Changes in Nucleosome Repeat Lengths Precede Replication in the Early Replicating Metallothionein II Gene Region of Cells Synchronized in Early S Phase†

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ABSTRACT: Previous investigations showed that inhibition of DNA synthesis by hydroxyurea, aphidicolin, or 5-fluorodeoxyuridine produced large changes in the composition and nucleosome repeat lengths of bulk chromatin. Here we report results of investigations to determine whether the changes in nucleosome repeat lengths might be localized in the initiated replicons, as postulated [D'Anna, J. A., & Prentice, D. A. (1983) *Biochemistry 22*, 5631–5640]. In most experiments, Chinese hamster (line CHO) cells were synchronized in G1, or they were synchronized in early S phase by allowing G1 cells to enter S phase in medium containing 1 mM hydroxyurea or 5 μg mL^{-1} aphidicolin, a procedure believed to produce an accumulation of initiated replicons that arise from normally early replicating DNA. Measurements of nucleosome repeat lengths of bulk chromatin, the early replicating unexpressed metallothionein II (MTII) gene region, and a later replicating repeated sequence indicate that the changes in repeat lengths occur preferentially in the early replicating MTII gene region as G1 cells enter and become synchronized in early S phase. During that time, the MTII gene region is not replicated nor is there any evidence for induction of MTII messenger RNA. Thus, the results are consistent with the hypothesis that changes in chromatin structure occur preferentially in the early replicating (presumably initiated) replicons at initiation or that changes in chromatin structure can precede replication during inhibition of DNA synthesis. The shortened repeat lengths that precede MTII replication are, potentially, reversible, because they become elongated when the synchronized early S-phase cells are released to resume cell cycle progression.

Synchrony of Chinese hamster (line CHO) cells in early S phase by allowing G1 cells to enter S phase in the presence of partial inhibitors of DNA synthesis (e.g., hydroxyurea, aphidicolin, 5-fluorodeoxyuridine) produces enormous changes in the composition and structure of bulk chromatin (D'Anna & Prentice, 1983; D'Anna & Tobey, 1984; D'Anna et al., 1985, 1986). The changes include (1) time-dependent depletion of ~30% of histone H1 in the cell, (2) failure of newly replicated chromatin to achieve longer mature nucleosome repeat lengths, (3) a slow reduction in the nucleosome repeat lengths of bulk chromatin, and (4) an increase in the relative quantities of a number of non-histone chromosomal proteins.

Those results are potentially important, because snychrony of cells in that way appears to produce an accumulation of initiated, partially elongated replicons that arise from early replicating DNA (Walters et al., 1976a,b; Heintz & Hamlin, 1982; D'Anna & Prentice, 1983; Decker et al., 1986). Although the depletion of histone H1 and changes in nucleosome repeat lengths might be occurring at random in early S-phase cells, the results can be rationalized if it is assumed that (1) loss of histone H1 and changes in chromatin structure normally occur specifically in replicons at initiation or (2) depletion of histone H1 and changes in chromatin structure can be uncoupled from DNA synthesis in the initiated replicons during inhibition of DNA synthesis (D'Anna & Prentice, 1983). If these models are correct, then measurements of chromatin structural changes between G1 and early S-phase cells should reveal the following features: (1) changes in chromatin structure should occur preferentially in normally early replicating regions of the genome, because those replicons should be initiated; (2) the chromatin structural changes in early replicating regions should exceed those observed for bulk chromatin, because changes in bulk chromatin are an average of changes in early and late replicating regions; (3) changes in chromatin structure should precede the replication forks in initiated replicons, and the distance will depend on whether the changes occur throughout the replicon at initiation or as a function of time during inhibition of DNA synthesis; (4) changes should not be observed in normally late replicating regions of the genone—at least during moderate periods of block.

We have begun to test these notions by measuring the nucleosome repeat lengths of bulk chromatin, the metallothionein II (MTII)[1] gene, and repeated sequences that are homologous to a cloned sequence called pHuR-093 in G1 cells and cells synchronized in early S phase. MTII is a single-copy or low-copy gene that is not expressed in wild-type CHO cells (Hildebrand et al., 1980); however, (1) it is replicated during the first half of S phase when cells are synchronized in early S phase and released to resume cell cycle traverse (Stallings et al., 1986), and (2) treatment of early S-phase CHO cells with 5-azacytidine increases the frequency of CHO transformation to the MT-active phenotype (Stallings et al., 1986). Thus, the unexpressed CHO MTII gene is part of an early

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[1] Abbreviations: APC, aphidicolin; BrdU, bromodeoxyuridine; EDTA, ethylenediaminetetraacetic acid; FCM, flow cytometry; HU, hydroxyurea; MT, metallothionein; NP-40, Nonidet P-40 (Shell); SDS, sodium dodecyl sulfate; SSC, 150 mM NaCl/15 mM sodium citrate, pH 7.2; Tris, tris(hydroxymethyl)aminomethane.

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replicating replicon that, potentially, is initiated when cells are synchronized in early S phase.

pHuR-093 is a hexameric repeated sequence that is located at or near the telomeres of human chromosomes and chromosomes from a variety of other organisms including CHO cells (Moyzis et al., 1988); however, at low stringency, pHuR-093 also hybridizes with large centromeric regions of some CHO chromosomes (J. Meyne and R. K. Moyzis, personal communication). Although the timing of pHuR-093 replication has not been precisely defined in CHO cells, its synthesis commences more slowly than that of MTII when CHO cells synchronized in early S phase are released to resume cell cycle traverse (see below).

Our results with these systems are consistent with the hypothesis that changes in chromatin structure occur preferentially in initiated replicons at initiation or that they can precede replication in those regions during the inhibition of DNA synthesis. The shortened repeat lengths that precede replication in the early S-phase cells are, potentially, reversible, because they become substantially longer when cells are allowed to resume cell cycle traverse. These results are similar to those reported for the histone H4 gene by Moreno et al. (1986); however, whereas the structural changes associated with the histone H4 gene may be related to transcriptional activity, the shortened repeat lengths of MTII appear to be associated solely with replication or the inhibition of DNA synthesis in initiated replicons.

Experimental Procedures

Cell Cultures and Cell Cycle Analyses. Chinese hamster (line CHO) cells and the cadmium-resistant CHO variant Cd^r 30F9-6 (Enger et al., 1981; Tobey & Seagrave, 1984) were grown in suspension cultures of "complete" F10 medium lacking calcium and supplemented with 15% heat-treated newborn calf serum, streptomycin, and penicillin (Tobey et al., 1966). Cells were synchronized in the G1 phase of the cell cycle by the isoleucine deficiency method (Tobey & Ley, 1971). They were further synchronized in early S phase by releasing G1 cells into complete medium containing 1 mM hydroxyurea, complete medium containing 5 $\mu g\ mL^{-1}$ aphidicolin (D'Anna & Tobey, 1984), or thymidine-deficient medium (F10 lacking calcium and thymidine, supplemented with 15% dialyzed newborn calf serum) containing 1 mM hydroxyurea and 20 μM bromodeoxyuridine to density-label newly replicated DNA. In some experiments, cells were subjected to prolonged block in early S phase by releasing G1 cells into complete medium containing 1.0 mM hydroxyurea for 24 h. In most of the synchrony experiments, exponentially growing cells were prelabeled for 24 h and during synchrony in G1 with 15 $\mu Ci\ L^{-1}$ [^{3}H]thymidine to radiolabel bulk or old DNA.

The distribution of cells in the cell cycle was determined from analysis of flow cytometry (FCM) histograms (Dean & Jett, 1974; Crissman et al., 1977; D'Anna & Prentice, 1983). Average DNA content per cell was computed from the histograms, assigning a relative DNA content of 1.0 to G1 cells and a relative DNA content of 2.0 to G2/M cells (D'Anna & Prentice, 1983).

Isolation and Digestion of Nuclei with Micrococcal Nuclease. Nuclei were isolated by using a nonionic detergent, NP-40, in 15 mM Tris·HCl/3 mM $CaCl_2$, pH 7.2 (D'Anna et al., 1982). Isolated nuclei were resuspended at 0.8–1.0 $\mu g\ \mu L^{-1}$ DNA in 15 mM Tris·HCl, 1.0 mM $CaCl_2$, and 1.0 mM $MgCl_2$, pH 7.2, and digested with 5 $\mu g\ mL^{-1}$ micrococcal nuclease. Digestions were stopped, DNA was isolated, and the percentages of acid-soluble DNA were determined as described (D'Anna & Prentice, 1983). Isolated DNA was further treated with 60 $\mu g\ mL^{-1}$ bovine RNase A and extracted 3 times with chloroform/isoamyl alcohol before recovery by ethanol precipitation.

Restriction Endonuclease Digestion of DNA. High molecular weight DNA was isolated from nuclei by using the general deproteinization procedures employed for nuclei digested with micrococcal nuclease, except the DNA was spooled on a glass rod before being placed in 10 mM Tris·HCl/1.0 mM EDTA, pH 8.0.

DNA was digested with restriction nucleases at 0.125 $\mu g\ \mu L^{-1}$ in the buffers recommended by Maniatis et al. (1982a) or by Bethesda Research Laboratories. Digested DNA was treated with EDTA and RNase before it was phenol/chloroform extracted and recovered by ethanol precipitation.

Electrophoresis, Southern Transfers, and Slot Blots. DNA digested with micrococcal nuclease was resolved by electrophoresis (40 V for 11 h) in 0.35 × 15 × 20 cm^3 1.5% agarose (SeaKem LE) flat-bed gels in 89 mM Tris, 89 mM borate, and 2.0 mM EDTA buffer (Maniatis et al., 1982b). Seventeen micrograms of DNA was loaded into 0.15 × 0.7 cm^2 wells.

Genomic DNA digested with restriction nucleases was separated in 0.6–0.8% agarose gels using the apparatus and buffer system described above, but loads of 3–6 μg were used. Mixtures of unlabeled and ^{32}P-end-labeled ϕX174, *Hae*III RF DNA or λ, *Hin*dIII RF DNA were run in separate lanes from the samples as size/mobility standards.

DNA from micrococcal nuclease digests was transferred from gels to Zetabind nylon membranes (AMF Cuno) by capillary transfer (Southern, 1975) with 0.4 N NaOH (Reed & Mann, 1985). Gels containing high molecular weight DNA were agitated 10 min in 0.25 M HCl before treatment and transfer with 0.4 N NaOH.

Genomic DNA for slot blotting was digested to completion with *Hin*dIII, or it was sheared by successive passage through 20- and 26-gauge hypodermic needles (Mariani & Schimke, 1984). The sheared or digested DNA was treated with RNase for 1 h and recovered by ethanol precipitation. DNA concentrations were determined from absorbance spectra in 10 mM Tris·HCl/0.1 mM EDTA, pH 8.0, or relative DNA concentrations were determined by ^{3}H scintillation spectrometry of radiolabeled DNA. Constant quantities of 0.63–2.5 μg of DNA or [^{3}H]DNA cpm were adjusted to 0.28 M NaOH and incubated 30 min at 65 °C before aliquots of 300, 150, and 75 μL were transferred to a row of slots in a Schleicher & Schuell Minifold II apparatus. The vacuum was then turned on, and each slot was rinsed with an equal volume of or, at least, 150 μL of 0.28 M NaOH.

DNA–DNA Hybridizations. MTII was hybridized with a 1076 bp cloned genomic probe that spanned from position −247 to 62 bp beyond the MTII-transcribed sequences (in active cell lines). The probe was excised by digestion with *Hin*dIII and *Pst*I from a 2.2 kb *Hin*dIII CHO genomic fragment cloned in pUC-8 (Grady et al., 1987). The excised probe was separated in an agarose minigel and recovered after electroelution onto Whatman DEAE paper (Dretzen et al., 1981).

Nucleosome ladders and slot-blotted DNA were also hybridized with the repeated sequence $(TTAGGG)_n$ that had been cloned into pBR322 from sheared human repetitive DNA (Moyzis et al., 1988). The complete plasmid was used for nick translation and hybridization.

Hybridization probes were radiolabeled with ^{32}P by nick translation using all four [α-^{32}P]dNTPs, a commercial mixture of DNA polymerase I/DNase I (BRL), and the Tris·HCl buffer system used by BRL in their nick translation kits. The

desalted nick-translated probes had specific activities of (2–4) $\times 10^9$ cpm μg^{-1}.

Zetabind blots and slot blots were agitated 1 h at 60–65 °C in 0.1 × SSC/0.5% SDS prior to mixing with prehybridization/hybridization buffers, as recommended by the manufacturer. Southern blots to be hybridized with MTII were prehybridized overnight at 42 °C in 5 × SSC, 0.020 M phosphate (pH 6.7), 5 × Denhardt's solution [i.e., 0.1% Ficoll, 0.1% poly(vinylpyrrolidone), and 0.1% bovine serum albumin], 1.0 mM EDTA, 0.10% SDS, 45% formamide, 10% dextran sulfate, and 125 μg mL^{-1} heat-denatured salmon sperm DNA (Meinkoth & Wahl, 1984; Crawford et al., 1985). Slot blots were prehybridized in the same solutions without dextran sulfate.

Southern and slot blots to be hybridized with pHuR-093 were prehybridized as described above except the concentration of formamide was reduced to 30%, and dextran sulfate was excluded. Blots in the prehybridization solution were hybridized with 10^6 cpm mL^{-1} NaOH-denatured probe at 42 °C (42–48 h for MTII and 20–24 h for the repeated sequence pHuR-093). Hybridized blots were agitated in 2.0 × SSC/0.10% SDS 3 times at room temperature (10 min each) and once at 58–60 °C (15 min). They were then agitated twice for 15 min in 0.1 × SSC/0.10% SDS (57 °C for MTII and 52 °C for pHuR-093). At these conditions, >95% of the hybridization signal is specific for MTII; the rest comes from hybridization with MTI. Most of the pHuR-093 hybridization signal comes from the cluster of repeats located in the vicinity of the centromeres in some CHO chromosomes. The use of salmon sperm DNA as a competitor should compete out the exact sequences located at the telomeres (Moyzis et al., 1988), and the low stringency (30% formamide) allows hybridization with the repeats at the centromeres.

Repeat Length Measurements. The migration of nucleosome ladders from ethidium bromide fluorescence and from autoradiograms was measured with an engineering ruler, and the repeat lengths were calculated by a linear regression method (Thomas & Thompson, 1977). Repeat lengths of bulk chromatin, MTII, and pHuR-093 were all plotted as a function of the percentage acid-soluble bulk DNA (D'Anna & Prentice, 1983). Comparison of repeat lengths between different populations of cells was determined from analysis in a single gel to avoid variations in the absolute values of repeat lengths from gel to gel. Whereas the values of the measured repeat lengths vary by about ±3 bp from gel to gel, the differences between repeat lengths on the same gel are only about ±1.5 bp.

Determination of the Percentage of Newly Replicated DNA in S-Phase Cells. As synchronized cells progress from G1 to be resynchronized in early S phase, some of the MTII DNA may be replicated. If so, then the average number of MTII genes per cell in the early S-phase population will be given by $N[(1 - f) + 2f]$ or by $N(1 + f)$ where N is the number of MTII copies in a G1 cell and f is the fraction of cells in which MTII has been replicated. It follows that (1) the ratio of the average MTII content per cell, R, between the early S-phase cells and G1 cells will be given by $R = 1 + f$ and (2) the fraction of newly replicated MTII in the S-phase cells, F, will be given by $F = 2f/(1 + f)$ or by $F = 2(R - 1)/R$.

We have used two methods to estimate the fractions, f and F, for different populations of cells in S phase. In the first method, we have estimated the relative MTII content between early S-phase cells and G1 cells, R, by hybridization of slot-blotted DNA from G1 cells and cells synchronized in early S phase. Equal weights of DNA from G1 or S-phase cells were slot-blotted onto Zetabind in a Schleicher & Schuell Minifold II apparatus as described above. After hybridization, the relative integrated intensities were quantified from absorbance measurements of the autoradiograms. A 4.0 mm wide rectangular beam of light in a Helena Quick Scan R&D gel scanner was used to quantify the autoradiographic density in the middle of the 8 mm wide rectangular band of slot-blotted DNA. The integrated intensities from the three different loads of a sample were then plotted to determine the dependence of density on DNA concentration in a single sample. Corrected relative intensities per unit weight of DNA were then multiplied by the average relative DNA content per cell (from FCM) to determine R, the average relative MTII content per cell between early S-phase and G1 cells.

In the second method, cells were synchronized in early S phase by releasing G1 cells into thymidine-deficient medium containing 1.0 mM hydroxyurea and 20 μM BrdU to density-label new DNA replicated during the entry of cells into S phase. The isolated high molecular weight DNA was sheared, adjusted to a density of 1.71 g mL^{-1} with CsCl, and centrifuged in a Beckman 70.1 TI rotor at 34.0 krpm for 47 h at 20 °C. The rotor speed was reduced to 31.5 krpm for 50 min before ending the run. Recovered DNA from the heavy and light fractions was analyzed in an agarose minigel to estimate the concentrations of each. Equal weights of the heavy and light fractions were slot-blotted onto Zetabind and hybridized with MTII. Values of f and F were calculated from the relative quantities of MTII in the heavy and light fractions and the relative quantities of total new and old DNA estimated from FCM.

Isolation and Analysis of RNA. Total cellular RNA was isolated by the method of Longmire et al. (1987). RNA concentrations were determined from the absorbance at 260 nm, and 7.5 μg of RNA was analyzed in 1.4% agarose/formaldehyde gels (Lehrach et al., 1975) as described by Maniatis et al. (1982c). RNA was transferred from the gel to Zetabind in 20 × SSC (Maniatis et al., 1982c).

RNA blots were prehybridized and hybridized in the 10% dextran sulfate solutions used for DNA, but the formamide concentration was adjusted to 50%. Prewashing, prehybridization, hybridization, and posthybridization washings were also performed as described for DNA, except prehybridization and hybridization were at 45 °C and the final posthybridization washes were at 57 °C.

Results

Changes in Nucleosome Repeat Lengths Occur Preferentially in the Early Replicating MTII Region. Synchrony of CHO cells in early S phase produces differential changes in the nucleosome repeat lengths of the early replicating MTII gene region, bulk chromatin, and the repeated sequence pHuR-093 (Figures 1 and 2). As G1 cells become synchronized in early S phase, the MTII repeat lengths are reduced 7–9 bp (Figure 2), and the nucleosome ladders are noticeably shifted (Figure 1). In contrast, the repeat lengths of bulk chromatin are reduced 1–3 bp, and those of pHuR-093 are essentially the same.

Prolonging inhibition of DNA synthesis in the early S-phase cells to 24 h (when 70–80% of histone H1 is depleted in the cell; D'Anna et al., 1985) produces further reductions in the repeat lengths of bulk chromatin (a total of 3–10 bp) but only 1–2 bp additional changes in the repeat lengths of MTII or the later replicating pHuR-093 (Figures 1 and 2). Thus, the results are consistent with the notion that changes in chromatin structure occur preferentially in the early replicating MTII gene region when cells are initially synchronized in early S phase.

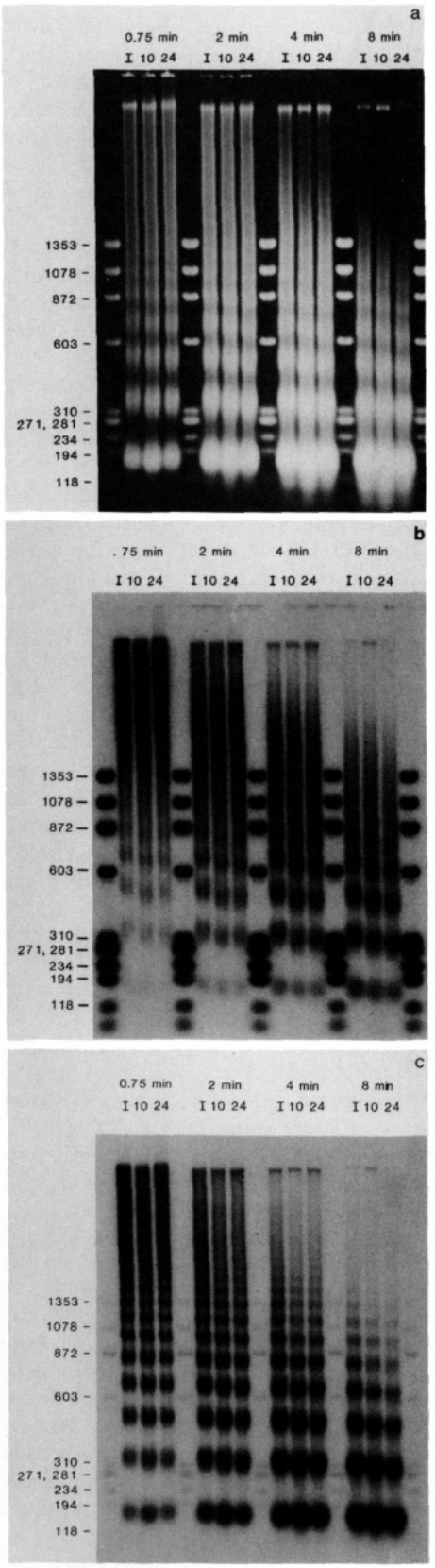

FIGURE 1: Electrophoretic analysis of DNA from the nuclei of synchronized cells digested with micrococcal nuclease. Cultures were synchronized in G1 by isoleucine deficiency (I) or in early S phase by releasing G1 cells into complete medium containing 5 μg mL^{-1} aphidicolin (APC) for 10 h (10) or subjected to prolonged block by releasing G1 cells into complete medium containing 1.0 mM hydroxyurea (HU) for 24 h (24). Nuclei were digested for 0.75, 2, 4, and 8 min as indicated at the top of each group of three lanes. DNA was detected by transillumination of ethidium bromide fluorescence (a), hybridization with MTII (b), or hybridization with pHuR-093 (c).

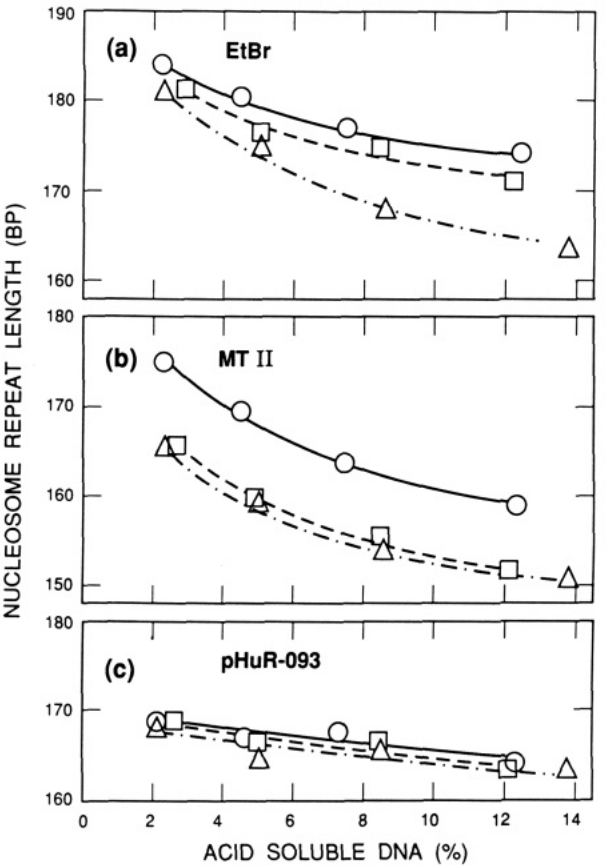

FIGURE 2: Nucleosome repeat lengths of bulk chromatin (a), MTII (b), or pHuR-093 (c) plotted as functions of the percentage acid-soluble bulk DNA in each digest. The data for G1 cells (○), early S-phase cells (□), and cells subjected to prolonged block (△) were measured from the results in Figure 1 and from plots of the percentage acid-soluble DNA as a function of the time of digestion with micrococcal nuclease (data not shown).

We have obtained similar results whether cells are synchronized in early S phase using aphidicolin in complete medium (Figures 1 and 2), hydroxyurea in complete medium (D'Anna et al., 1988), or hydroxyurea in thymidine-deficient medium containing BrdU (data not shown). The latter conditions were chosen for one set of experiments, because they have been used in conjunction with CsCl gradients to estimate the precentage of MTII that becomes replicated as G1 cells become synchronized in early S phase (Stallings et al., 1986; see below).

Changes in the MTII Repeat Lengths Precede MTII Replication. Previously, we showed that DNA replicated during synchrony of cells in early S phase had shortened repeat lengths, compared with mature bulk chromatin (D'Anna & Prentice, 1983; D'Anna & Tobey, 1984; D'Anna et al., 1986). Thus, potentially, MTII replication during synchrony might account for the shortened MTII repeat lengths in early S-phase cells. This is not the case, however.

Slot blot analysis of total DNA from synchronized G1 and early S-phase cells and of BrdU-labeled and unlabeled DNA from CsCl gradients (Table I) indicates that, under most conditions, only a small portion of the total MTII is replicated as G1 cells become synchronized in early S phase. Less than 10% of the total MTII is newly replicated when cells are synchronized in early S phase in the presence of 5 μg mL^{-1} aphidicolin in complete medium or in the presence of 1.0 mM hydroxyurea and 20 μM BrdU in thymidine-deprived medium. Since shortened MTII repeat lengths are observed at these conditions, the results indicate that the changes in MTII repeat

Table I: Percentage of Total MTII DNA That Is Newly Replicated after G1 Cells Have Become Synchronized in S Phase in the Presence of 1.0 mM Hydroxyurea or 5 $\mu g\ mL^{-1}$ Aphidicolin for the Indicated Periods of Time

conditions	method[a]	independent slot blots	MTII per unit DNA	DNA content per cell	relative MTII per cell	% new MTII in S-phase cells
10 h HU (BrdU)[b]	1	3	0.98 ± 0.03	1.03 ± 0.01	1.01 ± 0.04	2 ± 8
10 h HU (BrdU)[b]	2	1		1.03 ± 0.01	1.05	9 ± 1
10 h HU (BrdU)[b]	2	1			~1.03[c]	~5[c]
10 h APC	1	2	0.99 ± 0.05	1.00 ± 0.01	0.99 ± 0.05	0 ± 10
10 h HU	1	2	1.05 ± 0.03	1.04 ± 0.01	1.09 ± 0.04	17 ± 8
10 h HU	1	3	1.02 ± 0.03	1.03 ± 0.01	1.05 ± 0.04	10 ± 8
17 h HU	1	3	0.99 ± 0.03	1.08 ± 0.02	1.07 ± 0.05	13 ± 9
24 h HU	1	3	1.06 ± 0.03	1.11 ± 0.02	1.18 ± 0.05	31 ± 8

[a] Values were calculated by method 1 or method 2 as described under Experimental Procedures. [b] BrdU was present in these experiments as cells progressed from G1 to become synchronized in early S phase. [c] Estimated from the data of Stallings et al. (1986), assuming there was a 5% increase in DNA content per cell as G1 cells became synchronized in early S phase in the presence of 1.0 mM hydroxyurea.

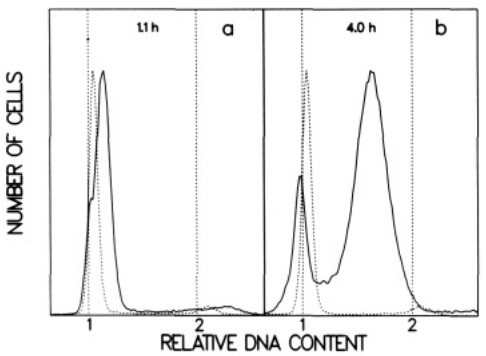

FIGURE 3: Flow cytometry histograms of cells synchronized in early S phase by releasing G1 cells in complete medium containing 1.0 mM hydroxyurea [(---) in (a) and (b)] and cells released into complete medium without hydroxyurea for 1.1 h [(—) in (a)] or 4.0 h [(—) in (b)] to resume cell cycle progression.

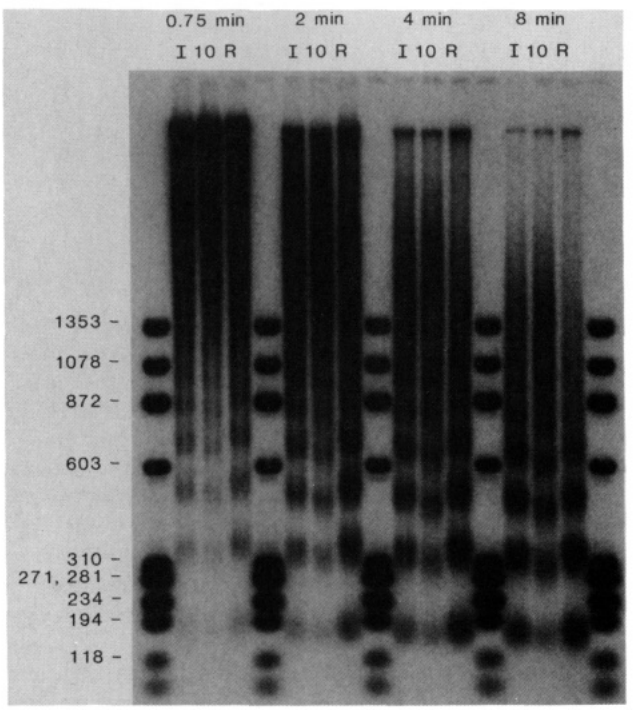

FIGURE 4: MTII nucleosome ladders of DNA from the nuclei of G1 cells (I), cells synchronized in early S phase by releasing G1 cells into complete medium containing 1.0 mM hydroxyurea for 10 h (10), and cells 4.0 h after release from synchrony in early S phase (R). Nuclei were digested for the times shown at the top of each set of three lanes. DNA was separated in agarose gels, blotted, and hybridized with the 1076 bp MTII probe.

lengths can occur before MTII is replicated in the early S-phase cells.

If cells are synchronized in early S phase using 1.0 mM hydroxyurea in complete medium, there may be somewhat more MTII synthesis. There is clearly more MTII synthesis when cells are subjected to prolonged block in complete medium containing 1.0 mM hydroxyurea for 24 h (Table I); yet, there is no further reduction in the MTII repeat lengths, compared with those of cells synchronized in early S phase.

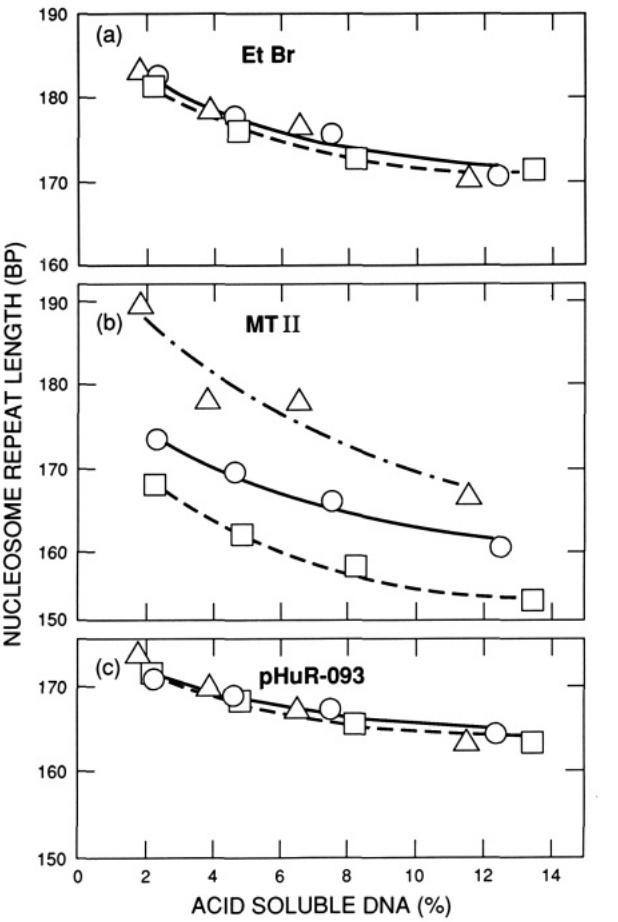

FIGURE 5: Nucleosome repeat lengths of bulk chromatin (a), MTII (b), or pHuR-093 (c) from G1 cells (○), early S-phase cells (□), and cells 4 h after release from synchrony in early S phase (△). MTII data were measured from the results in Figure 4; the other primary results are not shown.

Shortened Repeat Lengths That Precede Replication Are Potentially Reversible. To determine whether the shortened repeat lengths in the MTII region might be reversible, cells were synchronized in early S phase and released into drug-free medium to resume cell cycle progression. At 1.1 and 4.0 h after release (see Figure 3 for FCM histograms), cultures were harvested, and their nuclei were digested with micrococcal nuclease.

After 1.1 h, the MTII nucleosome ladders are already shifted so that they are nearly the same as those in G1 cells

(results not shown). By 4.0 h, when most cells are in late S phase and MTII is fully replicated (Figure 3), the MTII nucleosome ladders are substantially different (Figure 4), and the MTII repeat lengths are even longer than those measured in G1 cells (Figure 5). Again, there are relatively small or no changes in the repeat lengths of bulk chromatin or pHuR-093 between G1 cells, early S-phase cells, or late S-phase cells following release (Figure 5). Thus, whereas these results do not prove that the shortened MTII repeat lengths are thermodynamically reversible, they do show that they can be reelongated within the same cell cycle. Also, since 90–95% of the released cells exhibit long-term colony formation (D'Anna et al., 1985), we conclude that, for the most part, the changes in repeat lengths that precede or follow replication in the early S-phase cells are not lethal.

Synchrony of CHO Cells in Early S Phase Does Not Lead to an Accumulation of MTII mRNA. The increases in MTII repeat lengths that occur when early S-phase cells resume cell cycle traverse are reminiscent of the increased repeat lengths reported for histone H4 chromatin when HeLa cells were released from synchrony in early S phase (Moreno et al., 1986); however, the shortened repeat lengths of the transcriptionally active histone H4 gene could be attributed to variable rates of transcription (Moreno et al., 1986). Since synchrony of cells in early S phase (D'Anna et al., 1985) or transient inhibition of DNA synthesis (Johnston et al., 1986) leads to an accumulation of RNA and protein, we investigated whether synchrony of cells in early S phase might lead to an accumulation of MT messenger RNA that is not seen in exponentially growing wild-type CHO cells (Hildebrand et al., 1980).

Analysis of total cellular RNA from G1 and early S-phase CHO cells and from uninduced and Zn^{2+}-induced CHO Cd^r 30F9-6 variants (positive controls with MT expression) indicates that MT message is not detectable in the synchronized wild-type CHO cell (data not shown). Hence, it is unlikely that the shortened repeat lengths in early S-phase cells result from an increased rate of MT transcription.

Increased Cellular Contents of MTII and pHuR-093 as Synchronized Cells Traverse S Phase. At the time that pHuR-093 was chosen as a potentially late replicating region, it had been detected in the vicinity of the centromers of some CHO chromosomes by low-stringencey hybridization, but it had not yet been noticed in the telomeres (J. Meyne and R. K. Moyzis, personal communication). Since centromeric repeated sequences are often late replicating, we sought to test this expectation.

To avoid use of CsCl gradients, cells were prelabeled with [^{3}H]thymidine before and during synchrony in G1. They were then synchronized in early S phase in the absence of [^{3}H]-thymidine and released to resume cell cycle traverse. With these labeling/synchrony procedures, aliquots of DNA that contain a constant number of ^{3}H cmp also contain a quantity of DNA proportional to the average DNA content per cell for a constant number of undivided cells. Consequently, hybridization of the slot-blotted DNA with MTII or pHuR-093 should give an autoradiographic signal that is proportional to the average copy number per original undivided cell, within the limitations of film response and hybridization efficiency for different loads of DNA.

Figure 6 shows the relative integrated film densities of MTII and pHuR-093 hybridizations as functions of time after cells were released to resume cell cycle traverse. Although the increase in relative film density exceeds the theoretical value of 2.0 for complete replication, most of the increase in MTII

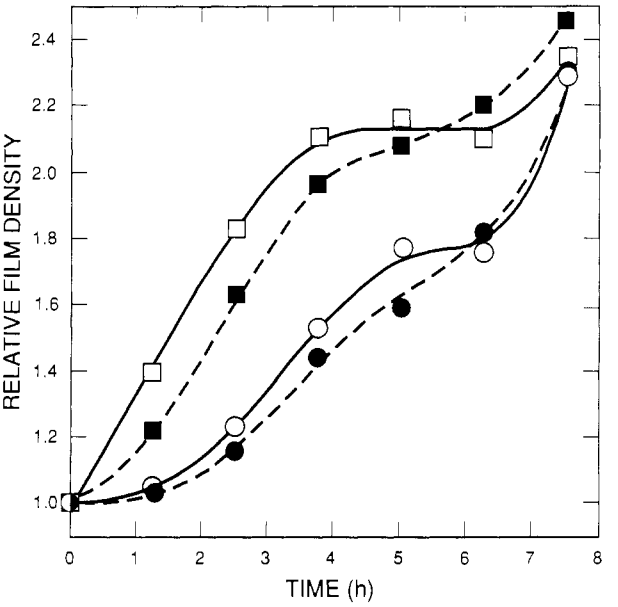

FIGURE 6: Relative integrated intensities of slot-blotted DNA hybridized with nick-translated MTII or pHuR-093. Cells were synchronized in early S phase by sequential use of isoleucine deprivation and treatment with 5 μg mL^{-1} aphidicolin in complete medium for 10 h. They were then released to resume cell cycle traverse. A quantity of DNA proportional to the DNA content per cell was slot-blotted at three relative concentrations and hybridized with MTII or pHuR-093. The relative integrated intensities were plotted for the two sets of lower DNA concentrations whose hybridization densities did not become saturated (as a function of the three concentrations) like those from the heaviest load: (□, ■) MTII at the lower and lowest concentrations; (○, ●) pHuR-093 at the lower and lowest concentrations.

intensity occurs during the first half of S phase as expected from the data of Stallings et al. (1986). In contrast, the signal for pHuR-093 is delayed before it increases at a more nearly constant rate, compared with MTII. Thus, whereas these experiments do not precisely define the replication timing of pHuR-093 centromeric sequences (see Experimental Procedures) in CHO cells, they do indicate that the majority of the pHuR-093 sequences is unlikely to be located in an early replicating region or regions in CHO cells.

DISCUSSION

We have measured nucleosome repeat lengths of bulk chromatin, the early replicating unexpressed MTII gene region, and a later replicating repeated sequence, pHuR-093, in synchronized CHO cells. The major results can be summarized as follows: (1) the repeat lengths of MTII become preferentially shortened, compared with those of bulk chromatin and pHuR-093, as G1 cells enter and become synchronized in early S phase; (2) the shortened MTII repeat lengths precede MTII replication in the early S-phase cells; (3) the shortened MTII repeat lengths are potentially reversible.

MT RNA does not accumulate during the time that G1 cells become synchronized in early S phase; however, prolongation of block in early S phase leads to increased replication of MTII compared with total DNA. Together, these observations and the results of Stallings et al. (1986) support the assumption that MTII is an unexpressed gene region that is part of an initiated replicon when cells are synchronized in early S phase.

Collectively, the results are consistent with previously postulated models (D'Anna & Prentice, 1983); i.e., (1) depletion of histone H1 and changes in chromatin structure occur throughout a replicon at initiation, or (2) depletion of histone H1 and chromatin structural changes can be uncoupled from

and precede replication in initiated replicons (e.g., such changes may be part of a multistep disassembly of chromatin in preparation for DNA replication). We note, however, that this consistency must be substantiated by further results and refinements of the system. First, we do not know that the MTII replicon is truly initiated when cells are synchronized in early S phase. We cannot exclude the possibility that the shortened repeat lengths that precede replication also precede initiation and are a preparatory requirement for initiation. Second, the precise structural changes that underlie the changes in nucleosome repeat lengths or the extent of their occurrence throughout the replicon have not been elucidated. Whereas changes in repeat lengths are sensitive indicators of structural change in this system, their interpretation is complex. They might arise from changes in the frequency of micrococcal nuclease cleavage in specific subregions, or they may simply reflect a change in the relative accessibility of part of or the complete MTII region. In regard to the underlying mechanisms, Droge et al. (1985) have reported that inhibition of simian virus 40 replicative synthesis by aphidicolin produces torsionally stressed replicative intermediates. Droge et al. (1985) suggested that the results reflect some degree of "fork movement" or separation of parent strands that continues to some extent ahead of replication. Thus, it is possible that DNA unwinding or torsional stress is a contributing factor to the shortened nucleosome repeat lengths in the early replicating MTII region.

Third, a late replicating gene region would be a better control than the pHuR-093 repeated sequence. If structural changes were restricted to a small part of a large replicon or replicons containing tandem repeats of pHuR-093, changes in only part of the sequences would not be detected by hybridization. This concern is mitigated, however, by consistent preliminary results obtained with a hybridization probe for a, reportedly, late replicating family of genes in CHO cells. Finally, it has not been shown that histone H1 is truly dissociated from those regions that exhibit changes in repeat lengths during inhibition of DNA synthesis.

We noted in the text that the potential reversibility of the shortened MTII repeat lengths in early S-phase cells was reminiscent of the apparently reversible changes of HeLa histone H4 chromatin reported by Moreno et al. (1986). Moreno et al. (1986) synchronized HeLa cells by two successive treatments with 2.0 mM thymidine, which also synchronizes cells in early S phase. They found that the nucleosome repeat lengths of the early replicating histone H4 gene were cell cycle dependent, going through a minimum in mid-S phase and then becoming longest when cells were in G2/M or in G1 of the next cell cycle. Moreno et al. (1986) suggested that the changes in the histone H4 repeat lengths were evidence for structural remodeling of chromatin that could be correlated with the cell cycle dependent expression of the histone H4 gene. In contrast to the histone H4 gene, however, MTII is unexpressed in CHO cells. Either the changes in HeLa H4 and CHO MTII are unrelated or the changes in HeLa H4 may reflect a complex superposition of both transcription and replication effects.

The preferential shortening of the MTII repeat lengths in early S-phase cells is potentially important for several reasons: (1) they may be normal replication events that cannot be observed in traversing cells, because of imperfect cell synchrony methods; (2) they may be reversible drug-induced perturbations that will provide insight to the disassembly or reorganization of replicons in preparation for DNA synthesis [see Heintz and Stillman (1988)]; (3) they and changes that occur in newly replicated DNA during inhibition of DNA synthesis (D'Anna & Prentice, 1983; Leffak, 1983; D'Anna & Tobey, 1984; D'Anna et al., 1986) may be useful models of reversible or irreversible changes that might occur during transient inhibition of DNA synthesis. These changes might alter gene regulation or induce processes leading to genomic rearrangements associated with transient inhibition of DNA synthesis, i.e., gene amplification [e.g., see Tlsty et al., (1984) and Schimke et al. (1986)], sister chromatid exchanges (Rainaldi et al., 1984; Calego et al., 1988), and chromosomal aberrations [e.g., see Hahn et al., (1986)].

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Lysophosphatidylcholine Metabolism to 1,2-Diacylglycerol in Lymphoblasts: Involvement of a Phosphatidylcholine-Hydrolyzing Phospholipase C†

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ABSTRACT: We have previously described the chemoattraction of lymphoblasts by lysophosphatidylcholine [Hoffman, R. D., et al. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3285-3289]. In studying the mechanism of chemoattraction it was found that lysophosphatidylcholine was metabolized to 1,2-diacylglycerol by the lymphoblastic cell line 6C3HED. One route of metabolism involves the acylation of lysophosphatidylcholine to phosphatidylcholine with subsequent hydrolysis to 1,2-diacylglycerol and phosphocholine by the action of phospholipase C. The increase in cellular 1,2-diacylglycerol was established by metabolic experiments using [^{14}C]glycerol-labeled lysophosphatidylcholine and by mass measurements of 1,2-diacylglycerol. The presence of a phosphatidylcholine-hydrolyzing phospholipase C was confirmed in 6C3HED cell homogenates. In intact cells, lysophosphatidylcholine induced a pattern of protein phosphorylation similar to those of 1,2-dioctanoylglycerol and phorbol 12-myristate 13-acetate, two known activators of protein kinase C. This pathway of lysophosphatidylcholine metabolism, which involves a phosphatidylcholine-hydrolyzing phospholipase C, may be important in the activation of protein kinase C independent of inositol phospholipid hydrolysis.

The activation of protein kinase C, a phospholipid- and calcium-dependent kinase, is believed to play an important role in receptor-mediated signal transduction [for review see Nishizuka (1984, 1986)]. In a variety of cells, activation of protein kinase C leads to numerous biological responses including secretion, ion fluxes, superoxide generation, proliferation, and differentiation (Nishizuka, 1986; Ashendel, 1985). One of the factors modulating the activation of protein kinase C is 1,2-diacylglycerol (DAG),[1] which is believed to operate by reducing the enzyme's requirement for calcium to a physiologic range (Kikkawa et al., 1982). In many cells DAG has been shown to be generated from inositol phospholipids

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[1] Abbreviations: DAG, diacylglycerol; LPC, lysophosphatidylcholine; PMA, phorbol 12-myristate 13-acetate; TPS, 2,4,6-triisopropylbenzenesulfonyl chloride; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; TLC, thin-layer chromatography; [^{14}C]FA-LPC, 1-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine; [^{14}C]choline-LPC, 1-palmitoyl-*sn*-glycero-3-phospho[N-*methyl*-^{14}C]choline; [^{14}C]glycerol-LPC, 1-palmitoyl-*sn*-[U-^{14}C]glycero-3-phosphocholine; MOPS, 3-(*N*-morpholino)propanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; PFB, phosphate-free buffer.

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