Replication initiation sites are distributed widely in the amplified CHO dihydrofolate reductase domain

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ABSTRACT

In previous studies, we utilized a neutral/neutral two-dimensional (2-D) gel replicon mapping method to analyze the pattern of DNA synthesis in the amplified dihydrofolate reductase (DHFR) domain of CHO 400 cells. Replication forks appeared to initiate at any of a large number of sites scattered throughout the 55 kb region lying between the DHFR and 2BE2121 genes, and subsequently to move outward through the two genes. In the present study, we have analyzed this locus in detail by a complementary, neutral/alkaline 2-D gel technique that determines the direction in which replication forks move through a region of interest. In the early S period, forks are observed to travel in both directions through the intergenic region, but only outward through the DHFR gene. Surprisingly, however, replication forks also move in both directions through the 2BE2121 gene. Furthermore, in early S phase, small numbers of replication bubbles can be detected in the 2BE2121 gene on neutral/neutral 2-D gels. In contrast, replication bubbles have never been detected in the DHFR gene. Thus, replication initiates not only in the intergenic region, but also at a lower frequency in the 2BE2121 gene. We further show that only a small fraction of DHFR amplicons sustains an active initiation event, with the rest being replicated passively by forks from distant amplicons. These findings are discussed in light of other experimental approaches that suggest the presence of a much more narrowly circumscribed initiation zone within the intergenic region.

INTRODUCTION

Thirty years ago, Jacob and Brenner proposed a model for the regulation of replication of simple genomes (1). In this model, a DNA binding protein (initiator) interacts with a specific sequence (replicator) to effect separation of the double-stranded DNA helix; nascent DNA strands then initiate at sites very near the replicator itself. This general model has since been shown to apply not only to bacterial and viral replicons (reviewed in

2), but also to the more complex genomes of lower eukaryotes such as *S.cerevisiae* (reviewed in 3) and *P.polycephalum* (4).

In the chromosomes of higher eukaryotes, the situation is less clear. The dihydrofolate reductase (DHFR) domain in Chinese hamster ovary cells, which has been scrutinized by more techniques than any other mammalian locus, is a case in point.

Initial studies took advantage of the methotrexate-resistant cell line, CHO 400, which has amplified one allele of the DHFR locus ~1,000 times (5). The major repeating unit (amplicon) in this cell line is 240 kb in length (6). Results of intrinsic labeling studies on synchronized CHO 400 cells were consistent with the presence of one (7,8), or possibly two (9,10), fixed initiation sites lying in the intergenic region between the DHFR and 2BE2121 genes [termed ori- β and ori- γ (10); Fig. 1]. The presence of two preferred start sites in the unamplified DHFR loci in CHO cells was also suggested by an assay that determines the approximate positions at which leading strands of replication switch from one template to the other (11).

In another study in which both CHO 400 and CHO cells were examined by a lagging strand assay (12), the majority of Okazaki fragments appeared to switch template strands within a 500 bp region located near the center of the upstream ori- β locus. In addition, a PCR-based analysis of the size distribution of nascent strands in the single copy DHFR locus suggested that a 2 kb region centered over ori- β contains a major start site (13). Unfortunately, neither of the latter two studies examined the downstream ori- β region, but both studies argue for the existence of at least one relatively circumscribed initiation zone in the neighborhood of ori- β . Thus, ori- β has been termed an *origin of bidirectional replication* (OBR; 12).

However, the situation became much less clear when the amplified DHFR domain in CHO 400 cells was analyzed by a two-dimensional (2-D) gel electrophoretic method that distinguishes between fragments containing replication bubbles (i.e. internal initiation sites) and those replicated passively by forks from outside origins (14). The principle of this 'neutral/neutral' 2-D gel technique is outlined in Fig. 2.

Surprisingly, by this criterion, initiation appears to occur at any of a large number of sites scattered throughout the 55 kb early-labeling, intergenic region (although the frequency is

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considerably higher in the central portion that encompasses the ori- β /OBR, MAR and ori- γ loci; 15,16). Moreover, although initiations are no longer detected in the intergenic region after the first 2.5 h of the S period, this region continues to display single replication forks for at least a further 3.5 h (16) and probably longer (7). Since it should take only ~ 50 min for two diverging forks moving at ~ 2.5 kb/min (10) to replicate a single 240 kb amplicon from its internal initiation site, this finding suggests that the majority of amplicons do not sustain initiation events and are replicated passively by forks emanating from active origins in distant amplicons. It follows that no template switch would occur in the ori- β locus in these amplicons. It is therefore surprising that a pronounced switch in lagging strand template bias was also detected when Okazaki fragments were isolated from asynchronous CHOC 400 cultures (12).

In an effort to understand this apparent dichotomy, we have carried out a detailed analysis of the DHFR locus in CHOC 400 cells by an independent, neutral/alkaline 2-D gel assay (17) that measures the direction of replication fork movement through a region of interest. If initiation in the CHOC 400 DHFR locus is, indeed, delocalized, as suggested by the neutral/neutral 2-D gel data, then there are testable predictions for the directions of fork movement at different positions within this locus. For example, if initiations occur at different positions within the intergenic region in different copies of the amplicon, then forks should be observed to move in both directions at any position within this region in the early S period. However, forks would be expected to travel only outward from the initiation zone at all other locations in the amplicon prior to the time when they could cross into adjacent repeating units. This assumes that there is only one major initiation zone per amplicon, which is the case for the major amplicon types in CHOC 400 cells (7).

In the present study, we show that the expected patterns of fork movement are, indeed, observed in the upstream DHFR gene and in the intergenic region, as assessed by the neutral/alkaline 2-D gel technique. Surprisingly, however, dual fork movement is observed in the downstream 2BE2121 gene in the early S period, and a small number of replication bubbles can be detected

in the 2BE2121 gene on neutral/neutral 2-D gels. These findings argue that initiation occurs not only in the intergenic region, but also in the 2BE2121 gene. We also show that, by the criterion of neutral/alkaline gel analysis, replication forks persist in the DHFR locus until at least the fourth hour of the S period, long after initiation in this locus has ceased. Thus, it appears that most amplicons are replicated passively by forks from active origins in distant amplicons.

MATERIALS AND METHODS

Cell culture and synchronization protocols

The methotrexate-resistant CHOC 400 cell line was developed as previously described (5) and was propagated in Minimal Essential Medium supplemented with non-essential amino acids (GIBCO/BRL) and 12.5% Bovine Serum Product (Hyclone). Cells were synchronized near the G₁/S boundary by incubation in isoleucine-free medium for 45 h, followed by exposure to 400 μ M mimosine (Aldrich) in complete medium for 14 h (16). Where appropriate, time-zero samples were taken; the remaining cultures were then released into drug-free medium, and additional samples were harvested at the indicated times thereafter.

Isolation of replication intermediates and two-dimensional gel electrophoresis

Replication intermediates were partially purified following the matrix-enrichment protocol described previously (18). Briefly, matrix-halo structures were prepared by extracting CHOC 400 nuclei with LIS detergent (19). The DNA was then digested with either *Eco*RI or *Xba*I (GIBCO/BRL) and the fraction attached to the matrix was isolated by centrifugation. This fraction was further enriched for replicating (partially single-stranded) DNA by passage over BND-cellulose (Sigma; 20) and was loaded onto a 2-D gel as described (18). For neutral/neutral gels, $0.5-1.0 \times 10^8$ cell equivalents were loaded into each well; for neutral/alkaline gel analyses, the corresponding numbers were $1.0-1.5 \times 10^8$. After separation, the DNA was transferred to Hybond N+ (Dupont/NEN) and the membranes were hybridized

Figure 1. Organization of DHFR amplicons in the CHOC 400 cell line. (A) Schematic representation of the head-to-head (star)/tail-to-tail (asterisk) arrangement of the major ($\sim 85\%$) amplicon type in CHOC 400. The DHFR gene and the co-amplified 2BE2121 gene are represented by the black and the hatched box respectively. (B) Map showing the positions of ori- β , ori- γ , and the MAR (M) within the intergenic initiation zone. Relevant *Eco*RI and *Xba*I fragments are indicated above and below the line.

with specific ^{32}P -labeled probes as described previously (18). Individual probes are described in the figure legends.

RESULTS

Organization of the amplified DHFR domain in CHO 400 cells

Fig. 1 shows the map of a 120 kb region from the DHFR locus that is common to the majority of amplicons in the CHO 400 cell line and which represents the arrangement in the unamplified DHFR locus in CHO cells. The *initiation zone*, as defined by previous *in vivo* labeling and neutral/neutral 2-D gel studies (7,15,16), corresponds to the region between the two genes. The positions of *ori-β/OBR*, *ori-γ*, and a prominent matrix attachment region (MAR; 21) are also indicated in the map in Fig. 1. Throughout this paper, the terms upstream and downstream (and 5' and 3') will be used to refer to the left and right ends of this map as drawn and not to the direction of transcription from the two genes.

Replication intermediates appear to originate in the intergenic region and to move outward into the flanking genes

When analyzed by the neutral/neutral 2-D gel technique (Fig. 2), restriction fragments harboring origins of replication should distinguish themselves from neighboring non-origin fragments in two respects: (1) they should display replication bubbles during the time of active initiation, and (2) they should be replicated prior to flanking fragments. In fact, in a previous study on synchronized CHO 400 cells (16), we found that every fragment examined from the intergenic region displayed bubble arcs in the early S period, and significant numbers of replication intermediates appeared in the two flanking genes only after a lag.

An example of such an experiment is presented in Fig. 3. CHO 400 cells were arrested in G_0 by isoleucine deprivation, the block was reversed, and cells were collected near the G_1/S boundary with the replication inhibitor mimosine (16,22,23). The mimosine block was then removed, allowing cells to enter S in a relatively synchronous wave starting ~40 min later (16,22,23). Samples were harvested at 80 and 160 min, and replication intermediates were isolated using *EcoRI* to digest the DNA. After separation on a neutral/neutral 2-D gel and subsequent transfer to a membrane, each digest was hybridized sequentially with probes for selected fragments (see map in Fig. 1). In this experiment, an attempt was made to normalize autoradiographic

exposures so that the $1n$ spots that represent linear, non-replicating fragments are approximately the same intensity when different probes are used to analyze the same DNA sample on the same transfer. This allows the relative amounts of replication intermediates to be compared at any given time point.

In the 80 min sample, two fragments from the initiation locus (RF' and RF in Fig. 3), as well as every other fragment examined within the intergenic region (data not shown), display composite patterns consisting of a complete bubble arc and a single fork arc. This is the pattern predicted for a fragment residing in a broad initiation zone, since initiations occurring within the fragment would give rise to the bubble arc, while initiations occurring elsewhere in the initiation zone would contribute to the fork arc. In contrast, probes specific for fragments in the DHFR and 2BE2121 genes (RH and RA) detect only a faint single fork arc at 80 min, suggesting that only a small number of forks have moved into the two genes at this time point (Fig. 3, upper panels).

By 160 min after removal of mimosine, all four fragments display strong single fork arcs, and only trace amounts of bubbles can now be detected in fragments RF' and RF from the initiation zone (lower panels). In previous studies, we have shown that prominent single fork arcs persist in the amplified DHFR domain until at least 6 h after release from mimosine (16).

Dual fork direction is observed in both the intergenic region and the 2BE2121 gene in the early S period

The data from the neutral/neutral 2-D gel analysis in Fig. 3 suggest that forks can originate at any of a large number of sites in the intergenic region and then move outward through the genes. This delocalized mode of initiation predicts that in the early S period, forks should move in both directions within the intergenic region, but only outward through the two genes. If initiation does not occur in every amplicon, then after sufficient time has elapsed to allow forks to cross into adjacent amplicons, forks should be observed to move in both directions at all locations within the amplicon (assuming that there are no termini to impede fork progression).

To directly test this proposal, we determined the direction of fork travel through the DHFR domain using the neutral/alkaline 2-D gel technique (18). In this method, replication intermediates are separated in the first dimension according to mass, as in the neutral/neutral analysis. However, the second dimension is run in alkali, which releases a diagonal of nascent strands and a unit-

Figure 2. Principle of the neutral/neutral 2-D gel replicon mapping technique (14). Idealized autoradiographic images obtained when a digest of replicating DNA is hybridized with probes for fragments that contain different intermediates. (A) A complete single fork arc (b) from a fragment replicated passively from an outside origin. Curve a represents the diagonal of non-replicating fragments from the genome as a whole. (B) A fragment with a centered origin of replication (curve c). (C) A fragment containing an off-centered origin.

sized template band (Fig. 4A). By hybridizing a transfer of the gel sequentially with probes from the two ends of a fragment, the direction of fork movement through the fragment can be ascertained, since the origin-proximal probe detects nascent strands of all sizes, while the distal probe detects only the largest ones (Fig. 4B). Thus, the neutral/alkaline 2-D gel method depends on nascent strand size, while the neutral/neutral method assesses differences in the shapes of replication intermediates.

In the experiment presented in Fig. 5, CHO 400 cells were synchronized near the G₁/S border with mimosine; samples were then harvested prior to drug removal (time 0), and 80 and 240 min later. After digestion with *Xba*I, replication intermediates were purified and separated on neutral/alkaline gels. Transfers of each digest were then hybridized sequentially with probes specific for the two ends of fragments from the DHFR gene (XA), the initiation zone (XD), and 2BE2121 (XK and XM) (see map, Fig. 1). Note that an *Xba*I digest was chosen (as opposed to *Eco*RI) because the end-labels for a larger percentage of fragments are free of repetitive sequence elements.

As shown in Fig. 5, virtually no replication intermediates can be detected in any of the fragments prior to drug removal (time zero), in agreement with earlier observations suggesting that mimosine is an extremely effective inhibitor of DNA replication (16,22,23). However, by 80 min, *both* end-probes for a fragment near the center of the initiation zone (XD) detect strong, complete diagonals of roughly equal intensity (Fig. 5B; note that for the 80 min sample, the autoradiogram of the 5' probe has inadvertently been somewhat overexposed). Thus, at a time in early S when replication is largely confined to the intergenic region based on neutral/neutral gel analysis (Fig. 3), forks are entering this fragment from both directions. As shown in Fig. 4C, this result is predicted for a fragment situated in a broad initiation zone. Furthermore, dual fork direction is consistent with the composite neutral/neutral gel patterns (15,16; Fig. 3) in which bubbles would result from internal initiations, while the single fork arcs would originate from initiation sites elsewhere in the intergenic region.

Figure 3. Replication initiates in the intergenic region in the early S period. CHO 400 cells were synchronized near the G₁/S boundary with mimosine, and samples were harvested 80 min and 160 min after release from mimosine. *Eco*RI digests of replication intermediates were separated on neutral/neutral 2-D gels, transferred to a membrane, and hybridized sequentially with probes specific for *Eco*RI fragments RH, RF', and RA. An attempt was made to approximately normalize all film exposures to the 1n spot of non-replicating fragments.

In the 80 min sample, fewer replication intermediates were detected with end-probes for the fragment from the DHFR gene (XA), as expected from the neutral/neutral 2-D gel data. However, it is clear that at this early time point, replication forks are entering this fragment only from the downstream direction. This result is consistent with the neutral/neutral gel data suggesting that initiation is largely confined to the intergenic region at this time point, but a small number of forks have moved outward into the two genes (Fig. 3).

Surprisingly, however, when fragment XK from the 2BE2121 gene was analyzed in the 80 min sample, *both* end-probes detected faint but complete diagonals (Fig. 5C). The same result was obtained with end-probes for the downstream fragment, XM, which overlaps the promoter of the 2BE2121 gene (Fig. 5D). Thus, forks are entering these two fragments from both upstream and downstream locations at a time in S when the majority of replication intermediates are confined to the initiation locus, based on the neutral/neutral 2-D gel data (Fig. 3).

In the 240 min sample, both end-probes for all four fragments (XA, XD, XK, and XM) detect strong and complete diagonals (Fig. 5, lower panels). This data is consistent with the presence of prominent single fork arcs in all three regions in neutral/neutral gels after ~160 min (Fig. 3; 16), and argues that, by this time, replication forks that originated in the initiation zones in active amplicons have crossed over into adjacent amplicons.

Analysis of the DHFR gene (fragment XA) and the initiation zone (fragment XD) in *asynchronous* CHO 400 cells clearly shows that these two regions are replicated by forks moving in both directions (Fig. 6A). Furthermore, the number of forks entering the gene from upstream and downstream locations appears to be approximately equal, which would be predicted if the majority of amplicons are replicated passively. This finding argues that dual fork movement is not the result of synchronization *per se*. In addition, the fact that most of the

Figure 4. Principle of the neutral/alkaline 2-D gel technique (17). (A) Idealized patterns of migration of double-stranded replication intermediates in the first (neutral) dimension and single-stranded nascent chains in the second (alkaline) dimension. (B) Three adjacent restriction fragments are demarcated by the vertical lines. An origin of replication is positioned at the hatchmark in the central fragment, and nascent strands of different sizes are indicated. The panels below show the autoradiograms that would be obtained when each of the indicated probes (hatched boxes) is used to illuminate its cognate restriction fragment. (C) As (B), showing three adjacent initiation sites.

amplicons are replicated passively explains why the bubble:fork arc ratio in fragments from the initiation zone is so low in asynchronous cell populations (e.g. compare fragments RF' in Fig. 3 and XL in Fig. 6B; the latter is almost entirely contained in RF'): bubbles would arise only from those amplicons that sustain initiation events, while the fork arc would derive from initiations that occur in the same amplicon as well as in distant amplicons.

Although the intergenic region is preferred, initiations also occur elsewhere in the amplified DHFR domain

The data from the neutral/alkaline 2-D gel analysis of the amplified DHFR domain show that a small number of replication forks move through the 2BE2121 gene from right to left in the early S period. To determine the origin of these replication forks, we reanalyzed this downstream region for the presence of replication bubbles by neutral/neutral 2-D gel analysis (Fig. 7).

Fragment RF' again displays a strong bubble arc and single fork arc, the characteristic pattern for fragments from the initiation zone in the early S period (Fig. 7). However, faint bubble arcs can now also be detected in fragments RB and RJ from the 2BE2121 gene. Note that a larger variant of fragment RJ from another amplicon arrangement also displays both a single fork arc and a bubble arc. However, due to the size of the variant fragment, the lower parts of fork and bubble arcs have not been approximately the same intensity as those in fragment RF' from the initiation zone (compare the 1n spots in the four panels in Fig. 7).

Fragment RF' again displays a strong bubble arc and single fork arc, the characteristic pattern for fragments from the initiation zone in the early S period (Fig. 7). However, faint bubble arcs can now also be detected in fragments RB and RJ from the 2BE2121 gene. Note that a larger variant of fragment RJ from another amplicon arrangement also displays both a single fork arc and a bubble arc. However, due to the size of the variant fragment, the lower parts of fork and bubble arcs have not been

resolved. Interestingly, the variant appears to represent a substantial fraction of the early replicating amplicons in this film exposure, though analysis of non-replicating genomic DNA on Southern blots showed that it represents fewer than 10% of the total (P.A.Dijkwel, unpublished observations). Apparently, this variant, on average, replicates earlier in S than does the predominant amplicon type. In agreement with earlier observations (15,16), no trace of a bubble arc is detectable in the DHFR gene at any film exposure. In samples taken 180 min after release from mimosine, only single fork arcs can be detected in all four fragments (data not shown), in agreement with the experiment in Fig. 3.

Thus, both of the complementary 2-D gel methods suggest that replication initiates preferentially in the intergenic region, although a low level of initiation also occurs within the body of the 2BE2121 gene itself.

DISCUSSION

Until a few years ago, it seemed that it might be possible to identify the approximate location of a replicator in the DHFR domain by localizing the positions of nascent strand start sites. Beginning with intrinsic labeling studies suggesting that replication initiates somewhere within a 28 kb region downstream from the DHFR gene (7), and culminating with the potentially high resolution lagging strand assay (12), it appeared that there was at least one highly preferred initiation locus (termed ori- β or OBR) situated about 15 kb to the right of the 3' end of the DHFR gene.

However, 2-D gel studies on this locus, as described herein and previously (15,16), have painted a somewhat different picture. Instead of narrowing down the position of the preferred initiation locus to within a few hundred base pairs, which both techniques are capable of doing when applied to genetic replicators in yeast (e.g. 14,17), these methods have provided compelling evidence that replication initiates throughout the entire

Figure 5. In the early S period, replication forks move in both directions in the intergenic region and in the 2BE2121 gene, but only outward through the DHFR gene. CHO 400 cells were released from a mimosine block and samples were harvested at the indicated times. Replication intermediates were digested with *Xba*I, separated on neutral/alkaline gels, and hybridized sequentially with end-probes for the indicated fragments (see map, Fig. 1).

55 kb intergenic region and, less frequently, in the body of the 2BE2121 gene itself.

The detection of dual fork direction in the 2BE2121 gene by neutral/alkaline analysis provided the first clue that initiation might be occurring even outside of the previously defined initiation zone (Fig. 5). In fact, early in the S period, a small but significant number of replication bubbles can be detected at two different locations in the 2BE2121 gene, including a fragment that straddles the promoter (Fig. 7) (we have not yet been able to isolate useful probes to determine how far downstream this initiation zone extends). Thus, the 2BE2121 gene contrasts sharply with the DHFR gene, which appears to be replicated exclusively by forks moving outward from the initiation locus in the early S period (Fig. 5), and in which bubble arcs have never been detected at any film exposure (Fig. 7; 15,16, and unpublished observations).

The suggestion that initiation might be a relatively delocalized event in mammalian replicons is, admittedly, a rather heterodox one. However, 2-D gel analyses of several different replicons in a variety of eukaryotic organisms show that nascent strand start sites need not be confined to a small, circumscribed locus; rather, initiations are detected in zones that range anywhere from 1 kb (24) to 40 kb in length (25,26). These replicons include: the chorion, histone, and chromosome II regions in *Drosophila* (25–28), the II/9A locus in *Sciara coprophila* (24), and the rDNA locus in man (29).

Furthermore, Harland and Laskey (30) showed that *Xenopus laevis* oocytes initiate semi-conservative replication on virtually any injected template and possibly at random positions (although see 31). Interestingly, however, replication is limited to one round per cell cycle, indicating that fixed initiation sites are not a prerequisite for all levels of control.

It has also been shown that virtually any mammalian genomic DNA fragment can replicate autonomously in a mammalian cell

background when cloned into a vector containing a nuclear retention signal from the Epstein–Barr viral genome (32). However, replication efficiency varies with the size of the inserted fragment (33), suggesting either that the density of replicators in mammalian genomes is very high, or that sequence requirements for origin function in this assay are minimal, but larger fragments are more efficiently retained.

Taken together, these observations suggest that, potentially, initiations can occur either at randomly selected sites or at a large number of genetically defined replicators positioned at frequent intervals throughout the genome. According to this model, certain chromosomal features must then favor or suppress the use of some initiation sites (or replicators) over others. Those features could include the presence of active transcription units, matrix attachment sites, developmentally mandated chromatin structure, etc.

For example, in the DHFR domain, the region between the DHFR and 2BE2121 genes could accumulate torsional stress as a consequence of convergent transcription; this could lead to increased accessibility of the chromatin to initiator proteins, perhaps because of altered interactions with nucleosomes. The MAR lying near the center of the initiation zone (Fig. 1) could lock in this stress; alternatively, it could bring the intergenic region into the matrix subcompartment that has been proposed to contain the replication machinery (34). Presumably, the DHFR gene itself would be inaccessible due to high levels of transcription in late G₁ and early S (35; T.-H.Leu, P.A.Dijkwel, and J.L.Hamlin, manuscript in preparation). The 2BE2121 gene, which appears to be far less active transcriptionally (36; T.-H.Leu, unpublished observations), could still support an occasional initiation event, accounting for the low level of replication bubbles that can be detected in the early S period after very long film exposures.

Presumably, other regions of the DHFR domain that have not yet been analyzed could also support a low, baseline level of initiation, and we are developing probes for these regions to test this hypothesis. In fact, most of the restriction fragments in the DHFR amplicon are labeled to some extent when CHO 400 cells are pulsed with [³H]thymidine in the early S period, even though the fragments defining the intergenic region are much more heavily labeled (7,10). In the past, we have assumed that this background represents a small number of cells that have escaped the synchronizing regimen and are located at many different positions in the S period; however, it is equally possible that the cells are, indeed, synchronized, but that initiations occur at low levels throughout most of the remainder of the amplicon.

Figure 6. Replication forks move through the DHFR gene and the initiation locus in both directions in unsynchronized cells. Replication intermediates were prepared from unsynchronized CHO 400 cells, using *Xba*I to digest the nuclear matrix preparations. (A) After separation on a neutral/alkaline 2-D gel, the digest was transferred to a membrane and hybridized sequentially with end-probes for fragments XA and XD. (B) After separation on a neutral/neutral 2-D gel, the digest was hybridized sequentially with probes for fragment XA in the gene and fragment XL in the initiation zone.

Figure 7. A low level of initiation can be detected in the 2BE2121 gene in the early S period. The transfer used in Fig. 3 was hybridized sequentially with the indicated probes, in each case using ~5 times more radioactive probe than in Fig. 3. The exposure times for the RH, RF', RB, and RJ probes were 8 days, 17 h, 6 days, and 11 days, respectively.

This general model is not precluded by recent observations suggesting the presence of a single genetic replicator near the promoter of a β -like globin gene in humans. An initiation locus had been roughly localized to this region and it has been shown that the 8 kb deletion encompassing this region in Hemoglobin Lepore patients results in the loss of origin activity (37). However, it may be that the replication of developmentally regulated genes such as globin is, indeed, regulated by single genetic replicators, while housekeeping genes such as DHFR may have more relaxed regulatory mechanisms characterized by multiple potential origins. Alternatively, there may be only one or two genetic replicators in the DHFR initiation zone corresponding to ori- β and ori- γ , but once initiation proteins bind these origins, the replication machinery can initiate daughter strands at many sites both near to and far from the replicator itself.

Regardless of the mechanism that operates to control replication of the DHFR locus, it is still necessary to reconcile the 2-D gel data on the DHFR locus with previous studies suggesting the presence of one, and possibly two, highly preferred initiation sites within this locus (7–13). Although it is not possible to accurately determine the relative numbers of replication bubbles in different fragments when analyzed on neutral/neutral 2-D gels (38), initiation rates do appear to be highest in the central 30 kb of the intergenic region (16). Importantly, this region encompasses the early-labeled fragments (including ori- β /OBR and ori- γ) detected in previous *in vivo* labeling studies (7–10). Given their semi-quantitative nature, the 2-D gel data can also accommodate the results of the leading strand assay in which template switches were approximately localized to the ori- β and ori- γ loci with a resolution of $\sim \pm 8$ kb (11). Furthermore, both 2-D gel techniques detect replication forks in the DHFR locus long after initiation has ceased; this result explains earlier observations that amplified restriction fragments can still be labeled with [3 H]thymidine as late as 8 h into the S period (7), and suggests that only 10–20% of amplicons actually sustain an initiation event in any one cell cycle. Thus, the 2-D gel results can be reconciled with much of the earlier work on this locus.

Is it possible to reconcile the 2-D gel data with the results of the lagging strand template bias assay (12) or with nascent strand size analysis (13), which suggest that most initiations are confined to a zone less than 500 bp and 2 kb in length, respectively? As with the leading strand assay, the lagging strand assay measures a hybridization *bias*, but significant hybridization usually occurs to both strands (11,12). Thus, instead of representing an unavoidable background, hybridization to the non-preferred template could represent genuine nascent strands with opposite polarity. The PCR-based nascent strand size analysis, on the other hand, is purposely designed to detect the locations of the most abundant small nascent strands, and would again give a 'background' of small DNA fragments from less preferred sites scattered at other locations.

It is also important to point out that 2-D gel methods analyze steady-state levels of replication intermediates, and would consequently favor detection of structures that turn over slowly; in contrast, the lagging strand assay and the nascent strand size analysis each depend on short-term labeling protocols that would favor molecules with short half-lives. This important difference could lead to over- or underestimates of the number of initiation events that occur in any given genomic region. For instance, it has been suggested that the bubble arcs detected in the DHFR domain by the neutral/neutral 2-D gel technique might represent futile cycles of initiation, the products of which are relatively

long-lived (12,39). In contrast, initiations occurring at the sites implicated in the lagging strand assay as well as by nascent strand size analysis might represent *bona fide* start sites; forks emanating from these sites would presumably travel too fast to be detected by physical mapping on 2-D gels. However, it is equally possible that the structures detected on 2-D gels actually give rise to full-length daughter replicons, while those that are preferentially labeled (i.e. ori- β /OBR and ori- γ) give artificially high values owing to differing thymidine contents or other technical reasons.

There is one aspect of the 2-D gel studies that we find difficult to reconcile with results of the lagging strand assay by any model. Both the neutral/neutral and the neutral/alkaline 2-D gel techniques argue strongly that replication initiates in the intergenic region only during the first 2.5–3 h of the S period. Yet replication forks persist in the DHFR amplicons for at least 6 h (Fig. 5; 16), and probably longer (7). Furthermore, replication forks are observed to move in both directions in the DHFR gene and in the intergenic region (and presumably everywhere else) in unsynchronized cells (Fig. 6). From these observations, one is led to the conclusion that most of the amplicons (probably 80–90%) never sustain an active initiation event, but are replicated by forks from active amplicons. Since there would be no switch in template bias at the proposed OBR in passively replicated amplicons, a very small bias should be observed in the asynchronous population as a whole, which contains cells in all stages of the S period. However, a dramatic switch in template bias was observed at the OBR in both synchronized *and* unsynchronized cells (12).

One final observation deserves comment. In the neutral/neutral gel experiment pictured in Fig. 3, there are very few single forks in the 3' end of the DHFR gene (fragment RH) in the 80 min time sample, even though there are already large numbers of both bubbles and forks in fragments RF' and RF from the initiation zone at this time. In fact, we have shown previously that the composite pattern of bubble and single fork arcs can be detected as early as 40 min after mimosine removal. Therefore, why have most of these bubbles not matured into the body of the DHFR gene by 80 min? Although we do not have the complete answer to this question, preliminary evidence suggests that a region near the 3' end of the DHFR gene may slow forks or prevent them from moving in the 3' $\rightarrow$ 5' direction in very early S (P.A.Dijkwel, unpublished observations). Perhaps this proposed traffic signal prevents replication through the DHFR gene at a time when maximum transcription rates are required, at the end of G₁ and the beginning of the S period (35; T.-H.Leu, P.A.Dijkwel, and J.L.Hamlin, manuscript in preparation). Indeed, a replication fork barrier has been detected by 2-D gel techniques at the 3' end of rRNA genes in *S.cerevisiae* (40,41).

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