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# Distribution of Globin Genes and Histone Variants in Micrococcal Nuclease-Generated Subfractions of Chromatin From Friend Erythroleukemia Cells at Different Malignant States

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**Abstract** The distribution of the α and β-globin genes and histone variants was examined in micrococcal nuclease-generated chromatin fractions of three Friend murine erythroleukemia cell types differing in malignant potential and inducibility to erythroid differentiation. A preferential concentration of globin gene sequences, as compared to satellite DNA, was noted in a physiological salt-soluble, histone H1-depleted, mononucleosomal chromatin fraction (Sup 120) in all Friend cell types, even those in which the globin gene was not capable of transcriptional activation by chemical induction. The level of globin gene enrichment in the Sup 120 fraction was highest in the most malignant and inducible cell type. The chemical induction of erythroid differentiation in this cell line did not change the distribution of globin genes in the chromatin fractions. The Sup 120 chromatin fraction prepared from mouse brain nuclei was not enriched in globin genes. Besides the previously reported low H2A.1/H2A.2 ratio [Blankstein and Levy: Nature 260:638–640, 1976], chromatin from the most tumorigenic cell type showed the lowest H2B.2 to H2B.1 ratio, highest levels of histone H4 acetylation, and the most pronounced change in relative amounts of two major electrophoretic bands of histone H1 variants as compared to the less malignant cell types. The histone variant content of the micrococcal nuclease-generated chromatin fractions from the three Friend cell types reflected the core histone variant differences for the respective intact nuclei. However, the electrophoretic separation of mononucleosomes by size revealed several classes with different H2A variant ratios. The results demonstrate the existence of structural differences in globin gene and histone variants in erythroleukemia cell chromatin associated with distinguishable phenotypes during malignant cell progression. © 1994 Wiley-Liss, Inc.

**Key words:** chromatin fractions, tumors, nucleosomes, micrococcal nuclease digestion, histone variant composition, erythroid differentiation

Three Friend virus-induced erythroleukemia cell types with increasing ability to form tumors emerged sequentially from an initial subcutaneous tumor [Levy et al., 1979]. Although showing distinct biochemical and growth characteristics [Levy et al., 1979], the cells were indistinguishable by light or electron microscopy [Woytowicz et al., 1983]. Only the most malignant Friend cell type (able to form tumors from only 10–100 cells) could be "induced" to differentiate into an erythroid cell by treatment with chemicals such as dimethylsulfoxide (DMSO) [Levy et al., 1979; Woytowicz et al., 1983]. The three tumor cell types differed in the relative amounts of the two major histone H2A variants, H2A.1 and H2A.2 [Blankstein and Levy, 1976; Blankstein et al., 1977], and displayed differences in their bulk chromatin organization as revealed by micrococcal nuclease digestion [Leonardson and Levy, 1980]. Studies of six newly isolated Friend tumors showed a common directional shift in their histone H2A and H2B variant ratios during passage over time, coordinate with increasing malignant potential [Leonardson and Levy, 1989].

Histone variant contents change during embryonic development and differentiation and differ among various adult tissues [Poccia et al., 1984; Brandt et al., 1979; Zweidler, 1984]. The genes coding for the core histones exist in 10–20

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nonallelic copies in the mouse genome [Jacob, 1976]. It is unclear whether the particular histone variant composition directly affects chromatin structure or cell function, or merely reflects the balance of histone genes which are active in a given cell type. However, recent studies implicate a restrictive role for H2A and H2B histones in gene expression [Hayes and Wolffe, 1992]. The relative affinity of the histone variants to form the core histone octamer could affect the accessibility of the associated gene to transcription factors.

We have extended the analyses of three Friend cell tumor types with distinct phenotypes and H2A variant compositions to other histones, nuclear proteins, and gene organization. We show that different relative amounts of histone variants of H2B and H1 are present in the three tumor types. The chromatin organization of the globin gene in the Friend cell types is also different. Globin gene sequences were enriched in a micrococcal nuclease-released salt-soluble fraction in all Friend tumor cell types, but the level of enrichment was greater in the more malignant and inducible tumor cell. The core histone variant composition was not different from bulk chromatin in the fraction enriched for globin gene sequences, although differences in nonhistone proteins, histone H1, and histone H4 acetylation levels were observed. Five mononucleosome species, detected in the low salt fraction, showed differences in nonhistone proteins, histone H1, and the histone H2A variants.

## MATERIALS AND METHODS

### Friend Cell Tumors, Cell Lines, and Mouse Brain

Friend cell tumors were propagated in adult DBA/2J mice by subcutaneous implantation of cells or tumor fragments (approximately $10^6$ cells). After 14 days, animals were sacrificed, and tumors excised, frozen on dry ice, and stored at −70°C until used for nuclear isolation. The derivation of Friend cell tumors MT1 (Cell Type II), A6 (Cell Type III), and C7D (Cell Type IV) has been described elsewhere [Levy et al., 1979; Blankstein, 1976]. MT1 was the least tumorigenic, A6 intermediate, and CD7 most tumorigenic as defined by the number of cells needed to form subcutaneous tumors in mice [Levy et al., 1979].

Tissue culture cell line C7D was propagated in Eagle's basal medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum and gentamicin sulfate (50 μg/ml, Schering-Plough, Kenilworth, NJ). Viability was assayed by Trypan blue exclusion. Cells were induced to differentiate along the erythroid pathway by addition of 1.5% dimethyl sulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO). After treatment for 4–5 days, 90% of the fully inducible Friend cell line (e.g., C7D) was positive for hemoglobin by the benzidine test [Levy et al., 1979]. Cells for the present studies were harvested after 40 h of DMSO treatment, when globin gene transcription was maximal [Salditt-Georgieff et al., 1984].

Mouse brain tissue was obtained from DBA/2J mice, frozen on dry ice, and stored at −70°C until used for isolation of nuclei.

All procedures adhered to the NIH guidelines for animal studies.

### Micrococcal Nuclease Digestion of Nuclei and Chromatin Fractionation

Preparation of cells and isolation of nuclei were performed as previously described [Leonardson and Levy, 1980, 1989; Levy et al., 1980], with solutions containing the protease inhibitors phenyl methyl sulfonyl fluoride (PMSF), tosyl lysyl chloride (TLC), tosyl phenyl chloride (TPC), and 5 mM sodium butyrate. Nuclei suspended in digestion buffer [Leonardson and Levy, 1989] at 100–130 $A_{260}$ units per ml were digested using 1 mM $CaCl_2$ and micrococcal nuclease (Worthington, Freehold, NJ) (1 U/$A_{260}$). The reaction was stopped with 1/10 volume of EDTA (15 mM, final concentration) and the nuclei were centrifuged at 1000 *g*. The supernatant (Sup 120), was removed and the pellet was resuspended to the initial volume with 10 mM Tris, 1 mM EDTA, pH 7.5, to lyse the nuclei. After 1 h at 4°C with intermittent mixing, the suspension was centrifuged at 10,000 *g*. This second supernatant (Sup 10) was removed and the residual lysed nuclear pellet (LP) saved for analysis. The percentage of chromatin which was solubilized was determined by using the absorbance at 260 nm of the fractions compared with the initial nuclear concentration. Samples of chromatin and nuclei were dissociated in 5.5 M urea–2.2 M NaCl for spectrophotometric reading. Greater than 70% of the $A_{260}$ material in the solubilized fractions was DNA. Less than 10% of the DNA was acid-soluble (i.e., broken down to small fragments) after 30 min of digestion.

### Preparation of Radioactively Labeled DNA Probes

Mouse α-globin gene probe consisted of 380 bp of cDNA of the globin sequence cloned in plasmid pCR1; the β-globin gene probe contained 500 bp of cDNA in pCR1 [Rougeon and Mach, 1977]. These plasmids were kindly provided by V. Volloch (MIT, Cambridge, MA). The mouse satellite DNA which was obtained from J. Maio (Albert Einstein College of Medicine, New York, NY) had been isolated from $AgSO_4$ gradient [Tobias et al., 1971]. The probes were labeled with $^{32}$P-α-ATP by nick translation [Rigby et al., 1977]. Specific activities for cloned probes were 1–5 × $10^8$ cpm/μg. Satellite DNA was nick-translated to a specific activity of 1–7 × $10^7$ cpm/μg.

### Dot Blot Hybridization

Nitrocellulose sheets [Schleichler and Schuell, Dassel, Germany] were prepared for DNA application by wetting in deionized water for 30 min and transferring to 20 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M Na citrate) for 30 min. DNA isolated from the chromatin fractions was applied to the paper using an S & S Minifold apparatus.

Individual baked nitrocellulose sheets containing bound DNA samples were placed in Seal-a-Meal bags (Sears and Roebuck) with 10 ml of wetting solution [50% formamide (Bethesda Research Labs, Gaithersburg, MD, ultrapure), 5 × SSPE (1 × SSPE = 0.18 NaCl, 10 mM $NaPO_4$, pH 7.1, 1 mM EDTA), 5 × Denhardts' reagent [Denhardt et al., 1978], 0.1% SDS] and placed for a minimum of 1 h in a 42°C shaking waterbath. Denhardts' reagent contained 0.02% bovine serum albumin (BSA), 0.02% polyvinyl pyrollidone (Eastman, Rochester, NY), and 0.02% Ficoll (Sigma). The wetting solution was then replaced with 10 ml of prehybridization solution [400 ng/ml sonicated denatured salmon sperm DNA (Sigma) added to wetting solution] and returned to the 42°C waterbath for at least 2 h. The hybridization solution (10 ml) contained 50% formamide, 5 × SSPE, 1 × Denhardts' solution, 10% dextran sulfate (Sigma), 200 ng/ml sonicated denatured salmon sperm DNA, 30 to 60 ng/ml denatured pBR322, and 0.4 ng of denatured nick-translated $^{32}$P-labeled probe. Hybridization at 42°C proceeded for 16 h.

Nitrocellulose sheets were washed twice at 42°C for 30 min in 200 ml of 2 × SSPE, 5 × Denhardts' solution, 0.1% SDS, 45% formamide containing 25 ng/ml denatured salmon sperm DNA, and then for two additional washes at 42°C for 30 min in 200 ml of 0.1 × SSPE, 0.1% SDS. Nitrocellulose sheets were dried briefly under a heat lamp. Autoradiography was carried out at −70°C in the presence of Dupont Lighting Plus intensifying screens using Kodak XO-Mat X-ray film. Quantitation of intensities was achieved by scanning films on a Gilford model 250 spectrophotometer with chart recorder. Serial dilutions of the unlabeled probes in the picogram range were applied to each nitrocellulose sheet to establish the intensity level over which the hybridization response was linear; densities outside the linear response range were not used for calculations.

### Triton-X100 Gel Electrophoresis

The histone variant composition of nuclear chromatin fractions was determined after electrophoresis in 6 mM Triton-X100, 5% acetic acid, 8 M urea gels containing 12% acrylamide (150:1 ratio acrylamide to bisacrylamide) (TX PAGE) using protamine sulfate to release the basic proteins from the chromatin samples [Froussard et al., 1981]. Tube and slab gels were stained with 0.5% amido black. Total Friend cell histones were isolated from nuclei by extraction with 0.4 N $H_2SO_4$ [Blankstein et al., 1977]. Calf thymus high mobility group (HMG) proteins were isolated using the method of Goodwin and Johns [Goodwin et al., 1977].

### SDS Polyacrylamide Gel Electrophoresis

Separation of the proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) utilized a 0.5% acrylamide stacking gel with a 7.5% acrylamide resolving gel. Five to 10 μg samples of chromatin fractions or nuclei were brought to 100°C for 2 min in sample buffer (2 mM Tris, pH 6.8, 1% SDS, 0.04% β-mercaptoethanol), then applied to the gel. Chromatin fractions were routinely examined for histone proteolysis using the SDS-PAGE system.

### Nuclear Protein/Triton X100 Two-Dimensional Gel Electrophoresis

Nucleoprotein gel electrophoresis was performed in tube gels as described [Leonardson and Levy, 1980], then applied to TX100 slab gels

for second dimension separation. The gel slabs were stained in 0.5% amido black.

### Protein Quantitation

Protein species resolved by two-dimensional separations were scanned by scanning positive film transparencies of the gels with a Joyce Lobel densitometer. Single-dimensional Triton gels were directly quantitated using a Gilford model 250 spectrophotometer with a gel scanner attachment or a film attachment (for SDS gels). The area under the curve of each protein "peak" was determined using a planimeter or by weighing the peaks cut out of photocopy paper [Blankstein and Levy, 1976].

## RESULTS

### Micrococcal Nuclease Fractionation of Chromatin

Nuclei isolated from the three Friend cell types and from mouse brain were digested with micrococcal nuclease followed by separation of chromatin into three fractions: a fraction soluble in buffer approximating physiologic salt conditions (Sup 120), a 10 mM salt-soluble fraction (Sup 10), and a residual insoluble pellet (LP). The amount of chromatin which fractionated into the Sup 10 at 15 min was about 50% for both C7D and mouse brain (Fig. 1). Release

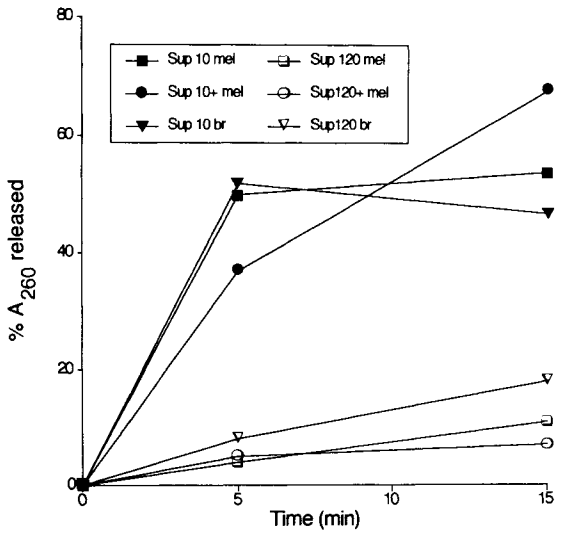



Fig. 1. Release of chromatin into soluble fractions by micrococcal nuclease digestion of nuclei from C7D ± DMSO and mouse brain. Nuclei from C7D MEL cells ± DMSO induction and brain (br) were digested with micrococcal nuclease at 37°C (see Methods). The percent chromatin solubilized into the Sup 120 and Sup 10 was calculated based on the initial $A_{260}$ of the nuclear suspension.

from the other cell types was between 42 and 65% [Leonardson and Levy, 1980] (data not shown). About 15% of the chromatin was found in the Sup 120 fraction after 15 min of digestion, whether nuclei were from the Friend tumors or mouse brain cells. Induction of erythroid differentiation by DMSO treatment of C7D cells increased the amount of chromatin released into the Sup 10 fraction at 15 min of micrococcal nuclease digestion (~70% after DMSO treatment compared to ~50% chromatin solubilized in untreated C7D) while the amount of chromatin released into Sup 120 was unaffected. DMSO treatment of the noninducible Friend cell type, A6, caused no detectable change in micrococcal nuclease digestion characteristics [Levy et al., 1980] (data not shown). We have previously reported an endogenous $Ca^{+2}$-, $Mg^{+2}$-dependent endonuclease activity in chemically induced Friend cells [Levy et al., 1980; McMahon et al., 1984] which may contribute to chromatin solubilization.

### Distribution of α and β-Globin Genes and Satellite DNA Sequences in Chromatin Fractions

We examined the three chromatin fractions for the distribution of α and β-globin genes. In chemically inducible Friend cells, globin genes are capable of transcription and are DNAse 1-sensitive or DNAse II-soluble [Salditt-Georgieff et al., 1984; Marks and Rifkind, 1978; Wallace et al., 1977]. Satellite sequences were used to represent transcriptionally inactive DNA [John and Miklos, 1979]. DNA isolated from the Sup 120, Sup 10, and LP fractions was bound to nitrocellulose and hybridized to $^{32}P$-labeled α-globin, β-globin, or mouse satellite DNA via the dot-blot technique. The Sup 120 DNA from all three Friend cell nuclei hybridized strongly to the globin gene probes, but weakly to the satellite DNA (see CTD in Fig. 2a). In contrast, Friend cell Sup 10 DNA hybridized strongly to satellite DNA but weakly to the globin gene probes, e.g., β-globin gene (Fig. 2a). This pattern was observed in Friend cell chromatin at 5 min of digestion as well as after 15 min (data not shown). The globin and satellite probes both produced strong signals with the LP DNA, with the satellite probe being the more reactive of the two (Fig. 2a).

In contrast to the Friend cell chromatin, the Sup 120 fraction from brain nuclei hybridized

weakly to the globin gene probes while the Sup 10 and LP fractions showed strong hybridization signals (Fig. 2a).

Transcription of globin genes in C7D cells in tissue culture was induced by treatment with DMSO for 40 h, followed by the isolation of chromatin fractions and DNA purification. To facilitate comparisons, the amount of hybridization of the labeled probes to DNA purified from each chromatin fraction on the "dot blot" was measured and converted to a value based on the relative signal intensities observed on the film. While DMSO treatment did cause an increase in the amount of chromatin solubilized into Sup 10 (Fig. 1), the relative distribution of α and β-globin genes and satellite DNA sequences into chromatin fractions did not change (Fig. 2b). Moreover, overt transcriptional activity did not significantly modify the enrichment of globin genes over satellite sequences in the Sup 120 fraction in the C7D cells (Fig. 2c). Under both conditions, the globin gene was greatly enriched over satellite DNA in the Sup 120 fraction.

The kinetics of release of α and β-globin genes at 5 and 15 min into different chromatin fractions was compared with satellite and bulk chromatin. The pattern of distribution of satellite gene hybridization followed the bulk chromatin (Fig. 3). The α and β-globin genes were distributed similarly to each other, but differently from satellite and bulk DNA. For example, there was a greater release of globin sequences relative to bulk or satellite DNA into the Sup 120 fraction (Fig. 3). About 20% of the total globin sequences were located in Sup 120 after 15 min of digestion. After 5 min of digestion the globin genes were underrepresented in the Sup 10 solubilized chromatin fraction but overrepresented in the LP fraction as compared to bulk/satellite DNA (Fig. 3).

We compared the globin gene enrichment in the Sup 120 fraction from the three Friend erythroleukemic cell types. Because MT1 tumor cells can only be propagated as a subcutaneous tumor and not in tissue culture, we used A6 and C7D cells grown as subcutaneous tumors for

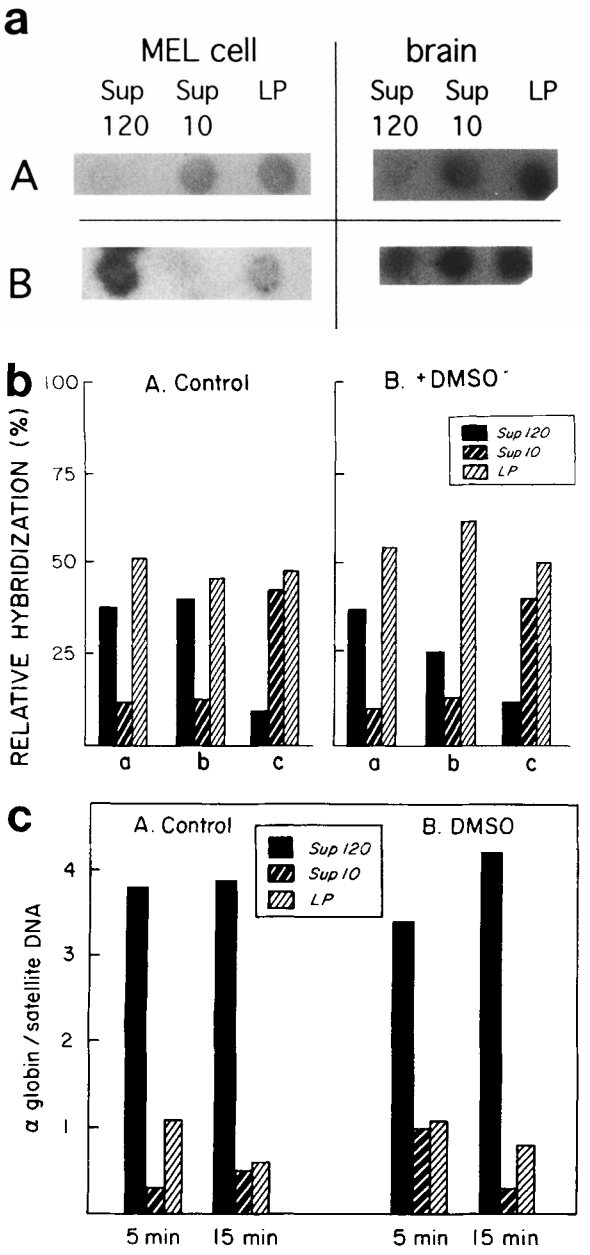



**Fig. 2.** **a:** Distribution of gene sequences in different micrococcal nuclease-generated chromatin fractions from nuclei of C7D erythroleukemia cells and mouse brain cells: DNA (2 μg), isolated from each chromatin fraction after 15 min digestion with micrococcal nuclease, was applied to nitrocellulose and hybridized to $^{32}$P-labeled satellite (A) or β-globin (B) DNA probes. **b:** Relative distribution of different genes in chromatin fractions of C7D cells ± DMSO after digestion with micrococcal nuclease. DNA (1 μg) isolated from the three micrococcal nuclease-generated chromatins fractions was hybridized by dot blot method to $^{32}$P-labeled DNA sequences. The intensity of each dot was measured by spectrophotometry. The sum of the intensities of Sup 120, Sup 10, and LP hybridization was made 1.0 and the relative hybridization of each fraction was then calculated. The source of the nuclei and the time of digestions were: A = untreated C7D cells, 5 min; B = DMSO-treated C7D cells, 5 min; a = α-globin gene; b = β-globin gene; c = satellite DNA. **c:** Ratio of α-globin to satellite DNA sequences in three chromatin fractions produced after 5 and 15 min of micrococcal nuclease digestion of nuclei from C7D ± DMSO. (A) Untreated C7D cells, (B) DMSO-treated C7D cells. Ratios were calculated from the relative hybridization of the two genes sequences (see Fig. 2b).

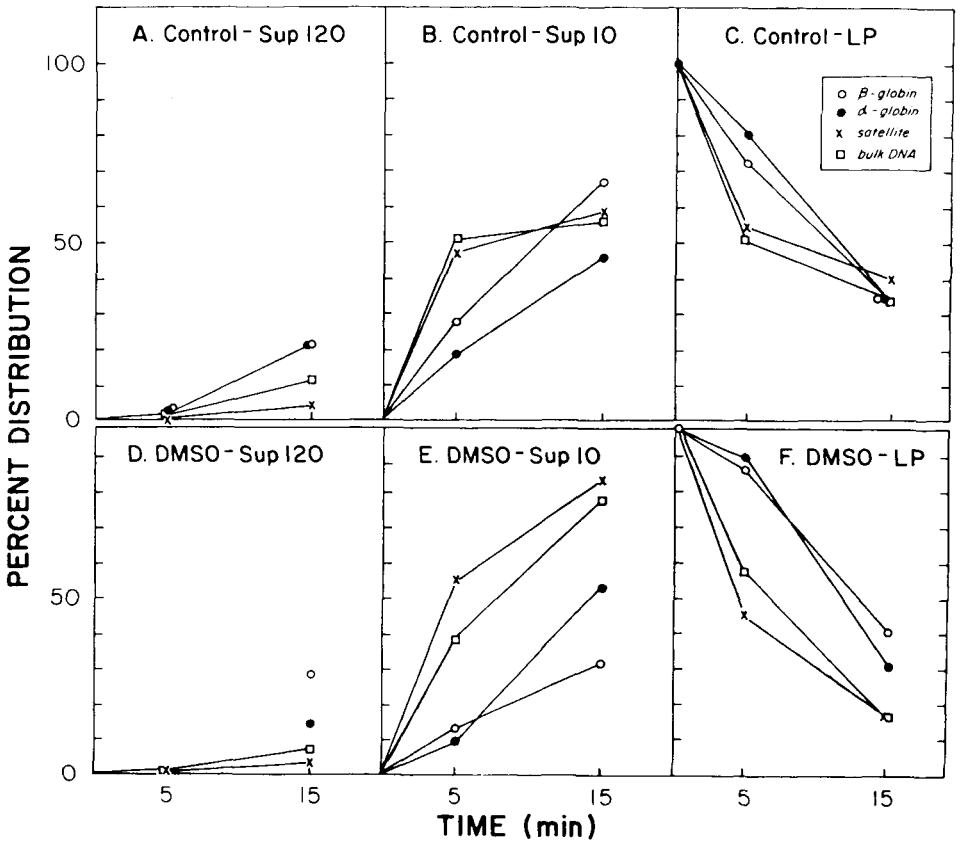



**Fig. 3.** Kinetics of release of bulk chromatin, satellite DNA sequence, and globin genes during micrococcal nuclease digestion of C7D Friend cells ± DMSO. Based on hybridization with specific DNA probes (see Figure 2A) and $A_{260}$ values for bulk chromatin, the release of different DNA sequences in relation to bulk chromatin was determined.

this analysis. The kinetics of micrococcal nuclease digestion of chromatin of C7D was the same whether the cells were propagated as a tumor or in vitro [Leonardson and Levy, 1980]. The ratio of globin sequences to satellite sequences in the Sup 120 fraction and the percent released was lowest in tumor MT1 (ratio = 1.9, 10%), intermediate in A6 (3.0, 15%), and highest in C7D (3.8, 20%) (Fig. 4). Examination of the signal intensity showed that this change was the result of an increase in the amount of globin gene DNA released into the Sup 120, while the level of satellite hybridization remained constant and low, reflecting bulk chromatin distribution (see Fig. 3). The step-wise increase in the level of globin gene enrichment (and in the amount of globin gene sequences) found in the Sup 120 paralleled the stepwise increase in malignancy and an increase in histone H2A.2 (see Table I). The results reveal that increased enrichment of the globin gene in the Sup 120 fraction occurs in Friend cell types that do not transcribe this sequence.

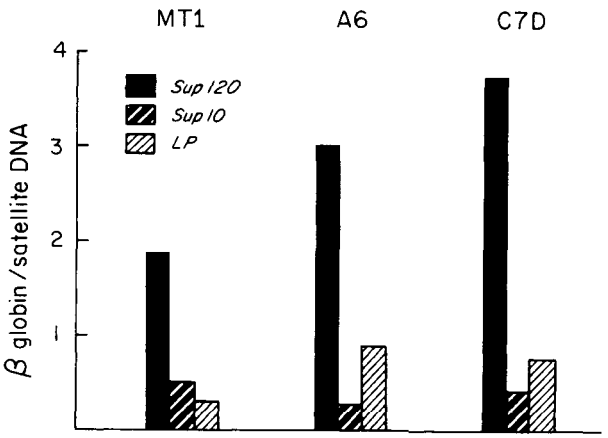



**Fig. 4.** Ratio of globin to satellite DNA sequences in chromatin fractions of Friend cell tumors MT1, A6, and C7D. DNA (1 μg) from the chromatin fractions (after 15 min micrococcal nuclease digestion) was hybridized by the dot blot method to $^{32}P$-labeled globin gene and satellite DNA sequences. The relative hybridization of each fraction to the probes was then converted into the ratio of globin to satellite hybridization signals, circumventing differences in hybridization efficiency of the mononucleosomal sized DNA of the Sup 120 compared to oligomeric length DNA in the Sup 10.

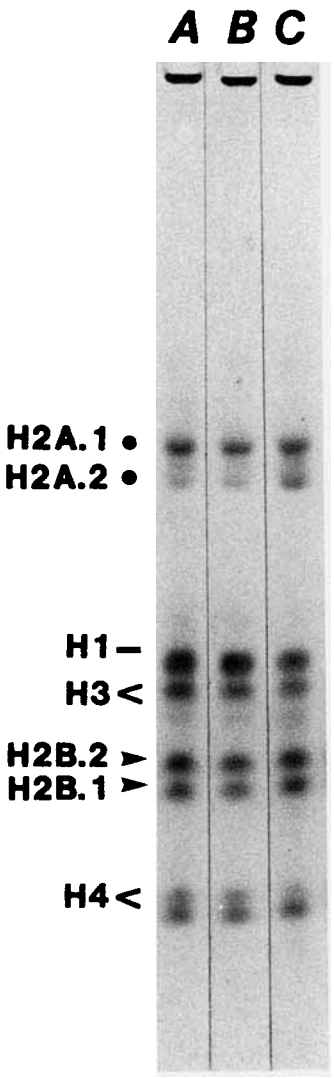



Fig. 5. Chromatin-associated proteins in different Friend cell types. Basic proteins separated by electrophoresis in Triton X 100 acetic acid-urea gels. The basic proteins from chromatin of three cell types were resolved by electrophoresis of intact nuclear samples (50 μg) dispersed in protamine sulfate + 6 mM Triton X 100, 8 M urea, 5% acetic acid gels [Froussard et al., 1981]. Gels were stained with amido black. The positions of the histone variants are indicated. A, MT1; B, A6; C, C7D.

## Histone and Nonhistone Protein Composition of Nuclei and Chromatin Fractions From the Three Friend Cell Types

Using the Triton X100-acetic acid-urea gel system, we separated the core histones into the variant forms, which differ by 2–4 conservative amino acid substitutions in the mouse (Fig. 5) [Zweidler, 1984, 1976]. The relative amount of the major H2A variant, H2A.1, and the H2B variant, H2B.2, was examined in relation to the minor variants in the cell types. The major/minor H2A histone variant ratios of the chromatins from each tumor type were found to be unchanged from that reported at earlier passages (MT1 = 2.5 ± 0.3; A6 = 1.9 ± 0.1; C7D = 0.9 ± 0.1) [Levy et al., 1980] (Fig. 5, Table I). As the malignant state increased, so did the relative amount of the minor variant form. The H2B variant content in the three tumors also changed (Fig. 5, Table I). Again, the minor variant H2B.1 increased with concomitant decrease in H2B.2, which paralleled increased levels of malignancy. The total amount of H2A measured relative to histone H4, was constant at 0.9–1.1 in all of the tumors (Table I, Fig. 5), in spite of the variant differences. The ratio of H3.2/H3.3 also showed a decrease with increased malignant state (Table I). The amount of acetylated histone H4 was lowest in C7D and highest in MT1, with intermediate levels in A6 (Table I).

Although histone H1 migrates as a single band in the Triton X100 gel system and separates into two major bands in SDS-PAGE (Fig. 6A), these patterns reflect the distribution of multiple H1 variants [Markose and Rao, 1989] which require other methods for complete resolution. The total amount of histone H1 was 0.7–0.8 molecules per H4 molecules in all tumors, but the C7D tumor contained considerably less of the top band of H1 variants than did the other cell types (Table I). SDS-PAGE re-

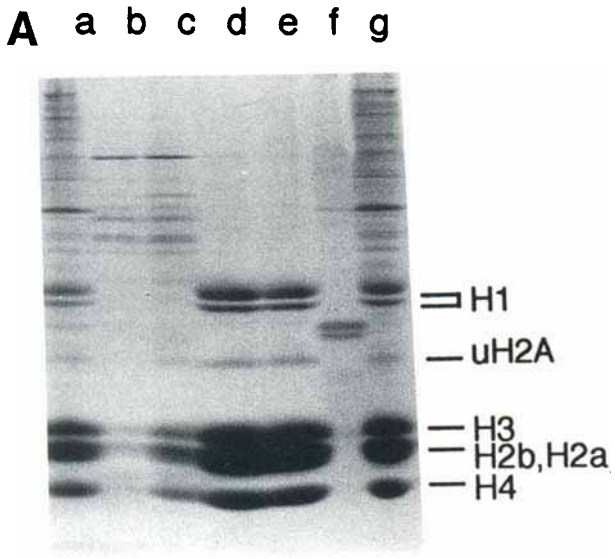
**TABLE I. Histone Variant Content in Chromatin From Three Friend Cell Types***

| Histone ratio | Tumor | | |
|---|---|---|---|
| | MT1 | A6 | C7D |
| H2A.1/H2A.2 | 2.5 ± 0.3 | 1.9 ± 0.1 | 0.9 ± 0.1 |
| H2B.2/H2B.1 | 2.0 ± 0.5 | 1.4 ± 0.2 | 1.2 ± 0.4 |
| H3.2/H3.3 | 5.6 | 4.8 | 3.3 |
| $H1^{t}/H1^{b\dagger}$ | 2.1 | 2.0 | 1.3 |
| $H4^{ac}/H4^{\ddagger}$ | 0.64 | 0.58 | 0.44 |
| H2A/H4 | 1.0 | 1.1 | 0.9 |
| H1/H4 | 0.7 ± 0.2 | 0.8 | 0.7 ± 0.2 |

*Isolated nuclear samples from three Friend tumors were applied to Triton X100 acetic acid-used polyacrylamide gels or SDS-PAGE, and separated by electrophoresis (see legend to Figs. 5 and 6). The protein bands revealed by amido black staining were scanned with a densitometer and the peaks, cut out of photocopy paper, were weighed to determined the relative amount. Average of two determinations or standard deviation (±) for determinations performed 3 or more separate times.

†Determined on SDS-PAGE; t = top band; b = bottom band (see ref.).

‡Determined from TX100 gels; acetylated/unacetylated H4.



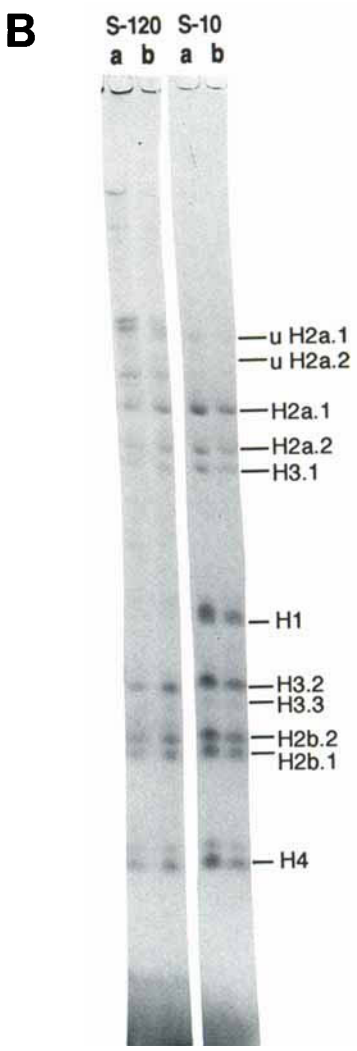




**Fig. 6.** **A:** SDS gel electrophoresis of proteins associated with Friend tumor cell MT1 nuclei and chromatin fractions. From each sample 20–50 μg was applied to the gel after denaturation by boiling briefly in the presence of SDS (see Methods). The gel was stained with amido black. The histones are designated. uH2A = ubiquinated H2A. H1 was resolved into two bands. The samples were: a: whole nuclei; b: Sup 120, 5 min; c: Sup 120, 15 min; d: Sup 10, 5 min; e: Sup 10, 15 min; f: calf thymus HMG preparation; g: LP, 15 min. The two major bands in lane f represent HMG 1, 2; HMG 14 and 17 are reported to migrate near H3 (a blurred area is visible). **B:** Triton X 100 acid-urea gel electrophoresis of micronuclease-generated chromatin fractions. Tumor cell MT1 was digested for 5 and 15 min (a,b) with micrococcal nuclease. The released fractions (Su-1 = Sup 120; Su-2 = Sup 10) were analyzed by gel electrophoresis and stained with amido black.

vealed a heterogeneous protein composition of the Sup 120 fraction (Fig. 6A). In contrast, the Sup 10 chromatin fraction was comprised almost exclusively of the 4 core histones and histone H1 (see MT1, Fig. 6A).

Triton X100 polyacrylamide gel analyses of the soluble chromatin fractions confirmed that the Sup 120 chromatin lacked histone H1, but contained all the variant forms of the core histones and a variety of nonhistone proteins. The Sup 10 chromatin fraction also contained all the major core histone variant forms as well as histone H1, but minimal nonhistone proteins (see MT1, Fig. 6B). Within each Friend cell type, the particular histone H2A and H2B variant compositions of the Sup 120 and Sup 10 fractions were not detectably different from values characteristic of intact nuclei (Table II). Preliminary analysis showed histone H4 acetylation, as

**TABLE II. Histone Variant Content of Chromatin Fractions From Micrococcal Nuclease Treated Nuclei ***

| Histone ratio | Chromatin fraction | Tumor cell MT1 | A6 | C7D |
|---|---|---|---|---|
| H2A.1/H2A.2 | Sup 120 | 1.7 ± 0.6 | 1.8 | 0.8 |
| | Sup 10 | 2.4 ± 0.7 | 2.2 | 1.0 |
| | LP | NA | 2.1 | 1.0 |
| H2B.2/H2B.1 | Sup 120 | 1.6 ± 0.4 | 1.4 | 1.2 |
| | Sup 10 | 1.6 ± 0.2 | 1.3 | 1.3 |
| | LP | 1.0 | 1.6 | 1.2 |

*Chromatin fractions were resolved in Triton X100-containing gels (see Fig. 6). The peaks from densitometric scans were cut out of photocopy paper and weighed to determine the relative quantity of each species (Blankstein and Levy, 1976). Nuclei were digested with micrococcal nuclease for 15 min; results were not different from fractions obtained at 5 min of digestion. Values represent average of two experiments or standard deviation (±) when three or more were performed.

revealed on Triton X100, was increased in the Sup 120 fraction and decreased in the Sup 10 compared to total nuclei (data not shown).

We separated the Sup 120 and Sup 10 mononucleosomes from MT1, A6, and C7D in a two-dimensional gel system. Five mononucleosomal species [Todd and Garrard, 1977] were resolved from the Sup 10 fraction. The histone H2A variant content of the mononucleosome subspecies in the Sup 10 fraction was measured in the three tumors (Table III). In tumor MT1, the MI and MV mononucleosome forms showed a lower H2A.1/H2A.2 variant content than total nuclear chromatin while the MII and MIII forms were slightly greater. Less difference was seen with A6, but again MI and MV followed the trend for lower values than total chromatin. No significant difference in the H2A variant ratio was found among the different C7D mononucleosomes, although the H2A variant ratio of the C7D nuclei was distinctly different from those of MT1 and A6. The Sup 120 fraction was not separable into mononucleosomal species, and instead formed a diffuse band of material in the second dimension (data not shown).

## DISCUSSION

In studies of Friend erythroleukemia cells, we have noted changes in a variety of cell characteristics which parallel malignant progression, including changes in esterase activity [Woytowicz et al., 1983], ability to differentiate upon chemical induction [Levy et al., 1979; Blankstein and Levy, 1976], and growth [Leonardson and Levy, 1989]. We have described a change in the relative amounts of the two major histone H2A variants, as cells assume states of greater malignancy [Blankstein and Levy, 1976; Blankstein et al., 1977]. Additional studies revealed differences in the kinetics of micrococcal nuclease solubilization of chromatin and nucleosome repeat lengths among the erythroleukemia cells at different malignant states [Leonardson and Levy, 1980, 1989].

**TABLE III. Histone H2A.1/H2A.1 Ratio of Mononucleosomal Subspecies in Sup 10***

| | Mononucleosomal subspecies | | | | | Total |
|---|---|---|---|---|---|---|
| Tumor | MI | MII | MIII | MIV | MV | Chromatin |
| MT1 | 2.1 | 2.9 | 3.0 | 2.7 | 2.1 | 2.7 |
| A6 | 1.7 | 1.7 | 2.0 | 1.8 | 1.6 | 1.9 |
| C7D | 1.0 | [a] | 1.1 | 0.8 | [a] | 1.0 |

*Five mononucleosomal subspecies from nuclei digested for 15 min were resolved by two-dimensional electrophoresis. The MI through MV designation follows Todd and Garrard [1977].

[a]Insufficient material in this area for accurate determination.

The present studies asked if these broad changes in chromatin structure and composition translated into an effect at the single gene level. We examined and compared the distribution of globin genes (which are transcriptionally inducible in the most malignant cell type) and the transcriptionally silent satellite sequences in Friend erythroleukemia cell chromatin. Transcriptionally active chromatin differs from inactive chromatin at many levels of organization including an altered association of histone H1 [Garrard, 1991], displacement of nucleosomes by activator proteins at regulatory sites [Kornberg and Lorch, 1991; Laybourn and Kadornaga, 1991], acetylation of histones H3 and H4 [Tazi and Bird, 1990; Lee et al., 1993], and the "splitting" of nucleosomes to assume an alternative conformation [Lee and Garrard, 1991].

The use of DNAse 1 has revealed nuclease hypersensitive sites at the 5′ end of transcribed genes [Gross and Garrard, 1988; Elgin, 1988], as well as moderate nuclease-sensitivity of genes undergoing and capable of transcription [Eissenberg et al., 1985; Kornberg and Lorch, 1992]. Mild micrococcal nuclease digestion has been used to produce chromatin fractions enriched in active genes [Ciejek et al., 1983; Bloom and Anderson, 1978; Saunders, 1978; Tata and Baker, 1978]. The susceptibility of globin sequences to DNAse I degradation in inducible Friend cells correlates with transcriptional competency of the globin gene [Marks and Rifkind, 1978]. The possibility that chromatin organization of the globin gene may be changed around globin genes before they are expressible (i.e., in noninducible Friend cells) has not been examined.

We analyzed the distribution of globin and satellite DNA sequences in chromatin fractions produced by micrococcal nuclease digestion. An early released physiological salt-soluble chromatin fraction (Sup 120), similar to fractions which were shown by others to be enriched for transcribable/transcribing genes [Ciejek et al., 1983; Bloom and Anderson, 1978; Saunders, 1978; Tata and Baker, 1978; Levy-Wilson and Dixon, 1979], was enriched for globin sequences and depleted in satellite sequences in all Friend cell lines tested (Fig. 4), including two cell types which are not inducible to erythroid differentia-

tion. The least malignant and uninducible tumor type showed the lowest enrichment for globin gene over satellite/bulk DNA in the Sup 120 fraction (i.e., 1.9), the intermediate (also uninducible tumor cell) had an enrichment of 2.8, while the most malignant and inducible cell type was enriched almost 4-fold for globin sequences (Fig. 4). This finding suggested that the potential for expression of the globin gene is preceded by incremental changes in the globin gene chromatin in the early non-inducible Friend erythroleukemia cell types. Perhaps this is analogous to a stem cell lineage whose developmental potential becomes restricted, although the cell is not yet capable of differentiation, i.e., the changes are necessary but not sufficient.

Chemical induction of globin gene transcription in the most malignant cell did not change the relative amount of the globin gene released into Sup 120 (Fig. 2b), nor were the kinetics of release of the globin gene altered, although there was an increase in total chromatin segregating into the Sup 10 in DMSO-treated cells (Fig. 1). Therefore, the physical parameter(s) responsible for the enrichment of globin sequences in the Sup 120 is not affected by globin gene transcription.

The tissue specificity of this particular globin gene organization was shown by the absence of such a globin gene enrichment in the Sup 120 chromatin fraction of mouse brain nuclei (Fig. 2a), while satellite sequences were distributed similarly into chromatin fractions from Friend cells and mouse brain (Fig. 2a). There was no detectable difference in the extent of overall micrococcal nuclease digestion between brain and Friend cell nuclei (Fig. 1).

The core histones, although highly conserved, exist in nonallelic variant forms, with the possible exception of histone H4. In mice, these variants differ by 2–4 conservative amino acid substitutions [Zweidler, 1976], while histone H1 forms are more diverse [Markose and Rao, 1989; Cole et al., 1990]. The existence of histone variants provides for compositional heterogeneity among nucleosomes. Although functional differences have been suggested for particular subsets of histone variants H1 [Simpson, 1981], this remains to be conclusively proven [Wu et al., 1986].

We have shown that three Friend cell types, distinguishable by their relative ability to form tumors [Levy et al., 1979], have different amounts of the two major histone H2A and H2B variants, as well as the variants of histone H1, and the H4 acetylation levels (Fig. 5, Table I). These histone differences were also observed among the micrococcal nuclease-generated chromatin fractions (Figs. 5, 6B; Table II) despite the non-random distribution of DNA sequences of different transcriptional abilities among these fractions. Moreover, the chromatin fraction (Sup 120) bearing a subset of potentially active genes had different histone variant compositions in different Friend cell types, and quantitatively different amounts of globin genes. Therefore, it is not clear how, or if, the overall quantitative changes in the histone variant composition of the total chromatin of these cells is related to the increased level of globin gene enrichment in the Sup 120 fraction (Fig. 4). However, the changes occurred concomitantly, suggesting that a general shift in protein composition may produce an effect on specific genes (e.g., globin), placing them in a transcriptionally different conformation.

Of note, satellite gene distribution did not change. This finding suggests that the chromatin alterations detected in these studies of Friend cells are manifested in a subset of the total genome, predetermined by the tissue cell type. The step-wise increase in H2A.1, H2B.2, and a decrease in H4 acetylation in tumors from low to intermediate to high levels of malignancy suggest some functional association. Since we have previously shown both H2A.1/H2A.2 and H2B.2/H2B.1 variant ratios decreased during early subcutaneous tumor establishment, with the greatest H2A.1/H2A.2 variant ratio decrease observed in the tumors of greatest malignancy [Leonardson and Levy, 1989], the association of histone change and malignant progression in Friend erythroleukemia appears meaningful in the Friend system.

Histone variant composition has been shown to alter the physical characteristics of nucleosomes [Simpson, 1981]. And, the nucleosome probably plays a much greater role in affecting alterations in chromatin structure than once believed [Croston et al., 1991; Grunstein, 1990; Clark and Felsenfeld, 1990; van Holde, 1993; Schild et al., 1993). Nucleosome positioning on DNA can block the binding of activation factors to regulatory sequences [Conconi et al., 1989; Hayes and Wolffe, 1992] and core histone acetylation can change histone H1's ability to condense chromatin [Ridsdale et al., 1990; Perry

and Annunziato, 1991]. The prominent difference observed in histone H1 variant contents in C7D as compared to A6 and MT1, may be an important step in making the transition to the chemical-inducibility of this cell type to erythroid differentiation.

Analysis of the globin genes has revealed alterations in their chromatin distribution among Friend erythroleukemia cell types with different growth and physiologic characteristics [Levy et al., 1979, 1980], as well as the ability for erythroid differentiation. This separation of chromatin change and transcriptional competence may be reflecting how general quantitative changes in chromatin constituents (histone variants, H4 acetylation, histone H1) may eventually be translated into a qualitative change, i.e., altered potential of expression, at the single gene level. The increased levels of globin gene segregation into the Sup 120 in the uninducible cell types suggest that a number of steps are necessary, but not sufficient alone, for the transcriptional competency which is observed in the inducible C7D tumor cell.

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