Kinetics of Accumulation and Depletion of Soluble Newly Synthesized Histone in the Reciprocal Regulation of Histone and DNA Synthesis

W. M. Bonner,* Roy S. Wu,‡ H. T. Panusz,§ and C. Muneses

Laboratory of Molecular Pharmacology, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Received January 27, 1988; Revised Manuscript Received May 3, 1988

ABSTRACT: Procedures are presented which permit the identification and analysis of cellular histone that is not bound to chromatin. This histone, called soluble histone, could be distinguished from that bound to chromatin by the state of H4 modification and the lack of H2A ubiquitination. Changes in the levels of newly synthesized soluble histone were analyzed with respect to the balance between histone and DNA synthesis in hamster ovary cells. Pulse–chase protocols suggeted that the chase of newly synthesized histone from the soluble fraction into chromatin may have two kinetic components with half-depletion times of about 1 and 40 min. When protein synthesis was inhibited, the pulse–chase kinetics of newly synthesized histone from the soluble fraction into chromatin were not significantly altered from those of the control. However, in contrast to the control, when protein synthesis was inhibited, DNA synthesis was also inhibited with kinetics similar to those of the chase of newly synthesized histone from the soluble fraction. There was a rapid decrease in the rate of DNA synthesis with a half-deceleration time of 1 min down to about 30% of the control rate, followed by a slower decrease with an approximate half-deceleration time of 40 min. When DNA synthesis was inhibited, newly synthesized histone accumulated in the soluble fraction, but H2A and H2B continued to complex with chromatin at a significant rate. Soluble histone in G1 cells showed the same differential partitioning of H4/H3 and H2A/H2B between the soluble and chromatin-bound fractions as was found in cycling cells with inhibited DNA synthesis. These results support a unified model of reciprocal regulatory mechanisms between histone and DNA synthesis in the assembly of chromatin.

Histones are among the most plentiful proteins in eucaryotic cells as well as among the major products of protein synthesis during the S phase of the cell cycle. These proteins are synthesized on cytoplasmic ribosomes and migrate into the nucleus where they assemble with DNA at a definite stoichiometry to form chromatin, the stoichiometric unit being the nucleosome, composed of two molecules of each of the four core histones species and about 200 base pairs of DNA [for a recent review, see Wu et al. (1986)].

In contrast to this rather rigid model of association between histone and DNA, there are also suggestions of more dynamic interactions. During the G1 phase of the cell cycle and the G0 state when there is no DNA replication, there is synthesis of certain histone isoproteins. These histones are incorporated into chromatin (Wu & Bonner, 1981; Wu et al., 1982), a process which can lead to changes in the bulk histone isoprotein pattern of tissues that have been quiescent for long periods of time (Zweidler, 1976). In addition, there are studies which suggest that during DNA replication, histones H2B and H2A may dissociate from and reassociate with the chromatin (Jackson & Chalkley, 1985; Louters & Chalkley, 1985). Finally, there are reports which suggest that during transcription, the histone octamer may be remodeled (Weintraub et al., 1976; Prior et al., 1983) or that some or all of the histones may be displaced from the transcribing unit (Lorch et al., 1987; Baer & Rhodes, 1983), although there are also reports to the contrary (Losa & Brown, 1987).

The assembly of chromatin involves a complex set of reciprocal regulatory constraints between histone and DNA synthesis. When DNA synthesis is inhibited, most but not all histone synthesis is inhibited, replication-linked histone mRNA becomes less stable, and its amount decreases (Butler & Mueller, 1973). Likewise, when protein synthesis is inhibited, most but not all DNA synthesis is inhibited (Gautschi & Kern, 1973; Venhatesan, 1977; Seale & Simpson, 1975; Annunziato & Seale, 1982), histone mRNA becomes very stable, and its amount increases (Stimac et al., 1983). Investigators in several laboratories have substantiated these changes in histone mRNA stability and amount (Baumbach et al., 1984; DeLisle et al., 1983; Graves & Marzluff, 1984; Heintz et al., 1983; Helms et al., 1984; Plumb et al., 1983; Sittman et al., 1983; Sive et al., 1984; Stimac et al., 1984; Sariban et al., 1985).

It has been suggested that newly synthesized histone in transit between the ribosomes and chromatin plays a definite role in the reciprocal regulation of histone and DNA synthesis. Butler and Mueller (1973) presented evidence that when DNA synthesis is inhibited, the concentration of soluble histone increases; they suggested that the increased soluble histone concentration leads to the degradation of the histone mRNA. More recently, Sariban et al. (1985) suggested that when protein synthesis is inhibited, a decreased soluble histone concentration could lead to the inhibition of DNA synthesis and protection of histone mRNA from degradation. In addition, they presented a unified hypothesis in which soluble histone regulated both the levels of replication-linked histone mRNAs and the rate of DNA synthesis so that the rates of histone and DNA synthesis remained in balance during changes in the rates of protein or DNA synthesis.

In order to more directly investigate the dynamic nature of

* Address correspondence to this author at NCI-NIH, Building 37, Room 5D19, Bethesda, MD 20892.

‡ Present address: Clinical Investigations Branch, Cancer Therapy Evaluation Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892.

§ Present address: Sloan Kettering Institute for Cancer Research, Rye, NY.

the histone interactions with DNA, we analyzed the soluble histone fraction in a variety of conditions. In this report, we present evidence that soluble histone can be isolated and reliably distinguished from chromatin-bound histone. We have characterized the kinetics of accumulation and depletion of newly synthesized histone in the soluble fraction during chromosome replicatin, during changes in the rate of chromosome replication due to the inhibition of DNA or protein synthesis, and in the G1 phase of the cell cycle.

Experimental Procedures

Cell Growth. Chinese hamster ovary (CHO) cells were grown in Hams F-10 with 10% fetal calf serum as previously described (Wu & Bonner, 1985). Typically, 3×10^6 cells were seeded in a T-75 tissue culture flask or 7×10^6 in a T-150 flask. Cells were allowed to grow 1–2 days before being labeled. Cells were routinely tested for mycoplasma contamination. Synchronized cells in G1 were obtained and labeled as previously described (Wu et al., 1982).

Preparation of Soluble Histone. Procedures were performed in a warm room with all components preequilibrated to 37 °C. Labeling for both continuous short-term labeling and pulse–chase experiments was performed with 10 μCl of [^{14}C]-lysine/mL in lysine-free HF-10 media containing 10% undialyzed fetal calf serum (5 mL for a T-150 flask and 2.5 mL for a T-75 flask). Flasks were incubated with gentle rocking. In continuous labeling protocols, aliquots of inhibitor stock solutions were added with gentle mixing directly to the T-flasks containing the labeled media. Final inhibitor concentrations were 100-fold diluted from the stock solutions (cycloheximide, 1 mg/mL in PBS; hydroxyurea, 1 M in PBS; aphidicolin, 1 mg/mL in DMSO). PBS was added to the mock-treated control. In pulse–chase protocols, the labeling medium was removed, and the chase medium (HF-10 with 10% fetal calf serum and 10-fold-increased lysine) containing inhibitors was added. Incubations were terminated by aspirating the media and adding ice-cold PBS containing 0.2 mg/mL lysine. The flasks were transferred to ice or a cold room for the preparation of soluble histone. From control experiments, it was found that after the cells were cooled, the continued presence of inhibitors was not necessary to maintain the altered soluble histone concentrations.

The following procedures were performed at 4 °C. The PBS was aspirated and trypsin added at 4 °C (5 mL of Gibco trypsin for a T-150 flask). After about 10 min, when the cells could be loosened by tapping, HF-10 medium with 10% FCS was added while simultaneously titurating the cells to a single cell suspension. The cells were transferred to a 15-mL centrifuge tube, pelleted at 300*g* for 5 min, and washed once more with PBS. The cells were then suspended in 0.5 mL of TM (10 mM Tris-HCl, pH 7.5, and 1 mM $MgSO_4$), transferred to a microfuge tube, and pelleted for 1–2 s. The cell pellet was resuspended well in 0.5 mL of TM, and a 10-μL aliquot was removed for cell counting with either a Coulter Counter or a haemocytometer. Triton-N101 was added to the cell suspension to a final concentration of 0.3% to lyse the cells. After a 5–10-min incubation, the lysed cells were centrifuged for 10 min in a microfuge with a horizontal rotor. The supernatant containing the soluble histone was carefully removed to another microfuge tube, leaving the nuclear pellet undisturbed. The nuclear pellet was resuspended in 0.5 mL of TE [10 mM Tris-HCl (pH 7.5) and 1 mM EDTA]. Histones were extracted from both the soluble and nuclear fractions by adding 12.5 M HCl containing 1% 2-mercaptoethanol to a final concentration of 0.5 M HCl. Samples were extracted for 1 h with occasional vortexing and then centrifuged in a microfuge for 10 min. The supernatants were removed and freeze-dried. Control experiments showed that cells lysed in isotonic PBS yielded the same soluble histone preparations as those prepared in TM.

Two-Dimensional Gel Electrophoresis. The freeze-dried samples were resuspended in AUT sample buffer (1 M acetic acid, 50 mM ammonia, and 5 M urea). Typical loadings were 5×10^6 cell equivalents for the soluble fraction and 1×10^6 for the nuclear. Gels were prepared, run, and stained as previously described (Wu & Bonner, 1981; Wu et al., 1982). Gels were stained for 15–30 min, destained for 15–30 min, washed in water for 15–30 min, soaked in a water-soluble fluorography solution for 30 min (Autofluor, National Diagnostics), and then dried and fluorographed (Bonner & Laskey, 1974; Laskey & Mills, 1975). Radioactivity in various histone species was quantitated by scintillation counting as previously described (Wu & Bonner, 1985).

Measurement of the Rate of DNA and Protein Synthesis. In order to compare changes in the rates of protein and DNA synthesis with the rapid changes in the soluble histone levels, a 1-min assay procedure was developed. The assays were performed in a warm room at 37 °C with all solutions equilibrated to that temperature. Cells were grown overnight in T-25 flasks. The growth medium was aspirated and replaced with 1 mL of HF-10 with 10% FCS. Inhibitors were added from a 10× stock solution directly to the growth medium in T flasks held so that the mixing was not over the cell monolayer. At zero time, the T-flasks were agitated so that the growth media wetted the cell monolayer. The T-flasks were placed on a slow rocker, and at the noted time, [^{3}H]-thymidine or [^{3}H]lysine was added from a 10× stock and mixed with the growth medium. Final concentrations were 100 μCi/mL for both labels. HF-10 contains 3 μM thymidine which ensures that the rate of [^{3}H]thymidine incorporation is proportional to the rate of DNA synthesis (Cleaver, 1967). After 1 min, 10 mL of ice-cold PBS containing 10× thymidine and 10× lysine was added to the T-flasks and mixed quickly with the labeling media. The contents of the T-flasks were aspirated, and 10 mL of ice-cold 20% perchloric acid containing 10× thymidine and lysine (PCA–TK) was added. The T-flasks were stored in the cold until the time course was finished and then washed 3 times with 10 mL of PCA–TK so that all the interior surfaces were rinsed. The T-flasks were then washed in the same manner twice with 10 mL of 100% ethanol and left to dry at room temperature. The dried attached cell layers were solubilized by wetting and digesting them with 1 mL of TM containing 5 μg/mL DNase I with slow rocking at 37 °C for 30 min. NaOH (0.1 mL of 5 M) and SDS (0.1 mL of 10%) were added, and the solubilization was continued for 1 h. The flasks were then tilted upright to collect the extract, and 0.1-mL aliquots were taken for scintillation counting. In some cases, the OD_{260} was determined to normalize the samples, but this was not necessary as the duplicates generally agreed within 20%.

Results

Identification and Kinetics of Labeling of Soluble Histone. Preliminary experiments demonstrated that newly synthesized histone could be detected and analyzed in the postnuclear supernatant of cells labeled with radioactive lysine. The supernatant histone, called soluble histone, could be reliably distinguished from chromatin-bound histone by the qualitatively different patterns of these two fractions in high-resolution two-dimensional gels (Figure 1, panels N and S). The most obvious and characteristic difference between the two fractions is the state of modification of histone H4. For newly syn-

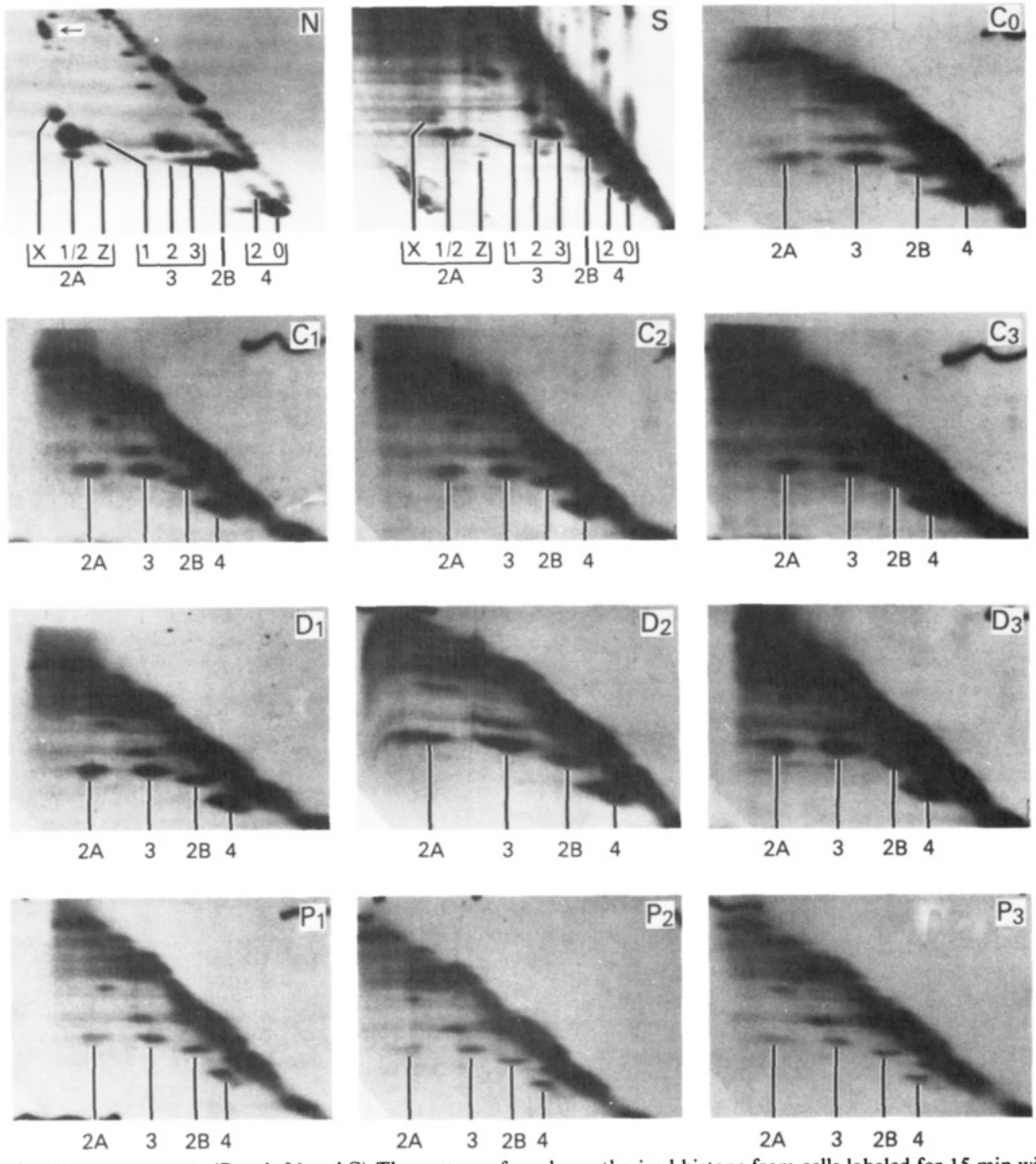

FIGURE 1: Soluble histone patterns. (Panels N and S) The pattern of newly synthesized histone from cells labeled for 15 min with [^{14}C]lysine. (N) Chromatin-bound histone. The arrow points to the uH2As. (S) Soluble histone. (Panels C_0–P_3) Continuous labeling protocol. Hamster ovary cells were prelabeled for 10 min with [^{14}C]lysine (C_0) as described under Experimental Procedures. Labeling was continued for 3 min (C_1, D_1, P_1), 10 min (C_2, D_2, P_2), or 30 min (C_3, D_3, P_3) in the presence of complete medium alone (C_1, C_2, C_3), with 10 mM hydroxyurea (D_1, D_2, D_3), or with 10 μg/mL cycloheximide (P_1, P_2, P_3). At the times indicated, the cells were harvested. Soluble and chromatin-bound histones were prepared, analyzed, and quantitated as described under Experimental Procedures.

thesized soluble histone H4 (panel S), the dimodified form predominates with at least 60% of the H4 label in that form (Ruiz-Carrillo et al., 1975), while for newly synthesized chromatin-bound H4 (panel N), the dimodified form accounts for only about 10% of the total. A second characteristic difference between the two is the apparent lack of ubiquitination of newly synthesized soluble histone H2A (panel N, arrow shows where uH2A migrates). Third, as became apparent as studies progressed, the kinetic parameters of the soluble histone are different than those of chromatin-bound histone.

In order to gain more insight into the reciprocal constraints between histone and DNA synthesis, the kinetic characteristics of the histones present in the soluble fraction were determined in cells labeled with radioactive lysine utilizing continuous and pulse–chase labeling protocols. Cell cultures in three states were studied in detail: (1) cultures with cycling cells in exponential growth; (2) cultures similar to the above in which protein synthesis had been inhibited; and (3) cultures similar to the above in which DNA synthesis had been inhibited. In addition, soluble histone was also isolated from cell cultures in the G1 phase of the cell cycle. Each of these states will be considered in turn.

When cycling cells were incubated continuously with label, (panels C_0–C_3 in Figure 1 and circles in Figure 2), label accumulated rapidly in soluble histones up to about 10 min (C_0 in Figure 1, zero minutes in Figure 2) and then more gradually so that after 40 min of labeling (C_3 in Figure 1, 30 min in Figure 2), there was about twice as much label in soluble histones as at 10 min. In other experiments, this gradual rate of labeling was found to persist for at least 2 h, the longest time used with this type of protocol (data not presented). Thus, label in soluble histone does not quickly reach a steady-state level as would be expected if soluble histone consisted of a homogeneous population of complexes with one input and one output. This result, which suggested that there may be some kinetic complexity in the soluble histone fraction, was substantiated by results from pulse–chase protocols; there

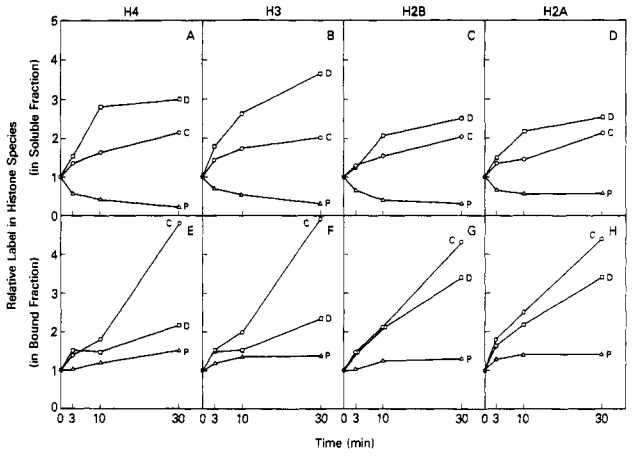

FIGURE 2: Continuous labeling protocol: kinetics of histone labeling. Quantitative data from the experiment shown in Figure 1 are shown for H4 (panels A and E), H3 (panels B and F), H2B (panels C and G), and H2A (panels D and H) in the soluble fraction (panels A–D) and the chromatin-bound fraction (panels E–H) after incubation of cells with complete medium alone (circles, C), with 10 mM hydroxyurea (squares, D), or with 10 μg/mL cycloheximide (triangles, P).

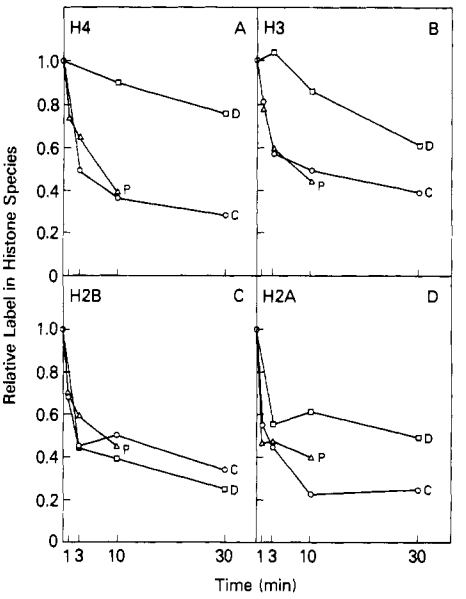

FIGURE 3: Pulse–chase protocol: kinetics of histone labeling. Hamster ovary cells were prelabeled for 20 min with [^{14}C]lysine as described under Experimental Procedures. Label was removed and incubation continued for 1, 3, 10, or 30 min in the presence of complete medium alone (circles, C), 10 μg/mL aphidicolin (Squares, D), or 10 μg/mL cycloheximide (triangles, P). At the times indicated, the cells were harvested. Soluble and chromatin-bound histones were prepared, analyzed, and quantitated as described under Experimental Procedures. Quantitative data from two-dimensional gels simlar to those shown in Figure 1 are shown for H4 (panel A), H3 (panel B), H2B (panel C), and H2A (panel D) in the soluble fraction. Experiments with 10 mM hydroxyurea yielded similar results as those with aphidicolin.

was a rapid decrease of label in soluble histone during the first few minutes of the chase followed by a much slower decrease (Figure 3, circles). Since these studies involve exponentially growing cell cultures, one possible explanation for these complex kinetics may be that cells in different phases of the cell cycle contribute different components to the overall kinetics. However, because the G1 pattern of newly synthesized histone in the soluble as well as in the chromatin-bound fraction is qualitatively different than that in S phase (as shown in Figure 5G,H), this possibility can be eliminated since the S-phase pattern present initially does not change during the chase. Another possible explanation is that there is contamination from chromatin-bound histone. This possibility can also be eliminated because the modification pattern of soluble H4, which differs from that of chromatin-bound H4, is retained throughout the chase (data not presented, but similar to that shown in Figure 1, panel P_3). Thus, these results do not seem to be consistent with the simplest models of soluble histone as a uniform population of molecules in transit from the ribosomes to the chromatin replication forks.

Changes in Soluble Histone Levels and the Rate of DNA Synthesis When Protein Synthesis Is Inhibited. DNA synthesis is known to be inhibited in a complex manner when inhibitors of protein synthesis are added to cultures of cycling cells (Gautschi & Kern, 1973; Seale & Simpson, 1975; Venhatesan, 1977). Generally, there seems to be an immediate decrease in the DNA synthetic rate to 20–30% of the control, followed by a further slow deceleration in the rate lasting for several hours. Sariban et al. (1985) suggested that the inhibition of DNA synthesis following the addition of inhibitors of protein synthesis might be due directly or indirectly to a lack of available histone for chromatin assembly. A necessary prediction of such a hypothesis is that the rate of DNA synthesis not decrease faster than the level of soluble histone in the relevant fractions.

In order to further investigate these possibilities, experiments were performed comparing changes in the levels of soluble histone and in the rate of DNA synthesis in cell cultures immediately after the termination of protein synthesis. These studies are shown in Figure 1 (C_0, P_{1-3}) and Figure 2 (triangles) for the continuous labeling protocol and in Figure 3 (triangles) for the pulse–chase protocol. In both protocols, there is a rapid initial decrease in the soluble histone levels followed by a much slower decrease. Because inhibition of protein synthesis prevents further labeling of soluble histone in the continuous labeling protocol (Figures 1 and 2), both protocols in this case are effectively pulse–chase protocols; thus, it is not surprising that the results from both protocols yield almost identical curves.

Changes in the rate of DNA synthesis immediately after the termination of protein synthesis were measured with 1-min pulses of labeled thymidine. Within 1 min after the addition of an inhibitor of protein synthesis, the rate of DNA synthesis decreased rapidly (Figure 4B, circles) to about 30% of the control rate and then continued to decrease more slowly (Figure 4A, circles). When plotted on semilogarithmic coordinates, the data fall along two straight lines. These results suggests that the decrease in the rate of DNA synthesis is consistent with a kinetic analysis in which there is a 1-min lag immediately following the addition of a protein synthesis inhibitor, then a rapid first-order deceleration down to about about 30% of the control rate at 5 min, and finally a slower but still approximately first-order deceleration. From these data, one can calculate that it takes about 10 s for the fast component to decrease 10% and about 1 min to decelerate to 50% of its initial rate. For the slow component, it takes about 7 min to decrease 10% and 40 min to decrease to 50% of its initial rate.

As shown in Figures 2 and 3 (triangles), upon the termination of protein synthesis, the level of labeled soluble histone decreases initially at a rapid rate followed by a much slower decrease. In order to more closely compare changes in the rate of DNA synthesis with the level of labeled soluble histone, the relevant data points as shown in those figures were averaged and also plotted in Figure 4 (along with some data from other experiments). The label in soluble histone decreases rapidly to about 50% of the initial level followed by a slower decrease. The rapidity of the initial decrease in the level of

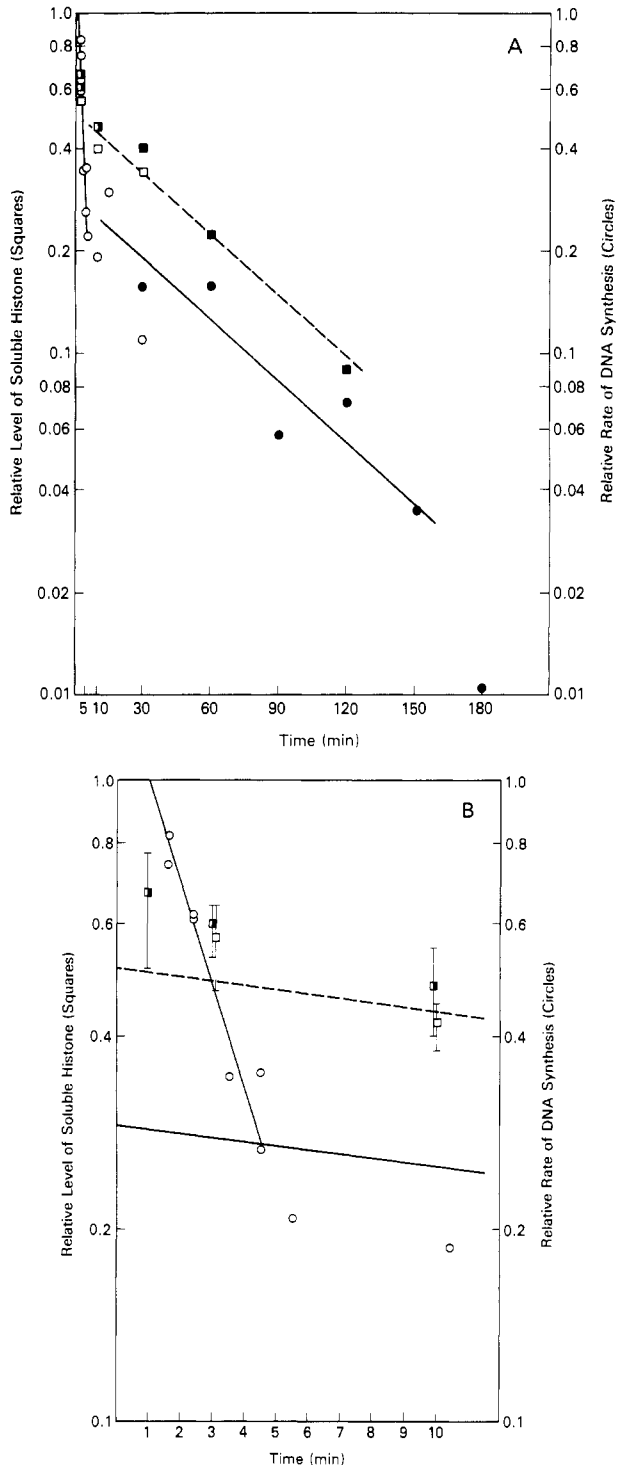

FIGURE 4: Soluble histone levels and the rate of DNA synthesis during inhibition of protein synthesis. The rate of DNA synthesis was measured at various times after the addition of cycloheximide (10 μg/mL) as described under Experimental Procedures. (Open circles) Short time course; (closed circles) longer time course. Values for the levels of the four histone species in the soluble fraction were taken from the cycloheximide-treated cultures in the experiments described above and from other similar experiments, averaged, and plotted. (Open squares) Continuous labeling protocol from Figure 2; (half-filled squares) pulse–chase protocol from Figure 3; (filled squares) pulse–chase protocol at longer times. (Panel A) Complete time course. Solid lines are fitted by eye to the data points for the rate of DNA synthesis (circles). The dashed line is fitted to the data points (10 min and after) for the level of soluble histone (squares). (Panel B) The initial 10 min of the time course shown in panel A, shown in larger detail. In panel B, the bars on each square show the range of values for the four histone species at each time point; the lines from panel A are extrapolated to zero time.

soluble histone makes it difficult to obtain accurate data; however, on the basis of these current limited data, it seems reasonable to describe the soluble histone system as having two kinetic components with half-times of depletion of about 1 and 40 min. More detailed studies will either confirm or refine this description. Thus, upon the termination of protein synthesis, both systems, the level of soluble histone and the rate of DNA synthesis, appear to decrease with two kinetic components of similar half-times.

These studies compare the level of soluble histone with the rate of DNA synthesis, even though mechanistically one would expect the rate of depletion of soluble histone to correlate with the rate of DNA synthesis. However, if the kinetics of depletion of soluble histone are approximately first order, there would be a direct proportionality between the rate of depletion and the level of soluble histone, thus accounting for the similarities in the slopes of the curves for the rate of DNA synthesis and the *level* of soluble histone. However, because of this type of comparison, an equality between the extents of the slow kinetic components, 50% for the level of soluble histone versus 30% for the rate of DNA synthesis, should not be expected and is not found.

In the pulse–chase protocol, the label in soluble histone appears to decrease at a similar rate whether or not protein synthesis has been inhibited, even though DNA synthesis is being inhibited in one case and not the other. This similarity is apparent in Figure 3 (circles and triangles) for the initial rapid decrease in the level of soluble histone. It has also been found for the slow kinetic component in which newly synthesized histone is chased from the soluble fraction in the presence of protein synthesis (data not presented) at almost the same rate as that found in the absence of protein synthesis (Figure 4A, closed squares). This result suggests that the depletion of histone molecules from the soluble fraction has not been affected by the inhibition of protein synthesis in general or the bulk level of soluble histone in particular, since that level is presumably decreasing in one case and not the other. Following from this and assuming that chromatin is the destination for soluble histone, it suggests that DNA synthesis still continues at a rate sufficient to complex with soluble histone when protein synthesis has been terminated to the same extent as when protein synthesis has not been inhibited. Lending support to this suggestion is the finding that both DNA synthesis and protein synthesis respond more rapidly to direct inhibition than DNA synthesis does to the inhibition of protein synthesis. Protein synthesis was found to be inhibited by 98% within 5 s after the addition of the inhibitor cycloheximide (data not presented). DNA synthesis was found to be 95% inhibited 2.5 min after and 98% inhibited 3.5 min after the addition of the inhibitor hydroxyurea (data not presented), compared to 40% inhibited 2.5 min after the addition of the inhibitor of protein synthesis, cycloheximide (Figure 4B).

Changes in Soluble Histone Levels When DNA Synthesis Is Inhibited. Inhibition of DNA synthesis is known to be followed by the inhibition of histone synthesis, while total protein synthesis remains unaffected. Butler and Mueller (1973) reported that histone mRNAs are degraded over a 15–30-min period after the onset of inhibition of DNA synthesis. They also obtained evidence that there was some histone accumulation in the soluble cellular fraction and proposed that this elevated concentration of soluble histone leads in some way to the degradation of the histone mRNAs. Continuous labeling kinetics of the accumulation of newly synthesized histones in the soluble fraction in the absence of

DNA synthesis are shown in Figure 1 (C_0, D_{1-3}) and Figure 2 (squares). Similar studies for the retention of pulse-labeled histone in the soluble fraction during the pulse–chase protocol are shown in Figure 3 (squares). In the continuous labeling protocol (Figures 1 and 2), there is an accumulation of labeled histone in the soluble fraction above that in the control. The accumulation is greater for H4 and H3 (Figure 2A,B) than it is for H2B and H2A (Figure 2C,D). Under the same conditions, there is also a very noticeable difference in the incorporation of the various histones into chromatin (Figure 2E–H); during the 30-min time course of this experiment, H2B and H2A continued to be incorporated into chromatin at a significant fraction of the control rate, while H4 and H3 are incorporated to a much smaller extent. Time courses as long as overnight in the absence of DNA synthesis show that H2A and H2B incorporation into chromatin also decreases to a small fraction of the control but is always noticeably greater than the incorporation of H4 and H3 (data not presented). The results of the pulse–chase protocol (Figure 3) show that at the onset of the chase in the presence of the inhibitor of DNA synthesis, pulse-labeled H4 and H3 are only very slowly chased from the soluble fraction, while pulse-labeled H2B and H2A are initially chased from the soluble fraction with kinetics similar to those in the control.

Thus, there are clear differences in the immediate and the short-term response of the two histone pairs to the inhibition of DNA synthesis. Newly synthesized H4 and H3 are only very slightly incorporated into chromatin (Figure 2E,F), are chased slowly from the soluble fraction (Figure 3A,B), and accumulate in the soluble fraction. However, the data present a more complex situation for newly synthesized H2B and H2A. These two histones continue to be incorporated into chromatin at a significant rate (Figure 2G,H) and initially are chased from the soluble fraction (Figure 3C,D) but also accumulate in the soluble fraction at times longer than 3 min (Figures 2C,D).

Interpretation of the results from the continuous labeling protocol in the absence of DNA synthesis in particular is complicated by the decreasing rate of histone synthesis due to histone mRNA destruction (Butler & Mueller, 1973). In order to investigate the different behavior of the H4–H3 and H2A–H2B histone pairs further, the relative rates of synthesis of the histones and their partitioning between the soluble and chromatin fractions were measured immediately and 30 min after the inhibition of DNA synthesis (Figure 5A–F). Quantitation of these data (Table I) shows that two factors seem to be important. The first is that the synthesis of H2A and H2B is not inhibited as rapidly as that of H4 and H3; during the first 15 min after the onset of DNA inhibition, about twice as much H2A and H2B as H4 and H3 is synthesized (Figure 5C,D; Table I, 0–15 HU), but by 30–45 min, the synthesis of all four histone species has been inhibited about 90% (Figure 5E,F; Table I, 30–45 HU). The second factor is the different partitioning of the two histone pairs between the soluble and chromatin-bound fractions. Under the labeling conditions of this protocol, 3–4% of the newly synthesized histone remains in the soluble fraction in uninhibited cells (Table I, control). However, during the first 15 min after the onset of inhibition of DNA synthesis, about 28% of the labeled H4 and H3 remains in the soluble fraction, and this increases to 35–40% during the 30–45-min time period. For H2A and H2B, about 3 times as much label remains in the soluble fraction at both 0–15 and 30–45 min compared to the control, but this is still only about 10% of the total labeled H2A and H2B. Thus, the difference in partitioning between the soluble and chromatin-bound fractions for the two histone pairs is maintained even though the total synthesis of both pairs is inhibited by about 90%. While the H4 and H3 incorporated into chromatin under these conditions could be due to exchange

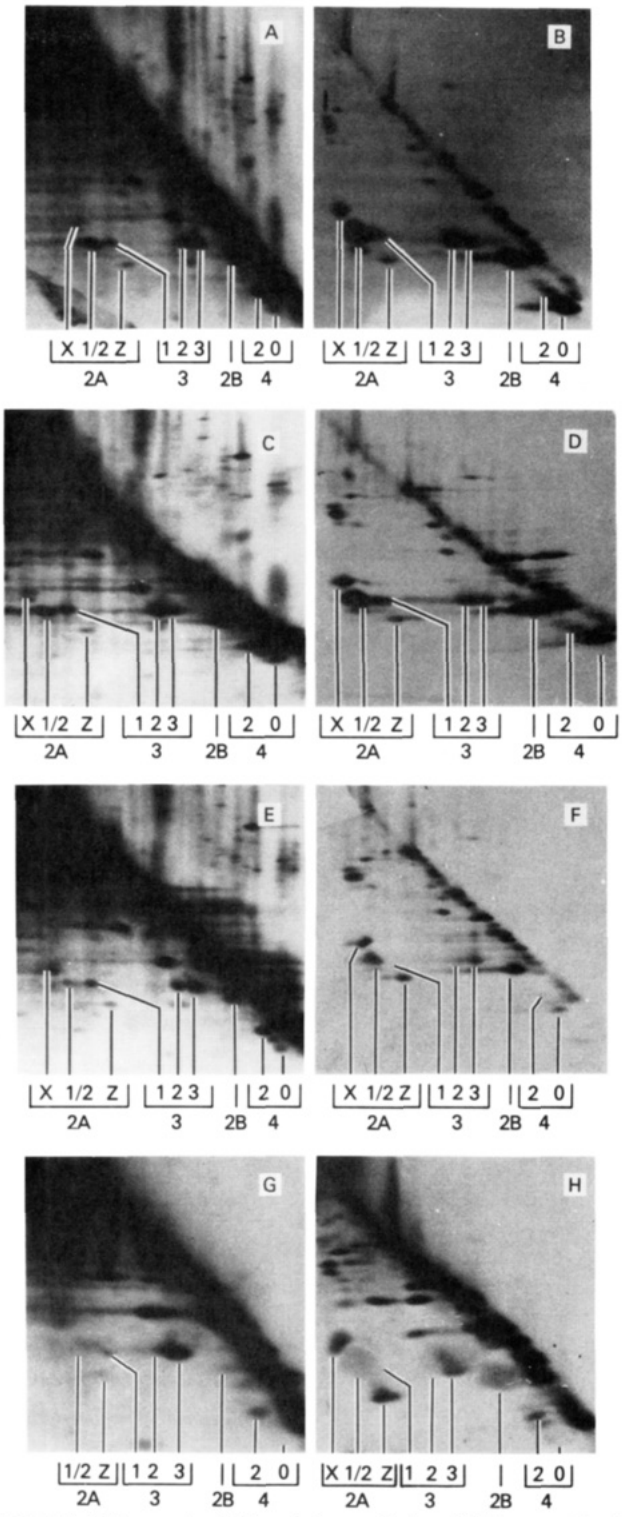

FIGURE 5: Patterns of soluble and chromatin-bound histone synthesis during the inhibition of DNA synthesis and in G1. Hamster ovary cells were labeled for 15 min with [^{14}C]lysine with no additions (panels A and B), coincident with the addtion of 10 mM hydroxyurea (panels C and D), or 30 min after the addition of 10 mM hydroxyurea (panels E and F). Hamster ovary cells in G1 (panels G and H) were obtained as described in Wu et al. (1982); the cells were labeled for 1 h starting 1 h after mitotic shake-off with [^{14}C]arginine. Soluble (panels A, C, E, and G) and chromatin-bound (panels B, D, F, and H) histones were prepared and analyzed as described under Experimental Procedures.

Table I: Characteristics of Soluble Histone in G1 Cells and Cells with Inhibited DNA Synthesis[a]

	control		0–15 HU		30–45 HU		G1	
	E/C	S/T	E/C	S/T	E/C	S/T	E/C	S/T
H4	100	4.3	21	28	6.5	42	5.6	60
H3	100	3.9	23	27	10.5	34	17	82
H2B	100	2.8	46	7.4	9.1	8.9	7.5	36
H2A	100	2.6	36	9.9	12.5	11.5	12	28

[a] Quantitative data for the experiments shown in Figure 5 are expressed as percent synthesis compared to the control (E/C) and the percent of the total labeled histone remaining in the soluble fraction (S/T).

or to residual amounts of replication, the excess incorporation of H2A and H2B suggests that some sort of exchange is occurring for this histone pair.

These results support and extend those reported by Louters and Chalkley (1985) concerning the possibility of exchange of H2A and H2B after inhibition of DNA synthesis. Jackson and Chalkley (1985) reported that while newly synthesized H4 and H3 segregated almost solely with newly replicated DNA, newly synthesized H2B and H2A also complexed with unreplicated DNA. In addition to exchange, Worcel et al. (1978) suggested that H2A and H2B may be incorporated into chromatin slightly later than H4 and H3. In such a case, there would still be chromatin sites available for H2A and H2B immediately after the onset of inhibition of DNA synthesis. Thus, this type of mechanism could account for the relatively greater rate of synthesis of H2A and H2B and their apparently unimpeded chase from the soluble fraction immediately after the onset of inhibition of DNA synthesis. However, it cannot account for the continued incorporation of H2A and H2B into chromatin during longer term inhibition of DNA synthesis.

Soluble Histone Levels in the G1 Phase of the Cell Cycle. Wu and Bonner (1981) showed that the isoprotein patterns of histone synthesis are very similar in G1 cells and in S-phase cells in which DNA synthesis has been inhibited. H2A synthesis is dominated by that of the H2A.X and H2A.Z isoproteins; H3 synthesis is dominated by that of the H3.3 isoprotein. The similarity of these patterns suggested that the mechanisms controlling replication-linked histone synthesis may also be similar in G1- and S-phase cells. This suggestion was further supported by the finding that quiescent cells (G0), which also are not replicating DNA, exhibit a pattern of histone isoprotein synthesis different than that found in G1 cells (Wu et al., 1982). In order to futher study the similarities between the control of histone synthesis in G1- and S-phase cells, the patterns and partitioning of newly synthesized soluble histone were compared in the two phases. The results for the G1 cells (Figure 5G,H; Table I, G1) and cells with inhibited DNA synthesis (Figure 5E,F; Table I, 30–45 HU) show that the similarity does extend to the pattern and partitioning of newly synthesized histone in the soluble and chromatin-bound fractions. For both populations of cells, H4 and H3 partition more to the soluble fraction while H2B and H2A partition more to the chromatin fraction. Incubation of G1 cells with an inhibitor of DNA synthesis (hydroxyurea) did not alter the nascent soluble and chromatin-bound histone patterns (data not presented), substantiating that these patterns in G1 cells were independent of DNA synthesis. Thus, these results show that the soluble histone responds to the inherent absence of DNA synthesis in G1 cells in the same way as it does to the induced absence of DNA synthesis in S-phase cells.

Discussion

The results presented here lead to findings concerning the relationship of soluble histone to the balance between histone and DNA synthesis and the nature of soluble newly synthesized histone.

In general, the newly synthesized histone appears to behave as an intermediate in a reaction chain, entering the soluble fraction after synthesis on cytoplasmic polyribosomes and leaving it to be incorporated into chromatin. When the utilization of newly synthesized histone is prevented with inhibitors of DNA synthesis, histone accumulates in the soluble fraction. During the same period, replication-linked histone mRNA is degraded (Butler & Mueller, 1973; Baumbach et al., 1984: DeLisle et al., 1983; Graves & Marzluff, 1984; Heintz et al., 1983; Helms et al., 1984; Plumb et al., 1983; Sittman et al., 1983; Sive et al., 1984; Sariban et al., 1985) apparently by a specific nuclease (Ross & Kobs, 1986; Ross et al., 1986), leading to decreased histone synthesis. When the synthesis of histone is blocked by inhibitors of protein synthesis, histone is depleted from the soluble fraction. Closely correlating with the depletion of soluble histone is the inhibition of DNA synthesis. The replication of DNA in the absence of protein synthesis is semiconservative (Seale & Simpson, 1975); the chromatin assembled under these conditions contains recently synthesized histones but may not have the same density or arrangement of nucleosomes as found in mature chromatin (Seale & Simpson, 1975; Annunziato & Seale, 1982). As protein synthesis is inhibited, histone mRNA levels are also known to increase due to a stabilization of the normally short-lived mRNA (Stimac et al., 1983, 1984). It has been proposed that this increase is a part of a mechanism to compensate for the decreased efficiency of histone synthesis during the chromosome cycle (Sariban et al., 1985).

As such, the findings are consistent with the model presented by Sariban et al. (1985) which attempts to unify the regulatory relationships between histone and DNA synthesis when either is inhibited. In this model, it was proposed that the level of soluble histone balances the synthesis of histone with the synthesis of DNA by leading to changes in the level of replication-linked histone mRNA on one hand and in the rate of DNA synthesis on the other. Mechanisms by which the depletion of soluble histone could lead to the inhibition of DNA synthesis fall into two categories. Depletion of soluble histone could by mass action lead to the synthesis of incomplete chromatin or free DNA, which could by various direct or indirect mechanisms then lead to the inhibition of DNA synthesis. On the other hand, depletion of soluble histone could inhibit some component of the replication complex directly by some sort of regulatory interaction. Both of these categories of mechanisms would be consistent with the model of Sariban et al. (1985). Other categories of mechanisms are also compatible with the present data. Both the depletion of soluble histone and the deceleration of DNA synthesis could be independently due to the inhibition of protein synthesis, coincidentally having similar kinetic parameters. One type of mechanism which is not supported by our studies is the type in which DNA synthesis is inhibited more quickly than the depletion of soluble histone. In order to differentiate between these and other possible mechanisms, it will be necessary to isolate and biochemically characterize the molecular components involved in soluble histone metabolism.

These results also reveal several complexities, of which two will be mentioned. The first concerns the complex kinetics associated with the chase or depletion of labeled newly synthesized histone from the soluble fraction under normal growth conditions as well as under conditions of inhibition of protein synthesis, and the similar kinetics of inhibition of DNA synthesis when protein synthesis is inhibited. The sets of data for both the depletion of soluble histone and the deceleration of DNA synthesis appear not to be consistent with single first-order processes; however, in hamster ovary cells, the two systems can be reasonably described as having two first-order kinetic components with half-completion times of about 1 min and about 40 min. These kinetics are compatible with physical models containing two types of soluble histone complexes with different routes of incorporation into chromatin as well as models containing one type of soluble complex which requires different factors for different routes of incorporation into chromatin. It is known than in addition to chromatin assembly during DNA replication, histones in nonreplicating chromatin are replaced or exchanged (Wu & Bonner, 1981; Wu et al., 1982; Zweidler, 1976). Biochemical studies will be necessary to describe the physical components of soluble histone and their interactions with other cellular components.

A second complexity concerns the different behavior of the H4/H3 and the H2A/H2B histone pairs when DNA synthesis is inhibited as in S-phase cells incubated with inhibitors of DNA synthesis or when DNA synthesis is naturally absent as in G1 cells. In these cases, H4 and H3 are retained in the soluble fraction much more than are H2A and H2B which continue to enter chromatin to a measurable extent for some time. These results support and extend those reported by other (Louters & Chalkley, 1985; Jackson & Chalkley, 1985; Worcel et al., 1978) suggesting spatial and temporal differences in the assembly of these two histone pairs into chromatin. Dilworth et al. (1987) and others (Kleinschmidt & Franke, 1982; Kleinschmidt et al., 1985) have reported that in *Xenopus* oocytes, which contain maternal stored histones, there are different storage/carrier complexes for H4/H3 and H2A/H2B. Cotten and Chalkley (1987) have purified similar components from *Xenopus* somatic cells.

Thus, the findings are consistent with the working hypothesis that the fast and slow kinetic components may reflect these biochemical processes of replication and exchange or replacement and that the different kinetics of the H4/H3 and H2A/H2B pairs may reflect the presence of multiple carrier proteins. However, many other models are possible, and verification of any such correspondences must await further biochemical studies.

A typical mammalian cell with a genetic complement of 6×10^9 base pairs of DNA would require about 10^5 H4 protein molecules per minute in a 10-h S phase. If soluble histone derived from the fast kinetic component supplies 75% of the histone for DNA replication and is half-depleted in approximately 1 min, it would need to contain about 1.5×10^5 molecules of each histone species per cell. This is about 0.1% of the total cellular histone depending on the fraction of chromatin that has been replicated. These calculated values agree quite well with those estimated experimentally from the amount of soluble H4 labeled in 10 min with lysine of known specific activity (data not presented). Using the methods described herein as a basis for analyzing soluble histone, we are investigating the biochemical characteristics of soluble histone complexes and their interactions with chromatin in its various structural and functional states.

REFERENCES

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

Baer, B. W., & Rhodes, D. (1983) *Nature (London) 301*, 482–488.

Baumbach, L. L., Marashi, F., Plumb, M., Stein, G., & Stein, J. (1984) *Biochemistry 23*, 1618–1625.

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

Butler, M. B., & Mueller, G. C. (1973) *Biochim. Biophys. Acta 294*, 481–496.

Cleaver, J. E. (1967) *Thymidine Metabolism and Cell Kinetics*, North-Holland, Amsterdam.

Cotten, M., & Chalkley, R. (1987) *EMBO J. 6*, 3945–3954.

DeLisle, A. J., Graves, R. A., Marzluff, W. F., & Johnson, L. F. (1983) *Mol. Cell. Biol. 3*, 1920–1929.

Dilworth, S. M., Black, S. J., & Laskey, R. A. (1988) *Cell (Cambridge, Mass.) 51*, 1009–1018.

Gautschi, J. K., & Kern, R. M. (1973) *Exp. Cell Res. 80*, 15–26.

Graves, R. A., & Marzluff, W. F. (1984) *Mol. Cell. Biol. 4*, 351–357.

Heintz, N., Sive, H. L., & Roeder, R. G. (1983) *Mol. Cell. Biol. 3*, 539–550.

Helms, S., Baumbach, L., Stein, G., & Stein, J. (1984) *FEBS Lett. 168*, 65–69.

Jackson, V., & Chalkley, R. (1985) *Biochemistry 24*, 6921–6930.

Kleinschmidt, J. A., & Franke, W. W. (1982) *Cell (Cambridge, Mass.) 29*, 799–809.

Kleinschmidt, J. A., Fortkamp, E., Krohne, G., Zentgraf, H., & Franke, W. W. (1984) *J. Biol. Chem. 260*, 1166–1176.

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

Lorch, Y., LaPointe, J. W., & Kornberg, R. D. (1987) *Cell (Cambridge, Mass.) 49*, 203–210.

Losa, R., & Brown, D. D. (1987) *Cell (Cambridge, Mass.) 50*, 801–808.

Louters, L., & Chalkley, R. (1985) *Biochemistry 24*, 3080–3085.

Plumb, M., Stein, J., & Stein, G. (1983) *Nucleic Acids Res. 11*, 7927–7945.

Prior, C. P., Cantor, C. R., Johnson, E. M., Littau, V. C., & Allfrey, V. G. (1983) *Cell (Cambridge, Mass.) 34*, 1033–1042.

Ross, J., & Kobs, G. (1986) *J. Mol. Biol. 188*, 579–593.

Ross, J., Peltz, S. W., Kobs, G., & Brewer, G. (1986) *Mol. Cell. Biol. 6*, 4362–4371.

Ruiz-Carrillo, A., Wangh, L. J., & Allfrey, V. G. (1975) *Science (Washington, D.C.) 190*, 117–128.

Sariban, E. R., Wu, R. S., Erickson, L. C., & Bonner W. M. (1985) *Mol. Cell. Biol. 5*, 1279–1286.

Seale, R. L., & Simpson, R. T. (1975) *J. Mol. Biol. 94*, 479–501.

Sittman, D. B., Graves, R. A., & Marzluff, W. F. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1949–1953.

Sive, H. L., Heintz, N., & Roeder, R. G. (1984) *Mol. Cell. Biol. 4*, 2723–2734.

Stimac, E., Groppi, V. E., Jr., & Coffino, P. (1983) *Biochem. Biophys. Res. Commun. 114*, 131–137.

Stimac, E., Groppi, V. E., Jr., & Coffino, P. (1984) *Mol. Cell. Biol. 4*, 2082–2090.

Venhatesan, N. (1977) *Biochim. Biophys. Acta 478*, 437–453.
Weintraub, H., Worcel, A., & Alberts, B. (1976) *Cell (Cambridge*, Mass.) *9*, 409–417.
Worcel, A., Han, S., & Wong, M. L. (1978) *Cell (Cambridge*, Mass.) *15*, 969–977.
Wu, R. S., & Bonner, W. M. (1981) *Cell (Cambridge, Mass.)* *27*, 321–330.
Wu, R. S., & Bonner, W. M. (1985) *Mol. Cell. Biol. 5*, 2959–2966.
Wu, R. S., Tsai, S., & Bonner, W. M. (1982) *Cell (Cambridge*, Mass.) *31*, 367–374.
Wu, R. S., Panusz, H. T., Hatch, C. L., & Bonner, W. M. (1986) *CRC Crit. Rev. Biochem. 20*, 201–263.
Zweidler, A. (1976) *Life Sci. Res. Rep. 4*, 187–197.

Positive and Negative Regulatory Elements Control the Steroid-Responsive Ovalbumin Promoter†

Michel M. Sanders‡ and G. Stanley McKnight*,§

Department of Pharmacology, University of Washington, Seattle, Washington 98195, and Departments of Medicine and Biochemistry, The University of Minnesota, Minneapolis, Minnesota 55455

Received February 29, 1988; Revised Manuscript Received May 5, 1988

ABSTRACT: Steroid hormones regulate the transcriptional activity of the chicken ovalbumin gene both in vivo and in cell culture. To identify the regulatory elements involved, primary oviduct cell cultures were transfected with constructs containing the promoter and 5′-flanking region of the ovalbumin gene fused to the bacterial chloramphenicol acetyltransferase (CAT) gene. Induction of the OvCAT genes by estrogen, progesterone, or corticosterone mimics that of the endogenous ovalbumin gene, indicating that the transfected DNA is accurately regulated. Deletion analysis revealed that a steroid response element (SRE) resides between nucleotide coordinates −880 and −585 and that a negative regulatory element (NRE) resides between −350 and −248 in the ovalbumin gene. Thus, an NRE represses expression of the ovalbumin gene unless steroid hormones relieve this negative control through interactions involving a more distal SRE. Neither the SRE nor the NRE alone regulates the heterologous thymidine kinase promoter, suggesting either that they function as a single entity or that they are conditional regulatory elements. The NRE is functional in MCF-7 cells, but the SRE cannot be activated by steroids in this heterologous estrogen-responsive cell line. These data indicate that the steroid–receptor complex induces the ovalbumin gene through direct or indirect actions at an SRE to relieve repression at an NRE.

For 2 decades, the chicken oviduct has served as a model to study the regulation of eucaryotic gene expression, in part because few gene regulatory molecules other than steroid receptors have been identified in higher eucaryotes. As a result, the biology of this system is well-defined [for a review, see Sanders and McKnight (1986)]. In sexually immature birds, estrogen initiates differentiation of the tubular gland cells of the oviduct and induces the mRNAs for the major egg white proteins, ovalbumin, transferrin or conalbumin, lysozyme, and ovomucoid, by enhancing both the transcription rates of these genes and the stability of the resultant mRNAs. In vitro experiments revealed that estrogen requires the permissive effects of insulin (Evans & McKnight, 1984) and corticosterone (Sanders & McKnight, 1986) to exert its effects on transcription. After primary exposure to estrogen in vivo, three other classes of steroids, the androgens, glucocorticoids, and progestins, can also induce the egg white genes, making this model unique for investigations of how the specificity of induction of target genes by different steroid hormones is achieved.

Relatively little is known about the regulatory elements controlling the ovalbumin gene. While the canonical TATA and CAAT boxes typical of highly active promoters have been characterized (Zarucki-Schulz et al., 1982), the sequences required for regulation by estrogen or other steroid hormones have not been defined. Competitive filter binding assays from two laboratories (Mulvihill et al., 1982; Compton et al., 1983) have identified areas in the 5′-flanking region that effectively compete with nonspecific DNA for partially purified progesterone receptor, but the strongest regions identified by each group do not overlap. Likewise, ovalbumin fusion genes were transfected into cultured oviduct cells, but no consensus was reached about the sequences required for regulation by estrogen or progesterone (Dean et al., 1983, 1984; Chambon et al., 1984; Gaub et al., 1987). These discrepancies may result from differences in receptor preparations, cell culture conditions, fusion genes, and assay systems.

To define the sequences in the ovalbumin gene required for regulation by estrogen, we have developed a primary tubular gland cell culture system for chicken oviduct in which the endogenous ovalbumin gene is induced by hormones to the same extent that it is induced in vivo (Sanders & McKnight, 1985). As described in this paper, a transfection protocol has been adapted to these cells that allows the assay of promoter fusion genes without disturbing the responsiveness of the en-

† This research was supported by a grant from the National Institutes of Health to G.S.M. (HD14412).
‡ The University of Minnesota.
§ University of Washington.

0006-2960/88/0427-6550$01.50/0