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Transformation abrogates an early G_1-phase arrest point required for specification of the Chinese hamster DHFR replication origin

Jia-Rui Wu[1], Susan M.Keezer and David M.Gilbert[2]

Department of Biochemistry and Molecular Biology, S.U.N.Y. Health Science Center, 750 East Adams Street, Syracuse, NY 13210, USA

[1]Present address: Shanghai Institute of Biochemistry, Chinese Academy of Sciences, 320 Yue-Yang Road, Shanghai 200031, P.R. China

[2]Corresponding author
e-mail: gilbertd@vax.cs.hscsyr.edu

The origin decision point (ODP) was originally identified as a distinct point during G_1-phase when Chinese hamster ovary (CHO) cell nuclei experience a transition that is required for specific recognition of the dihydrofolate reductase (DHFR) origin locus by *Xenopus* egg extracts. Passage of cells through the ODP requires a mitogen-independent protein kinase that is activated prior to restriction point control. Here we show that inhibition of an early G_1-phase protein kinase pathway by the addition of 2-aminopurine (2-AP) prior to the ODP arrests CHO cells in G_1-phase. Transformation with simian virus 40 (SV40) abrogated this arrest point, resulting in the entry of cultured cells into S-phase in the presence of 2-AP and a disruption of the normal pattern of initiation sites at the DHFR locus. Cells treated with 2-AP after the ODP initiated replication specifically within the DHFR origin locus. Transient exposure of transformed cells to 2-AP during the ODP transition also disrupted origin choice, whereas non-transformed cells arrested in G_1-phase and then passed through a delayed ODP after removal of 2-AP from the medium. We conclude that mammalian cells have many potential sites at which they can initiate replication. Normally, events occurring during the early G_1-phase ODP transition determine which of these sites will be the preferred initiation site. However, if chromatin is exposed to S-phase-promoting factors prior to this transition, mammalian cells, like *Xenopus* and *Drosophila* embryos, can initiate replication without origin specification.

Keywords: cell cycle/DNA replication/origin decision point/protein kinase/transformation

Introduction

The search for replication origins in the genomes of multicellular organisms has still not conclusively identified specific DNA sequences that serve to initiate replication in each cell cycle (for review see Gilbert, 1998). In cultured fibroblasts, replication initiates within defined chromosomal loci. However, these initiation loci are poorly defined and may encompass up to several tens of kilobases (DePamphilis, 1993; Little *et al.*, 1993; Hamlin *et al.*, 1994; Shinomiya and Ina, 1994; Dijkwel and Hamlin, 1995; Little and Schildkraut, 1995). Furthermore, during the rapid early cleavage stages of *Xenopus* and *Drosophila* development, replication appears to initiate at sites distributed randomly throughout chromosomes (Hyrien and Mechali, 1993; Shinomiya and Ina, 1993). In these species, preferential initiation at specific chromosomal sites does not occur until after the blastula stage of development, coincident with the appearance of a more functionally organized nucleus (Gilbert *et al.*, 1995; Hyrien *et al.*, 1995; T.Sasaki and T.Shinomiya, personal communication). When purified DNA templates are introduced into cell-free extracts from *Xenopus* eggs, cell-cycle regulated semi-conservative DNA replication also initiates within apparently random sequences (Coverley and Laskey, 1994, and references therein). However, these same extracts are capable of initiating replication specifically at the Chinese hamster ovary (CHO) dihydrofolate reductase (DHFR) origin locus if the substrate for replication is introduced in the form of an intact fibroblast nucleus (Gilbert *et al.*, 1995; Lawlis *et al.*, 1996; Wu and Gilbert, 1996, 1997). These observations suggest that initiation of DNA replication in higher eukaryotes is focused to specific genomic regions by the functional organization of the nucleus.

Recently it was shown that, in order for *Xenopus* egg cytosol to recognize the CHO DHFR origin, intact nuclei must be isolated from cells after a specific point during G_1-phase, designated the origin decision point (ODP) (Wu and Gilbert, 1996, 1997). When nuclei from cells synchronized prior to this point are introduced into *Xenopus* egg extracts, replication initiates efficiently but without any detectable preference for the DHFR origin. Since intact G_2-phase nuclei will not replicate in *Xenopus* egg extracts (Leno *et al.*, 1992), pre-ODP nuclei have acquired the potential to replicate (replication licensing), but have not yet undergone an additional regulatory step that is required to restrict initiation to specific sites. The ODP can also be uncoupled from the decision to enter S-phase; passage through the ODP is upstream of restriction point control and independent of serum mitogens (Wu and Gilbert, 1997). Thus, the ODP represents a distinct replication regulatory point that takes place during the early stages of G_1-phase.

Rapid early cleavage embryos, which initiate replication at random sites, do not have a G_1-phase, oscillating instead between mitosis and S-phase. We have previously suggested the possibility that origin selection in higher eukaryotes requires a proper G_1-phase, during which chromosome architecture is remodeled to accommodate the functional activities of the somatic cell (Lawlis *et al.*, 1996). This hypothesis implies that even in mammalian

fibroblasts, replication would initiate without the selection of specific origin sites if a means could be devised to specifically abrogate the ODP without interfering with progression toward S-phase. Since we have previously shown that passage through the ODP can be prevented by treating pre-ODP cells with 2-aminopurine (2-AP) (Wu and Gilbert, 1997), we investigated the possibility that 2-AP treatment of pre-ODP cells would cause them to enter S-phase without specifying the DHFR origin. We found that 2-AP administered prior to the ODP arrested CHO cells in early G_1-phase, precluding the possibility of mapping origin sites. Even when exposure to 2-AP was limited to the early stages of G_1-phase, cells were arrested and passed through a delayed ODP upon removal of 2-AP. However, transformation with simian virus 40 (SV40) abrogated this early G_1-phase arrest point, resulting in the entry of transformed CHO cells into S-phase without selection of the DHFR origin. These findings support the hypothesis that origin specification is not required for initiation of DNA replication *per se*. At the same time, they suggest that focusing initiation to specific sites at the ODP may be an important hallmark in the progression of normal somatic cells through G_1-phase.

Results

SV40 transformation can override a 2-AP arrest point in G_1-phase

In previous experiments, CHO (CHOC 400) cells were treated with 2-AP at various times after mitosis and then incubated in growth medium for a time sufficient to allow cells to proceed to the post-ODP stage of G_1-phase. When nuclei from cells treated with 2-AP prior to the ODP were introduced into *Xenopus* egg extract, replication initiated at sites distributed throughout a much broader region than with nuclei from cells treated with 2-AP after the ODP or with nuclei from untreated cells (Wu and Gilbert, 1997). We reasoned that if cultured cells could enter S-phase in the presence of 2-AP, or could be manipulated to do so, then these cells might enter S-phase without specification of the DHFR origin.

To determine whether CHOC 400 cells would enter S-phase in the presence of 2-AP, cell populations were first synchronized in metaphase by mitotic shake-off following a brief (4 h) and completely reversible exposure to nocodazole. 2-AP was added to separate culture dishes at each hour thereafter. At 16 h after metaphase, cells from all dishes were labeled with 5′-bromodeoxyuridine (BrdU) for 30 min, and the percentage of cells that had entered S-phase and incorporated BrdU into chromosomal DNA was evaluated by indirect immunofluorescence using an anti-BrdU antibody. Results (Figure 1D) revealed that CHOC 400 cells were completely prevented from entering S-phase when 2-AP was added prior to the ODP, precluding the possibility of mapping the sites that would be selected by S-phase-promoting factors in these 2-AP-treated cells.

Other investigators have reported that several protein kinase inhibitors arrest mammalian cells in G_1-phase (Crissman *et al.*, 1991; Gadbois *et al.*, 1992). These kinase arrest points were absent when these same cells were transformed with SV40 viral DNA. To determine whether SV40 transformation might also abrogate the 2-AP arrest point in CHOC 400 cells, cells were co-transfected with SV40 DNA and a neomycin expression vector, and G418 resistant colonies were expanded and stained by indirect immunofluorescence with an antibody directed against SV40 large T-antigen (gift of C.Gruss). This report describes studies carried out with one of these cell lines (CHOC 400/SV40) in which 70% of the cell population consistently stained positive for the presence of nuclear T-Ag and Southern blot analysis verified the presence of a single SV40 integration site (data not shown).

In Figure 1, the properties of CHOC 400/SV40 cells are compared with those of the parental CHOC 400 cell

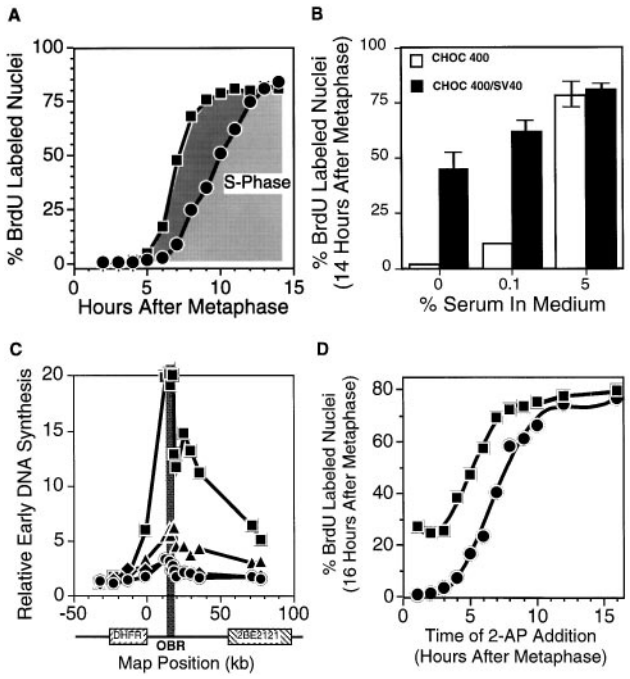

Fig. 1. Properties of SV40-transformed CHOC 400 cells. (**A**) Both non-transformed CHOC 400 (●) and CHOC 400/SV40 (■) were synchronized in metaphase and plated into fresh medium to allow progression into G_1-phase. At hourly intervals thereafter, aliquots of these cells were pulse-labeled for 30 min with BrdU and cells were stained by indirect immunofluorescence using an anti-BrdU antibody. (**B**) CHOC 400 (white) and CHOC 400/SV40 (black) were synchronized in metaphase and plated into fresh serum-free medium or medium supplemented with 0.1 or 5% serum. After 14 h, cultures were labeled for 30 min with BrdU and the percentage of cells in S-phase (BrdU-labeled) was determined as in (A). (**C**) CHOC 400/SV40 cells were synchronized in metaphase and plated into fresh medium. Aliquots of these cells were collected at 1 (●), 2 (◆), 3 (▲) and 4 (■) h thereafter. Intact nuclei from these cells were introduced into a *Xenopus* egg extract and the sites of initiation of replication were then evaluated using the ELFH assay, as described (Wu *et al.*, 1997). The relative amounts of hybridization of the earliest labeled nascent DNA to each unique probe from the DHFR locus are plotted against the map positions of each segment. The horizontal axis includes a diagram of the portion of the DHFR locus encompassed by these probes, including the positions of the DHFR and 2BE2121 genes (Hamlin and Dijkwel, 1995). The vertical shaded line shows the position of the previously mapped (Burhans *et al.*, 1990; Pelizon *et al.*, 1996) origin of bidirectional replication (OBR). Since the data are expressed as relative DNA synthesis values, it is the differences between the probes, not the area under the curve, that should be compared. (**D**) Both CHOC 400 (●) and CHOC 400/SV40 (■) were synchronized in metaphase and plated into fresh medium to allow progression into G_1-phase. At hourly intervals thereafter, 10 mM 2-AP was added to the culture medium. At 16 h after metaphase, all cultures were pulse-labeled with BrdU and the percentage of cells that had entered S-phase was evaluated as in (A) and (B).

line. To determine the length of G_1-phase, cells were synchronized in metaphase, released into G_1-phase and aliquots of these cells were pulse-labeled with BrdU at hourly intervals thereafter. Results (Figure 1A) revealed that the first CHOC 400/SV40 cells entered S-phase at approximately the same time as CHOC 400 cells, but that the overall population entered S-phase more synchronously than CHOC 400 so that by 8 h after metaphase, nearly three times as many CHOC 400/SV40 cells had entered S-phase as corresponding CHOC 400 cells. Furthermore, when cells were plated directly into medium lacking serum growth factors, CHOC 400 cells were completely prevented from entering S-phase, whereas entry into S-phase was largely independent of serum mitogens in CHOC 400/SV40 cells (Figure 1B). These results are consistent with an abrogation of restriction point (R-point) control (Zetterberg and Larsson, 1995) and a lack of requirement for retinoblastoma protein phosphorylation for the entry into S-phase in cells expressing SV40 T-Ag (Ludlow *et al.*, 1989).

To determine whether transformation with SV40 affected passage through the ODP, CHOC 400/SV40 cells were synchronized in metaphase and released into G_1-phase. At hourly intervals thereafter, intact nuclei were prepared from these cells and introduced into a *Xenopus* egg extract supplemented with aphidicolin. Within 40–60 min, *Xenopus* egg cytosolic factors initiate replication in nearly 100% of nuclei (Gilbert *et al.*, 1995; Wu and Gilbert, 1996, 1997). The presence of aphidicolin allows replication to initiate and short nascent strands to form, but arrests replication forks close to their initiation sites. Aphidicolin was then washed away and nascent replication forks were briefly labeled with [α-^{32}P]dATP. The positions of these short, pulse-labeled nascent strands can then be determined by hybridizing them to unique DNA sequences derived from specific positions along the DHFR locus. The relative amounts of hybridization of these labeled strands are then plotted against the map position of each DNA segment to give a distribution of the relative frequency at which replication initiated near each probe. This assay, termed the early labeled fragment hybridization (ELFH) assay, has been described in detail (Wu *et al.*, 1997). Results (Figure 1C) revealed that with nuclei from cells 1 and 2 h after metaphase, replication initiated with little or no preference for the DHFR origin locus, at 3 h a slight preference for the DHFR origin was observed, and by 4 h after metaphase most of the initiation sites were focused within the DHFR locus. These results are similar to those obtained in analogous experiments with CHOC 400 cells (Wu and Gilbert, 1996). Thus, we conclude that the ODP transition takes place in CHOC 400/SV40 at the same time as in CHOC 400 cells. Since SV40-transformed cells are R-point deficient (Figure 1B), these data also confirm and extend our previous finding that the ODP is independent of R-point control (Wu *et al.*, 1997).

To determine whether CHOC 400/SV40 cells could enter S-phase in the presence of 2-AP, cells were synchronized in metaphase and 2-AP was added to separate culture dishes at each hour thereafter, as described above for CHOC 400 cells. At 16 h after metaphase, cells were pulse-labeled with BrdU and the percentage of cells that had entered S-phase was evaluated. Results (Figure 1D) revealed that 24–28% of CHOC 400/SV40 cells were able to initiate DNA replication in the presence of 2-AP administered at various times prior to the ODP. At various times after the ODP, an increasing percentage of the remainder of the cell population could enter S-phase (Figure 1D). It is not clear why many CHOC 400/SV40 cells do not enter S-phase in the presence of 2-AP, but we speculate that it may be due to cell-to-cell variation in the level of expression of SV40-transforming proteins. Nonetheless, the fraction of 2-AP-resistant cells within the CHOC 400/SV40 population (24–28%) was sufficient to determine whether these cells were initiating replication at the DHFR origin.

Initiation of replication in culture without specification of the DHFR origin

To determine whether 2-AP treatment prior to the ODP prevents specification of the DHFR origin within those cells that enter S-phase, CHOC 400/SV40 cells were synchronized in metaphase and 2-AP was added at hourly intervals from 1–5 h thereafter. Aphidicolin was added to all cultures at 5 h after metaphase, and cells were incubated for an additional 11 h to allow entry of those cells that were not arrested in G_1-phase into S-phase and the consequent accumulation of *in vivo*-initiated nascent DNA strands close to their sites of origin. These cells were then washed free of aphidicolin, permeabilized, and nascent DNA strands were labeled briefly with [α-^{32}P]dATP. The sites of initiation of replication were then mapped by the ELFH assay. Results (Figure 2) revealed that 2-AP treatment of CHOC 400/SV40 at times prior to the ODP resulted in the initiation of replication at apparently random sites throughout the DHFR locus. By contrast, when 2-AP was added after the ODP, replication initiated specifically within the DHFR origin locus.

To verify that the nascent strands labeled in cells treated with 2-AP prior to the ODP resulted from the initiation of semi-conservative DNA replication, density shift experiments were performed in parallel with ELFH assays. CHOC 400/SV40 cells were treated with 2-AP prior to the ODP (1.5 h) and incubated in the presence of aphidicolin as in Figure 2. Control cultures received no 2-AP. At 16 h after metapahase, cells were transferred to fresh medium lacking aphidicolin and supplemented with [5′-^{3}H]dC and BrdU. Parallel cultures were processed for ELFH to verify the difference in origin specification as in Figure 2. After 30 min of labeling, DNA was purified from these cells and subjected to neutral pH density gradient centrifugation, to separate BrdU-substituted (HL) from unsubstituted (LL) DNA. Results (Figure 3) revealed that, under conditions in which ELFH assays demonstrated a lack of origin specification, these same cells initiated the synthesis of hybrid density BrdU-substituted DNA. To verify that this HL DNA consisted of a single strand of BrdU-substituted DNA hydrogen-bonded to a single strand of parental, unsubstituted DNA, an aliquot of this same BrdU/[5′-^{3}H]dC DNA was denatured in alkaline, and then subjected to alkaline pH density gradient centrifugation (Figure 3B). These results demonstrate that the DNA synthesized after 2-AP treatment resulted from the semi-conservative replication of nascent DNA strands.

To determine whether the nascent DNA labeled after 2-AP treatment was derived from active replication forks,

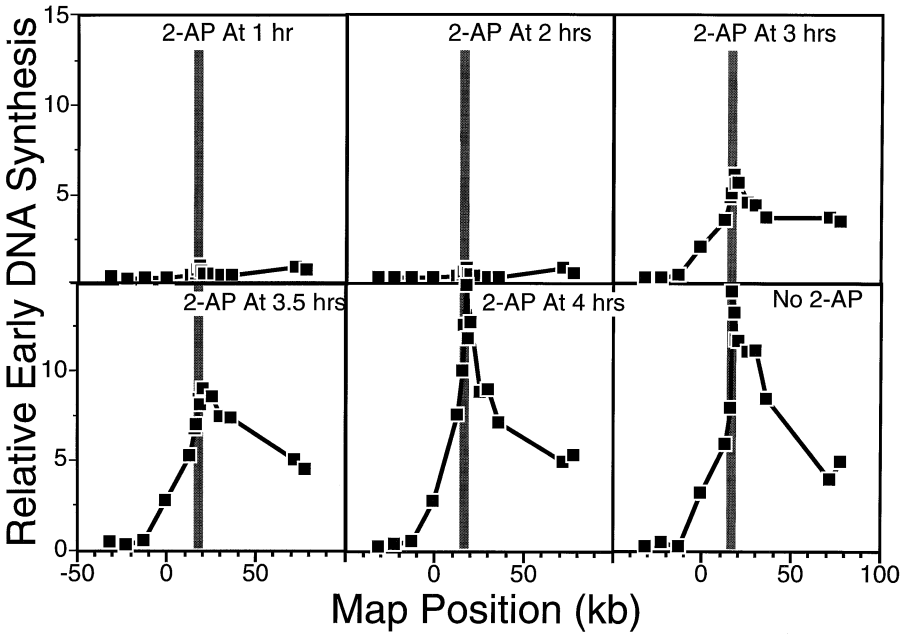

Fig. 2. SV40-transformed CHOC 400 treated with 2-AP prior to the ODP initiate replication in culture without specification of the DHFR origin. CHOC 400/SV40 cells were synchronized in metaphase plated into complete medium. At the indicated times, cells were transferred to medium containing 10 mM 2-AP. Aphidicolin was added to all cultures at 5 h after metaphase and cells were allowed to accumulate at the G_1/S-phase border in culture. At 16 h after metaphase, cells were washed free of aphidicolin, permeabilized with digitonin, and the earliest replicating nascent DNA chains were labeled briefly with [α-^{32}P]dATP. Sites of initiation of replication were mapped by the ELFH assay, described in Figure 1C. Similar results were obtained in three independent experiments. Five additional experiments were performed where 2-AP was added at 1.5 and 5 h after metaphase, with similar results.

we performed pulse–chase experiments and analyzed the size of replication products on alkaline agarose gels. CHOC 400/SV40 cells were incubated in the presence or absence of 2-AP added prior to the ODP (1.5 h), and in the presence of aphidicolin added at 5 h (Figures 2 and 3). At 16 h after metapahase, cells were washed free of aphidicolin, permeabilized, and nascent DNA strands were pulse-labeled with [α-^{32}P]dATP (Figure 2). Aliquots of these reactions were then chased with an excess of unlabeled dATP, and DNA products from these reactions were analyzed on alkaline agarose gels to determine the sizes of nascent DNA strands (Figure 4A). As described previously (Burhans *et al.*, 1990, 1991; Gilbert *et al.*, 1995), pulse-labeling of nascent strands from G_1/S-phase synchronized CHOC 400 cells reveals a mixture of small nascent strands (the size of mature Okazaki fragments) and larger strands presumably representing leading strands that have migrated variable distances (mostly 1–20 kb) away from their sites of initiation during the 11 h aphidicolin arrest. The largest leading strands may also result from small numbers ($<$5%) of contaminating S-phase cells that were present at the time of aphidicolin addition. As shown in Figure 4A–C, this distribution was nearly identical whether or not cells were treated with 2-AP prior to the ODP. Furthermore, during the chase period, small nascent strands were elongated to larger strands several kilobases in length. Conversion of these intermediates to higher molecular weight strands in permeabilized cells requires the addition of S-phase cytosolic factors (Anderson and DePamphilis, 1979). Importantly, the rate and extent of replication fork movement was nearly identical whether or not cells were treated with 2-AP (Figure 4A–C).

The results described above demonstrate that the overall size distribution of nascent replication intermediates is the same whether or not CHOC 400/SV40 cells were treated with 2-AP prior to the ODP. However, the possibility remained that the random initiation pattern obtained at the DHFR locus was due to an alternative type of DNA synthesis, represented in the higher molecular weight fractions of the pulse-labeled DNA that do not show an obvious precursor–product relationship. To address this concern, the smallest pulse-labeled nascent strands, which are clearly from growing replication forks, were purified and subject to the ELFH assay as described in Figure 2. Results (Figure 4D) confirmed that these small nascent replication intermediates were derived from sites distributed throughout the DHFR locus in cells treated with 2-AP prior to the ODP, whereas in the absence of 2-AP, the same population of small nascent strands was localized to the DHFR origin locus. Since the [α-^{32}P]-DNA strands used in this hybridization can be chased into higher molecular weight intermediates, we conclude that the administration of 2-AP prior to the ODP disrupts the pattern of initiation of genuine replication forks.

Although the detection of alternative start sites for replication required transformation with SV40, it is unlikely that their recognition is due to the initiation activity of the viral T antigen protein since: (i) initiation at apparently random sites required the administration of 2-AP prior to the ODP; (ii) Chinese hamster cells are not permissive for SV40 replication (Gilbert and Cohen, 1990); and (iii) stable binding of SV40 T antigen to DNA requires pairs of pentanucleotide recognition sites separated by approximately one turn of a DNA double helix, and positioned in a head-to-head orientation (Joo *et al.*, 1997), a configuration that is unlikely to occur at all in the CHO genome. A more likely interpretation of

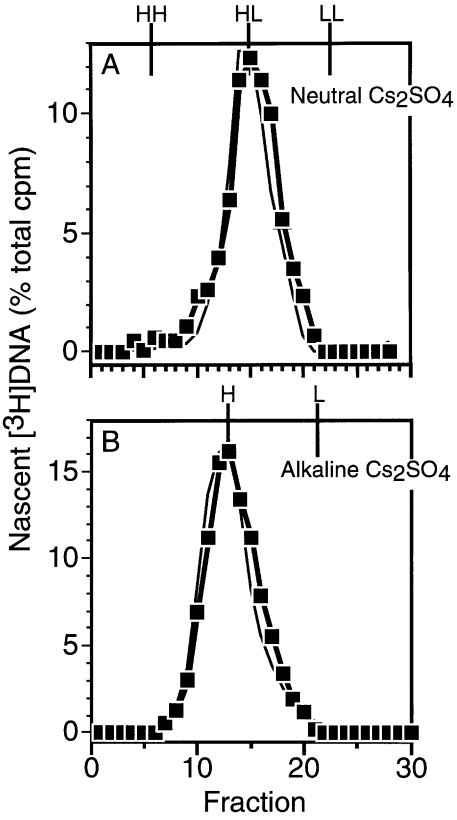

Fig. 3. DNA synthesis detected after 2-AP treatment results from semi-conservative DNA replication. CHOC 400/SV40 cells were synchronized in metaphase, released into G_1-phase, and 2-AP was administered 1.5 h later. Control cultures were not treated with 2-AP. Aphidicolin was added to all cultures at 5 h and cells were allowed to accumulate at the G_1/S-phase border up to 16 h after metaphase, as in Figure 2. Medium was changed to 2-AP- and aphidicolin-free medium supplemented with [5′-^{3}H]dC and BrdU. Duplicate cultures were processed for ELFH analysis as in Figure 2, to verify that replication initiated without specification of the DHFR origin in these experiments (data not shown). After 30 min of labeling, cells were lysed and their DNA was subjected to (**A**) neutral or (**B**) alkaline Cs_2SO_4 density gradient centrifugation. The percentage of c.p.m. present in each gradient fraction, out of a total of 29 000 and 122 000 c.p.m., incorporated into high molecular weight DNA for 2-AP-treated and untreated cells, respectively, is shown. Filled squares show the results from cells treated with 2-AP. Results with untreated cells processed in parallel are shown as a thin line. The positions of the peaks for marker DNA centrifuged to equilibrium in a separate gradient in the same experiment are indicated. Similar results were obtained in two independent experiments.

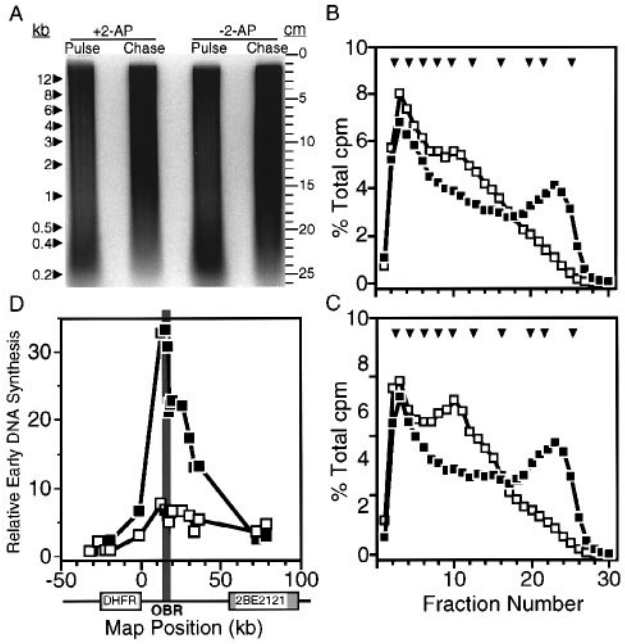

Fig. 4. Replication intermediates labeled after 2-AP treatment are derived from active replication forks. CHOC 400/SV40 cells were synchronized, treated with 2-AP at 1.5 h after metaphase, and allowed to accumulate at the G_1/S-phase border in the presence of aphidicolin exactly as described in Figures 2 and 3. Cells were then permeabilized with digitonin and the earliest replicating nascent DNA chains were labeled briefly with [α-^{32}P]dATP. After 2 min, part of each reaction was chased with unlabeled dATP for an additional 8 min. (**A**) DNA isolated from these reactions was then resolved in a 0.8% alkaline agarose gel. Aliquots of these reactions were also run on a 1.8% alkaline agarose gel to compare them directly with previously reported results (Burhans *et al.*, 1990, 1991; Gilbert *et al.*, 1995) (data not shown). Results from the 0.8% gel are shown because they reveal the sizes of nascent strands after the chase period more clearly. The left margin indicates the positions (arrowheads) and sizes (kb) of molecular weight markers run in parallel. Each lane of the gel was then divided into 1 cm fractions (shown on the right margin) and the percentage of total c.p.m. in each lane was plotted against the fraction number for cells either treated (**B**) or untreated (**C**) with 2-AP in the pulse (■) and chase (□) reactions. The arrowheads correspond to the molecular weight markers shown in (A). Total c.p.m. ($\times 10^{-6}$) in this experiment were: +2-AP (pulse), 6.9; +2-AP (chase), 12.7; –2-AP (pulse), 13.7; –2-AP (chase), 28.3. (**D**) The smallest population of α-^{32}P-labeled nascent strands in the pulse-labeling reaction (corresponding to fractions 19–26) after growth in the presence (□) or absence (■) of 2-AP was purified by electroelution and the genomic positions of these earliest labeled nascent replication intermediates were evaluated using the ELFH assay, as in Figure 2.

these results is that CHO initiation factors can initiate replication at many chromosomal sites. Normally, events taking place at the ODP select which of these sites will function as an origin. In the absence of transformation, inhibition of the ODP with 2-AP causes G_1-phase arrest, preventing initiation at these alternative sites. The transformation properties of SV40 allow CHOC 400 cells to overcome G_1-phase arrest, causing them to enter S-phase without passing through the ODP.

Transient 2-AP treatment delays passage of CHOC 400 cells through the ODP

The results described above show that chronic exposure of pre-ODP cells to 2-AP both inhibits the ODP and prevents CHOC 400 cells from entering S-phase. Since the specificity of 2-AP is not well defined, it was possible that G_1-phase arrest was not due to the presence of 2-AP during the ODP transition but resulted from the inhibition of a later step in the progression of cells through G_1-phase. For example, phosphorylation of the retinoblastoma tumor suppressor protein (Rb) takes place 6–8 h after metaphase in CHOC 400 cells (Wu and Gilbert, 1997), several hours after the ODP. G_1-phase arrest could be due to the inhibition of Rb phosphorylation and relief of this arrest due to the inactivation of Rb by SV40 T-Ag (Ludlow *et al.*, 1989). This possibility encouraged us to determine whether limiting 2-AP exposure to early G_1-phase would elicit G_1-phase arrest in CHOC 400. If G_1-phase arrest was due to the inhibition of a late G_1-phase kinase pathway, such as Rb phosphorylation, then transient exposure to 2-AP early in G_1-phase might allow CHOC 400 cells to enter S-phase after inhibition of the ODP, without requirement for SV40 transformation.

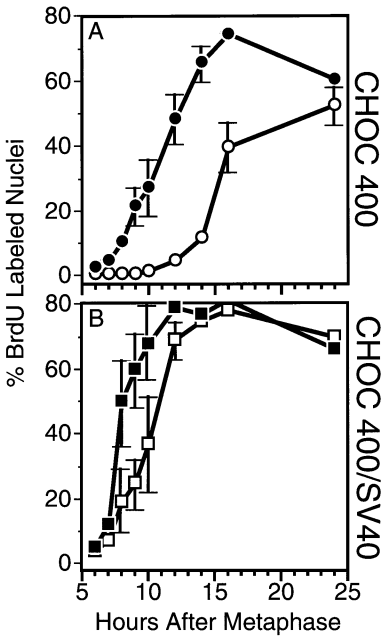

Fig. 5. An early G_1-phase arrest point is abrogated by SV40 transformation. Either (**A**) CHOC 400 (circles) or (**B**) CHOC 400/SV40 (squares) were synchronized in metaphase and plated to fresh medium. Half of the cultures were transferred to medium containing 10 mM 2-AP at 1.5 h after metaphase and transferred back to medium without 2-AP at 5 h after metaphase (open symbols). Control cultures (filled symbols) were untreated. Starting at 6 h after metaphase, aliquots of cells were pulse-labeled for 30 min with BrdU at the indicated times, and the percentage of cells in S-phase was evaluated by indirect immunofluorescence with an anti-BrdU antibody as in Figure 1A. Similar results were obtained when the entry of cells into S-phase was monitored by flow cytometry (data not shown).

To this end, both CHOC 400 and CHOC 400/SV40 cells were synchronized in metaphase and treated with 2-AP from 1.5–5 h after metaphase. At 5 h after metaphase, 2-AP was removed and at hourly intervals thereafter cells were pulse-labeled with BrdU for 30 min to determine the percentage of cells that had entered S-phase. Results (Figure 5A) revealed that although most CHOC 400 cells were able to recover from the 3.5 h 2-AP treatment, it resulted in a 5–6 h delay in their entry into S-phase. By contrast, progression of CHOC 400/SV40 cells into S-phase was barely affected by this transient 2-AP treatment (Figure 5B). Thus, brief exposure of CHOC 400 cells to 2-AP during early G_1-phase, 1–3 h prior to the phosphorylation of Rb, is sufficient to elicit G_1-phase arrest. SV40 transformation abrogates this G_1-phase arrest point.

To determine whether those CHOC 400 cells that recover from transient 2-AP treatment and enter S-phase initiate replication specifically at the DHFR origin locus, cells treated transiently with 2-AP from 1.5–5 h after metaphase were incubated in medium containing aphidicolin from 5–16 h after metaphase, and the sites of initiation of replication at the DHFR locus were mapped using the ELFH assay. Results revealed that although only 35% of the CHOC 400 cells were in S-phase at the time of release from aphidicolin (Figure 6A), replication initiated specifically within the DHFR origin locus (Figure 6B). Control cultures not treated with 2-AP entered S-phase efficiently and initiated replication specifically within the DHFR origin locus. These same cells were pulse-labeled with BrdU at 4, 8 and 12 h after release from the aphidicolin block to monitor their progression through S-phase (Figure 6A). These results demonstrated that the 2-AP-treated CHOC 400 cells were significantly delayed in their entry into S-phase, consistent with Figure 5A. Since we have shown that CHOC 400 cells treated with 2-AP during this same time interval do not pass through the ODP prior to the removal of 2-AP (Wu and Gilbert, 1997), CHOC 400 cells must pass through a delayed ODP after removal of 2-AP. In contrast, when CHOC 400/SV40 cells were exposed transiently to 2-AP, the same percentage of cells entered S-phase as with untreated cultures but selection of the DHFR origin was significantly inhibited (Figure 6C and D). Monitoring the progression of these cells through S-phase by BrdU labeling (Figure 6C) and by flow cytometry (data not shown) demonstrated an almost complete lack of delay after transient exposure to 2-AP, consistent with Figure 5B. From these results we conclude that SV40 transformation abrogates an early G_1-arrest point. The lack of G_1-phase arrest upon exposure of transformed cells to 2-AP allows entry into S-phase without specification of the DHFR replication origin.

Discussion

The results presented in this report demonstrate that the ODP in mammalian cells can be abrogated independently of the mechanisms that license chromatin and initiate DNA replication. This represents the first demonstration of chromosomal replication without origin specification in a system other than rapid early cleavage embryos, and supports the hypothesis that chromosome replication in higher eukaryotes does not require specific DNA sequences (Coverley and Laskey, 1994; Gilbert *et al.*, 1995; Lawlis *et al.*, 1996). These experiments also validate the utility of *Xenopus* egg extracts to study origin specification, since it was findings with nuclei introduced into these extracts that allowed us to predict conditions in which we could disrupt the pattern of replication initiation in proliferating cultured cells. We also provide evidence that origin specification requires a protein kinase pathway that is essential for progression of fibroblasts through early G_1-phase. When the ODP was inhibited by a transient 2-AP treatment during early G_1-phase, passage through the remainder of G_1-phase was delayed and removal of 2-AP was followed by passage through a delayed ODP. This G_1-phase arrest point was abrogated by SV40 transformation, resulting in the initiation of replication without passage through the ODP. Thus, the specification of origins may be an important G_1-phase hallmark for reasons other than to initiate semiconservative DNA replication.

Demonstration that the initiation of DNA replication at the CHO DHFR locus does not require specific origin sequences extends the dispensibility of origins beyond the specialized cases of *Xenopus* and *Drosophila* embryos. If replication origins are not necessary for replication, what then might be their function? We have monitored the fate of CHOC 400/SV40 cells after administration of 2-AP from 1.5–5, 1.5–16, and 5–16 h after mitosis by: (i) flow cytometry; (ii) following the changes in patterns of DNA replication foci that are characteristic of different stages

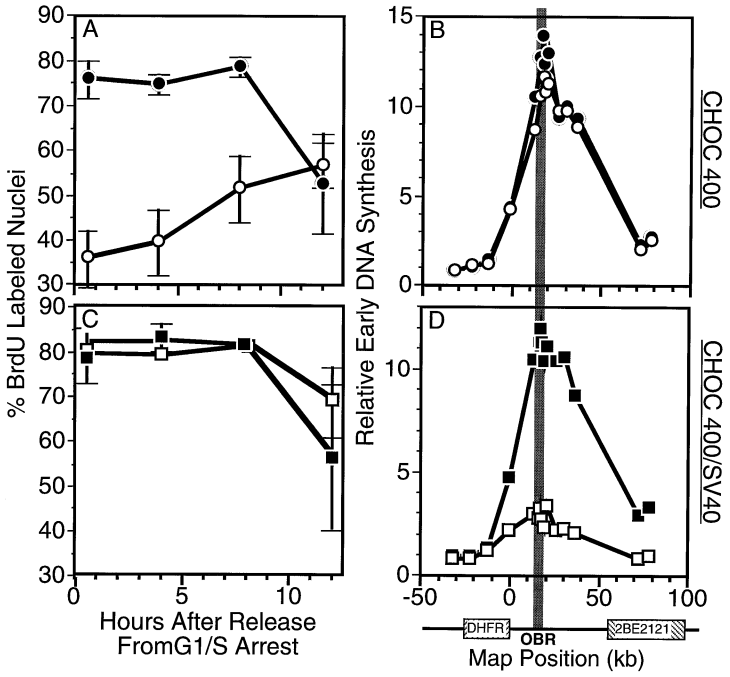

Fig. 6. CHOC 400 cells pass through a delayed ODP after transient 2-AP treatment. Either CHOC 400 (**A** and **B**) or CHOC 400/SV40 (**C** and **B**) were synchronized in metaphase and half of the cultures were transferred to medium containing 10 mM 2-AP from 1.5 to 5 h after metaphase (open symbols) as in Figure 4. Control cultures (filled symbols) were untreated. At 5 h after metaphase, aphidicolin was added to all cultures and cells were allowed to accumulate at the G_1/S-phase border to 16 h after metaphase. Aphidicolin was removed and cells were either pulse-labeled with BrdU (A and C) at the indicated times to evaluate the percentage of cells in S-phase as in Figure 4, or evaluated for the specificity of initiation using the ELFH assay (B and D) as in Figure 2. (A) and (C) show the mean of two experiments and the variation about the mean. The vertical shaded line in (B) and (D) shows the position of the previously mapped OBR. (B) shows the results of one of three independent experiments with similar results. (D) shows the results of one of six independent experiments with similar results. Some preference for initiation within the DHFR locus was reproducibly detected with cells transiently exposed to 2-AP (compare Figure 5D with the 1 and 2 h samples in Figure 2). This was expected, considering that a certain percentage of the CHOC 400/SV40 cells are not 2-AP resistant (Figure 4) and are likely to pass through a delayed ODP after 2-AP removal.

of S-phase (O'Keefe *et al.*, 1992); and (iii) determining the percentage of live and dead cells in the population (J.-R.Wu and D.M.Gilbert, unpublished). We found that prolonged treatments with 2-AP, whether administered before or after the ODP (1.5–16 or 5–16 h), severely delayed progression through S-phase and resulted in eventual cell death. This fatal phenotype is unlikely to be due to inhibition of the ODP, as those cells treated from 5–16 h initiate replication specifically at the DHFR origin (Figure 2). By contrast, CHOC 400/SV40 cells that were treated with 2-AP specifically during the ODP transition (1.5–5 h) progressed through S-phase into the next G_1-phase, and were passaged through several cell generations, without detectable loss of viability. Therefore, a single S-phase with aberrant origin selection is not sufficient to impair the viability of these SV40-transformed CHO cells in culture. This implies that origin specification serves a function that is not revealed within a single cell cycle, or is perhaps not realized at all in transformed cells grown in culture. For example, a disruption of initiation patterns could alter gene expression patterns in a way that is not lethal to cells in culture, but is essential for proper development.

This possibility offers a fresh interpretation for the contrast between random origin specification in the rapid early cleavage stages of *Drosophila* and *Xenopus* development and site-specific initiation at later stages of development and in cultured somatic cells (Figure 7). The selection of specific sites is likened to a check-point, such as the R-point, not required for proliferation *per se* but for the proper functioning of the nucleus in the context of differentiation. Passage through the ODP might reflect the completed re-assembly of the functional architecture of the nucleus after mitosis. The model implies that the transformed cell will still pass through the same cell-cycle transitions as the untransformed cell (i.e. ODP, R-point) when conditions are appropriate for normal cell proliferation, but will proliferate independently of those transitions if they are inhibited. By contrast, the nuclei of rapid early cleavage embryos are not engaged in activities that require a high degree of functional architecture. Thus, they have eliminated these controls points, oscillating between mitosis and S-phase, to facilitate rapid duplication of the genome.

We cannot say what aspects of the transformed phenotype allow entry into S-phase without passage through the ODP, however, the ability to proliferate in the presence of several kinase inhibitors is a property shared by cells transformed by a variety of means (Crissman *et al.*, 1991; Gadbois *et al.*, 1992). It will be interesting to examine whether origin choice can be abrogated in cells transformed by other oncogenes. We also cannot say whether G_1-phase arrest is a direct consequence of inhibition of origin specification, since the specificity of 2-AP is not well defined. It is possible that G_1-phase arrest is due to the inhibition of a parallel early G_1-phase kinase pathway which is required for passage through the ODP but is not directly associated with origin specification. Direct

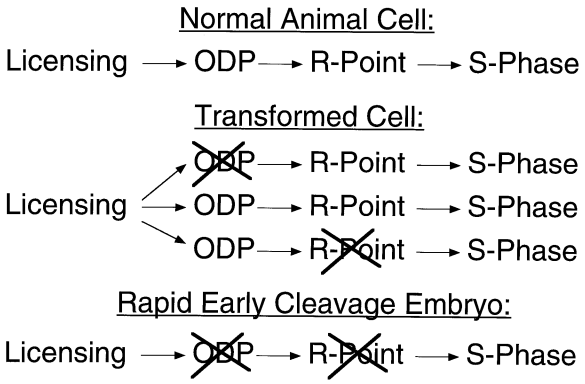

Fig. 7. Origin specification, like the R-point, may be required for G_1-phase progression but is dispensable for the initiation of DNA replication *per se*. It is hypothesized that specific DNA sequences are not required to initiate one round of semi-conservative DNA replication in higher eukaryotes. All that is needed is a signal to assemble pre-initiation complexes ('licensing') separated from and preceded by a signal to convert those pre-initiation complexes into active replication complexes (S-phase; see Gilbert, 1998). Origin specification may focus initiation to certain chromosomal loci for reasons that are important in the context of normal somatic cell function. In this model, a typical somatic cell must pass through both the ODP, and subsequently the R-point, in order to enter S-phase. The R-point responds to extracellular cues that dictate the choice between proliferation and withdrawal from the cell cycle (Zetterberg and Larsson, 1995). The ODP apparently responds to internal cues, since it proceeds on schedule in the complete absence of serum mitogens, or in nutrient-deficient medium (Wu and Gilbert, 1997). Inhibition of either of these steps will cause cells to arrest in G_1-phase. Entry into S-phase thereafter will be dependent upon a return to conditions that allow passage through these steps. Transformation of somatic cells by certain oncogenes can override either of these separate arrest points. Under conditions that favor the passage of normal somatic cells through G_1-phase, transformed cells also pass through the ODP and the R-point, specifying origins and phosphorylating Rb as would a non-transformed cell. However, the transformed cell will still be able to enter S-phase when these G_1-phase steps are inhibited. Finally, rapid early cleavage embryos do not have a G_1-phase, oscillating instead between alternating cycles of mitosis and S-phase. Thus, neither event takes place.

demonstration of a G_1-phase check-point associated with the selection of origins will require identification of the protein kinase pathway that is required for passage through the ODP.

Materials and methods

Cell culture and synchrony

CHOC 400 is a CHO cell derivative in which a 243 kb segment of DNA conatining the DHFR gene has been amplified ~500-fold by stepwise selection in methotrexate (Hamlin *et al.*, 1994). Complete medium for these cells consists of Dulbecco's modified Eagle's medium supplemented with non-essential amino acids and 5% fetal bovine serum. Cells were synchronized in metaphase by mitotic shake-off as described (Wu *et al.*, 1997) and, when appropriate, were subsequently synchronized at the G_1/S-phase boundary by incubation with 5 μg/ml aphidicolin (Calbiochem, 17823-T, dissolved in DMSO at 10 mg/ml) from 5 to 16 h after metaphase. For experiments with 2-AP, growth medium was replaced by medium containing 10 mM 2-AP (free base, Sigma A-3509) which was dissolved directly in medium and adjusted to pH 7.2. For transient treatment of 2-AP, after removal of medium containing 2-AP, cells were washed twice with phosphate-buffered saline (PBS) and once with growth medium, then incubated in growth medium. Transfer of metaphase cells to serum-deficient and serum-free medium was as described (Wu and Gilbert, 1997). Evaluation of the percentage of cells in S-phase by BrdU labeling and flow cytometry has been described (Wu *et al.*, 1997).

SV40 transformation of CHOC 400

2×10^5 CHOC 400 cells in 35 mm dishes were incubated at 37°C in 5% CO_2 for 24 h until the cell density reached 30–40% confluence. Cells were then co-transfected with a 10:1 mixture of SV40 DNA and the neomycin gene expression plasmid pcDNA3 (Gibco-BRL) using lipofectin reagent (Gibco-BRL) as instructed by the manufacturer. Cells were incubated in the presence of 1 mg/ml G418, and G418-resistant cells were selected. Thirty-five well isolated colonies were selected, expanded, and tested for T antigen expression by indirect immunostaining with monoclonal antibody PAB 101 (Gruss *et al.*, 1993) against SV40 T antigen (a gift from C.Gruss). Five of these cell lines stained positive for T-Ag. The laboratory designation of these cells lines is JH-1–JH-5. JH-1 is the cell line referred to as CHOC 400/SV40 in the text of this report.

Density gradient centrifugation

Cells synchronized at the G_1/S-phase border were washed twice with PBS and once with growth medium, then incubated in growth medium containing 30 μg/ml of BrdU (Sigma) and 8 μCi of [^{3}H]-cytidine (16.2 Ci/mmol; Amersham) for 30 min. Cells were lysed in 50 mM Tris, 10 mM EDTA, 0.4 M NaCl, 0.6% SDS, treated with RNAase A and Proteinase K and precipitated with isopropanol as described for the ELFH assay (Wu *et al.*, 1997). DNA samples were resuspended in 500 μl TE (10 mM Tris–HCl, 1 mM EDTA, pH 7.8), extracted once with phenol and once with chloroform, precipitated by adjusting the solution to 0.3 M sodium acetate and 70% ethanol, and pelleted in a microfuge. DNA samples were then resuspended in 100 μl *Eco*RI restriction enzyme buffer (New England BioLabs) and digested to completion with *Eco*RI. Density gradient centrifugation was performed as described (Gilbert and Cohen, 1987; Gilbert *et al.*, 1995).

Earliest labeled fragment hybridization (ELFH) assay

For mapping the sites of initiation when G_1 nuclei were introduced into *Xenopus* egg extract (Figure 1C), the ELFH assay was performed as described in detail elsewhere (Wu *et al.*, 1997). Briefly, intact nuclei were prepared by digitonin permeabilization and introduced into a *Xenopus* egg extract supplemented with aphidicolin. After 90 min, nuclei were washed free of aphidicolin and nascent DNA strands were labeled close to their sites of initiation with [α-^{32}P]dATP. Fifteen unique probes distributed over a 120 kb region that includes the DHFR ori-β were immobilized to nylon filters with a slot blot apparatus and hybridized to the ^{32}P-labeled early replication intermediates. Relative c.p.m. hybridized were obtained by phosphorimaging analysis and adjusted for variations in hybridization efficiency, dA content and probe size, as described (Wu *et al.*, 1997). These relative values were then plotted against the map positions of each probe.

For mapping the sites of initiation within cultured cells (Figures 2, 4 and 6), cells synchronized at the G_1/S-phase border were washed twice with PBS and once with growth medium, then incubated in growth medium for 5 min at 37°C. Cells were collected by trypsinization and permeabilized with digitonin (Wu *et al.*, 1997). Nuclei (typically 5×10^6) were resuspended with 7 μl 5× replication cocktail containing 50 μCi of lyophilized [α-^{32}P]dATP and replication intermediates were labeled for 2 min at 26°C as described (Gilbert *et al.*, 1995). Replication intermediates were isolated and hybridized to specific DHFR locus probes, as described above.

Alkaline gel electrophoresis and purification of small nascent strands

Replication intermediates were labeled with [α-^{32}P]dATP for 2 min at 26°C as described above. Part of that labeling reaction was then chased by supplementing the reaction with 100 μM dATP and 100 μM $MgCl_2$ for an additional 8 min at 26°C. Reactions were stopped with Lysis buffer (50 mM Tris, pH 7.8, 10 mM EDTA, 0.4 M NaCl, 0.6% SDS) containing Proteinase K (200 μg/ml) and incubated for 1 h at 65°C. [α-^{32}P]DNA was extracted once with phenol:chloroform:isoamyl alchohol (25:24:1) and once with chloroform:isoamyl alchohol (24:1), 0.35 volumes of saturated NaCl were added and the DNA was precipitated with 1 volume of isopropanol. The DNA pellet was washed twice in 70% ethanol and resuspended in 40 mM NaOH, 1 mM EDTA, 10% glycerol for 3 h at 65°C. Samples were then loaded onto both 0.8 and 1.8% alkaline agarose gels, prepared as described (Sambrook *et al.*, 1989). Both 0.8 and 1.8% gels were subjected to electrophoresis for 16 h at 40 volts. For analysis of the sizes of replication intermediates, gels were dried and exposed to a Molecular Dynamics Phosphorimager cassette. Using the Image Quant program, each lane of each gel was divided into thirty 1 cm fractions and the relative c.p.m. per fraction was determined. For preparation of

small nascent strands from the 0.8% gel, each lane of the wet gel was sliced manually into 1 cm fractions. The c.p.m. in each fraction was then determined by Cerenkov scintillation counting. [α-^{32}P]DNA from the fractions corresponding to the peak of smallest nascent strands (Figure 4) was isolated by electroelution using an Elutrap device (Schleicher and Schuell) as described by the manufacturer. This DNA was adjusted to 0.3 M Na acetate and 70% ethanol, precipitated and used as a probe in an ELFH assay, as described above.

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