Sequence and Context Effects on Origin Function in Mammalian Cells

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Abstract   Jacob and Brenner proposed a model for control of DNA replication in which a *trans*-acting initiator protein binds to a *cis*-acting replicator to effect initiation of nascent DNA chains at a fixed locus. Although replicators have been identified in prokaryotic and simple eukaryotic genomes, it has been much more difficult to demonstrate their presence in mammalian chromosomes. Owing to the lack of genetic approaches for identifying mammalian replicators, investigators have directed attention to localizing nascent strand start sites, which should lie close to replicators. Toward this end, a variety of clever techniques have been invented for analyzing replication intermediates, but only rarely have more than one of these techniques been applied to a single locus. However, virtually all have been used to analyze the dihydrofolate reductase locus in CHO cells. The picture that has developed in this locus is that initiation can occur at any of a large number of sites scattered throughout a broad zone, but somewhat more frequently near two sites that may correspond to true genetic replicators. Furthermore, it appears that local transcriptional activity, as well as appropriate torsional stress (as imparted by local attachment to the nuclear matrix), may have profound effects on origin activity.   © 1996 Wiley-Liss, Inc.

Since its inception more than 30 years ago, the *replicon model* [Jacob and Brenner, 1963] has provided the conceptual framework for studies on control of DNA replication. The model proposes that a site-specific DNA binding protein (*initiator*) interacts with a defined genetic element (*replicator*) in the template DNA to facilitate melting of the double helix and access by the replication machinery. In many bacterial and viral systems, both the initiator and the replicator have been identified. This, in turn, has led to reconstitution of the initiation reaction in vitro in many of these systems [e.g., Li and Kelly, 1984; Kaguni and Kornberg, 1984].

The replicon model has also been shown to translate very well to simple eukaryotes such as *Saccharomyces cerevisiae* and *Schizomyces pombe*. Candidate replicators were first identified as autonomously replicating sequence (ARS) elements [Stinchcomb et al., 1980; Chan and Tye, 1980] and, using two-dimensional gel replicon mapping techniques, it has been possible to show that a large subset of these ARS elements serve as true replicators when resident in their native chromosomal positions [Newlon and Theis, 1993]. More recently, an ARS-binding protein complex (the origin recognition complex or ORC) has been isolated, which is highly likely to represent or be part of the *initiator* for *S. cerevisiae* [Bell et al., 1993].

By comparison to prokaryotes and simple eukaryotes, identification of the key players controlling initiation in metazoans has lagged far behind. This can be attributed to the sheer complexity of mammalian genomes and, probably more importantly, to the absence of a reliable functional assay for defining candidate replicators (i.e., ARS elements). Without cloned replicators, it has not been possible to search for cognate initiator proteins that would recognize and bind to them. Consequently, the search for potential replicators has involved localization of the regions where nascent strands initiate in a chromosomal domain of interest, with the assumption that *cis*-regulatory sequences will lie close by.

However, different assays have painted conflicting views of the nature of initiation reactions in metazoans and, by in large, these assays have not detected highly preferred nascent strand start sites analogous to those that characterize replication origins in simpler chromosomes. Thus, it seems that only genetic ap-

proaches will answer unequivocally whether metazoan cells contain bona fide replicators. These experiments will have to take advantage of naturally occurring initiation mutants or will require in loco mutagenesis of selected chromosomal sequences with recently developed techniques for selecting homologous recombinants.

Surprisingly, recent data suggest that cis-regulatory sequences contributing to origin function comprise elements that are both proximal to and quite distant from nascent strand start sites. Thus, it appears that signals can be propagated over long distances in mammalian chromosomes. Furthermore, it seems that chromatin organization plays a key (and generally underestimated) role in the control of DNA replication. Once again, metazoans have elaborated on basic mechanisms that characterize simpler organisms in a way that probably reflects the special problems inherited by differentiated cells.

In this contribution, we will review the field of mammalian replication control, with emphasis on the Chinese hamster dihydrofolate reductase locus, which has been studied by more techniques than any other. We will then attempt to integrate the data into a unified model in which nuclear organization plays a vital role in proper origin function.

IDENTIFICATION OF INITIATION SITES

The replicon model predicts that a replicator should be able to direct autonomous replication of any DNA sequences to which it is linked. Replicators have been isolated by virtue of this property from a variety of prokaryotes and viruses and from the simple eukaryotic yeasts [for review see Kornberg and Baker, 1992]. However, only rarely have sequences been recovered that are capable of even limited autonomous replication when introduced into mammalian cells by transfection [Frappier and Zannis-Hadjopoulos, 1987; McWhinney and Leffak, 1990; Sudo et al., 1990; Krysan et al., 1989; Heinzel et al., 1991]. Part of the problem is that even if a cloned sequence were replicating autonomously once per cell cycle, it is likely to be lost during nuclear breakdown in mitosis because of the lack of a centromere.

In fact, the most convincing data for autonomous replication of mammalian DNA comes from experiments in which a nuclear retention signal from Epstein-Barr virus was included in the cloning vehicle [Krysan et al., 1989; Heinzel et al., 1991]. When random fragments from the human genome were inserted into this vector and introduced by transfection into mammalian cultured cells, virtually all such constructions replicated autonomously to some degree, with the largest inserts replicating best [Heinzel et al., 1991]. However, initiation was largely confined to the mammalian DNA insert and appeared to occur at any of a large number of sites throughout the insert, rather than in a narrowly circumscribed zone [Krysan and Calos, 1991]. This result suggests either that replicators occur at a very high frequency in mammalian genomes, or that initiation can occur at virtually any site without the need for a genetic replicator.

Another factor that may complicate attempts to isolate mammalian replicators in ARS assays is that, a priori, any effects of chromosomal context are not recreated in the plasmid. Even in yeast, it is clear that all cloned ARS elements do not function as replicators in their usual chromosomal contexts [Newlon and Theis, 1993; Brewer and Fangman, 1991]. Furthermore, the time in the S period that an origin fires can change depending upon its surroundings. For example, yeast replicators that normally fire late in the S period can transmutate to early firing when they are moved from the chromosome onto plasmids [Ferguson et al., 1991]; conversely, a normally early-firing origin can be caused to fire late in S by positioning it near a telomere [Ferguson et al., 1991].

Thus, attempts to identify potential replicators have relied instead on methods for localizing nascent strand start sites in a chromosomal domain of interest. As we will see, almost all of these techniques can be divided into two general categories: (1) those that label replicating DNA so that nascent strands can subsequently be isolated and somehow related back to their initiation sites; and (2) those that take advantage of the unique shape of expanding replication bubbles to determine the site of initiation.

At a fork rate of ~ 3 kb per minute [Huberman and Riggs, 1968], it should take only ~ 1 min out of a 24 h (1,500 min) cell cycle to replicate a 6 kb origin-containing restriction fragment; thus, less than $\sim 0.1\%$ of the copies of a particular restriction fragment will contain replication intermediates at any one time in an asynchronous cell population. These intermediates would be impossible to detect by Southern blotting and hybridization procedures without incorporating some prior enrichment step. One

approach has been to employ synchronizing regimens to increase the number of cells initiating replication at the time of sampling. Although the results obtained with such procedures have to be revisited at later times to verify their validity, synchronization strategies allowed the first glimpse into initiation reactions at defined loci. Enrichment has also been achieved by utilizing cell lines that contain high copy numbers of a given sequence, somewhat like multi-copy plasmids in *Escherichia coli*. Examples include: (1) the naturally occurring chromosomal rDNA locus, which is repeated several hundred times in mammalian cell genomes, and (2) amplified chromosomal domains containing genes that confer drug resistance.

The latter category is exemplified by the CHOC 400 cell line, which was developed by sequential selection over the period of a year in increasing concentrations of the antifolate, methotrexate [Milbrandt et al., 1981]. CHOC 400 cells contain 1,000 copies of one of the alleles encoding the target enzyme, dihydrofolate reductase (DHFR) [Milbrandt et al., 1981]. Largely because it was the earliest of such resistant variants to be isolated, this cell line has been subjected to virtually all of the extant methods for localizing replication initiation sites, which allows the results obtained with each method to be compared.

The majority of the amplified DHFR domains (amplicons) in CHOC 400 cells are 240 kb in length [Looney and Hamlin, 1987]. Since the average replicon in mammalian cells is ~100 kb, it seemed likely that each amplicon would contain at least one origin of replication. Conveniently, amplicon-specific restriction fragments from the CHOC 400 genome are visible as discrete bands in a digest separated on an agarose gel.

Mapping the initiation site in this amplified locus turned out to be relatively straightforward. Cells were synchronized at the G1/S boundary, released into the S period in the presence of radioactive thymidine, and a genomic restriction digest was analyzed to determine which bands were the first to incorporate label in the beginning of the S period [Heintz and Hamlin, 1982]. These initial studies showed that replication begins somewhere in a zone lying downstream from the DHFR gene. By using an in-gel renaturation method to rid single copy background sequences from non-amplified DNA, it was possible to obtain a higher resolution view of the initiation reaction; two preferred sites of initial labelling (ori-β and ori-γ) were identified by this approach [Leu and Hamlin, 1989]. These sites are located in the region between the convergently transcribed genes DHFR and 2BE2121 genes and lie ~22 kb apart (Fig. 1).

To obtain a more detailed view of the initiation reaction, physical mapping methods were applied to this locus. The neutral/neutral two-dimensional (N/N 2-D) gel replicon mapping technique takes advantage of the differences in
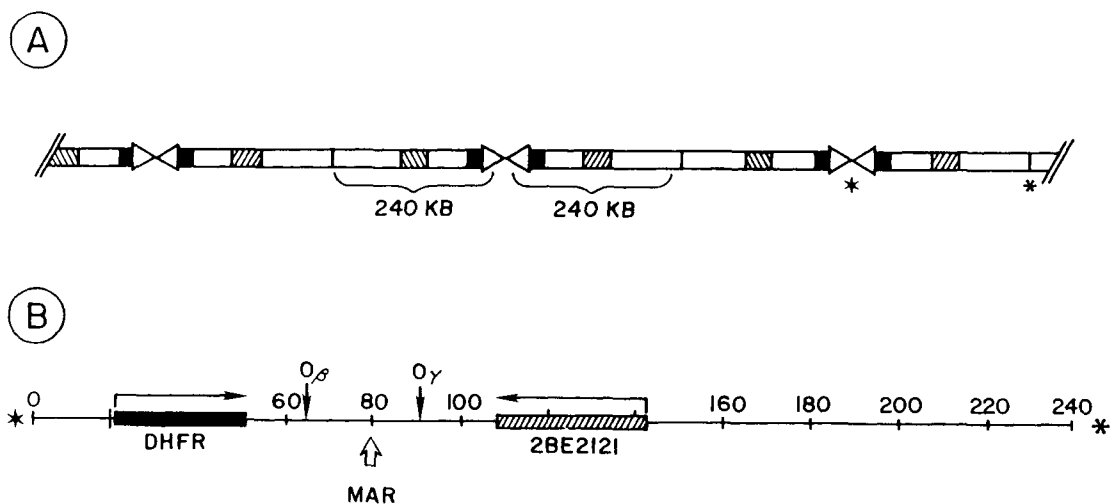

Fig. 1. Organization of DHFR amplicons in the CHOC 400 cell line. A: Schematic representation of the head-to-head (star)/tail-to-tail (asterisk) arrangement of the major amplicon type in CHOC 400 cells. The DHFR gene and the co-amplified 2BE2121 gene are represented by the black and the hatched boxes, respectively. B: Map showing the positions of ori-β, ori-γ, and the MAR (M) within the intergenic *initiation zone*.

the electrophoretic migration of restriction fragments containing either single-forked or bubble-like structures to characterize replication intermediates in a genomic region of interest (Fig. 2) [Brewer and Fangman, 1987]. Genomic DNA from proliferating cells is digested with a restriction enzyme and one or more steps are usually used to enrich for replication intermediates. The digest is then separated in a first dimension agarose gel under neutral conditions primarily according to molecular mass, which will vary from $1n$ to just less than $2n$ (where n is the size of the full-length non-replicating fragment). The lane is excised, turned through 90°, and run in a second, neutral gel under conditions that maximize the contribution of shape to migration rate. The digest is then immobilized on a filter and hybridized successively with radioactive probes for the regions of interest. The pattern traced by a fragment that is replicated passively by a single fork entering from a neighboring fragment (Fig. 2A) is distinct from the patterns of fragments that are replicated either from a centered (Fig. 2B) or an off-centered (Fig. 2C) start site.

Unexpectedly, when the amplified DHFR domain was analyzed by this technique in DNA isolated from early S phase cells, every fragment in the 55 kb intergenic region displayed a composite pattern consisting of both an intense single fork arc and a less prominent bubble arc [Vaughn et al., 1990]. In contrast, fragments from outside the intergenic region displayed only single fork arcs, as would be expected if they flank a bidirectional origin from which forks proceed

outward bidirectionally. By the end of the second hour of the S period, the bubble arcs disappeared from the intergenic region, confirming that the DHFR origin is, indeed, early-firing [Dijkwel and Hamlin, 1992]. However, single fork arcs persisted until at least the sixth hour of the S period, long after initiation was detectable in the intergenic region.

This unusual pattern is consistent with a model in which initiation can occur at any of a large number of potential initiation sites in each copy of the amplicon (Fig. 3). The result of this is that any given fragment contributes to the bubble arc when initiation occurs within that fragment, but contributes to the fork arc when initiation occurs elsewhere in the intergenic zone.

Two additional conclusions can be drawn from N/N 2-D analysis of the DHFR domain. The absence of appreciable numbers of termination structures in the intergenic region indicates that rarely does any one amplicon sustain more than one or a few initiation events. Possibly, multiple starts are suppressed by the same mechanism that restricts initiations on plasmids carrying multiple yeast ARS elements to only one [Brewer and Fangman, 1993].

A second important observation is that most of the amplicons in CHOC 400 cells do not appear to support active initiation events, with the consequence that they are replicated passively by forks emanating from active origins in adjacent amplicons (Fig. 3). Since single forks persist in the intergenic region for 3 additional hours after the last initiations are detected in
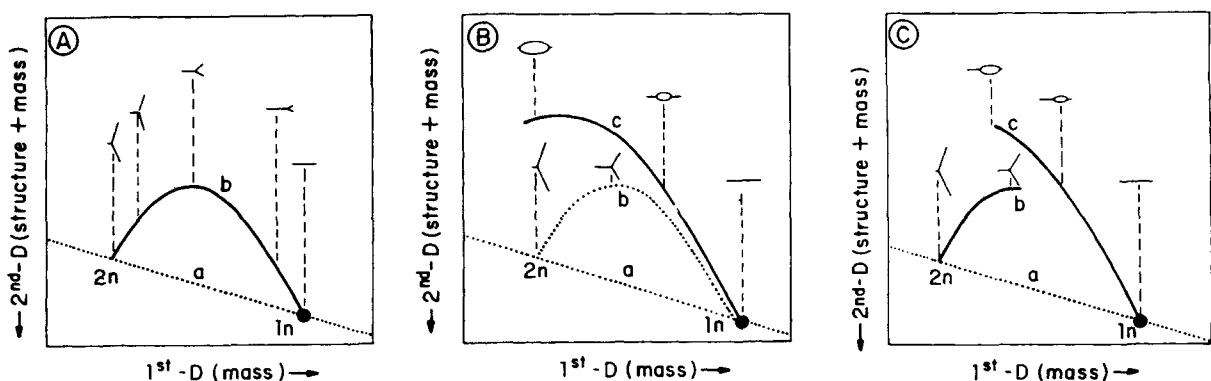

Fig. 2. Principle of the neutral/neutral 2-D gel replicon mapping technique (14). Idealized autoradiographic images obtained when a digest of replicating DNA is hybridized with probes for fragments that contain different intermediates. A: A complete single fork arc (b) from a fragment replicated passively 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.
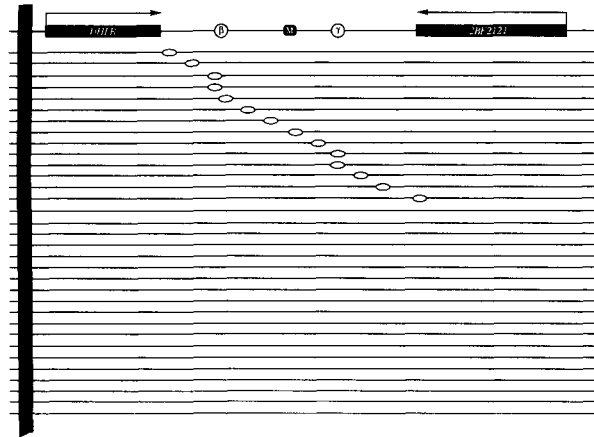
Fig. 3. Initiation in the DHFR locus. Several copies of the DHFR amplicon shown aligned side-by-side instead of end-to-end. Initiation is thought to occur at any of a large number of sites in the region between the two convergently transcribed genes, but possibly more often near ori-β and ori-γ. The amplicons without bubbles are replicated passively by forks from adjacent, active amplicons.

this region, we can calculate that probably only 10–20% of amplicons sustain an initiation event (Fig. 3). This interpretation is supported by the very low bubble-to-fork arc ratios detected in intergenic fragments in asynchronously growing cells, in which most copies of the amplicon would be undergoing passive replication and only a small proportion would actually contain bubbles at the time of sampling [Dijkwel and Hamlin, 1992; Vaughn et al., 1990]. Importantly, the results on unsynchronized cells show that this delocalized mode of initiation is not the consequence of synchronization per se.

Since the N/N 2-D method painted a rather unorthodox picture of initiation in this locus (and, indeed, one quite different than evinced by several other methods; see below), it was necessary to analyze this locus by additional, independent methods. A second, complementary 2-D technique was employed that directly measures the sizes of nascent strands and allows a determination of replication fork direction in a locus of interest [Nawotka and Huberman, 1988]. When the DHFR locus was analyzed by this technique, in the early S phases very short nascent strands were detected at all locations within the intergenic region, as predicted if initiation occurs at sites scattered throughout the region. Again as predicted, forks were observed to move in both directions within the intergenic region, but only outward unidirectionally through the DHFR gene [Dijkwel and Hamlin, 1992; Vaughn et al., 1990]. At later time points when initiation has ceased but the remaining amplicons are being replicated passively, forks are observed to move

in both directions even through the DHFR gene [Vaughn et al., 1990].

Thus, the 2-D gel replicon mapping methods both suggest that initiation can occur at any of a large number of sites in the region between the two genes. This raised the question whether this unusual mode of replication might be the consequence of amplification per se. New methods for purifying replication intermediates by more than 100-fold [Dijkwel et al., 1991] made it possible to extend both 2-D methods to the parental CHO cell line, which contains only the normal two copies of the DHFR locus. It is quite clear that initiation is also a delocalized phenomenon in the unamplified DHFR locus [Dijkwel and Hamlin, 1995]. Moreover, it appears that delocalized initiation is not particular to the DHFR domain, since a broad initiation zone has also been detected in the single copy rhodopsin locus in CHO cells (Dijkwel, unpublished data); nor is delocalized initiation unique to hamster cells, since initiation zones have been described in the multicopy human rDNA repeat [Little et al., 1993] and in both multi-copy and single copy loci in Drosophila [Shinomiya and Ina, 1991, 1993].

The data from 2-D analyses of the DHFR locus are in reasonable agreement with earlier, low-resolution intrinsic labelling studies showing that initiation occurs downstream from the DHFR gene, possibly from two preferred zones within the intergenic region [Leu and Hamlin, 1989]. However, several other studies that examined this locus by independent approaches initially appeared to support a more conventional model for initiation. At this juncture, it is impor-

tant to point out that both 2-D gel techniques are necessarily biased toward the most long-lived replication intermediates, since both methods examine steady-state intermediates. In contrast, methods that identify and/or isolate nascent DNA in brief pulse-labelling strategems with radioactive thymidine or the density label, bromodeoxyuridine (BrdU), are biased toward intermediates that have a short half-life. This difference would be important if, for example, some initiations were futile and only some mature to finished replicons.

In one such intrinsic labelling technique [Vassilev and Johnson, 1989], DNA is briefly labelled with BrdU in vivo, the BrdU-labelled DNA is isolated with anti-BrdU antibodies, and nascent DNA is fractionated according to size. The abundance of specific sequences in each size fraction is then estimated by quantitative polymerase chain reaction (PCR). When applied to the DHFR locus in CHO cells, a 2 kb region including and surrounding ori-β was found to be represented in nascent strands of all sizes (including the very shortest), which suggested the presence of a highly preferred initiation site in or near ori-β [Vassilev et al., 1990]. Unfortunately, only three closely spaced positions near ori-β were analyzed, making it difficult to fully evaluate the significance of this observation.

A different approach was taken in the *leading strand* assay, which determines the template preference of leading strands, which switches from one template strand to another at origins and termini [Roufa and Marchionni, 1982]. By exposing cycling cells to emetine for 24 h, it is possible to selectively inhibit lagging strand synthesis [Burhans et al., 1991], with the consequence that leading strands can be selectively labelled with BrdU. This BrdU-labelled DNA fraction is then isolated, immobilized on a membrane, and hybridized with a series of ³²P-labelled + and − template strands representing various positions in the locus of interest. The nascent DNA isolated from unsynchronized CHO cells by this approach was found to switch template preference approximately at the ori-β and ori-γ positions [Handeli et al., 1989]. Additional template switches were detected between the two origins as well as upstream from the DHFR gene which, in theory, should correspond to termini at these locations [Handeli et al., 1989].

At first glance, these data would appear to be most compatible with the presence of highly preferred initiation sites in the ori-β and ori-γ

loci, as suggested by the early in-gel renaturation experiments [Leu and Hamlin, 1989]. However, on close inspection, the data are more compatible with the delocalized model evoked by 2-D gel studies, in which initiations can occur at any of a large number of positions throughout the intergenic zone, but are slightly preferred near ori-β and ori-γ. For one thing, leading strand template biases were not detected when sequences within ∼ 5 kb of either ori-β or ori-γ were examined [Handeli et al., 1989], which is compatible with a dispersed zone of initiation rather than a more narrowly circumscribed locus. Secondly, although some hybridization bias was observed with almost every probe, in each case there also was significant hybridization to the opposite strand [Handeli et al., 1989].

The results of a *lagging strand* assay [Burhans et al., 1990] were initially more difficult to reconcile with the delocalized initiation model. This assay measures the bias in hybridization of Okazaki fragments to the separated template strands of a region of interest. Cells are first permeabilized and are then allowed to extend pre-existing nascent strands in vitro in the presence of ³²P-labelled deoxyribonucleotide triphosphates and BrdUTP. Okazaki fragments are isolated by size and enriched with antibodies to BrdU and are used to probe + and − strands as above. In an initial study on CHOC 400 cells and on CHO cells with only two copies of the DHFR locus, a very pronounced switch in template strand preference was detected between the two halves of a 500 bp fragment mapping at the approximate position of ori-β [Burhans et al., 1990]. Quantitative estimates of template bias suggested that more than 80% of initiations occur within this 500 bp fragment, a result that cannot be easily reconciled with the 2-D gel data suggesting a polydisperse choice of initiation sites in the region.

Interestingly, the pronounced switch at ori-β was also observed in exponentially growing CHOC 400 cells, which could only occur if all initiation sites are active in every S period. This result was in stark contrast to the 2-D gel data showing that only 10–15% of amplicons actually sustain an active initiation event in one S period, with the rest being replicated passively [Dijkwel and Hamlin, 1992].

However, discrepancies between the results of the lagging strand and 2-D gel approaches appear to have been reconciled in a later report [Gilbert et al., 1995]. Template bias was not

observed at any position within the DHFR locus in unsynchronized cells, presumably because in the earlier study, the cells had been inadvertently synchronized. Secondly, when synchronized CHOC 400 cells were reexamined, a much more gradual change in template preference for Okazaki fragment synthesis was detected, and the very sharp strand switch within the 500 bp fragment centered over ori-β was not observed [Gilbert et al., 1995]. Indeed, the bias shifted gradually from about six-fold in the DHFR gene to virtually zero in a region downstream from ori-β. This result is not compatible with a single, or even a highly preferred, initiation site; instead, it suggests the presence of a broad initiation zone.

In a more recent set of experiments, considerable insight was gained into the different views afforded by pulse-labelling and physical mapping techniques. A *nascent strand abundance* assay has been developed that measures not only the size of the nascent strands at any given position, but also their abundance [Yoon et al., 1995]. This analysis allows much more accurate quantitation of the number of initiations occurring at different positions within a genomic region of interest. When the human rDNA repeat was examined using this assay, initiations were found to occur at all positions within the non-transcribed spacer, in close agreement with results from 2-D gel analysis [Little et al., 1993]. However, the assay showed clearly that initiation frequency varies considerably throughout the non-transcribed spacer, a finding that is not apparent from the less quantitative N/N 2-D gel analysis of the same region. Presumably, this difference could reconcile the seemingly disparate views afforded by the two different approaches in the DHFR origin as well.

Several other higher eukaryotic initiation loci have now been analyzed by at least two different origin mapping techniques, and their characters are, by in large, similar to the DHFR loci in CHO cells. For example, an early-firing origin in the amplified adenosine deaminase domain in coformycin-resistant murine cells has been roughly localized by preferential labelling of amplified restriction fragments at the beginning of the S period [Carroll et al., 1993]. This result was confirmed by using the lagging strand assay to show that a switch in template bias occurs in the same position [Carroll et al., 1993]; however, given the relatively widely spaced probes and the fact that the Okazaki fragments hybridized

to both + and − strands even when biases were observed, the results can be construed to reflect an initiation zone with a slightly preferred locus in its center.

Interestingly, an initiation zone has been localized within the body of the CAD gene in both Chinese hamster and Syrian Hamster cells [Kelly et al., 1995]. This locus was identified initially be an early-labelling protocol, and its location was confirmed by both the leading and lagging strand assays [Kelly et al., 1995]. Again, it is not clear how circumscribed the zone of initiation is in this locus, owing to the spacing of the probes, but hybridization to both + and − templates in the two latter assays suggests at least a somewhat decentralized zone as in the DHFR locus.

There is one higher eukaryotic initiation locus in which the results from N/N 2-D gel analysis and nascent strand size analysis seem to agree almost perfectly with one another. The histone gene repeats in *Drosophila* cultured cells have been examined by both structural and intrinsic labelling techniques [Shinomiya and Ina, 1993]. It was found by both assays that initiation sites are dispersed throughout the 5 kb repeating units, with some evidence for pause sites at the ends of individual genes. This represents an unusual example of an almost total lack of sequence preference, and it will be interesting to learn whether other loci in which genes are tightly clustered together will behave this way.

If the data on the DHFR and other higher eukaryotic loci reflect an initiation reaction that is replicator-independent, then the original replicon model devised to explain replication control in bacteria would appear to have broken down. However, other explanations for delocalized initiation reactions are possible that still retain most aspects of the replicon model. For example, there might be a true replicator in each of these loci, but binding to the initiator might destabilize a much more extended region of the chromosome (as opposed to just the immediate surrounds of the replicator), allowing the replication machinery to access any region of the template in this extended region. Alternatively, a replicator/initiator interaction might melt the helix only in the immediate vicinity of the replicator, but the replication machinery could then load into this site and course along the template for various distances before laying down the RNA primer that begins a nascent chain. Clearly, both of these are variations on the replicon model. In principle, however, destabilization of

an origin region could be induced by other means that are replicator-independent, such as local changes in superhelical density induced, e.g., by transcription. This possibility will be discussed below.

Thus, all of the labelling and physical approaches now seem to converge on a relatively unified model in which eukaryotic initiation is confined to preferred zones but possibly not to the narrowly circumscribed loci that characterize initiation reactions in simple prokaryotes and in yeast. The replicon model can still accommodate the dispersive initiation mode provided that each zone harbors a replicator-like sequence that would abolish initiation if deleted. However, precise localization of replicators by mapping nascent strand start sites is not going to be a fulfilling approach. Just as with bacterial and viral replicons, genetic approaches will be required to identify components of the replicator in higher eukaryotic chromosomes. Such approaches are finally possible, since techniques have now been developed for deleting (knocking out) selected sequences in mammalian genomes by homologous recombination. In addition, with the development of much more sensitive assays that can detect initiation intermediates even in single copy loci, it should be possible to genetically engineer changes in cloned fragments containing potential replicators and then to insert these clones into the chromosomes at random positions by transfection and selection techniques. Using this combined negative/positive approach, it will no doubt be possible to identify critical cis-regulatory sequences in the very near future. Whether required sequence elements will be confined only to classical replicators remains to be seen. As discussed below, there are reasons to believe that other sequences—sometimes acting over large distances—may also be required for proper origin function.

STRUCTURE-FUNCTION RELATIONSHIPS IN THE NUCLEUS

It is no surprise that the mammalian nucleus is a highly organized cell organelle, and sophisticated new cytological techniques are revealing new aspects of overt structural organization. Chromosome painting techniques have shown that each chromosome occupies its own domain in the interphase nucleus [Gray et al., 1992; Lengauer et al., 1990; Lichter et al., 1988]. This can be visualized readily in CHOC 400 cells when the amplified DHFR locus is visualized by

fluorescence in situ hybridization (FISH) using a DHFR-specific probe: each of the three different chromosome regions bearing the multiple amplicons can be seen to occupy its own sector in the nucleus [Trask and Hamlin, 1989].

With the aid of fluorescent antibodies to various proteins involved in transcription and replication, it has been possible to show that mammalian nuclei have a sophisticated functional organization. This is inferred from the punctate (as opposed to diffuse) staining patterns exhibited by transcription, splicing, and replication factors [Jackson et al., 1993; Spector, 1990]. There is now considerable evidence that transcription and replication co-localize in nuclear foci, reopening the specter that transcription and replication may be intimately related processes [Hassan et al., 1994]. Focal patterns change in reproducible ways throughout the S period, suggesting that replication is not only programmed temporally but also spatially [Nakamura et al., 1986]. Interestingly, focal patterns seem to be defined in the G_1 period, when the various components of the factories assemble at future replication sites [Adachi and Laemmli, 1994; Yan and Newport, 1995].

The highly ordered appearance of the interphase nucleus and of metaphase chromosomes undoubtedly reflects the underlying higher order structure of chromatin itself. In metaphase, chromatin has been shown to be organized into loops that are radially attached to a central scaffolding structure [for review see Pienta et al., 1991]. This organization persists in a somewhat modified form in interphase. Chromatin loops are topologically constrained and typically contain 50 to 100 kb of DNA [Pienta et al., 1991]. Their quasi-circular behavior suggested that the loops must be anchored to some immobile support.

Indeed, when nuclei are depleted of histones and soluble proteins by any of a number of extraction methods, an insoluble residual structure remains. Electron microscopy has shown that this structure (termed the *nuclear matrix*) [Berezney and Coffey, 1974] consists of the nuclear lamina and residual nucleoli and is permeated by a fibrillar network [Belgrader et al., 1991; Gerace and Blobel, 1980; Kaufmann et al., 1981; Berezney, 1984]. Because DNA was shown to be attached periodically to this structure both internally and peripherally, the nuclear matrix (or scaffold) is a prime candidate for the organizing entity in the cell nucleus.

Much attention has been paid to the possible functional role, if any, of the sequences at the bases of the DNA loops. Matrix (or scaffold) attachment regions (MARs or SARs) are defined as sequences that are either preferentially retained on the matrix after removal of the majority of chromosomal DNA, or that exhibit high affinity for isolated matrices in vitro. Evidence has been provided that MARs can function as boundary elements that shield genes from positional effects [Kellum and Schedl, 1991; Fishel et al., 1993; Stief et al., 1989; Klehr et al., 1991; Loc and Stratling, 1988]. MARs have also been documented to serve as molecular switches in H1-dependent condensation and decondensation of chromatin [Kas et al., 1993; Zhao et al., 1993].

While it is conceivable that there may be different functional categories of MARs or SARs, all such sequences are thought to displace proximal functional sequences to the appropriate nuclear compartment [Mirkovitch et al., 1984]. This would reduce the search volume for trans-acting factors that could presumably enhance the efficiency of any nuclear process, including initiation of DNA replication. This view is supported by the finding that in S. cerevisiae, MARs and ARS elements, though juxtaposed, are separable [Amati and Gasser, 1988, 1990]. However, separation severely affects ARS function as determined by plasmid stability assays [Amati and Gasser, 1988].

In higher eukaryotes, several lines of evidence suggest that matrix attachment is important for origin function. When DNA synthesized at the onset of the S period is selectively labelled with BrdU and detected with fluorescent antibodies, the newly replicated DNA is distributed in a distinctive focal pattern [Nakamura et al., 1986; Nakayasu and Berezney, 1989]. This pattern persists in isolated matrices [Nakayasu and Berezney, 1989], corroborating earlier labelling studies showing that radioactive thymidine incorporated in the early S period is physically quite close to the nuclear matrix, and remains so throughout the S period; in contrast, radiolabel incorporated later in the S period eventually migrates out into the DNA loops [Dijkwel et al., 1986]. Electron microscopy also showed that replication bubbles are enriched in the DNA fraction that is tightly associated with the nuclear matrix [Valenzuela et al., 1983]. Furthermore, once-per-cell-cycle replication of exogenously introduced DNA or chromatin in Xeno-

pus eggs or egg extracts is contingent upon prior formation of functional nuclei [Blow et al., 1987]; interestingly, interference with matrix formation by topoisomerase II inhibitors prevents the onset of DNA replication in this system [Newport, 1987; Newport et al., 1990; Meier et al., 1991].

While the present evidence strongly implicates the nuclear matrix in DNA replication, it remains to be established that MARs are indispensable for origin function in metazoans. As mentioned above, MARs have been mapped close to sequences that function as ARS elements in yeast [Amati and Gasser, 1988], and several MARs have been isolated from DNA replicated in the earliest part of the S period. However, it has yet to be shown convincingly that any of these mammalian MARs actually behaves as a replication origin, either as an episome or in its native position in the mammalian chromosome. Somewhat stronger evidence for the involvement of at least some MARs in initiation was provided by an analysis of nascent DNA strands that were extruded in vitro from small replication bubbles formed in the beginning of the S period in monkey cells [Zannis-Hadjopoulos et al., 1985]. This fraction was shown to contain several fragments that were able to associate with the matrix in vitro [Mah et al., 1993]. In addition, a similarly prepared fraction was found to hybridize preferentially to restriction fragments containing a MAR in the upstream region of the chicken α-globin locus [Razin et al., 1986]; however, proof that the extruded sequences behave as initiation sites in the chromosome is still lacking.

The DHFR locus in CHO and CHOC 400 cells appears to contain a bona fide origin of replication and, interestingly, a prominent MAR was detected in the middle of the initiation zone in this domain, approximately midway between ori-β and ori-γ [Dijkwel and Hamlin, 1988]. As with all MARs identified so far, this one is AT-rich, the most prominent motif being a series of nine almost perfectly repeated AAAT-sequences (Mesner and Dijkwel, unpublished observations). Interestingly, only 10–20% of amplicons appear to be attached in CHOC 400 cells [Dijkwel and Hamlin, 1988], a number that corresponds closely to the fraction of initiation zones that are active in any one S period [Dijkwel and Hamlin, 1992]. In contrast, in the parental CHO cells in which the efficiency of origin usage is considerably higher [Dijkwel and Hamlin, 1995],

a much larger percentage of the MAR sequences in the initiation zone appear to be attached [Dijkwel and Hamlin, 1988].

As shown above (Fig. 1), the DHFR initiation zone is delimited by the convergently transcribed DHFR and the 2BE2121 genes, both of which are active in late G_1 and early S [Leu and Hamlin, unpublished observations]. This is an interesting arrangement for several reasons. A large body of work suggests that transcription and initiation of DNA replication may be linked. For example, euchromatic (active) chromatin normally replicates early in the S period, while heterochromatin replicates late [e.g., Holmquist, 1987]. Furthermore, active genes usually replicate early in the S period, but are often late-replicating in cells in which the gene is not expressed [e.g., Goldman et al., 1984]. As mentioned above, in very early S phase, fluorescent-labelled replication and transcription foci overlap, but only if nascent DNA is labelled very briefly with BrdU [Jackson et al., 1993].

Even though this and other evidence suggests that transcription and initiation of DNA replication are linked, little is known about underlying molecular mechanisms. One obvious model is that the more open chromatin configuration in and around the promoters of transcription units allows more ready access by the replication machinery to potential replicators in the template. It is therefore interesting that the organizational motif of the DHFR domain is reiterated at a second initiation site (ori-α) lying 200 kb upstream from ori-β/ori-γ in the much larger DHFR amplicons of a second methotrexate-resistant hamster cell line (DC3F/A3) [Biedler and Spengler, 1976]. In most of the amplicons in the DC3F/A3 cell line, ori-α is near the center of the amplicon and is located ~20 kb downstream from a transcription unit; in this amplicon type, ori-α fires in the middle of the S period [Ma et al., 1990]. However, 20% of the amplicons are arranged as giant inverted duplications with the gene being transcribed convergently toward ori-α. Consequently, an intergenic region was created that closely resembles the ori-β/ori-γ locus between the DHFR and 2BE2121 genes. Interestingly, initiations in this zone now occur at the very beginning of the S period, suggesting that convergent transcription in the early S period may somehow affect chromosomal architecture to allow initiation to occur in early S at this usually later-firing origin.

Preliminary data lends support to this hypothesis. In two hamster cell lines that are hemizygous for the DHFR gene, N/N 2-D gel studies detect an initiation pattern that is temporally and spatially indistinguishable from that in CHO cells (Dijkwel et al., unpublished observations). However, in variants containing γ-ray-induced deletions that remove the DHFR promoter but leave the downstream initiation locus intact, the DHFR locus is not replicated until several hours after entry into the S period (Levenson et al., unpublished observations). Preliminary data from N/N analysis suggests that the DHFR domain is replicated passively in the late S period by forks from distant origins, and not by late-firing of the DHFR origin itself. Therefore, it seems that silencing of the DHFR gene affects not only the timing of initiation in the DHFR domain but may also abolish initiation altogether.

CHROMATIN CONTEXT MODEL FOR INITIATION

This collection of observations suggests a general model for those early-firing initiation loci that are close to active genes. The most catholic form of the model starts by assuming that nascent strands can initiate at virtually any site in the mammalian genome, but that the actual rate of initiation at any given site depends on chromatin context and local transcriptional activity. According to the twin-supercoiled domain proposal [Liu and Wang, 1987], those regions of the DHFR locus that lie beyond the 5′ ends of the DHFR and 2BE2121 genes (Fig. 1) should accumulate negative supercoils as a consequence of converging transcription in the DHFR and 2BE2121 genes in late G_1 and in early S. Correspondingly, the intergenic region should accumulate positive supercoils, which could result in nucleosome splitting and a relatively more open chromatin configuration in the intergenic region [Lee and Garrard, 1991]. This would increase the accessibility of this region to incoming replication complexes, allowing replication forks to be established. An extension of the model assumes that origin usage will also be dictated by its location in the nucleus, regardless of whether or not it is positively supercoiled: only those origin regions that are brought closer to the matrix-affixed replication complex by permanent or transient attachment to the matrix will be recognized. This would explain why only ~15% of the DHFR amplicons in CHOC 400

cells actually support initiation [Dijkwel and Hamlin, 1992].

At face value, this model obviates the need for the classical replicator, since any sequence that is easily unwound [Natale et al., 1993], that finds itself in a perturbed region of the genome (i.e., at the beginning or end of genes), and that is attached to the nuclear matrix nearby, could function as an origin. However, the model could also accommodate the classical *cis*-regulatory replicator by assuming that it corresponds to an easily unwound region next to a binding site for a helix-destabilizing initiator protein. Again, the test of this or any other model will await systematic inactivation of MARs, potential unwinding elements, and promoters of genes by homologous recombination strategies to determine whether they contribute to origin function.

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