

# Rearrangements and Aberrant Expression of the Retinoic Acid Receptor $\alpha$ Gene in Acute Promyelocytic Leukemias

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## Summary

Although acute promyelocytic leukemias (APLs) are consistently associated with a reciprocal chromosome 15;17 translocation, the gene(s) directly affected by the breakpoints have never been isolated. The chromosome 17 breakpoint maps to near the retinoic acid receptor  $\alpha$  (RAR $\alpha$ ) locus. Investigation of 20 APLs and a large series of other neoplastic patients and normal controls revealed RAR $\alpha$  gene rearrangements and aberrant transcripts only in the APL cases. These findings suggest that the RAR $\alpha$  gene is involved in the APL chromosome 17 breakpoint, is implicated in leukemogenesis, and could be used as a marker for identifying leukemic promyelocytes.

Acute promyelocytic leukemia (APL;<sup>1</sup> M3 of the FAB classification) is a distinct, well-characterized clinical and morphological subtype of acute myeloid leukemia (AML) (1, 2). It is cytogenetically distinguished by a reciprocal chromosome 15;17 translocation, which is present in 70–90% of cases and never seen in other AMLs or other types of malignancy (3, 4). Although the high frequency and specificity of the t(15;17), and the fact that it is often the only karyotypic aberration present (4), is strong evidence that it plays a crucial role in the pathogenesis of APL, the gene(s) directly involved in the chromosome 15 and 17 breakpoints have never been identified. We have previously shown that the APL chromosome 17 breakpoint lies between the *c-erbB-2* and the retinoic acid receptor  $\alpha$  (RAR $\alpha$ ) loci (5). We report here the findings of RAR $\alpha$  gene rearrangement and aberrant expression in APLs.

## Materials and Methods

**Molecular Studies.** The K/S, IT, and P/R RAR $\alpha$  cDNA probes were obtained by subcloning portions of the p63 plasmid (6) in

<sup>1</sup> Abbreviations used in this paper: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; BM, bone marrow; RAR $\alpha$ , retinoic acid receptor  $\alpha$ .

the pGEM-3 vector. The K3 probe is a KpnI/BamHI 1.8-kb genomic fragment derived from the RAR $\alpha$ -Fix $\alpha$ 1 phage, isolated from a  $\lambda$ -FixII human genomic library (Stratagene, La Jolla, CA) (unpublished data). It includes the intron located between the RAR $\alpha$  exons 281–430 and 431–574 (exon nucleotide positions refer to the RAR $\alpha$  cDNA sequence of reference 6), Southern and Northern blot analyses were performed according to established procedures (7).

**Cytogenetic Analysis.** Chromosome preparations were obtained from unstimulated bone marrow (BM) cultures as previously described (5).

## Results

**The RAR $\alpha$  K/S cDNA Probe Hybridizes to Extra-fragments in 13 of 20 APL DNAs.** High molecular weight DNA was extracted from the blasts of 20 leukemia patients who met all the morphological criteria for the FAB M3 classification (2). 18 displayed the typical t(15;17), and two had a normal karyotype (Table 1). DNAs were digested with the EcoRI, HindIII, or BamHI restriction enzymes and hybridized to DNA probes representative of different portions of the RAR $\alpha$  cDNA in Southern blot experiments (probes are diagrammatically shown in Fig. 1). The K/S probe representative of the 5' end of the RAR $\alpha$  p63 cDNA revealed additional hy-

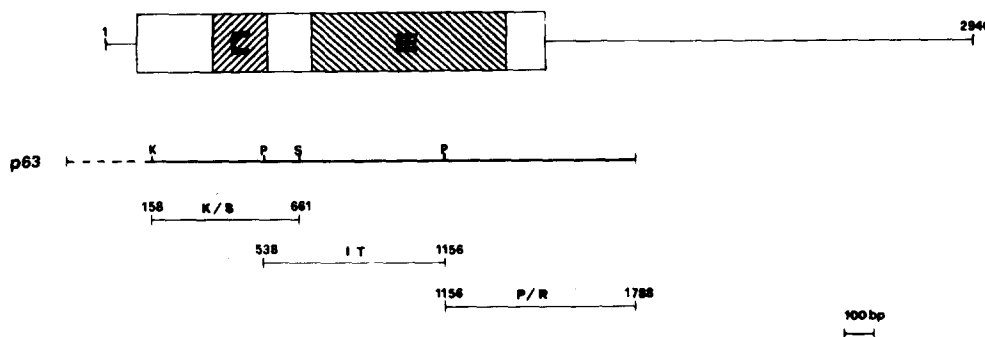
**Table 1.** Synthesis of Cytogenetic, and Southern and Northern Blot Results in APL Cases

APLs	Cytogenetic	Southern			Northern
		E	H	B	
1MPa	t15;17	G	G	G	ND
2MGu	t15;17	G	G	G	4.0; <u>3.8</u> ; 3.5; <u>2.8</u>
3MGa	t15;17	R(13.7)	R(5.5)	G	ND
4MCh	46,XX	G	G	G	ND
5MFe	t15;17	R(16.5)	R(23)	ND	ND
6MMa	t15;17	G	R(17)	ND	4.4; <u>3.8</u> ; <u>2.8</u>
7MPi	t15;17	G	G	G	4.0; 3.5; <u>2.8</u>
8MRo	t15;17	G	G	G	4.0; <u>3.8</u> ; 3.5; <u>2.8</u>
9MSp	t15;17	G	G	R(16.5)	ND
10MMo	t15;17	G	R(9)	R(9.5)	4.4; <u>3.8</u> ; <u>2.8</u>
11MGu	t15;17	G	R(18)	G	4.0; 3.5
12MVa	t15;17	G	G	G	ND
13MTr	t15;17	R(11.5)	R(6.6)	R(7)	ND
14MSa	t15;17	G	R(16)	G	4.4; <u>3.8</u> ; <u>2.8</u>
15MPa	t15;17	R(16.4)	R(6)	G	4.0; <u>3.8</u> ; 3.5; <u>2.8</u>
16RDe	t15;17	G	G	G	ND
17TGp	t15;17	R(3.6)	R(7.5)	G	ND
18RLa	t15;17	R(6)	G	R(9.4)	ND
19T48	t15;17	R(13.3)	R(8.5)	G	ND
20PRe	46,XY	R(9.7)	R(6.5)	R(6.7)	ND

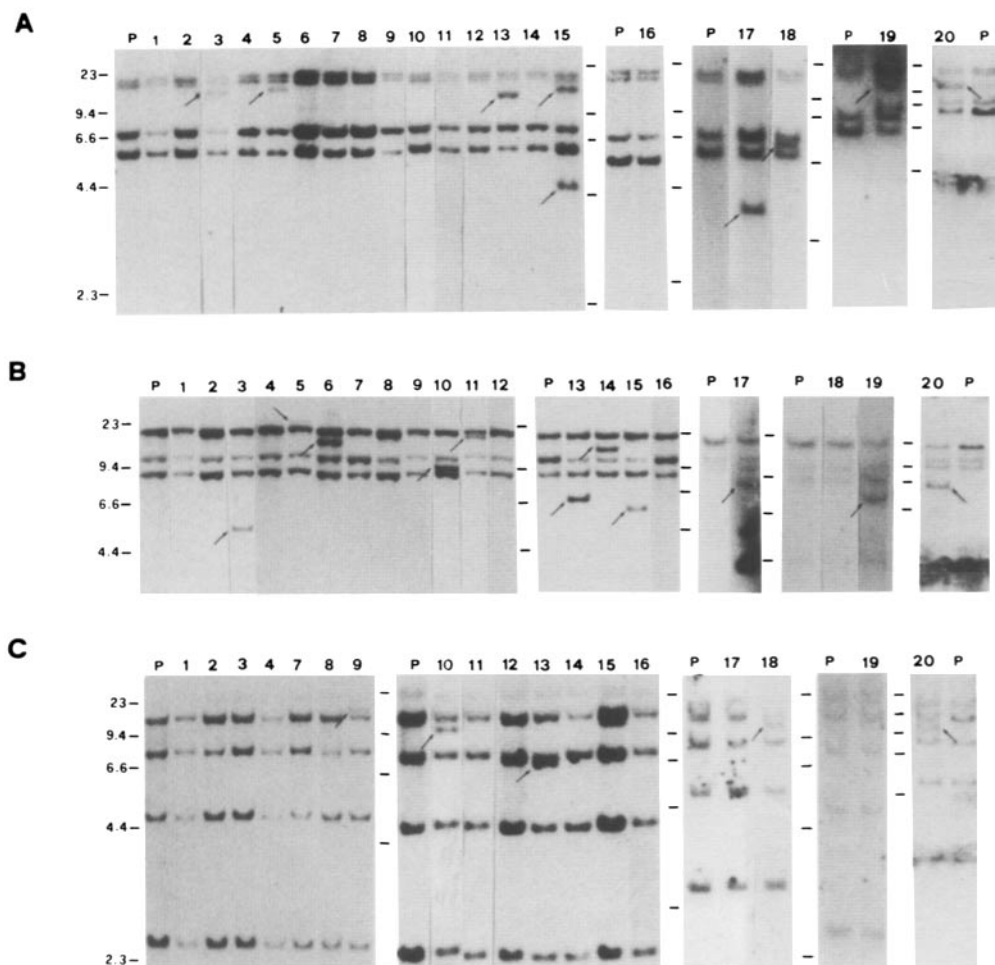
G, germline hybridization fragments; R, rearranged fragments (kilobase length is given between parentheses). Numbers in the Northern column indicate the length of RAR $\alpha$  transcripts (the typical 3.8- and 2.8-kb transcripts are underlined).

bridizing fragments that differed from the placental germline pattern with one (four cases), two (seven cases), or all three (two cases) of the restriction enzymes used (Fig. 2). The additional fragments were of a different size in each case (Table 1). No deviation from the germline configuration was seen in any case with either the RAR $\alpha$  IT or P/R cDNA probes, which contain the intermediate or the 3' portions of the RAR $\alpha$  p63 cDNA, respectively (not shown).

*The K/S-hybridizing Extra-fragments Are Not the Consequence of Genetic Variation.* 19 DNAs from normal PBL and 45 non-APL samples were digested with the EcoRI, HindIII, or BamHI restriction enzymes and hybridized to the K/S DNA probe. All cases showed the germline K/S hybridization pattern with all three restriction enzymes (not shown). In addition, DNA was extracted from the BM cells of two APL patients (3MGa and 14MSa) who had entered morpholog-



**Figure 1.** RAR $\alpha$  DNA probes. A near full-length RAR $\alpha$  cDNA is shown in the top line (1-2940) (10). The block is a schematic representation of the coding region. The DNA (C) and retinoic acid (E) binding domains are shown. A limited restriction map of the p63 plasmid (6) used to generate the RAR $\alpha$  probes is shown in the middle line. The broken line indicates that the sequence of the region has not been reported. Numbers on the bars below indicate the sequence limits of each probe with respect to the RAR $\alpha$  cDNA.

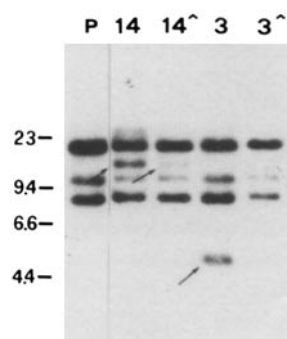


**Figure 2.** Southern blot analysis of the  $RAR\alpha$  gene in APLs. DNAs from 20 APLs were digested with EcoRI (A), HindIII (B), or BamHI (C) restriction enzymes and hybridized to the K/S probe. The cases are identified above the lanes by the same numbers as used in Table 1. P, placenta DNA. Arrows indicate  $RAR\alpha$ -rearranged bands. Molecular weight markers are indicated at the left of each panel.

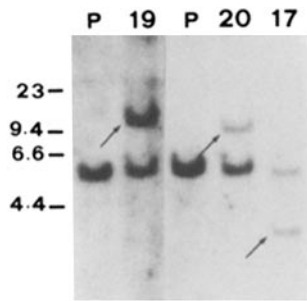
ical remission after conventional chemotherapy. These DNAs, and DNAs extracted from blasts of the same patients before remission, were digested with the HindIII restriction enzyme, hybridized to the K/S probe, and compared. The results showed that the K/S extra-fragments seen in the APL blasts had disappeared in one case (3MGa) and become fainter in the other (14MSa) (Fig. 3). Taken together, these data indicate that the K/S extra-fragments are not the consequence of RFLPs, but of somatic mutations that had occurred in the APL blasts. In the 14MSa case, where the K/S extra-fragment was less intense after chemotherapy, promyelocytes

may have persisted in the BM despite the diagnosis of morphological remission. It is well known that, due to the difficulty of establishing whether promyelocytes are normal or leukemic, it is far from easy to accurately assess remission in APL (8).

*The K/S-hybridizing Extra-fragments Are the Consequence of  $RAR\alpha$  Gene Rearrangements.* The  $RAR\alpha$  gene is a member of the steroid and thyroid hormone receptor family (9). The members of this family share certain structural features: they contain the highly conserved domain that mediates receptor binding to specific sequences of DNA in target genes and the less conserved domain required for ligand binding. As a consequence, the K/S probe, which includes the DNA binding domain, hybridizes to other than  $RAR\alpha$  fragments in Southern blot experiments (10). To exclude that the extra-bands seen in the APL blasts were due to crosshybridizations of the K/S probe, three APL DNAs were hybridized to the K3 probe, which includes intron sequences of the  $RAR\alpha$  locus (see Materials and Methods for a description of the K3 probe). The K3 probe hybridized to a single EcoRI fragment in placenta DNA and to extra-fragments in all three APL EcoRI-digested DNAs (Fig. 4). Note that the K3 EcoRI-hybridizing extra-fragments were of the same size as the K/S



**Figure 3.** Southern blot analysis of the  $RAR\alpha$  gene in two APL cases at diagnosis and during remission. DNAs from placenta (P), BM APL blasts of two patients (14MSa, 14; 3MGa, 3), and BM cells from the same two patients after chemotherapy (14, 3) were digested with the HindIII restriction enzyme and hybridized to the K/S probe. Arrows indicate  $RAR\alpha$ -rearranged bands.



**Figure 4.** Hybridization of the K3 genomic RAR $\alpha$  probe to three APL cases. Three APL (19T48, 19; 20PRE, 20; 17TGp, 17) and placental (P) DNAs were digested with the EcoRI restriction enzyme and hybridized to the K3 genomic RAR $\alpha$  probe.

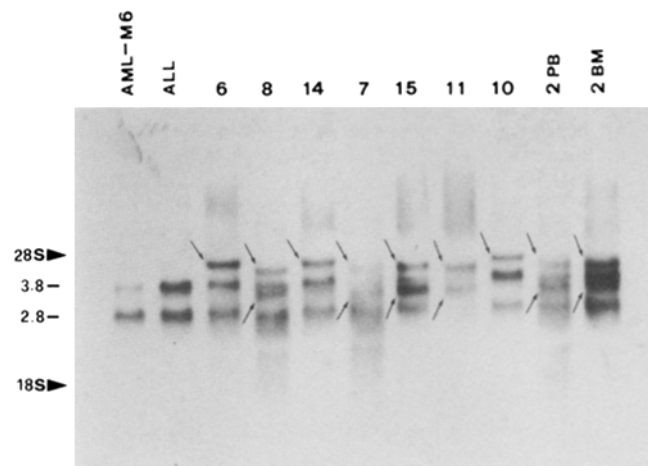
EcoRI extra-fragments in all three APL cases (Figs. 2 and 4). We conclude that RAR $\alpha$  rearrangements are responsible for the K/S-hybridizing extra-fragments.

**RAR $\alpha$  Gene Is Aberrantly Expressed in all APLs.** To determine whether the rearrangements of the RAR $\alpha$  locus interfere with its expression pattern, Northern blot experiments were performed on RNAs extracted from APL blasts. When the I/T and K/S probes were hybridized with RNAs from one AML-M6 and one acute lymphoblastic leukemia (ALL) case, the typical RAR $\alpha$  double transcript (3.8 and 2.8 kb) was seen (see Fig. 5 for IT hybridizations) (11). However, when the RNAs from eight APL cases were hybridized with the same two probes, the two typical RAR $\alpha$  transcripts appeared at an intensity that varied both within and between samples in seven cases. Neither transcript was detected in one case (11MGu). All eight cases exhibited one or two RAR $\alpha$ -aberrant transcripts of three different sizes: 4.4 kb in three cases (6MMa, 10 MMo, 14MSa); and 4.0 and 3.5 kb in five (2MGu, 7MPi, 8MRo, 11MGu, 15MPa) (Fig. 5 and Table 1). The eight APL cases included two (2MGu, 7MPi) that carried the germline configuration of the RAR $\alpha$  gene (Table 1).

**RAR $\alpha$ -aberrant Transcripts Are Specific to APLs.** To investigate the specificity of the aberrant transcripts, the RAR $\alpha$  expression pattern was analyzed in a variety of non-APL RNAs that included both normal (granulocytes, monocytes, lymphocytes, mesothelial cells, amnion cells) and neoplastic cells (21 AMLs, five chronic myelogenous leukemias, six ALL, one chronic lymphoid leukemia, one Burkitt lymphoma, one Hodgkin lymphoma, two lung cancers, two brain tumors, one cervix cancer, and one bladder cancer). Only the typical 3.8- and 2.8-kb transcripts were detected in all cases (not shown).

## Discussion

The RAR $\alpha$  locus maps in the proximity of the chromosome 17 breakpoint of the APL-specific t(15;17) (5, 12). However, the relationship between the chromosome 17 breakpoint and the RAR $\alpha$  locus is not known. 13 of the 20 APL DNA samples analyzed bore rearrangements of the RAR $\alpha$  gene, and eight of eight RNA samples showed aberrant RAR $\alpha$  transcripts. These findings provide insights into both the molecular architecture of the t(15;17) and the pathogenesis of APLs.



**Figure 5.** RAR $\alpha$  gene expression patterns in APLs. RNA samples from BM blasts of eight APLs, one AML-M6, and one ALL case were analyzed by Northern blotting using the IT probe. Both PBL and BM blasts were analyzed for patient 2MGu (2PB, 2BM). The patient numbers are the same as those used in Table 1. Ribosomal RNAs (28S and 18S) are given as size markers. The estimated length (3.8 and 2.8 kb) of the RAR $\alpha$  typical double transcript is also given. Arrows indicate aberrant RAR $\alpha$  transcripts.

As 12 of the 13 rearranged RAR $\alpha$  alleles were identified in APLs with the t(15;17), the rearrangements could be indicative of a breaksite within, or in the proximity of, the RAR $\alpha$  locus. The fact that the remaining case had a normal chromosome 17 at cytogenetic analysis does not contradict this proposal, since the RAR $\alpha$  rearrangement could be associated with submicroscopic chromosome 17 aberrations, similar to those encountered in chronic myeloid leukemias that bear bcr gene rearrangements in the absence of the typical 9;22 chromosome translocation (13). Although no RAR $\alpha$  rearrangements were detected in six APLs with the t(15;17), they could have occurred in regions of the gene not explored by presently available RAR $\alpha$  DNA probes. The finding of aberrant RAR $\alpha$  transcripts in every APL case examined, including two with no RAR $\alpha$  rearrangements, supports the hypothesis that structural abnormalities occur in the RAR $\alpha$  gene of all APLs. The physiological function of the RAR $\alpha$  gene product supports the hypothesis that such abnormalities are involved in the pathogenesis of APLs. RAR $\alpha$  is an intracellular receptor that functions to regulate gene expression in response to the binding of retinoic acid (RA) (6, 10). RA exerts profound effects on embryonic development and cell differentiation in many systems (9), and is also a potent inducer of the differentiation of myeloid leukemia blasts (14), including APL (15).

Finally, the combined Southern and Northern blot analyses of the RAR $\alpha$  gene provides us with a molecular marker for neoplastic promyelocytes. The clinical implications of such a marker are obvious, since it would both allow neoplastic promyelocytes to be unambiguously distinguished from normal promyelocytes at diagnosis, and would permit the monitoring of APL cases during induction chemotherapy; neither of which can be done by presently available methods.

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