

ENRICHMENT AND VISUALIZATION OF SMALL REPLICATION UNITS FROM CULTURED MAMMALIAN CELLS

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ABSTRACT

DNA from cultured Chinese hamster cells has been fractionated to yield a population of DNA enriched for replicating molecules. Molecules containing replication structures were analyzed by electron microscopy, and replicon size was estimated. The enrichment procedure takes advantage of single-stranded regions characteristic of replicating molecules, and the greater affinity of mercuric ion for single-stranded rather than native DNA. After interaction with low concentrations of HgCl_2 , DNA with bound mercury is separated from the bulk of the DNA by virtue of its increased buoyant density in an isopycnic Cs_2SO_4 gradient. When DNA from cells labeled with ^3H thymidine for 45 s is interacted with HgCl_2 and banded in Cs_2SO_4 , the DNA with the highest specific activity is found in a dense region of the gradient. The high specific activity DNA behaves kinetically like nascent DNA since the radioactivity can be chased into main band if the cells are incubated for a further 2 h in excess unlabeled thymidine.

Electron microscope analysis of the DNA in the enriched fraction confirmed that it contains a substantial fraction of molecules with replication structures. The level of enrichment is about 25-fold compared to unfractionated DNA or DNA taken from the main band of the $\text{Hg}^{++}/\text{Cs}_2\text{SO}_4$ gradient. Of the replicating molecules visualized, 85% possessed a single replication structure. All molecules with multiple replication forms contained replicon sizes less than 5 μm , ranging from 0.2 to 4.5 μm . Replicon size was determined by measuring the distance from the center of one replication structure to the center of the adjacent replication structure on the same molecule. The replicons observed in this study are far smaller than can be detected by DNA fiber autoradiography and are in the same size range as the very small replication units reported in embryonic systems.

KEY WORDS DNA · replication units · replicon size · cesium gradients · mammalian cells

Autoradiographic analysis of metaphase chromosomes from cells pulse-labeled with ^3H thymidine has revealed that DNA replication takes place at many regions along the length of a given chromosome (20). A corollary to this finding is that a chromosome must possess multiple replication

initiation sites. Hydrodynamic approaches and DNA fiber autoradiography have substantiated this observation, and have also been used to estimate the size of the replicating unit or replicon, i.e., the distance between adjacent replication origins (10, 16). Estimates of replicon size in mammalian systems have ranged from as high as 200 μm (14) down to 4 and 5 μm (10, 20; for a review, see reference 5). In cultured Chinese

hamster cells, Huberman and Riggs (10), using DNA fiber autoradiography, reported replicon sizes at both extremes of this range, with an average value of about 30 μm .

A major shortcoming of DNA fiber autoradiography is that it cannot resolve replicons much smaller than 4 or 5 μm . That smaller replicons do in fact exist in nonmammalian systems has been demonstrated by electron microscopy of DNA from early insect embryos. DNA from both *Drosophila* and *Cochliomyia* possesses tandem replication bubbles with "eye to eye" distances, i.e., the distance between adjacent replication origins, as small as 0.2 μm with average replicon sizes about 3 μm . The advantage of working with early embryonic systems is the brevity of the S phase, which can be as short as 10 min (1, 11, 23). Since the entire genome replicates during that 10 min, DNA preparations are enriched for replicating molecules. Analysis of replication by electron microscopy is therefore feasible. As the S phase lengthens with development (1), the probability of finding replicating molecules decreases. In cultured Chinese hamster V79 cells, the S phase lasts between 6 and 8 h (17) rendering a direct analysis of replication by electron microscopy very tedious, even in synchronized cells.

Visualization of replicating DNA by electron microscopy requires a method that enriches for replicating molecules. The technique developed takes advantage of two observations. The first relates to a physical property characteristic of replicating DNA, namely that single-stranded regions exist at replication forks. These single-stranded regions have been demonstrated by both biophysical means (8) and electron microscopy (12). The second observation is that heavy metals such as mercuric ion preferentially bind to single-stranded rather than native DNA (15).

Since mercuric ion preferentially associates with single-stranded DNA, it seemed reasonable that, by interacting DNA with low levels of HgCl_2 , molecules containing replication structures would preferentially bind with the mercuric ion because of their associated single-stranded regions. These molecules could then be separated from the bulk of the DNA by centrifugation in Cs_2SO_4 due to their increased buoyant density. When DNA from cells pulse-labeled with [^3H]thymidine is treated in this manner, the specific activity of the DNA in the dense region of the gradient is about four- to fivefold higher than that of the rest of the DNA. When the density-shifted peak of specific activity

was examined by electron microscopy, it was substantially enriched in structures consistent with replicating DNA molecules. In all cases where multiple replication forms were observed, replicons (eye to eye distances) were less than 5 μm , and some were as small as 0.2 μm .

MATERIALS AND METHODS

Cell Culture

Chinese hamster V-79 cells, originally derived by Ford and Yerganian (7), were cultured and maintained as monolayers. Cells were grown in minimal essential medium (MEM) supplemented with fetal calf serum (15%), lactalbumin hydrolysate (2%), glutamine (0.4 mM), trypsin (20.0 $\mu\text{g}/\text{l}$), 50 U/ml penicillin, and 50 $\mu\text{g}/\text{ml}$ streptomycin at 37°C in 5% CO_2 (18). In experiments involving cell synchronization, the cells were synchronized by mitotic selection after a short Colcemid treatment as previously described (18). The progress of cells through the cell cycle was monitored by autoradiographic determination of percent labeled nuclei after a 15-min pulse label with [^3H]thymidine, and the mitotic index at successive intervals after collection of mitotic cells (18).

Pulse Labeling and DNA Extraction

Cells were grown in 150 \times 15 mm tissue culture dishes (Lux Scientific Corp., Newbury Park, Calif.) containing 25.0 ml of supplemented MEM until they were in late exponential growth, and used when three-quarters confluent. The medium was decanted, and cells were pulse-labeled for 45 s with [*methyl*- ^3H]thymidine (100 $\mu\text{Ci}/\text{ml}$, 20 Ci/mmol-50 Ci/mmol, New England Nuclear Corp., Boston, Mass.). After 45 s the medium was quickly removed, the cells were immediately lysed by addition of 5.0 ml of lysing solution (0.15 M NaCl, 0.1 M EDTA, 2% sodium dodecyl sulfate, and 100 $\mu\text{g}/\text{ml}$ pronase, Calbiochem, San Diego, Calif.), and the DNA was extracted as previously described (16). The lysate was incubated at 37°C for 6 h and gently shaken overnight with an equal volume of phenol saturated with sodium acetate, pH 5.0. After centrifugation, the aqueous layer was recovered, dialyzed against 2 \times SSC (SSC = 0.15 M NaCl, 0.015 M Na citrate) and digested with 50 $\mu\text{g}/\text{ml}$ pancreatic RNase (Sigma Chemical Co., St. Louis, Mo.), and 50 U/ml T_1 RNase (Sigma), for 1 h at 37°C, followed by digestion with pronase (100 $\mu\text{g}/\text{ml}$) for 3 h at 37°C. The DNA was re-extracted with phenol and extensively dialyzed against 0.01 M, sodium borate, pH 9.2.

$\text{Hg}^{++}/\text{Cs}_2\text{SO}_4$ Gradient Centrifugation

Cs_2SO_4 was dissolved in 0.01 M sodium borate containing 100 μg of pulse-labeled DNA. After the addition of 0.1 mM HgCl_2 to a $\text{Hg}^{++}/\text{DNA}$ -phosphate ratio of 0.001, the solution was adjusted to a final vol of 9.0 ml

and a refractive index of 1.372 (equivalent to a starting density of 1.453 g/cm³). Samples were centrifuged in a Beckman no. 50 Ti fixed angle rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 34,000 rpm at 25°C for 48 h. Gradients were fractionated by pumping from the bottom of the tube yielding about 26 fractions per gradient. Absorbance at 260 nm was determined on a Zeiss spectrophotometer, and an aliquot of each fraction was spotted on Whatman 3 MM filter disks, precipitated with 5% trichloroacetic acid (TCA), batch-washed, and counted as described by Bollum (2).

S₁ Digestion

Pulse-labeled DNA was dialyzed against a buffer containing 0.2 M NaCl and 0.2 M sodium acetate, pH 4.2 and divided into two equal aliquots. ZnCl₂ (0.01 M) was added to one of the aliquots to a final concentration of 1×10^{-4} M, followed by addition of 2,000 U/ μ g DNA of the single-strand specific nuclease, S₁ (Miles Laboratories, Inc., Miles Research Products, Elkhart, Ind.). Both samples were incubated at 37°C for 15 min, and the digest was terminated by addition of 0.1 M sodium acetate, pH 5.50. The Zn⁺⁺ was removed by extensive dialysis against 0.1 M sodium acetate, pH 5.50. HgCl₂ was added to the DNA sample as described in the third section under Materials and Methods, and the sample was centrifuged as described above.

Electron Microscopy

DNA was spread by the formamide technique of Davis et al. (4). A solution containing 25 μ l of DNA (1.0 μ g/ml), 5 μ l of 0.01 M EDTA, and 0.1 M Tris HCl, pH 8.5, 5 μ l of cytochrome c (1 mg/ml), 5 μ l of PM2 DNA (0.5 μ g/ml), and 30 μ l of amberlite-filtered formamide was spread on a hypophase of 0.01 M Tris and 0.001 M EDTA, pH 8.50. The DNA was picked up from the hypophase on parlodion-carbon-coated grids, stained with either phosphotungstic acid or uranyl acetate, and rotary-shadowed with palladium-platinum, 80:20. Molecules were visualized in an RCA EMU-4 electron microscope.

Isolation of Mouse Satellite DNA

Mouse Satellite DNA was isolated from mouse liver by centrifugation in Ag⁺/Cs₂SO₄ as described by Corneo et al. (3), and further purified by banding in neutral CsCl.

RESULTS

Hg⁺⁺/Cs₂SO₄ Centrifugation of Pulse-Labeled DNA

When cells are labeled with [³H]thymidine for 45 s, most of the radioactivity is associated with nascent DNA. A sample enriched for replicating DNA prepared from pulse-labeled cells should

exhibit a higher specific activity compared to total DNA. Addition of HgCl₂ (Hg⁺⁺/DNA phosphate = 0.001) to pulse-labeled DNA followed by centrifugation in Cs₂SO₄ yields a component with a specific activity two- to fourfold higher than the bulk of the DNA (Fig. 1). This component bands at a denser position than the bulk of DNA and comprises less than 3% of the total sample. When HgCl₂ is omitted from the gradient, this component cannot be detected (Fig. 2). The high specific activity component banding on the dense side of the absorbance profile of Hg⁺⁺/Cs₂SO₄ gradients is subsequently referred to as the enriched sample.

Hg⁺⁺/Cs₂SO₄ Centrifugation of Pulse-Chased DNA

To demonstrate that the high specific activity component described in Fig. 1 behaves kinetically like nascent DNA, cells were labeled with [³H]thymidine for 45 s and were washed and cultured in excess nonradioactive thymidine for an additional 2 h. DNA was extracted and banded in Hg⁺⁺/Cs₂SO₄ gradient as before. The results depicted in Fig. 3 show that the dense, high-specific activity component is missing from the gradient. The specific activity of the pulse-labeled DNA in the gradient is constant throughout. This result is predicted if the high specific activity component is composed of nascent DNA molecules which can be chased into high molecular weight, double-stranded DNA.

Hg⁺⁺/Cs₂SO₄ Centrifugation of S₁-Treated DNA

The enrichment procedure is based on the arguments that mercuric ion preferentially binds to single-stranded DNA and that short, single-stranded regions are associated with replicating DNA. When HgCl₂ is added to DNA at the appropriate concentration, the mercuric ion preferentially binds the single-stranded regions contained in the replicating DNA which can then be separated from bulk DNA by isopycnic centrifugation in Cs₂SO₄ (Fig. 1). Removal of single-stranded DNA by enzymatic digestion should prevent enrichment of replicating DNA by Hg⁺⁺/Cs₂SO₄ centrifugation. This was demonstrated by incubating pulse-labeled DNA with *Aspergillus* S₁ nuclease (2,000 U/ μ g DNA) for 15 min at 37°C followed by centrifugation in a Hg⁺⁺/Cs₂SO₄ gradient (Hg⁺⁺/DNA phosphate = 0.001). The results, described in Fig. 4, reveal a constant specific

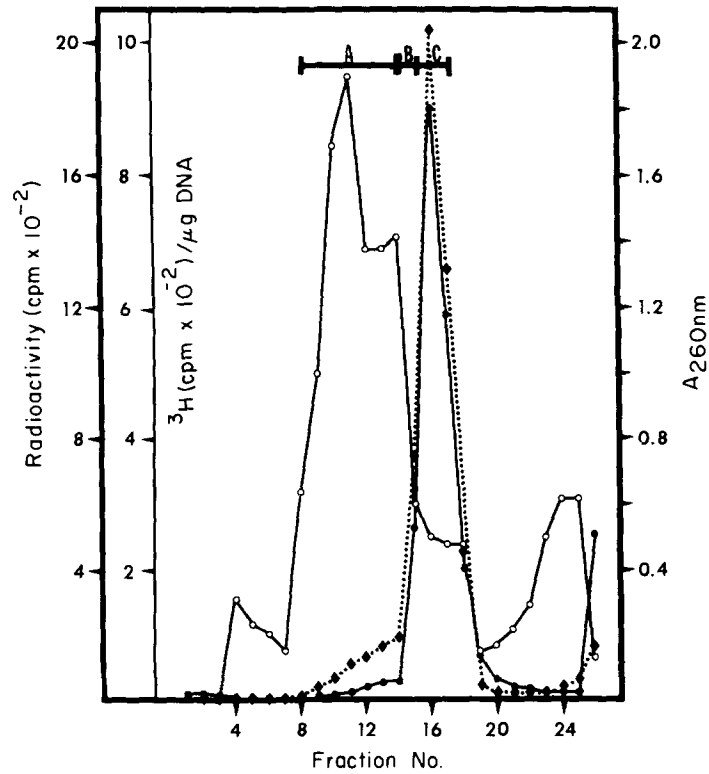


FIGURE 1 Centrifugation of pulse-labeled DNA in a $\text{Hg}^{++}/\text{Cs}_2\text{SO}_4$ gradient. Cells were labeled for 45 s with [^3H]thymidine (100 $\mu\text{Ci}/\text{ml}$, 20–50 Ci/mmol), and the DNA was extracted with phenol buffered with 0.1 M sodium acetate pH 5.0. HgCl_2 was added to 100 μg of DNA to a ratio of $\text{Hg}^{++}/\text{DNA-phosphate} = 0.001$, and the DNA was banded in a 9-ml Cs_2SO_4 gradient at 34,000 rpm for 48 h. The gradient was fractionated, the absorbance at 260 nm was determined for each fraction, and a 70- μl aliquot from each fraction was precipitated with TCA and counted. (◆····◆) ^3H cpm; (●—●) absorbance at 260 nm; and (○—○) specific activity (^3H cpm)/ μg DNA.

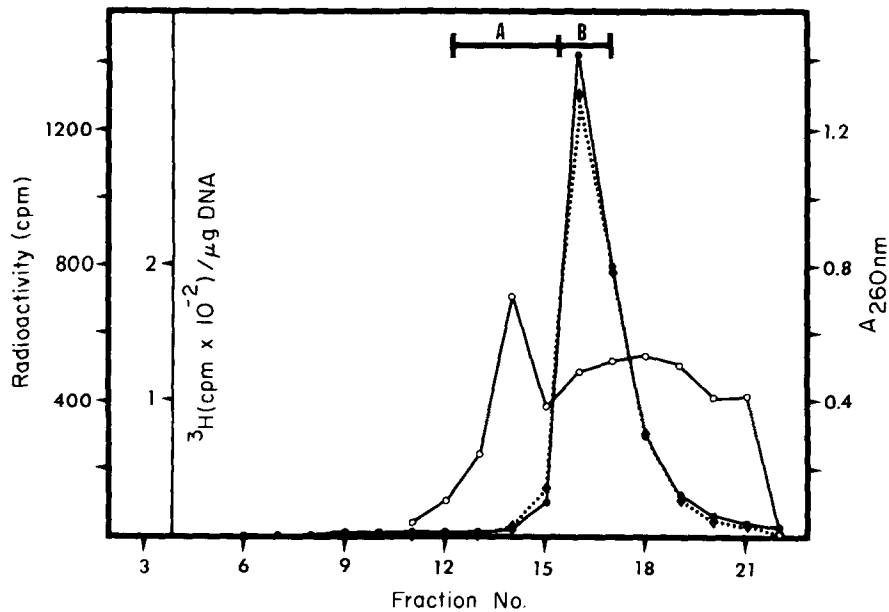


FIGURE 2 Centrifugation of pulse-labeled DNA in Cs_2SO_4 minus Hg^{++} . Cells were labeled for 45 s with [^3H]thymidine, and the DNA was extracted and centrifuged in Cs_2SO_4 for 48 h. at 34,000 rpm. The gradient was fractionated, the absorbance at 260 nm was measured for each fraction, and 70 μl from each fraction was precipitated with TCA and counted. (◆····◆) ^3H cpm, (●—●) absorbance 260 nm; and (○—○) sp act (^3H cpm)/ μg DNA.

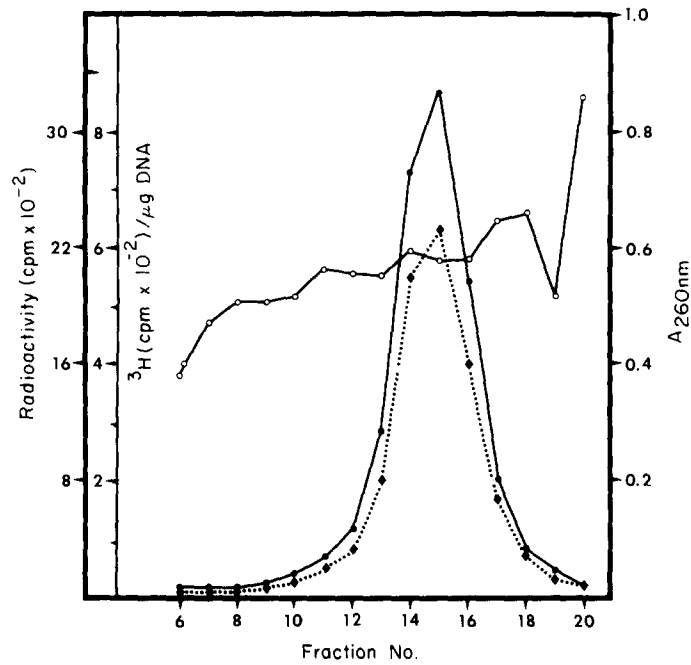


FIGURE 3 Centrifugation of pulse-chased DNA in a $\text{Hg}^{++}/\text{Cs}_2\text{SO}_4$ gradient. Cells were labeled with [^3H]thymidine for 45 s followed by culture in medium containing 0.1 mM unlabeled thymidine for 2 h. The DNA was extracted, and 100 μg was banded in Cs_2SO_4 after addition of HgCl_2 to a $\text{Hg}^{++}/\text{DNA}$ -phosphate ratio = 0.001. The DNA was centrifuged, the gradient was fractionated, and the DNA in each fraction was quantified and counted as described in Fig. 1. (\blacklozenge ···· \blacklozenge) ^3H cpm; (\bullet — \bullet) absorbance at 260 nm; and (\circ — \circ) sp act (^3H cpm)/ μg DNA.

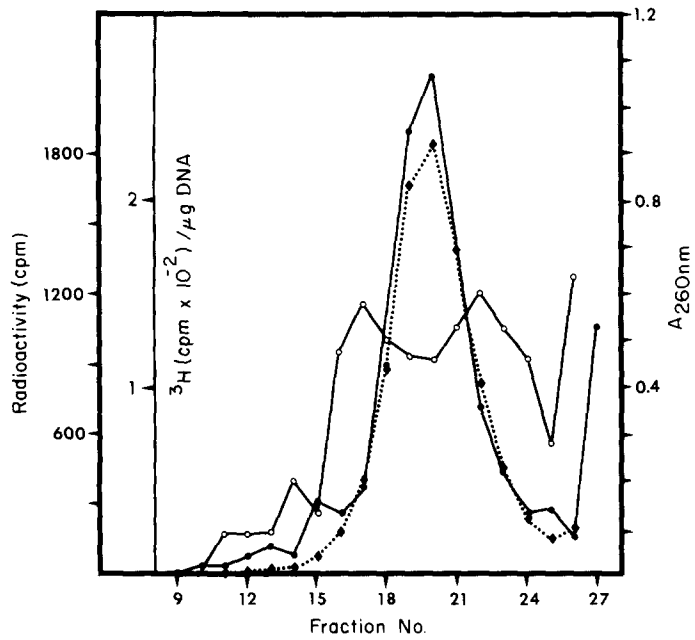


FIGURE 4 Centrifugation of S_1 nuclease-treated DNA in a $\text{Hg}^{++}/\text{Cs}_2\text{SO}_4$ gradient. Cells were labeled with [^3H]thymidine for 45 s, and the DNA was extracted. The purified DNA was incubated with 2,000 U S_1 nuclease/ μg DNA for 15 min at 37°C. After addition of HgCl_2 to the sample to a $\text{Hg}^{++}/\text{DNA}$ -phosphate ratio = 0.001, the DNA was centrifuged in Cs_2SO_4 , the gradient was fractionated, and the DNA in each fraction was quantified and counted as described in Fig. 1. (\blacklozenge ···· \blacklozenge) ^3H cpm; (\bullet — \bullet) absorbance at 260 nm; (\circ — \circ) sp act (^3H cpm)/ μg DNA.

activity throughout the gradient. There is no high specific activity component like that seen in Fig. 1.

Rebanding of DNA after Removal of Mercuric Ion

Rebanding of the enriched sample in CsCl after removal of the mercury demonstrates that its dense position in the gradient is due to its association with mercuric ion rather than its base composition or the presence of extensive regions of single-strandedness. When rebanded in CsCl, the radioactivity from the enriched sample (Fig. 1, region A) was redistributed to coincide with the absorbance profile of the added marker DNA (Fig. 5 A). In control experiments, DNA taken

from the ascending portion (Fig. 1, region B) and from the peak of the absorbance profile (Fig. 1, region C) of a $\text{Hg}^{++}/\text{Cs}_2\text{SO}_4$ gradient rebanded faithfully in CsCl after removal of the mercuric ion (Fig. 5 B and C).

As a further control, two fractions from a Cs_2SO_4 gradient from which mercury had originally been omitted were collected, and the DNA was rebanded in CsCl. In the first case, labeled DNA from a region of the gradient denser than the bulk DNA (Fig. 2, region A) was rebanded in CsCl. The radioactivity rebanded in the dense region of the gradient, in contrast to the enriched sample that redistributed itself after removal of the mercury (Fig. 5 D). In the second experiment, material taken from the peak of the absorbance

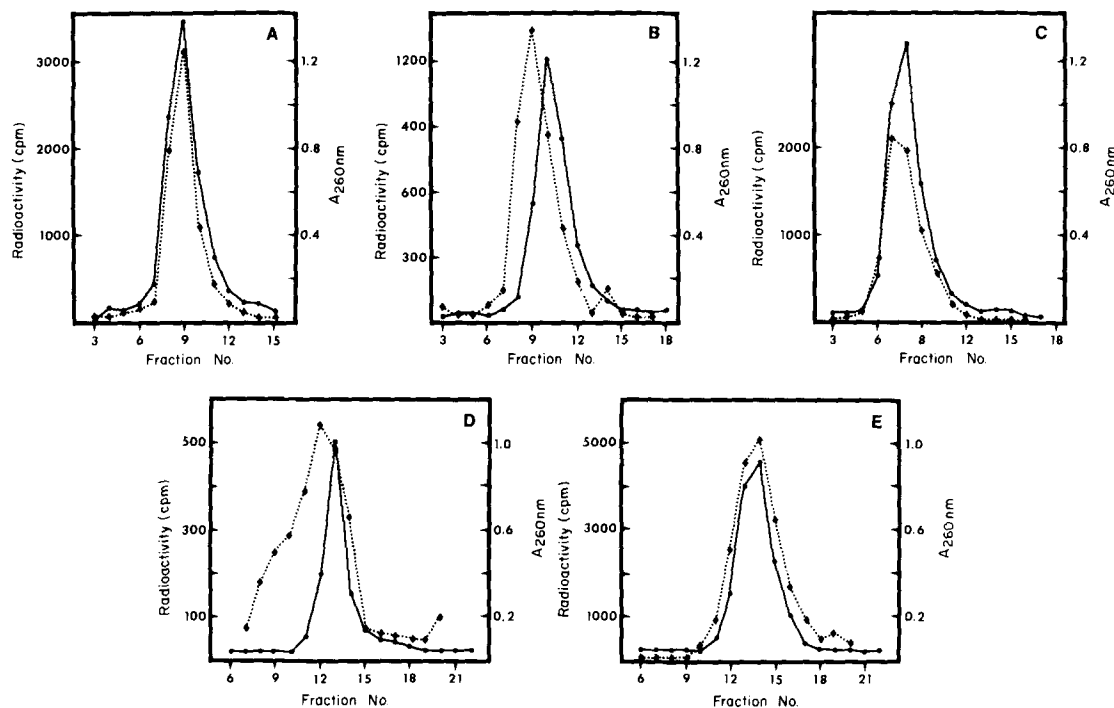


FIGURE 5 Rebanding of DNA in CsCl gradients after removal of mercuric ion. DNA from pulse-labeled cells was centrifuged to equilibrium in Cs_2SO_4 in the presence or absence of HgCl_2 . Fractions from specific regions of each gradient were pooled and dialyzed against 0.01 M sodium borate. After the addition of unlabeled DNA as a marker, the samples were recentrifuged in neutral CsCl for 48 h at 33,000 rpm. The gradients were fractionated, the absorbance at 260 nm of each fraction was determined, and the entire fraction was precipitated with TCA and counted. (\blacklozenge ···· \blacklozenge) ^3H cpm; (\bullet — \bullet) absorbance at 260 nm; (A) rebanding of the “enriched fractions” from the dense high specific activity sample of a $\text{Hg}^{++}/\text{Cs}_2\text{SO}_4$ (Fig. 1, region A); (B) rebanding of DNA from the ascending portion of the absorbance profile of a $\text{Hg}^{++}/\text{Cs}_2\text{SO}_4$ gradient (Fig. 1, region B); (C) rebanding of DNA from the peak of the absorbance profile of a $\text{Hg}^{++}/\text{Cs}_2\text{SO}_4$ gradient (Fig. 1, region C); (D) rebanding of DNA from the very dense region of the absorbance profile of a Cs_2SO_4 gradient minus HgCl_2 (Fig. 2, region A); and (E) rebanding of DNA from the peak of the absorbance profile from a Cs_2SO_4 gradient minus HgCl_2 (Fig. 2, region B).

profile (Fig. 2, region B) of a Cs_2SO_4 gradient rebanded faithfully upon recentrifugation in CsCl (Fig. 5 E).

Electron Microscopy

Although all of the biophysical data are consistent with an enrichment of replicating DNA, the ultimate proof lies in the visualization of replicating molecules by electron microscopy. When DNA from the enriched sample of $\text{Hg}^{++}/\text{Cs}_2\text{SO}_4$ gradients was examined by electron microscopy, 72 out of 1,003 molecules observed contained structures compatible with replication (Table I). Several classes of molecules were detected including molecules with a single bubble, a single fork (three arms), multiple forks, or multiple bubbles. Examples of such molecules are included in Figs. 6 and 7. Of the replication structures observed, 81% possessed a single fork and 4% a single bubble. The remaining 15% possessed multiple forks, multiple bubbles, or a combination of the two. In contrast to the enriched fraction, only 4 out of 547 molecules from unfractionated DNA contained replication structures (Table I). Likewise, less than 1% of molecules from the main band of a $\text{Hg}^{++}/\text{Cs}_2\text{SO}_4$ gradient or from the

heavy region of a Cs_2SO_4 gradient without HgCl_2 revealed replication forms. The DNA from a population of mitotic cells contained in the heavy region of a $\text{Hg}^{++}/\text{Cs}_2\text{SO}_4$ gradient yielded no replication structures (Table I); the DNA from cells in late G_1 yielded two replication structures out of 200 molecules examined from the density-shifted region (Table I). This result is expected since about 10–15% of the cells had entered the S phase at the time they were collected for analysis. To rule out the possibility that mercuric ion itself was inducing localized regions of denaturation, the A + T-rich mouse satellite DNA was examined by electron microscopy before and after incubation with HgCl_2 . Only 1 out of 400 molecules observed revealed a structure other than linear double-stranded DNA (Table I).

The enrichment effected by $\text{Hg}^{++}/\text{Cs}_2\text{SO}_4$ isopycnic centrifugation is slightly greater than eight-fold when calculated on a per molecule basis. The DNA contained in the enriched fraction, however, is about three times smaller than unfractionated DNA or DNA taken from the main band of the $\text{Hg}^{++}/\text{Cs}_2\text{SO}_4$ gradient. The actual enrichment calculated on a per mole nucleotide basis is about 20–30-fold.

Replicon size was approximated by measuring the distance between the centers of the two adjacent replication structures contained on molecules with multiple bubbles. In all cases, the bubbles contained arms of equal length. Circular PM2 DNA was included in each preparation as a size marker (6). Molecules with adjacent forks were included in the analysis only when it could be unambiguously demonstrated that they were generated from adjacent bubbles rather than from breakage of a single bubble. The measurements of replicon size, presented in Fig. 8, were all below 5 μm and ranged from 0.2 to 4.5 μm . These sizes, which are below the limit of resolution of DNA fiber autoradiography, are in the same range as replicon sizes observed in early insect embryos.

DISCUSSION

Analysis of DNA replication in eukaryotes by electron microscopy is limited by the low frequency of replicating structures within the DNA isolated from most proliferating cells. A major exception is the early insect embryo which provides a naturally occurring population of DNA enriched for replicating molecules due to the brevity of the S phase (1, 13, 23, 24). In this

TABLE I
Frequency of Replicating Molecules

Sample	No. of replicating structures	Total no. of molecules counted	Replicating molecules %
Enriched fraction			
$\text{Hg}^{++}/\text{Cs}_2\text{SO}_4$ (Fig. 1, Region A)	72	1,003	7.18
Unfractionated DNA	5	547	0.91
Main band			
$\text{Hg}^{++}/\text{Cs}_2\text{SO}_4$ (Fig. 1, Region C)	1	200	0.50
Heavy region (Fig. 2, Region A)			
Cs_2SO_4	3	341	0.88
Mitotic cells, heavy region ($\text{Hg}^{++}/\text{Cs}_2\text{SO}_4$)	0	200	0.00
Late G_1 cells			
Heavy region ($\text{Hg}^{++}/\text{Cs}_2\text{SO}_4$)	2	200	1.00
Mouse satellite DNA	1*	200	0.50
Mouse satellite DNA Plus HgCl_2	0	200	0.00

* This molecule was totally nonlinear and contained an ambiguous structure.

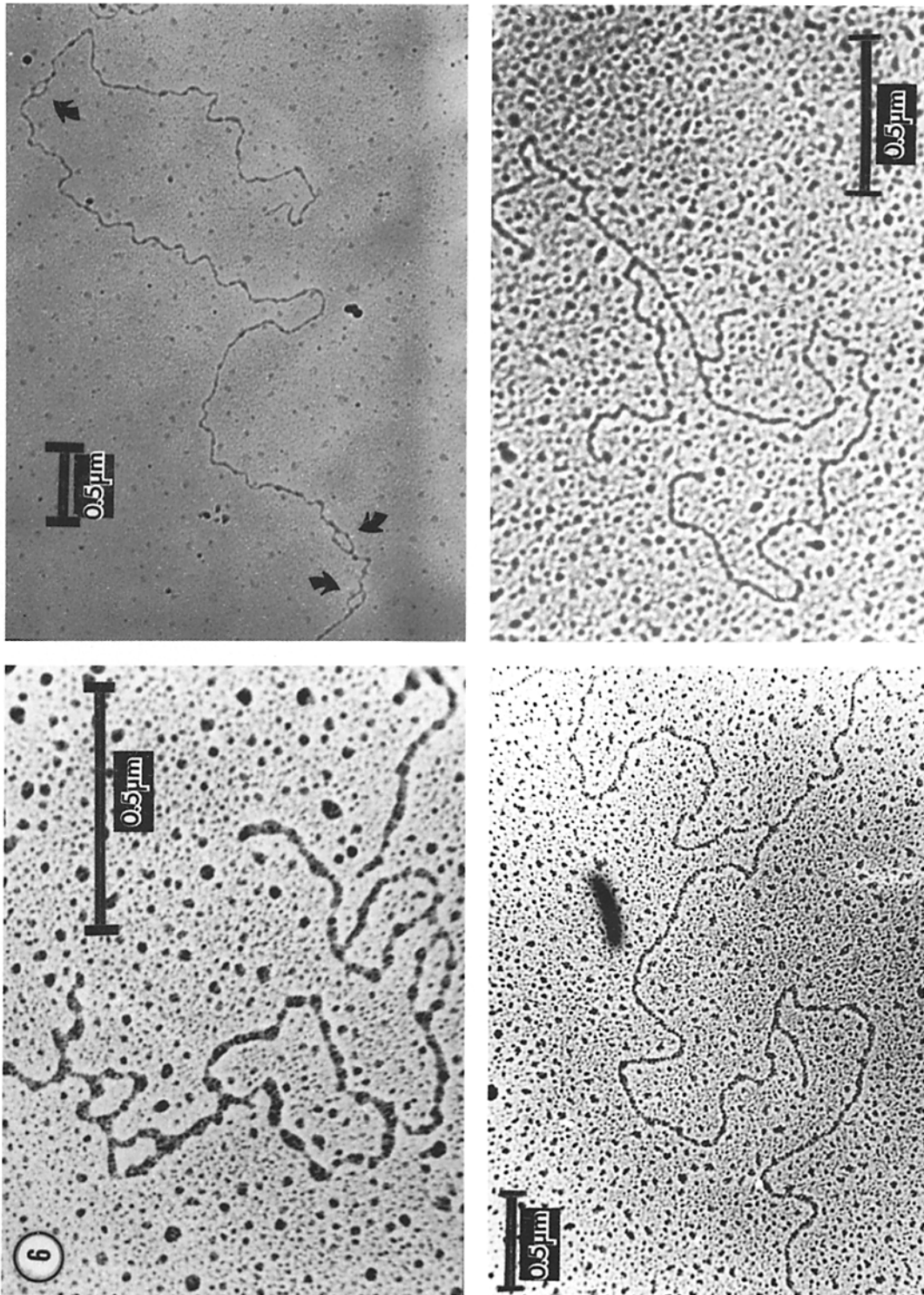


FIGURE 6 Molecules with multiple replication structures. Bar, 0.5 μm .

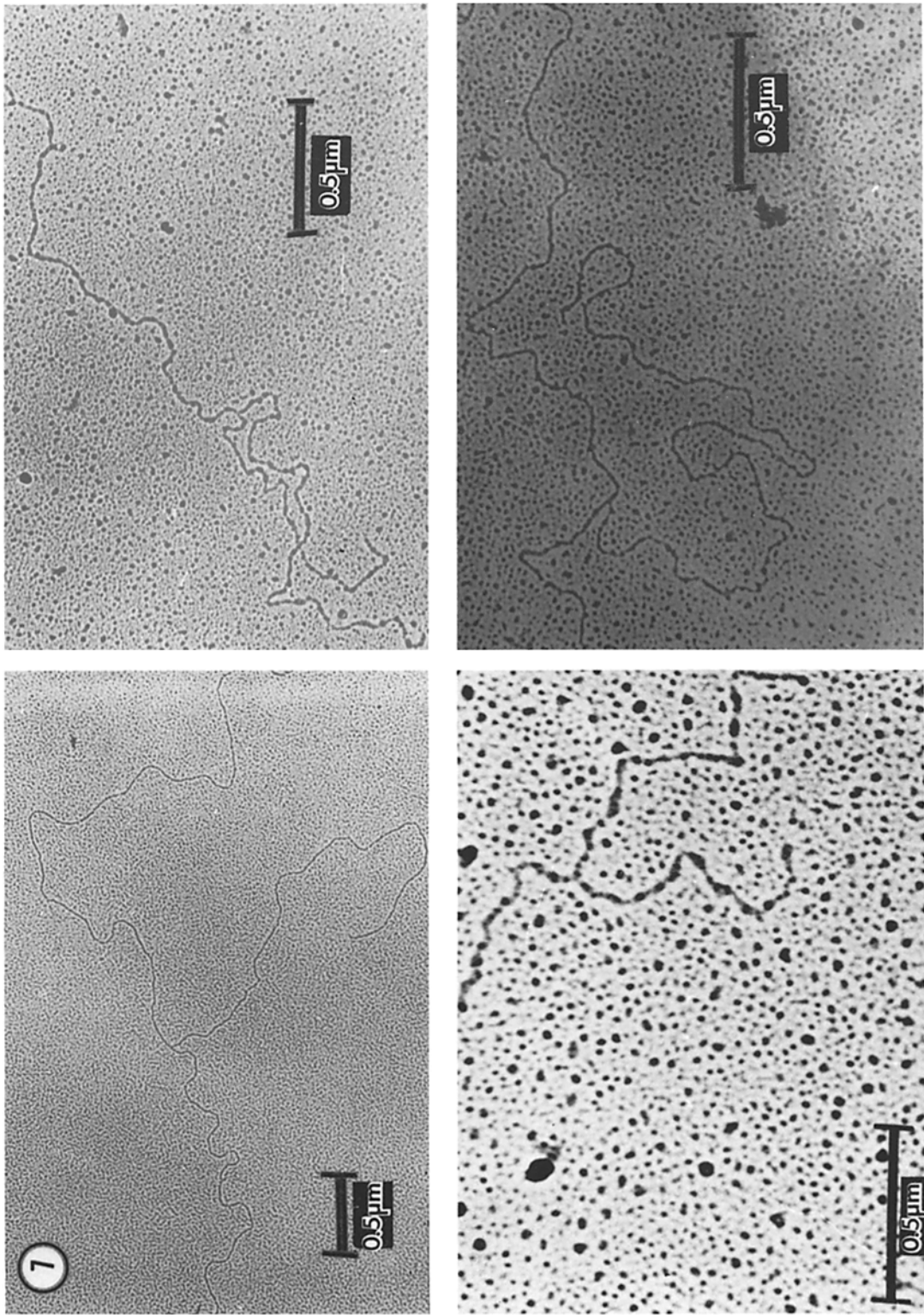


FIGURE 7 Molecules with a single replication fork. Bar, 0.5 μm .

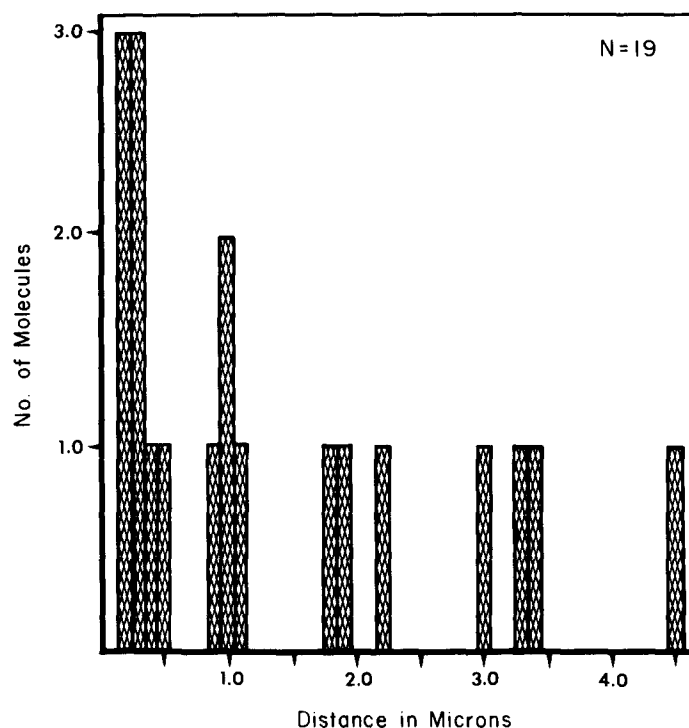


FIGURE 8 Distribution of replicon sizes. The distance between centers of adjacent replication structures was measured at a $73,000\times$ magnification, and absolute sizes were determined using circular PM-2 phage DNA as a size marker.

system, about 10% of the molecules visualized contain replication structures. In contrast, DNA from proliferating cultured mammalian cells yields less than 1% of molecules with replication forms (Table I). Such a low frequency requires that the DNA be enriched for replicating molecules before replication can be effectively studied by electron microscopy.

The enrichment procedure used in this study exploits both the single-stranded regions of DNA that are characteristic of replicating DNA (12) and the greater affinity of mercuric ion for single-stranded than native DNA. Molecules containing replication structures are separated from the bulk of the DNA by virtue of their increased buoyant density after interaction with low levels of HgCl_2 . The replicative nature of the enriched fraction was demonstrated by its behavior after a chase period with unlabeled thymidine, and was confirmed by electron microscopy. That Hg^{++} preferentially binds to replicating DNA was shown by the redistribution of the enriched fraction in CsCl after removal of the mercury. This experiment also revealed that molecules containing replicating

DNA were primarily double-stranded since upon redistribution they rebanded at the same density as the native DNA. The involvement of single-stranded regions in the enrichment procedure was demonstrated by pretreatment of DNA with S_1 nuclease and the subsequent loss of the enriched fraction upon centrifugation. The possibility that the bubbles observed by electron microscopy were due to localized regions of denaturation induced by the heavy metal ion rather than to replication was eliminated by processing the A + T-rich mouse satellite DNA for electron microscopy in the same way as the labeled Chinese hamster DNA, both with and without HgCl_2 . Only one of 400 molecules examined possessed an ambiguous structure that was not totally linear. No enrichment was observed by electron microscopy in DNA from cells collected in mitosis or the late G_1 phase.

When the DNA in the enriched fraction was visualized by electron microscopy, 72 of 1,003 molecules examined possessed replication structures. On a per molecule basis, this represents a six- to ninefold enrichment when compared to the

DNA in the main band of $\text{Hg}^{++}/\text{Cs}_2\text{SO}_4$ gradients or to unfractionated DNA. Since the DNA from the main band and from unfractionated DNA were both about three times as long as that from the enriched fraction, the actual enrichment on a per mole nucleotide basis is 20- to 30-fold.

The enrichment technique used would tend to select for molecules with the highest single-strand to double-strand ratio. These molecules would exhibit the greatest density shift after interaction with low levels of HgCl_2 , followed by centrifugation in Cs_2SO_4 . The predominant type of molecule contained in this population would tend to be smaller than the average, with one or more small replication forms. Larger molecules with large replication bubbles would have a lower single-strand to double-strand ratio, reducing its density shift and making its detection less likely. The reduced size is important since it limits our analysis to replication structures contained on smaller molecules (usually between 6 and 10 μm).

Although molecules with single replication structures were relatively common in the enriched fraction, example with multiple replication forms were rare. The low frequency of such molecules is due to at least three factors: (a) Tandem replicons less than 0.5 μm in size, such as those observed in this study (e.g., Figs. 6 and 8) would be very short-lived. Using current estimates of the rate of chain elongation, between 0.5 and 1.0 $\mu\text{m}/\text{min}$ (9, 10), and assuming bidirectional replication, such replicons would have a lifetime of less than 2 min before fusing to form a single large bubble. (b) Large tandem bubbles with "eye to eye" distances greater than 4 or 5 μm would not be detected by the procedures described because of the small size of the molecules examined. (c) When the molecules break during the enrichment procedure, they appear to preferentially break within replicating bubbles, yielding mainly linear molecules with arms extending laterally. This breakage is likely due to both the increased sensitivity of replication structures to shear (11) and to the nicking activity of mercuric ion upon DNA (22). Forklike structures have been observed together with bubbles in DNA molecules from embryonic systems (13), as well as in other studies in which replicating DNA from eukaryotic cells was visualized (21). A minority of the molecules containing replication structures possess intact bubbles.

The replicon size observed in this study is about an order of magnitude smaller than that described by DNA fiber autoradiography (10). The lower

limit of resolution of the latter technique restricts analysis of replication to replicating units larger than 4 or 5 μm . The smallest distribution of grain tracks thus far described was recently reported by Taylor and Hozier (19). Using synchronized Chinese hamster cells blocked at or near the G1/S boundary with fluorodeoxyuridine, they reported replicon sizes of 4 μm or multiples thereof upon release from the block with [^3H]thymidine. The tandem grain tracks observed in such autoradiographs may reflect true replicon sizes, i.e., distance between adjacent replication initiation sites. They may, however, represent an organization superimposed upon that of the replicon, such as the distribution of clusters of small replicons that have fused to generate the grain tracks detected by fiber autoradiography. Such an organization is consistent with the recent finding of Zakian (24) who reported that in *Drosophila virilis* embryos small replicons of less than 900 base pairs were often clustered, with cluster occurring at about 4,000 base pair intervals. When large eye-forms greater than 900 base pairs were seen, they were also frequently spaced at 4,000 base pairs or multiples thereof.

In contrast to fiber autoradiography, the procedure described in this report is restricted to the analysis of replicons smaller than 4 or 5 μm . The most common sizes observed were below 1 μm and most likely represent true replicon sizes. Whether such small replicons predominate throughout the entire genome of cultured mammalian cells cannot be resolved by this technique since larger replicon sizes would not be detected. The small tandem replicons may, in fact, not be representative of the majority of replicon sizes in mammalian cells but may reflect only the replication of selected repeated sequences such as satellites and tandemly repeated genes such as those coding for ribosomal RNA, transfer RNA, histone messenger RNA, or 5S RNA. Regardless of their distribution within the genome, replicons smaller than 1 μm can be detected in cultured mammalian cells and are not restricted solely to embryonic systems (1, 12, 23, 24).

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