Articles

Level of Ubiquitinated Histone H2B in Chromatin Is Coupled to Ongoing Transcription†

James R. Davie* and Leigh C. Murphy
Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Manitoba, 770 Bannatyne Avenue, Winnipeg, Manitoba, Canada R3E 0W3

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ABSTRACT: The relationship between transcription and ubiquitination of the histones was investigated. Previous studies have shown that ubiquitinated (u) histone H2B and, to a lesser extent, mono- and polyubiquitinated histone H2A are enriched in transcriptionally active gene-enriched chromatin fractions. Here, we show that treatment of T-47D-5 human breast cancer cells with actinomycin D (10 μg/mL) or 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole, inhibitors of heterogeneous nuclear RNA synthesis, selectively reduced the level of uH2B, but not uH2A, uH2A.Z, or polyubiquitinated H2A, in chromatin. Treatment of the cells with low levels (0.04 μg/mL) of actinomycin D slightly reduced the level of uH2B, suggesting that inhibition of ribosomal RNA synthesis does not have a profound effect on the level of uH2B in chromatin. The level of the ubiquitinated histones was not affected by treating the cells with inhibitors of DNA synthesis (sodium butyrate or aphidicolin), but heat-shock treatment resulted in the loss of all the monoubiquitinated histone species. These results demonstrate that maintenance of the levels of uH2B in chromatin is dependent upon ongoing transcription, particularly the synthesis of hnRNA. Thus, histone H2B would be ubiquitinated when the nucleosome was opened during transcription. Ubiquitination of histone H2B may impede nucleosome refolding, facilitating subsequent rounds of transcription.

Ubiquitin is a 76-residue protein that may be attached to proteins via an isopeptide linkage to the ε-amino group of the target protein. Proteins can also be polyubiquitinated by the attachment of a chain of ubiquitin units, joined via isopeptide linkages (Chau et al., 1989; Nickel & Davie, 1989). Polyubiquitination of cytoplasmic proteins appears to be necessary for ubiquitin-dependent degradation of proteins (Chau et al., 1989; Hershko, 1988). However, the attachment of single ubiquitin to a protein appears to have a function(s) other than targeting the conjugated protein for degradation. Proteins that have been found to be ubiquitinated include cell surface proteins (e.g., the lymphocyte homing receptor and the growth hormone receptor), ribosomal proteins, actin, and the histones (Goldknopf & Busch, 1977; Thorne et al., 1987; Siegelman et al., 1986; Yarden et al., 1986; Leung et al., 1987; Ball et al., 1987; Finley et al., 1989; Redman & Rechsteiner, 1989; Muller-Taubenberger et al., 1989).

Histones H2A and H2B and their variant forms can be ubiquitinated. The carboxyl end of ubiquitin is attached to the ε-amino group of lysine (Lys-119 in H2A and Lys-120 in H2B; Goldknopf & Busch, 1977; Thorne et al., 1987). Typically, histone H2A is ubiquitinated to a greater extent than H2B (~10% of H2A versus ~1% of H2B). These histones can also be multiubiquitinated, with histone H2A usually having the greater level of polyubiquitinated species (Davie et al., 1987; Nickel et al., 1989; Nickel & Davie, 1989). Interestingly, the linkage of ubiquitin units in polyubiquitinated H2A is the same as that for proteins targeted for turnover (Chau et al., 1989; Davie & Nickel, 1989).

Ubiquitinated (u) histones may have a role in maintaining chromatin in an "open" configuration. The disappearance of uH2A and uH2B at metaphase suggests that these modified proteins must be deubiquitinated for packaging of the chromatin to occur (Mueller et al., 1985; Raboy et al., 1986). There is evidence that uH2A is complexed to transcriptionally active DNA (Levinger & Varshavsky, 1982; Barsoum & Varshavsky, 1985), although not all reports agree with this association (Huang et al., 1986). We have demonstrated that uH2B is highly enriched in transcriptionally active gene-enriched chromatin fractions (Nickel et al., 1989). Moreover, the transcriptionally active macronucleus, but not the transcriptionally inert micronucleus, of *Tetrahymena* is associated with uH2B. Our results indicate that uH2B and, to a lesser extent, uH2A are associated with transcriptionally active DNA.

Ericsson et al. (1986) reported that inhibition of transcription does not affect the amount of uH2A in HeLa cell chromatin. In this report, we investigated whether the levels of uH2B and polyubiquitinated H2A in human breast cancer cell chromatin could be altered by inhibition of transcription, inhibition of replication, or heat-shock treatment. We show that the level of the ubiquitinated histones is not affected by inhibition of replication but the amount of uH2A and uH2B is reduced to very low levels in heat-shocked cells. Inhibition of transcription, particularly synthesis of hnRNA, leads to the almost complete disappearance of uH2B, but not uH2A, uH2A.Z, or polyubiquininated forms of H2A, in chromatin. These observations show that ubiquitination of histone H2B is dependent on the transcription process, supporting our other evidence that uH2B is complexed with transcriptionally active DNA.

Materials and Methods

Cells and Treatments. Stock cultures of T-47D-5 human breast cancer cells were maintained at 37 °C under a humidified atmosphere of 5% CO_2 in 5% fetal bovine serum containing medium as described previously (Murphy & Dotzlaw,

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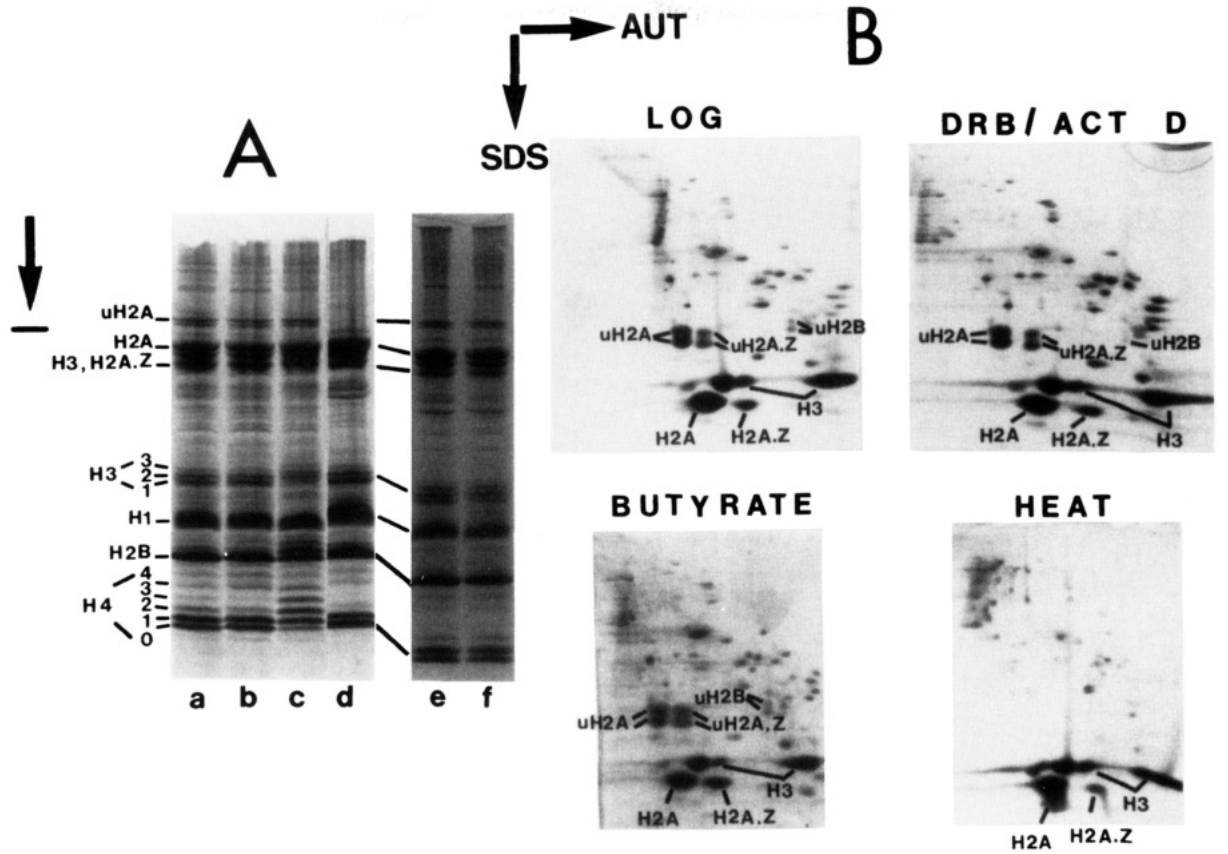

FIGURE 1: Effect of inhibition of replication, inhibition of transcription, and heat shock on levels of ubiquitin–histone conjugates. Panel A shows Coomassie Blue stained AUT 15% polyacrylamide gel patterns containing acid-soluble nuclear proteins isolated from T-47D-5 human breast cancer cells (lane a, 18 μg; lane e, 9 μg) or from cells incubated with DRB/actinomycin D (lane b, 18 μg), incubated with sodium butyrate (lane c, 18 μg), heat shocked at 44–45 °C for 4 h (lane d, 18 μg), or incubated with aphidicolin (lane f, 9 μg) as described under Materials and Methods. Panel B shows silver-stained two-dimensional gel patterns (AUT into SDS–polyacrylamide gel) containing acid-soluble nuclear proteins (36 μg) from the untreated or treated cells described above. The two-dimensional gel patterns are labeled as follows: LOG (untreated), DRB/ACT D (DRB/actinomycin D), HEAT (heat shock), and BUTYRATE (sodium butyrate). 0, 1, 2, 3, and 4 correspond to the un-, mono-, di-, tri-, and tetraacetylated species of histones H3 and H4, respectively. The ubiquitin adducts of histones H2A, H2A.Z, and H2B are denoted as uH2A, uH2A.Z, and uH2B, respectively.

1989). For experiments, cells were plated in 25 mL of the above medium at 2×10^6 cells per 150 mm diameter dish. Three to four days later, when the cells were still in the logarithmic phase of growth, the medium was renewed, and the cells were subjected to the various treatments described below. Following treatment, the medium was removed, and the cells were harvested off the monolayer by scraping with a rubber policeman and placed on ice. After centrifugation at 4 °C, the cell pellet was snap frozen and stored below −70 °C until further analysis. Different groups of cells underwent the following treatments: (a) 24 h with 5 mM sodium butyrate (Candido et al., 1978), (b) 0.04 μg/mL actinomycin D for 30 min followed by 75 μM 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) for 40 min (Ericsson et al., 1986), (c) heat shocked (44–45 °C for 4 h; Andrews et al., 1987), (d) 0.04 μg/mL actinomycin D alone for 70 min, (e) 10 μg/mL actinomycin D alone for 70 min, (f) 75 μM DRB alone for 40 min, (g) 75 μM DRB alone for 70 min, (h) 75 μM DRB for 40 min, following which the medium was removed, the cell monolayer was washed once with phosphate-buffered saline and fresh medium was added, and the cells were allowed to recover for 30 and 60 min without DRB, or (i) μM aphidicolin for 70 min (Sariban et al., 1985).

Isolation of Histones. The cell pellet was resuspended in buffer A [100 mM KCl, 10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 10 mM sodium butyrate, 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.1 μg/mL aprotinin, and 10 mM iodoacetamide, an inhibitor of isopeptidase (Matsui et al., 1982)] containing 0.1% NP-40 and homogenized. The nuclei were collected by centrifugation at 2000*g* for 10 min, and the pellet was resuspended in buffer A. Sulfuric acid was added to 0.4 N, and the suspension was incubated on ice for 30 min. Acid-soluble proteins were collected following centrifugation at 12000*g* for 20 min (Davie & Nickel, 1987).

Polyacrylamide Gel Electrophoresis and Immunochemical Detection of Ubiquitinated Histone Species. One-dimensional AUT gel electrophoresis [acetic acid/6.7 M urea/0.375% (w/v) Triton X-100/15% polyacrylamide gel] and two-dimensional gel electrophoresis [AUT into SDS (sodium dodecyl sulfate)/15% polyacrylamide gel] were performed as described (Nickel et al., 1989). The gels were stained with Coomassie Blue and silver stained or transferred to nitrocellulose and immunochemically stained for ubiquitin with an anti-ubiquitin IgG and ^{125}I-protein A as described previously (Nickel et al., 1987; Nickel et al., 1989).

RESULTS

Effect of Inhibition of DNA Synthesis on Levels of Ubiquitin–Histone Conjugates. T-47D-5 cells were incubated with sodium butyrate or aphidicolin. Sodium butyrate inhibits DNA synthesis, arresting the cells in the G1 phase of the cell cycle (D'Anna et al., 1980), and inhibits histone deacetylase (Candido et al., 1978), resulting in the hyperacetylation of the nucleosomal histones, H2A, H2B, H3, and H4. Aphidicolin, which is a potent inhibitor of DNA polymerase α, is an inhibitor of DNA replication (Pedrali-Noy & Spadari, 1979;

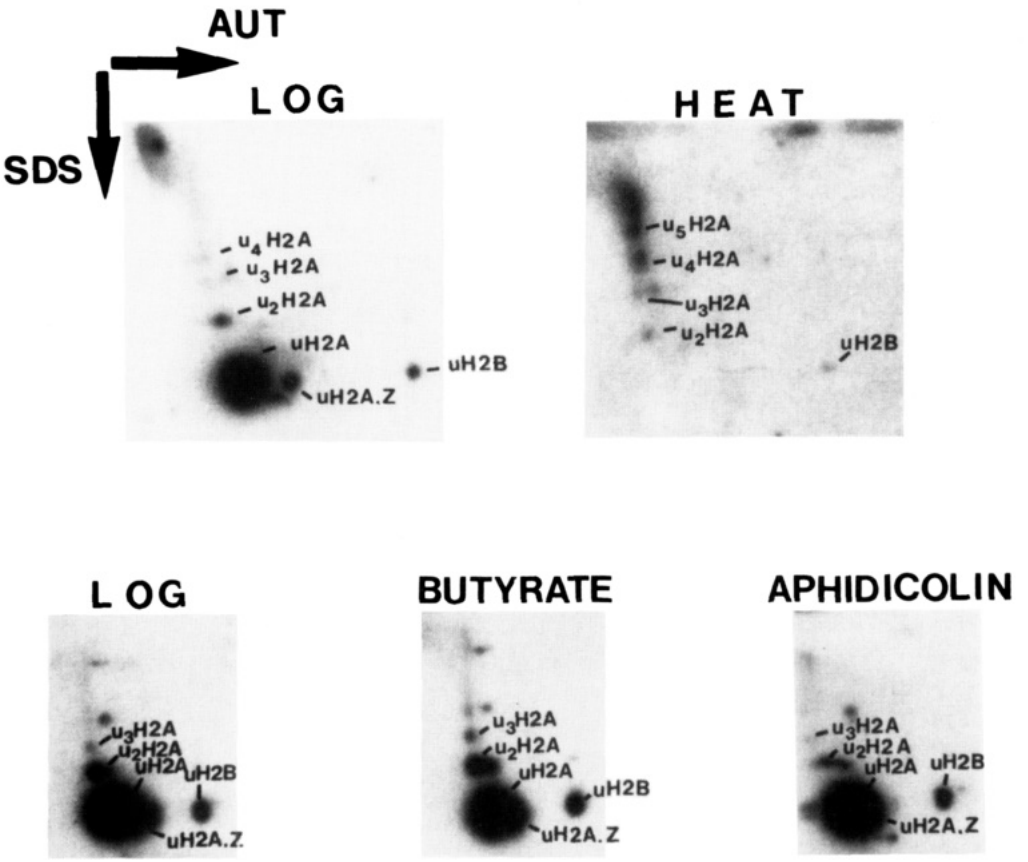

FIGURE 2: Effect of inhibition of replication and heat shock on levels of ubiquitinated histone species. Acid-soluble nuclear proteins (36 μg) of untreated (LOG), aphidicolin-treated (APHIDICOLIN), butyrate-treated (BUTYRATE), and heat-shocked (44–45 °C for 4 h; HEAT) cells were resolved by two-dimensional gel electrophoresis (AUT into SDS), electrophoretically transferred to nitrocellulose, and immunochemically stained for ubiquitin with anti-ubiquitin IgG and ^{125}I-protein A. The autoradiograms are shown. The ubiquitin adducts of histones H2A, H2A.Z, and H2B are denoted as uH2A, uH2A.Z, and uH2B, respectively. The polyubiquitinated histone species are labeled u_2, u_3, u_4, and u_5, representing the attachment of two, three, four, and five ubiquitins, respectively.

Arabshahi et al., 1988). Acid-soluble proteins isolated from the nuclei of the treated cells were electrophoretically resolved on AUT polyacrylamide gels (Figure 1A). Incubation of the cells in the presence of sodium butyrate elevated the levels of acetylated forms of histones H4, H2B, and H3 (Figure 1A lane c), but the amount of uH2A was not affected. Figure 1A (lane f) shows that aphidicolin treatment does not affect the level of uH2A in chromatin. The proteins were resolved further by two-dimensional gel electrophoresis (AUT into SDS–polyacrylamide gel). One of the distinctive features of the ubiquitinated histones is that they usually migrate as doublets in the second-dimension SDS gel (Nickel et al., 1989). In the two-dimension gel pattern, three ubiquitinated histone species uH2A, uH2A.Z, and uH2B, were detected [see Nickel and Davie (1989) and Nickel et al. (1989) for assignment of the ubiquitinated histones]. Figure 1B shows that treatment of the cells with sodium butyrate does not change the steady-state levels of uH2A, uH2A.Z, or uH2B.

The histones of control, aphidicolin-treated, or butyrate-treated cells were resolved by two-dimensional gel electrophoresis and transferred to nitrocellulose, and the ubiquitinated histone forms were detected by immunochemical staining with anti-ubiquitin IgG and ^{125}I-protein A (Figure 2). Treatment of the cells with aphidicolin or butyrate did not alter the levels of uH2A, uH2A.Z, uH2B, or polyubiquitinated species of H2A.

Effect of Heat Shock on Content of Ubiquitinated Histone Species. For a number of different cell lines it has been shown that heat shock reduces the levels of the acetylated histone species and uH2A (Parag et al., 1987; Bond et al., 1988, Arrigo, 1983). Figure 1A, lane d, shows the histone species of T-47D-5 human breast cancer cells incubated at 44–45 °C for 4 h. In agreement with the results of others, the levels of acetylated forms of histones H4, H2B, and H3 and of uH2A of heat-shocked cells were significantly lower than those of untreated cells. The two-dimensional gel pattern (Figure 1B, heat shock) shows that, in addition to uH2A, the levels of uH2A.Z and uH2B are very low.

Figure 2 shows that although the monoubiquitinated forms of histones H2A, H2A.Z, and H2B of heat-shocked cells have dropped to very low levels, uH2B is still detectable. The level of diubiquitinated H2A was also reduced, but detectable, in the histones of heat-shocked cells. In contrast to the levels of the other ubiquitinated histones, the amount of the polyubiquitinated forms of histone H2A in heat-shock cells was elevated.

Effect of Inhibition of Transcription on Levels of Ubiquitinated Histone Species. Incubation of cells with actinomycin D and DRB (treatment a under Materials and Methods) results in an inhibition of 60–75% of the hnRNA synthesis and most of the rRNA synthesis (Sehgal et al., 1976; Ericsson et al., 1986; Perry, 1963). Figure 1A (lane b) shows that inhibition of transcription does not alter the level of uH2A, in agreement with the results of Ericsson et al. (1986). However, Figure 1B (DRB/actinomycin D) shows that the amount of uH2B, but not uH2A or uH2A.Z, is reduced significantly when the cells are treated with these inhibitors of transcription.

DRB is a selective inhibitor of transcription by RNA polymerase II while actinomycin D at high concentrations is an

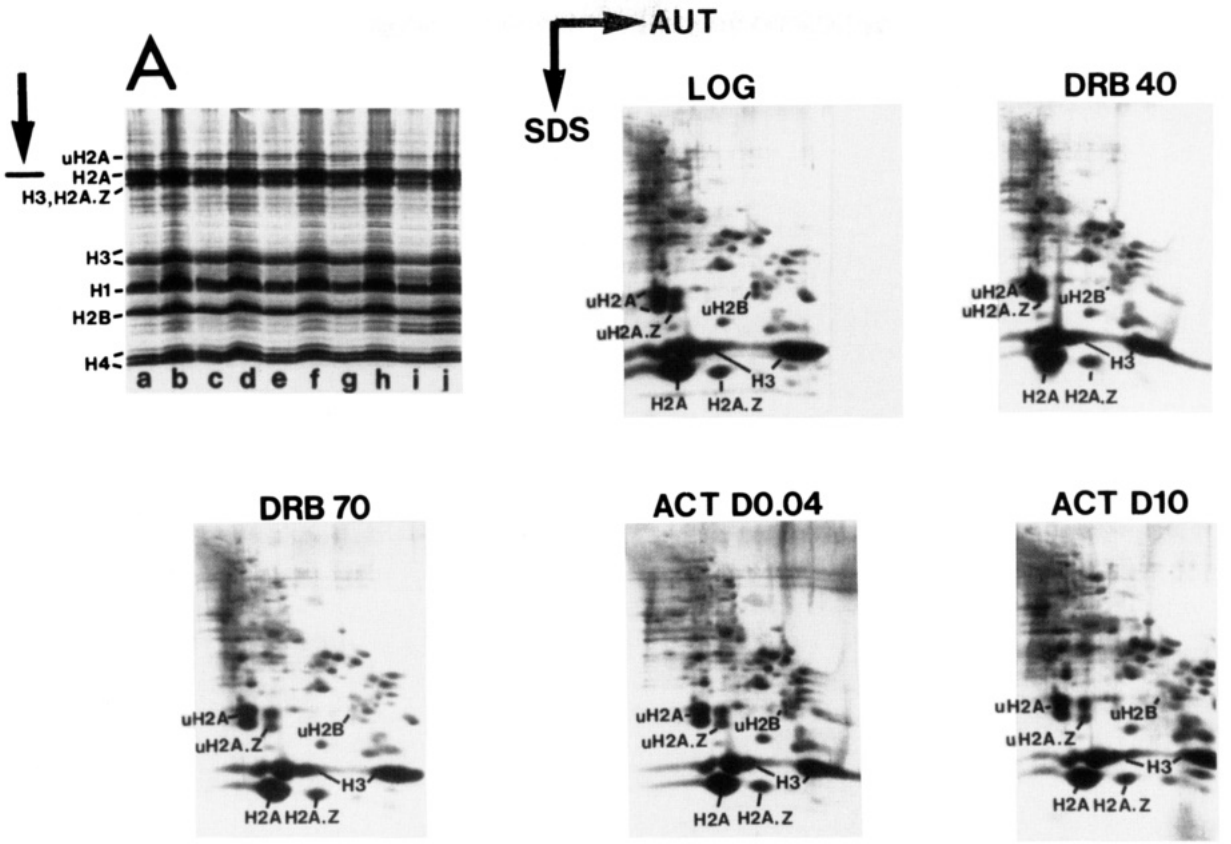

FIGURE 3: Effect of inhibition of RNA synthesis on levels of ubiquitinated histone species. Acid-soluble nuclear proteins were isolated from human breast cancer cells that were untreated (panel A, lanes a and b) or incubated with DRB for 40 min (panel A, lanes c and d), DRB for 70 min (panel A, lanes e and f), actinomycin D at 0.04 μg/mL for 70 min (panel A, lanes g and h), or actinomycin D at 10 μg/mL for 70 min (panel A, lanes i and j). Panel A shows a Coomassie Blue stained AUT 15% polyacrylamide gel pattern. Lanes a, c, e, g, and i contained 18 μg of protein while lanes b, d, f, h, and j contained 36 μg of protein. The lanes containing 36 μg of protein were resolved further by two-dimensional gel electrophoresis (AUT into SDS). The silver-stained two-dimensional gel patterns are labeled as follows: LOG (untreated cells), DRB40 (DRB, 40 min), DRB70 (DRB, 70 min), ACT D0.04 (actinomycin D at 0.04 μg/mL), and ACT D10 (actinomycin D at 10 μg/mL). The ubiquitin adducts of histones H2A, H2A.Z, and H2B are denoted as uH2A, uH2A.Z, and uH2B, respectively.

inhibitor of both RNA polymerase I and RNA polymerase II transcription (Chodosh et al., 1989; Perry, 1963). Concentrations of actinomycin D in the range of 10^{-7}–10^{-8} M strongly inhibit RNA polymerase I transcription while higher concentrations (10^{-6} M) are required to inhibit RNA polymerase II transcription (Perry, 1963). The human breast cancer cells were treated separately with these two inhibitors. Figure 3A shows that incubation of the cells with DRB (40 or 70 min) or actinomycin D (0.04 or 10 μg/mL, 70 min) does not significantly change the level of uH2A. However, two-dimensional electrophoretic patterns of the histones (shown in Figure 3) show that DRB (40 or 70 min) or actinomycin D at 10 μg/mL selectively reduces the level of uH2B, with the levels of uH2A and uH2A.Z being unaltered. Actinomycin D at 0.04 μg/mL only slightly reduced the amount of uH2B.

Immunochemical detection of the ubiquitin conjugates resolved by two-dimensional gel electrophoresis verifies the above results; i.e., incubation of the cells with DRB or high concentrations of actinomycin D selectively reduces the steady-state levels of uH2B while low concentrations of actinomycin D only slightly lower the amount of uH2B (Figure 4). Moreover, Figure 4 shows that the level of the polyubiquitinated forms of H2A is not appreciably altered by the presence of transcriptional inhibitors.

DRB is a reversible inhibitor of RNA polymerase II transcription. Thus, we asked whether the levels of uH2B are regained in DRB-treated cells when they are incubated in the absence of DRB. Cells incubated with 75 μM DRB for 40 min were placed in fresh medium, and the cells were allowed to recover for 30 min and 60 min. Within 30 min the levels of uH2B were back to normal (Figure 4).

Discussion

The major contribution of this study is the demonstration that inhibition of transcription selectively reduced the steady-state level of uH2B in human T-47D-5 breast cancer cell chromatin. The levels of uH2A, uH2A.Z, and polyubiquitinated forms of H2A were not affected. In preliminary studies, identical results were obtained when human fibroblast cells were used. These observations suggest that the reduction in the level of uH2B in chromatin is due to an inhibition of histone H2B ubiquitination rather than an increase in isopeptidase activity. It is possible that the decrease in uH2B levels during inhibition of transcription is secondary to inhibition of ubiquitin synthesis. However, this would require uH2B to be more rapidly turned over than uH2A or uH2A.Z.

The lowering of the uH2B concentration was most evident when the breast cancer cells were treated with inhibitors of RNA polymerase II mediated transcription (e.g., DRB, a specific inhibitor of RNA polymerase II activity, or high concentrations of actinomycin D). Incubation of the cells with low concentrations of actinomycin D, which inhibits the synthesis of rRNA, slightly lowered the level of uH2B. These results suggest that the majority of ubiquitination of H2B is directly coupled to ongoing synthesis of hnRNA.

Heat-shock treatment of cells represses the expression of most genes and induces the expression of a few genes. The repression of some of these genes is at the transcriptional level

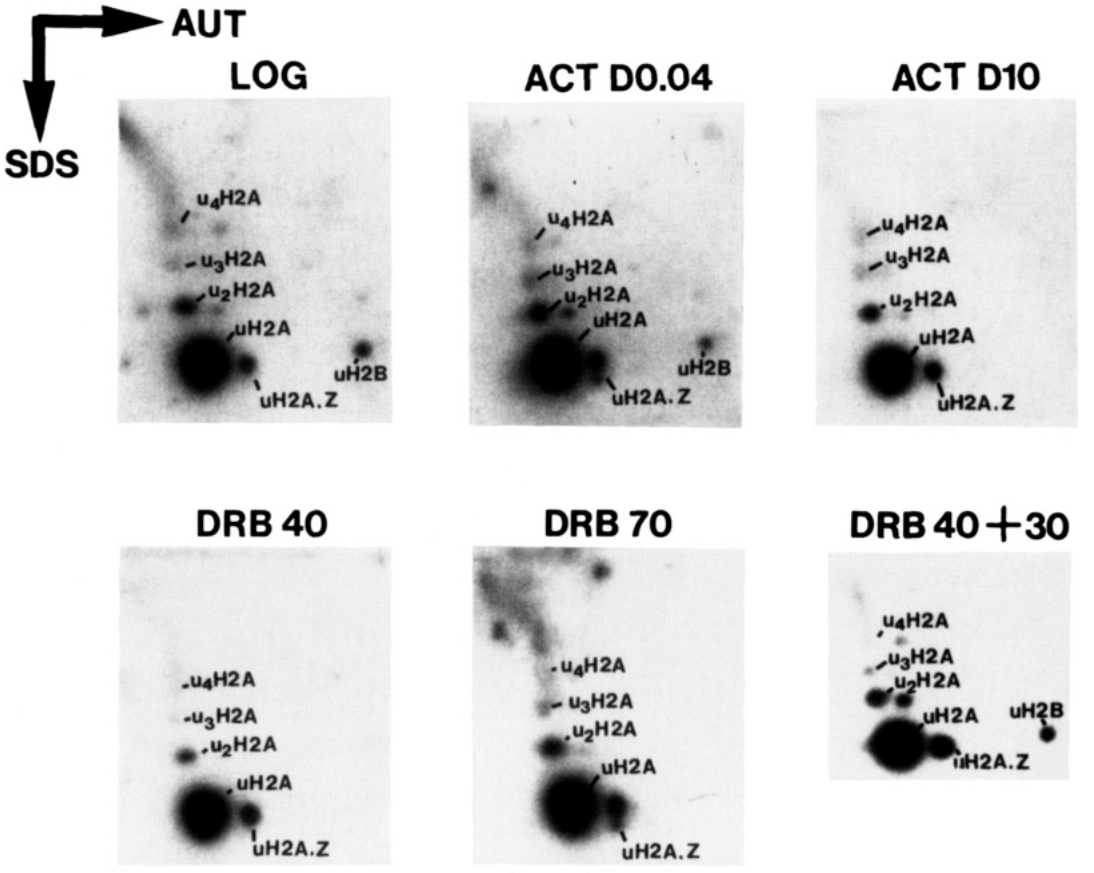

FIGURE 4: Effect of inhibition of transcription on levels of ubiquitin–histone conjugates. Acid-soluble nuclear proteins (36 μg) isolated from untreated cells (LOG) or cells incubated with actinomycin D at 0.04 μg/mL (ACT D0.04) or 10 μg/mL (ACT D10) for 70 min, DRB [40 min (DRB40) or 70 min (DRB70)], or DRB for 40 min and a 30-min recovery (DRB40+30) as described under Materials and Methods were analyzed by two-dimensional gel electrophoresis and immunochemical staining for ubiquitin conjugates as described in Figure 2.

(Voellmy et al., 1983; Gilmour & Lis, 1985). Heat shock also induces deacetylation of the nucleosomal histones, blocks methylation of histones H3 and H4, reduces the levels of uH2A, and induces the methylation of histone H2B (Camato & Tanguay, 1982; Arrigo, 1983; Parag et al., 1987; Bond et al., 1988). In agreement with these studies, we observed that heat shock led to the deacetylation of the histones and the disappearance of uH2A. There was also a reduction in the levels of uH2A.Z, uH2B, and di-uH2A and an increase in the amount of polyubiquitinated histone H2A species. Parag et al. (1987) reported that heat-shocked cells had increased levels of high molecular weight conjugates. This reflects the increased amount of protein degradation (Parag et al., 1987; Chau et al., 1989). Whether the increased level of polyubiquitinated histone H2A indicates that there is an increase in the degradation of H2A remains to be determined.

Butyrate has several effects on nuclear function, including inhibition of DNA synthesis, inhibition of phosphorylation of histones H1 and H2A, reduced methylation of lysine and arginine residues in nuclear proteins, and inhibition of histone deacetylase which results in hyperacetylation of the nucleosomal histones (Boffa et al., 1981; Candido et al., 1978). The chromatin of butyrate-treated cells has an increased sensitivity to DNase I digestion and is less condensed than the chromatin of control cells (Vidali et al., 1978; Birren et al., 1987; Tralka et al., 1979; Annunziato et al., 1988). However, there is no effect of butyrate on histone ADP-ribosylation (Boffa et al., 1981). Similarly, we observed that butyrate did not affect histone ubiquitination or polyubiquitination, suggesting that decondensation of the chromatin fiber does not increase the level of histone ubiquitination. Other inhibitors of DNA synthesis, e.g., aphidicolin, did not alter the levels of the ubiquitinated histones. Thus, in agreement with the results of Wu et al. (1981), we find that histone ubiquitination is not linked to DNA synthesis.

In previous studies, we have shown that uH2B is highly enriched in chromatin fractions enriched in transcriptionally active genes (Ridsdale & Davie, 1987; Davie & Nickel, 1987; Nickel et al., 1989; Delcuve & Davie, 1989). Enrichments were also observed for uH2A and poly-uH2A, but the degree of enrichment of these ubiquitinated histones was never as great as that for uH2B. Several other modified histone species are preferentially located in the active gene chromatin regions, including hyperacetylated and methylated histone species (Alonso et al., 1987; Hebbes et al., 1988; Hendzel & Davie, 1989). However, neither acetylation nor methylation of the histones is coupled to ongoing transcription (Ruiz-Carrillo et al., 1976; Arrigo, 1983; Hendzel & Davie, 1989). To our knowledge, the ubiquitination of histone H2B is the only histone modification that is linked to ongoing transcription.

We proposed that the preferential location of uH2B in active gene chromatin is a result of an alteration or disruption of nucleosome structure during transcription such that the COOH terminus of histone H2B becomes accessible to the enzymes catalyzing the addition of ubiquitin. It should be noted that the COOH-terminal sequence of H2B, but not H2A, is buried in the nucleosome [see Nickel et al. (1989) and references cited therein]. Thus, inhibition of transcription should prevent the ubiquitination of histone H2B but not H2A. Consistent with this hypothesis is the observation that the level of uH2B in chromatin is dependent upon ongoing transcription. Once the nucleosome is initially opened by transcription and histone H2B ubiquitinated, uH2B may impede nucleosome refolding. Thus, unless deubiquitination of uH2B were ex-

tremely rapid, nucleosomes opened for transcription would be kept in an open state until after transcription subsided.

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