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Isolation of newly replicated chromatin by using shallow metrizamide gradients

(nucleosome/histone deposition/histone acetylation)

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ABSTRACT **The properties of chromatin containing newly synthesized DNA and protein were investigated. A fraction of chromatin enriched in newly replicated DNA was isolated by means of its increased density in metrizamide relative to bulk chromatin. The DNA of this fraction appeared to be packaged into nucleosomes but at a reduced nucleosomal spacing. Although pulse-labeled DNA was present in this dense fraction, nucleosomes labeled with short pulses of arginine or acetate were of normal density. The data presented are consistent with the conclusion that newly replicated DNA is associated with preexisting histones in a short-lived, compact structure, whereas newly synthesized histones are deposited at normal spacing some distance from the replication fork.**

The events involved in the replication of eukaryotic DNA and its assembly into chromatin have been the subject of a number of recent investigations. These studies have utilized either chromatin whose proteins had been covalently bound to DNA by formaldehyde fixation (1–4) or chromatin assembled under conditions where protein synthesis had been inhibited (5, 6). Some of these investigations have yielded conflicting results. In addition, possible disruption of chromatin structure, resulting from the methods of sample preparation and analysis used, leaves the results of previous experiments open to some objection. To avoid many of these objections, we developed techniques by which unfixed, newly replicated chromatin can be isolated by using metrizamide density gradient centrifugation. In this paper we describe the isolation and properties of newly replicated chromatin and compare these results with those obtained previously.

MATERIALS AND METHODS

Cell Culture and Labeling. Friend leukemia cells, uninducible clone F4+, were grown in suspension culture as described (7). The generation time under our conditions was 15.2 hr. For various experiments, cells were labeled with [^{14}C]thymidine (61 Ci/mmol; Amersham) at 4 nCi/ml, [^{14}C]L-arginine (312 mCi/mmol; Schwarz/Mann) at 50 nCi/ml, [^{3}H]thymidine (22 Ci/nmol; Amersham) at 40 μCi/min/ml, [^{3}H]L-arginine (8.8 Ci/mmol; Amersham) at 250 μCi/min/ml, and [^{3}H]acetic acid (2 Ci/mmol, Moravek Biochemicals, Burbank, CA) at 300 μCi/min/ml (1 Ci = 3.7 × 10^{10} becquerels). All samples for scintillation counting were made 0.1% in sodium dodecyl sulfate with a final volume of 0.4 ml, and radioactivity was measured in 3 ml of Aquasol-2 (New England Nuclear). Under these conditions, the spillover from the ^{3}H window to the ^{14}C window was 4%, and the ^{14}C to ^{3}H spillover was 11%. No effect on the counting efficiencies or spillover ratios was seen with metrizamide from 0% to 50%. The presence of the sodium dodecyl sulfate eliminated almost all self-quenching of large molecules.

Preparation of Nuclei and Nucleosomes. Nucleosomes were prepared from purified nuclei as described (8), with the exception of micrococcal nuclease, which was purchased from Worthington (1 unit produces 1 A_{260} unit of acid-soluble material from DNA in 30 min at pH 8.0, 37°C). To facilitate comparison of different experiments, digestions were carried out for 5 min at 37°C at specified ratios of nuclease units to number of nuclei.

Metrizamide Gradient Centrifugation. Metrizamide was obtained from Myegaard, A/S, Oslow, Norway. All metrizamide solutions contained 2.5 mM Tris (pH 8) and 2.5 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA). In a 1.3 by 5 cm cellulose nitrate centrifuge tube, 2.3 ml of sample in 30% (wt/vol) metrizamide was layered over 1.7 ml of 60% (wt/vol) metrizamide and was overlayered with 1 ml of Tris/EGTA. This was centrifuged at 10,000 rpm for 16 hr at 4°C in the Sorvall TV-865 rotor, and gradient fractions were collected dropwise from the bottom. Fraction densities were determined from the refractive index at 25°C (9). The peak density and percentage of total material were determined by using a nonlinear least-squares Gaussian fitting program (10). DNA samples for electrophoresis were prepared by proteinase K (EM Laboratories, Elmsford, NY) digestion of gradient fractions.

Polyacrylamide Gel Electrophoresis. Tube gels of 2.5% polyacrylamide/0.5% agarose (0.6 × 15 cm) containing 89 mM Tris, 89 mM borate, and 2.5 mM EDTA (pH 8.3) were prepared essentially as described by Maniatis *et al.* (11). Gels were electrophoresed for 3 hr at 100 V and fractionated into 2-mm slices (Aliquogel fractionator, Gilson). Gel fractions were assayed for radioactivity as described above. Radioactivity of gels polymerized with [^{3}H]DNA and [^{14}C]DNA of various sizes showed no quenching by the polyacrylamide fragments, and there was minimal dependence of the counting efficiency and spillover on DNA length.

RESULTS

Buoyant Density of Pulse-Labeled Chromatin. In order to examine the buoyant density of unfixed, newly replicated chromatin, cells were labeled with [^{14}C]thymidine for 24 hr and then with [^{3}H]thymidine for 1, 10, or 100 min. After brief digestion with micrococcal nuclease, the total nucleosomal fraction was centrifuged in metrizamide. Fig. 1 shows that the 1-min-labeled nucleosomes were more dense than the long-term-labeled nucleosomes and that this density difference disappeared with longer periods of labeling. Table 1 gives the

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Abbreviations: bp, base pairs; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

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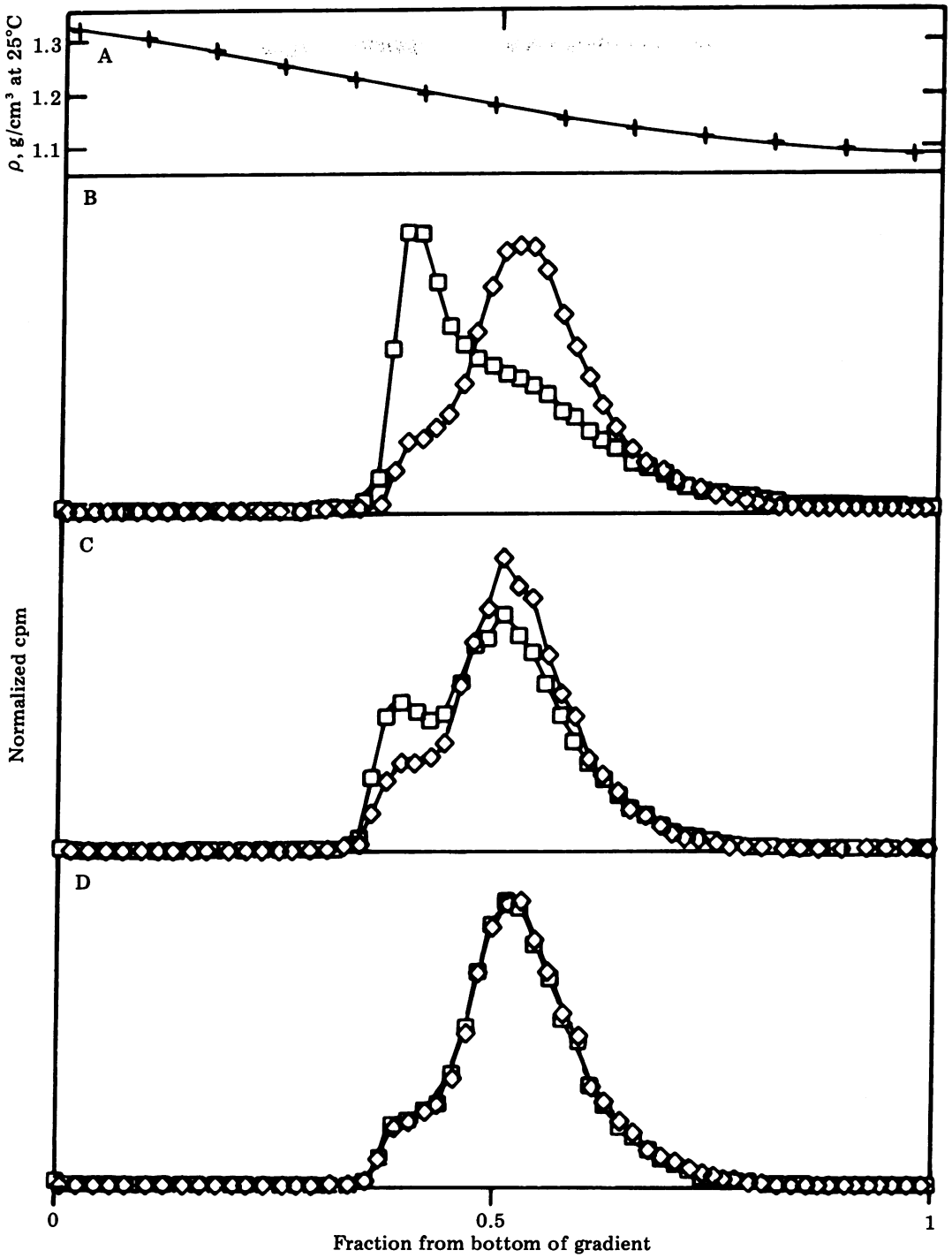

FIG. 1. Separation of newly replicated chromatin from bulk chromatin in metrizamide gradients (*A*). Cells were labeled for 24 hr with [^{14}C]thymidine and then for 1 min (*B*), 10 min (*C*), or 100 min (*D*) with [^{3}H]thymidine. After digestion with micrococcal nuclease at 5.33×10^7 units/nucleus and removal of acid-soluble material (2.4% ^{14}C; 8.8, 2.4, 2.4% ^{3}H), the total nucleosomal fraction was centrifuged in metrizamide. The data in *B–D* are plotted so that the total area of each panel represents 7 times the number of cpm in the gradient. ◇, ^{14}C cpm; □, ^{3}H cpm (see Table 1).

densities of the two chromatin fractions and shows that bulk chromatin had a dense shoulder constituting approximately 4% of the total DNA. Birnie *et al.* (12) found the density of native mouse DNA to be 1.118 g/cm^3 and the density of purified proteins to vary from 1.24 to 1.29 g/cm^3. The density of nuclear DNA (proteinase K-treated) in metrizamide is 1.12 g/cm^3 and that of nucleosomal protein (DNase I-treated) is 1.23 g/cm^3 (data not shown).

Removal of Density Difference by Extensive Nuclease Digestion. Since newly replicated chromatin is digested to nucleosomes more slowly than bulk chromatin is (8, 13), the density difference in Fig. 1 might be due to the presence of newly replicated DNA in higher nucleosome multimers. If this were the case, the density difference should disappear if soluble nucleosomes (i.e., low multimers) were isolated and analyzed. That this was not the case is demonstrated by the fact that 1-min-labeled soluble nucleosomes are more dense than long-term-labeled nucleosomes (Fig. 2). However, the density difference disappeared as the chromatin was digested to monomers. This suggests that the density difference is not due to a

Table 1. Buoyant density of chromatin fractions in metrizamide gradients of Fig. 1

Time, min	Heavy			Light		
	^3H	%T*	^{14}C	%T*	^3H	^{14}C
1	1.2034	28.2	1.2070	2.9	1.1712	1.1664
10	1.2106	10.4	1.2107	6.0	1.1730	1.1701
100	1.2097	3.6	1.2093	3.4	1.1678	1.1669
Mean	1.2079	—	1.2090	4.1	1.1707	1.1678
SD	0.0039	—	0.0019	1.7	0.0026	0.0020

* Percent of total cpm in gradient.

Table 2. Buoyant density of nucleosomes as a function of extent of digestion

Enzyme range*	Thymidine			Arginine		
	n†	ρ	SD	n†	ρ	SD
−8 to −7.1	5	1.1577	0.0024	3	1.1580	0.0078
−7 to −6.1	7	1.1517	0.0059	7	1.1547	0.0068
−6 to −5.1	5	1.1363	0.0049	2	1.1417	0.0007

* Logarithm of enzyme concentration (units/nucleus); lower and upper limits for grouped data.
† Number of gradients.

higher protein/DNA ratio in the nucleosome core of newly replicated chromatin compared with that of total chromatin but rather to a decreased internucleosome spacing.

Fig. 2 also suggests that there is a shift in density of bulk nucleosomes as nuclease digestion proceeds. This is further demonstrated by the data from a number of experiments summarized in Table 2.

Nucleosome Spacing of Isolated Newly Replicated Chromatin. To determine the nucleosome spacing of this fraction, DNA was isolated from the dense peak of chromatin that had been labeled for 24 hr with [^{14}C]thymidine and for 1 min with [^3H]thymidine, as illustrated in Fig. 2*C*. This DNA was analyzed by electrophoresis on a polyacrylamide gel, and the molecular weights of the peak materials were estimated from relative mobility by using ^{32}P-labeled pBR322 DNA digested with the restriction endonuclease *Hin*fI (Fig. 3*B*). For comparison, DNA was isolated from the chromatin after the brief micrococcal nuclease digestion but before centrifugation and was analyzed on a parallel gel (Fig. 3*A*). The distribution of the DNA of the newly replicated fraction (Fig. 3*B*) is dramatically different than that of the bulk nucleosomes (Fig. 3*A*). It appears that the unit spacing of the low multimers (dimer and trimer) is approximately 160 base pairs (bp) rather than the 200 bp observed for the bulk nucleosomes (Fig. 3*C*). The spacing for the tetramer peak approaches that for the bulk chromatin. The two peaks of DNA towards the top of the gel are unexplained but might represent some unique structure associated with chromatin labeled for such a brief period.

Buoyant Density of Newly Deposited Nucleosomal Protein. Some investigators have suggested that newly synthesized chromosomal protein is deposited on unreplicated DNA (3, 4, 15, 16). There has been some uncertainty about this conclusion since CsCl gradients of formaldehyde-fixed chromatin were used. To determine the degree of association of newly synthesized histone with newly replicated DNA by using unfixed chromatin, cells were labeled with [^{14}C]arginine for 24 hr and then with [^3H]arginine for 5 min. Tris/EGTA-soluble nucleosomes from these cells were run on metrizamide gradients after digestion to varying extents. Fig. 4*B* shows one of these gradi-

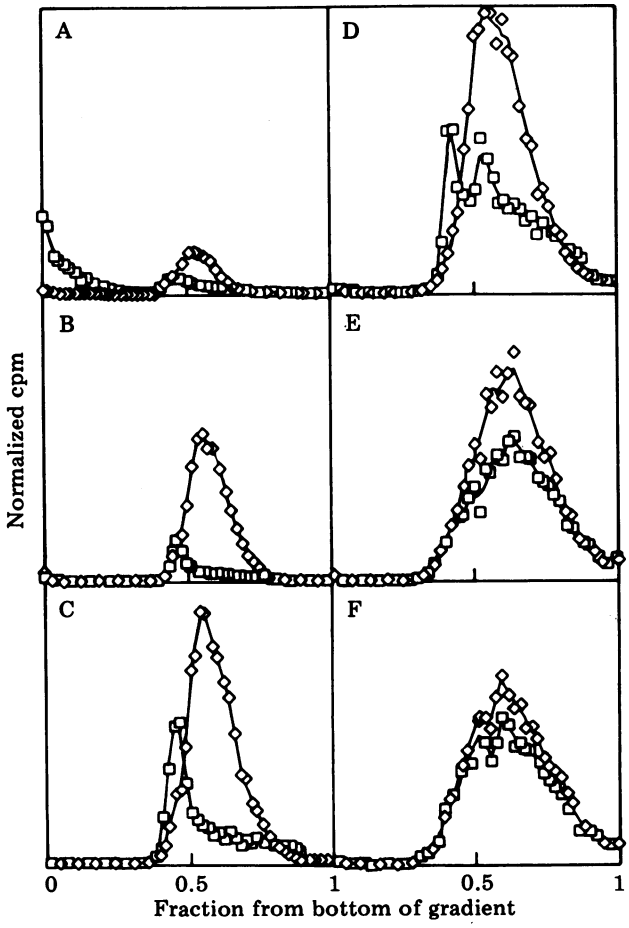

FIG. 2. Effect of extent of digestion on separation of newly replicated chromatin from bulk chromatin. Tris/EGTA-soluble nucleosomes from cells labeled for 24 hr with [^{14}C]thymidine (◇) followed by 1 min with [^3H]thymidine (□) were centrifuged in metrizamide. The total area of each panel is 6 times the total nuclear cpm. Digestions *A–F* were from 6.67×10^8 to 6.67×10^6 units/nucleus. Acid-soluble ^{14}C-labeled material increased from 0.6% to 23% and ^3H-labeled acid-soluble material, from 4% to 39%. The lines were drawn by 5-point quadratic smoothing of the data (10, 14).

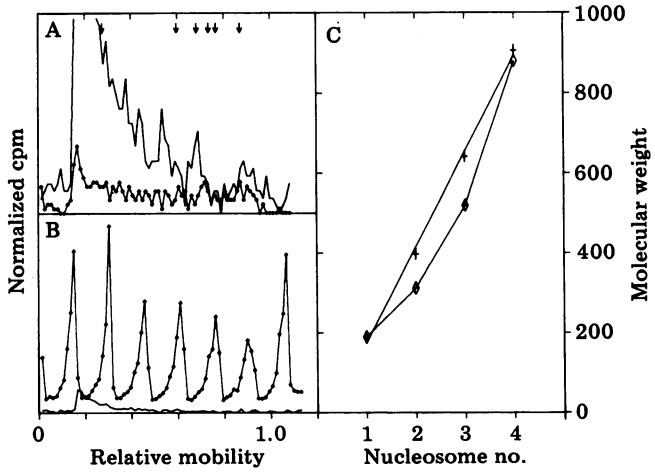

FIG. 3. Nucleosomal DNA spacing of dense fraction from metrizamide gradients. Samples from the gradient shown in Fig. 2*C* were analyzed by electrophoresis on 2.5% polyacrylamide/0.5% agarose gel (11). (*A*) Tris/EGTA-soluble fraction before centrifugation. (*B*) Heavy peak from gradient (fractions 25 and 26). —, ^{14}C; ◇, ^3H. Mobility is relative to bromophenol blue. The arrows mark the positions of ^{32}P-labeled pBR322 DNA digested with *Hin*fI and correspond to 1631, 516/506, 396, 344, 298, and 221/220 bp. (*C*) DNA size versus nucleosome number. +, ^{14}C peaks (from *A*); (◇), ^3H peak (from *B*).

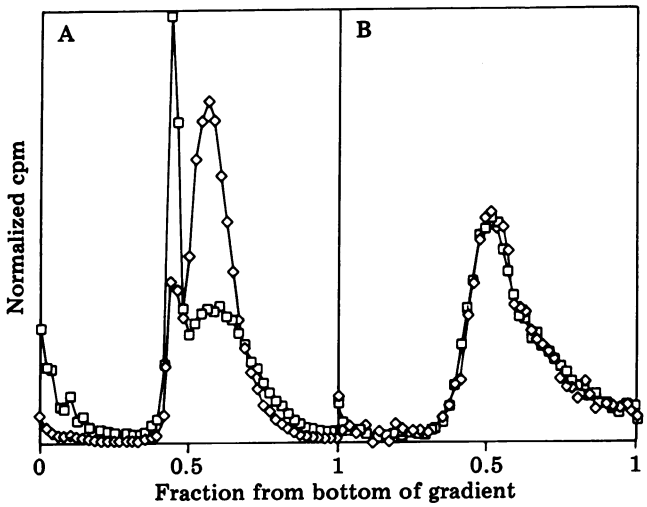

FIG. 4. Comparison of density distribution of thymidine- and arginine-labeled, Tris/EGTA-soluble nucleosomes. Digestion was with 1.0×10^7 units/nucleus. (*A*) Cells were equilibrated for 24 hr with [^{14}C]thymidine (◇) (2.8% acid-soluble) followed by 5 min with [^{3}H]thymidine (□) (3.7% acid-soluble). (B) Cells were equilibrated for 24 hr with [^{14}C]arginine (◇) followed by 5 min with [^{3}H]arginine (□). The total area of each panel is 6.6 times the total cpm in each gradient.

ents, along with a gradient of [^{14}C]thymidine-labeled (24 hr)/[^{3}H]thymidine-labeled (5 min), Tris/EGTA-soluble nucleosomes (at the same nuclease concentration). In no case was [^{3}H]arginine radioactivity detected in the dense region. Indeed, the ^{14}C and ^{3}H radioactivities were nearly identical. Similar results were obtained for cells labeled with arginine for 10 or 100 min and for cells labeled with acetate for 1, 10, or 100 min.

DISCUSSION

Metrizamide Fractionation of Chromatin. Because metrizamide is an inert, nonionic density-gradient material, it has been used to examine chromatin properties (e.g., ref. 9). Rickwood *et al.* (17) demonstrated the separation of chromatin into two fractions as a function of the extent of mechanical shearing and took this as an indication of the presence of protein-rich and protein-poor regions. The data of Fig. 2 and Table 2 suggest that the separation is due instead to the decreased density of low nucleosome multimers. This shift well may be due to the removal of nonhistone proteins/or histone H1 during digestion or shearing (18).

Buoyant Density of Pulse-Labeled Chromatin. In CsCl gradients of formaldehyde-fixed chromatin, a decreased density has been reported for pulse-labeled chromatin (1, 5, 15), cycloheximide-treated chromatin (5), and *in vitro* labeled chromatin (19). This decreased density in CsCl corresponds to the increased density in metrizamide demonstrated above, since protein is less dense than DNA in CsCl but more dense than DNA in metrizamide. Both increased (20) and decreased (21) densities in metrizamide have been reported for pulse-labeled chromatin that had been mechanically sheared. As Noll *et al.* (22) have pointed out, there is significant disruption of nucleosome structure during mechanical shearing; in addition, these results demonstrate the importance of controlling the extent of shearing during sample preparation. These facts make interpretation of the previous reports difficult.

Hancock (23) has shown that the preparation of nuclei and chromatin by methods similar to those used in these experiments do not allow exchange of histones between deoxyribonucleoprotein molecules. The lack of histone exchange, the fact that nuclease digestion was used to prepare nucleosome samples, and the use of the nonionic density-gradient material make it unlikely that the results are due to distortion or disruption of the chromatin structure.

Deposition of Newly Synthesized Histone. Having established a method for isolating native nucleosomes containing newly replicated DNA, we then examined the distribution of newly synthesized histone. Fig. 4 shows that no significant deposition of this histone occurs on new DNA during the lifetime of the closely packed nucleosomal structure. This is in agreement with the results reported for fixed chromatin (3, 4, 15, 16). Our data do not rule out the possibility that new histones are deposited on the unpackaged new DNA strand at normal spacing. However, previous results with fixed chromatin suggest that this is unlikely.

Consistency of Results with Eukaryotic Replication Rates. For an unsynchronized cell population with a generation time, t_g, and an S phase transition time, t_s, the fraction of cells engaged in DNA replication, f_s, is t_s/t_g. The average time to complete a replicon, t_r, is L/R, where L is the mean replicon length and R is the mean replication rate. With the assumption that the number of forks started per unit time is approximately constant during S, the fraction of the unsynchronized cell population actually synthesizing DNA is

$$f = \frac{t_r}{t_s} \cdot \frac{t_s}{t_g} = \frac{L}{R \cdot t_g}.$$

From the mean values for a number of eukaryotic cell types (determined by electron microscopy, autoradiography, and alkaline sucrose sedimentation), R is 0.6 ± 0.3 μm/min per replicon (1800 ± 900 bp/min per replicon) (24–29) and L is 14 ± 4 μm (42 ± 12 kilobases) (26–33). Thus, $t_r = 23 \pm 11$ min. Because the generation time for F4$^+$ cells is 15.2 hr, $f_r = 2.5 \pm 1.2\%$; because initiation of replication is probably not uniform throughout S (34, 35), the expected values of f_r are somewhat higher. The observed value of $4.1 \pm 1.7\%$ (Table 1) is thus quite reasonable. The time it takes newly replicated chromatin to mature to normal density (10–30 min) is close to t_r, which may indicate that completion of replication is a requirement for return to normal nucleosome configuration. This would be expected if the decreased nucleosome spacing occurs to accommodate the replication machinery. However, the increased protein/DNA ratio may be due, at least in part, to the presence of the "replication proteins" as has been suggested by Seale cited in ref. 8. The fact that protein synthesis is required for maturation to normal density in CsCl (5) may favor the former hypothesis. The agreement of the results presented in this paper with eukaryotic replication rates and replicon sizes determined by unrelated methods lends support to our current picture of the events involved in chromatin replication.

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