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Modified Intranuclear Organization of Regulatory Factors in Human Acute Leukemias: Reversal After Treatment

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Abstract Acute leukemias arise secondary to chromosomal aberrations that cause dysfunctions in gene regulation and regulatory factors. Significant differences in morphology between acute leukemic and nonleukemic hematopoietic cells are readily observed. How morphologic changes of the nuclei of acute leukemic cells relate to the underlying functional alterations of gene expression is minimally understood. Spatial modifications in the representation and/or organization of regulatory factors may be functionally linked to perturbations of gene expression in acute leukemic cells. Using in situ immunofluorescence microscopy, we addressed the interrelationships of modifications in nuclear morphology with the intranuclear distribution of leukemia-related regulatory factors (including ALL-1, PML, and AF-9) in cells from patients with acute leukemia. We compared the localization of leukemia-associated proteins with various factors involved in gene transcription and RNA processing (e.g., RNA polymerase II and SC-35). Our findings suggest that there are leukemia-associated aberrations in mechanisms that direct regulatory factors to sites within the nucleus. This misplacement of key cognate factors may contribute to perturbations in gene expression characteristic of leukemias. J. Cell. Biochem. 77:30–43, 2000. © 2000 Wiley-Liss, Inc.

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Acute leukemias arise secondary to chromosomal aberrations that cause dysfunctions in gene regulation and regulatory factors. Morphologic changes of the cytoplasm, and particularly of the nucleus and subnuclear structures in the nucleolus under light microscopy are one of the foundations of the current French-American-British (FAB) classification system for acute leukemias [Bennett et al., 1976, 1985a, 1991] Significant differences in morphology between acute leukemic and nonleukemic hematopoietic cells can be readily observed: number, size, and staining properties of cytoplasmic granules; size, shape, and staining of the cytoplasm and nucleus; and size and number of nucleoli. Just as these criteria set acute leukemic cells apart from chronic leukemic and normal hematopoietic cells, their physiologic properties also differ dramatically. Acute leukemic cells fail to display adherence to normal growth and cell cycle regulatory controls. Acute leukemia is one of the fastest-growing human malignancies, accounting for rapid onset and mortality.

An increasing number of chromosomal abnormalities, identified by cytogenetic testing, exists in the various acute leukemias [Rowley, 1990; Hogge, 1994; Heim and Mitelman, 1992; Rowley, 1998]. Genomic modifications can lead

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to over-expression of oncogenes, deregulation of key cell cycle checkpoints, or constitutive stimulation of growth. Recently developed testing modalities for establishing cellular identification, including immunohistochemical staining and flow cytometry, have enhanced our ability to characterize acute leukemias [Cheson et al., 1990; Catovsky et al., 1991]. These recent advances have improved the specificity of the classification system, as well as the sensitivity of detecting acute leukemia. This increased sensitivity is essential in assessing the therapeutic success or the presence of relapsed disease before overt clinical problems are manifest. However, although detailed definitions of cellular structure are available, how morphologic changes of the nuclei of acute leukemic cells relate to the underlying functional alterations of gene expression and subnuclear organization is minimally understood.

The nonchromatin structure of the nucleus, termed the nuclear matrix, has been shown to underlie nuclear morphology [Fey et al., 1984; Fey et al., 1986]. This internal architecture of the nucleus is associated with functional activities of nuclear components [Pienta et al., 1991; Stein et al., 1996]. It has been well established that the protein composition of the nuclear matrix is dramatically altered between normal and tumor cells. Indeed, nuclear matrix proteins provide markers for diagnosis and disease progression [Fey and Penman, 1988; Getzenberg et al., 1991; Mattern et al., 1996; Bidwell et al., 1994]. Recent investigations have suggested that the fidelity of gene expression is mediated by the precise placement of genes and their cognate gene regulatory factors within nuclear domains. Alterations in the spatial distribution of these nuclear components can lead to deregulation of gene expression. Modifications in the representation and/or organization of nucleic acids or regulatory factors may be functionally linked to perturbations of gene expression in acute leukemic cells [Stein et al., 1998]. For example, we have recently identified a nuclear matrix targeting signal (NMTS) in the acute myelogenous leukemia-related gene regulatory factor AML-1/CBFA2 [Zeng et al., 1997]. The NMTS represents a unique protein structure [Tang et al., 1998, 1999] that targets the regulatory protein to subnuclear domains that support gene expression [Zeng et al., 1998]. The gene locus encoding the AML1/CBFA2 factor frequently undergoes chromosomal rearrangements in acute myelogenous leukemias [Speck and Stacy, 1995; Meyers and Hiebert, 1995]. These rearrangements modify intranuclear trafficking to nuclear matrix associated sites [McNeil et al., 1999; Chen et al., 1998].

Acute promyelocytic leukemia (APL) provides another example of how modifications in the intranuclear distribution of regulatory factors relate to leukemias. Most APL cells display a translocation of chromosomes 15 and 17 [Rowley et al., 1977]. This translocation fuses the retinoic acid receptor-α (RARα) gene with the promyelocytic leukemia (PML) gene, thereby producing the chimeric protein PML-RARα. This fusion protein can be identified in >95% of APL cells [de The et al., 1990; Pandolfi et al., 1991; Grimwade et al., 1996; Li et al., 1997]. In nonleukemic hematopoietic cells, PML is localized to discrete nuclear matrix-associated domains (termed PML nuclear bodies) [Daniel et al., 1993; Dyck et al., 1994; Maul et al., 1995]. Normal cells contain from 1–10 large PML nuclear bodies with a range in diameter of 0.3–0.5 μm [Cho et al., 1998]. In acute promyelocytic leukemic cells, this PML immunostaining pattern becomes delocalized, resulting in an increased number of microspeckles (up to 100) with a decreased size (0.1 μm), and a change of PML immunostaining properties (weak versus strong) [Melnick and Licht, 1999]. The use of all-*trans*-retinoic acid (ATRA) or arsenic trioxide (As_2O_3) causes the dispersed PML pattern in APL cells to return to the larger, normal size pattern of nuclear bodies, with strong PML immunostaining [Daniel et al., 1993; Dyck et al., 1994; Weis et al., 1994; Koken et al., 1994; Duprez et al., 1996; Zhu et al., 1997]. This restoration of the PML nuclear bodies occurs concurrently with degradation of the PML-RARα oncoprotein and differentiation of the leukemic cells along normal hematopoietic pathways [Raelson et al., 1996; Yoshida et al., 1996]. Thus, intranuclear organization of PML bodies provides a paradigm for redistribution of nuclear matrix associated regulatory factors in tumor cells.

Changes in nuclear morphology are a diagnostic hallmark of acute leukemia. Consequently, in these studies we addressed the interrelationships of modifications in nuclear morphology with the in situ localization of regulatory factors associated with other nonpromyelocytic acute leukemias. The representation and distribution of endogenous levels of these regulatory

factors, including ALL-1, PML, and AF-9, within nuclear domains were examined. Parallel comparisons were made between acute leukemic and normal cells, as well as between acute leukemic cells before and after treatment. We additionally examined the colocalization of leukemia-associated proteins with various factors involved in gene transcription and RNA processing. Our findings suggest that there are leukemia-associated aberrations in mechanisms that direct regulatory factors to sites within the nucleus. This misplacement of key cognate factors relative to their target genes may contribute to perturbations in gene expression characteristic of leukemias.

METHODS

Cells and Cell Lines

The human acute lymphoblastic leukemia (ALL) cells used in these studies were obtained from a 20 year old woman. Cytogenetic analysis showed that all cells contained a heterozygous deletion in the short arm of chromosome 9 (del9;p21). This deletion is seen in 20% of patients with ALL [Takeuchi et al., 1997]. Peripheral blood and bone marrow samples were obtained at the time of diagnosis (ALL-Dx) and again at the time of complete remission (ALL-CR), after treatment with standard anthracycline-based induction chemotherapy. The human acute myelogenous leukemia (AML) cells used in this study were obtained from a 30 year old man. Cytogenetic analysis revealed monosomy for chromosomes 10 and 20; the significance in relation to acute leukemias is unknown. Peripheral blood and bone marrow samples were obtained at the time of diagnosis. The patient died during standard anthracycline-based induction chemotherapy treatment. Nonleukemic human peripheral blood was obtained from two different human subjects. Subject 1 is a 32-year-old man. Subject 2 is a 26-year-old woman. Both subjects are healthy without any known hematologic or oncologic disorders. Patients and normal subjects provided signed informed consent according to the guidelines of the Committee for the Protection of Human Subjects in Research.

The ML-2 human myeloblastic leukemia cell line used in this study contains a gene rearrangement involving the ALL-1 and AF-6 genes. This gene rearrangement is frequently found in acute myelogenous leukemias [Prasad et al., 1993].

Cellular Preparation and Fractionation

Slides for cytologic analysis were prepared from the ALL, AML, and nonleukemic control peripheral blood samples. All blood samples were processed within 1 h by placing 2 drops of blood on the center of a positively charged glass slide (Fisher Scientific, Springfield, NJ). Smears were performed in standard fashion. Slides were air-dried for less than 10 min at room temperature. ML-2 cells in suspension were deposited onto positively charged glass slides using Cytospin (Shandon Lipshaw, Pittsburgh, PA) at 800 rpm for 4 min. All cell samples on slides were processed immediately for in situ cellular fractionation.

Whole cell, cytoskeletal, and nuclear matrix preparations were made by stepwise extraction [Fey et al., 1984, 1986]. Whole cell preparations were obtained by fixing cells with 3.7% formaldehyde in phosphate-buffered saline (PBS) on ice for 10 min. Cells were permeabilized with 0.5% Triton X-100 in PBS at room temperature for 5 min. Soluble nuclear and cytoplasmic proteins were removed by extracting the cells on ice, twice using cytoskeletal buffer (CSK) (100 mM NaCl, 300 mM sucrose, 10 mM PIPES, 3 mM $MgCl_2$, 1 mM EGTA, and 0.5% Triton X-100) adding 2 mM vanadyl ribonucleoside complex immediately before use. Cells were fixed with 3.7% formaldehyde in CSK buffer on ice and warmed to room temperature over 10 min. Nuclear matrix-intermediate filament (NMIF) preparations were obtained by extracting the cells with digestion buffer to remove soluble cytoplasmic and nuclear proteins and chromatin. The digestion buffer was 50 mM NaCl, 300 mM sucrose, 10 mM PIPES, 3 mM $MgCl_2$, 1 mM EGTA, and 0.5% Triton X-100. Two mM vanadyl ribonucleoside complex and DNase I (50 µg/ml) were added just before incubation. For the leukemia cell line ML-2, incubation was at room temperature twice for 30 min each. For human leukemic and nonleukemic peripheral blood cells, the incubations were conducted at room temperature twice for 15 min, to minimize nuclear shrinkage. The NMIF preparations then underwent two extractions with 250 mM ammonium sulfate to remove any additional cytoskeletal proteins and undissociated chromatin. Fixation with digestion buffer containing 3.7% formaldehyde was conducted on ice and left to warm to room temperature over 10 min. Each extraction step was followed by two rinses in PBS and two rinses in PBS con-

taining 0.5% bovine serum albumin (PBS-A) to block nonspecific antibody binding.

In Situ Analysis of Nuclear Proteins

Primary antibodies were an anti-ALL-1 N-terminus affinity-purified rabbit polyclonal (169) (1:400 dilution) [Rozenblatt-Rosen et al., 1998], anti-ALL-1 C-terminus affinity-purified rabbit polyclonal (170) (1:400 dilution) [Rozenblatt-Rosen et al., 1998], anti-human PML N-terminus mouse monoclonal IgG_1 (PML; Santa Cruz Biotechnology, Santa Cruz, CA) (1:100 dilution), anti-human AF-9 C-terminus affinity-purified rabbit polyclonal (AF-9) (1:200 dilution) (T. Nakamura, unpublished observations), anti-Pol II_0 rat IgM monoclonal (Pol II_0; gift of Dr. Ronald Berezney, State University of New York at Buffalo, Buffalo, NY) [Mortillaro et al., 1996] (1:20 dilution), and anti-SC-35 mouse monoclonal (SC-35; Sigma Chemical Co., St. Louis, MO) (1:2,000 dilution). Secondary antibodies used were a fluorescein-labeled goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) (1:400 dilution) or a fluorescein-labeled goat anti-mouse IgM (FITC; Kirkegaard & Perry Laboratories, Gaithersburg, MD) (1:200 dilution); a Texas red-conjugated donkey anti-mouse IgG (1:200 dilution) or a fluorescein-labeled donkey anti-mouse IgG (DTAF) (Jackson ImmunoResearch Laboratories) (1:400 dilution); and a rhodamine-labeled affinity-purified goat anti-mouse IgM (Kirkegaard & Perry Laboratories) (1:10 dilution). All antibodies were prepared to their final concentrations in PBS-A. Slides were incubated at 37°C for 60 min, then washed 4 times in PBS-A. Efficiency of DNA extraction was determined by the use of 4′,6-diamidino-2-phenylindole (DAPI) staining (5 µg/ml) plus Triton 0.1% in PBS-A for 5 min, washed once in PBS-A with 0.1% Triton, and then mounted on a slide using Vectashield H-1000. Control slides were incubated with PBS-A instead of primary antibody. All control slides were incubated with a secondary antibody and then were stained with DAPI.

RESULTS

Modifications in Subnuclear Distribution of ALL-1 and Its Translocation Partner AF-9 in Acute Leukemic Cells

The ALL-1 gene on human chromosome 11q23 is frequently involved in acute childhood leukemias. Chromosome translocations that occur in the ALL-1 locus generate a chimeric molecule composed of ALL-1 and a partner gene [Rowley, 1998]. An unusual feature of ALL-1 gene rearrangements is the diversity of partner genes with more than 12 identified and cloned at this time. Of these, creation of an ALL-1-AF9 fusion protein in mice by virtue of knock-in methodology has been shown to trigger leukemia [Corral et al., 1996]. The ALL-1 gene encodes a 430-kDa nuclear protein; within the molecule, the region that possesses a potential to transport pyruvate kinase into the nucleus and the region that displays remarkable transactivation activity have been identified [Prasad et al., 1995; Yano et al., 1997]. These observations clearly show that ALL-1 is a transcription factor. Similarly, AF9 gene encodes the nuclear localization signal [Nakamura et al., 1993] as well as the transactivation domain [Prasad et al., 1995], indicating that the AF9 molecule is also a transcription factor. We addressed the subnuclear distribution of ALL-1 in cells from leukemic patients at diagnosis and during remission using two different antibodies that specifically recognize either the N- or C-terminus of ALL-1 [Rozenblatt-Rosen et al., 1998].

In nuclei from leukemic cells of patients with acute lymphocytic leukemia (ALL-Dx), and acute myelogenous leukemia (AML-Dx), both ALL-1 antibodies (i.e., against the N or C termini) exhibited a similar staining pattern (Fig. 1). This pattern consisted of a diffuse, fine background with distinct foci of brighter antibody staining distributed throughout the nucleus. The ALL-1 antibodies did not stain nucleoli in any of the cell samples. The number and size of the foci varied in each focal plane within the nucleus and did not correlate with the size of the cell or nucleus. In cells from the leukemia patient at time of remission (ALL-CR), nuclei retained the diffuse, fine background staining pattern, but did not show the individual foci observed before remission (Fig. 1). Nuclei from the patient at remission (ALL-CR) showed a pattern similar to that of the nonleukemic subjects (Fig. 1). Hence, the subnuclear organization of ALL-1 is modified in several distinct types of acute leukemic cells and additionally modified after treatment.

The patient with ALL in this study harbored a heterozygous deletion of part of chromosome 9 (del 9p21) in all leukemic cells; this chromosome 9 deletion was not present in patient samples at time of remission. We therefore evaluated the distribution of the chromosome 9-related AF-9 protein. For comparison, we also

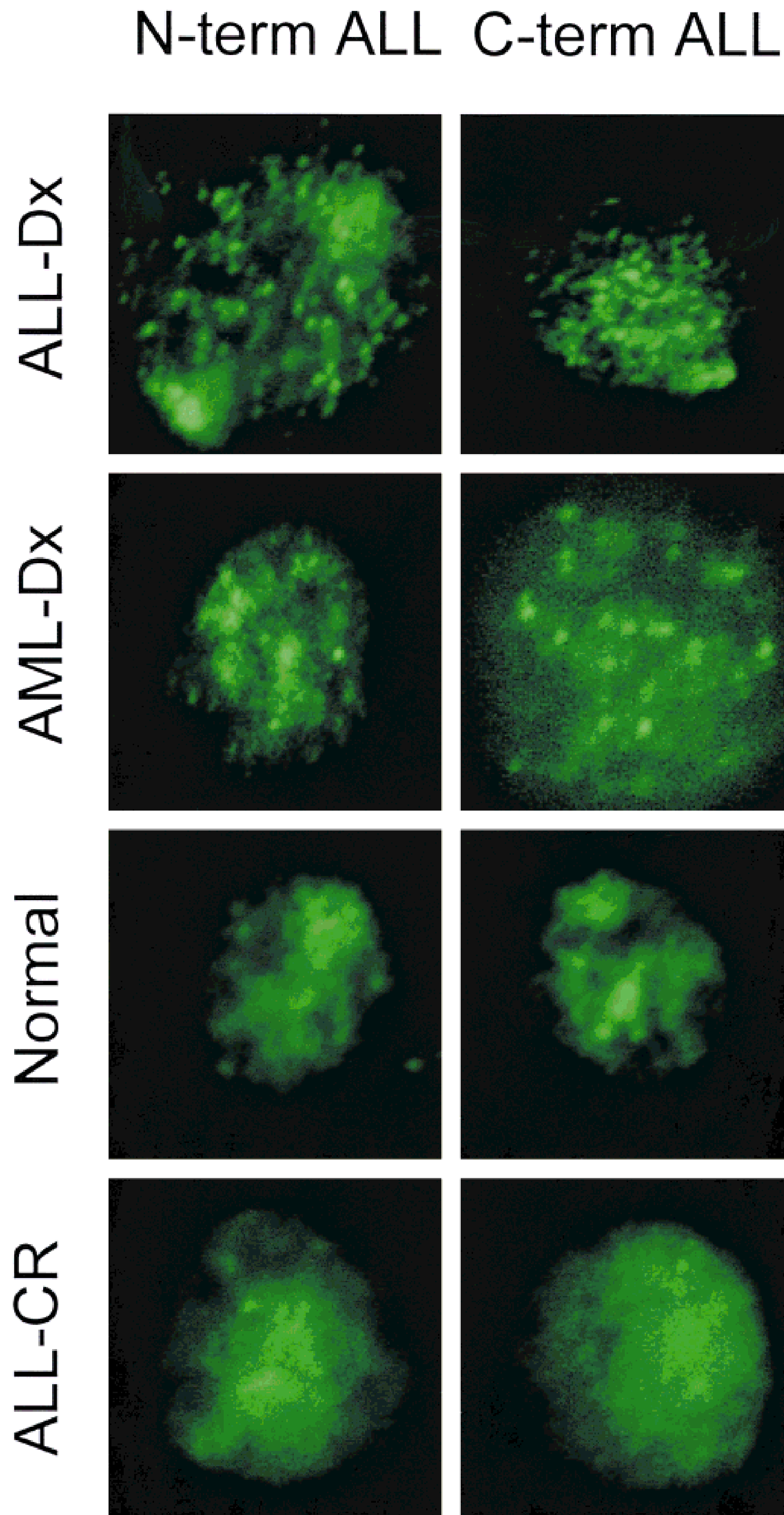


Fig. 1. Acute leukemia-related differences in the subnuclear organization of ALL-1. Nuclear matrix-intermediate filament preparations immunostained with either anti-ALL-1 N-terminus (**left**) or ALL-1 C-terminus (**right**) affinity-purified rabbit polyclonal antibody. A fluorescein-conjugated secondary antibody was used for in situ fluorescence of ALL-1 in cells from a patient with acute lymphocytic leukemia at diagnosis (ALL-Dx), with AML-Dx acute myelogenous leukemia at diagnosis (AML-Dx), a non-leukemic subject (normal), and a patient with acute lymphocytic leukemia during complete remission (ALL-CR).

analyzed AF-9 antibody staining in the acute leukemic cell line ML-2, which has a different chromosomal translocation involving AF-6 on chromosome 6 and does not involve chromosome 9. We observed that leukemic cells at diagnosis (ALL-Dx) contain multiple distinct AF-9 foci of varying size throughout the nucleus (Fig. 2). Leukemic cells from the patient with acute myelogenous leukemia at diagnosis (AML-Dx), which did not have chromosome 9 abnormalities, have a similar pattern of multiple distinct AF-9 foci of varying size (Fig. 2). Hence, the subnuclear distribution of AF-9 observed in acute leukemic patients (ALL-Dx, AML-Dx) is not directly related to a chromosome 9 deletion. The acute leukemic cell line ML-2 has an AF-9 staining pattern different from ALL-Dx and AML-Dx: multiple distinct foci that are of large size (Fig. 2). In contrast with the leukemic cells (ALL-Dx, AML-Dx, and ML-2), cells in remission (ALL-CR) showed a small number of foci, with most being of small size (Fig. 2). A decreased number of AF-9 foci was observed in leukemic cells at diagnosis (ALL-Dx), relative to normal cells. Interestingly, nonleukemic cells from normal subjects have more numerous and smaller foci than cells from the two patients (ALL-Dx or AML-Dx) (Fig. 2). AF-9 immunofluorescence studies thus demonstrate that this acute leukemia-associated factor displays several nuclear matrix distribution patterns that differ between (1) different types of leukemic cells, (2) between leukemic cells and nonleukemic cells, and (3) leukemic cells before and during complete remission.

ALL-1 Subnuclear Foci Are Distinct From SC-35 RNA Processing Domains and Are Infrequently Associated With Pol II RNA Transcription Domains

The extent to which ALL-1 is associated with either RNA polymerase II (Pol II_0) or SC-35 may reflect the activity of ALL-1 in gene regulation. Therefore, we examined in situ nuclear

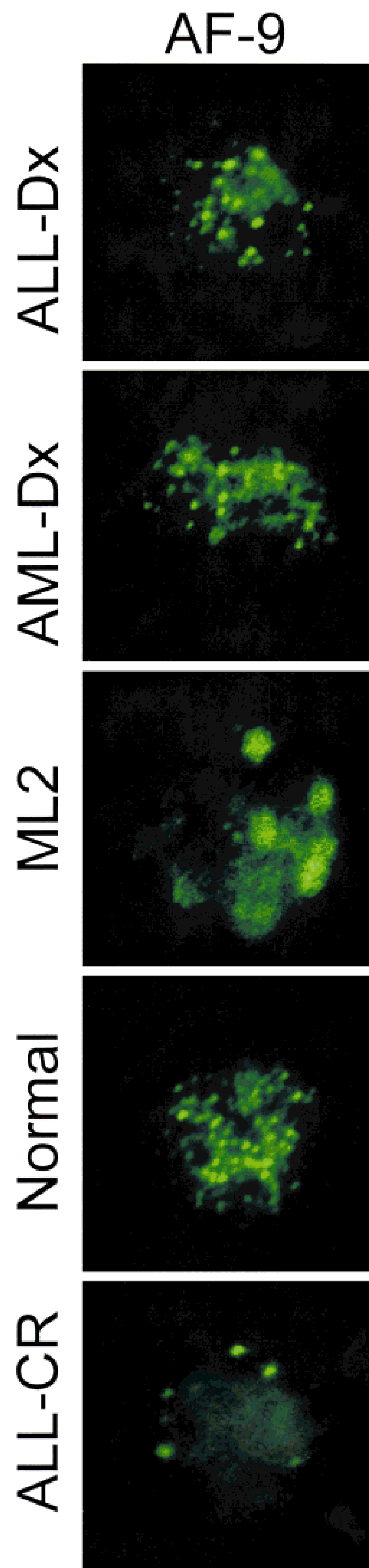


Fig. 2. Localization of AF-9 in subnuclear domains in leukemic and nonleukemic cells. Nuclear matrix-intermediate filament preparations immunostained with an affinity-purified rabbit polyclonal antibody directed against human AF-9 C- terminus. Fluorescein-conjugated secondary antibody was used for in situ labeling of cell samples from acute lymphocytic leukemia at diagnosis (ALL-Dx), acute myelogenous leukemia at diagnosis (AML-Dx), myelogenous leukemic cell line (ML-2), nonleukemic (normal), and acute lymphocytic leukemia during complete remission (ALL-CR).

matrix preparations using immunofluorescence microscopy with antibodies directed against ALL-1 and the transcriptionally active, hyperphosphorylated form of RNA Pol II_0. For comparison, we analyzed the spatial relationship of ALL-1 with SC-35, a nuclear matrix-associated protein involved in RNA processing. In examining Pol II_0 nuclear distribution, a diffuse, micropunctate staining pattern was observed throughout the nuclei of leukemic cells ALL-Dx, AML-Dx, and ML-2 (Fig. 3). In remission (ALL-CR) and nonleukemic cells, this micropunctate pattern was observed, although a limited number of more distinct foci was also evident in these normal cells (Fig. 3). Distinct large foci of Pol II_0 appear to be present in nonleukemic cells and do not appear in the leukemic cells; the significance of this finding remains to be established. Double-label immunofluorescence studies using both ALL-1 and Pol II antibodies reveal that ALL-1 is not significantly associated with Pol II_0 in all cells examined (data not shown).

Most leukemic cells (ALL-Dx, AML-Dx, and ML-2) displayed a typical speckled SC-35 immunofluorescence pattern (Fig. 4). Overall, SC-35 was absent from the nucleoli, although SC-35 signals can surround these structures. Occasionally, we observed large SC-35 foci that appeared to coalesce into reticular, amorphous structures of variable size and staining intensities. Cells at remission (ALL-CR) and nonleukemic cells displayed similar SC-35 distribution (Fig. 4). No defined colocalization pattern could be seen between SC-35 and either one of the ALL-1 antibodies, although occasional areas of signal overlap were observed between the large, reticular SC-35 foci and the ALL-1 foci (data not shown). These immunofluorescence results indicate that the spatial distribution of SC-35 RNA processing domains is not significantly altered in leukemic cells.

Nuclear Matrix-Associated PML Bodies Are Numerically Increased in Acute Myelogenous and Lymphoblastic Leukemic Cells

In acute promyelocytic leukemia (APL), cells display a dispersal of PML nuclear bodies com-

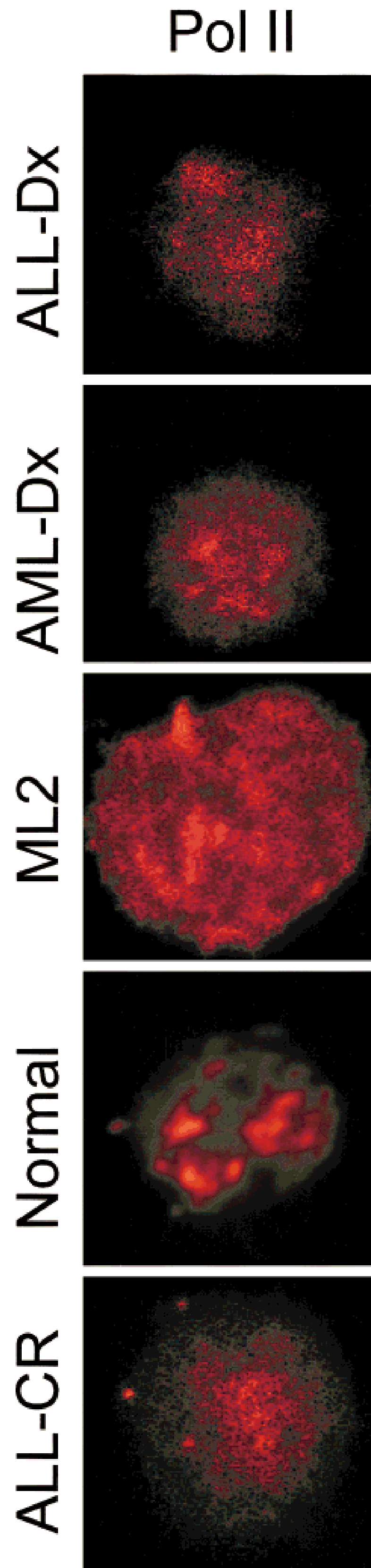
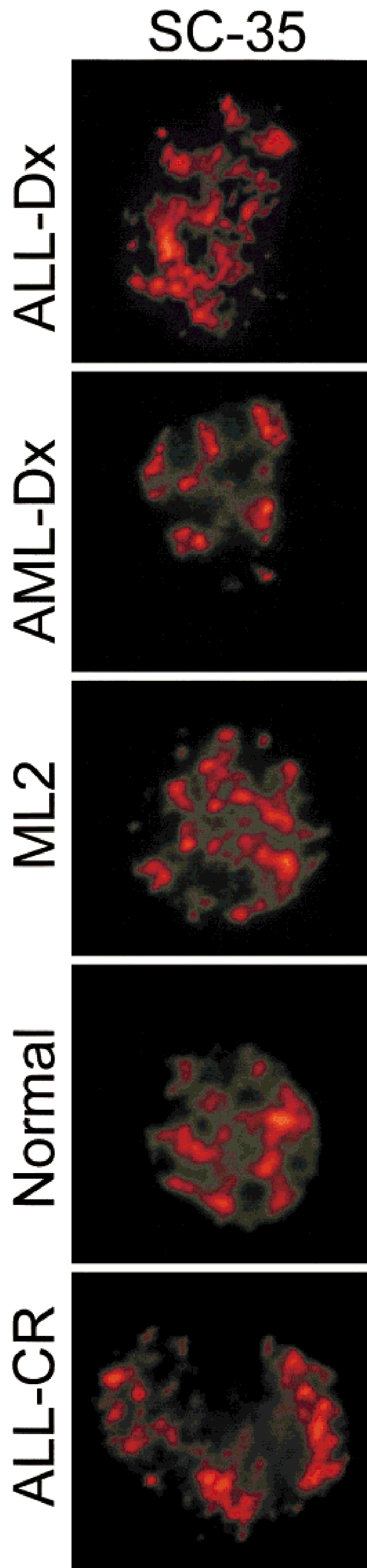


Fig. 3. Subnuclear distribution of transcriptionally active Pol II_0 (Pol II) in leukemic and nonleukemic cells. Nuclear matrix-intermediate filament preparations immunostained with anti-Pol II_0 rat monoclonal antibody. A rhodamine-conjugated antibody was used to label cell samples from acute lymphocytic leukemia at diagnosis (ALL-Dx), acute myelogenous leukemia at diagnosis (AML-Dx), ML-2, nonleukemic (normal), and acute lymphocytic leukemia during complete remission (ALL-CR).



pared with nonleukemic hematopoietic cells. Treatment of APL with all-*trans*-retinoic acid (ATRA) causes the PML nuclear body distribution in the nucleus to revert to the pattern observed in normal, nonleukemic cells [Daniel et al., 1993; Dyck et al., 1994; Weis et al., 1994; Koken et al., 1994; Duprez et al., 1996; Zhu et al., 1997; Shen et al., 1997]. We performed immunofluorescence studies on nonpromyelocytic acute leukemic cells (ALL-Dx, AML-Dx, and ML-2), as well as nonleukemic cells (ALL-CR and normal), using PML antibody to examine the spatial distribution of PML nuclear bodies in the nuclear matrix relative to the transcription factor ALL-1. We observed a correlation between the size and location of PML nuclear bodies. In ALL-Dx and AML-Dx cells, PML bodies of intermediate size were centrally located in the nucleus. PML domains of small size were distributed throughout the nucleus and were excluded from the nucleolus. All PML nuclear bodies were spherical, with larger bodies displaying increased signal intensity compared with smaller bodies, suggesting increased concentrations of the PML protein. The immunofluorescence pattern of PML nuclear bodies is distinct from the subnuclear distribution of ALL-1 (Fig. 5) and these proteins do not appear to colocalize.

Quantitation of PML nuclear bodies in 100 representative ALL-Dx leukemic cells revealed a mean of 5.2 and a median of 5 PML bodies per nucleus. We observed 1–6 PML bodies in 76% of cells, although as many as 15 nuclear bodies were present in some cells (Fig. 6). In most cells, we found 1–3 intermediate size PML bodies (mean: 530 nm; range: 420–720 nm), with the remaining bodies of smaller size (mean: 250 nm; range: 130–400 nm) (Fig. 6). Cells with a higher number of PML bodies retained a relatively constant number of intermediate size nuclear bodies, with the remaining being of the smaller size.

Quantitation of PML nuclear bodies in 100 representative AML-Dx leukemic cells showed a mean of 8.4 and a median of 8 nuclear bodies,

Fig. 4. Intranuclear localization of SC-35 RNA processing domains in leukemic and nonleukemic cells. Nuclear matrix-intermediate filament preparations immunostained with a SC-35 mouse monoclonal antibody followed by a Texas red secondary antibody. Cell samples are acute lymphocytic leukemia at diagnosis (ALL-Dx), acute myelogenous leukemia at diagnosis (AML-Dx), ML-2, nonleukemic (normal), and acute lymphocytic leukemia during complete remission (ALL-CR).

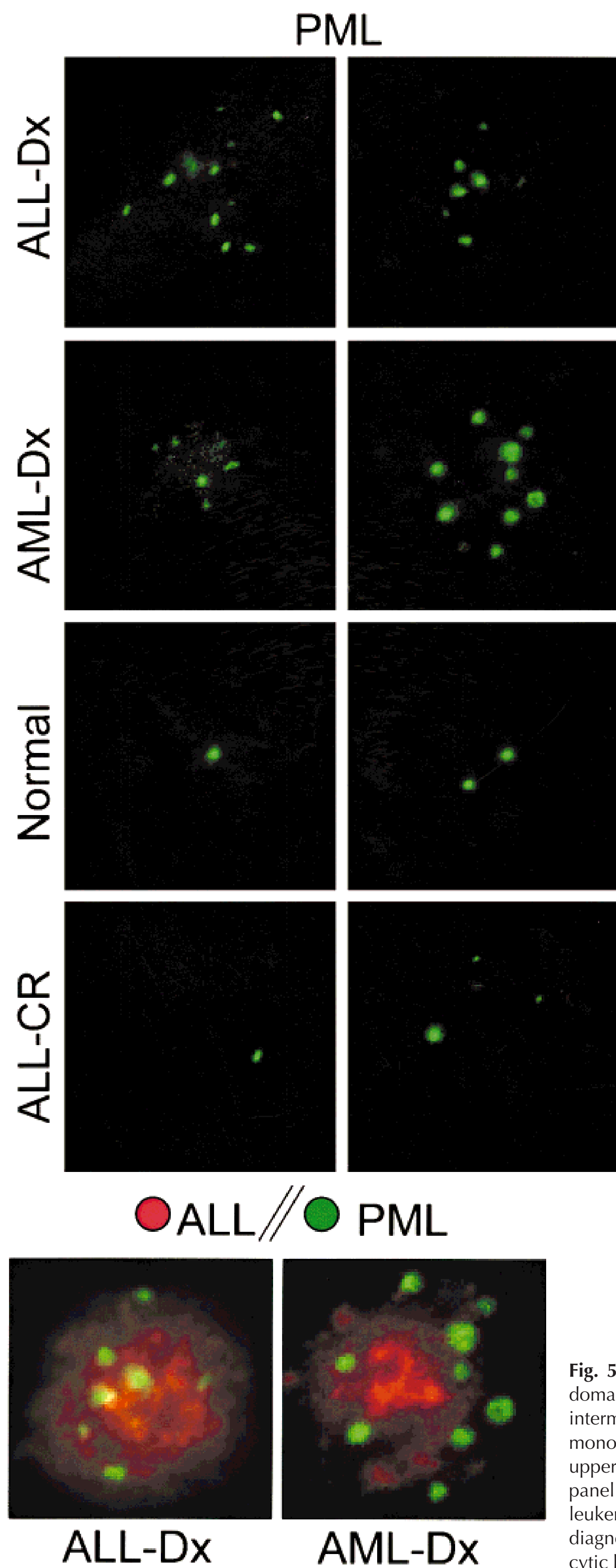


Fig. 5. Nuclear distribution of promyelocytic leukemia (PML) domains in leukemic and nonleukemic cells. Nuclear matrix-intermediate filament preparations immunostained with a mouse monoclonal antibody against human PML N-terminus (green; upper and lower) and anti-ALL-1 antibody (red; lower). The lower panel is a merged image. Cell samples from acute lymphocytic leukemia at diagnosis (ALL-Dx), acute myelogenous leukemia at diagnosis (AML-Dx), nonleukemic (normal), and acute lymphocytic leukemia during complete remission (ALL-CR).

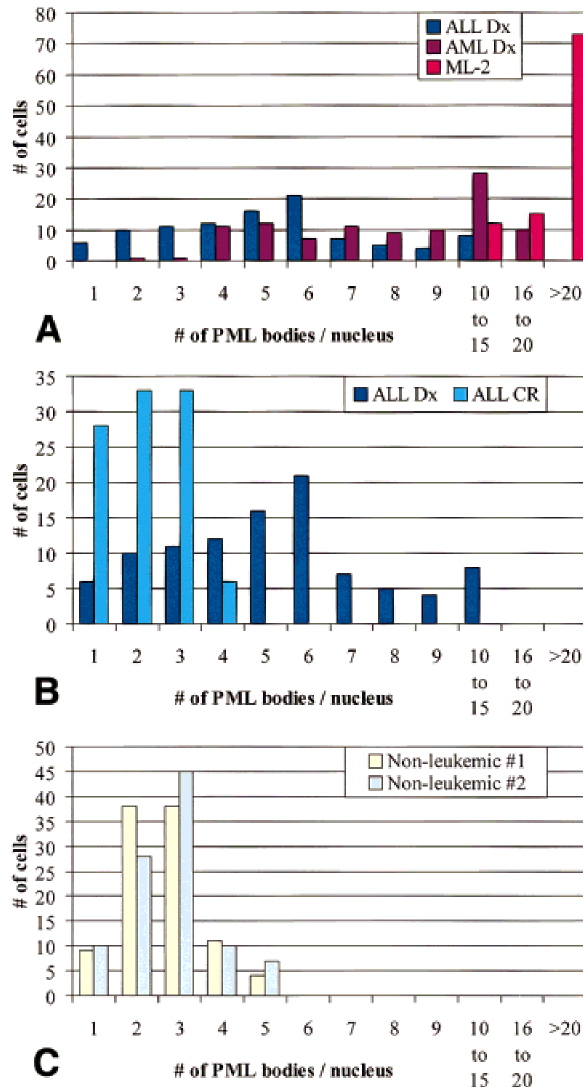


Fig. 6. Distribution of promyelocytic leukemia (PML) nuclear bodies in leukemic and nonleukemic cell samples. A total of 100 nuclei were analyzed for each subject or cell type. Horizontal-axis, number of PML bodies observed per given nucleus; vertical-axis, total number of nuclei observed to have a given number of PML domains. **A:** Acute leukemic cell samples. Acute lymphocytic leukemia at diagnosis (ALL-Dx); acute myelogenous leukemia at diagnosis (AML-Dx); myelogenous leukemia cell line (ML-2). **B:** Acute lymphoblastic leukemia patient cell samples at diagnosis (ALL-Dx) and acute lymphocytic leukemia during complete remission (ALL-CR). **C:** Peripheral blood cells from two nonleukemic subjects.

with a broad range from 1–20 nuclear bodies. We observed 6–15 PML bodies in 58% of cells, and 10% contained 16–20 nuclear bodies (Fig. 6). Most AML-Dx cells contain 4–6 intermediate-size PML bodies, with the remainder being small (Fig. 6). For comparison, we analyzed the leukemic cell line ML-2. These cells contained numerous PML bodies, with 88% having 16 or more nuclear bodies, and none having less than 11 (Fig. 6). We observed 4–10 PML bodies of intermediate to large size. Thus, in all these nonpromyelocytic acute leukemic cells, we observed a significant number of PML nuclear bodies of small and intermediate size.

In contrast with the leukemic cells, all nonleukemic cells examined exhibit a very limited number of PML bodies (Fig. 5). In the nuclei of nonleukemic cells from two human subjects, a mean of 2.6 and a median of 3 PML bodies were seen in each nucleus, with 83% of nuclei containing 1–3 nuclear bodies; nuclei contained no more than 5 PML bodies (Fig. 6). We detected 1–3 of these nuclear bodies of intermediate size, with the remainder being small. Occasionally, 1 or 2 large PML bodies (mean size: 1,050 nm; range: 850–1,200 nm) were present in the nucleus. Large nuclear bodies were generally located near the nuclear center, with smaller PML nuclear bodies distributed throughout the nucleus. These PML immunofluorescence studies reveal that the number of PML nuclear bodies in nonpromyelocytic acute leukemic cells is significantly increased as compared with normal hematopoietic cells.

Strikingly, the cells of the patient with acute lymphoblastic leukemia in remission (ALL-CR) displayed a smaller number of PML bodies compared with that seen in cells from the same patient at time of diagnosis (ALL-Dx) (Fig. 5). In ALL-CR cells, a mean of 2.2 and a median of 2 PML bodies were seen, with 94% of nuclei containing only 1–3 nuclear bodies; no nuclei contained more than 5. The size of the PML bodies in the ALL-CR cells were small to intermediate, with occasional cells displaying large nuclear bodies (Fig. 6). Consequently, our data suggest that when the ALL process has been treated successfully (i.e., complete remission), the number and size of PML nuclear bodies resemble the normal state.

DISCUSSION

We have identified a redistribution of key nuclear regulatory factors in cells of patients with leukemia that are reversed after treatment. In this study, we examined modifications in PML nuclear bodies and other nuclear matrix-associated factors in cells for the first time from patients with nonpromyelocytic acute leukemias. We evaluated subjects and patients with acute leukemias at the time of diagnosis (ALL-Dx and AML-Dx). The ALL-Dx patient was also analyzed at the time of complete remis-

sion (ALL-CR) and was compared with normal subjects and to the human leukemic cell line ML-2. The most striking finding is that the leukemic patients displayed multiple PML nuclear bodies of small to intermediate size, whereas the nonleukemic subjects or the remission patient have a limited number of larger PML bodies. Rearrangement of PML domains has been observed as a consequence of the chromosomal translocation involving chromosomes 15 and 17 [Rowley et al., 1977]. This translocation fuses the PML coding sequences to the RARα which may directly perturb PML trafficking by linking PML to RARα-related subnuclear trafficking signals. Neither patient had cytogenetic abnormalities involving translocations of chromosomes 15 and 17, indicating that the modifications in PML organization observed in this study are not related to genetic lesions involving the PML locus. Recently, we have shown that the subnuclear organization of PML domains can be perturbed independent of rearrangements of PML coding sequences. For example, expression of the AML/ETO fusion protein, which represents a frequently observed translocation product in AML, alters the subnuclear distribution of PML bodies [McNeil et al., 1999]. Thus, there appear to be two classes of chromosomal lesions that modify the intranuclear organization of PML bodies. In the first, the PML gene is the direct target of genetic instability resulting in fusion proteins that have lost competency for fidelity of subnuclear targeting. The second is a transacting mechanism that is mediated by other genetic lesions incurred in multiple leukemia-related genes that influence intranuclear trafficking of PML proteins. In a broader context, our studies and those of others suggest that reorganization of PML bodies is a prominent characteristic of several distinct types of acute leukemias. Furthermore, these observations offer striking examples of morphologic changes reflecting remodeling of nuclear matrix-associated PML domains, as well as reorganization of gene regulatory mechanisms in acute leukemias.

We observed other key differences in nuclear architecture between leukemic and nonleukemic cells. For example, nonleukemic and remission cells (ALL-CR) displayed immunofluorescence patterns that are clearly different from leukemic cells (ALL-Dx, AML-Dx, and ML-2) for many nuclear matrix-associated factors studied (ALL-1, POL II, and AF-9). We observed that ALL-1 subnuclear foci are distinct from SC-35 RNA processing domains and are infrequently associated with Pol II_0 RNA transcription domains. The nuclear matrix associated factors that we studied appeared to undergo changes in their subnuclear distribution in acute leukemic cells that range from subtle to dramatic. Taken together, our data indicate significant modifications in the subnuclear organization of key regulatory factors and are consistent with a leukemia-related nuclear architectural phenotype.

Our results show modifications in ALL-1 distribution in leukemic cells, which occur, independent of rearrangement in the ALL-1 gene locus. The increased representation of the ALL-1 protein in distinct subnuclear foci was not observed in nonleukemic cells or cells in complete remission (ALL-CR). These findings are markedly different from previous studies using leukemic cell lines in which modifications in ALL-1 organization were observed as a direct consequence of chromosomal translocations that result in expression of ALL-1 fusion proteins [Rogaia et al., 1997]. In these studies [Rogaia et al., 1997], wild-type cells were found to contain varying numbers of small to large ALL-1 foci, while cells expressing an ALL-1 fusion protein exhibited an increased number of foci. Subnuclear localization of wild-type ALL-1 was noted to be speckled throughout the nucleus of cells from several leukemia cell lines [Yano et al., 1997]. ALL-1 fusion proteins involving AF-9, similar to the fusion proteins created by the leukemia-related t(11:17) translocation, produced a similar speckled pattern. Changes in the size and number of ALL-1 foci were noted compared with the antibody staining pattern observed in the wild-type cell [Yano et al., 1997]. Although there appears to be considerable heterogeneity in the subnuclear distribution of ALL-1 proteins in leukemias, this ALL-1 distribution is frequently modified relative to normal cells.

The rules that govern nuclear structure-gene expression interrelationships in leukemic cells remain to be defined. Normal hematopoiesis follows an orderly progression of differentiation from immature bone marrow stem cells to mature peripheral blood cells. Acute leukemias represent maturation arrests along the myeloid

or lymphoid pathways of differentiation. However, the morphology of acute leukemic cells do not fully mirror that of their normal counterparts. Chronic leukemias, on the other hand, although harboring malignant elements, appear similar to their normal, mature counterparts. Evaluating nuclear matrix-associated regulatory factors in acute leukemia may permit evaluation of hematologic malignancies to progress beyond the basic morphologic observations that have been the hallmarks of acute leukemia diagnosis for over a century. The advent and practical use of cytogenetic, immunohistochemical, and flow cytometry studies were profound. The subnuclear organization of nuclear matrix-associated regulatory factors offers viable options for clinical diagnosis and treatment of acute leukemias. From a fundamental perspective, insight is provided into tumor-related perturbations of gene regulatory mechanisms that are functionally linked to hematologic malignancies within the three-dimensional context of nuclear matrix architecture.

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