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Initiation of DNA Replication in the Dihydrofolate Reductase Locus Is Confined to the Early S Period in CHO Cells Synchronized with the Plant Amino Acid Mimosine

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In previous studies, we used two complementary two-dimensional gel electrophoretic methods to examine replication intermediates in the 240-kb amplified dihydrofolate reductase (DHFR) domain of methotrexate-resistant CHOC 400 cells (J. P. Vaughn, P. A. Dijkwel, and J. L. Hamlin, Cell 61:1075–1087, 1990). Surprisingly, in both asynchronous and early-S-phase cultures, initiation bubbles were detected in several contiguous fragments from a previously defined 28-kb initiation locus. However, because of the low levels of bubblelike structures observed on gels, it has been suggested that these structures might represent artifacts, possibly unrelated to replication per se. In this study, we have achieved much more synchronous entry into S phase by using a novel inhibitor and have isolated replication intermediates by a new procedure that largely eliminates branch migration and shear. Under these conditions, we find that (i) the relative number of bubblelike structures detected in fragments from the initiation locus is markedly increased, (ii) bubbles are detected at multiple sites scattered throughout the region lying between the DHFR and 2BE2121 genes, and (iii) bubbles appear and disappear in this region with the kinetics expected of an early-firing origin. These data strengthen the proposal that in vivo, initiation can occur at any of a large number of sites scattered throughout a broad zone in the DHFR domain.

To study the replication pattern of a single mammalian chromosomal locus, several years ago our laboratory developed a methotrexate-resistant CHO cell line (CHOC 400) that has amplified one allele of the early-replicating dihydrofolate reductase (DHFR) locus ~1,000 times (19). The high copy number of the 240-kb amplicon in CHOC 400 cells has facilitated a number of studies designed to identify the replication start site(s) in each repeating unit.

When CHOC 400 cells were pulsed with [^{3}H]thymidine in the very early S period, only a small number of restriction fragments from the amplicon was shown to label preferentially (10). These early-labelled fragments were subsequently shown to map contiguously in the genome and defined a 28-kb initiation locus lying downstream from the DHFR gene (11, 15).

However, evidence for two separate initiation sites within this locus was obtained in a higher-resolution in vivo-labelling study employing in-gel renaturation (24) to rid of single-copy background sequences (15). One of the sites (termed ori-β) is located 15 kb downstream from the 3′ end of the DHFR gene, while the other (ori-γ) lies ~22 kb further downstream (indicated as I_β and I_γ in Fig. 1B). Furthermore, Handeli et al. (9) showed that leading and lagging nascent strands switch templates at the ori-β and ori-γ loci (but see reference 5 for a reinterpretation of the basis of these experiments). Interestingly, a prominent matrix attachment region is situated approximately midway between ori-β and ori-γ (6) (Fig. 1C).

None of these relatively low-resolution approaches can accurately locate a fixed initiation site. However, recently developed two-dimensional (2-D) gel electrophoretic replicon mapping methods do have this potential (2, 21). When these methods were applied to the DHFR domain, it was very surprising to detect initiation sites (replication bubbles) in every fragment examined within the 28-kb initiation locus but not in fragments outside this locus (25, 26). However, the same fragments that displayed bubbles also displayed single replication forks, suggesting that in some amplicons, these fragments were replicated passively from outside origins. This phenomenon was observed both in early-S-phase cells after release from an aphidicolin block and in asynchronous cultures.

The simplest explanation for this unexpected result is that initiation can occur at any of a large number of different sites within the previously defined initiation locus rather than at only two fixed sites, as suggested by earlier studies (9, 15). However, since it is not possible to accurately quantitate the number of replication bubbles per unit length of DNA in the 2-D gel experiments, we could not determine whether the ori-β and ori-γ regions might be preferred over other sites within the initiation locus, resulting in the preferential labelling that was observed in these two zones.

It was also troubling that the levels of bubblelike structures detected on 2-D gels were rather low in any given fragment from the initiation locus. It seemed possible that most of the fragile bubble structures might have been lost during isolation as a result of branch migration and shear and/or that a large fraction of origins may have already fired prior to removal of aphidicolin (the chain elongation inhibitor that was used to collect cells at the G_1/S boundary).

Other possibilities were raised by a high-resolution approach in which Okazaki fragments were labelled in an in vitro replication reaction and were then used to determine template strand bias in the neighborhood of ori-β (4). The results of this study suggested that initiation in the ori-β locus occurs predominantly within a single 500-bp fragment,

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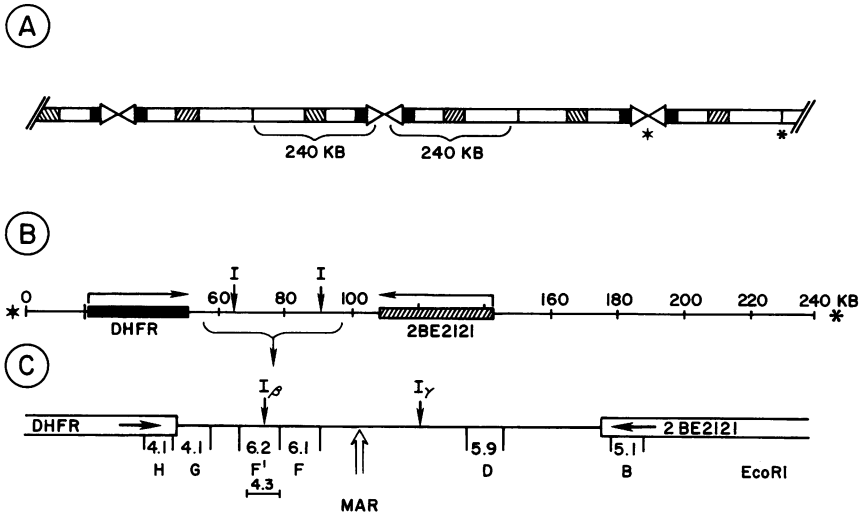

FIG. 1. Arrangement of the 240-kb DHFR amplicons in CHOC 400 cells. (A) Alternating head-to-head and tail-to-tail arrangement of the 240-kb DHFR amplicons in CHOC 400 cells. (B) Functional map of one repeating unit (amplicon), showing the DHFR and 2BE2121 genes and the two preferred zones of early labelling (the centers of which are labelled I_β and I_γ). The directions of transcription of the two genes are indicated with arrows above the scale. (C) Expansion of the region encompassing the initiation locus to show the *Eco*RI fragments discussed in the text (others are not shown). The 4.3-kb *Xba*I fragment discussed in the text is indicated below and is located within the 6.2-kb *Eco*RI fragment. The position of a prominent matrix attachment region (MAR) is shown with an arrow.

as opposed to the broad zone suggested by the 2-D gel studies.

Thus, it is possible that we failed to detect this major site in ori-β on 2-D gels because of loss or breakage of bubbles and instead were able to detect only a minor class of dispersed initiation sites. Alternatively, the bubblelike structures that we detected on 2-D gels may be artifacts (possibly unrelated to DNA replication) that for unknown reasons migrate as replication bubbles and occur only in the initiation locus of the DHFR amplicon.

In view of these possibilities, we have reanalyzed this locus to determine whether either the cell-synchronizing protocol or the DNA isolation procedure has resulted in the selective loss of replication intermediates from this proposed major site. We have achieved very synchronous entry into the S period by reversibly arresting cells at the G_1/S boundary with the novel inhibitor mimosine (13, 20a). In addition, we have purified replication intermediates from synchronized cells at various times in S by a newly developed procedure that largely eliminates branch migration and shear (7). The results of these studies are reported here.

MATERIALS AND METHODS

Cell culture, synchronization, and labelling protocols. The methotrexate-resistant CHO cell line CHOC 400 was maintained as previously described (19). Synchrony was achieved by isoleucine deprivation for 45 h to induce arrest in G_0, followed by release into complete medium containing either 30 μM (10 μg/ml) aphidicolin or 400 μM mimosine. After 12 or 14 h, each 15-cm-diameter dish was rinsed once with 50 ml of serum-free minimal essential medium, and 35 ml of complete medium was added. The efficacy of the synchronizing protocol was determined by fluorescence-activated cell sorter analysis of a small aliquot of each sample (22).

In some experiments, CHOC 400 cells growing in multiwell dishes were synchronized as described above, and the kinetics of traverse through the S phase were monitored by labelling with 1 μCi of [^{3}H]thymidine (85 Ci/mmol; New England Nuclear Corp.) per ml for 1 h at 2-h intervals, starting immediately after drug removal. Trichloroacetic acid-precipitable incorporation was determined as previously described (14).

Isolation of replication intermediates. Nuclear matrices were isolated as described previously (7, 20) and were digested with either *Eco*RI or *Xba*I. Matrix-attached DNA was isolated and fractionated on a benzoylated naphthoylated DEAE (BND)-cellulose (Sigma Chemical Corp.) column as previously described (7, 16). The replication intermediates from 0.5×10^8 to 1.0×10^8 cells were loaded into one well of a 0.4% agarose gel.

2-D gel electrophoresis. Neutral/neutral 2-D gel electrophoresis (2) was performed exactly as described previously (7). After electrophoresis in the second dimension, DNA was transferred to Hybond N+ (Amersham) by using an alkaline blotting procedure (23). Membranes were hybridized with appropriate ^{32}P-labelled probes as previously described (7).

RESULTS

Mimosine inhibits entry into the S phase more effectively than does aphidicolin. In our standard synchrony protocol, aphidicolin is used to collect cells near the G_1/S boundary. Since this drug is an inhibitor of DNA polymerase, it is expected to inhibit chain elongation but not initiation per se (8, 12). If aphidicolin were somewhat leaky, forks emanating from an early-firing origin could progress slowly out of a restriction fragment containing the origin, with the result that only a low level of initiations would subsequently be observed after the drug is removed. However, if the drug mimosine actually prevents entry into S, as has been suggested (13), then upon its removal, all initiations should subsequently be detectable on 2-D gels.

To address this possibility, cell populations were released from a G_0 block into either aphidicolin or mimosine for 12 h and were subsequently analyzed on the fluorescence-activated cell sorter to determine cell cycle position. As shown

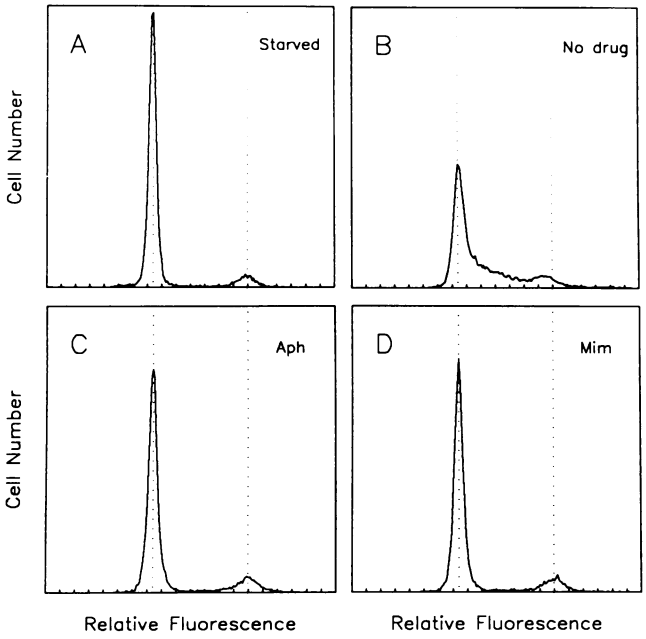

FIG. 2. Evidence that cells released from a G_0 block into either aphidicolin or mimosine appear to arrest at the G_1/S boundary. CHOC 400 cells were starved for isoleucine for 45 h (A) and were then released for 12 h into either drug-free medium (B), 30 μM aphidicolin (Aph; C), or 400 μM mimosine (Mim; D). The cells were then processed for fluorescence-activated cell sorter analysis (22). The two vertical lines indicate the channels occupied by cells with the G_1 and G_2 DNA contents. Note that ~50% of cells in the G_2 channel in each panel are probably tetraploids that are actually in the G_1 phase of the cell cycle, since a low level of cells with $>2n$ DNA content appear after release from either aphidicolin or mimosine (data not shown).

in Fig. 2C and D, populations arrested with either drug display approximately the G_1 DNA content (compare with the G_0-arrested population in Fig. 2A and that in Fig. 2B, in which the population was released into complete medium without drug for 12 h and many cells have entered S). Thus, by this criterion, both drugs appear to arrest cells near the G_1/S boundary.

However, an indication that aphidicolin may allow significant escape synthesis is demonstrated in the uptake study

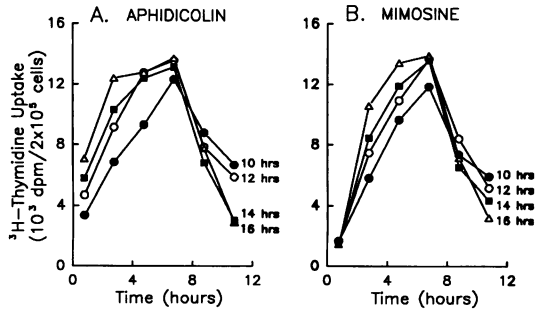

FIG. 3. Evidence that mimosine inhibits G_1/S traverse more effectively than does aphidicolin. CHOC 400 cells were arrested in G_0 and were released into 30 μM aphidicolin (A) or 400 μM mimosine (B) for 10, 12, 14, and 16 h. The cells were then released into drug-free medium and were pulsed with 1 μCi of [^{3}H]thymidine per ml for 60 min at the indicated times after release (time zero).

summarized in Fig. 3. CHOC 400 cells were collected in medium containing 30 μM aphidicolin for 10, 12, 14, or 16 h after release from isoleucine-deficient medium. The drug was then washed out, and the rate of [^{3}H]thymidine incorporation into DNA was monitored at 2-h intervals throughout the subsequent S period, beginning at time zero.

In Fig. 3A, it can be seen that the initial rate of DNA replication (i.e., [^{3}H]thymidine uptake in the first hour after release from the drug) is high, even after only 10 h in aphidicolin. Furthermore, this value increases markedly as cells are maintained in aphidicolin for longer time intervals and are then released into drug-free medium; the initial rate after 16 h in aphidicolin (7,060 dpm per 10^5 cells) is more than twice the rate observed after only 10 h (3,360 dpm per 10^5 cells), presumably because more replication forks were in operation at the time that aphidicolin was removed and label was added.

Quite a different result is obtained when the experiment is repeated with mimosine as the blocking agent (Fig. 3B). The initial rate of DNA synthesis after mimosine is removed is much lower and does not increase with the length of time that cells are maintained in mimosine after reversal of the G_0 block (Fig. 3B; 1,680, 1,670, 1,530, and 1,430 dpm per 10^5 cells after 10, 12, 14, and 16 h, respectively).

These data suggest either that mimosine prevents initiation in the beginning of the S period or that 400 μM mimosine inhibits chain elongation much more effectively than does 30 μM aphidicolin. However, we have previously found that aphidicolin levels higher than 10 μg/ml do not cause a further decrease in the rate of [^{3}H]thymidine incorporation into CHO cells (data not shown). We therefore consider it more likely that in the context of this experiment, mimosine actually prevents entry into the S period (20a).

The relative number of replication bubbles observed in the DHFR initiation locus in early S is greatly increased when cells are synchronized with mimosine. The arrangement of the DHFR amplicons in the CHOC 400 cell line and the map of a single 240-kb repeating unit are shown in Fig. 1A and B, respectively. The approximate centers of the two peaks of early labelling (ori-β and ori-γ), designated I_β and I_γ in Fig. 1C, lie between the DHFR and 2BE2121 genes (1, 15). In both asynchronous and early-S-phase cells, replication bubbles were observed on 2-D gels in several contiguous and overlapping fragments representing the 28-kb region encompassing both ori-β and ori-γ, although the relative number of bubbles was quite low (7, 25, 26).

In all of the previous experiments in which cells were synchronized, aphidicolin had been used as the blocking agent. Since aphidicolin appears to be quite leaky (Fig. 3A), we determined whether the number of replication bubbles in the initiation locus is higher in cultures entering the S phase more synchronously after release from mimosine.

CHOC 400 cells were arrested in G_0 and then released into complete medium containing either 400 μM mimosine or 30 μM aphidicolin. After 14 h, the cells were returned to drug-free medium, and DNA was prepared at various intervals in the ensuing S period (the time zero sample was taken prior to drug removal). *Eco*RI digests of each DNA sample were separated on 2-D gels according to the method of Brewer and Fangman (2), and the digests were transferred to a filter. The filter was then hybridized with a probe that recognizes a 6.2-kb *Eco*RI fragment centered over the upstream ori-β locus (fragment F'; Fig. 1C). The diagram in Fig. 4 shows the patterns expected for fragments that contain active origins (Fig. 4B and C), single forks (Fig. 4A), or termini (Fig. 4D) (2).

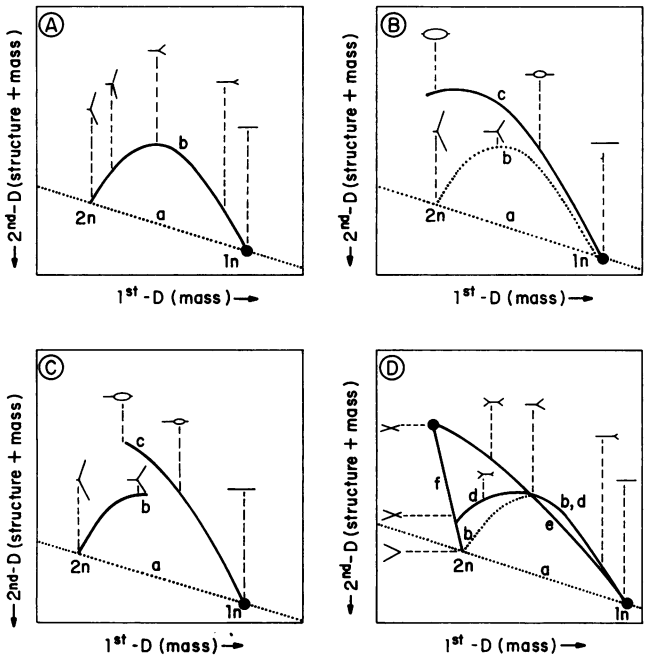

FIG. 4. Patterns of typical replication intermediates separated by 2-D neutral/neutral gel electrophoresis. Each panel shows an idealized autoradiographic image that would be obtained when a restriction digest of replicating DNA is hybridized with probes for fragments that contain different intermediates. (A) A complete simple Y or fork arc (b) resulting from a fragment that is replicated passively from an outside origin. Curve a represents the diagonal of nonreplicating fragments from the genome as a whole. (B) The pattern obtained when a fragment with a centered origin of replication is probed (curve c). Bubbles migrate more slowly at all extents of replication than do forks in a fragment of equal mass (b). (C) The presence of an off-centered origin in a fragment gives rise to an incomplete bubble arc (c), which then reverts to the fork arc when the bubble expands beyond the righthand restriction site, resulting in a fork arc break. (D) When two forks approach each other in a fragment either symmetrically or asymmetrically, curve e or d, respectively, is obtained. Note that curve e emerges from under the fork arc below its point of inflection. If there is a fixed terminus in a fragment, the collected X-shaped structures would result in a concentrated spot somewhere on curve f. Recombination structures would also fall along curve f (2). The triangle formed between the simple Y arc and curves e and f contains a collection of double-forked structures differing in the extents of replication and the positions of the fork within the fragment.

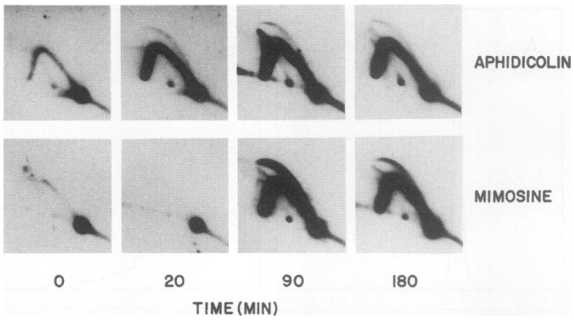

FIG. 5. Detection of replication intermediates in the presence of aphidicolin. CHOC 400 cells were arrested in G_0 and were then released for 14 h into complete medium containing either 30 μM aphidicolin or 400 μM mimosine. Cells were harvested either immediately (time zero) or at 20, 90, or 180 min after transfer into drug-free medium. Matrices were prepared and digested with *Eco*RI, and the matrix-attached replicating fractions were isolated, fractionated on BND-cellulose, and separated on 2-D gels. Transfers of the gels were hybridized with a probe specific for the 6.2-kb *Eco*RI fragment that straddles ori-β (see map in Fig. 3).

The autoradiogram of the time zero sample (Fig. 5, upper lefthand panel) shows that even before aphidicolin is removed, the 6.2-kb *Eco*RI fragment contains replication forks and barely detectable levels of replication bubbles (visible on the original film). Therefore, a significant amount of replication fork movement occurs during the incubation with aphidicolin. By 20 and 90 min after drug removal, the number of replication intermediates in this fragment increases considerably relative to the 1*n* spot, although there are fewer bubbles than single-forked structures at both time points. By 180 min after removal of aphidicolin, the single fork arc is still very strong, but the bubble arc begins to disappear. (Note that by 90 min, significant numbers of double-forked termination structures have accumulated in the 6.2-kb *Eco*RI fragment [see Discussion].)

The picture obtained when mimosine is used as the blocking agent is quite different (Fig. 5, lower panels). Very low levels of replication intermediates are detected either at time zero or by 20 min after removal of mimosine. What little there is can probably be explained by a small percentage of cells that escape the G_0 block and are still cycling at the time of drug addition (20a). By 90 min, both a prominent fork arc and a very strong bubble arc are detected. By 180 min, the fork arc is still very prominent, but the relative number of replication bubbles has decreased. Note that termination structures appear at 90 and 180 min in mimosine-synchronized cells as well.

Thus, relative to an aphidicolin block, there appears to be very little escape synthesis in the presence of mimosine. Furthermore, once mimosine is removed, the total amount of replication bubbles observed in this fragment is dramatically increased relative to the numbers detected after release from aphidicolin during an equivalent time interval (20 to 180 min). It is therefore possible that much of the initiation in this region has already occurred prior to removal of aphidicolin, leaving only those bubbles resulting from the remaining origins that have not yet fired at the time of drug removal. It is important to point out, however, that even though the relative amounts of replication intermediates observed after release from mimosine are significantly higher than after release from aphidicolin, the patterns are not qualitatively different.

Initiation in the ori-β locus occurs only during early S. To determine more precisely the time interval during which initiation occurs in the region of ori-β, CHOC 400 cells were synchronized at the G_1/S boundary with mimosine and were sampled at several different times after release from the drug. After separation of *Xba*I-digested replication intermediates on 2-D gels, the digests were transferred to a membrane and hybridized with a probe specific for a 4.3-kb *Xba*I fragment centered in the ori-β region (Fig. 1C). The resulting autoradiograms are shown in Fig. 6.

Twenty minutes after drug removal, very few replication intermediates are detected in the 4.3-kb *Xba*I fragment. By 40 min, some very small bubbles and single-forked structures are observed. By 60 min, both a strong fork arc and a modest bubble arc can be detected in this fragment. At 80, 100, and 120 min, the fork arc is more intense and the bubble

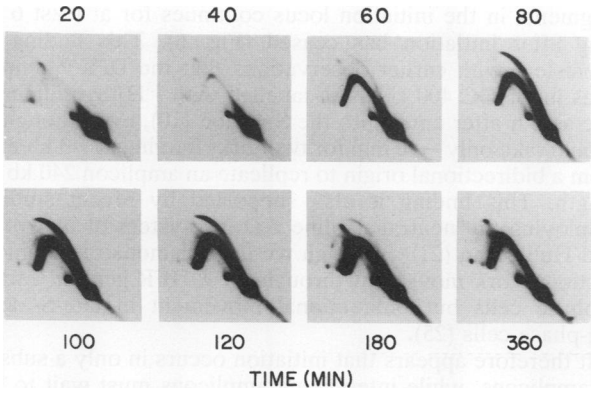

FIG. 6. Evidence that initiation in the DHFR locus is largely confined to a 1-h interval in the early S period. Matrices were isolated from synchronized CHOC 400 cells released from a mimosine block for increasing time periods. *Xba*I digests of replicating DNA were separated on 2-D gels, and transfers of the DNA were hybridized with a probe specific for the 4.3-kb *Xba*I fragment centered over the ori-β region (see map in Fig. 1C).

arc is very strong. After 120 min, there is a sharp decline in the bubble-to-fork arc ratio, owing to both the disappearance of the bubble arc and the retention of a strong fork arc until at least 6 h after removal of mimosine. Again note that termination signals appear by 120 min and are still observed at 180 and 360 min.

Therefore, most of the initiation in this region is confined to the interval between 60 and 120 min after release from mimosine. However, since simple fork arcs can still be detected in this region 360 min after drug removal, some of the DHFR amplicons must be replicated passively by forks emanating from active origins in distant amplicons.

Replication bubbles are detected in the early S period in a broad but circumscribed zone in the DHFR domain. It was conceivable that the delocalized form of initiation observed in earlier studies (7, 26) could somehow be related to the lengthy incubation in aphidicolin, during which time replication forks appear to leak slowly away from origins (Fig. 5). We therefore reexamined the replication pattern of the initiation locus after CHOC 400 cells were synchronized with mimosine. Samples were taken at 80 and 160 min after release from the drug, and *Eco*RI fragments containing replication intermediates were separated on a 2-D gel. The transfer was hybridized sequentially with probes for different fragments in the region between and within the DHFR and 2BE2121 genes. The resulting autoradiograms are shown in Fig. 7.

At 80 min (upper panels), when a large number of bubble-like structures are detected in the 6.2-kb *Eco*RI fragment centered over the ori-β region (fragment F'), a prominent bubble arc is also observed in the adjacent 6.1-kb fragment (fragment F). Thus, in two neighboring fragments of very similar size, the bubble-to-fork arc ratios are similar.

Less intense bubble arcs are detected in a 4.1-kb fragment lying just downstream from the 3' end of the DHFR gene (fragment G) as well as in a 5.9-kb *Eco*RI fragment downstream from ori-γ (fragment D). However, no bubble arcs are detected in fragments H and B, which lie just inside the DHFR and 2BE2121 genes, respectively, even after very long exposures (Fig. 7 and data not shown).

By 160 min after removal of mimosine, very few bubbles are detected in any fragment, even though the fork arcs are still strong in all of the fragments examined. Thus, bubble-like structures appear and disappear throughout the initiation locus with the same kinetics. Termination structures are again detected in the 160-min samples, albeit primarily in fragments F' and F.

DISCUSSION

In previous studies, we used the polymerase inhibitor aphidicolin to collect cells near the G_1/S boundary after release from G_0 arrest. However, we show here that the initial rate of DNA replication after release from aphidicolin increases with the length of the block (Fig. 3). Furthermore, significant numbers of replication forks are detected on 2-D gels in the initiation locus even before aphidicolin is removed (Fig. 5A). This finding indicates that (i) aphidicolin does not prevent initiation and (ii) aphidicolin allows replication forks

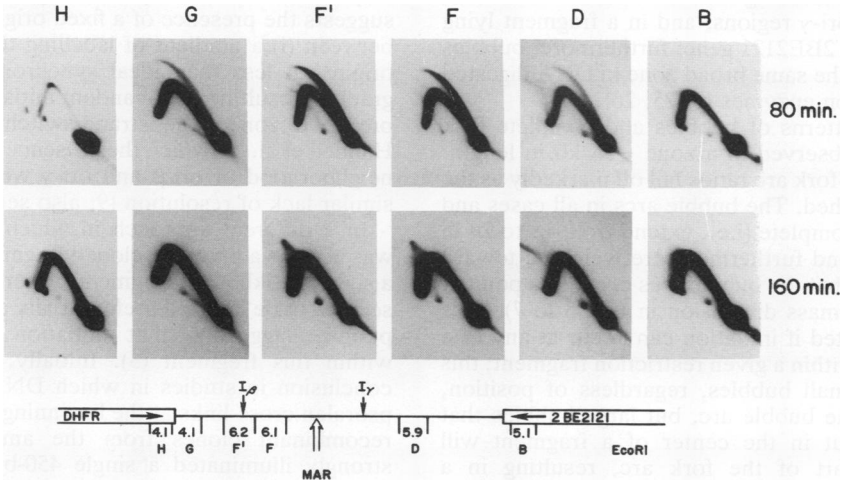

FIG. 7. Detection of initiation in the early S phase throughout a 55-kb zone between the two convergently transcribed genes. CHOC 400 cells were synchronized as described in the legend to Fig. 6. After removal of mimosine, the cells were allowed to grow in drug-free medium for 80 or 160 min. Matrices were isolated and digested with *Eco*RI. Transfers of replication intermediates were hybridized sequentially with probes specific for fragments B to H (indicated on the map below).

to move slowly away from origins even at a relatively high drug level.

Given that most of the population arrives at the G_1/S boundary anywhere between 5 and 13 h after release from the G_0 block, the amount of escape synthesis would be significant and the corresponding bubbles that developed in and moved out of each fragment during this lengthy time interval would therefore be lost from analysis. In combination with less than ideal DNA isolation procedures, this phenomenon probably accounts for the rather low level of replication bubbles that we detected in the DHFR initiation locus in earlier studies (7, 26).

In contrast, the initial rate of DNA synthesis after reversal of a mimosine block is very low and does not increase with the length of the block (Fig. 3). In addition, there is about a 40-min lag after release from mimosine before significant amounts of replication intermediates can be detected in the initiation locus (Fig. 5 and 6). When DNA replication does get under way, large numbers of replication bubbles appear in this locus in a very synchronous burst during the next 60 min, declining to negligible levels by 180 min. To our knowledge, this is the first study in which it has been possible to determine the precise interval in the S period during which initiation occurs in a defined chromosomal locus.

We estimate that at the peak of initiation (80 to 100 min after release from mimosine), the ratios of bubbles to single forks in the ori-β locus and in immediately adjacent fragments are in the range of 1:4 to 1:6 (estimated by eye from lower exposures of the autoradiograms shown in Fig. 5 to 7). Therefore, the number of bubbles detected in the initiation locus when DNA is isolated from highly synchronized cells by the matrix enrichment procedure is significant. The fact that replication bubbles are found only during a narrow window in the early S period further argues that bubble arcs are not artifacts unrelated to initiation per se.

In the experiment shown in Fig. 7, a composite pattern of replication bubbles and complete fork arcs is observed in *Eco*RI fragments G, F′, F, and D from the region between the two genes (see map in Fig. 1C). However, only simple fork arcs are detected in fragments H and B, which flank this intergenic region. These data support and extend our previous studies in which bubble arcs were found throughout the 28-kb initiation locus, including near the matrix attachment site, in the ori-β and ori-γ regions, and in a fragment lying just upstream from the 2BE2121 gene; furthermore, bubbles have been detected in the same broad zone in DNA digested with different restriction enzymes (7, 25, 26).

Thus, composite patterns of bubbles and complete fork arcs have now been observed in a zone ~55 kb in length, although the bubble-to-fork arc ratios fall off markedly as the two genes are approached. The bubble arcs in all cases and at all time points are complete (i.e., extend from 1*n* to 2*n* in the mass dimension) and furthermore are weighted toward smaller bubbles (note that the bubble arcs come to a point at the 2*n* position in the mass dimension in Fig. 5 to 7). This result would be expected if initiation can occur at any of a large number of sites within a given restriction fragment; this follows because all small bubbles, regardless of position, would contribute to the bubble arc, but large bubbles that originate anywhere but in the center of a fragment will eventually become part of the fork arc, resulting in a collection of fork arc breaks (the pattern illustrated in Fig. 4C). The complete single fork arc would then be composed of forks that initiated in neighboring fragments.

An important observation is that passive replication of fragments in the initiation locus continues for at least 6 h, long after initiation has ceased (Fig. 6). This finding is consistent with earlier observations that the DHFR amplicons in CHOC 400 cells are labelled with [^{3}H]thymidine as late as 8 h after entry into the S period (10), even though it should take only ~40 min for two forks moving at ~3 kb/min from a bidirectional origin to replicate an amplicon 240 kb in length. This finding is also supported by recent studies employing the neutral/alkaline 2-D gel system of Nawotka and Huberman (21), in which we have demonstrated unidirectional fork movement through the DHFR gene in early-S-phase cells but bidirectional movement in late-S- and log-phase cells (25).

It therefore appears that initiation occurs in only a subset of amplicons, while intervening amplicons must wait to be replicated by forks emanating from active origins in distant amplicons. These observations may explain why the ratio of bubbles to single forks in fragments from the initiation locus is 5- to 10-fold lower in DNA from log-phase cells than in DNA from early-S-phase cells (7, 25, 26).

The termination structures that are detected in the initiation locus ~100 min after release from mimosine could, in fact, be forks from distant amplicons that collide with forks close to the point of origin in the initiation locus itself (Fig. 6 and 7). However, since termination structures can be detected as late as 360 min into S (Fig. 7), they must be very long-lived or must result from some other phenomenon that we presently do not understand.

How can we reconcile the 2-D gel data suggesting a delocalized form of initiation with results of several previous studies suggesting that initiation in the DHFR locus may occur at a relatively fixed site? We will start with the assumption that, in fact, there may be two *cis*-regulatory elements (origins) located in the ori-β and ori-γ regions. Delocalized initiation could occur if the *cis*-acting elements function as loading sites for the replication complex, which can then migrate along the template and initiate a chain at random positions but, stochastically, more often near the two origins themselves.

On the basis of a model such as this, we believe that the 2-D gel data are not incompatible with earlier in vivo experiments in which the DNA was labelled intrinsically and examined on gels (10, 15). Although preferential labelling of only a subset of the amplified fragments in the early S period suggests the presence of a fixed origin, it cannot distinguish between (i) a gradient of labelling that results from a fixed origin but less than ideal synchrony and (ii) a bona fide gradient resulting from random initiation sites in a broad but preferred zone. The strand-switching approach used by Handeli et al., in which the presence of origins in the general neighborhood of ori-β and ori-γ were suggested, suffers a similar lack of resolution (9; also see reference 5).

In a different approach in which early-replicating DNA was used as a probe on cloned fragments from the amplicon, a single 4.3-kb *Xba*I fragment centered over the ori-β region seemed to be labelled preferentially at the beginning of the S period, suggesting that initiation occurs predominantly within this fragment (3). Initially, we reached a similar conclusion in studies in which DNA synthesized between psoralen cross-links at the beginning of S was used to probe recombinant clones from the amplicon (1); this DNA strongly illuminated a single 450-bp *Pvu*II fragment contained within the 4.3-kb *Xba*I fragment. However, we also showed that there are repetitive sequence elements in the 4.3-kb fragment that occur elsewhere in the genome. These sequences are enriched in early-replicating DNA, but their

synthesis is not confined to early S (1, 14). In view of the presence of these interfering repetitive elements, we do not presently understand the significance of either our own hybridization data (1) or those of Burhans et al. (3).

To our mind, it is more difficult to reconcile the results of 2-D gel analysis with more recent in vitro experiments suggesting that >80% of Okazaki fragments switch templates within a single 500-bp region centered in ori-β (4). However, several suggestions have been made to accommodate both sets of data into a unified model.

Burhans et al. (4) have pointed out the difficulty in quantitating the relative numbers of bubbles in 2-D gel patterns, owing to differences in fragment size and the vagaries of bubble loss, etc. (also see reference 17). However, even with this caveat, it is difficult to believe that 80% of initiations occur within the 6.2-kb *Eco*RI fragment that contains the 500-bp zone implicated by Burhans et al. (4), when the bubble-to-fork arc ratio in the adjacent 6.1-kb fragment is nearly equal (Fig. 7). Relatively strong bubble arcs are also observed in the same DNA preparation in fragments G and D and have been detected in intervening fragments as well (25).

It is more likely that the 2-D gels and the in vitro strand-switching assay are measuring different aspects of initiation reactions in mammalian chromosomes. The most obvious difference is that one assay analyzes replication intermediates synthesized in vivo, while the other utilizes Okazaki fragments labelled in vitro. A second, not so obvious difference is the static versus kinetic nature of the two approaches, as suggested previously (18): the intermediates observed on 2-D gels are necessarily weighted toward longer-lived structures, while the bromodeoxyuridine-labelled Okazaki fragments isolated from the strand-switching assay had to have been labelled during the 1.5-min incubation in vitro.

It is conceivable, for example, that origins of replication in mammalian chromosomes undergo futile, delocalized initiation cycles, the products of which never mature to full-grown replicons, but which have a longer half-life than do the successful initiations that occur at the real origin. The former would represent the delocalized bubbles that we detect in 2-D gels (7, 26), while the latter would be detected in the strand-switching assay (4).

Alternatively, the rapidly labelled material detected in the strand-switching assay could represent the futile cycle, while the longer-lived material detected on 2-D gels may actually mature to fully double-stranded daughter helices. In this regard, we have recently detected bubbles of all sizes in fragments as large as 15 kb (5a), and there obviously could be even larger bubbles that cannot be contained in a single restriction fragment. Thus, some of the long-lived structures mature to very large sizes.

Another possibility is that regardless of their respective modes of action, both aphidicolin and mimosine might be leaky, allowing initiation to occur and forks to move slowly away from origins. In a competing reaction, the nascent chains might then be digested preferentially at their 5′ ends to a position very close to (but not including DNA protected by) the replication complex. This would result in a series of microbubble pairs symmetrically arranged at random positions around the true origin of replication. Upon removal of the drug, each microbubble could expand unidirectionally into the full-fledged bubbles observed on 2-D gels. After a few minutes, there would again be a disparity in size between leading- and lagging-strand synthesis, resulting in the Okazaki template strand bias observed by Burhans et al. (4). (This is a modified version of a general model proposed by Linskens and Huberman [18].)

The fact that bubbles are detected in the initiation locus in asynchronous cultures (although at low levels) argues against this model. Furthermore, we do not observe a collection of small bubbles in the presence of mimosine (Fig. 5), although very small bubbles could be occluded by the 1*n* spot.

There are undoubtedly other models that could be used to reconcile most of the extant data. However, the fact that fragments are replicated passively by read-through replication from other amplicons points out a real discrepancy between the 2-D gel results, which suggest delocalized initiation, and the strand-switching assay, in which most initiations are suggested to occur within a 500-bp region. The latter result was obtained not only in early-S-phase cells but also in log-phase cultures of CHOC 400 cells (4).

However, in log-phase cells, if a large fraction of the amplicons are replicated passively from an active origin in a different amplicon, there will be no dramatic switch in Okazaki template strand bias even at a fixed origin; this is so because when an outside fork replicates an inactive origin, Okazaki fragments will be synthesized from the same template strand on both sides of the origin, resulting in no strand bias for that particular copy of the origin. Furthermore, this rule pertains regardless of whether amplicons are arranged head to head or tail to tail.

Thus, there appear to be fundamental differences in the DNA replication processes being measured in vivo by the 2-D gel techniques and in vitro in permeabilized cells. We believe that a fuller understanding of these differences will eventually reconcile two sets of data that are seemingly at odds.

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