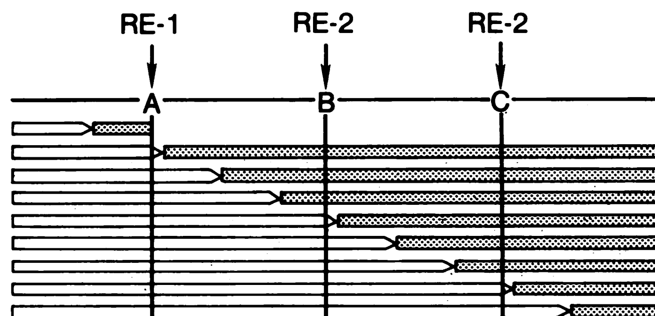

FIG. 1. IVR. Arrowheads indicate the direction and position of replication forks on region A-B-C in an asynchronous population of nuclei when in vitro replication and incorporation of BrdUTP begins. Symbols: ▭, light DNA; ▦, dense DNA synthesized in vitro.

2). Based on ethidium staining after gradient centrifugation, approximately 2% of the input DNA is replicated during a 60-min IVR reaction. During this hour, therefore, the overall rate of DNA synthesis is roughly 10% of the in vivo rate. Thus, in a 1-h incubation each in vivo-initiated replication fork should be extended nearly 15 to 20 kilobase pairs. This is consistent with the electrophoretic pattern of purified dense DNAs showing an average size greater than 10 kilobase pairs, with molecules as large as 20 to 25 kilobase pairs. When restriction enzyme was added to the IVR cocktail a decrease in the rate and extent of nucleotide incorporation was observed (Fig. 3A). As anticipated, this effect was greater for the 4-cutter *Hae*III than for the 6-cutter *Eco*RI, suggesting that truncation of the template DNA by restriction enzyme cleavage impedes progression of replication forks. (The relatively nonspecific staphylococcal nuclease further decreased incorporation, even at the earliest times of digestion.) The runoff nature of the IVR reaction should be optimized, therefore, by increasing the extent of RE-1 restriction enzyme cleavage (Fig. 1). We found that the efficiency of RE-1 inhibition could be increased simply by incubation of the nuclei with enzyme before the addition of cytosol and deoxynucleotides, i.e., before replication occurred in vitro (Fig. 3B), and we have included this step in our standard IVR protocol.

Assay sensitivity. To insure that blot hybridization was sufficiently sensitive to detect differences in the ratio of globin DNA signals, the plasmid α^D (45; provided by H. Weintraub) was cleaved with *Eco*RI and *Bam*HI to release 1.0- and 0.6-kb globin DNA fragments. The fragments were isolated by electroelution and mixed at various ratios before reelectrophoresis and blot hybridization (Fig. 4). The linear correspondence of densitometric signal to DNA input ratio demonstrates that small differences in the ratios of the two globin DNA fragments can be accurately measured by this procedure.

To determine whether light and IVR-replicated dense DNAs could be resolved on neutral CsCl gradients, MSB chicken lymphoblastoid cells (1) were prelabeled with [^{3}H]thymidine, and nuclei from these cells were then submitted to the IVR protocol in the presence of BrdUTP, [α-^{32}P]dCTP, and *Eco*RI. After purification the DNA was banded on CsCl. The prelabeled and IVR-replicated DNAs are adequately resolved to permit analysis of relatively pure light (1.68 to 1.70 g/ml) and dense (1.74 to 1.76 g/ml) fractions (Fig. 5). (Further experiments [data not shown] with alkaline CsCl gradients yielded better resolution of light versus dense DNAs, but resulted in more diffuse signals on blots. The conclusions from those experiments were, however, consistent with the data presented here.) To demonstrate directly

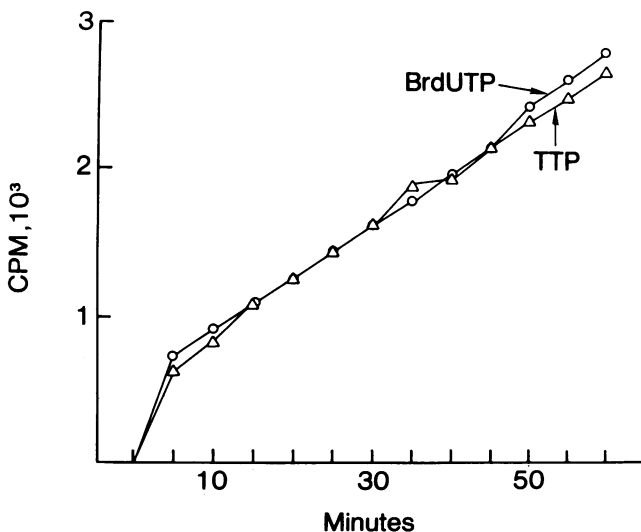

FIG. 2. Similar IVR kinetics in the presence and absence of BrdUTP. MSB nuclei were incubated in the IVR cocktail containing either 100 μM TTP (△) or 100 μM BrdUTP (○), and the incorporation of [^{3}H]dCTP into acid-precipitable DNA was measured as a function of time.

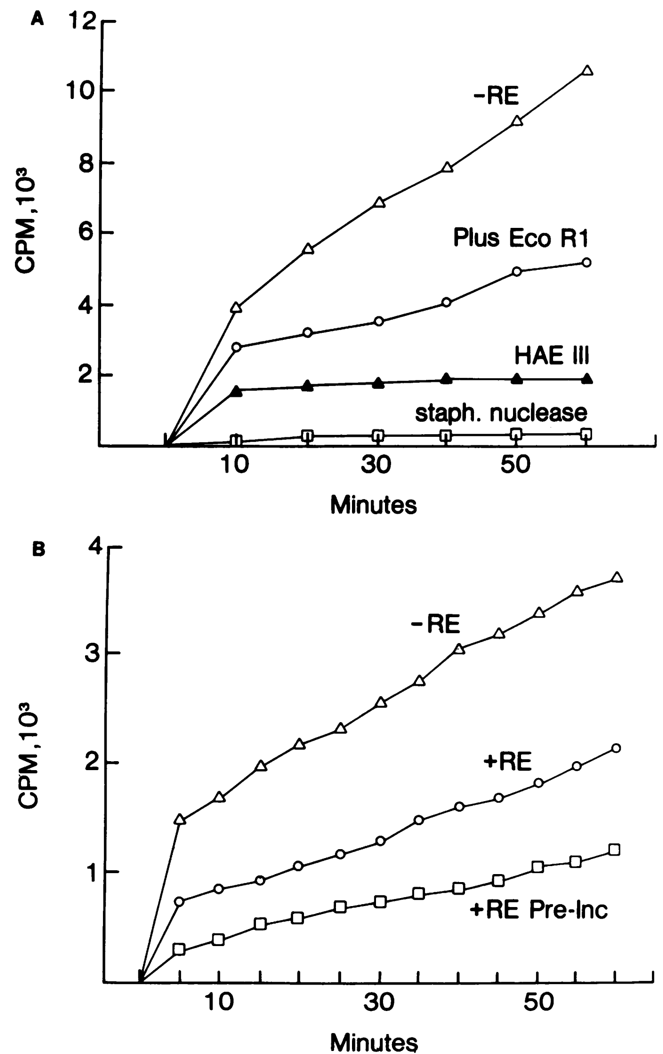

FIG. 3. Endonuclease digestion decreases replication in vitro. (A) MSB nuclei were incubated in the IVR cocktail in the presence of the indicated nuclease (without preincubation), and the incorporation of [^{3}H]dCTP into DNA was measured. −RE, IVR reaction without added nuclease. (B) +RE Pre-Inc, the kinetics of [^{3}H]dCTP incorporation were analysed when nuclei were preincubated in isolation buffer with *Eco*RI for 40 min before IVR; +RE, *Eco*RI was added concurrently with the initiation of IVR (no preincubation); −RE, nuclei were preincubated in isolation buffer without exogenous nuclease. The incorporation kinetics in this reaction were quantitatively similar to IVR reactions without preincubation.

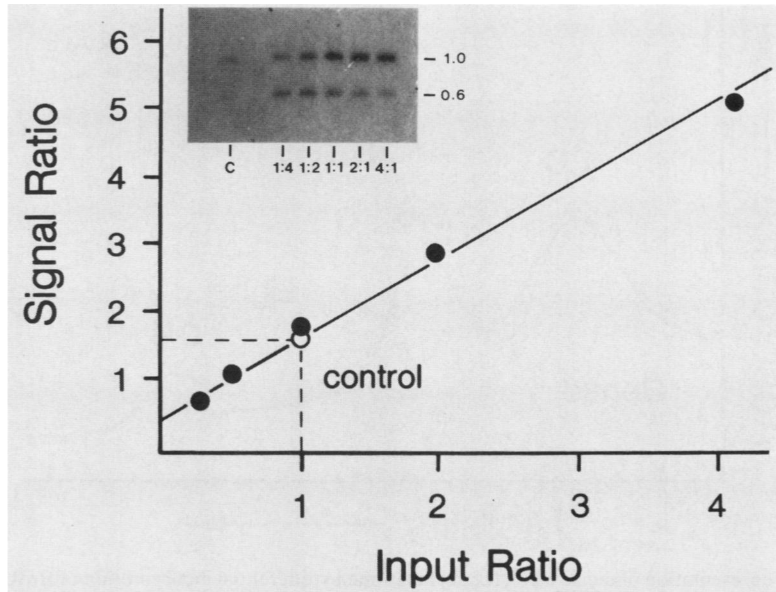

FIG. 4. Sensitivity of the blot hybridization assay. The α^D-globin plasmid contains the 1.6-kb *Bam*HI fragment denoted probe B (see Fig. 6) in pBR322. α^D was digested with *Eco*RI and *Bam*HI to release 1.0- and 0.6-kb fragments, which were gel purified and mixed in the indicated ratios. The mixtures (and an unfractionated control digest) were run on a 1.2% agarose gel and blot hybridized to the α^D insert. The resulting autoradiogram (inset) was scanned and the ratio of 1.0-kb/0.6-kb fragment signals plotted versus the molar input ratio of 1.0-kb/0.6-kb DNAs. Symbols: ●, DNAs mixed in vitro; ○, control DNA.

that the incorporation of the density label does not introduce hybridization artifacts the following experiment was performed. MSB cells were incubated for 6 h (approximately one-half cell generation) in medium containing BrdU. DNA was purified from these cells, digested with *Bam*HI, *Hin*dIII, and *Eco*RI, and centrifuged to equilibrium on CsCl. DNA was pooled into light, dense, and hybrid density (1.71 to 1.73 g/ml) fractions and submitted to gel electrophoresis and blot hybridization (30) to alpha-globin probes (Fig. 6). Densitometry of the film shows no difference in the ratios of the 1.0-, 0.6- (α^D), and 0.3- to 0.4-kb (α^π) fragments in light versus heavy DNA (Fig. 7). (We do not include hybrid density DNA in this analysis since the amount of a fragment in this fraction will be proportional to its length. This is not so for DNA banding in the light or heavy fractions when the length of DNA synthesized [either in vivo or in vitro] substantially exceeds the size of the domain under consid-

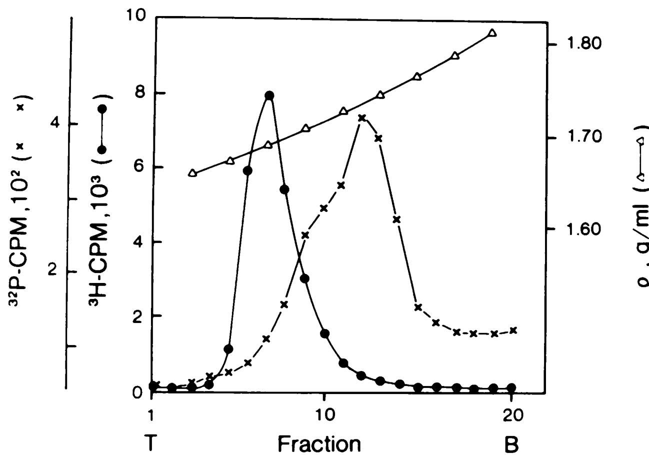

FIG. 5. Isopycnic separation of unsubstituted and unifilarly substituted DNA. MSB cells were prelabeled in vivo with [³H]thymidine and nuclei from these cells used for subsequent IVR in the presence of BrdUTP and [α-³²P]dCTP. DNA was isolated and digested with *Eco*RI before banding on a neutral CsCl gradient. Fractions were collected into vials, and radioactivity was measured by liquid scintillation spectrometry.

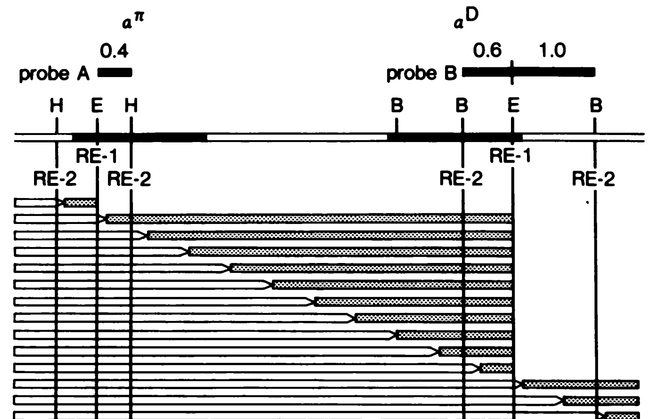

FIG. 6. Chicken alpha-globin locus. At the top of the figure is a map of the alpha-globin locus showing restriction sites pertinent to these experiments (redrawn from Stalder et al. [45]). Probes used for hybridization to the α^π- and α^D-globin sequences are indicated. Abbreviations: E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI. The bottom of the figure shows an interpretation of IVR results (see the text). Symbols are as in Fig. 1.

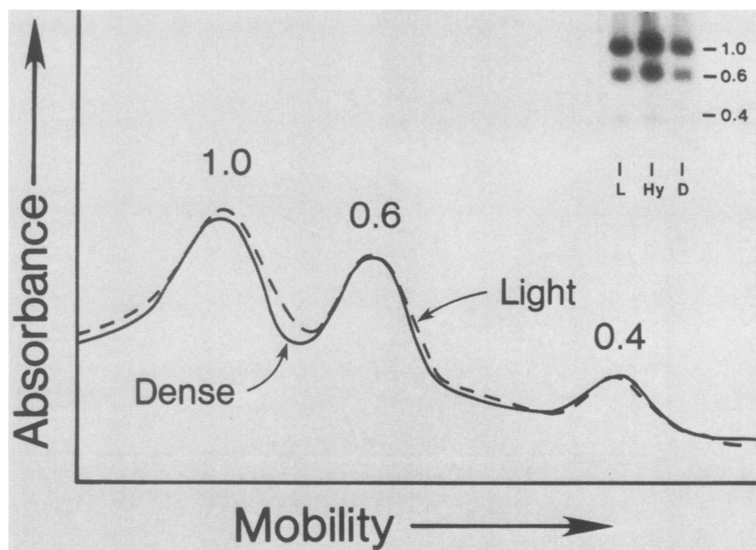

FIG. 7. Nonstoichiometric representation of dense DNA fragments is due to differential incorporation of BrdUTP. MSB cells were labeled in vivo for 6 h (ca. one-half cell generation) with BrdU (20 μg/ml) before DNA isolation and digestion with a combination of *Bam*HI, *Eco*RI, and *Hin*dIII. The restricted DNA was banded as in Fig. 5, fractionated, and run on a 1.2% agarose gel. The DNA was blot hybridized to a mixture of ^{32}P-labeled alpha-globin probes A and B (Fig. 6) and autoradiographed. The inset shows the film image which was quantitated by laser densitometry. Abbreviations: L, light (1.68 to 1.70 g/ml) DNA; Hy, hybrid (1.71 to 1.73 g/ml) density DNA; D, dense (1.74 to 1.76 g/ml) DNA.

eration, as in the present case.) This control not only confirms previous reports that BrdU substitution does not detectably alter DNA hybridization in this type of experiment (4, 19), but also is crucial in showing that sample quantity, selective transfer and hybridization, nucleotide sequence, differential cleavage of purified dense versus light DNAs, and DNA fragment size do not significantly affect the signal ratio when these data are normalized against an internal control. This approach is similar to the internal standard method used to quantitate the extent of chorion gene amplification during *Drosophila* embryogenesis (44).

Polarity of globin gene replication. MSB cell nuclei were incubated in the IVR cocktail in the presence of *Eco*RI (RE-1) and BrdUTP. DNA was purified before *Bam*HI-*Hin*dIII (RE-2) digestion and CsCl centrifugation. Dense and light DNA was blot hybridized to a mixture of probes A and B (Fig. 6) complementary to the α^{π}- and α^{D}-globin genes, respectively. Quantitation by densitometry shows an increase in the normalized ratio of 0.6-kb/1.0-kb fragments (complementary to probe B) in dense DNA (Fig. 8). In each of four repeats of this experiment the 0.6-kb band was consistently enriched (1.8- to 2.3-fold) over the 1.0-kb band in newly replicated DNA. (Because restriction enzyme cleavage does not go to completion in nuclei [29], it is important to note that both the 0.6- and 1.0-kb fragments are generated by the same *Eco*RI cut [Fig. 6]; therefore incomplete digestion at this site in situ cannot be responsible for the change in stoichiometry of these fragments.) We conclude that the 0.6-kb *Bam*HI-*Eco*RI fragment is at the end of an *Eco*RI fragment distal to its origin of replication, whereas the 1.0-kb *Eco*RI-*Bam*HI fragment is at the end of an *Eco*RI fragment proximal to its origin. This result indicates that the α^{D} gene in MSB cells is replicated preferentially from an origin 5′ to this sequence. The depletion of the 0.4-kb α^{π} fragment relative to the 0.6-kb fragment (Fig. 8) is consistent with this interpretation, i.e., that the α^{π} gene lies closer to an origin than does the α^{D} gene. The 0.4- and 0.6-kb bands are generated by distinct *Eco*RI cuts, however, and a change in the ratio of their signals in light versus dense DNA could conceivably be due to unequal *Eco*RI digestion of these two sites in replicating versus bulk chromatin.

To address this question, MSB cell nuclei were submitted to the same IVR protocol, except that *Eco*RI was omitted from the in vitro incubation. Subsequently, the DNA was purified, digested with a combination of *Eco*RI, *Hin*dIII, and *Bam*HI, resolved on CsCl, and blot hybridized as described above. Under these conditions no significant enrichment of the 0.6-kb band relative to the 1.0-kb band was detected (Fig. 9), presumably due to the contiguity of these sequences in the genome. The enrichment for the 0.6-kb band seen in Fig. 8 therefore must result from endonuclease cutting during the IVR reaction. Interestingly, the data of Fig. 9 show that even in the absence of RE-1 (*Eco*RI) digestion the 0.4-kb band was depleted relative to the 0.6-kb band in newly replicated DNA. This depletion cannot be due to unequal *Eco*RI digestion in situ, but reflects the presence in vivo of a site, possibly a replication origin, close enough to the α^{π} gene that DNA polymerase molecules effectively run off from this point. The potential significance of a replication origin sufficiently close to the alpha-globin domain to be detected by this assay remains to be elucidated.

To determine the polarity of replication through the alpha-globin locus in cells expressing these genes, erythrocyte nuclei from 5-day chicken embryos were tested in the IVR reaction. The 0.6-kb fragment was again enriched relative to both the 0.4- and 1.0-kb fragments (Fig. 10), implying the same direction of replication through this region in embryonic erythrocytes as in lymphocytes.

DISCUSSION

The selective activation of eucaryotic replication origins and the nonrandom nature of chromatin assembly led to the

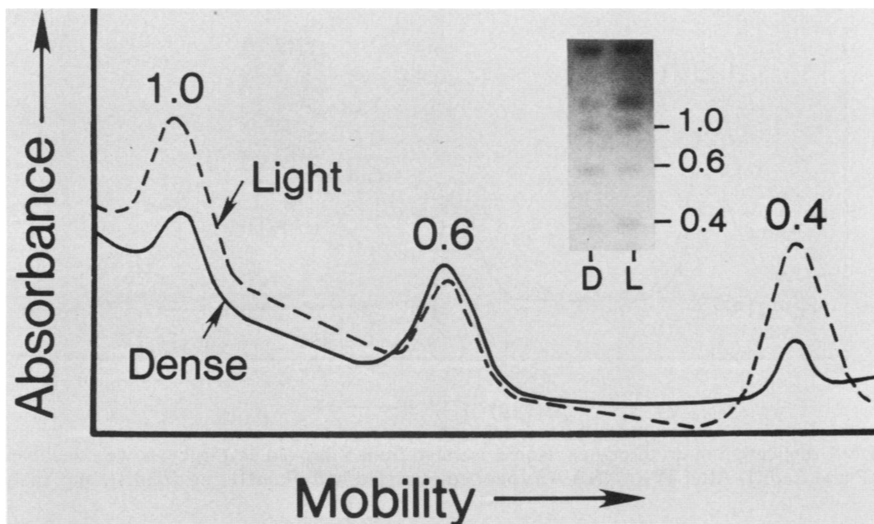

FIG. 8. Alpha-globin DNA replication in MSB cells. MSB cell nuclei were submitted to the IVR reaction in the presence of *Eco*RI and BrdUTP (see the text). After IVR, DNA was purified and digested with *Bam*HI and *Hin*dIII. The DNA was banded in CsCl, electrophoresed, hybridized, and analyzed by densitometry.

formulation of a model by which replication origin selection could influence chromatin structure and gene activity (49). This proposal has recently been restated by Smithies (42), who suggested that a gene must be replicated from an upstream origin to be expressed in the following cell generation. Thus, replication in the transcriptional direction would allow subsequent activation of the gene, whereas replication from a downstream origin would result in a chromatin conformation precluding transcription. To test this hypothesis we have used in vitro replication to determine the direction of replication of the alpha-globin gene region in chicken cells, where this locus exists either in a transcriptionally active or inactive state.

The IVR assay relies on the graded incorporation of nucleotide analog across a region of DNA when polymerases are limited to running off the template; polymerase molecules are prevented from entering (and uniformly labeling) the region by virtue of double-stranded DNA cuts introduced by restriction endonuclease. Although it has not been shown directly, the inverse relationship between the extent of DNA synthesis in vitro and frequency of endonuclease cutting and the observation that the differential incorporation of label into contiguous segments of DNA is dependent on the addition of restriction enzyme to the IVR cocktail are consistent with the idea that truncation of the DNA template stops the progress of replication forks and limits replication to the completion of in vivo-initiated chains by polymerase molecules downstream of the RE-1 cut site.

Assuming a random distribution of polymerases, during IVR in the presence of *Eco*RI the α^D-globin 0.6-kb *Bam*HI-*Eco*RI fragment would be traversed by polymerases which had passed the *Eco*RI site approximately 5 kb upstream, whereas the 1.0-kb fragment would be traversed only by polymerases downstream of the *Eco*RI site within the α^D gene. This would predict a minimum four- to fivefold increase in the normalized 0.6-kb/1.0-kb signal ratio in dense

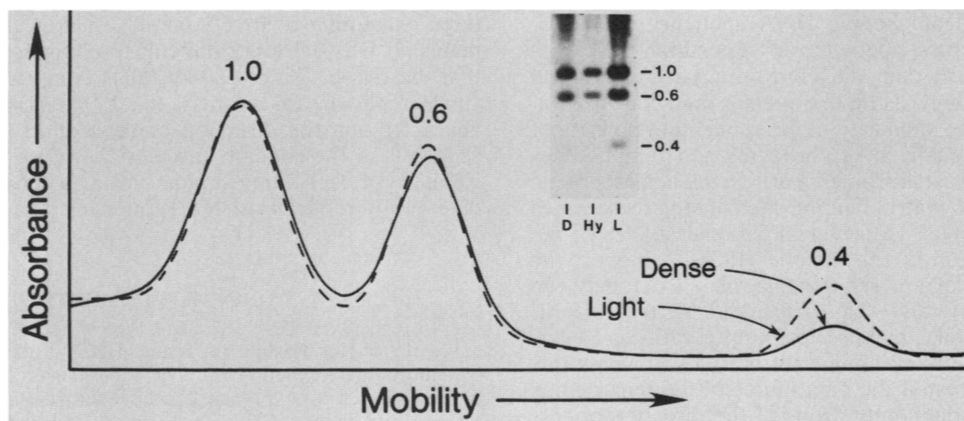

FIG. 9. Replication in vitro without RE-1. MSB cell nuclei were incubated in the standard IVR cocktail (including BrdUTP) without *Eco*RI. After purification the DNA was digested with *Eco*RI, *Hin*dIII, and *Bam*HI and analyzed as in Fig. 8.

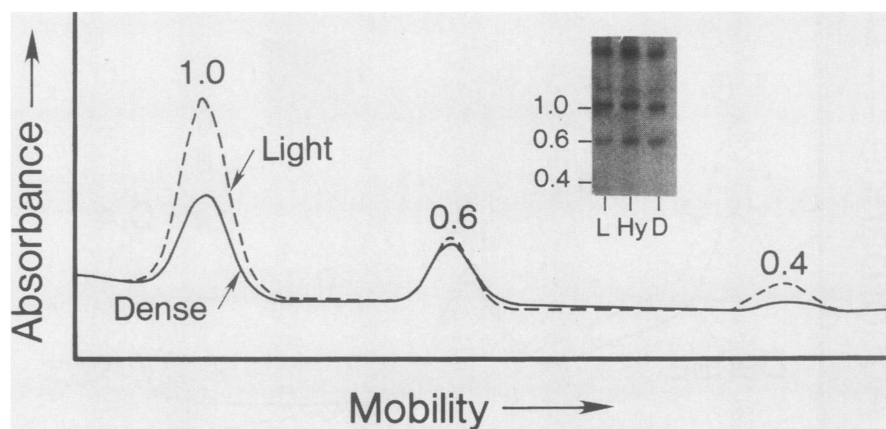

FIG. 10. Alpha-globin DNA replication in erythrocytes. Nuclei isolated from 5-day-old erythrocytes were incubated in the IVR reaction cocktail containing BrdUTP and *Eco*RI. After IVR, DNA was purified, digested with *Bam*HI and *Hin*dIII, and analyzed as in Fig. 8.

DNA. By densitometry, however, the observed enrichment is only about twofold. It is unlikely that this difference arises from inaccuracy in quantitation, since we have shown that the detection of a twofold difference in signal ratio is within the sensitivity of our assay. It is more likely that the failure to attain the theoretical maximum enrichment is due to the inefficiency of restriction enzyme cutting in situ and the probability that the RE-1 restriction enzyme will cut after versus before the passage of the replication fork. We stress, therefore, that the interpretation of replication polarity rests on whether the normalized signal ratio of two DNA fragments is greater or less than unity, rather than on the actual quantitative enrichment. Nevertheless, since we do not reach the theoretical enrichment, it is possible that not all cells in the population replicate this region in the same direction and that we detect only the preferred polarity.

Based on the selective incorporation of BrdUTP into origin-distal DNA during runoff replication, we conclude that the alpha-globin locus is preferentially replicated in the transcriptional direction in cells where these genes are active or inactive. These findings are in agreement with the hypothesis that replication from an upstream origin is a necessary but not sufficient condition for gene activity. However, an alternative possibility is suggested by reports that DNA replication initiates at the nuclear matrix (2, 37), and that active (and potentially active) genes are anchored to the matrix (10, 13, 38) at specific DNA sequences (31, 41); namely, that the correspondence of replication and transcription polarities is a coincidence resulting from DNA and RNA polymerases both using the nuclear matrix as a chromatin entry site. The similarity in the replication polarity of the alpha-globin domain in erythrocytes and lymphocytes could arise from the establishment early in the hematopoietic lineage of a common matrix binding site flanking these genes in these two cell types. However, additional studies of the polarity of replication of the histone H5 gene support the former view, i.e., that a relationship may exist between replication polarity and transcriptional activity (J. P. Trempe and M. Leffak, manuscript in preparation). Nevertheless, due to the limited number of systems in which this proposal has been tested the data must still be regarded as correlative. Additional comparisons of the direction of replication of specific genes in transcriptionally active and inactive configurations and analysis of the transcriptional activity of a given DNA sequence in both orientations relative to its replication origin are necessary before a general conclusion can be drawn.

Insight into one component of the connection between replication and transcriptional potential may come from the recent results of Chiu and Blau (9). These workers suggested that a decrease in the level of DNA methylation was responsible for the observation that the activation of human muscle-specific gene expression in HeLa × mouse muscle cell heterokaryons required a prior round of replication of the HeLa cell donors in the presence of azacytidine. In those systems where a quantal cell division (3, 14, 24) was not necessary to intervene between the presentation of an activating stimulus and enhanced gene expression (8, 9, 15, 26, 33), it is tempting to speculate that the sequences in question may have been poised in an activatable configuration resulting from a previous replication.

Based on the differential incorporation of BrdU into portions of the murine immunoglobulin heavy-chain constant region during selected intervals of S phase, Braunstein et al. (4) demonstrated that the temporal order of replication of these sequences in MEL cells corresponds to their $3' \rightarrow 5'$ linear order in the genome. Although their data were consistent with the replication of this region from a single origin, these authors indicated that the resolution of the elutriation method for obtaining synchronized cell populations necessitated examining relatively large (>50-kb) chromosome domains. It was therefore difficult to eliminate the possibility that the entire 170- to 200-kb locus comprised more than a single replicon. In contrast, the IVR assay is effective in characterizing the direction of replication through smaller segments of the genome and may therefore be useful in the isolation of replication origins through the identification of divergently replicated DNA sequences.

ACKNOWLEDGMENTS

We thank Jim Trempe for many useful discussions during the planning of these experiments, Joel Huberman for his critical reading of the manuscript, and Mark T. Muller for assistance with the IVR reaction and the use of the densitometer (LKB Instruments, Inc.) at Ohio State. The alpha-globin probes were obtained from J. D. Engel through H. Weintraub.

This work was supported by National Science Foundation grant PCM8302916 and by the Miami Valley Branch of the American Cancer Society (to M.L.).

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