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Sites of Replication of Chromosomal DNA in a Eukaryotic Cell

(chromatin/nuclear membrane/replication complex/autoradiography)

STANISLAV FAKAN, GAIL N. TURNER, JOSEPH S. PAGANO*, AND RONALD HANCOCK

Department of Cell Biology, Swiss Institute for Experimental Cancer Research, 1011 Lausanne, Switzerland

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ABSTRACT In mouse cells (line P815), newly synthesized DNA labeled for 20–30 sec during exponential growth is found by electron microscope autoradiography at sites throughout the cell nucleus. These sites are relatively more concentrated in the peripheral region of the nucleus (averaged over a random population of S-phase cells), probably reflecting a higher local concentration of DNA in this region. Newly synthesized DNA is not preferentially associated with purified nuclear envelopes, but is found in a fraction of the chromosomal deoxynucleoprotein whose buoyant density in CsCl after formaldehyde treatment is about 1% lower than that of the deoxynucleoprotein peak. Kinetics experiments suggest that this material is a precursor of mature deoxynucleoprotein; it may represent regions of deoxynucleoprotein containing replicating DNA and the additional proteins involved in DNA replication. Other complexes of newly replicated DNA that are found in the interphase after phenol extraction of nuclei are formed during the extraction procedure, probably due to the partially single-stranded nature of replicating DNA, and do not appear to exist *in vivo*.

The many studies on the location of replication sites of chromosomal DNA in the eukaryotic nucleus have led to divergent conclusions. Electron microscope autoradiography (1–3) shows that replication sites of chromosomal DNA are distributed throughout eukaryotic nuclei, and do not appear to be associated with any specific morphological structure. The peripheral replication sites in cells released from synchronizing inhibitors (4) may not represent normal S-phase initiation (5).

In contrast, most (6, 7, 8) [but not all (9)] biochemical studies show that newly replicated DNA is preferentially associated with the nuclear envelope. Newly replicated DNA is often (6, 10–12), but not always (13, 14), localized in the interphase after phenol or chloroform–isoamyl alcohol extraction. It sometimes associates preferentially with crystals of detergent in sucrose gradients (7, 30). The interpretation of these observations has been influenced by models of membrane-associated replication of bacterial (15) and phage (16) DNA; the phage DNA–membrane association may, however, be related to transcription rather than replication (17).

We present here studies designed to resolve these discrepancies and to identify by two independent methods, electron microscope autoradiography and cell fractionation, the sites of chromosomal DNA synthesis in exponentially growing mouse cells. Eukaryotic chromosomal DNA replicates at 0.5 (18) to 1–2 (19) μm/min; within the nucleus, therefore, a labeled precursor newly incorporated into DNA could be displaced as much as 1 μm away from its site of incorporation after 1 min. We have used labeling periods of 20–30 sec, which are about the lower practical limit for present techniques of electron microscope autoradiography; labeled molecules incorporated at the beginning of this period are unlikely to be displaced more than 0.5 μm from the sites of DNA replication. Our results show that replication sites occur throughout the nucleus and are not preferentially associated with the nuclear envelope.

Abbreviations: DNP, deoxynucleoprotein; SDS, sodium dodecyl sulfate.

* On leave from the Departments of Medicine and of Bacteriology and Immunology, University of North Carolina, Chapel Hill, N.C. 27514 (present address).

MATERIALS AND METHODS

Cells of mouse line P815 (20) were grown in suspension culture in medium MEM (Gibco) containing 10% calf serum, and unlabeled thymidine (dT) (0.2 μg/ml).

Labeling of DNA and Nuclear Envelope. Cellular DNA was labeled where indicated with [^{14}C]dT (22 Ci/mol, 0.5 μCi/liter: New England Nuclear) and the nuclear envelope with [^{14}C]choline (choline [*methyl*-^{14}C]chloride, 10.5 Ci/mol, 0.015 μCi/ml: New England Nuclear), both during at least two generations of growth. Newly replicated DNA was pulse-labeled during exponential growth (3×10^5 cells/ml); [*methyl*-^{3}H]dT (2.5 mCi, 50–55 Ci/mmol: New England Nuclear) was injected into a 50-ml spinner culture from a syringe during 3–4 sec. After the desired labeling time (20 or 30 sec), the culture was poured rapidly onto half the volume of crushed frozen buffer (150 mM NaCl–25 mM Tris·HCl, pH 7.5) containing sodium azide (10 mM), with rapid stirring. The incorporation of [^{3}H]dT into Cl_3CCOOH-precipitable material stopped within 5 sec. In some experiments a form of chase was performed after pulse-labeling, by addition of a 100-fold excess of unlabeled dT to the culture; growth and DNA synthesis continued at the normal rates for at least 1 hr.

Preparation and Fractionation of Nuclei. At 4°, nuclei were prepared (20), purified to remove nonnuclear membranes by centrifugation through 2 M sucrose in TKM buffer (20) (Spinco SW65 rotor, 35,000 rpm, 90 min, 4°), and washed twice in a solution containing 20 mM EDTA–80 mM NaCl (pH 8) to remove RNA and soluble proteins (20). For separation of the nuclear envelope from deoxynucleoprotein (DNP), the nuclei were broken gently (loose Dounce homogenizer, 10 strokes) in 0.2 mM EDTA (pH 7.2). Soluble DNP was extracted from sheared nuclei, treated with formaldehyde, and centrifuged to equilibrium in CsCl (Spinco rotor 40, 33,000 rpm, 65 hr, 20°) as described (20, 21). The nuclear envelope was separated by equilibrium density centrifugation; sheared nuclei were either mixed with CsCl to give a density of 1.20 and centrifuged (Spinco rotor SW65, 50,000 rpm, 24 hr, 4°), or layered on a preformed gradient of sucrose (15–60%) containing 10 mM Tris·HCl buffer (pH 7)–1.5 M NaCl and centrifuged (Spinco SW65 rotor, 50,000 rpm, 36 hr, 4°).

Samples (0.5 ml) were collected from the bottom of the tube (CsCl gradients) or from 2 cm above the bottom (sucrose gradients); in the latter case the material remaining in the lower part of the tube was considered as one fraction.

Extraction of Nuclear DNA. For examination of the distribution of DNA during phenol extraction of nuclei from pulse-labeled cells, nuclei (about 10^7/ml) were treated with sodium dodecyl sulfate (SDS) and phenol (10), and the aqueous and phenol phases and the interphase were removed separately. For radioactive counting, aliquots were precipitated as described below; duplicates of each sample were prepared with internal standards of labeled DNA, added before Cl_3CCOOH-precipitation, to allow correction for quenching. For analysis in CsCl gradients, the interphase and aqueous phases were dialyzed to remove phenol against a solution containing 0.02 M trisodium citrate–0.5% SDS–0.3 M NaCl (the calculated concentrations in the aqueous phase). CsCl was added to a density of 1.65; after centrifugation (Spinco rotor 50.1, 38,000 rpm, 48 hr, 20°), samples were collected from the bottom of the tube. SDS–phenol-treated samples showed a pellicle on the surface, which remained in the tube and was dissolved in water by brief sonication; this material was considered as the top fraction of the gradient. For preparative purposes, chromosomal DNA was extracted from nuclei by phenol (10), precipitated from ethanol, and banded to equilibrium in CsCl gradients. Samples of this DNA in 0.15 M NaCl–0.015 M trisodium citrate were denatured by heat (15 min in a boiling water bath, followed by cooling in ice) or in alkali (30 min at pH 12 followed by neutralization). Radioactivity was measured by precipitation of samples with 10% Cl_3CCOOH after addition of carrier DNA (100 μg); the precipitates were collected on glass fiber filters (Whatman GF/C) and washed with 10% Cl_3CCOOH and water.

Electron Microscope Autoradiography. At 4°, the cells were washed twice in medium without serum, fixed in 1.6% glutaraldehyde in Sörensen's phosphate buffer (pH 7.4), and washed for about 24 hr in frequent changes of this buffer containing 0.2 M sucrose to eliminate unincorporated soluble precursor molecules (22); one part was then postfixed with osmium tetroxide. The cells were then dehydrated in acetone and embedded in Epon.

To detect possible extraction of acid-precipitable radioactive material during preparation of cells for autoradiography, samples taken from the pulse-labeled culture, from the several fixing and dehydrating solutions, and from the cell preparation immediately before embedding in Epon, were precipitated with Cl_3CCOOH and prepared for determination of radioactivity as described above. Autoradiographs of sections (about 900 Å thickness) were prepared by the dipping technique (23) with either Ilford L4 or Gevaert NUC 3.07 emulsion; after 3–6 months they were developed in D-19 developer, stained by uranyl acetate and lead citrate, and examined in the Philips EM300 microscope. For calculation of autoradiographic grain densities, the relatively few nuclear sections cut peripherally or containing less than 10 grains were excluded. With prints at a magnification of 14,000, grains were counted in the peripheral region of each nucleus extending on both sides of the nuclear envelope for a distance of 0.6 μm [this value represents *twice* the error limiting the autoradiographic resolution, calculated (24) as the radius of the circle around the radioactive source within which fall 50% of developed grains] and in the remaining central region of the nucleus. The areas of these regions were determined by tracing onto paper and weighing.

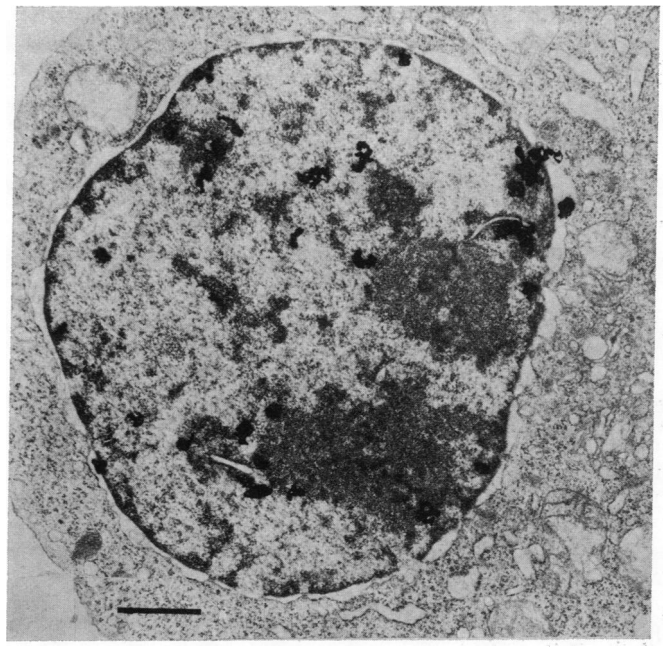

FIG. 1. Sites of incorporation of [^{3}H]dT in the nucleus of a P815 cell during a 30-sec labeling period. In this section, the total number of autoradiographic grains and their distribution (nuclear periphery compared with central region) differ by less than 20% from the statistical average, determined as described in *Methods*. The *bar* represents 1 μm.

RESULTS

Electron Microscope Autoradiography. During a 30-sec labeling period. [^{3}H]dT is incorporated at sites throughout the nucleus (Fig. 1). We compared in a population of cells the autoradiographic grain density in the region adjacent to the nuclear envelope with that in the remaining internal region of the nucleus, taking into account the resolution of the autoradiographic technique. This comparison (Table 1) shows that although newly synthesized DNA is located somewhat more frequently in the peripheral region than in the nuclear interior, more than half is found at sites located throughout the interior of the nucleus. These sites are often located on or near regions of dense chromatin (Fig. 1).

Among some artifacts that could affect the interpretation of these experiments, we considered first a limited rate of penetration of [^{3}H]dT into the interior of the nucleus. When a large excess of unlabeled dT was added after labeling, incorporation of [^{3}H]dT continued at a much lower rate during the next hour (Table 1). It is unlikely that the flow of labeled precursor did not reach equilibrium in all regions of the nucleus during this relatively long period; nevertheless, the sites of continued incorporation are still somewhat more frequent at the nuclear periphery. These results suggest that the higher concentration of sites at the nuclear periphery reflects a higher concentration of DNA in this region; indeed, microspectrophotometric determinations show that in nuclei of mouse-liver cells over half the DNA is contained in the peripheral 1-μm shell (25) (see *Note added in proof*).

TABLE 1. *Distribution within nuclei of sites of [^{3}H]dT incorporation during a 20- or 30-sec labeling period, determined by electron microscope autoradiography*

Experiment	Labeling time (sec)	No. of nuclear cross sections analyzed	Mean grains/ nuclear cross section	Grains per area in peripheral region/those in central region	% of total grains in central region
1	20	29	27	1.8 ± 1.2*	59
2	30	28	23	2.2 ± 1.6	59
3	30	18	32	1.9 ± 1.3	57
	30 followed by 1 hr chase	22	93	1.7 ± 1.0	63

The topological distribution of autoradiographic grains corresponding to sites of [^{3}H]dT incorporation was determined (see *Methods*). The regularly circular cross section of the nuclei indicates that they are about spherical, with a mean diameter of about 8 μm. Since the thickness of the electron microscope sections is about 900 Å, relatively few nuclei are sectioned through the periphery; these are easily identified and were eliminated from counting.

* Standard error.

Of the total acid-precipitable radioactivity in pulse-labeled cells, about 90% could be recovered as DNA in CsCl gradients (see Fig. 4*A*), and over 90% was recovered in the fixed, dehydrated cells immediately before embedding for autoradiography. Thus, the label seen in autoradiographs is in DNA: no significant loss of labeled DNA or acid-precipitable biosynthetic intermediates from certain regions of the nucleus occurs during preparation for autoradiography. The possibility that DNA repair contributes significantly to the incorporation under these conditions may, we believe, be excluded. Repair incorporation in interphase cells is negligible compared with synthesis, even after irradiation, and occurs also in noninterphase cells (26), which in our experiments showed no labeling [we found almost 50% of cells labeled, the remainder completely unlabeled; this corresponds to the proportion of cells synthesizing chromosomal DNA in this cell line that has an S-phase of about 5 hr and a doubling time of 9 hr (27)]. Pulse-labeling with [^{3}H]dT of cells previously grown for one generation time with BrdU, in which the chromosomal DNA was completely in the hybrid density form, resulted in incorporation of radioactivity equally into light and hybrid DNA (R. Hancock, unpublished results) as expected for semiconservative replication. Repair incorporation results in predominant labeling of hybrid DNA under these conditions in irradiated HeLa cells (26).

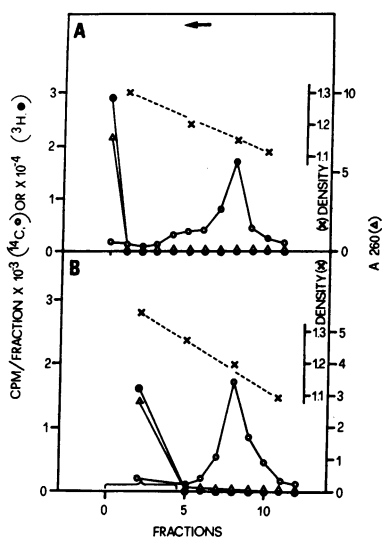

FIG. 2. Separation of pulse-labeled DNA from nuclear envelope in equilibrium density gradients of CsCl (*A*) or sucrose–1.5 M NaCl (*B*). Cells were previously labeled with [^{14}C]choline and pulse-labeled for 30 sec with [^{3}H]dT, and nuclear lysates were prepared and analyzed as in *Methods*. ●, [^{3}H]dT radioactivity in pulse-labeled DNA; ○, [^{14}C]choline radioactivity in nuclear envelope; △, A_{260}; ×, density. The sedimentation in this and succeeding figures was from *right* to *left*.

Distribution of Newly Synthesized DNA in Subnuclear Fractions. We investigated the distribution of newly synthesized DNA between the small fraction of DNA that remains associated with the nuclear envelope [usually less than 1% of the total nuclear DNA (28, 29)] and the chromosomal DNA. The nuclear envelope (labeled with radioactive choline) was separated from chromosomal DNA by centrifugation of sheared nuclei through gradients of sucrose containing 1.5 M NaCl, or of CsCl. A high ionic strength is necessary to dissociate chromatin and prevent nonspecific aggregation between chromatin and the nuclear envelope (28, 29); gradient centrifugation permits separation of nucleoli from the nuclear envelope. Under these conditions the nuclear envelope bands at its equilibrium density, while the chromosomal DNA sediments to the bottom of the gradient (Fig. 2). Newly synthesized DNA sediments also, and is clearly separated from the nuclear envelope (Fig. 2). The newly synthesized DNA sedimented in this way shows the equilibrium density of free DNA when banded again in CsCl gradients; thus, it is not attached to non-choline containing envelope constituents. We conclude that the DNA associated with the nuclear envelope is not preferentially enriched in newly synthesized DNA.

A Fraction of DNP Containing Newly Replicated DNA. It has been proposed that newly-synthesized DNA may be associated with other cellular macromolecules as a replication complex (7, 10, 30). We searched for such complexes with the object of localizing them in morphologically characterized subnuclear structures. Nuclei of cells pulse-labeled with [^{3}H]dT were opened by gentle shearing, and the nuclear membrane and nucleoli were sedimented out (20). Essentially all the newly synthesized DNA remains in the supernatant, together with DNP. The small amount (about 10%) of chromatin that also sediments is somewhat enriched in pulse-

labeled DNA (specific activity about four times higher than in chromatin), probably due to the slightly higher sedimentation coefficient of DNP containing newly synthesized DNA (13). Equilibrium centrifugation in CsCl of the supernatant DNP after treatment with formaldehyde (21) shows that the newly synthesized DNA is not free, but is present in material having a buoyant density only slightly lower than that of DNP (Fig. 3*A*); it passes from this intermediate into DNP with a half-time of about 2 min (Fig. 3*B*; R. Hancock, unpublished results). Reconstruction experiments suggest that newly synthesized DNA does not become artifactually associated with DNP during the fractionation procedure. When native or heat-denatured [^{14}C]DNA was added at any step during the isolation of DNP containing [^{3}H]DNA, it did not become associated with the DNP to any significant extent but separated from it as free DNA on banding in CsCl (Fig. 3*C*).

We conclude that newly synthesized DNA does not exist within the nucleus as free DNA, but in a complex having a buoyant density in CsCl very close to that of chromosomal DNP. Labeled choline is not incorporated in material in this region of the gradient (R. Hancock, unpublished results); this constitutes further evidence that nuclear envelope phospholipids, most of which contain choline (28), are not associated with the regions of chromatin containing replicating DNA. As a working hypothesis we think that newly synthesized DNA is already associated with proteins as DNP, but made slightly less dense than mature DNP by the additional proteins associated with DNA replication; the difference in density is compatible with a ratio of protein/DNA about 5% higher than that in DNP. A DNP precursor of similar nature has been found in rat liver nuclei (A. Ya. Varshawsky, personal communication).

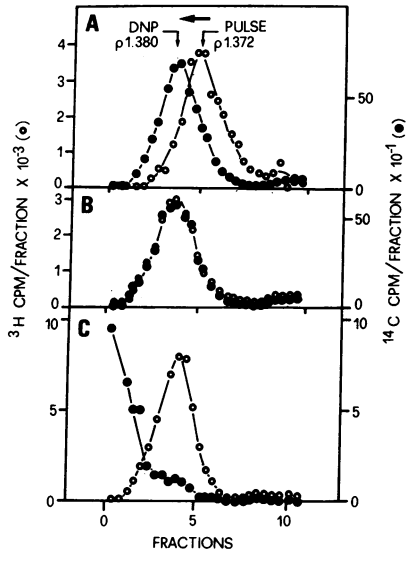

FIG. 3. CsCl equilibrium gradients of formaldehyde-treated DNP from nuclei of (*A*) cells previously labeled with [^{14}C]dT and pulse-labeled for 30 sec with [^{3}H]dT; (*B*) cells labeled as in (*A*) but after 30 sec a 100-fold excess of unlabeled dT was added, and incorporation continued for 30 min. (*C*) Reconstruction experiment in which purified [^{14}C]DNA, sonicated to a mean size of 12 S and heat denatured, was added to nuclei prelabeled in this case to contain [^{3}H]dT-labeled DNP. Essentially similar results were obtained when the [^{14}C]DNA was added before cell rupture or during washing of nuclei, except that less than 5% of the added DNA was recovered in the DNP fraction. ●, [^{14}C]dT radioactivity in previously labeled DNP; ○, [^{3}H]dT radioactivity in pulse-labeled DNA (*A*, *B*). In (*C*), the added DNA (●) is labeled with [^{14}C]dT and DNP is labeled with [^{3}H]dT (○).

TABLE 2. *Localization of pulse-labeled DNA or denatured DNA in the interphase after extraction of nuclei with SDS–phenol*

Material treated with SDS–phenol	% of labeled DNA recovered in the interphase
A. Nuclei of labeled cells	
Total DNA (^{14}C)	38
Pulse-labeled DNA (^{3}H)	89
B. Purified chromosomal [^{14}C]DNA mixed with unlabeled nuclei	
Native DNA	8
Heat-denatured DNA	86
Alkali-denatured DNA	99

DNA was extracted by SDS–phenol treatment (10) from (*A*) nuclei of cells first labeled with [^{14}C]dT and pulse-labeled for 30 sec with [^{3}H]dT; or (*B*) unlabeled nuclei to which was added native or denatured chromosomal [^{14}C]DNA.

Formation of Low-density Complexes of Newly Replicated DNA During Treatment of Nuclei with SDS and Phenol. In these experiments, newly synthesized DNA was never found in material of low density in CsCl, such as that observed by Friedman and Mueller (10) in phenol-extracted nuclei. We therefore attempted to relate our observations to these and other studies of newly synthesized DNA treated with phenol or chloroform–isoamyl alcohol. After phenol extraction of nuclei from cells pulse-labeled for 30 sec with [^{3}H]dT, we found newly replicated DNA located preferentially in the interphase material, as observed for HeLa cells (10) (Table 2, *A*). Newly replicated DNA in eukaryotic cells shows a "destabilized" structure that confers on it under some conditions characteristics of single-stranded DNA (31); this property may arise from closing of denatured template regions with expulsion of nascent single strands as proposed for replicating DNA of phage T4 (32). Prompted by this observation, we performed reconstruction experiments in which purified chromosomal DNA, native or denatured by heat or alkali, was mixed with unlabeled nuclei. After processing with SDS and phenol, denatured DNA was now preferentially recovered from the interphase, although native DNA remained in the aqueous phase (Table 2, *B*). Thus, the localization of newly synthesized DNA in the interphase upon SDS–phenol treatment does not necessarily indicate attachment to other cellular components, but represents further evidence that newly synthesized DNA possesses some properties of single-strandedness, as shown also by its behavior towards nitrocellulose (14, 33), hydroxyapatite (31, 34), and methylated albumin–Keiselguhr (33).

We also confirmed the observation (10) that newly synthesized DNA, dissolved from the interphase after phenol extraction, bands in CsCl gradients at a lower buoyant density than free DNA. However, in reconstruction experiments in

which labeled denatured DNA was added to unlabeled nuclei followed by SDS–phenol treatment, the DNA recovered from the interphase also showed an abnormally low buoyant density (Fig. 4*C*).

That the low density complexes containing newly synthesized DNA are formed during SDS-phenol treatment is shown in Fig. 4. One part of a preparation of nuclei containing pulse-labeled DNA was treated with SDS and phenol; after removal of the phenol layer, the aqueous phase and the interphase, containing together all the radioactivity, were combined. Before SDS–phenol treatment, essentially all the newly synthesized DNA in the nuclear lysate banded at its normal equilibrium density in CsCl (Fig. 4*A*); but after SDS–phenol treatment, over half had become complexed in material that floated on the CsCl gradient and was enriched in newly synthesized DNA (Fig. 4*B*). Complexes of newly synthesized DNA, probably with SDS- and phenol-denatured proteins, are thus generated nonspecifically under these conditions; we find no evidence for their existence *in vivo*.

DISCUSSION

In consideration of the results of autoradiographic experiments, three general models can be envisaged for the topology of DNA replication. Chromosomal DNA could remain fixed relative to the nucleus while the replication site moves along it; the replication site could remain fixed relative to the nuclear structure while the DNA is displaced relative to it; or both the replication site and the DNA could move freely. Only one special case of the second mechanism can be distinguished by electron microscope autoradiography, when the replication site remains associated with an identifiable and topologically fixed structure. Thus, although our results show that replication sites are not preferentially associated with the nuclear envelope, they do not allow us to discriminate between replication at other fixed, but ultrastructurally unidentifiable, sites and replication at moving sites.

Our conclusion that replication sites are not associated with the nuclear envelope is compatible with the observation (35) that DNA polymerase activity of liver nuclei is restricted to the chromatin and is not detectable in purified nuclear membranes. We believe that the difference between our conclusions and those of others drawn from biochemical experiments (6, 7, 10, 30, 8) is due to the following factors. First, considering the unusual properties of newly synthesized DNA during extraction with SDS–phenol, the artifacts that occur in these procedures make untenable conclusions drawn about the molecular associations *in vivo* of newly synthesized DNA. [The possibility that similar association between newly synthesized DNA and other cellular material may occur when other detergents are used for cell fractionation should be noted (7, 30).] Second, considering other biochemical studies in which it has been claimed that the DNA associated with the nuclear envelope behaves kinetically as a precursor of chromosomal DNA in regenerating liver (6) and in HeLa cells (8), it may be calculated in both cases that although the specific activity of membrane-associated DNA was somewhat higher than that of the bulk chromosomal DNA at the earliest time studied, over 80% of the newly synthesized DNA was already in chromosomal DNA. Further, the kinetics of labeling of intranuclear precursor pools were not known; these results are not, in our view, adequate to establish a precursor-product relationship.

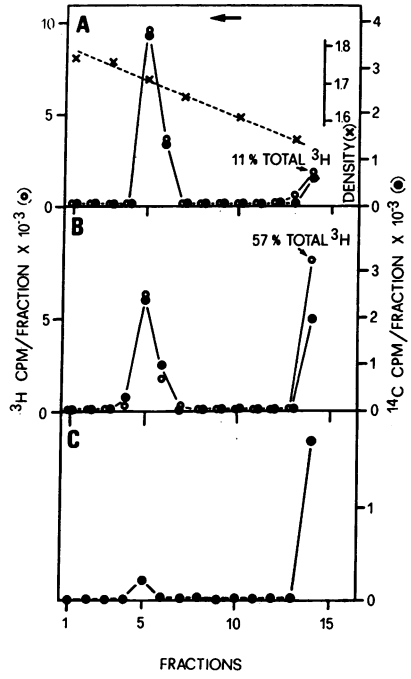

FIG. 4. Formation of DNA-containing complexes of low buoyant density during treatment of nuclei with SDS–phenol. Nuclei from cells first labeled with [^{14}C]dT and pulse-labeled for 30 sec with [^{3}H]dT were suspended (Dounce homogeniser, 20 strokes) in 0.05 M trisodium citrate (10). In half of the suspension (*A*), solid CsCl was dissolved by gently inverting the tube. The other half (*B*) was treated with SDS and phenol (10); the interphase and aqueous phase, containing together all the radioactivity, were pooled and dialyzed, and CsCl was added. (*C*) Reconstruction experiment in which heat-denatured chromosomal [^{14}C]DNA was added to unlabeled nuclei, followed by SDS–phenol treatment and dialysis as in (*B*). The density of the samples was adjusted to 1.65, and they were centrifuged to equilibrium and fractionated. Native DNA, mixed with unlabeled nuclei and treated as in (*C*), retained its normal density in CsCl. ●, [^{14}C]dT radioactivity in prelabeled DNA; ○, [^{3}H]dT radioactivity in pulse-labeled DNA; ×, density.

The proximity to the nuclear envelope of heterochromatin containing late-replicating DNA, and the relatively high dT content of the DNA of heterochromatin (36) may under certain conditions, for example late in S-phase, lead to preferential autoradiographic labeling of peripheral chromatin with [^{3}H]dT (2, 9). The results presented here show that, during the major part of S-phase, chromosomal DNA replicates at many sites distributed throughout the chromatin and not specifically associated with the nuclear envelope.

NOTE ADDED IN PROOF

We found that the distribution of total DNA–thymidine between peripheral and central regions of the nuclei of P815 cells, measured by electron microscope autoradiography of cells grown during several generations with [^{3}H]dT, is essentially the same as that of [^{3}H]dT incorporated during a 30-sec pulse. This finding further supports the conclusion that the higher density of DNA replication sites near the nuclear periphery is a consequence of the higher concentration of DNA in this region.

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