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The Chinese Hamster Dihydrofolate Reductase Origin Consists of Multiple Potential Nascent-Strand Start Sites

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Previous two-dimensional gel replicon-mapping studies on the amplified dihydrofolate reductase (DHFR) domain in CHOC 400 cells suggested that replication can initiate at any of a large number of sites scattered throughout a 55-kb region lying between two convergently transcribed genes. It could be argued that this unusual distributive initiation mode is unique to amplified chromosomal loci. In this paper, we report the first application of the two-dimensional gel techniques to the analysis of a single-copy locus in mammalian cells. Results obtained with both synchronized and exponentially growing CHO cells suggest that (i) initiation can also occur at any of a large number of sites distributed throughout the intergenic region in the nonamplified DHFR locus, (ii) initiation is confined to the first 2 to 2.5 h of the S period, and (iii) initiation occurs only in a fraction of the DHFR loci in each cell cycle.

Twenty-five years after DNA fiber autoradiographic studies established that the mammalian genome is replicated from multiple, bidirectional growing points (termed origins [24]), the nature of these origins remains obscure. Progress has been hampered by the lack of a reliable approach for identifying genetic elements (replicators) in mammalian genomes, such as the autonomously replicating sequence assay that was instrumental in isolating and characterizing replicators in bacteria and yeasts (10, 45, 49, 50). An alternative strategy is to identify the positions of nascent-strand start sites relative to the template, which, by analogy to viral, prokaryotic, and yeast origins, would be expected to map very close to the replicators themselves. An intrinsic difficulty with this approach is the sheer genetic complexity of mammalian genomes and, as a consequence of the long cell cycle times, the relatively small percentage of origins that would be firing at the time of sampling even in synchronized cultures.

To reduce the complexity problem, we developed a methotrexate-resistant CHO cell line (CHOC 400 [36]) that has amplified one allele of the early-replicating dihydrofolate reductase (DHFR) locus about 1,000 times. The majority of repeating units (amplicons) in this cell line are 240 kb in length and are arranged in tandem at three different chromosomal locations. Given the large size of each amplicon, it seemed likely that each would contain at least one origin of replication. In fact, a variety of different replicon-mapping strategies suggests the presence of at least one preferred initiation locus in the DHFR domain (1, 6, 7, 12–14, 20–22, 28, 47, 48). However, the details of the initiation reaction, as evidenced by these different strategies, are not entirely consistent with one another.

When synchronized CHOC 400 cells were incubated at the beginning of the S period with radioactive thymidine, only a small subset of restriction fragments from the 240-kb DHFR amplicon was preferentially labelled (21). These fragments defined an initiation locus at least 28 kb in length that lies between the DHFR and 2BE2121 genes (22). Subsequent higher-resolution labelling studies suggested that there might actually be two preferred initiation sites or zones within this region (1, 28). These sites (termed ori-β and ori-γ [28]) are separated by ~22 kb and straddle a prominent matrix attachment region (M; see Fig. 1) (11).

More recently, the DHFR locus has been examined by two other replicon-mapping procedures that determine the template preference of either the leading or lagging strands of replication, which should switch at an origin. Since both assays are more sensitive than intrinsic labelling protocols, they have been used to analyze replication intermediates in the single-copy DHFR loci in CHO cells (7, 20).

In the leading-strand assay (26, 41), cells were treated with emetine, which blocks lagging-strand synthesis (8), and bromodeoxyuridine-labelled leading-strand DNA was purified and used to probe the plus and minus strands of M13 subclones from the DHFR locus. This study detected template strand switches in the vicinities of ori-β and ori-γ with a resolution of 6 to 8 kb (20). In the lagging-strand assay, which measures the template bias of bromodeoxyuridine-labelled Okazaki fragments synthesized in vitro, a dramatic shift in template strands was observed to occur within a 500-bp fragment centered in the ori-β region (ori-γ was not examined in this study [7]). On the basis of the measured strand biases, it was estimated that more than 80% of all initiations in the DHFR locus occur within this 500-bp region. ori-β has thus been termed an origin of bidirectional replication (7). ori-β was further implicated as a highly preferred nascent-strand start site in a study employing PCR to measure the size distribution of bromodeoxyuridine-substituted nascent strands, the smallest of which should be centered over origins (47).

In aggregate, these experimental approaches suggest that ori-β is a highly preferred locus for nascent-strand start sites and, by inference, should contain or be near a genetic replicator. Unfortunately, no cloned fragments containing this sequence, regardless of size, have demonstrable autonomous replication sequence activity when introduced into mammalian cells by transfection (7, 9, 35a). However, evidence has been presented that a ~17-kb fragment containing ori-β can direct initiation from any ectopic chromosomal site after transfection into CHO cells (20). Thus, by several criteria, ori-β would appear to represent a bona fide origin.

However, independent approaches have suggested that

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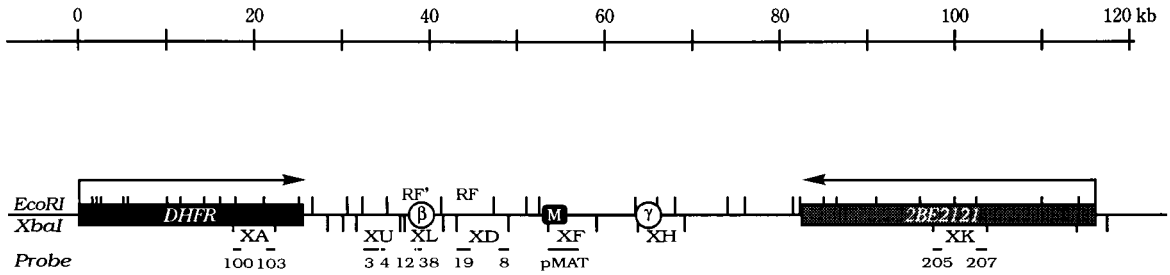

FIG. 1. Map of the initiation locus and flanking genes. The initiation locus corresponds to the region lying between the DHFR and 2BE2121 genes. Arrows above the map indicate the direction of transcription. The positions of ori-β, ori-γ, and the MAR (M) are indicated. The *Eco*RI and *Xba*I restriction fragments that were analyzed in 2-D gel experiments are shown above and below the map, respectively. The hybridization probes that were used to detect these fragments are numbered and are shown below the map. Probes were as follows: 100, a 1.2-kb *Xba*I-*Kpn*I fragment; 103, a 1.2-kb *Eco*RI-*Xba*I fragment; 3, a 2.0-kb *Eco*RI-*Bam*HI fragment; 4, a 0.6-kb *Bam*HI-*Eco*RI fragment; 12, a 0.3-kb *Pvu*II-*Bam*HI fragment; 38, a 0.5-kb *Pvu*II-*Xmn*I fragment; 19, a 1.0-kb *Hin*dIII-*Kpn*I fragment; 8, a 0.9-kb *Pvu*II-*Eco*RI fragment; pMAT, a 3.4-kb *Pvu*II fragment; 205, a 0.8-kb *Xba*I-*Taq*I fragment; and 207, a 0.7-kb *Sau*3a-*Hin*dIII fragment.

things are not quite that simple. When the amplified DHFR locus in CHOC 400 cells was analyzed by a neutral/neutral two-dimensional 2-D gel replicon-mapping method (3), replication bubbles were detected at a large number of sites scattered throughout the 55-kb intergenic region (although more frequently in the central two-thirds of this zone encompassing ori-β, ori-γ, and the matrix attachment region [12, 14, 48]). A very low level of starts could even be detected in the 2BE2121 gene (14), but bubbles have never been observed in the DHFR gene (12, 14, 48). Analysis of the DHFR domain in early-S-phase CHOC 400 cells by a neutral/alkaline 2-D gel method (25, 38) further showed that forks move in both directions through the intergenic region and the 2BE2121 gene but only outward through the DHFR gene (14). In all locations analyzed within the intergenic region, replication bubbles were detected only in the first 2.5 h of the S period, as predicted for an early-firing origin (12).

Thus, 2-D gel analyses of the amplified DHFR domain argue that nascent-strand start sites are distributed widely, with the 35-40 kb region encompassing the ori-β and ori-γ loci being preferred. While this view of initiation in the DHFR locus is reasonably consistent with results of some of the lower-resolution intrinsic-labelling studies on the amplified DHFR domain (1, 6, 21, 28), it is difficult to reconcile with the very circumscribed zone or site suggested by the lagging-strand assay and by PCR-based nascent-strand size analysis of the single-copy locus in CHO cells (7, 47).

There are several potential explanations for these rather different views. However, since we ultimately want to understand initiation reactions in a typical mammalian origin, an important first question is whether initiation in the single-copy (nonamplified) DHFR locus is also delocalized by 2-D gel criteria. It is conceivable, for example, that amplification of the DHFR domain in CHOC 400 cells has somehow deranged the initiation process, e.g., by altering chromatin architecture or the balance of other factors required for origin function.

In the present study, we have adapted both the neutral/neutral and the neutral/alkaline 2-D gel techniques to an analysis of replication intermediates in the DHFR domain of CHO cells. This represents the first successful application of these techniques to a single-copy locus in a mammalian cell line. Our results show that in almost all respects, the replication patterns of the amplified and single-copy DHFR loci are indistinguishable. Thus, the distributive initiation mode is not the consequence of amplification per se.

MATERIALS AND METHODS

Cell culture and synchronization protocols. CHO cells (the parental line from which the methotrexate-resistant CHOC 400 variant was derived) were grown as monolayer cultures in minimal essential medium supplemented with 10% bovine serum product (Hyclone). To synchronize cells at the G_1/S boundary, exponentially growing cultures were deprived of isoleucine for 45 h and then incubated for 14 h in complete medium containing 200 μM mimosine (12, 29, 37). The drug was then removed, and the cells were washed once with prewarmed serum-free medium and allowed to grow in complete medium for the indicated times before harvest.

Isolation of replication intermediates. Nuclear matrices were prepared as described previously (12, 13), using either *Eco*RI or *Xba*I to detach loop DNA from matrix-attached DNA. Matrix-attached DNA was isolated and fractionated on benzoylated-naphthoylated DEAE-cellulose (Sigma Chemical Corp.) as described previously (13). For neutral/neutral gels, the replication intermediates obtained from 1.0×10^8 to 1.5×10^8 cells were loaded into one well of a 0.4% agarose gel; for neutral/alkaline gels, the corresponding number was 2×10^8.

2-D gel electrophoresis. Neutral/neutral 2-D gel electrophoresis was performed exactly as described previously (13). For neutral/alkaline gel analysis, the first-dimension gel was run under the same conditions, but the lane was then excised, turned through 90°, and placed at the origin of a 1% agarose gel poured in water. This composite gel was then soaked in 0.4 M NaOH–5 mM EDTA for at least 2 h to denature the DNA. Electrophoresis in the second dimension was performed at room temperature in 0.04 M NaOH–0.001 M EDTA for 48 to 60 h at 0.5 V/cm. DNA was transferred to Hybond N+ (Amersham), and membranes were hybridized with the appropriate ^{32}P-labelled probes and washed as previously described (12, 13). Exposures to Kodak X-Omat AR film ranged from 5 to 15 days.

RESULTS

Organization of the DHFR domain in CHO cells. More than 600 kb of contiguous DNA sequence from the DHFR locus in CHO cells has been cloned in overlapping cosmids (34, 35). A map of the ~120-kb region lying between the promoters of the convergently transcribed DHFR and 2BE2121 genes is shown in Fig. 1. The major initiation zone, as defined by 2-D gel-mapping studies on the amplified DHFR domain in CHOC 400 cells (12, 14, 48), corresponds to the 55-kb intergenic region. The ori-β and ori-γ loci lie within this zone and straddle a prominent matrix attachment region (M in Fig. 1) (11). In the remainder of this report, we will use the terms 5′ and 3′ (or upstream and downstream) to refer to this map as drawn.

Initiation occurs in the early S period, but not all DHFR initiation zones are active. To determine the temporal pattern of initiation in the single-copy DHFR locus, we first analyzed the DNA from synchronized CHO cells by the neutral/neutral 2-D gel method. Cultures were arrested near the G_1/S boundary with mimosine, and the block was then removed, allowing cells to enter the S period in a synchronous wave (12, 29, 37). The CHO cells analyzed in this study traverse the S period in

~9 ± 1 h (data not shown). Samples were harvested at various times after drug removal, and replicating DNA was purified ~200-fold by a method described previously (13), using *Eco*RI to detach nonreplicating DNA from the nuclear matrix. Replication intermediates were separated on a neutral/neutral 2-D gel, transferred to a nylon membrane, and hybridized with a probe specific for a 6.2-kb *Eco*RI fragment (RF′) that contains the ori-β locus (Fig. 1). The principle of the neutral/neutral 2-D gel method (3) is summarized in Fig. 2, and the results of this analysis are presented in Fig. 3.

In the time zero sample (prior to drug removal), a strong 1*n* spot corresponding to contaminating nonreplicating DNA is present, but almost no branched, replicating molecules can be detected in the ori-β region, confirming earlier observations with CHOC 400 cells that mimosine is an extremely effective inhibitor of DNA replication that appears to prevent the formation of replication forks (29, 37). At 30 min after the drug was removed, a complex pattern of replication intermediates was detected in fragment RF′ that consisted of both a bubble arc and a fork arc. Since each of these arcs was incomplete, consisting only of smaller bubbles and forks, replication must have initiated in this region only in the preceding few minutes.

By 60 min, both a strong and complete bubble arc and an intense, complete fork arc were detected in fragment RF′. This pattern is virtually identical to the composite signal observed in the early S period in the amplified DHFR initiation locus in CHOC 400 cells (12, 14, 48). As we will show below, the fork arc arises from initiations that occurred at other locations within the intergenic region in the early S period.

As with the amplified locus, the relative number of replication bubbles was maximal 80 to 90 min after drug removal. By 180 min, the bubble arc was greatly reduced, suggesting that initiation had almost ceased; however, a single fork arc persisted that could still be detected in the 360-min sample. The same phenomenon was observed in CHOC 400 cells (12, 14) and implies that a sizeable percentage of DHFR domains in CHO cells does not sustain an initiation event but is replicated passively by forks emanating from origins in distant chromosomal domains.

If this were the case, then fragment RF′ from an unsynchronized cell population (which contains cells in all stages of the S period) should display a much lower bubble-to-fork arc ratio. This follows because only the fraction of DHFR loci that sustains active initiation events would contribute to the bubble arc in fragment RF′, whereas the fork arc would arise from two sources: (i) copies of the DHFR locus in which initiation occurred at a different position within the intergenic zone, and (ii) all of the other DHFR loci that were replicated passively by forks from an origin in a neighboring domain. As shown in Fig. 4A, the bubble-to-fork arc ratio in fragment RF′ is, indeed, greatly reduced in unsynchronized cells, supporting the contention that initiation occurs in only a subset of DHFR loci in CHO cells.

Initiation sites are dispersed throughout the intergenic region in the single-copy DHFR locus. Since only small bubbles were detected in fragment RF′ in the 30-min sample, it is likely that initiation has just begun; however, fragment RF′ also contained small replication forks at this time point, most of which must have arisen from initiation sites lying outside of this fragment. Small bubbles and small single forks were also present in the neighboring 6.2-kb fragment RF in the same 30-min sample (Fig. 3, right-hand panel). These findings are remarkably similar to those obtained in previous studies on CHOC 400 cells and suggest that in CHO cells as well, replication can initiate at any of a large number of sites in a broad initiation zone.

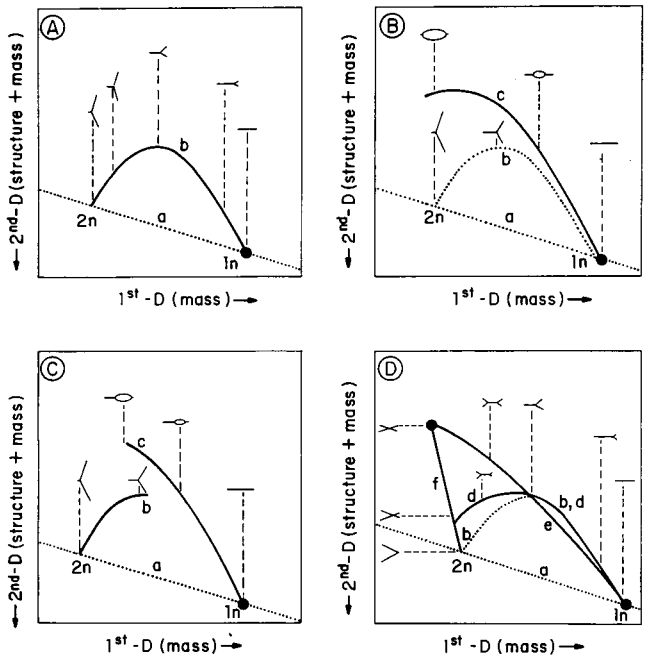

FIG. 2. Principle of the neutral/neutral 2-D gel replicon-mapping method (3). The figure shows the autoradiographic images that would be obtained when a digest of replicating DNA is hybridized with probes for fragments that are replicated from internal or external initiation sites. (A) A single fork arc (b) would be obtained with a fragment replicated passively from an outside origin. Curve a represents the diagonal of linear, nonreplicating fragments from the genome as a whole. (B) A fragment replicated from a centered origin (curve c). (C) A fragment containing an off-center origin. (D) A fragment containing two forks approaching one another either symmetrically or asymmetrically, generating curves e and d, respectively. If there is a fixed terminus in a fragment, the collected X-shaped structures result in a concentrated spot somewhere on curve f. Recombination structures would also fall along curve f (3). 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.

To investigate this proposal further, we surveyed several different fragments from the DHFR locus in DNA isolated near the peak initiation period (80 min after release from mimosine) and at a point when initiation has ceased in this locus (210 min). The DNA samples in this experiment were digested with *Xba*I, which yields a collection of similar-sized fragments in and around the intergenic region. The digests were separated on neutral/neutral 2-D gels, and the transfers for each time point were hybridized sequentially with probes specific for several different fragments (see the map in Fig. 1). An attempt was made to adjust the film exposures so that the 1*n* spots were of approximately equal intensity, allowing the number of replication intermediates in different fragments in the same sample to be roughly compared (this assumes that the recovery of similar-sized fragments is nearly equal, which appears to be the case [10a]).

As shown in Fig. 5, fragments XU, XL, and XF from the intergenic region all displayed prominent and complete bubble arcs and strong fork arcs at the 80-min time point (note that the film for fragment XF is slightly underexposed relative to the others). Complete bubble arcs can also be detected at the 80-min time point in fragments XD and XH from the intergenic region (data not shown). Therefore, seven different fragments in two overlapping sets (RF′, RF, XU, XL, XD, XF, and XH) from the intergenic region display complete bubble arcs

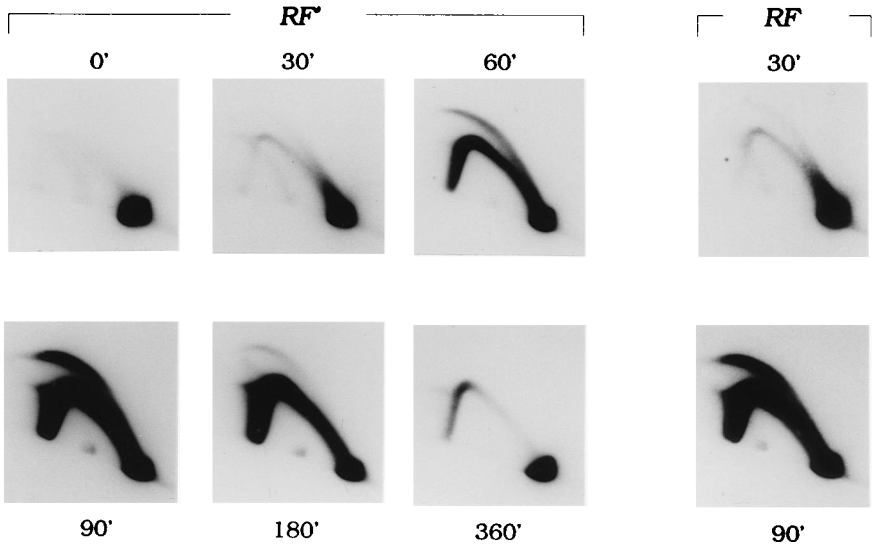

FIG. 3. The DHFR locus initiates replication only in the early S period. CHO cells were collected near the G_1/S boundary as described in Materials and Methods, and samples were harvested at the indicated times for analysis by neutral/neutral 2-D gel electrophoresis (the 0 time point refers to harvesting of the samples prior to removal of mimosine). Replication intermediates were isolated on the nuclear matrix, using *Eco*RI to detach nonreplicating DNA. After electrophoresis, the DNA digest was transferred to HyBond N+ and hybridized sequentially with probes specific for fragments RF′ (probes 12 and 38) and RF (probe 19). Films were exposed for 7 and 11 days, respectively.

as well as complete fork arcs in the early S period (Fig. 3 and 5, and data not shown).

Our interpretation of these composite patterns is that potential initiation sites are scattered throughout the intergenic zone. Thus, for any given fragment within this zone, the bubble arc results from internal initiations at different sites within the fragment (some of which are near the center), while the Y arc results largely from forks that initiated at other sites in neighboring fragments within the initiation zone as well as from initiation sites near the ends of the fragment. Moreover, since the relative number of bubbles near the ori-β locus does not appear to be markedly higher than in neighboring fragments, ori-β does not appear to be a highly preferred initiation site by the criterion of neutral/neutral 2-D gel analysis (e.g., compare fragments RF′ and RF in Fig. 3, as well as overlapping fragments XU and XL in Fig. 5, in which both the bubble arcs and the fork arcs are within the linear response range of the film).

In contrast, neither the DHFR gene (fragment XA) nor the 2BE2121 gene (fragment XK) displayed significant numbers of replication intermediates at the 80-min time point (Fig. 5). Thus, at early times in the S period, the single-fork arcs detected in fragments from the intergenic region appear to have arisen from start sites within the initiation zone itself and not from other origins in upstream or downstream chromosomal domains.

By 210 min, replication bubbles were undetectable in fragments XU, XL, and XF (as well as in XD and XH [data not shown]). Thus, initiation appears to have ceased in this locus ~3.5 h after release from the mimosine block. However, all seven *Xba*I fragments examined displayed strong single-fork arcs (Fig. 5 and data not shown). Conceivably, some of these forks could have originated from the very last initiations at other locations within the intergenic zone itself just minutes before sampling. However, it is likely that the majority arose from other origins in upstream or downstream chromosomal domains. Therefore, it appears that a substantial fraction of DHFR domains are replicated passively, in agreement with the presence of single-fork arcs in the 6-h sample in the experiment presented in Fig. 3.

Dual-fork direction in the early S period also suggests a very broad initiation zone in the CHO DHFR locus. If initiation occurs at different sites within the intergenic region in different copies of the DHFR locus in the early S phase, then in the cell population as a whole, replication forks should be observed to travel in both directions through any fragment within this zone when initiation is occurring but only outward from the initiation zone in flanking regions. Later in the S period, the directions of fork movement will depend upon whether every copy of the DHFR replicon actually sustains an active initiation event: if all DHFR origins are active, replication intermediates should clear from the locus shortly after initiation has ceased; however, if only some DHFR origins fire, then replication forks will be traversing some copies of the DHFR replicon from upstream and/or downstream origins well into the S period.

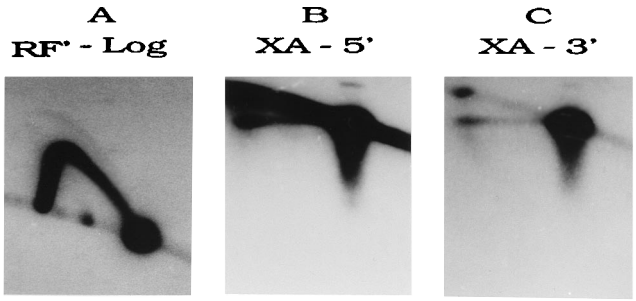

FIG. 4. Replication patterns in nonsynchronized cells. (A) Nonsynchronized CHO cells were harvested, and replication intermediates were prepared by using *Eco*RI to detach nonreplicating DNA from the matrix. After separation on a neutral/neutral 2-D gel, the digest was hybridized with probes specific for fragment RF′ (probes 12 and 38). Exposure was continued for 14 days. (B and C) Replication intermediates were prepared from asynchronous CHO cells, using *Xba*I to detach the DNA loops from the matrix. The digest was subjected to neutral/alkaline 2-D gel analysis (see the legends to Fig. 6 and 7 for details); after transfer to Hybond N+, the digest was hybridized sequentially with probes for the 5′ (B) and 3′ (C) ends of fragments XA (probes 100 and 103, respectively). Exposures were continued for 15 and 14 days, respectively.

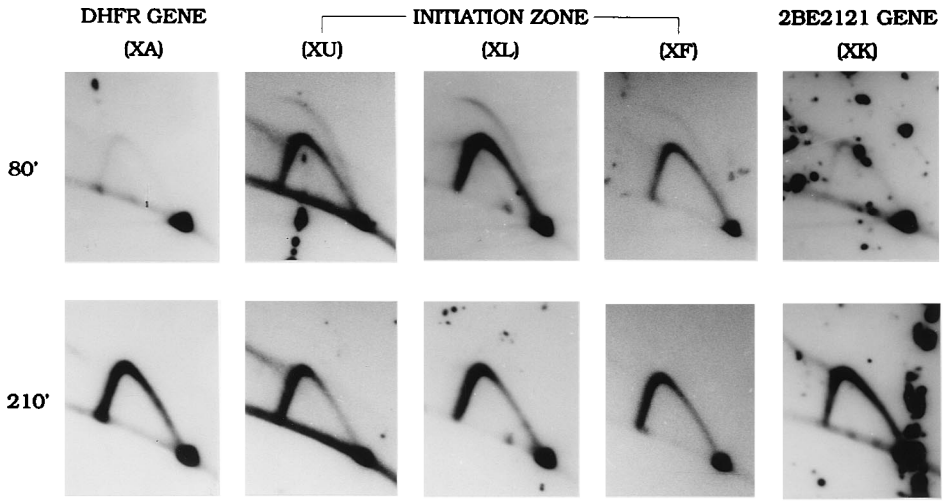

FIG. 5. Replication can initiate at multiple locations in the CHO DHFR locus. CHO cells were synchronized near the G_1/S boundary, and samples were taken 80 and 210 min after release from mimosine. An *Xba*I digest of each sample was prepared, run on a neutral/neutral 2-D gel, and hybridized with probes for the indicated *Xba*I fragments (Fig. 1). The probes for each fragment were as follows: XA, 100 plus 103; XU, 3 plus 4; XL, 12 plus 38; XF, pMAT; and XK, 205 plus 207.

To address these issues, the same DNA preparations that were subjected to neutral/neutral 2-D gel analysis in Fig. 5 were analyzed by a neutral/alkaline 2-D gel electrophoresis assay that determines the direction of fork movement through any given fragment (25, 38). In this method (Fig. 6), a digest of replicating DNA is separated by size in the first-dimension gel just as in the neutral/neutral 2-D gel technique. However, the second dimension is run in alkali, which releases nascent DNA from the 1*n* template strand to form a diagonal of nascent strands ranging from full length to only a few nucleotides. When a transfer of such a gel is hybridized with a probe from the origin-proximal end of a given fragment, nascent strands of all sizes are detected, while an origin-distal end probe detects only the largest nascent DNA strands (Fig. 6B). The results of this analysis on the CHO DHFR locus are shown in Fig. 7 (note that the signals for the 3′ XA probe and the 5′ and 3′ XD probes are not as intense as in the other three 210-min samples, because the specific activity of these probes was not as high; thus, it is necessary to compare the intensities of the horizontal 1*n* template and the diagonal with the 1*n* spot to estimate the approximate quantities of replication intermediates in each fragment; also note that in practice, it is very difficult to detect the smallest nascent strands in the diagonal owing to the correspondingly small hybridization signals).

In agreement with the data in Fig. 5, most of the replication intermediates were concentrated in the initiation zone in the 80-min sample, as indicated by the relatively intense horizontal 1*n* template signals obtained with probes for fragment XD (compare with the very weak template signals relative to the 1*n* spot with probes for fragments XA and XK from the two genes [Fig. 7]). Importantly, both end probes for fragment XD detected complete diagonals, indicating that replication forks are entering this fragment from both directions. This result is predicted if initiation sites are dispersed throughout the initiation zone, including sites upstream and downstream from fragment XD. Fragment XD was chosen for analysis because it is in the center of the intergenic region. We were unable to analyze either fragment XL or XH, which contain ori-β and ori-γ, respectively, because of the slightly repetitive nature of the end probes for these fragments. However, in the amplified DHFR locus in CHOC 400 cells, in which the greater signal-to-noise ratio can tolerate some repetitiveness in the probes, forks are observed to move through fragments XL and XH in both directions in the early S period (14).

Owing to the paucity of replication intermediates in the DHFR gene in the 80-min sample, hybridization signals were very weak, although there is some suggestion on the original film of a faint diagonal with the 3′ probe. However, faint diagonals were detected with both end probes for fragment XK in the 2BE2121 gene, suggesting that a low level of initiation is occurring within and/or downstream from this gene. Because of the small number of replication intermediates in the CHO 2BE2121 gene at the 80-min time point (Fig. 5), we have not been able to determine whether fragment XK contains replication bubbles on neutral/neutral 2-D gels (Fig. 5). However, faint bubble arcs can be detected in fragment XK in DNA isolated from early-S-phase CHOC 400 cells (14).

By 210 min, significant numbers of replication intermediates were observed in all of the regions analyzed. Dual-fork direction was again detected in fragment XD from the initiation zone and in fragment XK from the 2BE2121 gene. As noted above, most of these forks probably originated earlier from origins in neighboring domains, since initiation appeared to have ceased in the DHFR locus at 210 min by the criterion of neutral/neutral 2-D gel analysis (Fig. 3). Surprisingly, however, forks still appeared to traverse the DHFR gene only in the 3′-to-5′ direction, since a diagonal of nascent strands could be seen only with the 3′-end probe.

To attempt to rule out the possibility that the synchronizing regimen could somehow alter the pattern of fork movement, replication intermediates were isolated from unsynchronized cells and the fork directions through fragment XA from the DHFR gene were assessed. As shown in Fig. 4B, the 5′ probe detected a strong horizontal template band at the 1*n* position, indicating the presence of significant numbers of replication intermediates in fragment XA; however, the 5′-end probe detected only the largest nascent strands. Therefore, forks do not appear to enter this fragment from upstream locations at any time in S. The signal from the 3′ probe is very weak, even after a 14-day exposure, but the upper part of a faint diagonal can be discerned in the original autoradiograph. We tentatively conclude that replication forks probably enter this fragment only

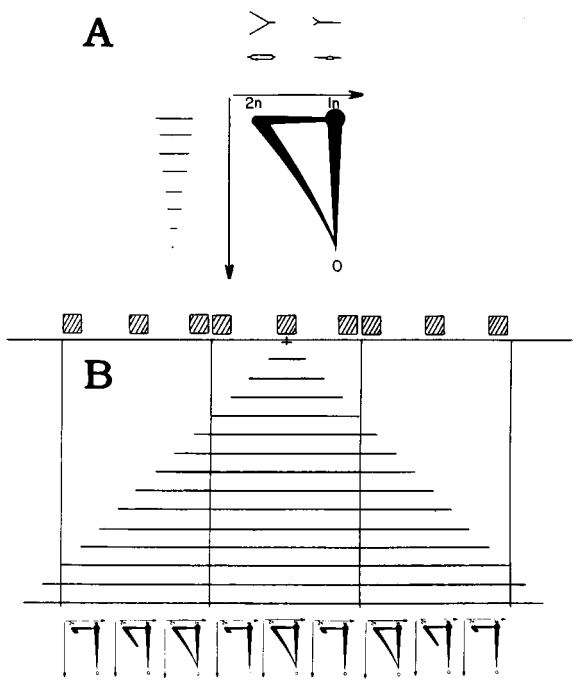

FIG. 6. Principle of the neutral/alkaline 2-D gel replicon-mapping method. (A) The idealized migration patterns of double-stranded replication intermediates in the first, neutral dimension and of single-stranded nascent chains in the second, alkaline dimension. (B) Diagram of three adjacent restriction fragments, with central and end probes denoted by hatched boxes above the horizontal line. An origin of replication is positioned in the middle of the central fragment, and nascent strands of different sizes are shown below. The three autoradiograms at the bottom of each fragment show the patterns that would be obtained with each probe.

from the direction of the downstream initiation locus (i.e., 3′ to 5′). Thus, the patterns of fork movement appear to be similar in synchronized and in asynchronous CHO cell populations.

DISCUSSION

When we previously analyzed the amplified DHFR domain in CHOC 400 cells by two different 2-D gel mapping techniques, the results suggested that initiation can occur at any of a large number of sites scattered throughout the 55-kb intergenic region, with the central 35 to 40 kb being somewhat preferred (12, 14, 48). These data are reasonably consistent with most of the previous low-resolution in vivo labelling results that identified an initiation zone in the region lying downstream from the DHFR gene (1, 6, 20, 21, 28). However, they are quite difficult to reconcile with results of two other origin-mapping approaches that measured either lagging-strand template bias (7) or nascent-strand size distributions (47): both of these assays suggest that initiation is confined to a zone encompassing ori-β that is less than a few thousand base pairs in length. In fact, results of the lagging-strand assay suggested that more than 80% of initiations occur within a single 500-bp fragment centered over ori-β (7).

Presumably, such dissimilar views of initiation in this locus could arise from several causes, including (i) differences in cell lines and/or modes of propagation, (ii) artifacts in some or all of the assays, or (iii) a bona fide novel mode of initiation in this locus, different facets of which are preferentially illuminated by each assay.

We have largely ruled out the first possibility in a collaborative study in which replication intermediates were prepared for 2-D gels with the same CHOC 400 cells and media that suggest a narrowly circumscribed initiation zone in the lagging-strand assay or by nascent-strand size analysis. Under these circumstances, initiation was also observed to be distributive in the amplified DHFR locus (10b). Furthermore, delocalized initiation reactions have now been observed at other loci in different eukaryotic organisms in which the DNA was prepared, in most cases, by very different methods; these loci include an amplifying chromosomal puff in *Sciara coprophila* (30), the rRNA genes in human cells (33), the rhodopsin gene in CHO cells (10c), and the histone and α-polymerase loci in *D. melanogaster* (43, 44). In the histone and α-polymerase loci, dispersed initiation sites were suggested by both 2-D gel and nascent-strand size analyses (43, 44).

We have also evaluated our method of preparing replication intermediates for selective loss or enrichment of one or the other kind of branched structure and have not detected any bias introduced at any step of the purification procedure (10a, 13). The material that we subjected to 2-D gel analysis also appears to represent normal replication bubbles and forks in the electron microscope (19) and by direct comparison with fragments containing a yeast autonomously replicating sequence element on 2-D gels (5a). In addition, we have recently shown that broken bubbles migrate to unique positions on neutral/neutral gels and are easily distinguishable from simple Y-shaped structures (25a); thus, the lack of a much more prominent bubble arc over the ori-β locus, as predicted by the lagging-strand assay, cannot be explained by the conversion of bubbles to Y-shaped structures via breakage (32, 42).

By purifying replication intermediates several hundred-fold from starting DNA preparations in the present study, we have been able to adapt both 2-D gel methods to an analysis of the nonamplified DHFR locus in CHO cells. The results of the two approaches can be summarized as follows: (i) in the early S period in CHO cells, replication intermediates are confined largely to the intergenic region and bubbles can be detected at all locations within this region; (ii) in early S phase, replication forks move in both directions throughout the intergenic region and in the 2BE2121 gene but appear to move only outward through the DHFR gene (presumably, dual-fork direction in the 2BE2121 gene reflects a low level of dispersed initiation sites in and downstream from the gene that cannot be detected in CHO DNA on neutral/neutral gels, since both dual-fork direction and replication bubbles have been detected in the 2BE2121 gene in CHOC 400 cells); and (iii) 2.5 h after removal of mimosine, initiation has ceased in the DHFR locus but single-fork arcs can still be detected for at least another 3 h, suggesting that a substantial number of DHFR origins in CHO cells are not activated in any one cell cycle and are replicated passively by forks from distant chromosomal domains. By all of these criteria, initiation is remarkably similar in the single-copy and amplified DHFR loci.

There is one rather puzzling difference between the replication patterns that we observed in the single-copy and amplified DHFR loci. In CHOC 400 cells, replication forks are observed to move only outward through the DHFR gene in the early S period by neutral/alkaline 2-D gel analysis but in both directions at later times in S when forks originating from an adjacent amplicon have had time to reach the DHFR gene in inactive amplicons (12, 14, 48). However, when we studied the single-copy locus in CHO cells, we were unable to detect replication forks moving from 5′ to 3′ through the DHFR gene 210 min after removal of mimosine, when initiation in the DHFR locus had basically ceased but some copies of the locus were still being replicated passively by forks from neighboring

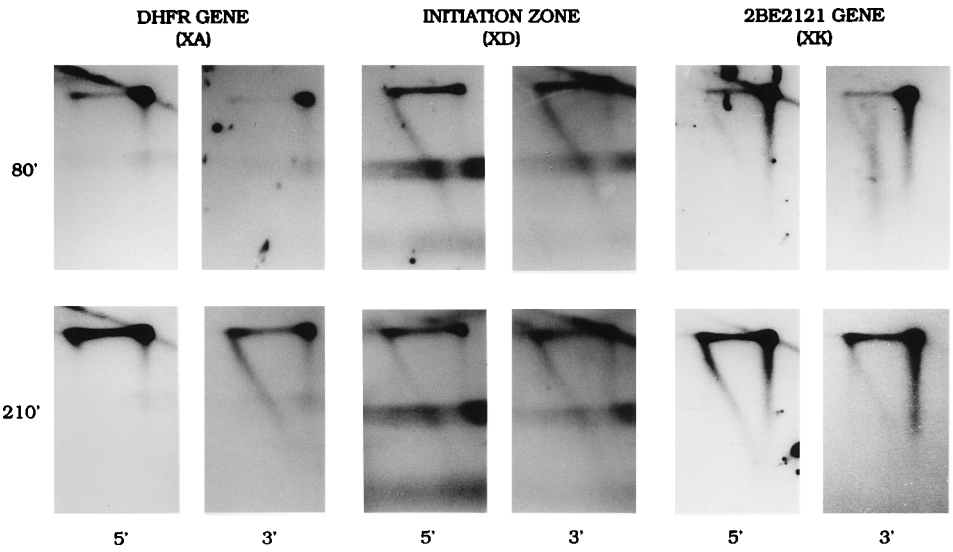

FIG. 7. Neutral/alkaline 2-D gel analysis suggests dispersed initiation sites in the intergenic zone but unidirectional fork movement through the DHFR gene. Half of the *Xba*I digest that was analyzed in the experiment in Fig. 5 was subjected to neutral/alkaline 2-D gel analysis and was hybridized with end probes specific for the 5′ and 3′ ends of the indicated fragments (Fig. 1). Probes for each fragment were as follows: XA, 100 followed by 103; XD, 19 followed by 8; XK, 205 followed by 207. Autoradiographic exposures for the six probings were 7, 9, 11, 14, 17, and 11 days, respectively.

loci (Fig. 4 and 7). In addition, analysis of an asynchronous population, in which cells at all positions in the S period are present, suggested that replication forks do not travel through the DHFR gene in the 5′-to-3′ direction (Fig. 4B).

There are probably many potential explanations for this apparent unidirectional fork movement through the gene in passively replicated copies of the locus. We can think of two, neither of which is entirely satisfactory. One possibility is the presence of a replication fork barrier or pause site upstream from the DHFR gene. Such a barrier has been discovered in the rRNA locus in *Saccharomyces cerevisiae* (4, 31), and evidence has been presented for pause sites at the junction between the nontranscribed spacer and the 3′ end of some copies of human rRNA genes (33). Indeed, there is some evidence for a replication terminus lying 5 to 10 kb upstream from the DHFR transcription start site in CHO cells (20), while the presence of a terminus has not been detected in the DHFR amplicons in CHOC 400 cells (12, 14, 48). However, at the same time in the S period that forks were observed to move only outward through the gene in CHO cells (e.g., 210 min [Fig. 7]), significant numbers of forks were still moving 5′ to 3′ through fragment XD. These forks had to have initiated somewhere upstream from fragment XD, but replication bubbles could not be detected in neutral/neutral 2-D gels either upstream in the initiation zone (fragments XU and XL) or in the 3′ end of the DHFR gene (XA) in the very same DNA preparation (Fig. 5). Although longer exposures of the neutral/neutral 2-D gels in Fig. 5 (were they possible) might reveal a low level of replication bubbles in fragments from the initiation zone in the 210-min sample, the signals from the single-fork arcs in fragment XD are almost comparable to those in the 80-min sample, in which bubbles are clearly visible. Thus, it is difficult to understand the origin of forks moving 5′ to 3′ through fragment XD if they did not originate somewhere upstream from the DHFR gene.

Another possible explanation is that in CHO cells, in which both copies of the DHFR gene are likely to be transcribed (46), forks may move more rapidly through the gene in the direction of transcription, so that they are greatly underrepresented in the steady-state population of intermediates that is examined on 2-D gels. In the 2BE2121 gene, which is far less active transcriptionally (18, 28a), there may not be enough transcripts at any given time to alter the rate of fork travel. If it were further supposed that only a minority of the amplified DHFR genes in CHOC 400 cells are actively transcribed (perhaps owing to the chromosomal rearrangements that occur during the amplification process), then forks could move at equal rates in both directions in most amplicons, as suggested by the results of neutral/alkaline 2-D gel analysis on nonsynchronized CHOC 400 cells (14, 48).

The latter possibility comes back to the issue of whether different assays afford different views of a complex initiation process. As pointed out previously (7), the 2-D gels are necessarily biased toward long-lived, steady-state replication intermediates, while the lagging-strand assay and nascent-strand size analysis are biased toward short-lived intermediates that can be labelled with a brief bromodeoxyuridine pulse. It could be that either the short-lived or the long-lived intermediates represent a futile cycle of initiation; the other species would then represent the real intermediate that eventually matures into finished replicons. We are presently attempting to address these issues experimentally, but as yet we have no evidence for either model. However, replication forks as large as 15 to 18 kb have been detected on neutral/neutral 2-D gels (19, 33), suggesting that if most of this material is destined to be recycled, the replication process is very wasteful.

There is a second important difference between the 2-D gel approaches on the one hand and the lagging-strand assay and nascent-strand size analysis on the other. The 2-D gel methods are capable of detecting low levels of either replication bubbles or replication forks moving in opposite directions. In contrast, methods that measure hybridization bias cannot distinguish nonspecific background hybridization from genuine hybridization that results from a small number of strands with opposite polarity; for example, in the lagging-strand assay, there is a relatively large amount of hybridization of the labelled Okazaki fragment preparation to the vector in the test plasmids, which presumably does not contain any mammalian sequences

(7). This relatively high background precludes detection of small numbers of initiations at any site other than a highly preferred one. Thus, these assays may be better at detecting somewhat preferred start sites (which could correspond to true genetic replicators), while the 2-D gel techniques may paint a more detailed, qualitative picture of initiation reactions.

If one accepts the possibility that initiation is distributive in the CHO DHFR locus, a model could be invoked in which mammalian replicators are very abundant and rather closely spaced. This suggestion was made earlier by Calos and coworkers, who showed that virtually any fragment of human DNA can replicate autonomously when cloned into a crippled Epstein-Barr virus-derived vector that provides a nuclear partition function (23, 27). To accommodate our data, the argument would have to further suppose that in the context of the chromosome, the frequency with which any replicator is used is dictated, in part, by the local milieu. For instance, high levels of transcription early in the S period could prevent initiation in the body of the DHFR gene and/or could cause torsional stress to accumulate in the intergenic region, rendering its replicators more accessible to the replication machinery. Interaction with initiation machinery might be further enhanced by the presence in this region of a prominent matrix attachment site (11, 40).

It is important to point out here that we did not detect significant levels of the triangular spike that characterizes termination structures in the intergenic zone in the early S period (Fig. 2, 3, and 5). Thus, it is likely that only one or a few initiations occur in any one intergenic zone in each cell cycle. If this is the case, the question arises how initiations at other potential sites are suppressed. Interestingly, it has been shown recently that when two yeast origins are positioned within a few kilobases of one another, interference occurs such that each origin fires less often than when it is more isolated from local origins (5, 15). It is conceivable that the same kind of interference operates in the CHO DHFR locus as well; however, the responsible signals would seem to have to operate over distances greater than a few kilobases to explain why there appear to be no more than one or a few initiation events in any given 55-kb intergenic zone in one S period.

We would also like to add that none of our 2-D gel data on either the CHO or the CHOC 400 DHFR locus discriminate between a relatively large number of potential genetic elements within the intergenic zone (as has been suggested previously [5]) and essentially randomly chosen initiation sites that are confined to the intergenic zone because of other factors related, e.g., to chromatin configuration, convergent transcription, or other unknown causes. However, we have recently obtained evidence that if start sites are dictated by fixed genetic elements, these elements must be situated at intervals no greater on average than ~500 to 700 bp (25a).

Even if nascent-strand start sites were shown to be chosen almost at random, our own preferred model is that ori-β and ori-γ do, in fact, correspond to true genetic replicators. Once one or both of these sites are activated by interaction with an initiator protein, it may be that a large region surrounding each replicator could be destabilized and that sites at almost any position within the destabilized zone could then serve as a template for the initiation of nascent strands by the replication machinery (2). Alternatively, once the ori-β and ori-γ loci are melted by the initiator protein, they could serve as loading sites for the replication machinery, which could then translate along the template to initiate new chains at any of a large number of sites. In both models, the regions near the replicators would be expected to be somewhat preferred.

Finally, the data from both 2-D gel approaches suggest that only a fraction of DHFR origins fire in any given cell cycle, with the remainder being replicated passively for at least an additional 3 h. A possible explanation is that in one DHFR allele, the origin has been inactivated for some reason. However, in a recent study on a CHO cell line that has lost one copy of the DHFR gene by deletion (UA41) (46), it was quite clear that the origin in the remaining locus was also used inefficiently (28b). Several autonomously replicating sequence elements in *S. cerevisiae* have also been shown to be relatively inefficient, while others are used virtually 100% of the time (16, 39). The factors that contribute to and modulate origin activity in mammalian cells are still unknown, but presumably, as in yeast cells (see, e.g., reference 17), chromosomal context plays a role.

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