Parental Nucleosomes Segregated to Newly Replicated Chromatin Are Underacetylated Relative to Those Assembled de Novo†

Carolyn A. Perry,‡ C. David Allis,§ and Anthony T. Annunziato*,‡

Department of Biology, Boston College, Chestnut Hill, Massachusetts 02167, and Department of Biology, Syracuse University, Syracuse, New York 13244-1220

Received May 4, 1993; Revised Manuscript Received August 31, 1993®

ABSTRACT: Antibodies specific for acetylated histone H4 were used to examine the acetylation state of parental histones that segregate to newly replicated DNA. To generate newly replicated chromatin containing only segregated parental nucleosomes, isolated nuclei were labeled with [^{3}H]TTP *in vitro*; alternatively, whole cells were labeled with [^{3}H]thymidine in the presence of cycloheximide. Soluble chromatin was prepared by micrococcal nuclease digestion, and subjected to immunoprecipitation with "penta" antibodies (Lin *et al.*, 1989). In sharp contrast to nucleosomes containing newly synthesized, diacetylated H4 (Perry *et al.*, 1993), chromatin replicated *in vitro* was only marginally susceptible to immunoprecipitation. Control experiments established that *bona fide* acetylated chromatin was selectively immunoprecipitated by the same techniques and that segregated nucleosomes were not disassembled during treatment with "penta" antibodies. When replication was coupled to an *in vitro* histone acetylation system, the enrichment for segregated nucleosomes in the immunopellet increased approximately 3-fold, demonstrating that changes in the acetylation state of segregated histones can be detected immunologically and that parental histones on new DNA are accessible to acetyltransferases during, or immediately after, DNA replication. *In vivo* pulse–chase experiments, performed in the presence of cycloheximide, confirmed these results. Uptake experiments further established that concurrent histone acetylation did not alter the rate of DNA synthesis *in vitro*. Our results provide evidence that replication-competent chromatin is not obligatorily acetylated, and indicate that the acetylation status of segregated histones may be maintained during chromatin replication. The possible significance of this, with respect to the regulation of chromatin higher order structures during DNA replication, and the propagation of transcriptionally active vs inactive chromatin structures, is discussed.

There are two pathways involved in packaging new DNA into nucleosomes: *de novo* nucleosome assembly (requiring newly synthesized H3 and H4), and the recycling (or segregation) of preexisting histones onto nascent DNA. Although the mode of nucleosome segregation, conservative or dispersive, has been a matter of debate [reviewed in Annunziato (1990)], the segregation of parental histones to new DNA has been confirmed by analyses of chromatin replicated in the absence of concurrent histone synthesis, using either (1) inhibitors of translation (Weintraub, 1974, 1976; Seale & Simpson, 1975; Seale, 1976; Crémisi *et al.*, 1978; Jackson & Chalkley, 1981; Cusick *et al.*, 1984), (2) a *ts* mutant (Roufa, 1978), or (3) isolated nuclei *in vitro* (Seale, 1978). DNA synthesized under these conditions is approximately twice as sensitive to nuclease digestion than control chromatin, and the nuclease-resistant fraction is in typical nucleosomes that contain histone H1 and HMG proteins (Annunziato & Seale, 1982). Recent evidence has supported the dispersive model of segregation (Bonne-Andrea *et al.*, 1990; Burhans *et al.*, 1991; Krude & Knippers, 1991; Randall & Kelly, 1992; Sugasawa *et al.*, 1992), in agreement with earlier findings (Annunziato & Seale, 1984; Cusick *et al.*, 1984; Jackson & Chalkley, 1985; Sogo *et al.*, 1986).

The ability of the replication complex to negotiate nucleosomal DNA raises questions concerning the structure and composition of prereplicative chromatin. Electron microscopic studies have shown that, as far as can be determined by this technique, typical nucleosomes immediately precede the replication fork (McKnight & Miller, 1977; Crémisi *et al.*, 1978; Sogo *et al.*, 1986). It nevertheless seems likely that the 30-nm fiber must somehow unfold to allow for strand separation and DNA synthesis. Whether this is accomplished passively (as a result of fork migration) or actively (through histone modifications, transient loss of H1, *etc.*) is unknown: although it is well established that newly synthesized histones are acetylated, and possibly phosphorylated (Ruiz-Carrillo *et al.*, 1975; Jackson *et al.*, 1976), surprisingly little is understood concerning parental histones that segregate to new DNA. To the extent that the related process of transcription can be used as a model for replication, it might be predicted that chromatin regions active in DNA synthesis contain acetylated *parental* histones, since acetylation has been linked to both ongoing transcription and transcriptional competence (for example, Ridsdale *et al.*, 1987; Ip *et al.*, 1988; Lin *et al.*, 1989; Hebbes *et al.*, 1988, 1992; Boffa *et al.*, 1990; Walker *et al.*, 1990; Turner, 1991; Lee *et al.*, 1993).

In this paper, we have analyzed the acetylation state of segregated nucleosomes. To eliminate the contribution of newly synthesized histones, replication reactions were performed in each of two complementary systems: (1) in intact cells *in vivo*, in the presence of cycloheximide; or (2) *in vitro*, using isolated HeLa cell nuclei. Under these conditions, endogenous replication enzymes synthesize DNA, while preexisting histones are transferred to nascent chromatin (Seale, 1976, 1977, 1978). In experiments paralleling those

† Supported by grants from the National Institutes of Health to A.T.A. (GM 35837) and to C.D.A. (HD16259).

* Correspondence should be addressed to this author. Telephone: (617) 552–3812. Fax: (617) 552–2011.

‡ Boston College.

§ Syracuse University.

® Abstract published in *Advance ACS Abstracts,* November 15, 1993.

0006-2960/93/0432-13615$04.00/0

presented in the preceding paper (Perry et al., 1993), newly replicated chromatin was analyzed using antibodies specific for acetylated H4. However, unlike chromatin assembled *in vivo* (containing newly synthesized, diacetylated H4), nascent nucleosomes comprising only segregated histones were not preferentially immunoprecipitated with "penta" antibodies. In control experiments, it was found that our results were not an artifact of the replication conditions and that demonstrably acetylated chromatin could be precipitated with high efficiency. We therefore find no evidence that histone acetylation is required to generate "replication-competent" chromatin regions in advance of the replication fork. If chromatin unfolding prior to DNA synthesis is actively achieved, modifications other than core histone acetylation may be involved. However, experiments in which acetylation accompanied replication revealed that parental histones are subject to acetylation during, or immediately after, DNA synthesis.

EXPERIMENTAL PROCEDURES

Cell Culturing, Nuclear Isolation, and Labeling. HeLa S3 cells were cultured in MEM (Joklik modification) plus 5–10% calf serum; nuclei were isolated in buffer A (10 mM Tris-HCl, pH 7.6, 3 mM $MgCl_2$, 2 mM 2-mercaptoethanol, and 5 mM sodium butyrate) as described previously (Annunziato & Seale, 1984). To label bulk chromatin DNA, cells were incubated for approximately one generation (18–24 h) with 0.01 μCi/mL [^{14}C]thymidine (55 mCi/mmol; New England Nuclear). Labeling of cells *in vivo* with [*methyl*-^{3}H]thymidine (90 Ci/mmol; New England Nuclear) in the presence of 50 mM butyrate was as described (Perry & Annunziato, 1989, 1991). Cycloheximide was added as needed at 200 μg/mL. Labeling of cells with [^{3}H]thymidine in the presence of cycloheximide was performed at 10 μCi/mL, following a 10-min preincubation in cycloheximide (plus butyrate for the last 5 min).

The reaction conditions for labeling newly replicated DNA *in vitro* were essentially those of Seale (1977), modified to include all of the ribonucleoside triphosphates, an ATP regenerating system, and uracil. Typically, cells were pretreated for 8 min with cycloheximide in the presence of 50 mM sodium butyrate, to interpose a region of unassembled DNA between the last fully assembled nucleosomes (containing newly synthesized H3/H4) and the labeled chromatin (Annunziato & Seale, 1984); this pretreatment did not affect subsequent DNA replication, or the immunoprecipitation of acetylated nascent chromatin. Nuclei were then isolated in buffer A and resuspended at 30 A_{260} units/mL [A_{260} measured in 1% sodium dodecyl sulfate (SDS)][1] in replication buffer: 50 mM dextrose, 12 mM $MgCl_2$, 10 mM ATP, 1 mM EDTA, 0.5 mM each of CTP, GTP, and UTP, 0.1 mM each of dATP, dCTP, and dGTP, 2 mM uracil, 2 mM dithiothreitol, 2 mM sodium butyrate, 50 mM phosphocreatine, 100 μg/mL creatine phosphokinase, 75–150 μCi/mL [^{3}H]TTP (75 Ci/mmol, in Tricine buffer; New England Nuclear), and 60 mM HEPES·Na_2, pH 8.0. Uracil was included to inhibit the potential activity of uracil–DNA glycosylase (Brynoff *et al.*, 1978). Labeling was performed at 37 °C for 20 min, and terminated by placing the reaction mixture on ice. Nuclei were then mixed with an equal volume of buffer A containing 50% glycerol, and collected by centrifugation (1000*g*, 8 min). The rate of incorporation *in vitro* was approximately 6.3 pmol of total nucleotide min^{-1} (10^7 nuclei)$^{-1}$ (as determined from the dpm incorporated and the specific activity of the isotope); this represents ~3–4% of the *in vivo* rate (Fraser & Huberman, 1977). Given a typical HeLa fork migration rate of 1.5–3.0 kb/min (Painter & Schaefer, 1969), it can thus be calculated that during a 20-min reaction *in vitro* each replication fork will advance about 1–2 kb. There is no initiation *in vitro* (Seale, 1977), and so radiolabeled nucleosomes will be contiguous with (unlabeled) segregated nucleosomes generated *in vivo* during the cycloheximide preincubation. Pretreatment with cycloheximide therefore ensured that the labeled nucleosomal oligomers would not be immediately adjacent to fully assembled (acetylated) new nucleosomes. The rate of replication in the presence of cycloheximide was ~20% of the control rate (Seale & Simpson, 1975).

To label acetylated histones *in vitro*, nuclei were resuspended in replication buffer containing 0.1 mM unlabeled TTP, and an acetyl-CoA generating system: 10 μM CoA, 0.04 unit/mL acetyl-CoA synthetase (Boehringer-Mannheim), and 5 mCi/mL [^{3}H]acetate (4 Ci/mmol; New England Nuclear). Under these conditions, [^{3}H]acetyl-CoA is produced, and endogenous histone acetyltransferases label acetylated histones (Vu *et al.*, 1987); electrophoresis and fluorography of isolated chromatin confirmed that the core histones were the only chromatin-bound proteins to incorporate radiolabel. When the replication and acetylation systems were coupled, 500 μM sodium acetate (unlabeled) was substituted for [^{3}H]acetate.

Nuclease Digestion and Preparation of Nucleosomes. Nuclei were resuspended in digestion buffer (10 mM PIPES, 20 mM sodium butyrate, 80 mM NaCl, and 0.5 mM $CaCl_2$, pH 7.0) at 40 A_{260}/mL, and digested with 5 units/mL micrococcal nuclease (Sigma) at 4 °C, producing a soluble chromatin fraction (termed S1) as described previously (Perry & Annunziato, 1989). Under these digestion conditions, most unassembled "non-nucleosomal" nascent DNA (generated during replication in the absence of histone synthesis) remains bound in the digested nucleus, possibly through association with the nuclear matrix, while newly replicated nucleosomes are released into the S1 (Annunziato & Seale, 1982); thus, following replication in the absence of concurrent protein synthesis, the S1 contains new DNA associated with segregated histones. Tritium-labeled nuclei (containing labeled newly replicated chromatin) were digested for 0.75–2 min, releasing 35–45% of newly replicated chromatin DNA into the S1; ^{14}C-labeled nuclei were digested for 10–15 min, yielding ~40% of bulk chromatin DNA in the S1. By altering the digestion times for ^{3}H- and ^{14}C-labeled nuclei in this manner, the greater sensitivity of nascent chromatin to nuclease digestion was compensated for (see Figure 2). Chromatin labeled *in vivo* (plus butyrate, minus cycloheximide) for greater than 20 min was mixed with control ^{14}C-nuclei prior to nuclease digestion, as these two types of chromatin are equally sensitive to micrococcal nuclease (Annunziato & Seale, 1983). [^{3}H]-Acetate-labeled nuclei were digested with micrococcal nuclease for 2–4 min. Nuclear digestions were halted with 5 mM EDTA; an additional 1 mM EGTA was added immediately prior to immunoprecipitation. Purified labeled DNA was obtained from micrococcal nuclease digested chromatin by treatment with proteinase K and phenol/chloroform, from cells incubated overnight with [^{14}C]thymidine.

Immunoprecipitation, Gel Electrophoresis, and Fluorography. For all immunoprecipitation experiments, the "penta"

[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PPO, 2,5-diphenyloxazole; DMSO, dimethyl sulfoxide; TCA, trichloroacetic acid.

antiserum generated by Lin *et al.* (1989), specific for acetylated H4, was used as previously described (Perry *et al.*, 1993). For the analysis of chromatin replicated *in vitro*, ^{3}H- and ^{14}C-labeled nucleosomes were mixed at a 2:1 (v/v) ratio prior to immunoprecipitation. Following immunoprecipitation, chromatin in the unbound supernatant, all washes, and the immunopellet was precipitated with 10% TCA containing 10 mM sodium pyrophosphate, and analyzed by scintillation counting; spill of ^{14}C radioactivity into the tritium channel was corrected for by the external standard method. Alternatively, chromatin samples were adjusted to 10 mM $MgCl_2$, ethanol-precipitated, and resuspended in electrophoresis sample buffer. As required, unbound chromatin remaining in the supernatant following immunoprecipitation was redigested with micrococcal nuclease for 3 min on ice, after adjustment to an additional 12 mM $CaCl_2$ (*i.e.*, twice the concentration of EGTA in the supernatant) and adding 0.5 unit of micrococcal nuclease; the reaction was stopped with EGTA (25 mM final concentration). In control experiments, mixtures of purified labeled DNA and unlabeled soluble chromatin were digested under identical conditions.

Chromatin proteins were subjected to electrophoresis in polyacrylamide gels containing SDS, and stained with Coomassie Blue (Thomas & Kornberg, 1975). For DNA size analysis, chromatin DNA was separated in 4% polyacrylamide gels in the presence of 0.1% SDS as described (Annunziato & Seale, 1982), stained with ethidium bromide, and photographed under ultraviolet illumination. Gels were treated with PPO/DMSO, dried, and exposed to preflashed film in preparation for fluorography (Bonner & Laskey, 1974; Laskey & Mills, 1975). Fluorographs were scanned at 580 nm with a GS 300 densitometer (Hoefer); the peaks were excised and weighed for quantitation.

RESULTS

Immunoprecipitation of Chromatin Replicated in Vitro. Chromatin that is replicated *in vivo* (during concurrent histone synthesis and deposition) can be efficiently immunoprecipitated with antibodies specific for acetylated H4 (Perry *et al.*, 1993). It is possible that this is due solely to the presence of new, acetylated H4 on nascent chromatin; alternatively, segregated parental histones may also be acetylated during replication. To examine the acetylation state of segregated nucleosomes, it is necessary to eliminate newly synthesized histones from the analysis. To accomplish this, chromatin was replicated *in vitro*, using isolated HeLa cell nuclei. Under these conditions, true DNA replication (as opposed to repair) continues within replicons initiated *in vivo* (Seale, 1977). In addition, no new histones are available for *de novo* nucleosome assembly, while parental histones segregate to newly replicated DNA (Seale, 1977, 1978).

Following *in vitro* replication in the presence of [^{3}H]TTP and sodium butyrate (included to prevent histone deacetylation), soluble nucleosomes were prepared by mild micrococcal nuclease digestion. Because chromatin replicated *in vitro* is more sensitive to nuclease digestion than bulk chromatin (Seale, 1978), digestion conditions were adjusted to produce a high yield of oligomeric nascent chromatin. To provide an internal standard for the immunoprecipitation experiments, bulk chromatin (prelabeled with [^{14}C]thymidine for one generation, and digested with micrococcal nuclease in a separate reaction) was mixed with the soluble nascent nucleosomes. By independently preparing bulk and nascent nucleosomes in this manner, it was thus possible to compensate for the preferential nuclease sensitivity of newly replicated

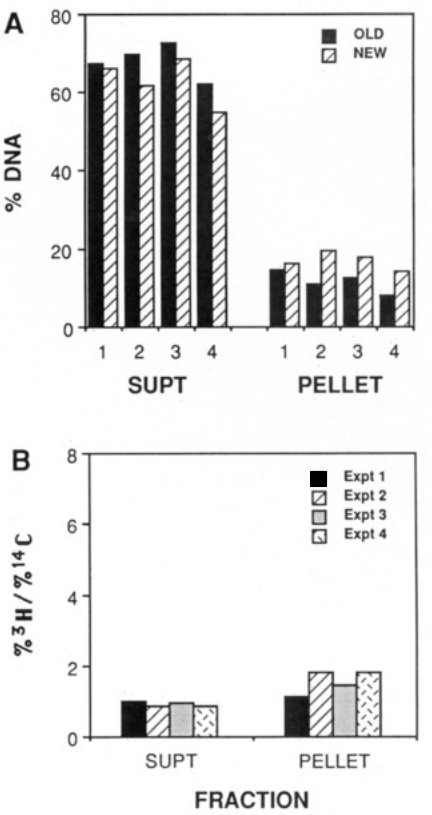

FIGURE 1: Segregated nucleosomes are not preferentially immunoprecipitated by antibodies specific for acetylated H4. (A) Newly replicated chromatin was labeled in isolated HeLa cell nuclei in the presence of [^{3}H]TTP and sodium butyrate (NEW). Soluble nucleosomes were mixed with control chromatin that was prelabeled for one generation with [^{14}C]thymidine (minus butyrate) (OLD) and immunoprecipitated with immobilized penta antibodies, generating supernatant and pellet fractions as described under Experimental Procedures. Data from four independent experiments are expressed as the percent of the total ^{3}H or ^{14}C radioactivity applied to the beads. For experiments 1–3, cells were preincubated for 8 min with butyrate and cycloheximide; for experiment 4, cells were preincubated in butyrate for 1 h (including cycloheximide for the last 8 min) prior to nuclear isolation and replication *in vitro*. Radioactivity in the washes is not shown. Note: [^{3}H]chromatin was digested with micrococcal nuclease on ice for either 0.75 min (Expt 1), 1 min (Expt 2 and 4), or 2 min (Expt 3); [^{14}C]chromatin was digested for either 10 min (Expt 1, 3, and 4) or 15 min (Expt 2). (B) The ^{3}H/^{14}C ratios in the supernatant and pellet fractions from panel A are given.

chromatin [see Figure 2A (lane 1) and Figure 2B]. The mixed samples were then subjected to immunoprecipitation using antibodies specific for acetylated H4 (Figure 1).

In marked contrast to chromatin replicated and assembled *in vivo*, segregated parental nucleosomes were only marginally immunoprecipitated by "penta" antibodies. Newly synthesized DNA was not depleted in the supernatant fraction, and only 10–20% of the nascent chromatin appeared in the immunopellet (Figure 2A). Consequently, there was little enrichment for new DNA in the pellet (^{3}H:^{14}C ratios = 1.1–1.8, Figure 1B), even when the extent of digestion of nascent chromatin was varied over a 2–3-fold range. Doubling the amount of beads and antiserum used did not alter these results (experiment 2), and less than 2% of the new chromatin remaining in the supernatant was immunoprecipitated following reincubation with freshly prepared beads (not shown); thus, the low degree of immunoprecipitation cannot be ascribed to less than optimum conditions. Moreover, pretreating cells for 60 min with sodium butyrate prior to nuclear isolation did not increase the immunoprecipitation of chromatin subsequently replicated *in vitro* (experiment 4). Substituting

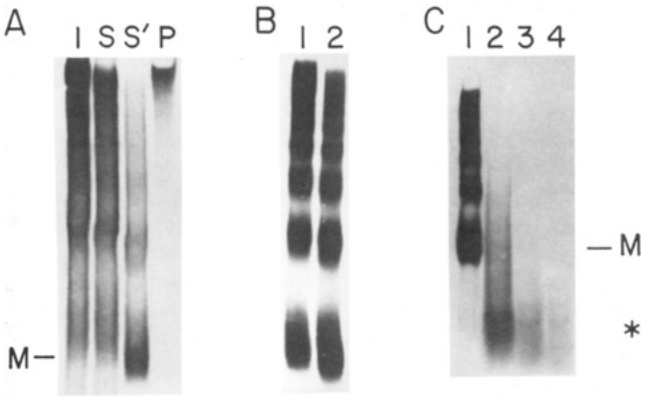

FIGURE 2: Unbound fraction of chromatin replicated *in vitro* is in nucleosomes. (A) Newly replicated chromatin was labeled with [^{3}H]-TTP *in vitro*, digested with micrococcal nuclease for 1 min (see input chromatin, lane I), and immunoprecipitated with penta antibodies. Half of the supernatant fraction was left untreated (lane S); the remaining half was redigested with micrococcal nuclease for 3 min (lane S′). The immunopellet (lane P) is also shown. (B) Control bulk chromatin (labeled with [^{14}C]thymidine for one generation) was digested with micrococcal nuclease for either 10 min (lane 1) or 15 min (lane 2). (C) Labeled naked DNA (1.25 μg, obtained by micrococcal nuclease digestion and phenol/chloroform extraction; lane 1) was redigested with micrococcal nuclease either alone (lane 2) or in the presence of an 8–10-fold excess of unlabeled HeLa chromatin (lanes 3 and 4), under the identical conditions used in panel A. Digestion times were 3 min (lanes 2 and 3) or 6 min (lane 4). For panels A–C, DNA samples were subjected electrophoresis and analyzed by fluorography; only the fluorograph is shown; thus, the unlabeled chromatin present in panel C (lanes 3 and 4) is not visible. The position of mononucleosomal DNA is indicated (M). The asterisk (panel C) denotes the ion front of the gel, and comigrating fragments of digested DNA.

preimmune serum for the "penta" antibodies essentially eliminated the immunoprecipitation of both new and old chromatin (data not presented). Because the new:old ratio in the immunopellet was between 1 and 2 for all experiments, it is concluded that the occurrence of acetylated H4 among segregated nucleosomes approximately equals that found in bulk chromatin. As was shown in the preceding paper (Perry *et al.*, 1993), chromatin replicated *in vivo* for only 5 min (containing diacetylated new H4) is immunoprecipitated with high efficiency. Thus, the level of acetylation in segregated histones must be significantly less than that occurring in nucleosomes assembled *de novo*.

One possible explanation for our results is that nascent nucleosomes were disassembled during treatment with immobilized antibodies, or during the subsequent washes. Although this was clearly not the case for chromatin replicated *in vivo*, nucleosomes replicated *in vitro* might be particularly unstable. To test for this, a standard immunoprecipitation reaction was performed; half of the unbound supernatant was left without further treatment, and the remaining half was redigested with micrococcal nuclease (in this experiment only the new DNA was labeled, to facilitate the analysis of nascent chromatin). The redigested supernatant, together with the remainder of the unbound fraction and the immunopellet, was subjected to electrophoresis and analyzed by fluorography (Figure 2A).

In agreement with the data of Figure 1, relatively little newly replicated chromatin appeared in the immunopellet (Figure 2A, lane P). In contrast, the unbound supernatant contained new DNA ranging from monomers to higher oligomers (lane S). Upon redigestion (lane S′), new DNA in the supernatant yielded monomers, dimers, trimers, *etc.*, as predicted if the unbound fraction of nascent chromatin remained nucleosomal during the immunoprecipitation reaction. As a control, purified labeled DNA (panel C, lane 1) was mixed with unlabeled chromatin, and digested with micrococcal nuclease under identical conditions. Even in the presence of excess chromatin, the naked DNA was rapidly degraded, and no trace of a ladder was seen (panel C, lanes 3 and 4). Thus, histones do not exchange onto naked DNA under our redigestion conditions. Newly replicated DNA in the unbound supernatant (panel A) must therefore be packaged into nucleosomes. Moreover, in no case (in more than 20 experiments) have we observed that chromatin replicated *in vitro* is preferentially released from the beads during washing of the immunopellet. Taken together, these results demonstrate that parental nucleosomes are not selectively disassembled or lost during immunoprecipitation.

Throughout all of these experiments, nuclei were isolated and replicated *in vitro* in the presence of sodium butyrate, to preserve the acetylation level of replicating chromatin. To ensure that the conditions of *in vitro* replication were not inhibiting the immunoprecipitation of acetylated nascent nucleosomes, a further control experiment was performed. HeLa cells were labeled for 20 min *in vivo* with [^{3}H]thymidine, to produce acetylated newly replicated chromatin as described previously (Perry & Annunziato, 1989, 1991). Nuclei were then isolated, and incubated *in vitro* under standard conditions, with the exception that unlabeled TTP was substituted for the usual [^{3}H]TTP. Soluble nucleosomes were then produced by micrococcal nuclease digestion, and immunoprecipitated with "penta" antibodies in the presence of ^{14}C-labeled bulk chromatin (Figure 3).

Subjecting prelabeled nuclei to the *in vitro* replication conditions did not prevent the preferential immunoprecipitation of acetylated newly replicated chromatin (Figure 3A,B). As typically observed, the unbound supernatant was markedly depleted of new DNA (^{3}H:^{14}C ratio = 0.21), and the pellet was correspondingly enriched for newly replicated nucleosomes (ratio = 8.4). A comparison of the ratios in the two fractions revealed a ~40-fold difference in enrichment for new DNA in the pellet, as compared to the supernatant, when "penta" antibodies were used. In contrast, less than 3% of either new or old chromatin was precipitated when control preimmune serum was substituted for the antiserum (not shown). Thus, the *in vitro* conditions *per se* do not inhibit the immunoprecipitation of acetylated nascent nucleosomes.

Immunoprecipitation of Acetylated Chromatin. In light of our results with segregated nucleosomes, it was important to demonstrate that acetylated chromatin could be effectively immunoprecipitated with "penta" antibodies. To approximate the *in vitro* replication conditions as closely as possible, acetylated histones were labeled with [^{3}H]acetate in isolated nuclei, in standard replication buffer, using the method of Vu *et al.* (1987). Under these conditions, histones are acetylated (and labeled) by endogenous histone acetyltransferases. Following *in vitro* labeling, soluble chromatin was prepared by micrococcal nuclease digestion, and immunoprecipitated with "penta" antibodies. Chromatin in the supernatant and pellet was then subjected to electrophoresis in the presence of SDS, and analyzed by Coomassie Blue staining (Figure 4A) and fluorography (Figure 4B).

As shown in the stained gel, little bulk chromatin was precipitated by the antiserum (Figure 4A, PENTA, lane P). In the fluorograph (Figure 4B, lane I), it was seen that core histones had efficiently incorporated acetate label, with the bulk of acetylation occurring on H3 and H4. Notably, ~50% of the acetylated H3/H4 was immunoprecipitated, causing acetylated histones to be markedly enriched in the immu-

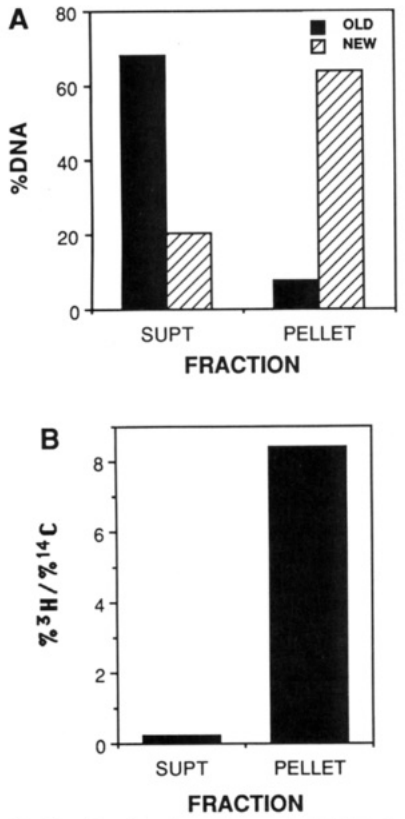

FIGURE 3: Replication *in vitro* does not abolish the preferential immunoprecipitation of acetylated nascent chromatin. (A) HeLa cells were labeled for 20 min *in vivo* with [^{3}H]thymidine in the presence of sodium butyrate (NEW). ^{3}H-Labeled nuclei were isolated, incubated *in vitro* under standard replication conditions for 20 min (using unlabeled TTP), and then mixed with control nuclei from cells prelabeled for one generation with [^{14}C]thymidine under normal conditions (OLD). Soluble nucleosomes were incubated with immobilized penta antibodies, yielding supernatant and pellet fractions; data were analyzed as in Figure 1. (B) The ^{3}H/^{14}C ratios in the supernatant and pellet fractions from panel A are presented.

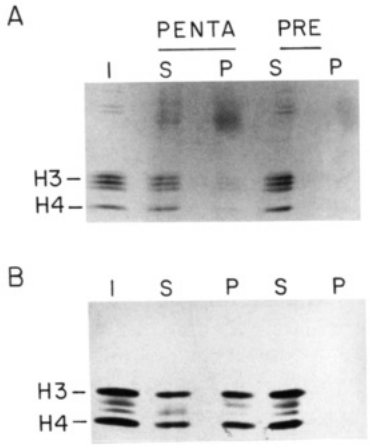

FIGURE 4: Immunoprecipitation of acetylated chromatin. Nuclei were incubated with [^{3}H]acetate *in vitro* (to label acetylated histones), as described under Experimental Procedures. Soluble nucleosomes (lane I) were immunoprecipitated with immobilized penta antibodies (PENTA), or were incubated with protein A–Sepharose treated with control preimmune serum (PRE), yielding supernantant (lane S) and pellet (lane P) fractions. Chromatin proteins were subjected to electrophoresis in the presence of SDS, stained with Coomassie Blue (A), and analyzed by fluorography (B). The core histones were the only chromatin-bound proteins to incorporate acetate label, and thus only this portion of the gel is shown.

nopellet. Double-label experiments (which included [^{14}C]-thymidine-labeled bulk chromatin as an internal standard)

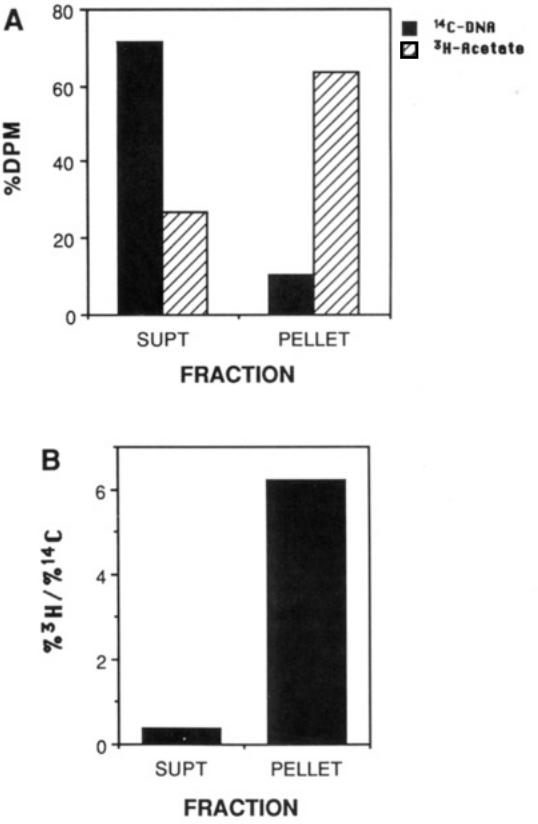

FIGURE 5: Enrichment in the immunopellet for acetylated chromatin. (A) Nuclei were incubated with [^{3}H]acetate *in vitro*, to label acetylated chromatin as in Figure 4 (^{3}H-Acetate, right-hand legend). ^{3}H-Labeled nuclei were isolated, mixed with control nuclei prelabeled for one generation with [^{14}C]thymidine (^{14}C-DNA), and digested with micrococcal nuclease; soluble chromatin was immunoprecipitated with immobilized penta antibodies, yielding supernatant and pellet fractions, and analyzed as in Figure 1. (B) The ^{3}H/^{14}C ratios from the data in panel A are presented.

confirmed that under these conditions up to 65% of the acetate label was selectively immunoprecipitated (Figure 5). The presence of labeled H3 in the immunopellet (Figure 4B, PENTA, lane P) also confirmed that acetylated nucleosomes remain intact during the immunoprecipitation reaction. Thus, genuinely acetylated chromatin can be effectively, and preferentially, immunoprecipitated by "penta" antibodies.

Concurrent Acetylation and Replication. It remained possible that segregated histones were susceptible to acetylation during, or immediately after, the passage of the replication fork. To examine this question, the replication and acetylation systems were coupled, with the modification that nonradioactive sodium acetate was substituted for [^{3}H]acetate; as usual, newly replicated DNA was labeled with [^{3}H]TTP to trace segregated parental nucleosomes. It was reasoned that if parental nucleosomes can be acetylated during or immediately after replication, the enrichment for newly replicated DNA in the immunopellet should increase. Following replication/acetylation *in vitro*, soluble nascent chromatin was mixed with control bulk chromatin, and precipitated with "penta" antibodies (Figure 6).

When histone acetylation accompanied *in vitro* replication, the enrichment for new DNA in the immunopellet was elevated 2–3-fold (Figure 6B), demonstrating that segregated histones are not refractory to acetylation, and that our techniques can detect changes in the acetylation state of parental nucleosomes on new DNA. This increase in enrichment stands in contrast to the effects of preincubating cells in butyrate (Figure 1, experiment 4), which did not heighten the immunoprecipitation

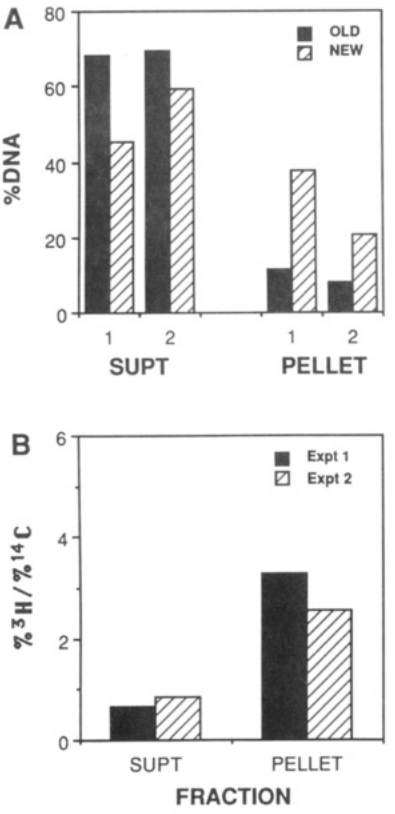

FIGURE 6: Segregated nucleosomes can be acetylated during replication. (A) Nascent chromatin was labeled with [^{3}H]TTP *in vitro* (NEW) under conditions in which nucleosomal histones are acetylated (cf. Figure 4), but using unlabeled sodium acetate (see Experimental Procedures). Soluble nucleosomes were mixed with chromatin from control cells prelabeled with [^{14}C]thymidine (OLD), and immunoprecipitated with penta antibodies, yielding supernatant and pellet fractions. The results of two independent experiments are shown. (B) The ^{3}H/^{14}C ratios in the supernatant and pellet fractions of panel A are given.

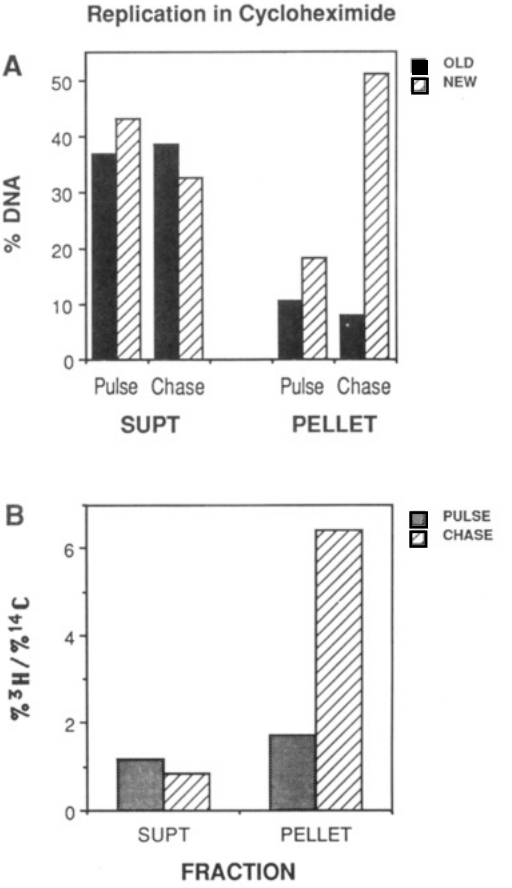

FIGURE 7: Acetylation state of nucleosomes segregated *in vivo*. (A) Cells were preincubated in cycloheximide for 10 min; during the last 5 min of preincubation, 50 mM sodium butyrate was also added. Cells were then labeled with [^{3}H]thymidine for 5 min in the continuous presence of cycloheximide and butyrate (NEW). Half of the cells was then harvested for immediate chromatin preparation (Pulse); the remaining half was further chased for 30 min in the presence of cycloheximide, butyrate, and 1.5 μM unlabeled thymidine (Chase). Soluble nucleosomes were mixed with chromatin from control cells prelabeled with [^{14}C]thymidine (OLD), and immunoprecipitated with penta antibodies, yielding supernatant and pellet fractions. (B) The ^{3}H/^{14}C ratios in the supernatant and pellet fractions of panel A are given.

of segregated nucleosomes. It therefore appears that following (or during) replication, nascent chromatin becomes more susceptible to acetylation, perhaps due to a more "open" structure. Similar observations have been made for newly synthesized histones (Cousens & Alberts, 1982).

Analysis of the Acetylation Level of Histones Segregated in Vivo. To further examine the acetylation of segregated histones, and to ensure that our results were not an artifact of the *in vitro* replication system, experiments were performed on chromatin replicated in living cells. To eliminate the contribution of newly synthesized histones, cells were preincubated in cycloheximide (plus butyrate) and then labeled with [^{3}H]thymidine for 5 min in the continuous presence of cycloheximide and butyrate (the latter was added to preserve the acetylation state of segregated chromatin). Because the rate of replication in cycloheximide is about 20% the normal rate (Seale & Simpson, 1975), a 5-min pulse permitted the synthesis of approximately 1.5–3.0 kb of new DNA on each arm of the fork. The cells were then divided into two equal fractions. Nuclei were immediately isolated from one half; the other half was further chased for 30 min in the continuous presence of cycloheximide, butyrate, and 1.5 μM unlabeled thymidine (to dilute the radiolabel). Soluble nascent chromatin was then prepared from both groups of cells, mixed with control bulk chromatin, and immunoprecipitated with "penta" antibodies (Figure 7).

In complete accord with the results obtained using the *in vitro* system, less than 20% of the nucleosomes segregated *in vivo* were immunoprecipitated (panel A, Pulse), resulting in a ^{3}H:^{14}C ratio of only 1.7 in the immunopellet (panel B, Pulse). However, a sharp increase in precipitability was observed following continued replication in the presence of butyrate (Chase), paralleling the increase in immunoprecipitation seen when replication and acetylation were coupled *in vitro* (Figure 6). It has previously been demonstrated that following 5 min of replication in cycloheximide, new DNA contains segregated histones of parental origin (Annunziato & Seale, 1982). Although we find no evidence that all segregated nucleosomes are acetylated in advance of replication, both the *in vivo* and *in vitro* data show that parental histones are subject to acetylation once replication has occurred.

Effects of Histone Acetylation on DNA Synthesis. Although our results argued against the specific involvement of histone acetylation in generating replication-competent chromatin, it remained possible that acetylation could facilitate DNA synthesis. To examine this, nuclei were isolated in the presence of sodium butyrate (to preserve native histone acetylation) and then replicated *in vitro* for various times in the presence or absence of concurrent acetylation (as in Figure 6); as an added variable, replication was performed plus or minus sodium butyrate. The data are presented in Figure 8.

No marked differences in the incorporation of [^{3}H]TTP were observed under any of the conditions tested (Figure 8A).

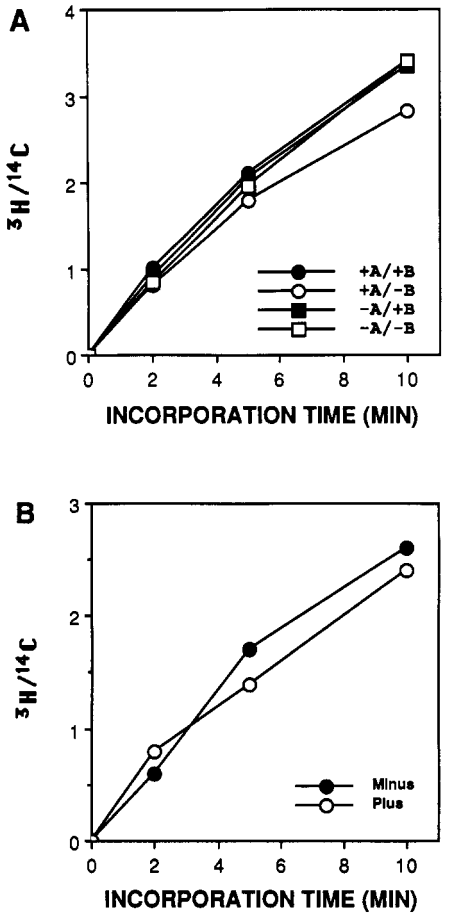

FIGURE 8: Effects of acetylation on chromatin replication. (A) HeLa cells were prelabeled with [^{14}C]thymidine for one generation; nuclei were isolated and labeled *in vitro* with [^{3}H]TTP in the presence (circles) or absence (squares) of the concurrent acetylation system (±A), and in the presence (closed symbols) or absence (open symbols) of sodium butyrate (±B). Uptake of [^{3}H]TTP is expressed as ^{3}H/^{14}C. (B) Cells either were left untreated (closed symbols) or were preincubated in 50 mM sodium butyrate for 1 h (open symbols), prior to nuclear isolation and replication *in vitro* in the presence of sodium butyrate and [^{3}H]TTP. Data are expressed as in panel A.

Identical results were obtained when cells were preincubated in butyrate for 1 h prior to nuclear isolation and replication *in vitro* (Figure 8B), or when the experiments were repeated using nuclei isolated in the absence of butyrate (not shown). Therefore, while it is clear that at least some parental histones can be acetylated (Figures 6 and 7), such acetylation alters neither the rate nor the extent of DNA synthesis, at least *in vitro*.

DISCUSSION

By replicating chromatin in an endogenous nuclear system, in which there are no newly synthesized histones, we have examined the acetylation state of parental histones that are segregated to newly replicated DNA. Immunoprecipitation experiments (using antibodies specific for acetylated H4) revealed that the occurrence of acetylated histones in segregated nucleosomes is about the same as it is in bulk chromatin. This is of course very different from nucleosomes assembled *de novo*, all of which contain diacetylated H4 (Ruiz-Carrillo *et al.*, 1975; Jackson *et al.*, 1976; Chicoine *et al.*, 1986), and which can be efficiently immunoprecipitated within minutes of DNA replication and histone deposition (Perry *et al.*, 1993). Control experiments established that the "penta" antiserum was capable of selectively recognizing acetylated nucleosomes and that the *in vitro* replication conditions did not inhibit the preferential immunoprecipitation of acetylated nascent chromatin. Data from *in vivo* experiments, in which cycloheximide was used to block histone synthesis, fully supported the results obtained *in vitro*, providing additional evidence that segregated nucleosomes are underacetylated relative to those containing new H4.

When histone acetylation was coupled to chromatin replication *in vitro*, it was found that segregated histones are accessible to acetyltransferases after the passage of the replication fork. A similar time-dependent increase in the acetylation of parental nucleosomes was observed during replication *in vivo* in the presence of cycloheximide. However, neither acetylation nor butyrate altered the rate of DNA replication *in vitro*, consistent with previous reports on the effect of sodium butyrate on the rate of replication in whole cells (Littlefield *et al.*, 1982; Annunziato & Seale, 1983). If acetylation occurs along with DNA synthesis, it apparently does not facilitate the replication process, at least *in vitro*. In light of both this and the immunoprecipitation data, we conclude that acetylation is not required to generate replication-competent chromatin, or to permit the transit of DNA polymerase through nucleosomal DNA.

Given the well-documented correlation between transcriptional competence and histone acetylation, it is perhaps surprising that acetylation does not appear to have a more prominent role in DNA replication. Nevertheless, an examination of the literature lends support to our observations. In two recent studies of nucleosome segregation, circular chromatin templates were either reconstituted (Bonne-Andrea *et al.*, 1990) or assembled (Randall & Kelly, 1992) *in vitro* prior to replication in a defined system. In neither case were acetylated histones necessary to generate replication-competent chromatin, and in one study (Randall & Kelly, 1992), histones from butyrate-treated cells were specifically avoided.

In a more direct analysis of acetylation, two unique antibodies specific for acetylated H4 (Lin *et al.*, 1989) were used to examine chromatin replication in the ciliated protozoan *Euplotes* (Olins *et al.*, 1991). In this organism, macronuclear DNA synthesis is confined to a migrating nuclear organelle, the replication band. Ultrastructural analysis of the replication band (Olins *et al.*, 1988) has shown that this region is organized into a forward zone (FZ), immediately preceding replication, and a near zone (RZ), the area of ongoing DNA synthesis and histone deposition (Prescott & Kimball, 1961). Although acetylation-specific antibodies strongly recognized the RZ, little reaction with the FZ was detected. In contrast, both the FZ and the RZ could be stained with antibodies against *un*acetylated H4. Because chromatin in the FZ is organized into 40-nm fibers [consistent with low levels of histone acetylation (Annunziato *et al.*, 1988)], it remained formally possible that acetylated H4 epitopes in the FZ were merely inaccessible to antibodies, due to steric hindrance. However, acetylated H4 can be detected immunohistochemically in condensed *Drosophila* chromatin (Turner *et al.*, 1990), and in intact metaphase chromosomes (Jeppesen *et al.*, 1992; Jeppesen & Turner, 1993), making it unlikely that acetylated H4 N-termini in the FZ were masked through chromatin folding. The simplest explanation for the *Euplotes* results, therefore, is that nucleosomes immediately preceding the replication fork are significantly underacetylated relative to those assembled *de novo*, in complete agreement with the biochemical data presented in this report.

If parental histones need not be acetylated for chromatin replication, then the acetylation status of prereplicative

chromatin may persist unaltered during normal DNA synthesis and histone segregation. If so, this may aid in differentiating potentially active from inactive chromatin structures. It has been shown that histone acetylation facilitates the binding of transcription factors to DNA (Lee *et al.*, 1993), as does stepwise chromatin assembly [Almouzni *et al.*, 1991; reviewed in Saver and Chalkley (1991) and Wolffe (1991a,b)]. A further level of gene regulation can be imposed by chromatin higher order structures (Hansen & Wolffe, 1992) mediated by histone H1 (Weintraub, 1984, 1985; Wolffe & Brown, 1988; Shimamura *et al.*, 1989; Wolffe, 1989, 1991b; Bresnick *et al.*, 1992; Zlatanova & van Holde, 1992; Garrard, 1991). In this regard, we have found that histone acetylation limits the stable interaction of H1 with newly assembled chromatin, thereby reducing H1-mediated nucleosome interactions (Perry & Annunziato, 1989, 1991). As a consequence of dispersive segregation, both arms of the replication fork receive nascent H4, which is always diacetylated (Ruiz-Carillo *et al.*, 1975; Jackson *et al.*, 1976). Thus, all newly replicated chromatin, whether destined to be transcriptionally active or inert, will be "50% acetylated" (more or less), at least initially. If, as discussed by Wolffe and colleagues, the assembly and subsequent deacetylation of newly replicated chromatin establish a chromatin structure that inhibits gene activation (Almouzni *et al.*, 1990; Lee *et al.*, 1993), then the segregation of unacetylated parental histones may help to identify chromatin that is to be fully deacetylated, and thus rendered inactive. Conversely, the segregation of acetylated histones may foster the generation of potentially active chromatin, by promoting further acetylation (Cousens & Alberts, 1979, 1982; Chicoine *et al.*, 1986) and H1 depletion (Bresnick *et al.*, 1992) [most likely as a secondary mechanism, following the binding of specific *trans*-activating factors (Felsenfeld, 1992; also above references)].

Despite the apparent absence of selective acetylation, chromatin fibers undergo transitions in structure that allow for the replication of DNA. It is difficult to imagine how this could be accomplished without unfolding the 30-nm fiber. If chromatin decondensation is actively achieved (not merely passively, by fork migration), modifications other than acetylation may play a role. One possibility is the phosphorylation of histone H1, which is known to increase in S phase [Ajiro *et al.*, 1981; reviewed in Hohman (1983)]. It has recently been suggested that H1 phosphorylation may induce chromatin unfolding, by weakening the interaction of linker histones with DNA and/or chromatin (Roth & Allis, 1992). If this is the case, then phosphorylation may serve to decondense replicating chromatin, without interfering with the acetylation status of segregated nucleosomes. Transient nucleosome dissolution (Jackson, 1990) might then provide replication enzymes access to the DNA template.

REFERENCES

Ajiro, K., Borun, T. W., & Cohen, L. H. (1981) *Biochemistry 20*, 1445–1454.

Almouzni, G., Mechali, M., & Wolffe, A. (1990) *EMBO J. 9*, 573–582.

Almouzni, G., Mechali, M., & Wolffe, A. (1991) *Mol. Cell. Biol. 11*, 655–665.

Annunziato, A. T. (1990) *The Eukaryotic Nucleus: Molecular Biochemistry and Macromolecular Assemblies* (Strauss, P., & Wilson, S., Eds.) pp 687–712, Telford Press, Caldwell, NJ.

Annunziato, A. T., & Seale, R. L. (1982) *Biochemistry 21*, 5431–5438.

Annunziato, A. T., & Seale, R. L. (1983) *J. Biol. Chem. 258*, 12675–12684.

Annunziato, A. T., & Seale, R. L. (1984) *Nucleic Acids Res. 12*, 6179–6196.

Annunziato, A. T., Frado, L.-L. Y., Seale, R. L., & Woodcock, C. L. F. (1988) *Chromosoma 96*, 132–138.

Boffa, L. C., Walker, J., Chen, T. A., Sterner, R., Mariani, M. R., & Allfrey, V. G. (1990) *Eur. J. Biochem. 194*, 811–823.

Bonne-Andrea, C., Wong, W., & Alberts, B. M. (1990) *Nature 343*, 719–726.

Bonner, W. M., & Laskey, R. A. (1974) *Eur. J. Biochem. 46*, 83–88.

Bresnick, E. H., Bustin, M., Marsaud, V., Richard-Foy, H., & Hager, G. L. (1992) *Nucleic Acids Res. 20*, 273–278.

Brynoff, K., Eliasson, R., & Reichard, P. (1978) *Cell 13*, 573–580.

Burhans, W. C., Vassilev, L. T., Wu, J., Sogo, J. M., Nallaseth, F. Z., & DePamphilis, M. L. (1991) *EMBO J. 10*, 4351–4360.

Chicoine, L. G., Schulman, I. G., Richman, R., Cook, R. G., & Allis, C. D. (1986) *J. Biol. Chem. 261*, 1071–1076.

Cousens, L. S., & Alberts, B. M. (1979) *J. Biol. Chem. 254*, 1716–1723.

Cousens, L. S., & Alberts, B. M. (1982) *J. Biol. Chem. 257*, 3945–3949.

Crémisi, C., Chestier, A., & Yaniv, M. (1978) *Cold Spring Harbor Symp. Quant. Biol. 42*, 409–416.

Cusick, M. E., DePamphilis, M. L., & Wasserman, P. M. (1984) *J. Mol. Biol. 178*, 249–271.

Felsenfeld, G. (1992) *Nature 355*, 219–224.

Fraser, J. M. K., & Huberman, J. K. (1977) *J. Mol. Biol. 117*, 249–272.

Garrard, W. T. (1991) *BioEssays 13*, 87–88.

Hansen, J. C., & Wolffe, A. P. (1992) *Biochemistry 31*, 7977–7988.

Heebes, T. R., Thorne, A. W., & Crane-Robinson, C. (1988) *EMBO J. 7*, 1395–1402.

Heebes, T. R., Thorne, A. W., Clayton, A. L., & Crane-Robinson, C. (1992) *Nucleic Acids Res. 20*, 1017–1022.

Hohman, P. (1983) *Mol. Cell. Biol. 57*, 81–92.

Ip, Y. T., Jackson, V., Meier, J., & Chalkley, R. (1988) *J. Biol. Chem. 263*, 14044–14052.

Jackson, V., & Chalkley, R. (1981) *Cell 23*, 121–134.

Jackson, V., & Chalkley, R. (1985) *Biochemistry 24*, 6930–6938.

Jackson, V., Shires, A., Tanphaichitr, N., & Chalkley, R. (1976) *J. Mol. Biol. 104*, 471–483.

Jeppesen, P., & Turner, B. M. (1993) *Cell 74*, 281–289.

Jeppesen, P., Mitchell, A., Turner, B., & Perry, P. (1992) *Chromosoma 101*, 322–332.

Krude, T., & Knippers, R. (1991) *Mol. Cell. Biol. 11*, 6257–6267.

Laskey, R. A., & Mills, A. D. (1975) *Eur. J. Biochem. 56*, 335–341.

Lee, D. Y., Hayes, J. J., Pruss, D., & Wolffe, A. P. (1993) *Cell 72*, 73–84.

Lin, R., Leone, J. W., Cook, R. G., & Allis, C. D. (1989) *J. Cell. Biol. 108*, 1577–1588.

Littlefield, B. A., Cidlowski, N. B., & Cidlowski, J. A. (1982) *Exp. Cell Res. 141*, 283–291.

McKnight, S. L., & Miller, O. L., Jr. (1977) *Cell 12*, 795–804.

Olins, A. L., Olins, D. E., Derenzini, M., Hernandez-Verdun, D., Gounon, P., Robert-Nicoud, M., & Jovin, T. M. (1988) *Biol. Cell 62*, 83–93.

Olins, D. E., Olins, A. L., Herrmann, A., Lin, R., Allis, C. D., & Robert-Nicoud, M. (1991) *Chromosoma 100*, 377–385.

Painter, R. B., & Schaefer, A. W. (1969) *J. Mol. Biol. 45*, 467–479.

Perry, C. A., & Annunziato, A. T. (1989) *Nucleic Acids Res. 17*, 4275–4291.

Perry, C. A., & Annunziato, A. T. (1991) *Exp. Cell Res. 196*, 337–345.

Perry, C. A., Dadd, C. A., Allis, C. D., & Annunziato, A. T. (1993) *Biochemistry* (preceding paper in this issue).
Prescott, D. M., & Kimball, R. F. (1961) *Proc. Natl. Acad. Sci. U.S.A. 47*, 686–693.
Randall, S. K., & Kelly, T. J. (1992) *J. Biol. Chem. 267*, 14259–14265.
Ridsdale, J. A., & Davie, J. R. (1987) *Nucleic Acids Res. 3*, 1081–1095.
Roth, S. Y., & Allis, C. D. (1992) *Trends Biochem. Sci. 17*, 93–98.
Roufa, D. J. (1978) *Cell 13*, 129–138.
Ruiz-Carrillo, A., Wangh, L. J., & Allfrey, V. G. (1975) *Science 190*, 117–128.
Seale, R. L. (1976) *Cell 9*, 423–429.
Seale, R. L. (1977) *Biochemistry 16*, 2847–2853.
Seale, R. L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2717–2721.
Seale, R. L., & Simpson, R. T. (1975) *J. Mol. Biol. 94*, 479–501.
Shimamura, A., Sapp, M., Rodriguez-Campos, A., & Worcel, A. (1989) *Mol. Cell. Biol. 9*, 5573–5584.
Sogo, J. M., Stahl, H., Koller, Th., & Knippers, R. (1986) *J. Mol. Biol. 189*, 189–204.
Sugasawa, K., Ishimi, Y., Eki, T., Hurwitz, J., Kikuchi, A., & Hanaoka, F. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 1055–1059.
Svaren, J., & Chalkley, R. (1990) *Trends Genet. 6*, 52–56.
Thomas, J. O., & Kornberg, R. D. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2626–2630.
Turner, B. M. (1991) *J. Cell Sci. 99*, 13–20.
Turner, B. M., Franchi, L., & Wallace, H. (1990) *J. Cell Sci. 96*, 335–346.
Vu, Q. A., Zhang, D., Chroneos, Z. C., & Nelson, D. A. (1987) *FEBS Lett. 220*, 79–83.
Walker, J., Chen, T. A., Sterner, R., Berger, M., Winston, F., & Allfrey, V. G. (1990) *J. Biol. Chem. 265*, 5736–5746.
Weintraub, H. (1974) *Cold Spring Harbor Symp. Quant. Biol. 38*, 247–256.
Weintraub, H. (1976) *Cell 9*, 419–422.
Weintraub, H. (1984) *Cell 38*, 17–27.
Weintraub, H. (1985) *Cell 42*, 705–711.
Wolffe, A. (1989) *EMBO J. 8*, 527–537.
Wolffe, A. P. (1991a) *Trends Cell Biol. 1*, 61–66.
Wolffe, A. P. (1991b) *J. Cell Sci. 99*, 201–206.
Wolffe, A., & Brown, D. (1988) *Science 241*, 1626–1632.
Zlatanova, J., & van Holde, K. (1992) *J. Cell Sci. 103*, 889–895.