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# Cloning of Nascent Monkey DNA Synthesized Early in the Cell Cycle

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**To study the structure and complexity of animal cell replication origins, we have isolated and cloned nascent DNA from the onset of S phase as follows: African green monkey kidney cells arrested in G1 phase were serum stimulated in the presence of the DNA replication inhibitor aphidicolin. After 18 h, the drug was removed, and DNA synthesis was allowed to proceed in vivo for 1 min. Nuclei were then prepared, and DNA synthesis was briefly continued in the presence of Hg-dCTP. The mercury-labeled nascent DNA was purified in double-stranded form by extrusion (M. Zannis-Hadjopoulos, M. Perisco, and R. G. Martin, Cell 27:155–163, 1981) followed by sulfhydryl-agarose affinity chromatography. Purified nascent DNA (ca. 500 to 2,000 base pairs) was treated with mung bean nuclease to remove single-stranded ends and inserted into the *Nru*I site of plasmid pBR322. The cloned fragments were examined for their time of replication by hybridization to cellular DNA fractions synthesized at various intervals of the S phase. Among five clones examined, four hybridized preferentially with early replicating fractions.**

DNA replication in eucaryotic cells is initiated at multiple origins separated by tens to hundreds of kilobase pairs of DNA (reviewed in references 11, 15, 17, and 21). Clusters of such origins and the DNA stretches whose replication they control (replicons) are activated and replicated in a defined spatial and temporal order (2, 3). In recent studies with specific gene probes, it has been demonstrated that unique DNA sequences replicate at defined S-phase intervals (4, 5, 13, 16, 20) and that the order of replication is colinear with the genomic arrangement of the genes or gene segments. However, a change in the genomic position of a given gene also changes its replication time.

The principles which govern the spatial and temporal organization of cellular replicons are not well understood. It remains to be established which DNA sequences serve as cellular replication origins and how activation of the origins is coordinated. Origins activated simultaneously might (i) share a common DNA sequence (24) or secondary structure or both recognized by cognate regulatory proteins or (ii) occupy a nuclear position favorable to activation at a given time (11). Such models are based on the assumption that different classes of replication origins are successively activated throughout the S phase (12, 14, 26). Nonetheless, activation of all origins at the onset of the S phase cannot be excluded. For example, the replication time of a given DNA sequence could be determined by the distance (5, 10) and, perhaps, by the presence of attenuation barriers traversed by a replication fork activated at the onset of the S phase. More information about the structure, function, genomic distribution, and nuclear location of individual and groups of replication origins will be required to examine these ideas.

Considerable progress towards understanding replication origins has been achieved by studying yeasts, in which presumptive chromosomal replication origins (*ars* elements) have been isolated by virtue of their ability to serve as replication origins for plasmid DNAs replicating extrachromosomally (6, 27, 28). The estimated number of *ars* elements, 400 per haploid yeast genome, is in close agreement to the estimated number of yeast replicons (7). Most of the *ars* elements are unique and dispersed within unique DNA sequences. On the other hand, repetitive *ars* elements are also found distributed among ribosomal genes and telomeres (8, 29).

Thus, one approach to the isolation of mammalian replication origins is based on the ability of DNA from various eucaryotic organisms to serve as a source of *ars* elements (27). Recently, Montiel et al. (22) identified several human DNA fragments which can serve as *ars* elements in yeast cells. These elements, which contain the yeast *ars* concensus sequence, were found at low frequency in human DNA and could only account for a small fraction of human replicon origins.

Another approach to the isolation of mammalian origins of DNA replication has been to use defined genomic probes such as the genes for hamster dihydrofolate reductase (16) and mouse immunoglobulin heavy chain (4, 5) to identify early replicating fragments within the corresponding replicons. This approach seems promising for the identification of specific origin sequences.

We have begun studies on replicon organization in mammalian cells by analyzing fragments of nascent DNA synthesized at the very onset of S phase. Because this DNA should be enriched for replication origins, its analysis ought to reveal any structural features common to origins. Moreover, it should reveal the genomic complexity of the earliest origins and whether they constitute a subset or include the entire set of origins. Here, we describe a procedure for isolating nascent DNA synthesized in G1/S-synchronized monkey kidney cells by use of extrusion (32) and mercury affinity labeling (9). We show that cloned segments derived from such a nascent DNA fraction contain early replicating sequences. Elsewhere (M. Zannis-Hadjopoulos, G. Kaufmann, S. S. Wang, J. Hesse, and R. G. Martin,

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submitted for publication; M. Zannis-Hadjopoulos, G. Kaufmann, S. S. Wang, and R. G. Martin, manuscript in preparation), we show that they share unique sequence properties.

## MATERIALS AND METHODS

**Materials.** Gene-Screen Plus paper and radioactive nucleotides were purchased from New England Nuclear Corp. (Boston, Mass.) or Amersham Corp. (Arlington Heights, Ill.); restriction enzymes, nick-translation kit, and T4 DNA ligase were purchased from Bethesda Research Laboratories, Inc. (Rockville, Md.) or New England Biolabs, Inc. (Beverly, Mass.); deoxynucleotides, bromodeoxyuridine, 5-Hg-dCTP, mung bean nuclease, and polynucleotide kinase were purchased from PL Biochemicals (Milwaukee, Wis.); and sulfhydryl-agarose was purchased from Pierce Chemical Co. (Rockford, Ill.). Elutip-d columns were obtained from Schleicher & Schuell, Inc. (Keene, N.H.) and used as described in the specifications of the manufacturer. Proteinase K and ribonuclease A were obtained from Boehringer Mannheim (Indianapolis, Ind.). The RNase was heated to 100°C for 5 min to inactivate any contaminating DNase. Ficoll 400 was obtained from Pharmacia Fine Chemicals, Inc. (Piscataway, N. J.). Aphidicolin was kindly provided by M. Suffness, National Institutes of Health. CV-1 monkey cells were grown in Dulbecco-Vogt medium supplemented with 5% fetal calf serum as previously described (32). The Fisher rat FR3T3 cells were obtained from Roland Seif.

**Synchronization of cells.** Cells were synchronized by a modification of published procedures (16, 23). CV-1 or Fisher rat 3T3 cells were plated at $10^6$ per 150-mm plate and incubated at 37°C in Dulbecco-Vogt medium supplemented with 5% fetal calf serum. (When chronically labeled cells were desired, medium was also supplemented with [$^3$H]thymidine [0.05 μCi/ml, 6.8 Ci/mmol]). After 3 days, when the monolayer reached ca. 30% of confluence, the medium was replaced with serum-free medium, and incubation was continued for 24 h. Next, medium supplemented with 20% fetal calf serum and aphidicolin (2 μg/ml) was added, and the cells were incubated for an additional 18 to 20 h. The drug-containing medium was then removed.

After removal of the aphidicolin, the plates were chilled and washed twice with phosphate-buffered saline (PBS) at 0°C. Next, the cells were either treated with bromodeoxyuridine (see below) or were incubated with 2 ml of PBS or medium without or with (for pulse-labeling) [$^3$H]-thymidine (100 μCi/ml, 78 Ci/mmol) for 1 min at 37°C by floating the plates in a water bath. The radioactive medium was removed, the monolayers were again washed with ice-cold PBS, and nuclei were prepared.

**Preparation of nuclei and cytosol.** Slight modifications were introduced into previously published protocols (1, 19). To accomodate the use of mercurated nucleotides, dithiothreitol was replaced with β-mercaptoethanol (9). The cells were washed twice with hypotonic buffer (10 mM sodium-HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.8], 1 mM $MgCl_2$, 2 mM β-mercaptoethanol, 5 mM KCl) at 0°C. The plates were drained well, and the cells were scraped with a rubber policeman and collected in 1 ml of hypotonic buffer per plate. The cell suspension was homogenized in a Dounce homogenizer (six strokes with pestle B). The suspension was centrifuged for 5 min at 1,000 × *g* (2,500 rpm in an HB-4 rotor) to separate the cytosol from the nuclei. The supernatant was brought to 0.1 M in KCl and centrifuged for 1 h at 100,000 × *g* (50,000 rpm in an SW 50.1 rotor). To this supernatant 0.6 g of solid ammonium sulfate per ml was added, and the precipitate was collected by centrifugation at 15,000 rpm in the SS34 rotor, redissolved in a minimal volume of buffer B (0.4 M ethylene glycol, 2 mM β-mercaptoethanol, 20 mM sodium-HEPES [pH 7.8], 50 mM KCl, 0.5 mM $MgCl_2$), and dialyzed against the same buffer. Meanwhile, the nuclear pellet from the first low-speed centrifugation was suspended in 1 ml per starting plate of Triton buffer (0.02% Triton X-100, 0.25 M sucrose, 0.5 mM $MgCl_2$, 2 mM β-mercaptoethanol, and 10 mM sodium-HEPES [pH 7.8]). The suspension from 20 plates was underlayered with 7 ml of the same buffer without Triton but with 15% Ficoll and centrifuged for 15 min at 4,200 × *g* (4,500 rpm in the HB-4 rotor). The supernatant, including the collar above the Ficoll layer, was aspirated, and the pellet was suspended first in 5 ml and then in 40 ml of potassium chloride-sucrose buffer (50 mM KCl, 0.25 M sucrose, and 2 mM β-mercaptoethanol, 0.5 mM $MgCl_2$, 10 mM sodium-HEPES [pH 7.8]) and centrifuged for 5 min at 2,000 × *g*. The pellet was suspended in a final volume of 300 μl of potassium chloride-sucrose buffer.

**In vitro DNA synthesis and isolation of nascent DNA.** Nuclei were incubated with cytosol, salts, and nucleotides in a reaction mixture (1.0 ml) containing the following: 1 mM ATP, 100 μM CTP, 100 μM GTP, 100 μM UTP, 10 μM dATP, 10 μM 5-Hg-dCTP, 10 μM TTP, 0.5 μM [α-$^{32}$P]dGTP (100 Ci/mmol), 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 65 mM KCl, 5 mM $MgCl_2$, 2 mM β-mercaptoethanol, 0.25 M ethylene glycol, 0.15 M sucrose, 10 to 15 mg of cytosol protein per ml, and 0.5 × $10^8$ to 1 × $10^8$ nuclei. The mixture was shaken for 10 min at 30°C, and the reaction was stopped by addition of 25 ml of potassium chloride-sucrose buffer without β-mercaptoethanol but with 0.1 mM nonradioactive dGTP. Control experiments indicated that this treatment resulted in the elongation of the nascent strands by <50 nucleotides. The nuclei were pelleted by centrifugation in the SS34 rotor, resuspended in another 25 ml of the above buffer, repelleted, and finally suspended in 1 ml of 10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA–RNase A (100 μg/ml) at 0°C. All subsequent procedures were carried out at 0°C unless specified otherwise. An equal volume of lysis buffer (1.2% sodium dodecyl sulfate, 0.2 M Tris-hydrochloride [pH 7.5], 20 mM EDTA, 0.01% proteinase K) was added, and after incubation overnight, the lysate was extracted with aqueous phenol and then with chloroform-isoamyl alcohol (24:1). The DNA was then incubated for 4 to 16 h at 50°C to extrude the nascent DNA (32). The DNA solution was layered over a 5 to 30% neutral sucrose gradient (in 0.2 M NaCl, 10 mM Tris-hydrochloride [pH 7.5], 1 mM EDTA) and centrifuged at 4°C in an SW27 rotor for 20 to 24 h at 25,000 × *g*. Fractions of 1.5 ml were collected from 5 ml above the bottom of the tube, and acid-insoluble radioactivity was determined in 0.05-ml samples. In parallel experiments, greater than 30% of the pulse-labeled nascent DNA and less than 1% of the chronically labeled parental DNA appeared in the top two-thirds of the gradient. Fractions from that region of the gradient corresponding to molecular weights of ca. 3 × $10^5$ to 1 × $10^6$ were pooled and concentrated by ethanol precipitation. The pellet was dissolved in 1.0 ml of TN buffer (50 mM NaCl, 50 mM Tris-hydrochloride [pH 7.5]) and the solution was applied to a 10-ml sulfhydryl agarose column at room temperature equilibrated with TN buffer. The column was washed with 3 volumes of the same buffer, and the effluent was collected in 2.5-ml fractions. The nascent DNA was then eluted with TN buffer containing 0.1 M β-mercaptoethanol. (We have found that for best results, the

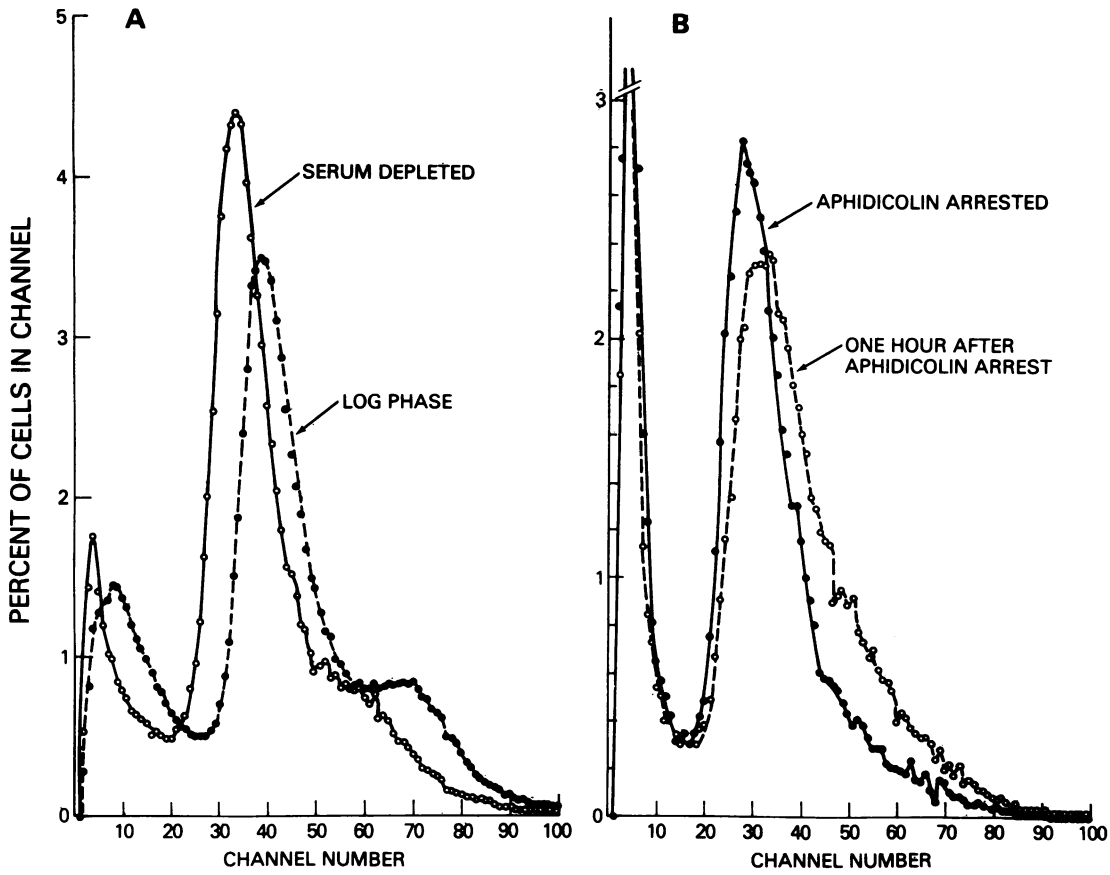


FIG. 1. Microfluorometric analyses of CV-1 cells before and after aphidicolin treatment. (A) CV-1 cells were analyzed during log-phase growth or after 48 h of serum depletion. (B) The medium was then supplemented with serum plus aphidicolin for 10 h, and the cells were analyzed immediately or 1 h after removal of the aphidicolin.

column should be stored in 1 mM β-mercaptoethanol buffer, rinsed with the 0.1 M buffer before use, and finally washed with at least 20 volumes of mercaptan-free buffer.) The eluate was concentrated on an Elutip-d column (Schleicher & Schuell), and eluted with 1 M NaCl.

**Cloning of the nascent DNA chains into the *Nru*I site of pBR322.** pBR322 DNA was extensively digested with *Nru*I, deophosphorylated, and rebanded in cesium chloride-ethidium bromide gradients to eliminate any undigested DNA. The linearized pBR322 DNA was added to the agarose-thiol column eluate. The mixture was precipitated by addition of 3 volumes of ethanol, dried, redissolved in 50 μl of digestion buffer, and digested for 30 min at 0°C with 1 U of mung bean nuclease. After treatment with nuclease, the DNA solution was again extracted with phenol and then with ether, precipitated with alcohol, redissolved in ligase and buffer for blunt-end ligation, and incubated for 16 h at 15°C. The mixture was used to transform *Escherichia coli* HB 101 as previously described (30, 32).

**Isolation of DNA at different stages in S phase and dot blotting.** Generally, three flasks of cells (150 $cm^2$ each) were used for each time point. The cells were grown and starved as above. In some experiments, the cells were synchronized with aphidicolin as above. Alternatively, the medium was supplemented with 20% fetal calf serum for 8 h and then further supplemented with 1 mM hydroxyurea for an additional 10 h. Finally, the monolayers were rinsed twice with PBS and again incubated with medium containing 20% serum. At different intervals, the medium was supplemented with 0.2 mM bromodeoxyuridine. After incubation with bromodeoxyuridine, the plates were washed with PBS and covered with 2 ml of lysis buffer (50 mM Tris-hydrochloride [pH 7.5], 10 mM EDTA, 0.6% sodium dodecyl sulfate. The lysates were combined, digested overnight with proteinase K (0.5 mg/ml), extracted with phenol and chloroform-isoamyl alcohol, precipitated by addition of 3 volumes of alcohol, rinsed with 70% ethanol, dried, and digested to completion with the restriction endonuclease *Bgl*I. The DNA solutions were then brought to a density of 1.70 g/ml with CsCl. Ethidium bromide (1 μg/ml) was added for visualization of the banded DNA, and the samples were centrifuged to equilibrium in a Vt 65 vertical rotor. Heavy-light (HL) DNA was separated from light-light (LL) DNA, and each was rebanded to equilibrium. The DNA fractions were diluted with 3 volumes of water and concentrated by alcohol precipitation. Equal samples of DNA (0.1 to 1 μg in different experiments) were applied to Gene-Screen Plus paper. The papers were hybridized and washed at 65°C as recommended by the manufacturer. Nick-translated probes with high specific activity (25) were prepared to the different *ors* sequences. The portion of each origin-enriched sequence (*ors*) used as probes were as follows: *ors*1, nucleotides 119 to 880 (*Hin*fI to *Pvu*II, a 760-bp fragment); *ors*2, nucleotides 169 to 460 and 460 to 703 (*Stu*I to *Bgl*II, 243- and 291-bp fragments); *ors*3-L, nucleotides 163 to 332 (*Bgl*II, a 170-bp fragment), and *ors*3-R, the *Alu* fragment, nucleotides 462 to

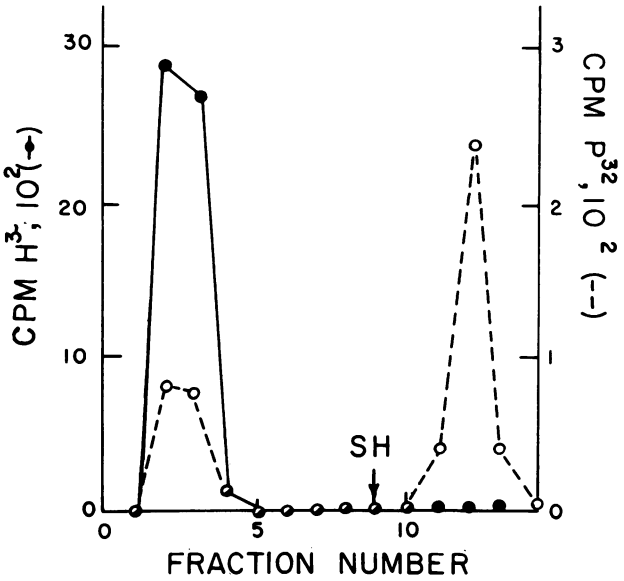


FIG. 2. Selective retention of nascent mercurated DNA on sulfhydryl agarose. Monkey CV-1 cells chronically labeled with [$^3$H]thymidine were synchronized with aphidicolin. Nuclei prepared from these cells were pulse-labeled with [$\alpha$-$^{32}$P]dGTP and Hg-dCTP. The nascent DNA was extruded and fractionated by sedimentation in a neutral sucrose gradient as detailed in the text. The extruded DNA fractions of 10S to 20S were chromotographed on a sulfhydryl agarose column as described in the text. Symbols: ●, [$^3$H]DNA; ○, [$^{32}$P]DNA.

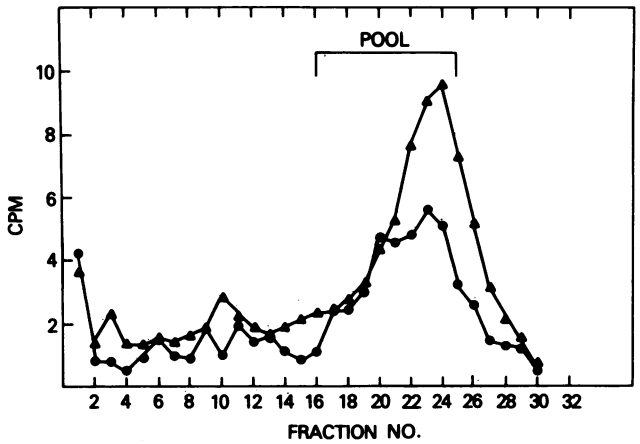


FIG. 3. Sucrose gradient fractionation of extruded DNA. After pulse labeling with [$^3$H]thymidine in vivo (●), nuclei were prepared, and the nascent DNA was extended with $^{32}$P-dGTP (▲) in the presence of Hg-dCTP. The DNA was purified, and the extruded nascent strands were fractionated by sucrose gradient centrifugation as described in the text. Fractions are quantified as times 10 for $^3$H and as times $10^2$ for $^{32}$P.

1162 (*Hin*fI, a 700-bp fragment); *ors*4, nucleotides 416 to 819 (*Bam*HI to *Sma*I, a 403-bp fragment); and *ors*5, nucleotides 95 to 428 (*Xba*I to *Hae*III, a 333-bp fragment), (Zannis-Hadjopoulos et al., manuscript in preparation).

## RESULTS

**Isolation of nascent DNA by extrusion and affinity chromatography.** CV-1 (monkey) and FR3T3 (rat) cells were synchronized with aphidicolin (16, 23). Briefly, 30 to 40% confluent cell layers were starved for serum to arrest the cells in G1. After 24 to 48 hours, serum and aphidicolin were added to stimulate entry into the S phase; both were added at the same time to prevent the onset of DNA replication. Upon removal of the drug, greater than 70% of the cells incorporated [$^3$H]thymidine during a subsequent 8-h period, as judged by autoradiography. After this period, incorporation subsided. When [$^3$H]thymidine was added for 1 h before removal of the drug, no incorporation of radioactivity into acid-precipitable material was detected. When [$^3$H]thymidine was added for 1 h intervals after removal of the aphidicolin, an initial low incorporation was found, followed by a dramatic rise during the second and third hours of S phase and then a rapid decline (data not shown). Rates of DNA synthesis based upon [$^3$H]thymidine incorporation are, of course, only crude estimates, since pool sizes may vary during S phase. We estimated that the amount of nascent DNA synthesized during the first minute of S phase was less than 0.1% of the bulk DNA. This estimate was based on the following: (i) S phase lasts 6 to 8 h (<500 min), (ii) the rate of DNA synthesis during the first hour of S phase was two- to threefold lower than the average value during the 8 h after removal of the aphidicolin block, and (iii) <30% of the cells failed to enter S phase. Hence, a $10^3$ or somewhat higher degree of purification seems to be needed to achieve purity of this nascent DNA fraction.

The reliability of the synchrony technique was also judged by flow microfluorometry (Fig. 1). Serum depletion led to a dramatic drop in the number of cells in the S and $G_2$ phases. (Curve-fitting analyses to estimate the precise percentages of cells in $G_1$, S, and $G_2$ phases are of little significance, since CV-1 cells tend to form multinucleate cells in low levels of serum.) After 10 h in 20% serum plus aphidicolin, the great majority of viable cells were still in $G_1$ but commenced DNA synthesis within 1 h after removal of the aphidicolin.

The method developed to purify the nascent DNA was based on two procedures: extrusion (32) and mercury affinity labeling (9). Extrusion is a process in which the parental strands of a (linearized) replicating DNA molecule reassociate while at the same time the newly synthesized daughter strands are displaced in double-stranded form, probably through rotational diffusion of the interstrand crossing or Holliday intermediate. The nascent DNA can subsequently be separated from the bulk on the basis of its low molecular weight (see below). The other procedure, incorporation of a mercurated DNA precursor, allows selective retention of the

TABLE 1. Purification of nascent DNA by extrusion and mercury-affinity chromatography

| DNA fraction | $^3$H (cpm) | $^{32}$P (cpm) | Fold purification[a] | Yield (%) |
|---|---|---|---|---|
| Crude from nuclei | 10,600,000 | 100,000 | 1 | 100 |
| Extruded DNA (10S to 20S) | 22,000 | 9,950 | 48 | 10 |
| Post SH-Sepharose | 460 | 6,980 | 1,620 | 7 |

[a] Purification is calculated as $^{32}$P activity/$^3$H activity relative to that of the crude DNA fraction from nuclei taken as 1.

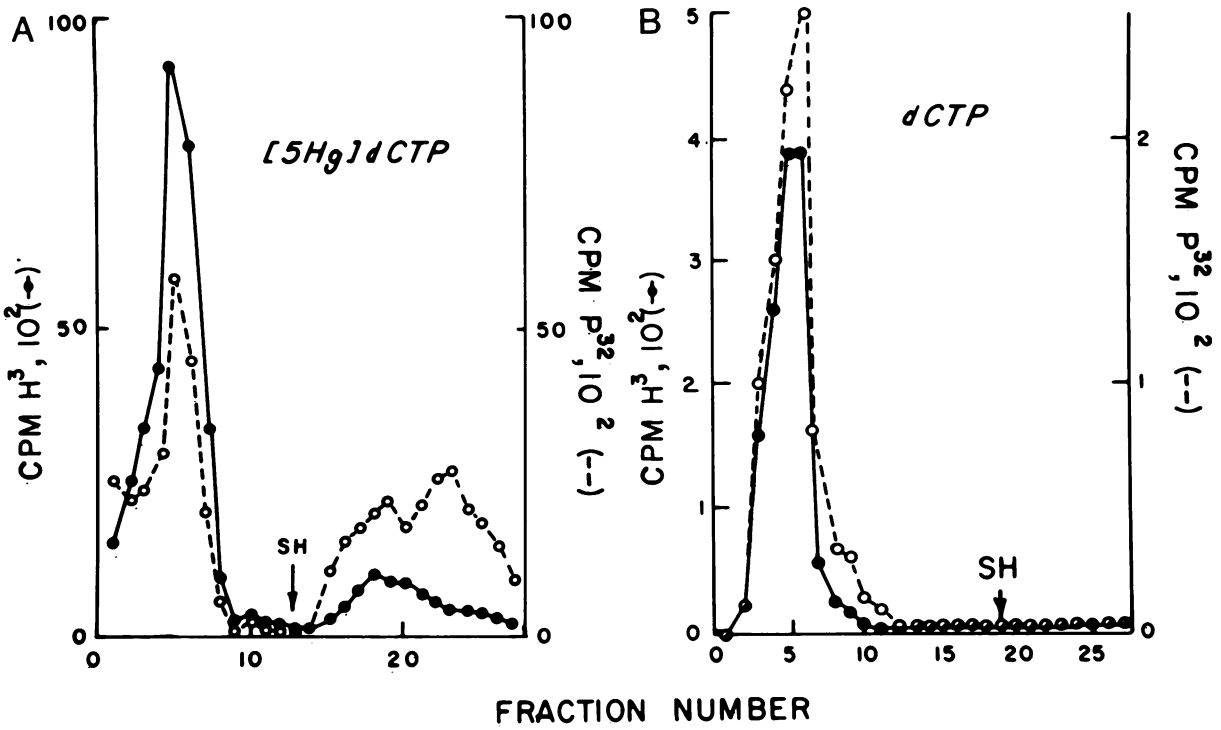


FIG. 4. Retention of in vivo-synthesized DNA on sulfhydryl agarose through in vitro extension with a mercurated precursor. Extruded DNA fractions, pulse-labeled in vivo with [$^3$H]thymidine and in vitro with [$\alpha$-$^{32}$P]dGTP and Hg-dCTP (A) or dCTP (B), were chromatographed on sulfhydryl-agarose as described in the text.

nascent DNA on sulfhydryl-agarose from which it is quantitatively recovered with a thiol-containing buffer (Fig. 2).

The effectiveness of these steps was examined with cellular DNA chronically labeled in vivo with [$^3$H]thymidine and subsequently pulse-labeled in isolated nuclei with [$\alpha$-$^{32}$P]dGTP and Hg-dCTP. The overall degree of purification of the $^{32}$P-labeled nascent DNA compared with that of the $^3$H-labeled bulk DNA was about 1,600-fold (Table 1). Since the precise amount of nascent DNA synthesized in the isolated nuclei was not known, it was not possible to establish whether absolute purification of the nascent DNA was actually achieved. Nevertheless, the degree of purification attained suggests that the procedure is adequate for the isolation of pure or nearly pure nascent DNA synthesized during the first minute of the S phase.

To isolate DNA synthesized during the onset of the S phase, cellular DNA synthesis was released from arrest, and the DNA was pulse-labeled for 1 min at 37°C with [$^3$H]thymidine in vivo and subsequently labeled with [$\alpha$-$^{32}$P]dGTP and Hg-dCTP in isolated nuclei. After extrusion and separation by sucrose gradient centrifugation (Fig. 3), DNA ca. 500 to 2,000 base pairs (bp) in length was chromatographed on sulfhydryl-agarose. About 20% of the $^3$H-labeled DNA and 40% of the $^{32}$P-labeled DNA were retained on the affinity column (Fig. 4a). In contrast, no labeled DNA was bound when a control preparation made with dCTP rather than Hg-dCTP was used (Fig. 4b). The DNA which bound to sulfhydryl-agarose was eluted and then concentrated by passage through an Elutip-d column, which retains single-stranded DNA. This step increased the ratio of the in vivo label to the in vitro label, probably due to selective loss of contaminating $^{32}$P-labeled Okazaki fragments.

**Cloning of the G1/S-synchronized DNA.** Synchronized nascent DNA (both CV-1 and FR3T3) purified as described above was treated with the single-strand-specific mung bean nuclease to generate blunt ends, ligated into the *Nru*I site of plasmid pBR322, and cloned in *E. coli*. The transformation frequency for the vector plasmid DNA ligated in the absence of the nascent DNA was ca. $2 \times 10^3$ colonies per $\mu$g, and that for vector plasmid DNA ligated in the presence of the nascent DNA was $2 \times 10^4$ colonies per $\mu$g. All of the following data refer to clones obtained from the CV-1 cells; the clones from the FR3T3 cells will be discussed in a subsequent communication. Approximately 4,000 colonies resistant to ampicillin but sensitive to tetracycline were isolated. Of the first 600 examined, 43 (ca. 7%) were found to contain inserts, as determined by digesting plasmid DNA preparations with *Bgl*I nuclease, which cleaves the plasmid DNA at two sites (928 and 1162) flanking the *Nru*I site at 971. These cloned segments were termed *ors* segments.

Probes were prepared from the five plasmids containing segments of >500 bp in length, *ors*1 through *ors*5, and used to analyze the 43 initial isolates. Only one, *ors*2, gave a positive signal with any colony other than itself. The positive isolate was designated *ors*6.

**Replication time of *ors* sequences.** To examine whether the *ors* fragments were actually derived from DNA replicated early in the S phase, we hybridized these fragments to bulk monkey kidney DNA synthesized at various time intervals of the S phase. The bulk DNA fractions were prepared by pulse-labeling aphidicolin-synchronized or hydroxyurea-synchronized cells with bromodeoxyuridine at intervals at 1.5 h. The HL and LL DNA fractions were banded twice on CsCl density gradients, and samples of the purified DNAs were denatured and spotted on nitrocellulose filters. The HL DNA fractions contained DNA replicated at the given S-phase interval. However, the LL DNA fractions were not devoid of these DNA sequences, since not all of the cells

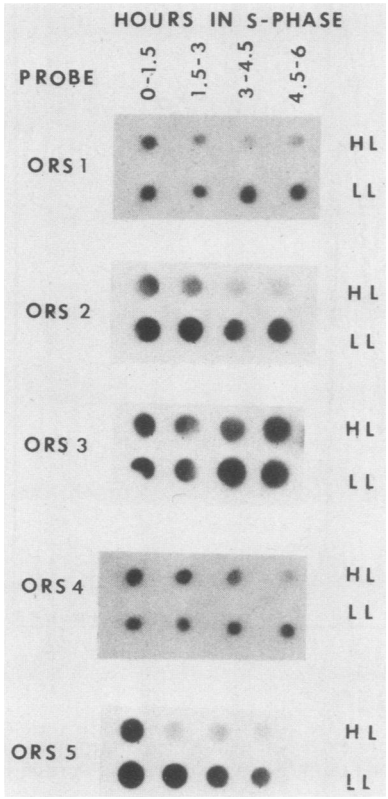


FIG. 5. Replication time of *ors* sequences. HL and LL DNA fractions corresponding to the indicated S-phase intervals were dotted on Gene-Screen Plus membranes and probed with nick-translated DNA probes derived from the *ors* sequences as detailed in the text.

synthesized DNA after the treatment with aphidicolin (Fig. 5).

The dot blots were probed with $^{32}$P-labeled DNA (25) from the plasmids containing *ors*1 through *ors*5. These plasmids had CV-1 DNA inserts ranging from 509 to 1,172 bp (Zannis-Hadjopoulos et al., submitted for publication; Zannis-Hadjopoulos et al., in preparation). As shown (Fig. 5), the five *ors* probes hybridized with the HL DNA of the interval from 0 to 1.5 h. However, only *ors*1, *ors*2, and *ors*5 exhibited a clear preference for this early DNA. The *ors*3 sequence hybridized evenly with all HL DNAs replicated up to 6 h into the S phase. The *ors*4 sequence hybridized with HL DNAs of up to 3 to 4.5 h but not to the later fraction. Thus, all sequences but *ors*3 hybridized preferentially with CV-1 DNA replicated early in S phase. Elsewhere, we show that clone *ors*3 contains an *Alu* sequence (18) as well as another reiterated AT-rich sequence (Zannis-Hadjopoulos et al., submitted for publication).

## DISCUSSION

We have described here a procedure for purifying nascent animal cell DNA suitable for cloning in a plasmid vector and the application of this procedure to the cloning of DNA synthesized at the onset of the S phase. Although it remains to be proven that this method can yield bona fide early replicating DNA enriched for origins (*ori*), the properties of the clones already isolated encourage further exploration of this approach.

Four of the five sequences examined hybridized with various degrees of preference to an early replicating CV-1 DNA fraction. The incomplete preference for the earliest fraction shown by the *ors* probes can be attributed to some extent to the presence of reiterated sequences in these clones (Zannis-Hadjopoulos et al., submitted for publication; Zannis-Hadjopoulos et al., in preparation). Although the later HL DNA fractions could be contaminated with earlier replicating DNA due to imperfect synchrony, Iqbal and Schildkraudt have also found some late replication with *ors*2 and *ors*5 sequences by using HL DNA from human cells synchronized by elutriation (personal communication). Further analyses should establish how effective the procedure is in isolating early replicating DNA. Nevertheless, the fact that most of the *ors* probes we examined preferentially hybridized to the early replicating DNA fraction indicates that the collection is enriched for nascent DNA synthesized at the onset of the S phase and does not represent a random collection of contaminating bulk DNA. Clearly, the synchronization procedures themselves may be introducing artifacts with regard to the timing of activation of cellular origins. Based on the average rate of mammalian fork progression, we expect that these cloned fragments are origin proximal and perhaps, if not disrupted during the isolation and cloning steps, contain replication origins at or close to their centers (30).

Without a functional test for animal cell replication origins, we can only examine the possibility that the *ors* sequences are replication origins by considering common features in *ors* DNA sequences and by analogy with known procaryotic and papovavirus origin structures. The following are some common sequence properties of the *ors* clones; these properties are presented in detail elsewhere (Zannis-Hadjopoulos et al., submitted for publication; Zannis-Hadjopoulos et al., in preparation). (i) Two of the 43 clones contained an identical 196-bp nucleotide sequence which is moderately reiterated in the genome. Hence, its selection cannot be considered random. (ii) Six *ors* sequences out of nine (including *ors*2 and *ors*6) contain a 21-bp concensus sequence. (iii) There is an abundance of AT-rich regions. (iv) There is a high frequency of potential stem-loop structures. We have previously reported that extruded nascent DNA from G1/S-synchronized cells is also enriched for "snap-back" sequences; the extruded DNA is expected to be enriched for replication origins (31). This finding suggested to us that the presence of potential stem-loop structures may be a characteristic in which mammalian replication origins are similar to procaryotic and papovavirus origins.

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