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Antigens in Chromatin Associated With Proliferating and Nonproliferating Cells

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Xenoantisera were raised to total chromatin from the leukemia cell line K562, or materials released through limited deoxyribonuclease I digestion of nuclei or during the control incubation of nuclei without enzyme. The peroxidase-antiperoxidase method of antibody-antigen detection was employed to visualize individual antigens resolved on one-dimensional polyacrylamide gels following transfer to sheets of nitrocellulose (immunotransfers). Each antiserum contained multiple antigen specificities as evidenced by the diverse patterns of reactive bands displayed on the immunotransfers. The most striking difference in antigens recognized between the antisera was observed in the molecular weight region below 50,000, where two highly reactive bands were seen mainly with antiserum to nuclear materials released by deoxyribonuclease I digestion. The antigens detected with all of the antisera were present in chromatins prepared from proliferating cells, while the levels of antigens present in chromatin from non-proliferating peripheral blood lymphocytes were greatly reduced or not detected. Antigens in chromatin from proliferating cells that migrated with apparent molecular weights of 37,000 and 100,000 were not lost once the activities to antigens in lymphocyte chromatin were absorbed out. These two activities were absorbed from antisera with the same amount of chromatins from proliferating cells. Two antigens migrating at molecular weight 52,000 and 76,000 appeared more active in the chromatin from unstimulated lymphocytes than in chromatin from proliferating cells.

Key words: chromatin, nuclear antigens, proliferation, lymphocytes, leukemia, lectins

The functions of some of the eukaryotic cell chromosomal proteins are being elucidated, most notably those proteins in the greatest numbers (histones) or with properties facilitating isolation (high mobility group chromosomal proteins (HMGs)). The role of histones in chromatin structure is generally recognized. Recent evidence suggests a possible association of HMG 14 and 17 with nucleosomes in transcriptionally active gene regions [1,2]. A number of enzymes active in the nucleus have been isolated and characterized through the use of standard biochemical methodology as was recently done with a DNA topoisomerase [3]. This approach has also been

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successfully employed to reveal the presence of a regulator protein binding a site within the 5S RNA gene [4–7]. Many more of the hundreds of proteins isolated as constituents of chromatin have not been associated with specific function. Because of the importance placed on elucidating gene regulation mechanisms at the transcriptional level, efforts to sort out the roles of proteins associated with the nuclear DNA (isolated as constituents of chromatin) continue.

One approach to this complex area of study is the application of immunochemical methods that are especially suited to facilitate the identification of specific chromatin proteins and the assigning of a possible function. Many immunological activities within the nucleus have been described [reviewed in 8], and progress is being made in the identification, isolation, and characterization of the antigens involved. Some of the activities have been localized to certain nuclear structures (nuclear envelope, chromatin, scaffold, nucleolus, and ribonucleoprotein particles) and the species and tissue or cellular distributions determined. Some nuclear antigens are ubiquitous, while others are specific for cell types, stages of differentiation or functional state. The information on distribution combined with the results of localization studies in the cell and nucleus can suggest functions that usually establishes a priority for continued investigation.

In the present study we have employed antisera containing multiple specificities to survey chromatins from a number of cell types in order to identify components that may be involved in cell proliferation in normal and leukemic blood cells. Activities associated with this process have been identified through the use of protein transfer methods combined with highly sensitive immunochemical detection. Reduced levels of many other chromosomal protein antigens in nondividing normal cells were observed along with the elevation of two activities in nonproliferating normal cells. Two low molecular weight antigens were major reacting species detected with antisera raised to materials released from nuclei by limited deoxyribonuclease (DNase) I digestion.

METHODS

Cells

The leukemic cell lines (HL-60, HL-60 blast, KG-1, KG-1a and K562) and the lymphoblastoid cells (F265) were grown as a suspension in RPMI 1640 supplemented with 10% fetal calf serum. The HL-60 cell line was provided by R.C. Gallo (National Institutes of Health, Bethesda, MD), and the HL-60 blast cell line was provided by P.P. Major (Sidney Farber Cancer Institute, Boston, MA). The KG-1 and KG-1a cell lines were obtained from H.P. Koeffler (University of California, Los Angeles, CA). The K562 and F265 cell lines were provided by J.T. Forbes (Vanderbilt University, Nashville, TN). Peripheral blood cells were obtained from normal donors and provided by the Nashville Regional Red Cross center. The mononuclear cells were obtained after separation on Ficoll-Paque^R (Pharmacia) and diluted to 3×10^6 cells/ml in Joklik-modified minimum essential medium (JM-MEM) with penicillin and streptomycin. Following culture overnight at 37°C in 5% CO_2, 0.2 ml of phytohemagglutinin P (PHA-P) (Difco) was added to each 100-ml culture flask. Optimal conditions for the stimulation of lymphocytes and monitoring the stimulation in subsequent preparations was done using flow cytometry. Unfixed cells were stained with propidium iodide using a one-step procedure [9] and DNA distributions analyzed to determine the relative percentages of G1/G0, S, and G2/M phase cells using the

cytofluorograf[R] 50H and 2150 computer system (Ortho Instruments). The clumps of lymphocytes were harvested by pouring from the flasks leaving the adherent cells. Flasks were gently washed with PBS and cells collected by centrifugation (200g, 5 min). Concanavalin A (Con A) (Miles) was also used to stimulate mitogenesis at 2 μg/ml under the same culture conditions.

A specimen of acute leukemia was obtained by leukapheresis of an untreated patient presenting with hyperleukocytic syndrome. The tumor was morphologically undifferentiated, markedly block periodic acid-Schiff (PAS) positive, minimally Sudan black positive, and had intracytoplasmic IgM suggesting primitive lymphoid lineage.

All procedures followed were in accordance with ethical standards of the Committee on Human Experimentation at the Vanderbilt University School of Medicine.

Isolations

Cells were suspended by vortex mixing in 60 vol (to initial packed cell volume) and collected by low-speed centrifugation (200g, 5 min). These cells were then suspended in 10–30 vol (depending on cell type) of 10 mM Tris-HCl, pH 7.5, 1.0 mM $MgCl_2$. Phenylmethylsulfonyl fluoride (PMSF) was added just before use to all solutions at a concentration of 0.2 mM. Some cells broke immediately, and nearly complete breakage was achieved with additional vortex mixing following a 15–20-min incubation on ice. The cells retained their normal morphology during the washing in sucrose and 5 mM $MgCl_2$. On washing in Tris buffer with 1 mM $MgCl_2$, the viable cells became swollen and eventually broke with the vortex mixing. Eliminating the Mg^{+2} from the Tris buffer facilitated cell breakage, but also allowed the nuclei to break which led to aggregation with cytoplasmic materials. In the presence of 1 mM $MgCl_2$ the cells broke, but the nuclei did not as long as mechanical homogenization was avoided. Once complete cell breakage was achieved in the Tris buffer, the suspended nuclei were diluted 1:1 with 2.2 M sucrose, 5 mM $MgCl_2$. The nuclei were then sedimented through a pad of 2.2 M sucrose, 5 mM $MgCl_2$ (100,000g, 1 hr). The pelleted nuclei were completely separated from cytoplasmic components and unbroken cells in this step.

The purified nuclei were washed with 5 vol of 0.25 M sucrose; 10 mM Tris-HCl, pH 7.5; 0.5% Triton[R] X-100 using a glass homogenizer with a motor driven Teflon pestle. Nuclei were collected by low-speed centrifugation (200g, 5 min) and chromatin prepared as described [10]. The detergent-washed nuclei were suspended in 5 vol (ml of original packed cells) of 80 mM NaCl, 20 mM ethylenediaminetetraacetic acid (EDTA), pH 6.3. A Teflon glass homogenizer with a 0.008-in clearance was mechanically driven to achieve good dispersion of nuclei in the first extraction. Nuclei were collected by centrifugation (3,000g, 10 min) and the pellet resuspended in 2.5 vol of the same buffer. Homogenization was repeated to disperse nuclei. Microscopic examination using phase-contrast optics at this point revealed some intact nuclei and many broken nuclei as evidenced by the presence of long strands of nuclear material. This material was collected by centrifugation as above and the pellet resuspended in 1 vol of the same buffer. Homogenization was continued until the majority of nuclei were broken. This material was collected by centrifugation as above and the pellet was suspended in 5 vol of 1/100 SSC (1.4 mM NaCl, 0.14 mM Na_3 citrate pH 7.0). The suspension was incubated on ice for 10 min and hydrated chromatin sedimented at 30,000g, 20 min. This pellet was dispersed in deionized

water (18 MΩ, specific resistance). The concentration of chromatin was estimated by A_{260} reading or by fluorescence determination of propidium iodide binding nucleic acid after ribonuclease digestion [11].

In some cases, the whole cell was fractionated by differential centrifugation. Once cells were broken while suspended in 10 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, a crude nuclear pellet was obtained by low-speed centrifugation (200g, 10 min). The supernatant fraction was used to obtain a 10,000g pellet (10,000g, 10 min), then a 100,000g pellet (100,000g, 1 hr) and a final supernatant fraction. The cell concentration was determined in the initial suspension and final supernatant volume recorded. The pellets were diluted with dH_2O and solubilized for electrophoresis so that each ml contained the amount of that fraction present in 3×10^7 cells. The volume of the 100,000g supernatant was adjusted to represent the same concentration of material as with the pellets and solubilized for electrophoresis. These samples were electrophoretically separated as sets on slab gels so that the amount of a particular protein or antigen in each lane could be directly compared between lanes to determine its distribution within the fractionated cell.

The method of Weintraub and Groudine [12] was employed for the isolation and DNase I digestion of K562 cell nuclei. Cells were harvested by low-speed centrifugation, washed twice in phosphate-buffered saline (PBS), and suspended in reticulocyte standard buffer (RSB) (0.01 M Tris-HCl, pH 7.4; 0.01 M NaCl; 5 mM $MgCl_2$) containing 0.5% NP-40 to release nuclei. Nuclei were collected by low-speed centrifugation and washed twice in RSB before suspending (DNA 1 mg/ml) in RSB. The DNA concentration was determined by measuring absorbancy at 260 nm after solubilization of nuclei in 5 M urea (1 mg/ml = 20 A_{260}). Deoxyribonuclease I (20 μg/ml) was added to the suspended nuclei and incubated at 37°C. At the completion of the digestion an aliquot was removed for determination of acid solubility using perchloric acid and the nuclei sedimented from the remaining material by low speed centrifugation. The supernatant fractions were aliquoted and stored (-80°C) for use in immunizations.

Antisera

Male New Zealand white rabbits were immunized with the nuclear and chromatin preparations. Total chromatin from K562 cells (250 μg as DNA) was used to immunize one rabbit (B2). The supernatant fraction from the DNase I digestion (37°C, 3 min) (2 A_{260} units) was used to immunize one animal (B8), and the same volume of supernatant from a control DNase I digest (no enzyme) was used on another animal (B7). The rabbits were given four weekly injections that included toe pads and multiple intradermal sites. The samples were emulsified in Freund's Complete Adjuvant for the first two injections and then with Freund's Incomplete Adjuvant for the last two injections. The rabbits were bled from the ear vein 1 wk after an intravenous injection of one-fourth the regular amount of immunogen mixed with saline. Serum was obtained, aliquoted, and stored at −20°C. These antisera were thawed, diluted (1:100), and used directly in the immunotransfer staining procedure. In some cases, the antisera were absorbed with total chromatin preparations prior to use with immunotransfers. Antisera were diluted and mixed with chromatin (1 mg/ml of 1:25 diluted antiserum) and incubated at 4°C for 2–4 hr. The chromatin was separated from the mixture by centrifugation (30,000g, 20 min). The supernatant was mixed with fresh chromatin (1 mg/ml) and allowed to incubate overnight (4°C). The

chromatin was again removed by centrifugation and the process repeated. The supernatant was diluted and employed in transfer staining. The absorption process was repeated until the level of activity detected on the transfers in chromatin used for absorbing was not further reduced or when no detectable activity remained.

Electrophoresis and Transfers

The chromosomal proteins were electrophoretically separated using the discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis method of Laemmli [13]. Chromatin preparations were adjusted to 1 mg/ml of DNA in distilled water and digested for 1 hr (37°C) with 20 μg/ml of DNase I in order to allow electrophoretic separation of proteins in the presence of DNA. Samples were then mixed with 0.9 vol of a stock solubilizing solution (0.139 M Tris-HCl, pH 6.8; 4.44% SDS; 22.2% glycerol; 25 μg/ml pyronin Y) and 0.1 vol of 2-mercaptoethanol. These samples were heated at 100°C for 3 min. Molecular weight standards (Bio-Rad) and the migration characteristics of H1 histones were used for molecular weight comparisons. Samples were applied to 1.5-mm thick, 7.5% slab gels. The gels were stained with Coomassie Brilliant Blue [14] or transferred to nitrocellulose sheets [15]. The transferred proteins were stained with amido black or reacted with antibodies [15].

The peroxidase-antiperoxidase reagent method of antigen-antibody visualization was employed with the transfers; a detailed description of this methodology used with chromatin is available [15].

RESULTS

Total chromatin was prepared from the blood cell lines F-265, KG-1, K562, and HL-60. The proteins in these preparations were separated on one-dimensional, sodium dodecyl sulfate (SDS)-polyacrylamide gels and then electrophoretically transferred to sheets of nitrocellulose. Following incubation with antisera, the antigens interacting with rabbit antibodies were visualized using the peroxidase-antiperoxidase reagent method of Sternberger (Fig. 1). Since these samples were run, transferred, and stained at one time, conditions were essentially the same, and the multitude of bands appearing on the immunotransfers with different relative migrations, intensities, and shapes are most likely indicative of the variety of different antigens detected with each of these antisera.

Antibodies were elicited to three different nuclear fractions, total chromatin (B2) (Fig. 1A), supernatant from a 3-min (37°C) DNase I digest of nuclei (B8) (Fig. 1b), and supernatant from a control 3-min DNase I digest (without enzyme) (37°C) (B7) (Fig. 1C) from the K562 cell line. The antigens detected with antiserum to total chromatin seemed to differ substantially in all molecular weight regions from those antigens detected with either antiserum B7 or B8. Two antigens migrating at molecular weight 40,000 and 44,000 were mainly detected with B8 (Fig. 1B), the antiserum to the DNase I digest supernatant. The remaining activities detected by B7 (control digest supernatant) and B8 appeared to be similar and most likely represent components easily extracted from nuclei in RSB buffer.

Although each antiserum obtained to a different chromatin or nuclear fraction from K562 cells appeared to recognize a different group of antigens in chromatin, these antigens were present in similar amounts in all of the cell lines investigated including a fresh specimen of acute leukemia (Fig. 2). Some minor differences in

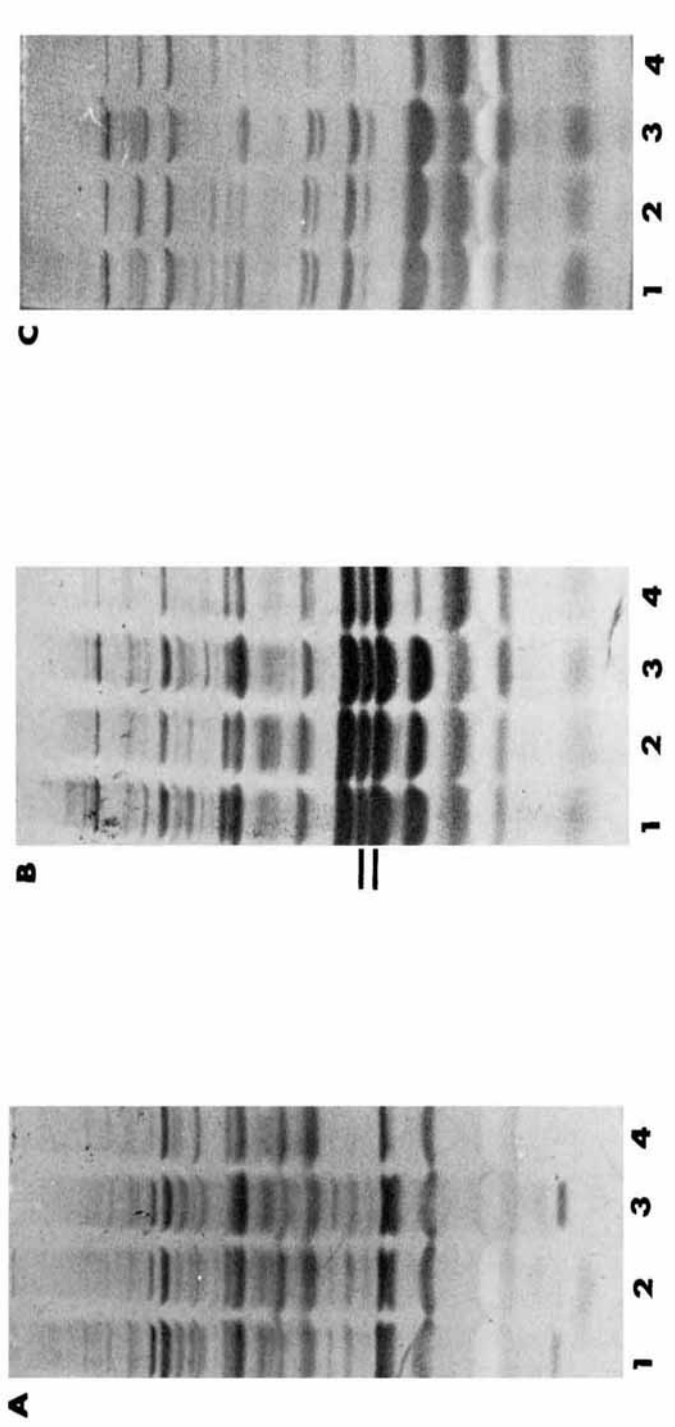

Fig. 1. Immunotransfer staining reactions of antisera with total chromatin protein preparations from F265 (lane 1), KG-1 (lane 2), K562 (lane 3), and HL-60 (lane 4) cells. Antiserum B2 to K562 cell total chromatin (A), antiserum B8 to the supernatant from a 3-min (37°C) DNase I digest of nuclei (B), and antiserum B7 to the supernatant from a control digest (37°C) without enzyme (C). The solid lines identify the activities at M_r 40,000 and 44,000. Each lane contains an equivalent amount of chromatin as DNA.

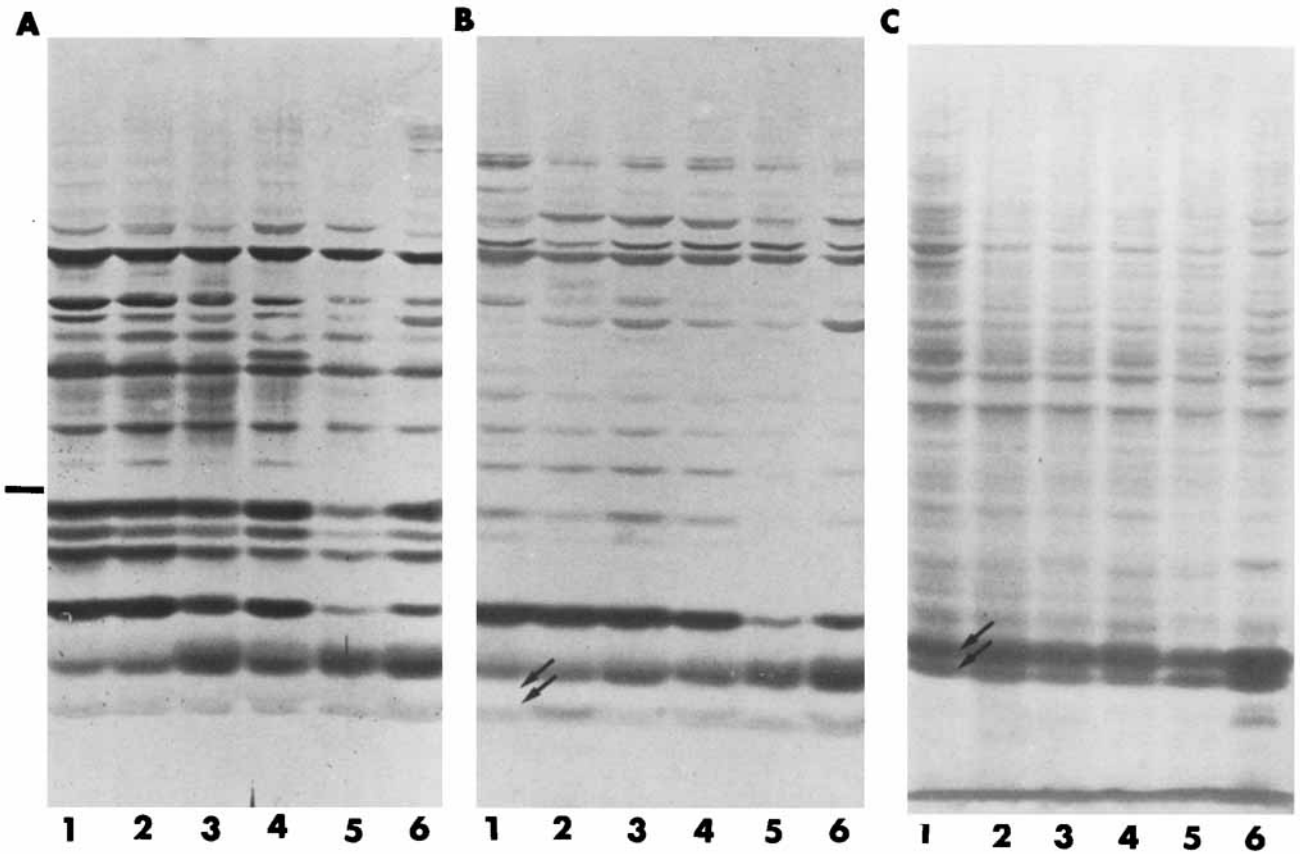

Fig. 2. Immunotransfer staining reaction of antisera B8 (A) and B7 (B) to total chromatin proteins from K562 (lane 1), KG-1a (lane 2), KG-1 (lane 3), HL-60 clone (lane 4), HL-60 (lane 5), and acute leukemia cells (lane 6). The same total chromatin preparations transferred to nitrocellulose sheets in A and B were stained with amido black in C. Note that the two H1 histone proteins strongly stained by amido black (arrows) (C) are not immunologically active in A and B (arrows in B). Also, the antigen bands below M_r 50,000 (line in A) in A and B do not seem to correspond to major protein species present in the amido-stained transfer (C) and apparently do not just represent the most prominent protein species present. Each lane contains an equivalent amount of chromatin as DNA. The similar intensity of the amido black-stained H1 histone bands in C further attests to the equivalence of chromatin in the lanes. It should be noted that the H1 bands in Figure 2C are not aligned with those in Figure 2B due to shrinking of the nitrocellulose in methanol during the amido black staining.

antigens appear between the cell lines, and these differences including major antigen differences between cell lines detected with other antisera are addressed in a separate report [16]. The HL-60 cell chromatins consistently exhibit an overall lower level of antigen activities (Figs. 1,2) that has been traced to sensitivity to mechanical shearing involved with nuclei and chromatin preparation. When less shearing was applied to the preparation of HL-60 materials, the levels of antigen detected were similar to amounts that were contained in chromatins prepared from the other cell lines or fresh tissue (data not shown). Since the electrophoretic separations are based on equivalent DNA, the staining intensity differences reflect differences in the amount of antigen in chromatin.

Chromatins isolated from unstimulated peripheral blood lymphocytes appeared *not* to contain many of the antigens observed in the cell lines (Figs. 3A, 4). The antiserum (B7) was absorbed with total lymphocyte chromatin in order to reveal which of the determinants on the antigens in the proliferating cell chromatin were not represented in lymphocyte chromatin. Alterations in protein modifications or antibody specificity for a modification group that appears on different proteins could result in an antigen's migrating to a different position in the acrylamide gel and subsequently appearing at a different location on the transfer. After three absorptions the antiserum contained activity to only one component in the lymphocyte total chromatin that migrated with an apparent molecular weight of 35,000. In an attempt to block or eliminate the activity to the M_r 35,000 antigen that remained, the antiserum was absorbed again with lymphocyte total chromatin and retested (Fig. 3C). The activity to the M_r 35,000 antigen remained (Fig. 3C, lane 5) at a similar intensity of reaction as observed with the unabsorbed antiserum (Fig. 3A, lane 5). No additional activities were detected in the unstimulated lymphocyte chromatin proteins (Fig. 3C, lane 5). However, activity to two antigens migrating with apparent molecular weights of 37,000 and 100,000 in the proliferating cell chromatin remained detectable with the absorbed antiserum (Fig. 3C, lane 4). The M_r 37,000 and 100,000 activities detected with the absorbed antiserum appear slightly diminished in level of activity from those observed with the unabsorbed antiserum (Fig. 3A). The reduced activity probably results from the small increase in dilution incurred during the absorption process or through inactivation during the extended incubations. A number of faint bands of activity appear above the M_r 100,000 band (Fig. 3C, lane 4) in the proliferating cell chromatin. The reduced staining intensity of these bands from that observed with the unabsorbed antiserum (Fig. 3A, lane 4) is another indication of the overall loss of antibody activity incurred during the absorption procedure. However, the complete absence of bands of activity in the lymphocyte chromatin (Fig 3C, lane 5) above M_r 35,000 attests to the effectiveness of the absorption with lymphocyte chromatin. One of the bands of activity observed in lymphocyte chromatin is more strongly reacting than the bands above M_r 100,000 in the HL-60 chromatin (Fig. 3A, lane 4), and after absorption no evidence of this activity remains. The general reduction in antibody activity could not account for these results. The activities to the M_r 37,000 and 100,000 antigens are apparently not absorbed by the lymphocyte chromatin. A number of other lesser reactive antigens detected in proliferating cells may not be represented in the unstimulated lymphocyte, however, they are not easily identified due to the overall loss of staining activity occurring during the absorption process.

The activity to the M_r 35,000 antigen is detected in all chromatin preparations and indicates that this activity in a total chromatin preparation is inaccessible to the

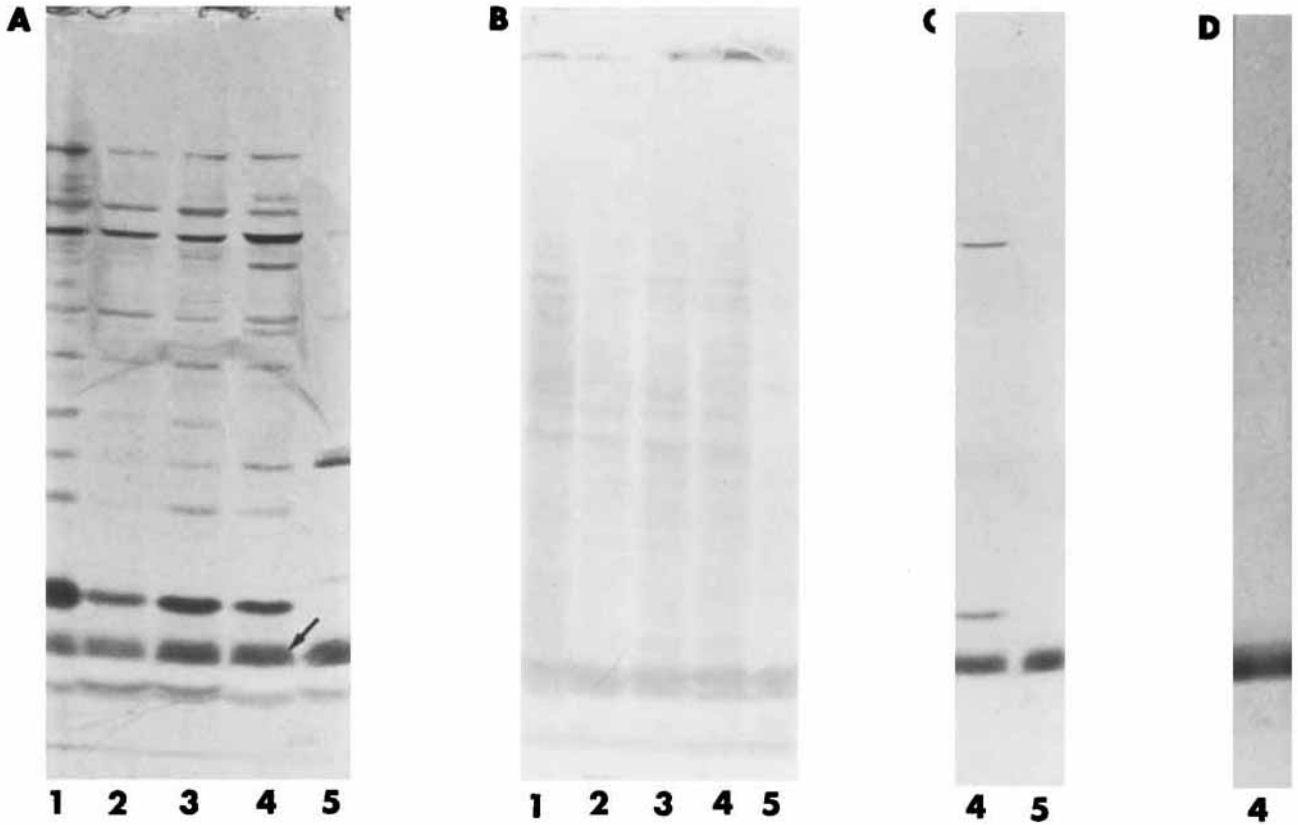

Fig. 3. Immunotransfer staining reaction of antiserum B7 with total chromatin proteins from the F265 lympho-blastoid cell line (lane 1), two preparations of PHA-stimulated peripheral blood lymphocytes (lanes 2 and 3), HL-60 cells (lane 4) and unstimulated lymphocytes (lane 5). Antiserum B7 reaction in A. Amido stained transfer in B. Absorption of B7 antiserum with unstimulated lymphocyte total chromatin four times in C. Absorption of B7 antiserum with KG-1a total chromatin three times in D. Note the lower level of non-histone proteins in the unstimulated lymphocyte chromatin (B, lane 5) compared to other proliferating cell chromatins. The M_r 35,000 antigen (arrow) is apparently present in all chromatin preparations but is not accessible to antibody in the chromatin preparation, and the activity to it cannot be absorbed out. Each lane contains an equivalent amount of chromatin as DNA and the similar amounts of amido black staining H1 histone bands (B) confirms this. In addition, the establishment of equivalent amounts of chromatin in stimulated and unstimulated lymphocytes was also done using propidium iodide staining of nucleic acids combined with ribonuclease treatment.

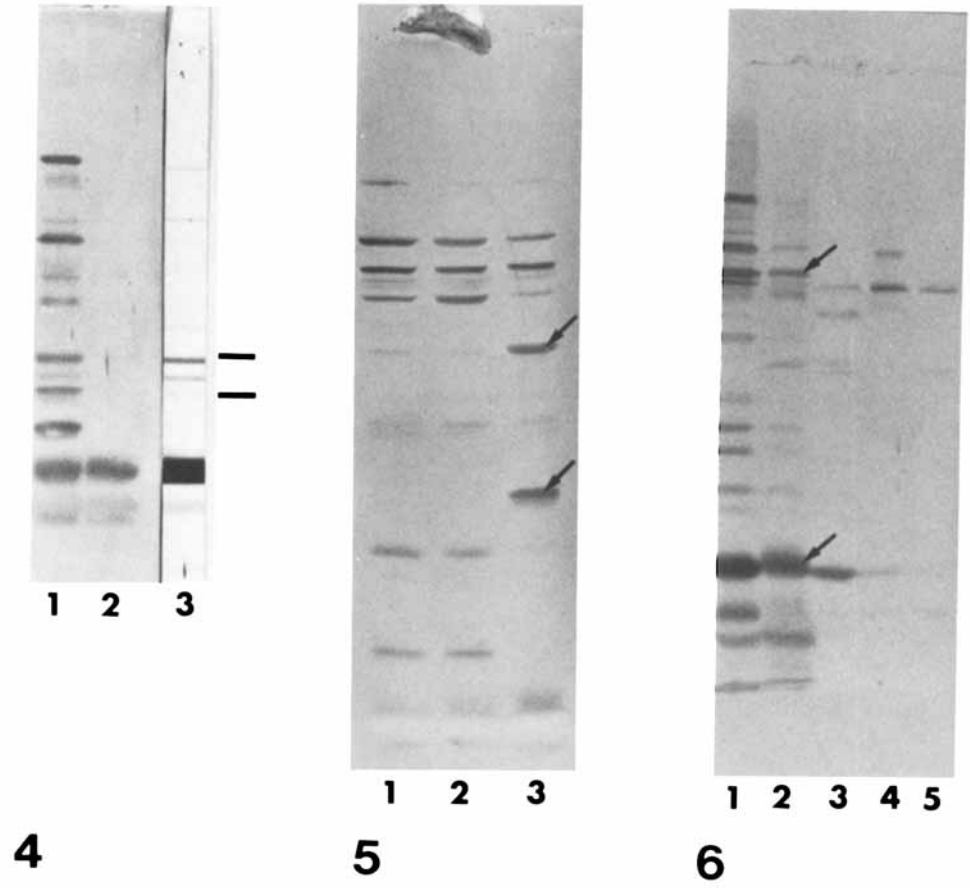

Fig. 4. Immunotransfer staining reaction with the B8 antiserum. Total chromatin protein preparations from PHA-stimulated lymphocytes (lane 1) and unstimulated peripheral blood lymphocytes (lanes 2 and 3). Only the activity at M_r 35,000 was detected in lymphocyte chromatin when equivalent amounts (25 μg DNA) of chromatin from stimulated (lane 1) and unstimulated lymphocytes (lane 2) were examined. When a greater amount of stimulated lymphocyte chromatin was reacted with the B8 antiserum (lane 3), weakly reacting bands of activity appear in the M_r 40,000–48,000 (lines) region. The more intense staining activity observed at M_r 35,000 in lane 3 is an indication of the increased amount of lymphocyte chromatin required to observe the antigens in the M_r 40,000–48,000 region.

Fig. 5. Immunotransfer staining reactions with antiserum B7. Total chromatin protein preparations from Concanavalin A-stimulated (lane 1), PHA-stimulated (lane 2), and unstimulated peripheral blood lymphocytes (lane 3). Antigens at M_r 76,000 and 52,000 (arrows) appear elevated in the unstimulated lymphocytes. Samples were adjusted to equivalent amounts based on DNA content.

Fig. 6. Immunotransfer staining reaction of antiserum B7 with cell fractions from HL-60 cells. Total chromatin (lane 1), nuclei (lane 2), 10,000g pellet (lane 3), 100,000g pellet (lane 4), and 100,000g supernatant (lane 5). Samples were adjusted and loaded to represent the amount of that particular fraction in 3×10^6 cells. Activities at M_r 100,000 and 37,000 (arrows) are found mainly in nuclear fractions.

antibody and not effectively absorbed or blocked from reacting. This antigen is reactive once the proteins are denatured and the polypeptides electrophoretically separated and transferred to nitrocellulose. This activity was also not absorbed out by chromatin from proliferating cells (Fig. 3D) whereas the M_r 37,000 and 100,000 activities were eliminated after three absorptions with chromatin from HL-60 cells (Fig. 3D) or phytohemagglutinin (PHA)-stimulated lymphocytes (data not shown).

Three absorptions of the B8 antiserum left weak activities to the M_r 37,000 and 100,000 antigens (data not shown). The three strongly reacting antigens between M_r 40,000 and 48,000 detected with unabsorbed B8 antiserum were not detected after absorbing with the lymphocyte chromatin. No antigens were observed in the lymphocyte chromatin on direct testing of equivalent amounts of lymphocyte and HL-60 cell chromatin with the B8 antiserum (Fig. 4, lane 2) except for the very strongly reacting antigen at M_r 35,000. However, when approximately double the amount of lymphocyte chromatin was reacted with the B8 antiserum, weak activities in the M_r 40,000 to 48,000 region were observed in the lymphocyte chromatin (Fig. 4, lane 3). The presence of these activities most likely accounts for the ability of this material to absorb out those activities from the B8 antiserum while being ineffective at absorbing out the activity just below this region at M_r 37,000.

Although the nondividing lymphocytes appear to be depleted of many chromatin antigens, they are elevated in two activities migrating at M_r 52,000 and 76,000 (Fig. 5). Once these cells are stimulated with lectins, the chromatin displays an antigen composition similar to the proliferating transformed cells (Figs. 3A, 5). The antigens detected by immunotransfer staining with the B2 antiserum also do not appear to be represented in lymphocyte chromatin (data not shown). The same complement of antigens detected in proliferating cells is also observed in chromatin from lectin stimulated lymphocytes. Unlike the B7 antiserum (Fig. 5), B2 (not shown), and B8 (Fig. 4) showed very little activity with lymphocyte chromatin on direct testing.

The distribution of the antigens detected with the B7 antiserum within the whole cell fractions was determined (Fig. 6). Those fractions containing predominantly nuclear components are enriched in all of the antigens including the proliferation associated activities migrating at M_r 37,000 and 100,000 (Fig. 6, lanes 1 and 2).

Use of the B8 antiserum on whole cell fractions confirmed the predominant nuclear location of M_r 37,000 and 100,000 activities (data not shown) and revealed that the antigens between M_r 40,000 and 48,000, mainly detected with the B8 antiserum, were also enriched in the nuclear fractions.

DISCUSSION

All of the antigens revealed in this study were detected in the same standard preparation of total chromatin and are therefore considered chromatin proteins. The results with the cell fractions confirm the nuclear origin of nearly all the activities observed and in particular the M_r 37,000 and 100,000 antigens. However, some activities appear to be more prominent in other cellular fractions indicating that these proteins are present in more than one intracellular compartment or that the fractionation is incomplete. It is also possible that proteins relocate during tissue processing [17]. These considerations are better addressed when monospecific reagents are available as was done previously [17], and the cell fractionation data should be considered only a preliminary indication of intracellular location.

The reaction specificities of the three antisera investigated appeared to vary widely, suggesting that the type of immunogen preparation employed determines which of the chromatin components will elicit antibodies. Two antigens migrating at M_r 44,000 and 40,000 were more strongly reactive with antiserum to materials released with a light DNase I digests of nuclei. The conditions for digestion were similar to those used to selectively digest specific DNA regions programmed for transcription [12]. Additional experiments are required to investigate the possible association of these antigens with transcriptionally active genes.

The much lower level of these activities in unstimulated lymphocyte chromatin is consistent with this possibility. The antigens associated with proliferating cells (M_r 37,000 and 100,000) and those elevated in nonproliferating cells (M_r 52,000 and 76,000) were all detected with an antiserum (B7) to nuclear materials released from nuclei during a control digestion without DNase I. The M_r 37,000 and 100,000 antigens were also detected with the B8 antiserum. These four antigens are probably loosely associated with chromatin and dissociated from the nucleus during the incubation or released through the action of an endogenous enzyme. These protein components seem to be more immunogenic when obtained in this manner than when isolated in chromatin.

The two antigens (M_r 37,000 and 100,000) that are only found in lectin-stimulated normal lymphocytes, and transformed blood cells appear to be proliferation associated components. The lectin-stimulated lymphocytes are frequently used to investigate events associated with the induction of cells to divide. Upon stimulation with lectins these normally quiescent cells undergo a series of rapid changes in the synthesis of RNA [18] and chromosomal nonhistone proteins [19,20]. Both the histone and nonhistone proteins undergo modification changes [18,21] at early times, and the level of chromosomal nonhistone proteins increases greatly as a result of an influx of preexisting proteins in the cytoplasm at the early times [22] and from newly synthesized proteins at later times. These dramatic nuclear events precede DNA synthesis, which begins at 20 hr after stimulation, and subsequent mitosis. Our use of cells stimulated for 72 hr with lectins helped to insure that the cells passed through these preparative stages and that the synchromy due to the simultaneous induction has deteriorated so that possible cell cycle phase specific events will not be a factor in our comparisons. Those changes detected in the stimulated lymphocytes were also observed in transformed cells of both myeloid (HL-60, KG-1) and probably erythroid (K562) cell lineages using antiserum to K562 cell nuclear materials, thereby eliminating the possibility that the antigen changes observed in lectin-stimulated lymphocytes were associated with immune response or selective expansion of the T-lymphocyte population [20]. Therefore, the two antigens not detected in unstimulated lymphocytes appear at this time to be specific for continuously proliferating cells. The examination of the antigens in chromatin from metabolically active cells that are not proliferating (ie, liver or kidney) will be completed when the materials are available.

The overall higher level of nonhistone protein content observed in the lectin-stimulated normal lymphocytes and leukemic cells noted previously [19,23,24] was also observed in the present study. The levels of proteins or antigens present in chromatin samples were established relative to an equivalent amount of DNA loaded on the gels. The differences in staining level suggest actual quantitative differences in chromatin. Unfortunately chromatin is only operationally defined, and even standardization of method with different cells and tissues does not insure a uniformly consis-

tent product. The effects of shearing on the HL-60 chromatin preparation not only demonstrated this problem, but also showed that it can be controlled once identified.

Some of the previous studies on the lectin-stimulated lymphocyte system revealed information about certain chromosomal nonhistone proteins. Polypeptide bands of molecular weight 43,000 and 150,000 found in a phenol-soluble chromosomal protein fraction showed preferential quantitative increases beginning at 2 hr after stimulation with lectin [22]. This same fraction contained a M_r 70,000 polypeptide that displayed an elevated labelling activity over all other components present [22]. Increased phosphorylation of a M_r 52,000 polypeptide was observed in this same fraction following lectin stimulation [22]. Using a more refined system that involved the examination of only the stimulated T cells, increased labelling was observed in polypeptides of M_r 40,000 and 95,000 present in the 200-mM phosphate buffer fraction of hydroxylapatite-separated total chromating proteins [20]. Another study employing hydroxylapatite-separated total chromatin proteins revealed a general quantitative increase in nonhistone proteins along with the specific enhancement of M_r 22,000, 24,000, 30,000, and 38,000 polypeptides [25]. Interestingly, polypeptides of M_r 40,000, 80,000, 100,000, and 110,000 decreased in amount out after 64 hr of stimulation.

Studies by another group, using two-dimensional gel electrophoresis methods, also revealed a reduction in some polypeptides in chromatin from lectin stimulated cells compared to normal lymphocytes [26,27]. Additional spots (polypeptides) were decreased in chromatin from leukemic cells compared to unstimulated lymphocyte chromatin, and four polypeptides were elevated in lectin-stimulated and leukemic cell chromatins compared to the normal lymphocyte material [26,27]. Through the use of two-dimensional gels a M_r 35,000 nuclear membrane glycoprotein was recently found to be specific for nonproliferating B cells [28]. It is unlikely that this protein would have been detected in the present study due to the low numbers of B cells present in the lymphocyte preparations used. In addition, the nuclear fractions used were not enriched in nuclear envelope proteins.

The present results are in general agreement with those previously reported. Most changes in specific polypeptides noted in prior studies do not appear to correspond to the major antigen changes observed. The M_r 38,000 protein that increased in amount in the stimulated lymphocyte [25] may be related to the M_r 37,000 antigen detected in the proliferating cell chromatins. Other of the lesser reactive antigens in stimulated lymphocyte chromatin could be related to previously noted polypeptide changes.

Although previous studies showed decreases in some proteins occurring with stimulation none of these seemed to be related to the reduction in the M_r 52,000 and 76,000 antigens. Since these two activities are detected with antiserum to K562 cells their elevation in unstimulated lymphocytes reflects only a quantitative change.

The results obtained using the one-dimensional electrophoretic separation combined with the immunochemical analysis with polyclonal antisera demonstrates the sensitivity of this approach for detecting changes in a large number of chromatin components accompanying functional changes in cells. This technique also eliminates some of the complications involved with changes in electrophoretic migration associated with modification changes. Once the observations have been made through this initial screening, individual activities can be selected for more in-depth study that involves monospecific antibody production and antigen isolation and characterization.

Each of these objectives is more easily achieved by having a specific immunochemical activity to follow. When monospecific reagents are available, the use of more sophisticated two-dimensional electrophoretic methods is desirable for examining additional heterogeneity. The antigens specific for the proliferating cells and ones specific for nonproliferating cells are candidates for further study to elucidate possible involvement in nuclear events associated with cell division. Activities preferentially detected with antiserum to the DNase I extract also warrant additional investigation.

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