
N-Butyrate incubation of immature chicken erythrocytes preferentially enhances the solubility of β^A chromatin

Catherine R.Ferez and Daniel A.Nelson

Department of Biochemical and Biophysical Sciences, University of Houston, University Park, Houston, TX 77004, USA

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ABSTRACT

The solubility of adult β -globin chromatin (β^A chromatin) from immature chicken red blood cells can be controlled by the presence or absence of n-butyrate in a cell incubation medium. In the absence of n-butyrate, only a small percentage (~4%) of the total β^A chromatin is in a soluble chromatin fraction following micrococcal nuclease digestion and centrifugation. This percentage increases to approximately 40-45% of the β^A chromatin if cells are incubated 1 hour in the presence of 10 mM sodium n-butyrate. The highest yield and enrichment of solubilized β^A chromatin is attained when 1-4% of the DNA is rendered acid soluble, and in buffers containing 1.5 - 5 mM $MgCl_2$. The soluble β^A nucleohistone is nucleosome oligomer size (contains DNA 250-600 bases in length) and can be separated from soluble, transcriptionally inert mononucleosomes by agarose A-5m exclusion chromatography.

The enhanced solubility appears to be specific for transcriptionally active chromatin. Whereas 40-45% of the β^A chromatin is recovered in the supernatant fraction from n-butyrate incubated immature erythrocytes, nucleohistone containing ovalbumin DNA sequences remains insoluble.

INTRODUCTION

Structural changes in chromatin are presumed necessary for the expression and replication of higher cell genomes. Nonhistone protein binding (1,2), histone modification (3-8) and the presence of histone variants (9) alter the properties of nucleosomes and chromatin *in vitro* and these compositional changes represent potential mechanisms for the functional modulation of nucleosome and nucleosome higher order structure.

The solubility of nucleohistone may be directly related to specific structural and compositional features of chromatin. Numerous reports suggest that transcriptionally active chromatin is more soluble in a variety of buffers compared to inactive chromatin, allowing fractionation of chromatin into active and inactive components (10-20). The increased solubility of transcriptionally active chromatin suggests a decrease in nucleosome-nucleosome interactions, reflecting a more open conformation. However, a portion of transcriptionally active nucleohistone is also highly

insoluble (21-23) and is likely to be tightly associated with transcriptional apparatus and the nuclear matrix.

We propose that in higher eukaryotic cells, a loosening of chromatin structure in regions of transcriptional activity is achieved by transient and rapid acetylation and deacetylation of the histones. It is established that n-butyrate incubation of cells enhances the extent of histone acetylation by the inhibition of histone deacetylases (24-28), increasing the solubility of chromatin (6-8). As a result, we predict a dramatic enhancement of the solubility of transcriptionally active chromatin when histone deacetylation is inhibited by a short incubation of cells with n-butyrate. In this study, we monitored the solubility characteristics of β^A chromatin from immature chicken red blood cells. Cloned cDNA probes are available for chicken adult globin gene sequences (29), and the adult globin genes are transcriptionally active in this cell type (30). DNA replication, an event which may also require histone acetylation and deacetylation (31,32), does not occur in the terminally differentiated erythrocyte (33), reducing the background of acetylation and deacetylation and simplifying the investigation of the relationship between gene expression and chromatin solubility. Under optimal conditions, soluble chromatin becomes 11 fold enriched for β^A DNA sequences as a result of a short n-butyrate incubation of the immature erythrocytes.

MATERIALS AND METHODS

Treatment of chickens and isolation of immature red blood cells: White leghorn chickens (retired layers) were made anemic by fasting for 48 hours, followed by daily injections of 1.0 ml of 1% phenylhydrazine (1% phenylhydrazine hydrochloride in 10 mM phosphate buffer; the solution adjusted to pH 7.2). After seven days, maturity of the red blood cells was assayed by microscopic examination of brilliant cresyl blue stained cells (1% brilliant cresyl blue in 0.85% saline, filtered and used 1/1 (v/v)). Ribosome aggregates are observed in the stained immature erythrocytes; clear cytoplasm in the mature cells (34). Birds were sacrificed when at least 98% of the erythrocytes were immature. Blood was collected by heart puncture using heparinized syringes (approximately 50 units of heparin (Sigma) per ml of whole blood). Whole blood was centrifuged at 1500 rpm for 10 minutes and the plasma removed from the pelleted cells. Cells were resuspended and washed 2x in Swim's S-77 medium (pH 7.2). Following the first or second centrifugation, the white blood cell layer above the pelleted erythrocytes was removed.

Treatment of immature chicken red blood cells: Routinely, cells pooled

from two chickens were incubated at 37°C for 60 minutes in 250 mls of Swim's S-77 medium (pH 7.2) in the presence or absence of 10 mM sodium n-butyrate. For some experiments, nuclei were prepared directly after removal of the white blood cells from the immature erythrocytes. No differences were observed between cells which were processed immediately or incubated for 1 hour in Swim's medium without n-butyrate.

Isolation and micrococcal nuclease digestion of nuclei: Nuclei were isolated from the untreated control and n-butyrate incubated cells as described previously (6). As required, oviduct nuclei were isolated by standard methods (21). Nuclear digestions were with micrococcal nuclease at 37°C using 1 or 2 units of enzyme per A_{260} unit of chromatin (50 μ g DNA). Micrococcal nuclease is the enzyme of choice since it cleaves between nucleosomes and nucleosome oligomers without preference for regions of the genome containing highly acetylated histones (5,7,32). Standard digestions consisted of 2-4 mls of nuclei at a concentration of 70 A_{260} units of chromatin per ml. The nuclear digestion buffer (NDB) contains 0.25 M sucrose, 60 mM KCl, 15 mM NaCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Na n-butyrate, 15 mM MES (2-N-morpholinosulfonic acid), 0.1 mM PMSF (phenylmethylsulfonyl fluoride), pH 6.6 (NaOH). For some nuclease digestions, nuclear concentrations and magnesium concentrations were varied. Reactions were terminated by the addition of 1/50 volume of 0.1 M EGTA (pH 7.4). Digestion kinetics were monitored by the release of acid soluble oligonucleotides (35) and release of nucleohistone into the soluble chromatin fraction.

Fractionation of the chromosomal material into soluble and insoluble components: Following nuclease digestion, the samples were centrifuged at 9,000 x g and the soluble material designated S (supernatant) and pelleted nucleohistone designated P (pellet). Buffers used during the nuclear isolation, digestion and fractionation included 10 mM sodium n-butyrate to prevent in vitro histone deacetylation (36).

Agarose A-5m exclusion chromatography of the soluble chromatin fraction: Soluble nucleohistone from n-butyrate incubated cells was fractionated at 0-4°C on a 2.5 cm dia. x 45 cm agarose A-5m column (BioRad A-5m). The column was equilibrated with 10 mM MES, 10 mM Na n-butyrate, 2 mM EDTA, 0.1 mM PMSF, pH 6.6 (Tris). Supernatant nucleohistone was dialyzed into the same buffer prior to loading on the column.

Isolation, electrophoresis, transfer and hybridization of DNA: Samples (2-6 ml volume) for DNA electrophoresis were adjusted to 20 mM EDTA, 0.1 M Tris (pH 8.5), 1% SDS and digested for 3 hours at 60°C with 50 μ g/ml

Proteinase K (Sigma, Type XI). Nucleic acid was extracted with equal volumes of saturated phenol (0.2 M Tris, 20 mM EDTA, pH 8) and chloroform/isoamyl alcohol (24/1, v/v), precipitated with two volumes of ethanol, dried and resuspended in 2 mls of 0.3 N NaOH. Alkali treatment to remove RNA was at 37°C for 18 hours. Samples were neutralized, precipitated with ethanol and resuspended by boiling for ten seconds in electrophoresis tray buffer containing 20% glycerol. The concentration of each sample was determined by measuring the absorbance at 260 nm.

DNA was electrophoresed on 1% agarose gels (Sigma, Type V) containing 20 mM Tris, 5 mM NaOAc, 1 mM EDTA, pH 7.8 (HOAc). 30 µg of chicken DNA was loaded on each lane except as otherwise noted in the figure legends. Gels were stained with ethidium bromide (1 mg/liter), photographed under ultra-violet light and the DNA transferred to nitrocellulose paper according to Southern (37). Transfer to nitrocellulose was for 18 hours in 20X SSC (SSC = standard saline citrate). Filters were vacuum dried at 80°C for 2.5 hours.

Prehybridization and hybridization of the nitrocellulose filters were at 68°C according to Stalder *et al.* (38). pHB1001 (W. Salzer, Molecular Biology, UCLA) containing a cDNA clone of the chicken adult β -globin gene and pOV230 (M. Tsai, Cell Biology, Baylor College of Medicine) containing an ovalbumin cDNA sequence were nick translated to $1-4 \times 10^8$ cpm/µg by standard methods (39) using α -³²P labeled dCTP (New England Nuclear, 800 Ci/mmol). Following hybridization, filters were washed at 65°C over a period of 16-20 hours as follows: two washes with 2x SSC, 0.5% SDS, two washes with 0.1 x SSC, 0.5% SDS, and two washes with 0.1 x SSC, 0.1% SDS. Autoradiography was at -80°C for 3-10 days using Kodak XAR-5 x-ray film and Dupont Lightning Plus intensifying screens.

DNA dot blotting: Dot blotting was as described by Kafatos *et al.* (40). DNA samples were denatured with 0.3 N NaOH, diluted with an equal volume of 2 M ammonium acetate and 0.5 to 5 µg of DNA dotted onto nitrocellulose filters. The filters were washed as described by Kafatos *et al.* (40), vacuum dried at 80°C for 2 hours and prehybridized and hybridized as described by Stalder *et al.* (38). After hybridization, filters were washed and autoradiograms prepared as described above. Differences in the β^A content/µg DNA of the various samples were determined by analysis of variance (one way) and Tukey's test (41).

RESULTS

Nuclease Digestion Kinetics

Figure 1 presents an overview of the time course of the release of

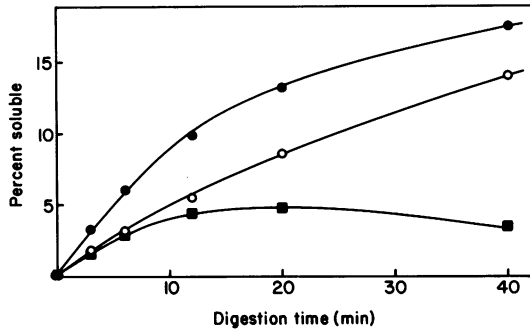


Figure 1: Kinetics of digestion of nuclei from immature chicken erythrocytes. Nuclei were digested with micrococcal nuclease (2 units/50 μ g DNA) at 37°C and the time course of digestion monitored by the release of chromatin into the supernatant and by the percentage of DNA rendered acid soluble. (●) Total percentage of A₂₆₀ nm absorbing material solubilized. (○) Percentage of DNA rendered acid soluble. (■) Approximate percentage of chromatin solubilized.

soluble chromatin and acid soluble oligonucleotides during incubation of the immature erythrocyte nuclei with micrococcal nuclease. The line drawn through the closed squares in figure 1 was obtained by subtracting the percentage of DNA rendered acid soluble from the total material absorbing at 260 nm in the soluble fraction. This line gives a reasonable estimate of the amount of soluble chromatin as assayed by independent measurements of the percentage of DNA in the soluble and insoluble chromatin fractions. Only a maximum of 4% of the total chromatin becomes soluble using our digestion and fractionation procedure. We were unable to detect differences in the percentage of material released from the nuclei of untreated cells, cells incubated in the presence of n-butyrate, or in the absence of n-butyrate.

To obtain the results in figure 1, 2 units of micrococcal nuclease per 50 μ g of DNA were used to illustrate that the amount of soluble chromatin plateaus during the digestion. We observed a rapid release and subsequent degradation of the β^A chromatin during this time course and accordingly, the enzyme concentration was decreased to 1 unit of micrococcal nuclease per 50 μ g of DNA for subsequent experiments. At the reduced enzyme to substrate ratio, about 20 minutes of digestion is required (4% of the DNA rendered acid soluble) to solubilize 4% of the total chromatin.

Kinetics of β^A Chromatin Solubilization

The relationship between the extent of micrococcal nuclease digestion and the solubility of β^A chromatin was monitored for untreated and 1 hour n-butyrate incubated cells. Nuclei were digested from 1 to 40 minutes with

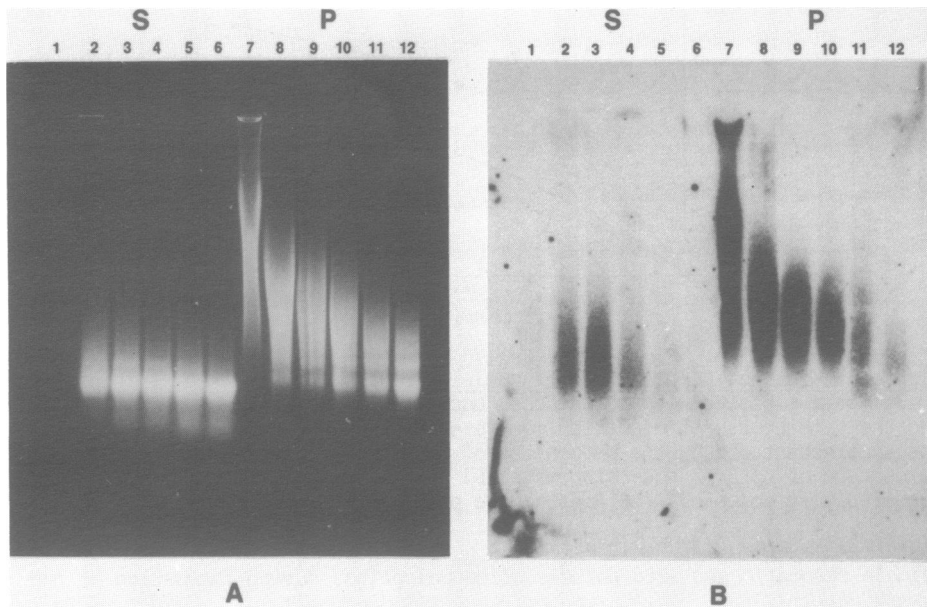


Figure 2: Solubility of β^A chromatin during a time course of micrococcal nuclease digestion of nuclei from untreated immature chicken erythrocytes. Red blood cells and nuclei were isolated as described in the Experimental Procedures and nuclei incubated with micrococcal nuclease at 1 unit/50 μ g DNA for 1, 3, 5, 10, 20 and 40 minutes (lanes 1 and 7; 2 and 8; 3 and 9; 4 and 10; 5 and 11; 6 and 12, respectively). Nuclei were fractionated into supernatant (S) and pellet (P), DNA isolated, alkali treated and electrophoresed on a 1% agarose gel (panel A). The DNA was transferred to nitrocellulose, hybridized to pHb1001, and an autoradiogram prepared (panel B). Note that lane 1 contains only a trace amount of DNA.

micrococcal nuclease (0 to 8% of the DNA rendered acid soluble), the chromatin fractionated by centrifugation and DNA isolated, electrophoresed and hybridized to pHb1001 as described in the Material and Methods. The results of these experiments are illustrated in figures 2 and 3.

As shown in figure 2, we do not observe enrichment of β^A chromatin in the supernatant fraction from untreated immature red blood cells. The observed ratio of β^A chromatin in the pellet to that in the supernatant is equal to or greater than one for all digestion times. The 5 minute digestion time (1-1.5% of the DNA rendered acid soluble) releases the largest amount of observable β^A chromatin (figure 2, panel B, lane 3) and it is evident that the length of digestion is critical to observing β^A chromatin in the soluble fraction. Adult β -globin chromatin is not detected in the supernatant after 20 minutes of digestion of the untreated cells (3-4% of the DNA rendered acid soluble).

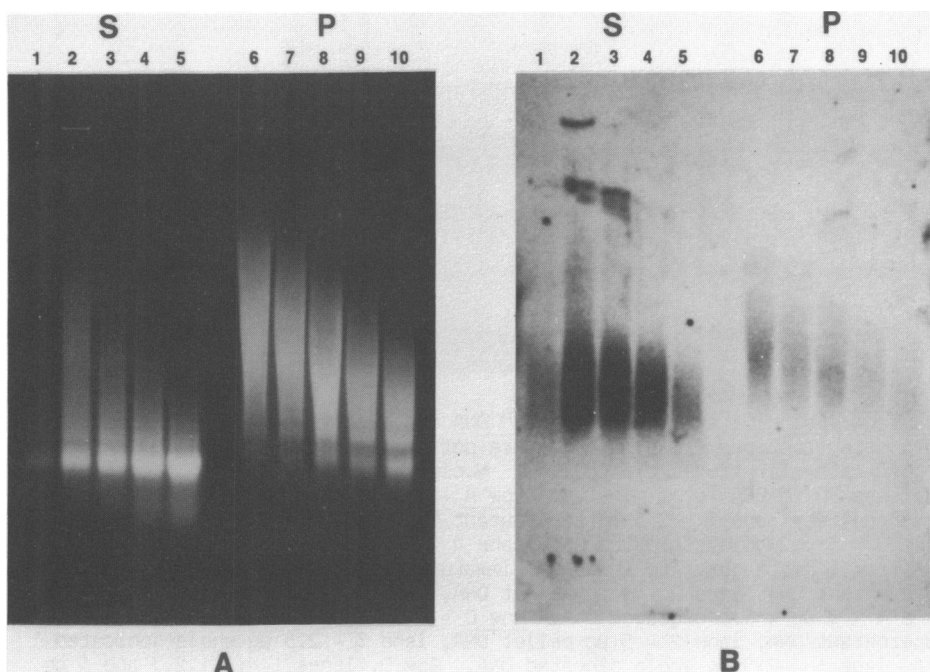


Figure 3: Solubility of β^A chromatin during a time course of micrococcal nuclease digestion of nuclei from immature red blood cells incubated 1 hour with 10 mM n-butyrate. Nuclei were incubated with micrococcal nuclease at 1 unit/50 μ g DNA for 3, 5, 10, 20, and 40 minutes (Lanes 1 and 6; 2 and 7; 3 and 8; 4 and 9; 5 and 10, respectively). Chromatin was fractionated into supernatant (S) and pellet (P), DNA isolated, alkali treated and the samples electrophoresed on a 1% agarose gel (panel A). DNA was transferred to nitrocellulose and hybridized to pHB1001. The autoradiogram is shown in panel B. Note that lane 1 contains 3 rather than 30 μ g of DNA.

The β^A chromatin solubility observed in figure 2, panel B, for the untreated cells is in striking contrast to that observed for the same cells incubated 1 hour in Swim's medium plus 10 mM n-butyrate (figure 3, panel B). After 5-20 minute digestion times (1-4% of the DNA acid soluble), the amount of observed β^A chromatin in the supernatant is markedly increased over that in the pellet fractions. An analysis of densitometer scans of the supernatant and pellet lanes of the 5 and 10 minute digestion times in figure 3, panel B, indicate that the soluble chromatin is 10-12 fold enriched for β^A sequences. Note that because of the 10-12 fold enrichment, the autoradiogram in figure 3, panel B was obtained after a shorter exposure time than the autoradiogram in figure 2, panel B. Thus the intensities seen in the autora-

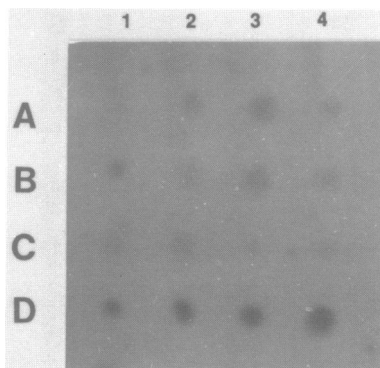


Figure 4: Dot blot analysis of the β^A DNA content of soluble and insoluble chromatin fractions. DNA samples were dot blotted onto nitrocellulose and probed with nick-translated pHB1001. Nuclei were digested with 1 unit nuclease/50 μ g DNA for 5 minutes. Row A - DNA from untreated immature erythrocytes: lane 1 - 2.5 μ g supernatant DNA, lane 2 - 5 μ g pellet DNA, lane 3 - 5 μ g whole sonicated DNA, lane 4 - 5 μ g whole digested DNA. Row B - DNA from 1 hr. n-butyrate incubated immature erythrocytes: lane 1 - 0.5 μ g supernatant DNA, lane 2 - 5 μ g pellet DNA, lane 3 - 5 μ g whole sonicated DNA, lane 4 - 5 μ g whole digested DNA. Row C - oviduct DNA: lane 1 - 2.5 μ g supernatant DNA, lane 2 - 5 μ g pellet DNA, lane 3 - 2.5 μ g whole sonicated DNA, lane 4 - 2.5 μ g whole digested DNA. Row D, lanes 1-4: 1,2,3 and 5 μ g of pHB1001 DNA.

diagram of figure 3 cannot be directly compared to the intensities in figure 2. Autoradiogram exposures were chosen to accurately depict the percentage of β^A chromatin in the supernatant relative to pellet chromatin fraction for each cell treatment.

After 40 minutes of digestion, a reduction in the amount of soluble β^A nucleohistone from the n-butyrate incubated cells is observed (figure 3, panel B, lane 5). This loss of β^A chromatin in the soluble fraction during digestion of nuclei from both untreated and n-butyrate incubated cells is the result of continued endo- and exonucleolytic cleavage (42) of the chromatin DNA.

Prior to the addition of micrococcal nuclease to the nuclear digestion buffer, nuclei are equilibrated at 37°C for 10 minutes. The endogenous nuclease activity present (43,44) does not solubilize an appreciable amount of chromatin or adult β -globin chromatin. This is evident from the one minute digest for untreated cells (figure 2, panel B, lane 1) and the 3 minute digest of 1 hour n-butyrate incubated cells (figure 3, panel B, lane 1). In separate experiments, incubation of nuclei from both untreated and n-butyrate incubated cells for 40 minutes at 37°C did not result in measurable

release of adult β -globin nucleohistone (data not shown).

Percentage of Soluble β^A Chromatin

The enrichment for β^A chromatin in the supernatant from n-butyrate incubated cells was determined by dot blotting (40) as depicted in figure 4. Varying amounts of DNA from both chicken erythrocyte and oviduct fractions, and from pH1001 were applied in triplicate onto separate filters, the samples hybridized to pH1001 and autoradiograms prepared. Increasing amounts of pH1001 DNA on the filter resulted in a relatively uniform increase in intensity on the autoradiogram (figure 4, row D). When 0.5 μ g of supernatant DNA from the n-butyrate incubated erythrocytes (row B, column 1) and 5 μ g of pellet DNA (row B, column 2) are applied, similar intensities are observed on the autoradiogram. This indicates an approximate 10 fold enrichment for β^A sequences in the soluble fraction as a result of the n-butyrate incubation, and this enrichment is not observed when 2.5 μ g of soluble DNA from untreated red blood cells is applied to the filters (row A, column 1).

Each dot from the series of autoradiograms was scanned with a densitometer to obtain the relative content of β^A DNA as expressed in arbitrary integration units. These units were divided by the amount of DNA per dot to yield the relative amount of β^A DNA per μ g of total DNA. Sonicated whole DNA samples were dotted on each filter for normalization and a standard deviation determined for each set of samples. From 16 measurements using whole sonicated DNA from oviduct, immature erythrocytes and mature erythrocytes, a relative value of 11 ± 5 was arrived at for whole sonicated DNA, with a range of values of 5 to 19. This is in contrast to a value of 125 ± 27 for the soluble DNA from the n-butyrate incubated cells, values ranging from 100 to 153. The difference in β^A DNA content is highly significant ($P < 0.001$) and we conclude that the soluble DNA isolated from the n-butyrate incubated cells is approximately 11 fold enriched for β^A sequences. This level of enrichment is retained even after 20 minutes of digestion, when the optimal amount (~4%) of the total DNA is in the soluble fraction (see Figure 3, panel B, lanes 4 and 9). We estimate that 40-45% of the total β^A chromatin can be solubilized by the n-butyrate incubation protocol.

The relative amount of β^A DNA per μ g of total DNA in the supernatant from untreated cells (7 ± 3) is not significantly different from the value obtained for whole sonicated DNA (11 ± 5). We therefore do not observe enrichment for β^A sequences in the soluble fraction from untreated erythrocytes. In addition, we do not detect any β^A enrichment or depletion in whole digested DNA, pellet fraction DNA from all cell types, or in the supernatant

DNA from oviduct. However, it is important to note that the pellet DNA from n-butyrate incubated immature erythrocytes should be approximately two fold depleted in β^A sequences compared to whole sonicated DNA samples. It is evident that our dot blot assay cannot detect 2 fold enrichment or depletion in these DNA sequences.

In separate experiments, the dot blotting method was assayed for the retention of nucleosome monomer size (160 nucleotide length) DNA, as has been described previously by Thomas (45). Nick-translated monomer size single stranded DNA was dot blotted onto nitrocellulose by the method of Kafatos *et al.* (40) and the flow through monitored with a second sheet of nitrocellulose paper. The radiolabel is quantitatively retained on the first filter. From this experiment we conclude that although some small DNA passes through the nitrocellulose filter during Southern transfers (46), this does not account for the lack of enrichment for β^A DNA in the soluble chromatin from untreated immature erythrocytes. The quantitative retention of small DNAs on the filter in the dot blot analysis augments the conclusion that the supernatant from untreated cells is not particularly enriched for β^A sequences.

Dependence of the Solubility Fractionation on N-butyrate, DNA and $MgCl_2$ Concentration

N-butyrate concentration: 10 mM sodium n-butyrate was chosen for the cell incubations since it completely inhibits histone deacetylation at this concentration (Ferez and Nelson, manuscript in preparation). The 1 hour n-butyrate incubation time allows the histones of the rapidly acetylated and deacetylated form to become preferentially hyperacetylated (6,48).

Nuclear concentrations: Nuclei, at concentrations of 30 to 130 A_{260} units per ml (1.5-6.5 mg/ml DNA), were digested at 1-2 units of micrococcal nuclease per 50 μg of DNA. The fractionation can be employed successfully with a variety of nuclease/DNA ratios and DNA concentrations. We observe a loss in the percentage of solubilized nucleohistone at elevated nuclear concentrations, probably as a result of the substantial volume of the pellet. Thus although enrichment for β^A chromatin remains unchanged, yields in the supernatant are reduced with increased nuclear concentration. We also find that at the higher nuclease to DNA ratio (2 units enzyme/50 μg DNA), adult β -globin sequences disappear more rapidly from the soluble fraction than would be predicted by the results at 1 unit/50 μg of DNA. We attribute this to an increased rate of exonucleolytic degradation of the solubilized chromatin at the elevated enzyme to substrate ratio.

Magnesium chloride concentration: Figure 5 illustrates that the $MgCl_2$

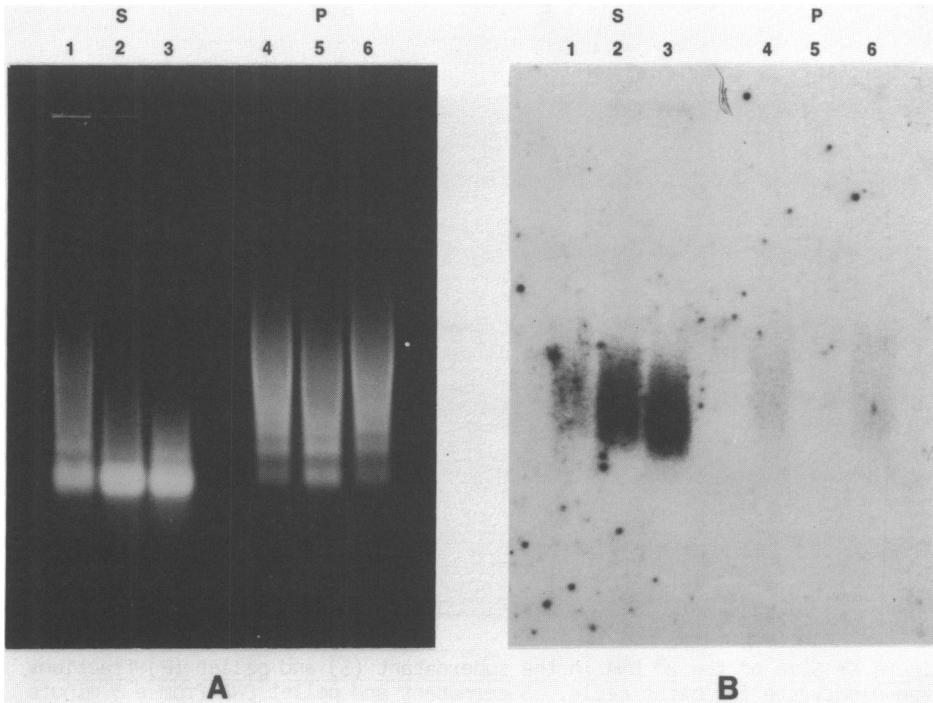


Figure 5: β^A DNA content in the soluble and insoluble chromatin fractions isolated in the presence and absence of $MgCl_2$. Nuclei from n-butyrate incubated immature red blood cells were digested with micrococcal nuclease for 5 minutes at 1 unit/50 μg DNA, and fractionated in digestion buffer containing zero (lanes 1 and 4) or 5 mM $MgCl_2$ (lanes 3 and 6). The supernatant chromatin isolated in the absence of $MgCl_2$ was made 5 mM in $MgCl_2$ to yield a second supernatant and pellet fraction (lanes 2 and 5, respectively). Supernatant (S) and pellet (P) DNA was isolated, alkaline treated, electrophoresed on a 1% agarose gel and stained with ethidium bromide (panel A). After transfer to nitrocellulose, the filter was hybridized to pH β 1001 (panel B; autoradiogram).

concentration in the digestion and fractionation buffers is critical to observing the enhanced solubility of the β^A chromatin. This is first shown by isolating, digesting and fractionating nuclei from n-butyrate incubated cells in the absence of $MgCl_2$. In the absence of $MgCl_2$, approximately 18-23% of the total chromatin is in the supernatant fraction. Most of the DNA in the supernatant is of nucleosome oligomer size and is not enriched in adult β -globin sequences (figure 5, panels A and B, lane 1). In contrast, digestion and fractionation in the presence of 5 mM $MgCl_2$ reduces the percentage of total DNA in the supernatant to 2.5%, and the enrichment for β^A DNA

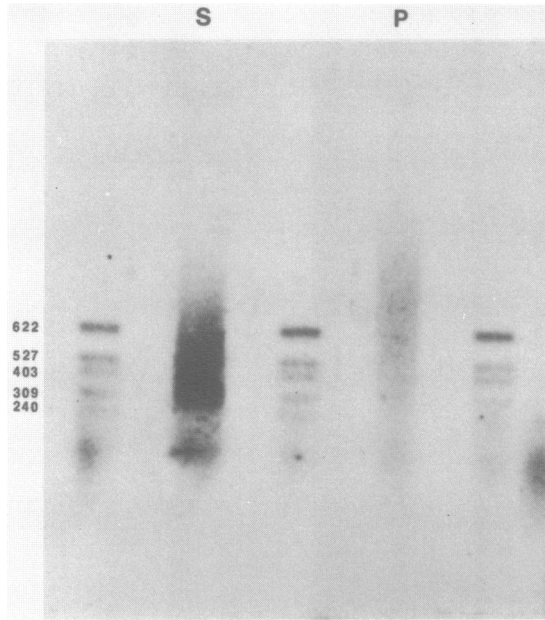


Figure 6: Size of the β^A DNA in the supernatant (S) and pellet (P) fractions from n-butyrate incubated cells. Supernatant and pellet DNA from a 5 minute micrococcal nuclease digest at 1 unit/50 μ g of DNA were electrophoresed on a 1.5% agarose gel with 20 pg of Hpa II digested pBR322 (left, center and right hand lanes). All DNA samples were denatured prior to electrophoresis. DNA was transferred to nitrocellulose and hybridized to co-nick-translated pHb1001 and pBR322. Only the autoradiogram is shown.

sequences is evident (figure 5, panel B, lane 3). The concentration of $MgCl_2$ in the digestion buffer has virtually no effect on the kinetics of micrococcal nuclease digestion (figure 5, panel A, lanes 4 and 6). Thus the addition of 5 mM $MgCl_2$ to the soluble chromatin isolated in the absence of $MgCl_2$, followed by re-centrifugation, yields a new supernatant (figure 5, lane 2) approximately ten fold enriched for adult β -globin DNA (compare lanes 1, 2 and 3 in panel B, figure 5).

The dependence of the chromatin fractionation on $MgCl_2$ concentration is explained as follows: The β^A chromatin is soluble at 0-5 mM $MgCl_2$, but due to the solubilization of other chromatin components, enrichment is reduced in the absence of $MgCl_2$. In separate experiments, elevated (7-10 mM) $MgCl_2$ concentrations result in a partial loss of soluble adult β -globin chromatin (data not shown). The critical range for the high yield and enrichment of the β^A chromatin in the soluble fraction is 1.5-5 mM $MgCl_2$.

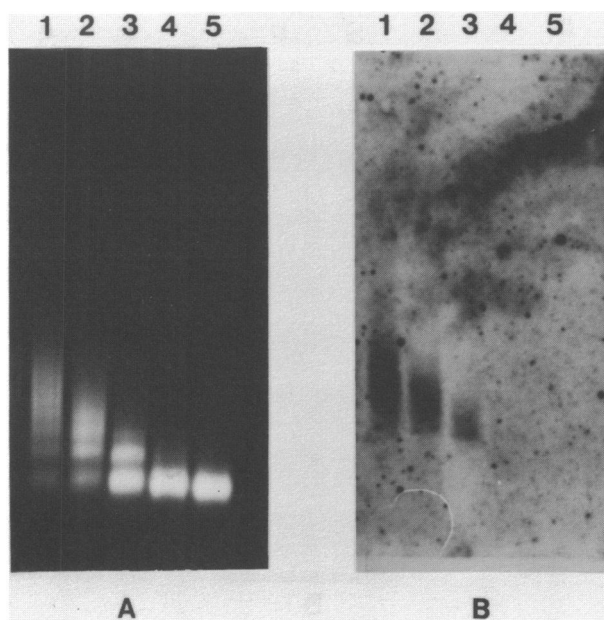


Figure 7. Localization of adult β -globin chromatin in nucleosome oligomer column fractions. Nuclei were isolated from 1 hour n-butyrate incubated immature chicken erythrocytes, digested with micrococcal nuclease and fractionated into supernatant and pellet. Approximately $10 A_{260\text{ nm}}$ units of supernatant chromatin were dialyzed against column buffer and fractionated by agarose A-5m exclusion chromatography. 3 ml fractions were collected, and pooled samples designated 1-5. DNA was prepared from the pooled samples, treated with alkali and 5 $\mu\text{g/lane}$ electrophoresed on a 1% agarose gel (panel A). The DNA was transferred to nitrocellulose and hybridized to pH β 1001 (autoradiogram, panel B).

Size of the β^A DNA and β^A chromatin in the Supernatant

Although the DNA in the supernatant is predominantly nucleosome monomer size as has been demonstrated previously (6,12,47), the β^A DNA is somewhat larger. This is a reproducible characteristic of the adult β -globin DNA in the soluble chromatin fraction from n-butyrate incubated cells. The size of the β^A DNA in the supernatant and pellet fractions was determined by the inclusion of DNA standards during electrophoresis on a 1.5% agarose gel. The autoradiogram in figure 6 demonstrates that the adult β -globin single-stranded DNA sizes in the supernatant fraction are approximately 250 to 600 nucleotides in length. The nucleosome oligomers containing this 250-600 base length DNA can be isolated by exclusion chromatography. As shown in figure 7, soluble nucleosome oligomers contain the bulk of the β^A sequences. The mononucleosomes eluting from the column do not appear enriched for β^A

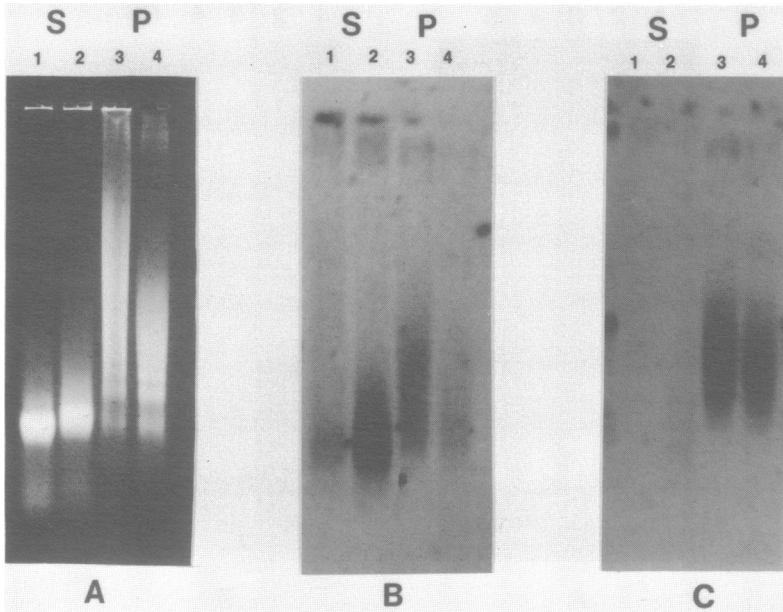


Figure 8. Specificity of the solubilization for β^A chromatin. Supernatant (S) and pellet (P) DNA from a 5 minute micrococcal nuclease digest (1 unit/50 μ g DNA) was isolated after fractionating nuclei from immature chicken red blood cells incubated 1 hour in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 10 mM n-butyrate. The DNA was incubated with alkali, electrophoresed on a 1% agarose gel and stained with ethidium bromide (panel A). Two sets of samples were then electrophoresed in parallel, transferred to nitrocellulose and the filter cut in half for hybridization with pHb1001 (panel B; autoradiogram) or pOV230 (panel C; autoradiogram).

sequences and, for the short digestion times used in these experiments, this is verified by a dot blot analysis (data not shown). Thus exclusion chromatography effects a further purification of the β^A chromatin.

Specificity of the solubilization for transcriptionally active globin genes

In the absence of $MgCl_2$, soluble chromatin fractions containing oligomer size DNA are not enriched for adult β -globin DNA (figure 5, panel B, lane 1). The enrichment for β^A DNA is augmented, however, by obtaining a new supernatant fraction after the addition of $MgCl_2$ (figure 5, panel B, lane 2). We conclude that in the presence of $MgCl_2$, the enrichment for "larger than monomer" size β^A chromatin in the supernatant must be related to some unusual feature of this nucleohistone. The following experiments evaluate the relationship between transcriptional or potential transcriptional activity of chromatin within the chicken immature red blood cell genome, and the solubi-

lity of this nucleohistone after the n-butyrate incubation. The results favor the notion that enhanced chromatin solubility and transcriptional and/or potential gene activity are related.

First, hybridization of filters with the probe pHB1003 (29) containing the chicken adult α -globin cDNA sequence yields the same results as shown in the figures for the adult β -globin DNA (data not shown). Secondly, autoradiograms from hybridizations using pOV230, a chicken ovalbumin cDNA containing plasmid (49), are quite distinct from those obtained with the transcriptionally active adult α and β -globin gene probes. Identical sets of DNA samples (figure 8) were electrophoresed on a 1% agarose gel, transferred to nitrocellulose, and the filter cut in half for hybridization with pHB1001 (figure 8, panel B) or pOV230 (figure 8, panel C). The patterns on the autoradiograms clearly demonstrate that the n-butyrate treatment results in a dramatically enhanced solubility of β^A chromatin (panel B, lane 2), but has no effect on the solubility of chromatin containing ovalbumin DNA sequences (panel C, lane 2).

DISCUSSION

Induced anemia in adult chickens results in a population of red blood cells active in adult globin RNA synthesis (30). Since immature red blood cells are no longer engaged in replication (33), this is a suitable cell type for relating changes in chromatin structure to adult β -globin DNA transcriptional activity. We demonstrate that a short n-butyrate incubation of immature erythrocytes results in an 11 fold increased solubility of the transcriptionally active adult β -globin nucleohistone and that 40-45% of the β^A chromatin is now in a soluble form. The enrichment for soluble adult β -globin chromatin is observed after a 5-20 minute digestion with micrococcal nuclease (1 unit/50 μ g of DNA; 1-4% of the DNA rendered acid soluble) in fractionation buffers containing 1.5-5 mM $MgCl_2$. The nuclear digestion buffer used for the fractionation contains 60 mM KCl and 15 mM NaCl, thus the presence of monovalent cations alone does not ensure enrichment for β^A chromatin in the supernatant. In the absence of $MgCl_2$, the supernatant from n-butyrate treated cells is not highly enriched for adult β -globin chromatin. Although the fractionation is dependent on the presence of magnesium, we have not explored the relationship between adult β -globin chromatin solubility and the concentrations of other di- or multivalent cations.

Although numerous reports demonstrate an enhanced solubility of transcriptionally active chromatin in the absence of any cell treatment

(10-20), we were unable to observe this enhanced solubility. However, other reports indicate a tight complexing between active chromatin and the nuclear matrix (21-23). Hentzen, Rho and Bekhor (23) report a 24 fold enrichment for β^A sequences in a matrix DNA preparation from mature chicken erythrocytes. Approximately 1-2% of the total nuclear DNA is recovered in the matrix chromatin fraction and we calculate that 25-50% of the β^A chromatin must be in an insoluble form. Robinson et al. (22) report a selective increase in the amount of matrix bound ovalbumin sequences in the oviduct of estrogen stimulated chicks (ie, when ovalbumin is transcriptionally active), and it is therefore possible that an enhanced amount of β^A chromatin may be matrix bound or in an insoluble chromatin form in the immature, compared to mature chicken erythrocyte. Our digestion and fractionation conditions may allow a portion of the transcriptionally active chromatin to remain insoluble or fixed to the matrix, explaining our inability to observe enrichment for β^A chromatin in the soluble fraction from untreated cells. It may also explain why the n-butyrate incubation solubilizes only 40-45% of the total β^A chromatin.

The DNA in the soluble chromatin fraction from both untreated and n-butyrate incubated immature red blood cells is predominantly nucleosome monomer size, in agreement with other solubility studies (6,12,47). Nucleosome cores are more soluble than nucleosomes or nucleosome oligomers containing H1 (8), and the soluble nucleosome monomers described in this report lack H1 and H5 (data not shown). Our dot blot analysis demonstrates that the core size DNA isolated from the soluble fraction from untreated cells is not enriched for β^A sequences. As shown in figure 7, the soluble chromatin component from n-butyrate incubated cells can be fractionated by exclusion chromatography into oligomer and monomer components, and an analysis of this DNA also indicates that the soluble monomers are not enriched for β^A sequences. Since the solubility of nucleosome monomers is independent of the presence or absence of n-butyrate in the incubation medium, and since they are not enriched for β^A sequences, we conclude that they are soluble by virtue of their lack of H1 and H5. They are in effect a contamination of the oligomer β^A chromatin sequestered into the supernatant by the n-butyrate incubation.

Histone acetylation and deacetylation may play multiple, universal roles in the functional modulation of chromatin structure. This histone modification occurs in yeast (50,51), protozoan (52), plants (53) and mammals (54,55). In higher eukaryotic cells, histone H4 is acetylated prior to depo-

sition on the DNA (56), and histone acetylation (31) and deacetylation (32) are necessary for replication, and for proper packaging of chromatin structures subsequent to replication. The modification is proposed to be necessary for DNA repair (8) and transcription (57). The 250-600 nucleotide length of the adult β -globin DNA sequences in the supernatant isolated from n-butyrate incubated cells is consistent with the "nucleosome oligomer" sizes expected to be soluble if the n-butyrate incubation results in local histone hyperacetylation (6-8). Although the effects of n-butyrate are known to be pleiotropic (58,59), the latter result reinforces our notion that rapid histone acetylation and deacetylation are preferentially occurring on the histones associated with the transcriptionally active β -globin gene in the immature erythrocyte.

The importance of our finding that the n-butyrate incubation preferentially enhances the solubility of β^A chromatin is that it provides a method for further evaluating the relationship between rapid histone acetylation and deacetylation and gene expression. For example, we can now ask the following questions: Is the solubility of the β^A chromatin rapidly reduced upon removal of the n-butyrate from the incubation medium? Is the enhanced solubility localized to regions of active transcription only, or does it occur over the entire potentially active chromatin domain? Moreover, the soluble nucleosome monomers can be removed from the nucleohistone solubilized by the n-butyrate incubation, resulting in a more highly enriched β^A chromatin preparation and allowing compositional characterization of this material.

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