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# The Retinoic Acid Receptor $\alpha$ Gene Is Rearranged in Retinoic Acid-Sensitive Promyelocytic Leukemias

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**All-trans retinoic acid (RA), the active metabolite of vitamin A, has recently been demonstrated to be an efficient alternative to chemotherapy in the treatment of acute promyelocytic leukemia (M3 subtype of the French-American-British cytological classification). Complete remission is obtained by inducing terminal granulocytic differentiation of the leukemic cells. To elucidate whether the effect of retinoic acid on the differentiation of M3 leukemic cells was related to any specific characteristics of its receptor, we analyzed the structure and expression of retinoic acid receptor (RAR) genes in 16 M3 patients. Abnormal RAR  $\alpha$  transcripts were detected in 13 cases. In nine patients, the genomic DNA was analyzed by Southern blotting and evidence for a rearranged RAR  $\alpha$  gene was found generated in four cases. Normal RAR transcripts and germline restriction fragments were found in samples from normal or other leukemic cells, suggesting that this alteration of the RAR  $\alpha$  gene is specifically seen in M3 leukemias. These results suggest that alteration of the retinoic acid receptor alpha may be implicated in M3 leukemogenesis.**

## INTRODUCTION

ACUTE myelogenous leukemias represent a clonal expansion of malignant myeloid cells blocked at a specific stage of differentiation. Intensive chemotherapy achieves remissions in 60–80% of the patients, but long-term survival rate is still very low. Clearly, new therapeutic approaches to these malignancies are required. Since *in vitro* studies have shown that myeloid leukemic cells may be induced to pursue their differentiation (1), clinical and biological research has focused on the screening of leukemic differentiating agents (2–4). In leukemic cell lines, the most potent effects (5–8) have been observed with all-trans retinoic acid (RA), a vitamin A derivative (9). In primary cultures, a specific and consistent response was noted only in the blasts of patients with acute promyelocytic leukemia (M3 leukemia of the French American British cytological classification (10)): these cells differentiated to mature granulocytes expressing differentiation antigens, capable of respiratory burst function and loss of self-renewal (11–17). *In vitro*, differentiation was correlated to the structure and dose of the retinoid used: all-trans RA being more potent than either the 13-cis stereoisomer or the ethyl ester (17–22). Likewise, striking results have been obtained *in vivo* with the all-trans isomer only (22–29). We have shown that oral all-trans RA 45mg/m<sup>2</sup>/day alone, achieved complete remission in 64% of patients with M3 leukemia in a median time of 45 days. Se-

quential blood and bone marrow studies showed progressive differentiation of the promyelocytic leukemic cells to terminally differentiated granulocytes (16, 22, 23).

The exact mechanism by which retinoic acid induces differentiation in human promyelocytic leukemic cells is unknown. Three receptors for retinoic acid have been cloned and identified as members of the nuclear hormone receptor superfamily (30–34). The known ligands of this group of receptors that control the expression of a number of genes implicated in cell differentiation and embryogenesis (35) include steroids and thyroid hormones, vitamin D<sub>3</sub>, retinoids. The genes encoding the retinoic acid receptors (RARs) are differentially expressed in tissues; in particular, RAR  $\alpha$  is highly expressed in hematopoietic cells (36).

We initiated a study to determine whether the preferential effect of RA in promyelocytic leukemic cells was linked to any specific characteristics of a retinoic acid receptor. We report here that M3 leukemic cells have one rearranged RAR  $\alpha$  gene transcribed into at least one novel transcript. We suggest that these molecular abnormalities, found in most patients, relate to the pathogenesis of this disease.

## METHODS

**Cell Samples.** Bone marrow or blood samples from 24 patients with acute leukemia were collected in heparinized tubes. Diagnoses were performed according to the FAB cytological classification criteria (10): AML1: one case; AML2: three cases, AML3: 16 cases, AML4: two cases, AML5: one case, ALL: one case. The hematological characteristics of the 16 M3 patients are summarized in Table 1. All patients involved in this study were advised of the methods and possible risks of marrow aspiration in accordance with institutional guidelines and gave informed consent. Mononuclear cells (MNC) were removed from the interface after Ficoll-Hypaque (Eurobio, Les Ulis, France) density gradient. The leukemic cell fraction was enriched after monocyte adherence to plastic surfaces. The leukemic cells were then immediately prepared for differentiation assay (7, 23) and RNA and DNA extractions. Peripheral blood lymphocytes (PBL) were isolated from heparinized normal blood samples after Ficoll-Hypaque density and adherence of monocytes to the plastic surface. Polymorphonuclear cells were then isolated in the remaining pellet from the red blood cells by density gradient with a concentrated glucose solution (5g/liter glucose and 5 g/liter gelatin). Cells from myeloid human leukemic cell lines (HL-60, U-937) (gift of Dr T. R. Breitman, Los Angeles, CA, and Dr. C. Rosenfeld, Paris, France), maintained in RPMI 1640 supplemented with 15% fetal calf serum, were washed three times in cold sodium phosphate buffer before RNA and DNA extractions.

**Nucleic Acid Extraction and Electrophoresis.** RNA was isolated by the guanidium isothiocyanate-cesium chloride centrifugation method described by Davies et al. (37). Poly-A<sup>+</sup> RNA was obtained by oligo-dT cellulose chromatography. Total RNA (15  $\mu$ g per lane) or poly-A<sup>+</sup> RNA (5 $\mu$ g per lane) was electrophoresed on a 1.1% agarose-formaldehyde gel after denaturation in formamide loading buffer. DNA was extracted from the human samples by proteinase K digestion and repeated phenol/chloroform extractions (38). DNA was then digested

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LEUKEMIA

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with *EcoRI*, *HindIII*, *BglII* at 37°C (Boehringer, Mannheim, F.R.G.) according to the supplier's recommendations. Horizontal gel electrophoresis using 0.6% agarose was performed according to standard procedures with 10 µg of restricted DNA per lane (37). After electrophoresis, DNA and RNA fragments were transferred onto a nitrocellulose filter (Hybond C, Amersham, U.K.) as previously described (38).

**DNA Probes and Hybridization Techniques.** Filters of Northern transfers were hybridized with a cDNA probe spanning the coding region of the RAR alpha gene (see probe 1, Fig. 2A)(31) labeled by random priming to a specific activity of  $5 \times 10^8$ cpm/µg. Specific cDNA probes of the RAR α gene (*EcoRI*-*Kpn* I 5' fragment; positions 1–345 in the cDNA (31)) (probe 2; Fig. 2A) and of the RAR β gene (*EcoRI*-*Xho* 5' fragment; positions 1–551 in the cDNA (32)), which are less than 35% similar, were subcloned in M13mp18 to generate high specific activity ( $>10^9$ cpm/µg) single stranded probes (36) to ensure that the hybridization signal seen with the RAR α cDNA probe corresponded to a RAR α mRNA and not to cross-hybridization with other RARs. Filters of Southern transfers were hybridized with a cDNA probe (probe 1) of the coding region of the RAR α gene and/or with a shorter cDNA probe of a *MstII/BglI* fragment; positions 368–612 (31)(probe 3; Fig. 2A), labeled by random priming to a specific activity of  $5 \times 10^8$ cpm/µg. A rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was used to rehybridize the filters and normalize RNA expression. Prehybridizations and hybridizations were performed in solutions containing 6XSSC (1XSSC = 0.15M sodium chloride, 0.15M sodium citrate), 0.2% sodium dodecyl sulfate and 10X Denhart's solution at 65°C. DNA probes were labeled following the different protocols described above. After hybridization, the filters were washed under stringent conditions in 2X SSC, 0.1% sodium dodecyl sulfate for 10 min at room temperature, followed by 0.2 xSSC, 0.1% sodium dodecyl sulfate at 65°C for 15 min and finally in 0.1xSSC, 0.1% SDS at 65°C for another 15 min. The filters were subjected to autoradiography using Kodak-XAR films at –80°C with an intensifying screen.

## RESULTS

**Transcription of the RAR α Gene in M3 Patients.** Fresh hematopoietic samples from 24 patients with acute leukemia were analyzed. Sixteen were M3 leukemias and are described in Table 1. Abnormal transcripts ranging from 4 kb to 1.1 kb were detected in 13 of these M3 patients (patients 1, 7, 8, 10, 12 (Fig. 1A), 2 (Fig. 1B), 3 (Fig. 1C), and data not shown), along with the two major RAR α transcripts of 3.2 and 2.3 kb found in every human tissue (36). The presence of these abnormal transcripts were confirmed on poly-A<sup>+</sup>RNA of three patients (1, 2, and 7 (Fig. 1B)). These results are summarized in Table 2. The number of abnormal transcripts per sample

varied from 1 to 4 and their intensity, relative to their normal counterparts, was variable. By contrast, a normal pattern of expression was noted in RNA extracted from myelomonoblastic cell lines (HL-60, U-937; Fig. 1B), eight samples from other fresh leukemic subtypes (M1, M2, M4, M5 (Fig. 1C) and data not shown), and five normal hematopoietic cells (polymorphonuclear cells: PMN, monocytes: Mo and peripheral blood lymphocytes: PBL; Fig 1C; and data not shown). Thus, the alteration in the RAR α gene expression is specifically seen in M3 leukemias. Interestingly, compared with other normal hematopoietic cells, RAR α transcripts were consistently more abundant in polymorphonuclear cells after normalization with respect to GAPDH, (Fig. 1C). No expression of RAR β was found in any of these cell samples (data not shown), extending the results obtained in leukemic cell lines (36). The presence of an abnormal transcript did not impede RA's differentiating effect in these cells (Table 2).

**Genomic Structure of the RAR α Gene in M3 Patients.** These results prompted us to analyze the genomic DNA of normal and leukemic samples by Southern blotting. After *EcoRI*, *HindIII*, *BglII* digestion, and hybridization with a cDNA probe consisting of the coding region of the RAR α gene (probe 1), four restriction fragments (RF) were detected in mononuclear cells (MNC; Fig. 2B). Using a shorter *MstII/BglI* cDNA probe, only three of the previous RF were found in polymorphonuclear or mononuclear cells after digestion with the same enzymes (Fig. 2C and D). Eight non-M3 leukemic samples and 12 controls also gave germline RF with either probes (M2: Fig. 2B; M2 and M4: Fig. 2E and data not shown). However, when DNA samples from patients 6 and 7 were analyzed using probe 1, an additional RF was found with all three enzymes (Fig. 2B). DNA from patient 7 was subsequently analyzed with the shorter *MstII/BglI* probe (probe 3) (Fig. 2C). Again the same additional hybridizing RFs were noted in DNA restricted with *EcoRI*, *HindIII*, or *BglII*. Alteration of the restriction pattern of several enzymes makes restriction length polymorphism (RFLP) unlikely. Moreover, it should be emphasized that the probes used have been reported not to exhibit RFLP with these enzymes (39). Such an alteration of the restriction pattern, without disappearance of a normal RF, indicates that one copy of the RAR α gene is rearranged. Interestingly, this gene alteration was detected with a probe that corresponds to the DNA-binding domain, a key region of the protein. Genomic DNA from seven other patients were subsequently analyzed by Southern blotting using probe 3, among which two patients (9 and 10) showed a RAR α gene rearrangement (Fig. 2D). On the other hand, the five remaining patients did not display any gene alteration as assessed by hybridization of an *EcoRI* digest to probe 3 (Fig. 2E and data not shown). Thus, in four of nine M3 patients analyzed, one copy of the RAR α gene is rearranged (Table 2). No obvious correlation was observed between the presence of RAR α gene alteration and clinical parameters such as M3 subtype, in vitro or in vivo response to RA therapy; the small number of patients with a detectable rearrangement precluding statistical analysis.

## DISCUSSION

This study was initiated to analyze the retinoic acid receptors in RA-sensitive leukemic cells. Abnormal RAR α gene transcripts were detected in the cells of 13 of 16 patients with M3 leukemia and was not found in eight other leukemic samples.

**Table 1. Clinical and Hematological Data of the 16 M3 Patients Studied for RAR α Gene Expression and Structure**

Patient	Sex/Age (yrs)	ANLL Subtype*	Disease Status	% Bone marrow Blasts
1	M/81	M3v	de novo	100
2	F/76	M3v	de novo	100
3	F/29	M3	de novo	80
4	M/48	M3v	de novo	95
5	F/31	M3	Induction failure	58
6	M/48	M3	2nd relapse	32
7	F/28	M3	2nd relapse	65
8	M/80	M3v	de novo	100
9	F/37	M3	de novo	99
10	F/73	M3v	de novo	92
11	F/66	M3	de novo	66
12	M/28	M3	de novo	79
13	M/35	M3	de novo	65
14	M/52	M3	1st relapse	65
15	F/30	M3	de novo	91
16	M/47	M3	1st relapse	100

\*FAB morphological classification (10).

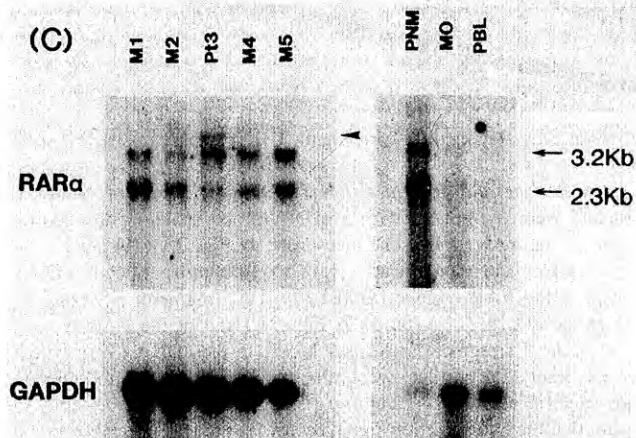
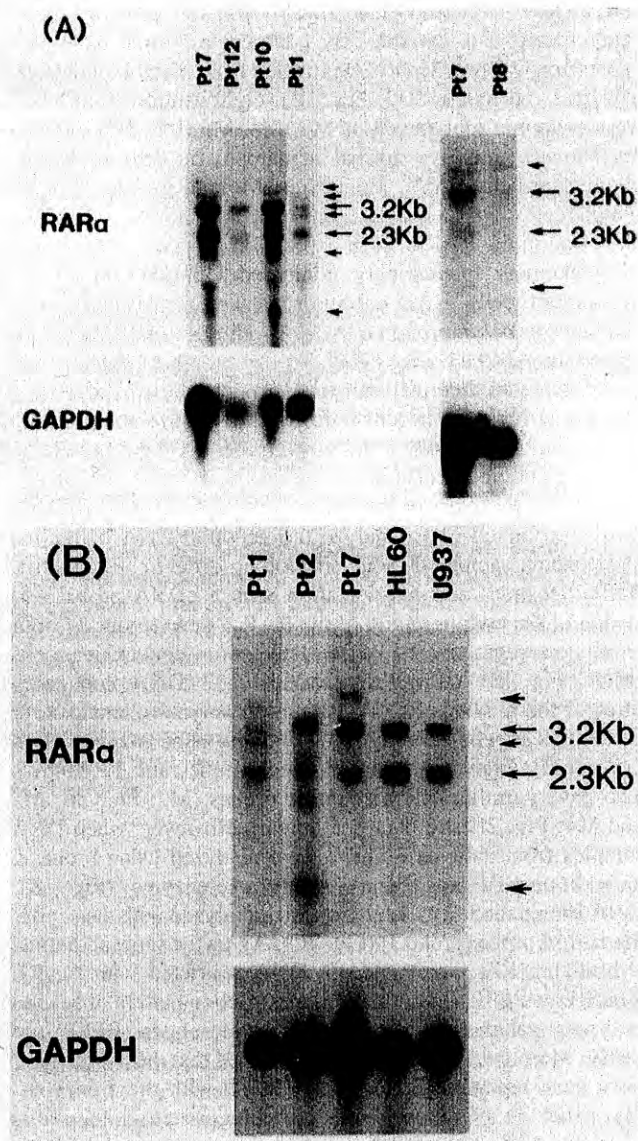


Figure 1. Analysis of RAR alpha gene expression in RNA from normal and malignant hematopoietic cells. A. Total RNA (15  $\mu$ g) from fresh human leukemic cells of M3 patients. B. Poly-A<sup>+</sup> RNA samples of fresh human M3 leukemic cells, and human myeloid leukemic cell lines (HL-60, U-937). C. Total RNA (15  $\mu$ g) from fresh human leukemic cells of patients with different subtypes of AML and from human peripheral blood polymorphonuclear cells (PMN), monocytes (MO), and lymphocytes (PBL). Lanes 1 to 4 of figure 1A were hybridized with a M13 derived single-stranded probe (probe 2); lanes 5 and 6 and filter B and C with a RAR  $\alpha$  cDNA probe (probe 1) labeled by random priming. Blots were rehybridized with a GAPDH probe for RNA expression normalization. The size of the normal transcripts are indicated. Short arrows point to the novel transcripts.

Table 2. Summary of the RAR  $\alpha$  Gene Anomalies detected in the 16 M3 Patients of the Study

Patient	Novel Transcripts	Gene Rearrangement	In Vitro response*	In Vivo response*
1	+	-	+	Early death
2	+	-	+	Early death
3	+	ND	+	CR
4	-	ND	+	NT
5	+	ND	+	Early death
6	+	+	+	CR
7	+	+	+	PR
8	+	ND	+	NT
9	+	+	ND	NT
10	+	+	ND	NT
11	+	ND	+	CR
12	-	-	+	NT
13	+	-	+	CR
14	+	ND	+	CR
15	-	-	+	CR
16	+	ND	+	CR

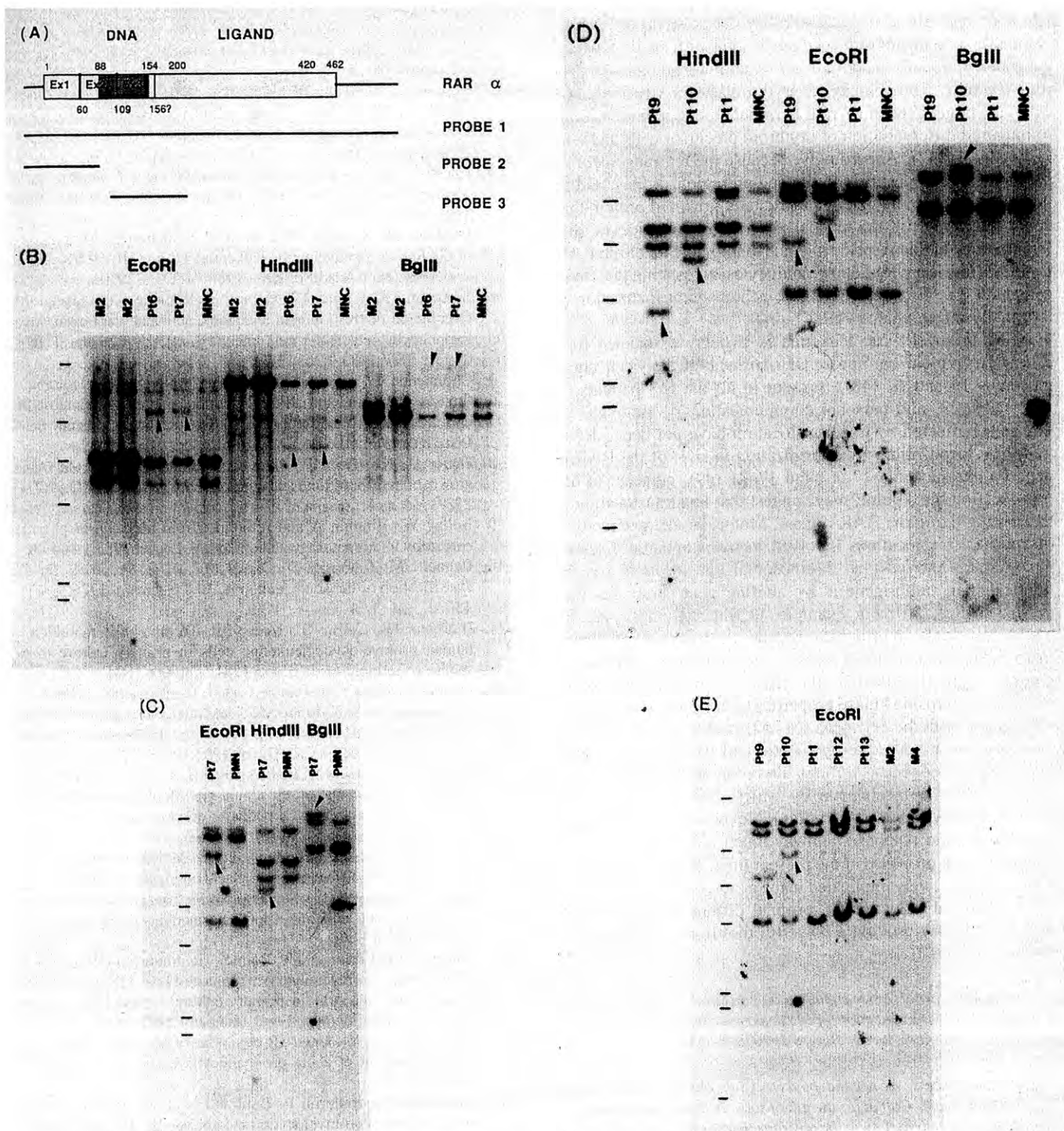
+ = presence; - = absence; ND = not done.

\*In vitro and in vivo response to all-trans RA differentiating effect (described in Refs. 17 and 23). CR = complete remission; TR = transient remission; NT = not treated with all-trans RA.

The subsequent analyses performed on the genomic DNA demonstrated that one copy of the RAR  $\alpha$  gene was rearranged in four of nine tested M3 patients, using the present experimental strategy (enzymes tested and probe used). Three previous studies have addressed the question of the RAR  $\alpha$  expression in M3 leukemia and one of its structures (40-42). None of these reported positive results. This discrepancy may be due to several reasons such as: number of patients, type of probes used, and labeling.

Thus the Southern analysis of this study demonstrates that the RAR  $\alpha$  gene is rearranged in M3 leukemias. This alteration of the RAR  $\alpha$  gene is likely to be the cause of the RAR  $\alpha$  mRNA abnormalities. The data reported in this study raises important questions: Is the rearrangement of the RAR  $\alpha$  gene in M3 patients related to the arrest of granulocytic differentiation and/or to the leukemic process? Is there a cytogenetic basis to this rearrangement?

The RAR  $\alpha$  gene is a nuclear hormone receptor that binds to retinoic acid responsive elements present in the promoters



**Figure 2.** Analysis of RAR  $\alpha$  gene structure in normal and malignant hematopoietic cells. **A.** Schematic representation of the RAR  $\alpha$  cDNA used in these experiments. The DNA and ligand binding domains are shaded. The localization of only exons 1, 2, and 3 are indicated. Thick vertical lines show known exons boundaries (32, 33, and unpublished results). The thin line indicates an exon boundary highly conserved in nuclear receptors (53-55) but presumptive in the case of RAR  $\alpha$ . Amino acid positions are indicated. **B-E.** Genomic DNA of seven M3 leukemic samples, four other leukemic controls, and three normal controls (polymorphonuclear cells (PMN) and mononuclear cells (MNC)), were digested with different restriction enzymes as described in Methods. The filters were hybridized to probe 1 (Fig. 2B) or to probe 3 (Fig. 2, C, D, and E). The position of the sizes of the 23, 9.5, 6.6, 4.5, 2.2, 2 and 0.5 kg fragments of a HindIII-cut phage  $\lambda$  DNA are indicated on the left by harsh marks. Short arrows point to abnormal RFs.

of target genes and thus activates or represses their transcription in a ligand dependent fashion (43). Altered expression of nuclear receptors has been proposed as one of the multiple steps leading to carcinogenesis (44): in a human hepatoma, for ex-

ample, the RAR  $\beta$  gene is truncated by the insertion of a hepatitis B viral genome (45), and the cellular homologue of the retroviral oncogene, *v-erbA* is a thyroid hormone receptor (46). The molecular mechanism of transformation by *v-erbA*

has been recently elucidated and appears to involve binding competition on the thyroid hormone response elements between the transcriptionally inactive *v-erb-A* and the endogenous thyroid receptor. Thus, in avian erythroblastosis virus infected cells, the target gene of thyroid hormones are repressed leading to impaired differentiation of erythroid precursor cells and leukemogenesis (47). Accordingly, because the rearrangement of the RAR  $\alpha$  gene occurred in the coding region, it may lead to the production of an altered, functionally impaired protein that could interfere with the normal program of granulocytic differentiation. In this respect, it is relevant to note that high RAR  $\alpha$  mRNA levels are found in granulocytes, suggesting that these cells have a requirement for RA to achieve their maturation to terminally differentiated cells.

Acute promyelocytic leukemia is strongly associated to a distinct reciprocal chromosomal translocation between chromosomes 15 and 17 (48), (present in all the M3 patients of this study (23, and personal communications)), although, so far, genes affected by this translocation have not been identified. The localization, by hybridization in situ, of the RAR  $\alpha$  gene on chromosome 17 at band 17q21 (49), adjacent to the translocation breakpoint, may suggest that this translocation is the event altering the RAR  $\alpha$  gene. Molecular characterization of specific translocations involved in hematopoietic diseases have already proved that chromosomal translocation may be implicated in tumorigenesis by altering gene structures (reviewed in Ref. 50). For example, in Burkitt lymphoma, the t(8;14) translocation brings *c-myc* under the transcriptional control of the immunoglobulin heavy chain enhancer whereas in chronic myeloid leukemia, the t(9;22) translocation confers constitutive tyrosine kinase properties to the *c-abl* gene product after fusion with the *bcr* locus (51, 52). Similarly, the t(15;17) translocation in acute promyelocytic leukemia may be implicated in leukemogenesis through alteration of the RAR  $\alpha$  gene.

The molecular mechanism by which these leukemic cells show a phenotypic reversion upon RA exposure is obscure and the retinoic acid response pathway much too complicated for a scheme to be proposed at the present time. If the altered RAR  $\alpha$  gene is causatively implicated in the genesis of M3 leukemias, the paradoxical induction of terminal differentiation of these cells by RA may provide a powerful tool to study this malignancy.

*Note added in proof: Since submission of this article, we have shown that the t(15;17) translocation of M3 leukemia fuses the RAR $\alpha$  gene to a novel transcribed locus (Nature 1990;347:558).*

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