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Translocation breakpoint of acute promyelocytic leukemia lies within the retinoic acid receptor α locus

(leukemogenesis/rearrangements)

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ABSTRACT **Acute promyelocytic leukemias (APLs) are characterized by a reciprocal balanced translocation that involves chromosomes 15 and 17 [t(15;17)]. We report the isolation and characterization of one of the two reciprocal break sites and demonstrate that the chromosome 17 breakpoint lies within the retinoic acid receptor α locus. Nucleotide sequencing of the 15;17 cross-over junction on 15q+ showed that the retinoic acid receptor α gene is truncated within its first intron, 370 base pairs upstream from the splicing donor site of exon II. Such a recombination would be expected to generate abnormal RARα mRNA and protein. Southern blot analysis of a number of APLs with chromosome 15- and 17-derived DNA probes revealed similar 15;17 recombinations in the majority of other APLs. Our data are strong evidence that the retinoic acid receptor α gene plays a crucial role in the leukemogenesis of APL.**

Acute promyelocytic leukemia (APL) is a distinct well-characterized clinical and morphological subtype of acute myeloid leukemia (1, 2). It is cytogenetically distinguished by a balanced reciprocal 15;17 chromosome translocation [t(15;17)] that results in the formation of two marker chromosomes: 15q+ and 17q−. The t(15;17) is present in 70–90% of APLs but is never seen in 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) are 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 reported that the APL chromosome 17 breakpoint and the retinoic acid receptor α (RARα) locus map to the same cytogenetic band (5) and that the RARα gene is translocated (5) and rearranged in APLs (6). The RARα gene product is a nuclear receptor that acts as a transcription enhancer in response to the binding of retinoic acid, a physiological metabolite of vitamin A with strong cell-differentiating and morphogenic potential (7–13). We herein report the isolation and sequencing of the recombination site of the RARα gene[‡‡] and chromosome 15-derived material from one APL patient and provide direct evidence that the RARα gene rearrangements in APLs are the consequence of chromosome 17 breaking within the RARα locus.

MATERIALS AND METHODS

Pathologic Samples and Cytogenetic Analysis. Involved bone marrow was collected from untreated patients during the course of diagnostic procedures. Diagnosis of APL was established in each patient by standard clinical and cytologic criteria, according to the FAB (French–American–British) recommendations (2). All 10 APL patients included in this study carried the typical t(15;17).

Isolation of Genomic λ Clones. Clones λ8C, λ2A, and λα1 were isolated from a commercially available genomic library obtained from human embryo lung fibroblasts (WI-38) in the λ-FIX-II vector (Stratagene) by screening with the K/S probe. The λW6A clone was isolated from the WI-38 genomic library by screening with the RH15 DNA probe. Clones λR2 and λR13A were isolated with the HB probe from a genomic library constructed by cloning partially *Mbo* I-digested DNA from patient 10 into the *Xho* I site of the λ-Fix-II vector (Stratagene). Genomic library screening and plaque purification were performed by standard procedures (14). Hybridization was at 37°C in 50% (vol/vol) formamide/3× SSC/5× Denhardt's solution/10% (wt/vol) dextran sulfate containing sonicated and denatured salmon sperm DNA (100 μg/ml). (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0; 1× Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin.) The stringency of the final wash was 0.2× SSC/0.2% SDS at 60°C. Isolated plaques were grown and phage DNA was analyzed by restriction enzyme mapping (14). Inserts were subcloned in plasmid vector pGEM-3 (Promega Biotec) for further analysis.

DNA Sequencing. The 2.7-kilobase (kb) *Kpn* I–*Eco*RI fragment from λα1 and the 2.1-kb *Bgl* I–*Bam*HI fragment from λR13A were subcloned into pGEM-3 (Promega Biotec) and sequenced. The 3′ deletion mutants of all subclones were generated by the Erase-a-Base system (Promega Biotec). DNA sequence analysis was performed on overlapping 3′ deletion mutants by the Sanger dideoxynucleotide-mediated chain-termination method (14). An alignment program was used to analyze nucleotide sequences (MicroGenie Sequence Software, Beckman, CA).

Somatic Cell Hybrids. The somatic cell hybrids were obtained as described (15). Briefly, human lymphocytes or human fibroblast cells were fused with HPRT$^-$ CHO cell lines YH-21 (16) or RJK88 (17), according to established procedures (18). The hybrid clones and subclones were characterized for human chromosome content by cytogenetic analysis based on Q-banding.

DNA Extraction and Southern Blot Analysis. DNAs were prepared by cell lysis, proteinase K digestion, extraction with

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Abbreviations: APL, acute promyelocytic leukemia; RARα, retinoic acid receptor α.
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‡‡The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M61110 and M61111).

phenol, and precipitation with ethanol. DNA (10 μg) was digested with the appropriate restriction endonuclease, electrophoresed in a 0.8% agarose gel, denatured, neutralized, and transferred to nylon membranes. Southern blot filter prehybridization and washings were performed as described for isolation of genomic λ clones.

***In Situ* Hybridization.** The RH15 DNA probe was labeled by nick-translation using [^{3}H]dCTP to a specific activity of 2 $\times$ 10^7 cpm/μg. Probe hybridization to metaphase chromosomes was performed by the method of Gerhard *et al.* (19) as slightly modified (20).

RESULTS

Isolation of the 5′ Portion of the Normal RARα Locus and Mapping of the RARα Gene Rearrangements. Previous Southern blot experiments with the K/S probe, which represents the 5′ portion of the RARα cDNA (Fig. 1*A*), revealed RARα gene rearrangements in 70% of the APL patients studied (6). To map the rearrangements within the RARα locus, the segment containing the corresponding K/S exons was isolated and characterized and APL DNAs were analyzed with genomic RARα probes. Fig. 1*B* gives a limited restriction enzyme map and the exon–intron organization of the 5′ portion of the normal RARα locus as derived from the analysis of the K/S complementary λ8C, λ2A, and $\lambda\alpha$1 clones isolated from a λ phage library prepared from normal human embryonic lung fibroblast DNA. The λ8C, λ2A, and $\lambda\alpha$1 clones overlapped in a 38-kb genomic region and contained six RARα exons, including all K/S sequences. DNAs from 10 APL patients were digested with the *Eco*RI or *Hin*dIII and hybridized to the H18, X5, HB, and E1 RARα genomic DNA probes (Fig. 1*B*) in Southern blot experiments (representative results are shown in Fig. 2). This combination of restriction enzymes and probes allows $\approx$45 kb of the 5′ portion of the RARα locus to be explored. After digestion with *Eco*RI, DNAs displayed rearranged fragments that hybridized to the H18 DNA probe in patients 1–4, to the X5 probe in patients 5–7, and to the HB probe in patients 8–10. Therefore, RARα gene rearrangements cluster in the 6.6-kb *Eco*RI fragment that contains exon I (patients 1–4), in the 12-kb *Eco*RI fragment from intron I (patients 5–7), or in the 5.5-kb *Eco*RI fragment containing exon II (patients 8–10).

Isolation of a Rearranged RARα Gene from a Patient with APL and Demonstration That It Contains One of the Two 15;17 Translocation Break Sites. The origin of the RARα rearrangements was sought by isolating the rearranged RARα gene from APL patient 10 with a t(15;17). A λ phage library was prepared from genomic DNA of patient 10 and screened with DNA probe HB. Two types of clones were obtained. As one type overlapped extensively with the restriction enzyme map of the normal RARα gene, it probably included part of the normal RARα allele from chromosome 17. The restriction

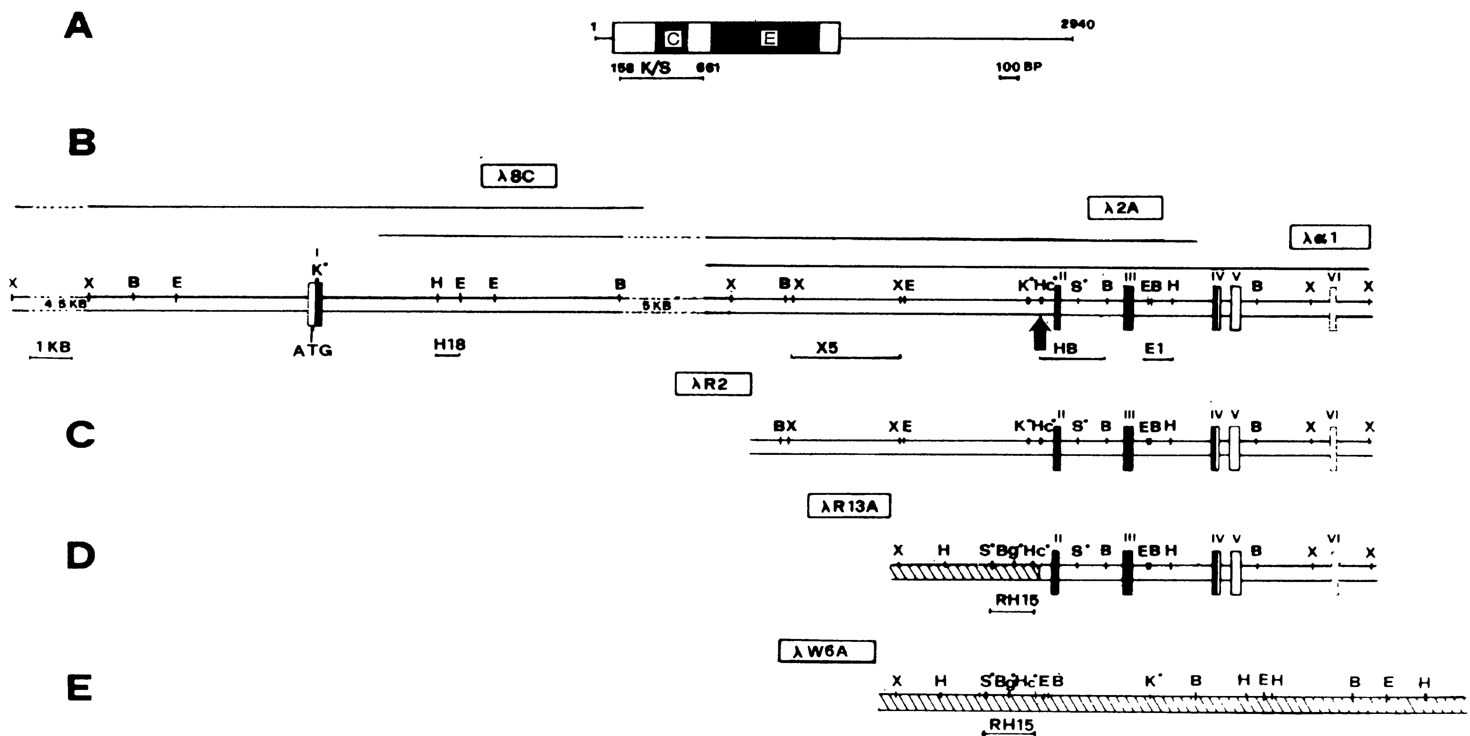

FIG. 1. Schematic representation of the RARα cDNA (*A*), genomic organization of the germ-line RARα locus 5′ portion (*B*), the normal (*C*) and recombined (*D*) RARα genes from one APL patient, and the normal counterpart of the chromosome 15-derived sequences involved in the RARα recombinations (*E*). (*A*) A near full-length RARα cDNA, from Giguère *et al.* (8), is shown (residues 1–2940). The block is a schematic representation of the coding region. DNA (domain C) and retinoic acid (domain E) binding domains are also shown. Numbers (158–661) on the bar below indicate the sequence limits of the K/S probe with respect to the RARα cDNA. BP, base pairs. (*B*) The restriction enzyme map of the 5′ portion of the germ-line RARα gene is derived from the analysis of the λ8C, λ2A, and $\lambda\alpha$1 clones, as indicated. The arrow shows the site of the chromosome 17 breakpoint in APL patient 10. Boxes indicate RARα exons as characterized by homology with RARα cDNA probes and by nucleotide sequence analysis (unpublished results). Exon VI was mapped by homology with RARα cDNA probes. Solid exons indicate the corresponding K/S sequences. The 5′ border of exon I was mapped by comparing the genomic (unpublished data) and cDNA (8) sequences and, therefore, does not necessarily correspond to the 5′ end of this exon. Consequently, the exon numbering is provisional. Exon I contains the translation initiation codon (ATG), exons II and III encode domain C of the putative RARα protein, and exon IV is the most 5′ exon encoding domain E. (*C* and *D*) The restriction enzyme map of the normal and rearranged RARα genes from APL patient 10 is derived from the analysis of the λR2 and λR13A clones, respectively. The shaded section of the λR13A map refers to chromosome 15-derived sequences. (*E*) The restriction enzyme map of the normal counterpart of the chromosome 15-derived sequences involved in the RARα recombinations is derived from the λW6A clone. Probes used in this study are indicated as solid lines beneath the maps. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; X, *Xba* I; I, *Sma* I; K, *Kpn* I; Hc, *Hin*cII; Bg, *Bgl* I. The dots after K, S, Hc, and Bg restriction enzyme sites indicate that they were only partially mapped.

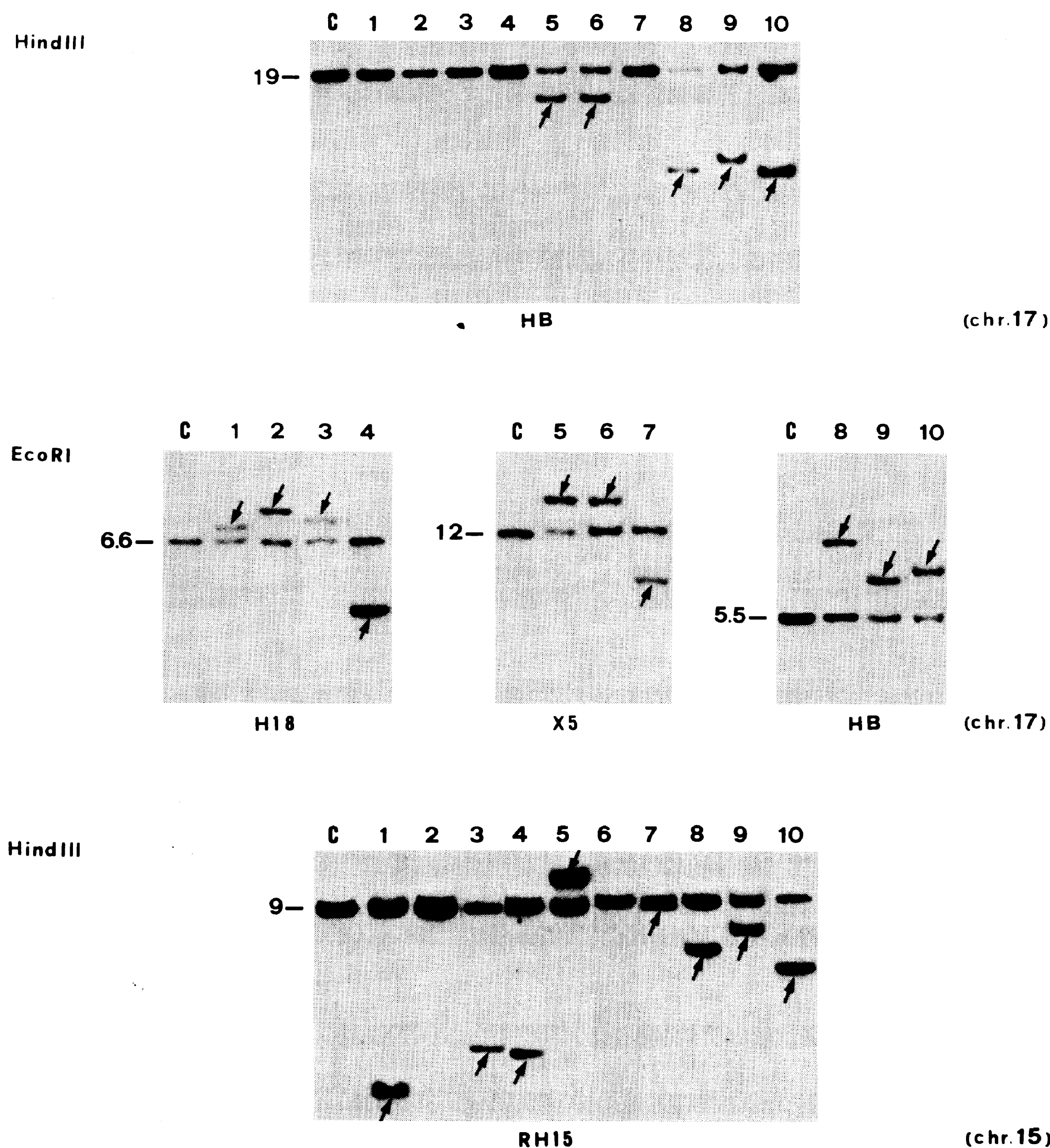

FIG. 2. Southern blot analysis of APL DNAs with RARα and chromosome 15-derived probes. APL DNAs (lanes 1–10; numbers correspond to patient's numbers used in text) and human placental DNA (lane C) were digested with the appropriate restriction enzymes and hybridized to the indicated DNA probes. The chromosome (chr.) origin of each probe is indicated. Arrows indicate rearranged bands. The length of germ-line hybridizing fragments (in kb) is given on the left.

enzyme map of the representative λR2 clone is shown in Fig. 1*C*. The second type of clones contained rearranged RARα sequences reminiscent of those disclosed in Southern blot analysis of total chromosomal DNA. Restriction enzyme map analysis and hybridization to RARα genomic and cDNA probes of one representative clone (λR13A) showed that it matched the germ-line RARα gene 3′ of the RARα exon II but diverged 5′ of RARα exon II (Fig. 1*D*). Nucleotide sequencing of the 2.1-kb *Bgl* I–*Bam*HI restriction fragment containing the site of divergence from the normal RARα locus revealed a stretch of 1670 nucleotides identical to germ-line RARα exon II and its 5′ and 3′ flanking sequences but interrupted within RARα intron I, 370 base pairs upstream from the splicing donor site of exon II (Fig. 3). To determine the chromosome origin of the nonhomologous RARα sequences in clone λR13A, human–rodent somatic cell hybrids were probed with the RH15 *Sma* I–*Hin*cII DNA fragment, representative of the nonhomologous sequences, in Southern blot experiments. Only hybrids containing the human chromosome 15 hybridized to the RH15 probe (Fig. 4*A*). *In situ* hybridization of the same probe to human metaphases from normal peripheral blood lymphocytes showed that RH15 sequences map to the 15q23–q24 chromosome bands (Fig. 4*B*), which correspond to the APL chromosome 15 breakpoint region (4). Thus these data indicate that the λR13A clone contains one of the two break sites of the 15;17 translocation from APL patient 10. As earlier *in situ* hybridization studies of RARα probes to APL chromosomes demonstrated that the RARα gene is translocated to the derivative chromosome 15q+ (5), the λR13A clone probably derives from the chromosome 15q+.

Isolation of the Normal Counterpart of the Chromosome 15 Sequences Involved in the 15;17 Recombination and Identification of a Chromosome 15 Breakpoint Cluster Region. Molecular cloning of one of the two break sites of the t(15;17) in APL patient 10 showed that the chromosome 15;17 recombination occurred between RARα intron I and the region of chromosome 15 identified by the RH15 DNA probe (Fig. 1*D*). Having demonstrated that this type of chromosome 17 recombination is common to the majority of APLs (Fig. 2), the next question we asked was whether the type of chromosome 15 recombination found in patient 10 is common to all APLs. The answer was sought by isolating the normal counterpart of the chromosome 15-derived sequences identified in patient 10 and mapping the chromosome 15 breakpoint from the other 9 APL patients by Southern blot analysis using the RH15 DNA probe. A human genomic library from normal embryo lung fibroblasts was screened with the RH15 probe. The restriction enzyme map of a representative RH15 complementary clone (λW6A) is shown in Fig. 1*E*. To map the translocation breakpoints within the isolated chromosome 15 DNA region, the RH15 DNA probe was hybridized to *Hin*dIII-digested APL DNAs. The RH15 probe hybridized to a *Hin*dIII 9-kb fragment in human germ-line DNA and to one rearranged fragment in 8 of the 10 APL patients tested (Fig. 2). This suggests that the APL chromosome breakpoints cluster within the *Hin*dIII 9-kb

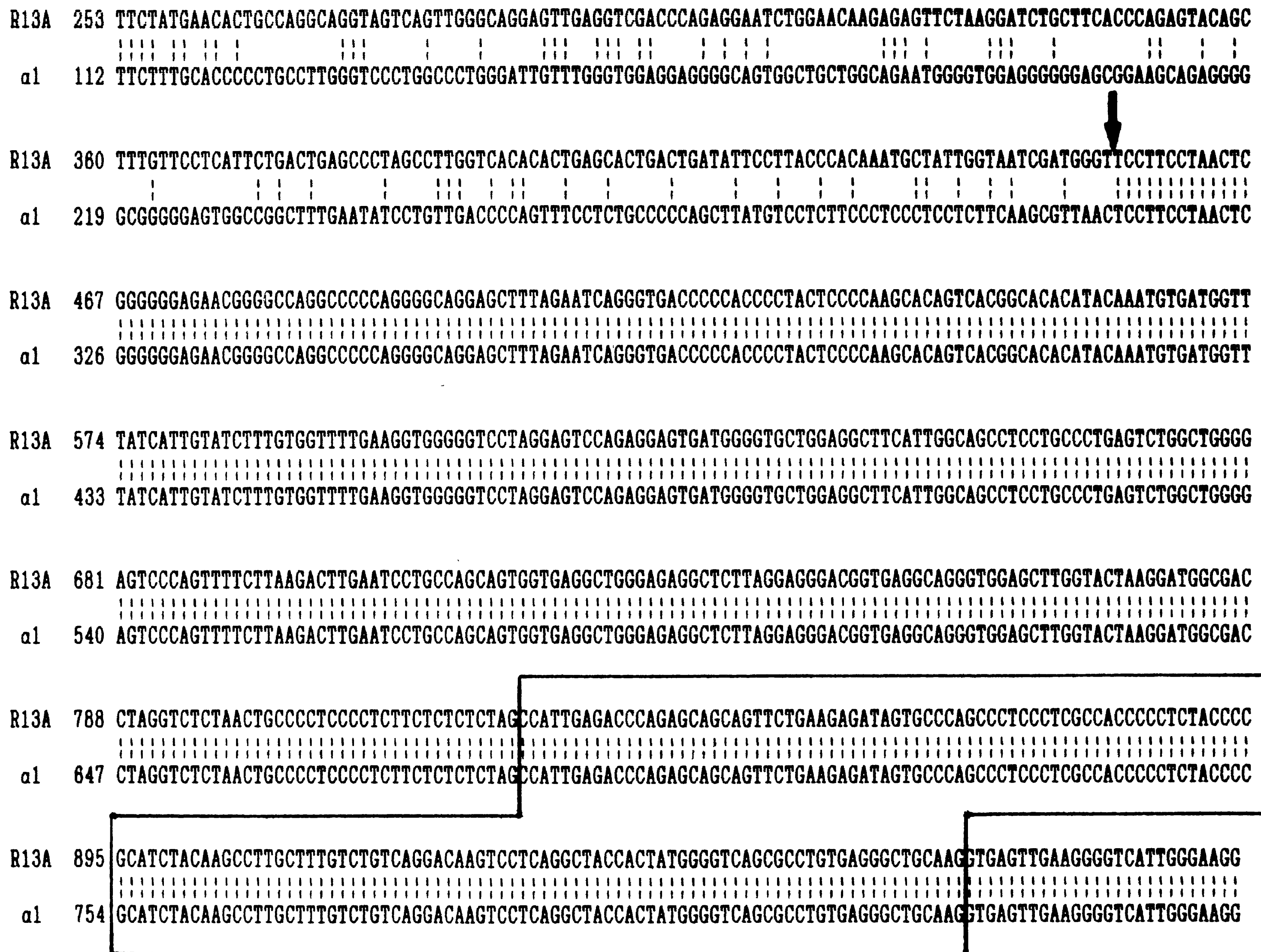

FIG. 3. Nucleotide sequencing of the chromosome 15;17 cross-over junction (R13A) and comparison with the normal RARα gene (α1). The 2.7-kb *Kpn* I–*Eco*RI fragment of the normal RARα gene (α1), which contains exons II and III, and the 2.1-kb *Bgl* I–*Bam*HI fragment of the rearranged RARα gene (R13A), which spans exon II and the point of divergence from the normal counterpart, were sequenced. Comparison of the two sequences showed (*i*) that the fragment from nucleotide 454 to the 3′ end of the *Bgl* I–*Bam*HI (R13A) segment was identical to that from nucleotide 312 to the end of segment *Kpn* I–*Eco*RI (α1) and (*ii*) that there was no homology between the stretches 5′ to nucleotide 452 of the *Bgl* I–*Hin*dIII fragment and nucleotide 311 of the *Kpn* I–*Eco*RI fragment. The arrow signals the divergence point. The box delimits exon II that is present in both segments. Only part of the sequence is given. Vertical lines indicate nucleotide identity.

RH15 homologous DNA fragment and recalls the picture seen in the chromosome 9 breakpoint cluster region in the t(9;22) of chronic myelogenous leukemias (21). When DNAs from patients 2 and 6, which were shown to be germ-line after *Hin*dIII digestion and RH15 probe hybridization, were analyzed with the *Eco*RI and *Bam*HI and the RH15 probe, they were found to be germ-line (data not shown). These findings indicate that the chromosome 15 breakpoint in a minority of APL patients lies outside the above mentioned chromosome 15 breakpoint cluster region.

DISCUSSION

This investigation provides direct evidence that the chromosome 17 breakpoint of APL patient 10 t(15;17) lies within the RARα locus and Southern blot data suggests that this occurs in all patients. The translocation site in patient 10 occurred at the 3′ end of the RARα intron I, 370 base pairs upstream from the splicing donor site of exon II, and separated the first coding exon (exon I) from the rest of the gene. In such a recombination, the translocated RARα gene would not be expected to generate a normal mRNA and our finding of aberrant RARα mRNAs in all APL patients analyzed supports this prediction (6). Molecular isolation and nucleotide sequence of cDNAs representative of the APL RARα aberrant transcripts showed that they resulted from the synthesis of a fusion mRNA composed of chromosome 15-derived sequences and a RARα segment from exon II to the 3′ end (unpublished results). The translocated RARα gene could, however, still contain the exons encoding the domains required for receptor binding to specific sequences in target genes (domain C) and the retinoic acid binding (domain E). We have, in fact, observed that domain C is encoded by exons II and III and domain E is encoded by exons from exon V onward (unpublished results). Based on these findings, one could speculate that the 15;17 recombination in APL patient 10 would lead to the production of a RARα protein whose N-terminal region is deleted and substituted by the chromosome 15-encoded amino acids. As the corresponding N-terminal domain of steroid receptors has been shown to be involved in transactivation and target gene specificity (22–

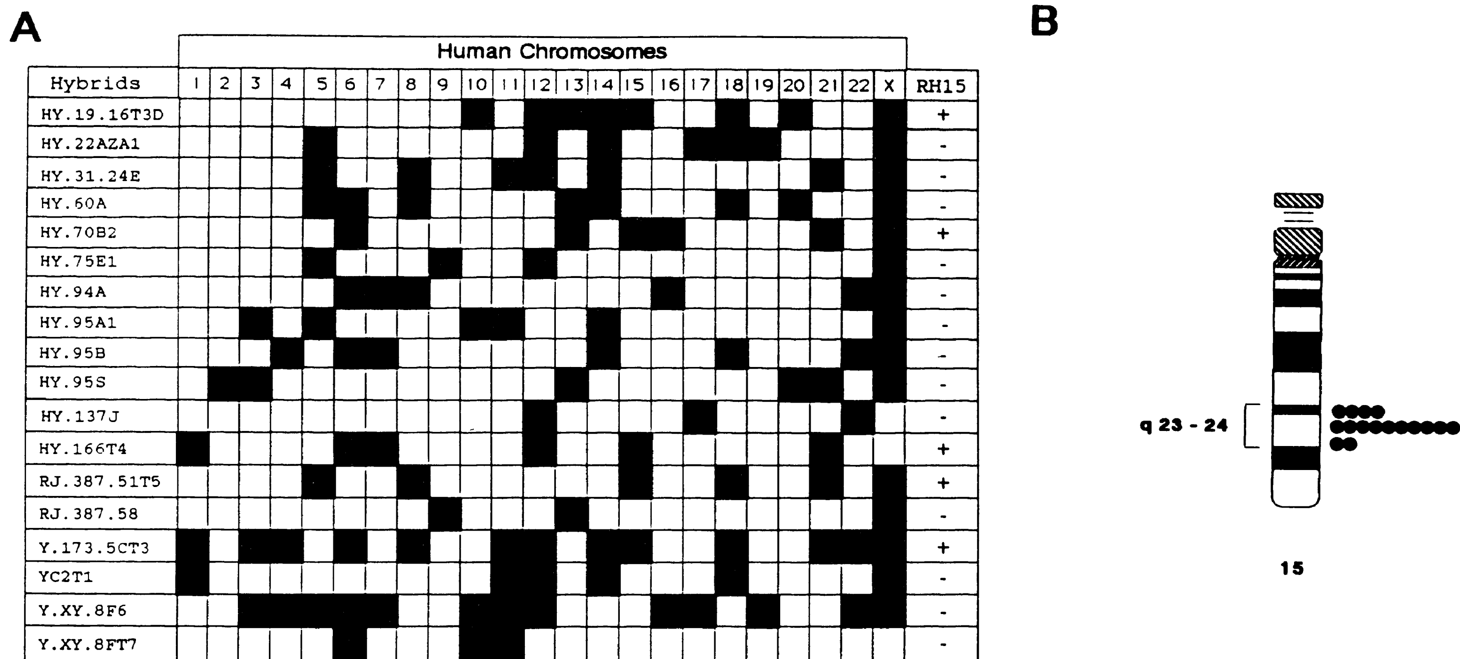

FIG. 4. Chromosome mapping of the RH15 sequences. (*A*) Presence of human RH15 homologous sequences in a panel of human–rodent hybrid clones. A solid square indicates that the hybrid clone named in the left column contains the indicated chromosome. An open square indicates that the hybrid clone has lost the human chromosome identified. + and − refer to the results of Southern blot hybridization of *Eco*RI-digested DNAs to the RH15 DNA probe. (*B*) Idiogram of chromosome 15 showing the distribution of RH15 hybridization grains in 122 normal metaphases. The RH15 plasmid was hybridized to human metaphases obtained from normal peripheral blood lymphocytes. One hundred twenty-two of 160 metaphases analyzed contained a total of 138 labeled sites. Sixteen grains (12% of total grains) were observed on chromosome 15 ($P < 0.001$); all were clustered on the q23–q24 region. There was no statistically significant labeling at any other karyotypic site.

24), the APL receptor N-terminal domain would, presumably, influence both functions. The fact that Southern blot data revealed similar chromosome 17 (10/10 rearranged within or in the proximity of RARα intron I) and 15 (8/10 rearranged with a chromosome 15-derived DNA probe) recombinations in most APL patients tested suggests that the aberrant RAR protein is a common feature of APLs.

The identification of a translocation site within the RARα gene is strong evidence that it is implicated in the leukemogenesis of APL. The RARα protein is an intracellular receptor that functions to regulate gene expression in response to retinoic acid (7–9). Retinoic acid not only exerts potent effects on development (it may be the natural morphogen for generating digit pattern in the chicken limb bud) but also is indispensable for the control of growth and differentiation of many cell systems, including myeloid blast cells (10). The most dramatic and consistent effects have been noted in APLs where retinoic acid stimulates extensive terminal granulocytic differentiation of fresh blasts (25). A plausible hypothesis is that the abnormal product from the translocated RARα gene interferes with the normal differentiation program of promyelocytes and so contributes to the transformed APL phenotype.

Note. Since this report was submitted, two papers have been published that report the isolation of the APL translocation breakpoint and demonstrate that it occurs within the RARα locus (26, 27).

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1. Sultan, C., Heilman-Gouaulyme, M. & Tulliez, M. (1973) *Br. J. Haematol.* **24,** 255–260.
2. Bennett, J. M., Catowsky, D., Daniel, M. T., Flandrin, G., Galton, D. A. G., Gralmick, H. R. & Sultan, C. (1976) *Br. J. Haematol.* **33,** 451–458.
3. Rowley, J. D., Golomb, H. M. & Dougherty, C. (1977) *Lancet* **i,** 549–554.
4. Mitelman, F. (1988) *Catalog of Chromosome Aberrations in Cancer* (Liss, New York), 3rd Ed.
5. Longo, L., Donti, E., Mencarelli, A., Avanzi, G., Pegoraro, L., Alimena, G., Tabilio, A., Venti, G., Grignani, F. & Pelicci, P. G. (1990) *Oncogene* **5,** 1557–1563.
6. Longo, L., Pandolfi, P. P., Biondi, A., Rambaldi, A., Mencarelli, A., Lo Coco, F., Diverio, D., Pegoraro, L., Avanzi, G., Tabilio, A., Zangrilli, D., Alcalay, M., Donti, E., Grignani, F. & Pelicci, P. G. (1990) *J. Exp. Med.* **172,** 1571–1576.
7. Petkovich, M., Brand, N. J., Krust, A. & Chambon, P. (1987) *Nature (London)* **330,** 444–450.
8. Giguère, V., Ong, E. S., Segui, P. & Evans, R. M. (1987) *Nature (London)* **330,** 624–629.
9. Evans, R. M. (1988) *Science* **240,** 889–895.
10. Breitman, T. R., Selonick, S. E. & Collins, S. J. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 2936–2940.
11. Collins, S. J., Robertson, K. A. & Mueller, L. (1990) *Mol. Cell. Biol.* **10,** 2154–2163.
12. Tickle, C., Summerbell, D. & Wolpert, L. (1982) *Nature (London)* **296,** 564–566.
13. Sporn, M. B. & Roberts, A. B. (1983) *Cancer Res.* **43,** 3034–3040.
14. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
15. Rocchi, M., Roncuzzi, L., Santamaria, R., Archidiacono, N., Dente, L. & Gianni, R. (1986) *Hum. Genet.* **74,** 30–33.
16. Rosenstraus, M. & Chasin, L. A. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 493–497.
17. Fuscoe, J. C., Fenwick, R. G., Ledbetter, D. H. & Caskey, C. T. (1983) *J. Mol. Cell. Biol.* **3,** 1086–1096.
18. Davidson, R. L. (1976) *Somatic Cell Genet.* **2,** 165–176.
19. Gerhard, D. S., Kawasaki, E. S., Carter Bancroft, F. & Szabo, P. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 3755–3759.
20. Donti, E., Lanfrancone, L., Huebner, K., Pascucci, A., Venti, G., Pengue, G., Grignani, F., Croce, C. M., Lania, L. & Pelicci, P. G. (1990) *Hum. Genet.* **84,** 391–395.
21. Groffen, J., Stephenson, J. R., Heisterkamp, N., de Klein, A., Bartram, C. R. & Grosveld, G. (1984) *Cell* **36,** 93–99.
22. Tora, L., Gronemeyer, H., Turcotte, B., Gaub, M. P. & Chambon, P. (1988) *Nature (London)* **333,** 185–188.
23. Hollenberg, S. M., Giguere, V., Segui, P. & Evans, R. M. (1987) *Cell* **49,** 39–46.
24. Kumar, V., Green, S., Stack, G., Berry, M., Jin, J. R. & Chambon, P. (1987) *Cell* **51,** 941–951.
25. Breitman, T. R., Collins, S. J. & Keene, B. R. (1981) *Blood* **57,** 1000–1004.
26. de Thè, H., Chomienne, C., Lanotte, M., Degos, L. & Dejan A. (1990) *Nature (London)* **347,** 558–561.
27. Borrow, J., Goddard, A. D., Sheer, D. & Solomon, E. (1990) *Science* **249,** 1577–1580.