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Nucleosome segregation at a defined mammalian chromosomal site

(DNA replication/simian virus 40-transformed cells/chromatin/replication origin)

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ABSTRACT **When animal cells replicate chromatin under conditions precluding new histone biosynthesis, half of the daughter DNAs are devoid of nucleosomes and are sensitive to staphylococcal nuclease. DNA sequences resistant to nuclease are associated with preexisting nucleosomes, which redistribute to progeny DNA duplexes during replication. We labeled newly replicated DNA sequences in a simian virus 40 (SV40)-transformed Chinese hamster cell clone with 5-bromodeoxyuridine (BrdUrd) in the presence and absence of a protein biosynthesis inhibitor, emetine. We resolved single-stranded BrdUrd- and dT-DNA sequences protected from nuclease digestion by nucleosomes and determined from which strands of the integrated viral DNA parental template (dT) and newly replicated progeny (BrdUrd) sequences were derived. Because we knew that the cell clone studied contained all of its integrated SV40 DNA at a single chromosomal site, we were able to determine that preexisting nucleosomes segregated to only one of the two daughter duplexes containing the integrated viral sequence. Additionally, in the presence of emetine, the integrated viral origin of replication, ORI_{sv}, appeared not to function as a chromosomal replication origin, perhaps reflecting the drug's effect on synthesis of SV40 large tumor antigen.**

Papovavirus (simian virus 40 and polyoma virus) minichromosomes consist of supercoiled viral DNA complexed with 21 octameric histone units arranged as nucleosomes (1). Replication of simian virus 40 (SV40) minichromosomes appears to be a semidiscontinuous process (2, 3). On the leading side of diverging viral replication forks (5′ → 3′ in the direction of fork progression), progeny DNAs are synthesized processively. On the retrograde side of replication forks (5′ → 3′ opposite the direction of fork movement), SV40 DNAs are synthesized via short Okazaki fragments. In contrast, replication of polyoma virus minichromosomes appears fully discontinuous, in that DNA synthesis on both sides of the replication fork has been shown to involve Okazaki intermediates (4, 5).

During replication, nucleosomes distribute to daughter DNAs as intact octameric units (6–8). In the presence of drugs or conditional mutations inhibiting *de novo* protein (histone) biosynthesis (6, 9), only half of the newly replicated DNA is associated with nucleosomes. Preexisting nucleosomes appear to segregate preferentially to the leading, processive side of SV40 DNA replication forks in cells treated with protein biosynthesis inhibitors (10). Presumably, these drugs do not affect the pattern of nucleosome segregation.

Detailed analysis of papovavirus replication has been possible primarily because the structures of viral replicons, the locations of their replication origins, and their transcriptional organizations are known precisely. To obtain similarly detailed information regarding replication forks that traverse an animal cell's chromosome, one must have equally precise knowledge of specific chromosomal DNA sequences. One also must be able to distinguish progeny DNA sequences that derive from each of the two parental DNA strands. We have developed such a system based on SV40 DNAs integrated as single-copy sequences into various clone-specific sites of SV40-transformed Chinese hamster cell chromosomal DNAs. We used restriction endonucleases and the Southern blot procedure to map integrated SV40 DNA in several transformed cell lines (11) and characterized a pair of single-stranded nick-translated [^{32}P]DNA hybridization probes that rigorously discriminate between DNA sequences deriving from SV40 early (E) and late (L) DNA strands (12).

The experiment we describe was designed to assess segregation of nucleosomes complexed with a specific sequence of integrated SV40 DNA. The data we obtained indicate that segregation of preexisting histones is asymmetric and, in the clone of cells analyzed, is directed toward the progeny duplex containing newly synthesized E-DNA sequences exclusively. This result suggests that the integrated viral origin of replication (ORI_{sv}) in one SV40-transformed cell line does not function as a chromosomal replication origin when the cell cannot synthesize new proteins.

METHODS

Cells. The tsA209 SV40-transformed Chinese hamster lung fibroblast cell clone, CHLA209L5, was obtained originally from R. G. Martin (National Institutes of Health). The transformed character and growth properties of this cell line have been described by others (13). We determined that each CHLA209L5 cell contains 0.85–0.90 viral genome equivalents of SV40 DNA sequence integrated at a single chromosomal site and that the integrated viral sequence is not excised to replicate autonomously under the culture conditions used in our experiments (11, 12). Fig. 1 summarizes the restriction endonuclease map determined for this integrated SV40 DNA sequence (11). Assignment of E- and L-strand 5′ → 3′ polarities in the integrated sequence was based on the orientation of the viral early gene (extending from the *Bgl* I site through the *Taq* I site to the *Bam*HI site). CHLA209L5 was grown in monolayer cultures at 33°C in Dulbecco's modification of Eagle's medium containing 10% (vol/vol) fetal calf serum (GIBCO).

Preparation of BrdUrd-DNAs. Subconfluent monolayer cultures of CHLA209L5 (4×10^7 cells) were incubated at 33°C in medium containing 10 μM 5-bromodeoxyuridine (BrdUrd), 10 μM 5-fluorodeoxyuridine, and, where indicated, 2 μM emetine·HCl. This concentration of emetine blocks Chinese hamster cell protein biosynthesis virtually completely (9). Under these conditions, Chinese hamster cells fully substitute BrdUrd for thymidine (dT) in progeny DNA (9, 11). After 24 hr, cells were harvested and washed in phosphate-buffered sa-

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Abbreviations: SV40, simian virus 40; E and L, early and late (referring to time of synthesis of DNA); BrdUrd, 5-bromodeoxyuridine; ORI_{sv}, viral origin of replication; T antigen, tumor antigen.

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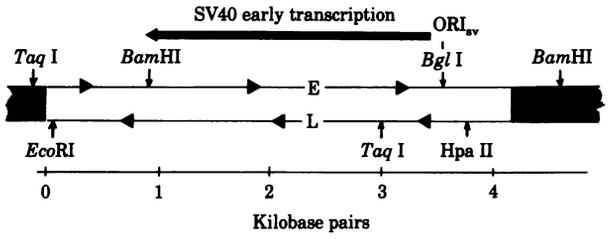

FIG. 1. Physical map of the SV40 DNA sequence integrated into CHLA209L5 chromosomal DNA. Several "one-cut" restriction endonuclease cleavage sites are located within the integrated sequence, and the location and polarity of the SV40 early gene transcription product are indicated (11). 5′ → 3′ polarities of the integrated E- and L-DNA strands have been inferred from SV40 DNA physical and transcription maps (14) and are indicated (→). The SV40 origin of DNA replication, ORI$_{sv}$, has been positioned at the unique *Bgl* I cleavage site. Chinese hamster cell DNA sequences flanking the integrated viral DNA are represented in black.

line, and nuclei were isolated as described (9). Staphylococcal nuclease (Worthington, 15, 200 units/mg of protein) was added, and suspensions were incubated with shaking at 37°C for 40 min. As illustrated in Fig. 2, most of the DNA extracted from nuclei after incubation with staphylococcal nuclease for 40 min was in chromatin fragments containing a single nucleosome ($n = 1$). Much smaller amounts of the DNA were found in chromatin fragments containing two to four nucleosomes. After digestion, mixtures were adjusted to 0.01 M EDTA/0.1% Sarkosyl. Nuclear lysates were treated with heat-inactivated RNase A (25 μg/ml) at 37°C for 60 min and then with autodigested Pronase (100 μg/ml) for an additional 18 hr (12). Preparations were extracted repeatedly with chloroform/isoamyl alcohol, 24:1 (vol/vol), and the DNA fragments were precipitated in 67% (vol/vol) ethanol at −20°C.

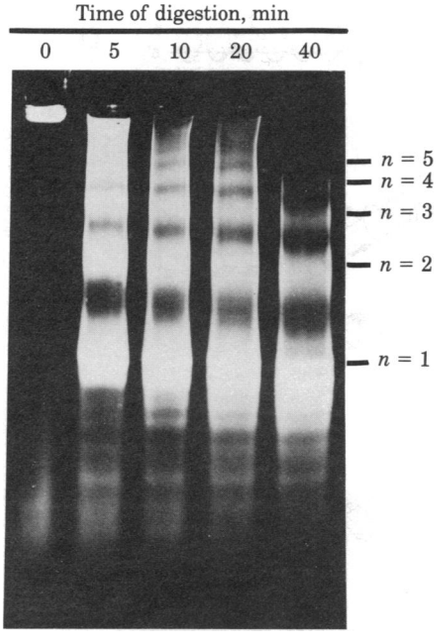

FIG. 2. Digestion of CHLA209L5 chromatin with staphylococcal nuclease. Chinese hamster cell nuclei were treated with 4.8 units of nuclease per 10^8 nuclei per ml at 37°C for the times indicated at the top of the figure. At each time, an aliquot of the reaction mixture was removed, and the digestion was terminated by adding EDTA and Sarkosyl (see text). DNAs were purified and analyzed by electrophoresis on a composite gel of 3.5% (wt/vol) acrylamide plus 0.6% (wt/vol) agarose at 75 V for 2 hr. The gel was stained with ethidium bromide (0.5 μg/ml) in water and photographed.

To obtain radiolabeled density marker DNA fragments, 10^7 Chinese hamster cells, previously grown for 3 days in medium containing 1.8 μM [^{14}C]dT (0.1 μCi/ml; 1 Ci = 3.7×10^{10} becquerels), were cultured for 24 hr at 33°C in medium supplemented with 10 μM [^{3}H]BrdUrd (1 μCi/ml) plus 10 μM 5-fluorodeoxyuridine. Nuclei were isolated and digested with staphylococcal nuclease. The resulting DNA fragments were purified as described above.

BrdUrd-containing (progeny) and dT-containing (parental) DNA strands were resolved from duplex fragments by isopycnic centrifugation in alkaline Cs_2SO_4 gradients. The precipitated DNA fragments were dissolved in 4.5 ml of 0.1 M NaOH/0.01 M EDTA with a small amount of radioactive tracer DNA ([^{14}C]dT-DNA and [^{3}H]BrdUrd-DNA). Solutions were adjusted to a density of 1.479 g/cm^3 by adding solid Cs_2SO_4, and the DNAs were centrifuged to equilibrium in a vertical rotor (Sorvall, TV865). Gradients were harvested from the bottom, and 0.2-ml fractions were collected dropwise. Trichloroacetic acid-insoluble DNA (^{3}H and ^{14}C) was analyzed in an aliquot of each fraction (20 μl) by double-label scintillation counting procedures (9). A typical gradient profile is illustrated in Fig. 3. Distributions of DNA within gradients invariably were broad, likely reflecting the size range of the DNA fragments (150–800 base pairs; see Fig. 2). However, in all cases we were able to obtain reasonably pure fractions of heavy BrdUrd-DNA and light dT-DNA, as illustrated by the shaded areas in Fig. 3. DNAs in pooled gradient fractions were twice precipitated as −20°C in 67% ethanol.

Hybridization-Titration of SV40 E- and L-DNA Sequences. SV40 form I DNA was labeled with ^{32}P to a specific activity of $1.5–2 \times 10^8$ cpm/μg by nick-translation (12). Labeled DNA was denatured and the strands were separated by using SV40 complementary RNA formed by transcription *in vitro* and hydroxyapatite column chromatography. Resulting E- and L-DNA probes contain SV40 DNA sequences derived from all regions of the viral genome (12).

BrdUrd- and dT-DNA fragments isolated from Cs_2SO_4 gradient fractions (see above) were prepared for hybridization-titration as described (12). The samples were incubated at 68°C with an excess of SV40 E- and L-DNA hybridization probes in a total volume of 10.1 μl under mineral oil. After 48 hr, hy-

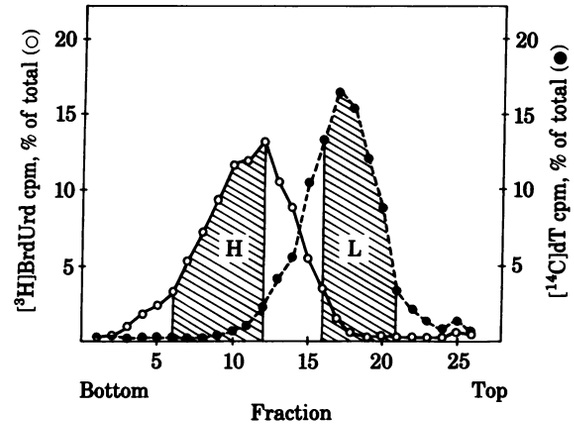

FIG. 3. Isopycnic centrifugation of Chinese hamster cell DNA fragments on an alkaline Cs_2SO_4 gradient. Duplex BrdUrd-DNA fragments were mixed with a small amount of radiolabeled tracer DNA ([^{3}H]BrdUrd plus [^{14}C]dT), denatured in alkali, and centrifuged in a Sorvall TV865 rotor to equilibrium (50,000 rpm, 20°C, 24 hr) in an alkaline Cs_2SO_4 gradient. Fractions of heavy, BrdUrd-containing, DNA (H) and light, dT-containing, DNA (L) were pooled as indicated to minimize cross-contamination.

bridization-titration mixtures were analyzed by hydroxyapatite column chromatography (12).

RESULTS AND DISCUSSION

The design of this experiment was adapted from one used by Seidman *et al.* (10) to investigate nucleosome segregation in autonomously replicating SV40 minichromosomes. In our experiment, cells were cultured in medium containing BrdUrd; nucleosomal particles were prepared; and newly synthesized, progeny BrdUrd-DNA was purified from parental DNA on the basis of its density (see above). As indicated in Fig. 4, two results were possible: If preexisting nucleosomes segregated to only one side of the replication fork(s) traversing the integrated viral sequence (i.e., conservative segregation), SV40 BrdUrd-DNA sequences surviving nuclease digestion (boldface lines) would have derived from only one strand of SV40 DNA (Fig. 4 *A*, *B*, and *C*). In contrast, if preexisting nucleosomes distributed to both sides of the replication fork (random distribution), nuclease-resistant SV40 BrdUrd-DNA sequences would have derived equally from both strands of the integrated viral DNA (Fig. 4 *D*, *E*, and *F*). Both models illustrated predict that SV40 sequences within unsubstituted, nucleosomal dT-DNAs (thin lines, Fig. 4) will include parental duplex DNAs in front of the replication fork and template single strands in daughter duplex DNA fragments. Hence, light dT-DNAs should include sequences derived from both SV40 DNA strands.

That we study an integrated SV40 DNA sequence offers additional opportunities to interpret this experiment. The SV40 sequence integrated into CHLA209L5's DNA includes a replication origin (ORI_{sv}) known to function during the virus's lytic growth cycle (see Fig. 1) and presumed to function as a chromosomal replication origin in CHLA209L5 at 33°C (11). ORI_{sv} is located 600–900 base pairs from the rightmost virus DNA–cell DNA junction illustrated in Fig. 1. If ORI_{sv} functions as a chromosomal replication origin in the presence of a protein synthesis inhibitor, two replication forks will diverge from ORI_{sv}. On the basis of the restriction endonuclease map in Fig. 1, we expected that a replication fork moving from integrated ORI_{sv} toward the viral *Taq* I site would contain SV40 L-strand BrdUrd-DNA sequences on its leading side and E-strand BrdUrd-DNA sequences on its retrograde side. Similarly, we expected that a replication fork moving from integrated ORI_{sv} toward the viral *Hpa* II site would contain SV40 E-strand BrdUrd-DNA on its leading side and L-strand BrdUrd-DNA sequences on the retrograde side. Thus, if preexisting nucleosomes were to segregate asymmetrically between daughter DNA duplexes in Chinese hamster cell chromosomes (Figure 4 *A*, *B*, and *C*), nucleosomal BrdUrd-DNAs would include both E- and L-strand SV40 sequences in a ratio dictated by both the direction of nucleosome segregation and the position of the functional replication origin.

Logarithmic phase CHLA209L5 cells were cultured for 24 hr in BrdUrd medium at 33°C, as described in *Methods*. One group of cultures contained emetine (2 μM), and a control group did not. Emetine blocks progression of Chinese hamster cells throughout early and mid-G_1 phase (unpublished data). Thus, only cells beyond late G_1 incorporate BrdUrd into DNA after emetine is added to medium (9). Because the integrated SV40 sequence is replicated by CHLA209L5 exclusively during very early S at 33°C (11), only cells within the late G_1 to early S interval of the growth cycle when emetine was added were ex-

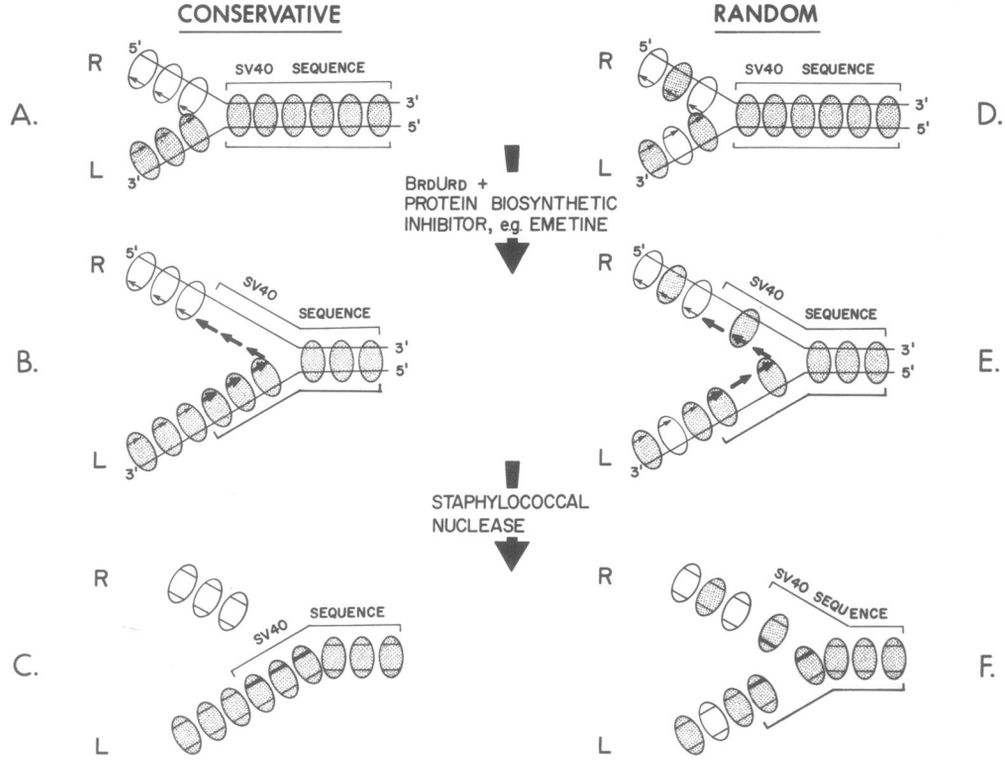

FIG. 4. A region of the CHLA209L5 chromosome containing the integrated viral sequence is represented schematically as it might replicate according to alternative experimental predictions: conservative segregation of preexisting nucleosomes (structures *A*, *B*, and *C*) versus random distribution of preexisting nucleosomes (*D*, *E*, and *F*). In each structure depicted, parental duplex DNA is drawn as a pair of continuous lines, and progeny DNAs synthesized by a fully discontinuous process are represented by short line segments. Polarities of DNA strands are indicated by labeling parental 5′ and 3′ termini and by arrows in progeny DNA (5′ → 3′). DNAs synthesized in the presence of BrdUrd are drawn in boldface. Nucleosomes are represented by oval structures; stippled nucleosomes decorated the parental DNA duplex, and unshaded nucleosomes are newly synthesized. In all structures, progression of the replication fork is from left to right; and leading (L) and retrograde (R) sides of the fork are indicated.

pected to incorporate BrdUrd into the integrated viral DNA. In medium lacking emetine, of course, all cycling cells should have replicated the SV40 DNA sequence, because the labeling period (24 hr) was approximately equal to the cells' generation time at 33°C (unpublished data).

Cells were harvested; nuclei were isolated and digested with staphylococcal nuclease; and the resulting nucleosome DNA fragments were purified as described in *Methods*. Two preparations of DNA (purified from cells treated with BrdUrd in the presence and absence of emetine) were denatured and resolved into their component BrdUrd- and dT-DNA single strands. This yielded four fractions of single-stranded DNA whose contents of SV40 sequence were determined by hybridization-titration. Titrations on each fraction were carried out with both SV40 E-strand and L-strand [^{32}P]DNA probes. The former provided a measure of each fraction's L-DNA sequence content; the latter, of each fraction's E-DNA sequence content. Hybridization-titration data are summarized in Table 1.

From the ratios of BrdUrd-DNA to dT-DNA within Cs_2SO_4 gradient samples we estimate that CHLA209L5 cells replicated almost all (98%) of their DNA during the 24-hr BrdUrd treatment in the absence of emetine (Table 1). In contrast, during the 24 hr of BrdUrd treatment in the presence of emetine, only 19% of the cells' DNA was replicated. This confirmed our expectations (see above), because only cells between late G_1 and late S at the time of emetine addition should have incorporated BrdUrd into their DNA; and on the average those cells synthesized approximately one-half a genome's equivalent of BrdUrd-DNA. In the absence of emetine, approximately equal amounts of SV40 E- and L-DNA sequences were detected in the BrdUrd- and dT-DNA strands purified from nucleosomes (Table 1). Both E- and L-DNA sequences of SV40 were distributed equally between newly synthesized BrdUrd-DNA strands and parental dT-DNA strands. These data also confirmed predictions ascribed to this experiment (see above).

When emetine was added to culture medium during treatment with BrdUrd, only E-strand SV40 DNA sequences were detected in the progeny BrdUrd-DNA strands. No L-strand SV40 DNA sequences were observed (Table 1). The assay was sensitive enough to have titrated as little as 1 pg of SV40 DNA sequence—i.e., 1 part in 10^7 of the 10-μg samples tested. In contrast, parental dT-DNAs isolated from cultures treated with emetine contained comparable amounts of SV40 E- and L-DNA sequences.

Table 1. Effect of emetine on the stability of newly replicated SV40 E- and L-DNA sequences integrated into the chromatin of Chinese hamster cell clone A209L5

Sample	Hybridization-titration: cpm in duplex L-[^{32}P]DNA	E-[^{32}P]DNA	E-DNA/L-DNA
Without emetine, 98% of DNA replicated			
BrdUrd-DNA (102.6 μg)	23,559	21,532	1.09
dT-DNA (106 μg)	22,291	22,634	0.99
With emetine, 19% of DNA replicated			
BrdUrd-DNA (10.06 μg)	5,232	<100	>52.30
dT-DNA (117 μg)	23,099	16,551	1.40

The extent of DNA replication in each cell population was estimated from the relative amounts of BrdUrd- and dT-DNAs obtained from a Cs_2SO_4 gradient, as described (15). The amounts of DNA in hybridization-titration samples were measured spectrophotometrically, assuming that 1 A_{260} unit = 40 μg of single-stranded DNA. E- and L-DNA probes (350 pg) labeled with ^{32}P by nick translation (1×10^8 cpm/μg) were added to each sample as a 20- to 200-fold excess over the amount of SV40 sequence anticipated. Data are presented as cpm of ^{32}P probe rendered double stranded by the sample of single-stranded DNA. In a control experiment, 0- to 60-pg aliquots of sonicated SV40 form I DNA were mixed with 20 μg of *Escherichia coli* DNA, and these were titrated by using both E- and L-DNA hybridization probes (data not shown).

Data in Table 1 support two conclusions directly: (*i*) Digestion of CHLA209L5 chromatin with staphylococcal nuclease selectively destroyed daughter duplex DNA fragments containing progeny SV40 L-strand sequences when chromatin replication involved only preexisting nucleosomes, and thus segregation of preexisting nucleosomes at this chromosomal site was conservative (see Fig. 4). (*ii*) Because we did not observe an 8:1 or 1:8 mixture of SV40 E- and L-DNA sequences among the progeny BrdUrd-DNA fragments protected by preexisting nucleosomes, ratios predicted from the restriction map of the integrated SV40 DNA sequence (Fig. 1), the integrated ORI_{sv} appeared not to function as an initiation site for chromosomal DNA replication in the presence of emetine. Because only progeny E-strand BrdUrd-DNA sequences were protected from nuclease by preexisting nucleosomes, the replication origin used to initiate synthesis of the integrated SV40 sequence appeared to have been located outside the viral sequence. Moreover, it appeared that all replication forks traversed the integrated viral DNA in the same direction, protecting progeny E-DNAs with preexisting nucleosomes exclusively. Initiation of DNA replication at ORI_{sv} during virus lytic growth requires an interaction between SV40 large tumor (T) antigen and the ORI_{sv} site (16, 17); and SV40 T antigen is synthesized by transformed cells primarily as they enter S phase (18). Thus, in the presence of emetine, S phase CHLA209L5 cells were not able to synthesize T antigen as they entered S and for this reason, perhaps, did not initiate replication at the integrated SV40 replication origin. This interpretation is consistent with observations suggesting that newly synthesized T antigens exhibit the greatest binding affinity for ORI_{sv} DNA sequences (19, 20).

In autonomous SV40 minichromosomes, preexisting nucleosomes segregate toward the leading side of replication forks (10). It was possible to deduce that fact from an experiment similar to the one reported here because the position of ORI_{sv}, the direction of replication fork progression, and the 5′ → 3′ polarities of the SV40 E- and L-DNA strands all were known. Prior to our experiment, we also knew the 5′ → 3′ polarities of the integrated SV40 E- and L-DNA strands (Fig. 1). However, because chromosome replication appeared not to initiate at the integrated ORI_{sv}, our data do not establish the direction of fork progression through this region of the Chinese hamster chromosome unambiguously. If we assume that nucleosome segregation was toward the leading side of the replication fork, as observed in autonomously replicating SV40 DNA (10), we deduce from the integrated SV40 DNA strands' polarities that replication initiates to the left of the viral sequence depicted in Figure 1 when protein (T antigen?) biosynthesis is inhibited by emetine.

Seidman *et al.* (10) pointed out that preexisting nucleosomes segregate to the leading side of replication forks and protect parental DNA transcriptional templates selectively in replicating SV40 and chicken cell chromatins. They suggested that organization of a DNA sequence thus might reflect both its transcription and replication activities. Our data (Table 1) indicate that progeny, not parental, SV40 E-DNA, the transcriptionally active template in SV40-transformed cells (14), segregated with preexisting nucleosome cores in a CHLA209L5 chromosomal replication fork in the presence of emetine. Previously we observed that temperature affects the replication program of in-

tegrated viral DNA in this clone, presumably by influencing the interaction between the SV40 tsA209 gene product, thermolabile T antigen, and integrated ORI_{sv} (11). Similarly, it is likely that altered replicon organization, such as appears to have been mediated by inhibition of T antigen biosynthesis in this experiment, might affect transcriptional activity of the SV40 early gene.

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