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Characterization of a Fusion cDNA (RARA/*myl*) Transcribed from the t(15;17) Translocation Breakpoint in Acute Promyelocytic Leukemia

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A nonrandom chromosomal translocation breakpoint, t(15;17)(q22;q21), is found in almost all patients with acute promyelocytic leukemia (APL). Most of these breakpoints occur within the second intron of the retinoic acid receptor-alpha (RARA) gene. We screened a cDNA library of APL and have identified and sequenced a cDNA transcribed from the t(15;17) translocation breakpoint. The 5′ end of cDNA p1715 consists of 503 bp of the RARA exon II sequence. A 1.76-kb cDNA without homology to any known gene available in GenBank was found truncated downstream. This cDNA sequence was assigned to chromosome 15 by dot blot hybridization of the flow cytometry-sorted chromosomes. We designate this fusion cDNA RARA/*myl*, which is different from *myl*/RARA reported by de The et al. (H. de The, C. Chomienne, M. Lanotte, L. Degos, and A. Dejean, Nature (London) 347:558–561, 1990). This result demonstrates that the two different types of hybrid mRNA can be transcribed from this breakpoint. We screened a non-APL cDNA library and identified a 2.8-kb *myl* cDNA. This cDNA is able to encode a polypeptide with a molecular weight of 78,450. Alternative splicing of the *myl* gene which resulted in *myl* proteins with different C terminals was found. Southern blot analysis of the genomic DNA isolated from 17 APL patients by using the *myl* DNA probe demonstrated that the *myl* gene in 12 samples was rearranged. Northern (RNA) blot analysis of RARA gene expression in two APL RNA samples showed abnormal mRNA species of 4.2 and 3.2 kb in one patient and of 4.8 and 3.8 kb in another patient; these were in addition to the normal mRNA species of 3.7 and 2.7-kb. The *myl* DNA probe detected a 2.6-kb abnormal mRNA in addition to the normal mRNA species of 3.2, 4.2, and 5.5 kb. Using the polymerase chain reaction, we demonstrated that both RARA/*myl* and *myl*/RARA were coexpressed in samples from three different APL patients. From this study, we conclude that the t(15;17) translocation breakpoint results in the transcription of two different fusion transcripts which are expected to be translated into fusion proteins.

Acute promyelocytic leukemia (APL) is a clonal proliferation of abnormal promyelocytes. A nonrandom chromosomal translocation breakpoint, t(15;17)(q22;q21), occurs in almost all patients with APL (39). A significant number of these patients achieve complete clinical remission after all-*trans* retinoic acid treatment (9, 12, 26). Since the retinoic acid receptor-alpha (RARA) gene has been localized to chromosome 17q21, in proximity to the breakpoint of APL (33), this gene was thought to play a critical role in the pathogenesis of APL. Indeed, Borrow et al. (3), de The et al. (15), and our studies (10) have shown that the RARA gene is involved in the t(15;17) chromosomal abnormality that results in transcription of abnormal mRNAs. It was thought that the majority of the breakpoints were in the first intron of the RARA gene. However, when Brand et al. (5) characterized the promoter region of the RARA gene, an additional exon was found 12 kb further upstream of exon I. Therefore, it is now clear that most APL breakpoints occur within the second intron of the RARA gene.

The retinoic acid receptor (RAR) is a member of a nuclear receptor superfamily that includes the thyroid and steroid hormone receptors. Four different forms of the human RAR have been identified, and its cDNA has been cloned and characterized (6, 22, 30, 32, 37). Retinoic acid (RA) can induce differentiation of a number of cell lines (29, 38, 44, 46), including the human leukemia cell line HL-60. RA is a morphogen, as has been demonstrated in chick limb experiments (7). Recent evidence indicates that purified RAR protein can interact directly with RA without cellular RA-binding protein (42). Therefore, RAR may be directly involved in mediating the RA concentration in the nucleus. Since the translocation breakpoint occurs within the RARA gene in APL, this may partially impair the normal function of RA and result in disruption of normal differentiation. In addition, chromosome translocations can produce potentially oncogenic fusion proteins (8, 25).

We report the characterization of a cDNA transcribed from the t(15;17) breakpoint region. This hybrid cDNA consists of exon II of the RARA gene and is truncated downstream by an unknown sequence from chromosome 15 (the *myl* gene). This hybrid cDNA (RARA/*myl*) differs from the *myl*/RARA fusion gene reported by de The et al. (15) in that the arrangement of the two genes involved in the breakpoint is reversed. Therefore, this finding demonstrates that the nonrandom translocation breakpoint in APL can result in the transcription of two different types of hybrid mRNAs, as shown by the transcription activities of the RARA and *myl* genes. We have also identified and characterized the *myl* cDNA.

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MATERIALS AND METHODS

Construction of a cDNA library from poly(A) RNA of an APL patient and screening for the hybrid mRNA. Total RNA was isolated from leukemic promyelocytes of an APL patient by using guanidinium isothiocyanate as described previously (11). Poly(A) RNA was selected by the method of Aviv and Leder (1). Ten micrograms of poly(A) RNA was used to synthesize cDNA by the method of Gubler and Hoffman (23); the cDNA was ligated into a lambda Zap II vector (Stratagene, La Jolla, Calif.). The library was screened by using an exon II probe (10) of RARA cDNA in accordance with established procedures (41). Dideoxy-DNA sequencing of a plasmid subclone was performed by the method of Zagursky et al. (48). DNA sequence analysis and comparison with the GenBank sequences were performed by using MicroGenie software (Beckman Instruments, Inc., Fullerton, Calif.).

Southern and Northern (RNA) blot analysis. Genomic DNA was isolated from human leukemic cells by an established method (10). Ten micrograms of total genomic DNA was restriction digested to completion and electrophoresed on a 0.75% agarose gel. DNA samples were transferred to a Nytran membrane in accordance with the manufacturer's instructions (Schleicher & Schuell, Inc., Keene, N.H.). Poly(A) RNA from patient samples was selected as described above. Two micrograms of the poly(A) RNA was denatured by glyoxalation, electrophoresed on a 1.2% agarose gel, and transferred to a Nytran membrane. The filters were hybridized to ^{32}P-labeled probes (10^9 cpm/μg of DNA), washed, and exposed to Kodak XAR-5 film.

Chromosomal assignment. Chromosomes from diploid human lymphoblastoid cells (GM-130A) or rodent-human hybrid cell lines (35) were sorted in an EPICS V flow cytometer and spotted onto nitrocellulose filters. Each spot contained 30,000 chromosomes, except the 9-12 spot from GM 130A, which contained 120,000 chromosomes. Chromosomes 9, 10, 11, and 12 were also sorted individually from hybrid cell lines. The chromosome-specific DNA on the filters was denatured and hybridized as previously described (14). A polymerase chain reaction (PCR)-amplified, 1.2-kb cDNA fragment isolated from the unknown portion of p1715 (nucleotides 504 to 1704 [see Fig. 2]) and not including the RARA sequence was ^{32}P labeled to 10^9 cpm/μg of DNA and hybridized to the filters as described previously (19). Filters were washed and exposed to Kodak X-ray film. Since the Southern blot analysis described above indicated that both *Eco*RI and *Bgl*II digestions yield a single hybridizing band, this unknown gene was likely a single human gene. Therefore, cross-hybridization to DNA sequences from other chromosomes was not expected.

PCR. Synthesis of cDNA and PCR amplification of total RNA were performed as previously described (17, 28), with some modifications. A 20-μl reverse transcriptase reaction mixture contained 1 mM of deoxynucleoside triphosphates (dNTP), 20 U of RNasin (Promega Corporation, Madison, Wis.), 20 ng of primer, 5 μg of total RNA, and 1× PCR buffer (Perkin Elmer-Cetus). The reaction was started by adding 200 U of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Gaithersburg, Md.). After incubation at room temperature for 10 min, the reaction mixture was transferred to 42°C for 60 min, boiled for 5 min, and quick chilled on ice. An 80-μl PCR reaction mixture was added (0.3 mM dNTP, 2.5 mM $MgSO_4$, 0.2 μg of each of the two primers, 50 mM KCl, 20 mM Tris-HCl, pH 8.4, and 1 mg of nuclease-free bovine serum albumin). The mixture was overlaid with mineral oil, and PCR was performed for 35 cycles in a thermal cycler (Perkin Elmer-Cetus). Each cycle was carried out at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min.

Sequencing of the PCR-amplified DNA fragments. DNA fragments were first purified by passing them through a Centricon-100 (Amicon, Beverly, Mass.) to remove primers. Approximately 0.1 μg of the purified DNA was used in a 25-cycle PCR using a single primer (24). After PCR was performed, the single-stranded DNA was purified by using a Centricon-100 and used directly in the DNA-sequencing reaction. DNA sequencing was performed in the presence of about 0.5 μg of single-stranded DNA and 20 ng of internal primer (R1 or R4) by using the DNA-sequencing kit obtained from the United States Biochemical Corporation (Cleveland, Ohio).

Nucleotide sequence accession number. The nucleotide sequences of p1715 and pMYL211 have been submitted to GenBank and have been assigned the accession numbers M73958 and M73840, respectively.

RESULTS

Identification and characterization of the fusion cDNA RARA/*myl* encoded from the t(15;17)(q22;q21) breakpoint of APL. The cDNA library constructed from poly(A) RNA isolated from the promyelocytes of an APL patient (see Fig. 7, lane 6) was screened with the RARA second-exon probe (10). Several cDNA clones were obtained. A brief restriction map of each clone was determined to further identify clones that were different from the RARA cDNA. One of the clones, p1715, had a restriction pattern different from that of RARA (Fig. 1). This clone was further characterized by DNA sequence analysis. Results revealed that the 503-bp DNA sequence on the 5′ end of p1715 matched exactly the second exon of the RARA cDNA sequence. However, DNA sequence homology stopped once it passed the second exon/intron splice junction (Fig. 1B).

Figure 2 shows the nucleotide sequence of p1715, with the junction of the exon II/intron II splice site (31) indicated by an arrowhead. The 1.75 kb of the cDNA sequence truncated downstream of the RARA second exon is unrelated to the RARA gene. This cDNA sequence was compared with the GenBank sequences, and no homology to any gene sequence was identified. Intron II of the RARA gene splits the ACC codon of amino acid 60 into exon II and exon III after translocation. The first nucleotide of the amino acid codon AAT is derived from the RARA cDNA. According to our DNA sequence analysis, the hybrid mRNA can encode a polypeptide of 178 amino acids, with a predicted molecular weight of 18,500. The other two reading frames of the *myl* cDNA fail to translate a long polypeptide. At least 1.5 kb of the DNA sequence remains beyond the stop codon. In order to understand whether the correct reading frame of the *myl* mRNA is retained after the translocation, it was necessary to characterize the normal *myl* cDNA.

Isolation and characterization of the normal cDNA of the *myl* gene. A cDNA library was constructed from poly(A) RNA isolated from an AML blast sample without the t(15;17) translocation and with high-level expression of *myl* mRNA. The cDNA library was screened with the *myl* cDNA probe (a 1.2-kb PCR-amplified DNA fragment of plasmid p1715 nucleotides 504 to 1704 [Fig. 2]). A 2.8-kb cDNA clone, pMYL211, was identified and completely sequenced. This cDNA clone may be close to full length, since the major

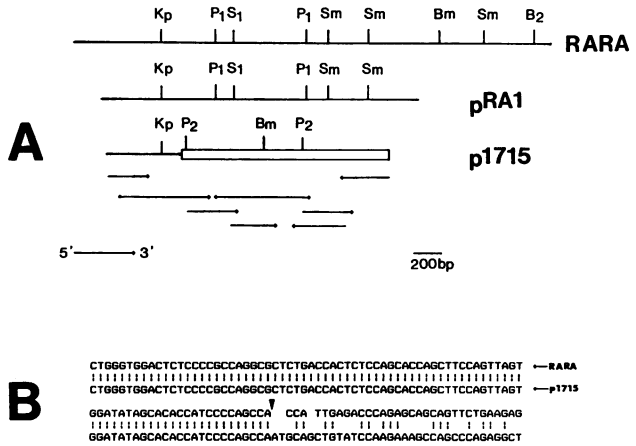

FIG. 1. Restriction map of RARA and p1715 cDNAs and DNA sequencing strategy of p1715. (A) The restriction map of RARA was derived from the report of Giguere et al. (22). pRA1 is a RARA cDNA clone of 2.1 kb. A poly(A) tail was found 850 bp upstream of the reported RARA cDNA (22). DNA sequencing of p1715 was performed by dideoxy sequencing of the plasmid DNA by using the Sequenase sequencing kit obtained from United States Biochemical. Restriction endonuclease sites indicated are abbreviated as follows: Kp, *Kpn*I; P1, *Pst*I; S1, *Sac*I; Sm, *Sma*I; Bm, *Bam*HI; B2, *Bgl*II; P2, *Pvu*II. The open box region represents the gene sequence derived from chromosome 15. (B) DNA sequences of RARA and p1715 cDNAs indicating the region of sequence homology between the two sequences. Homology stops after the exonII/exonIII splice junction. The inverted triangle indicates the RARA exon II/exon III splice site, which was determined as described by the report of Leroy et al. (31).

band of the *myl* mRNA was 3.2 kb according to the Northern blot analysis (see below).

Figure 3 shows the DNA sequence and the predicted amino acid sequence of the pMYL211 cDNA. This cDNA is able to translate a polypeptide of 713 amino acids with a predicted molecular weight of 78,450. The solid vertical arrow at nucleotide 1042 indicates the fusion junction of the RARA/*myl* cDNA, as shown in Fig. 2. The amino acid sequences predicted from the cDNA sequences of pMYL211 and p1715 downstream of the fusion junction are identical. This demonstrates that the correct amino acid-coding frame of the *myl* mRNA was utilized by the fusion transcript. However, as shown in Fig. 2, a termination codon (TGA) which is not found in the *myl* cDNA (Fig. 3) was found at nucleotides 856 to 858 of p1715. DNA sequence comparison between pMYL211 and p1715 at this region indicates an 8-bp insert in the p1715 cDNA (nucleotide 832 to 840 [Fig. 2]) which is not present in the pMYL211 cDNA. The presence of this additional DNA fragment alters the amino acid-coding frame. To determine whether this 8-bp insert is the result of cloning artifact, a set of oligonucleotide primers (RAR1, nucleotides 441 to 460, and mylD6, nucleotides 1142 to 1167 [Fig. 2]) was designed for PCR amplification by using total RNA isolated from the APL patient. The PCR-amplified DNA fragment was subcloned into pUC18 and sequenced. The result of this experiment demonstrated that the 8-bp insert is not a result of a cloning artifact, since cDNA sequences with and without the 8-bp sequence were found (data not shown). These same oligonucleotide primers were used to amplify the genomic DNA of the APL patient and of an acute myelogenous leukemia patient without the t(15;17) breakpoint. A DNA fragment of about 1.5 kb was amplified. This PCR-amplified DNA fragment was subcloned into pUC18. DNA sequences from both ends of the DNA fragment were determined. As shown in Fig. 4, line 3, the 8-bp insert was derived from the intron immediately adjacent to the intron/exon splice junction. Interestingly, a sequence that agreed with the splice donor consensus in seven of nine places was found at the 5′ end of the 8-bp DNA fragment. This result suggests that alternative splicing is responsible for generating different *myl* mRNAs with or without the eight additional bases. This differential splicing could result in the translation of different *myl* proteins with considerably different C terminals.

By comparing the cDNA sequences of pMYL211 and p1715, we have also found a 144-bp DNA sequence between nucleotides 1113 and 1257 of pMYL211 (Fig. 3) which is not found in p1715. This DNA fragment encodes a short polypeptide of 48 amino acids and does not affect the open reading frame of the *myl* mRNA. According to the Southern blot analysis shown in Fig. 6, a single band was detected by the *myl* probe with *Bgl*II and *Hin*dIII digestion. This suggests that the *myl* gene represents a single gene in the human haploid genome. It is likely that this 144-bp additional DNA sequence is also the result of alternative splicing. Differences in the nucleotide sequences of the cDNAs of p1715 and pMYL211 were also found at the 3′ ends. The polyadenylation signal AATAAA was found 16 bp upstream of the poly(A) tail of the pMYL211 cDNA. However, the 3′ end of the p1715 cDNA extended 160 bp further downstream of the poly(A) tail of pMYL211. Interestingly, the last 22 nucleotides upstream of the poly(A) tail of pMYL211 (Fig. 3), including the polyadenylation signal AATAAA, did not match the 3′ end sequence of p1715 (Fig. 2). It is possible that the mismatched portion of p1715 is the result of a cloning artifact. An oligonucleotide primer set was synthesized in accordance with the DNA sequences of nucleotides 1868 to 1888 and 2230 to 2250 (antisense) of p1715 (Fig. 2). PCR amplification of the genomic DNA resulted in a DNA fragment of 380 bp. Sequence analysis of the DNA fragment demonstrated that the genomic DNA fragment exactly matched the p1715 sequence. Furthermore, the 22-bp nucleotide sequence present in the *myl* cDNA was not found within this exon. It is possible that this 22-bp sequence is the result of alternative splicing. We are currently in the process of studying the genomic organization of the *myl* gene. Information obtained from this study should help us to understand the mechanism of alternative splicing occurring within the *myl* gene.

RARA/*myl* is transcribed from the t(15;17) breakpoint of APL. Since the RARA gene has been assigned to chromosome 17 (33), if the cDNA clone p1715 is a hybrid transcript encoded from the t(15;17) translocation breakpoint, the DNA fragment downstream of the RARA gene should be localized to chromosome 15. Figure 5 shows the dot blot hybridizations of the flow cytometry-sorted chromosomes. The 1.2-kb DNA sequence derived from the unknown portion of p1715 clearly hybridizes to the chromosome 15 spot. Two separate sets of dot blot filters were used in the experiment, and similar hybridization signals to chromosome 15 were observed for each. This demonstrates that the unknown cDNA truncated downstream of the RARA gene exon II is derived from chromosome 15. Therefore, the cDNA of p1715 is transcribed from the t(15;17) translocation breakpoint of the APL patient.

Rearrangement of the *myl* gene in acute promyelocytic leukemia. Southern blot analysis of 17 DNA samples isolated from APL patients with the t(15;17)(q22;q21) chromosomal

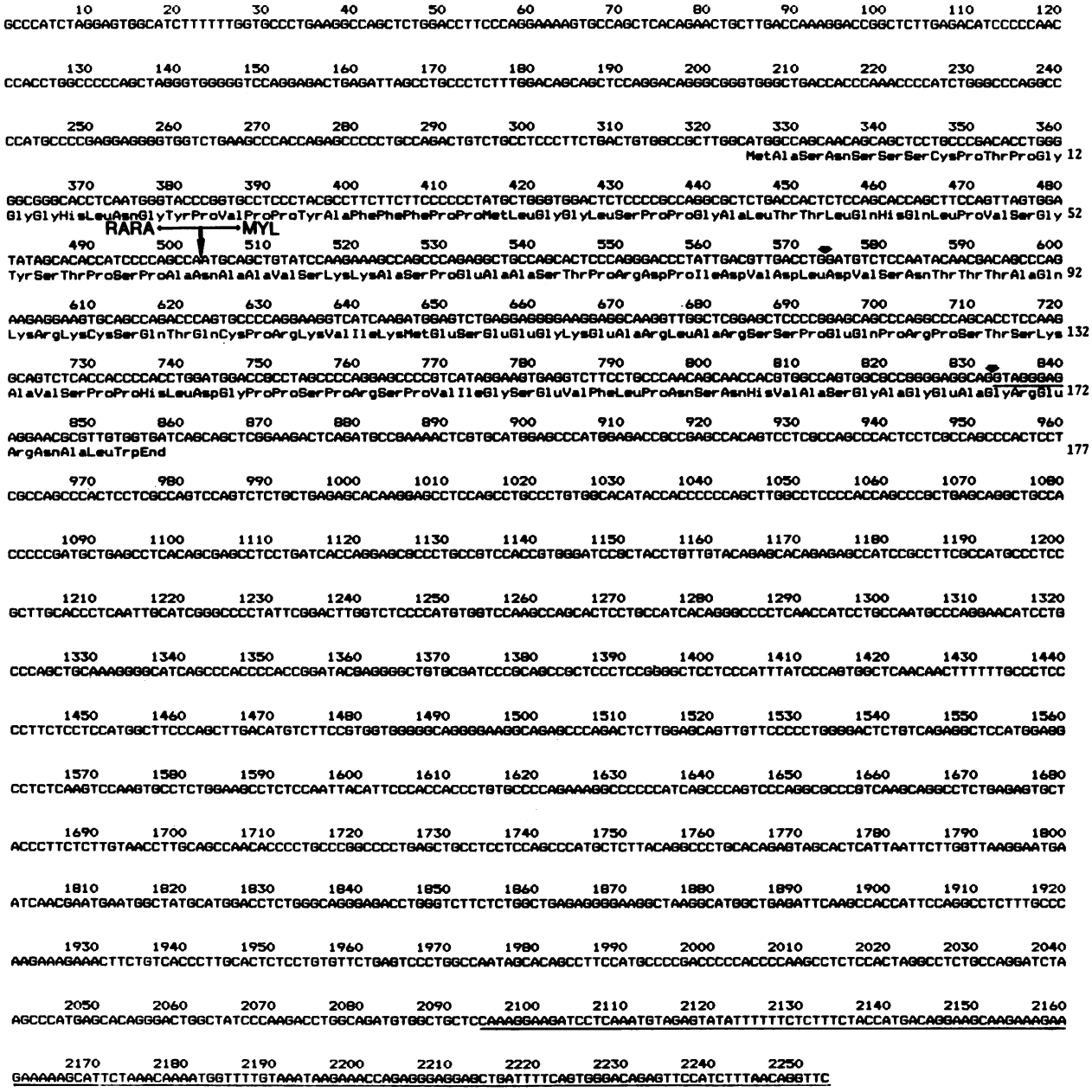

FIG. 2. Nucleotide sequence of p1715. The DNA-sequencing strategy of p1715 is outlined in Fig. 1. The fusion junction of the hybrid message is located between nucleotides 503 and 504, as indicated by the arrow. MYL and RARA designate the cDNA regions derived from chromosome 15 and chromosome 17, respectively. The two arrowheads indicate sites of alternative splicing.

abnormality was performed to determine whether there was rearrangement of the unknown gene sequence. A PCR-amplified 1.2-kb DNA fragment (nucleotides 504 to 1704 [Fig. 2]) was used as the probe for hybridization. As shown in Fig. 6A and B, 12 of the 17 APL DNA samples show rearranged bands. DNAs from HL-60 cells and patients without the t(15;17) translocation breakpoint did not show rearranged bands. This result indicates that translocation breakpoints in chromosome 15 cluster within the same gene. In addition, samples from patients 9 and 10 (Fig. 6B) represent DNAs isolated from the same patient in acute phase and in remission. A rearranged band was found in the acute-phase DNA sample but not in the remission sample. This result suggests that the rearrangement is not a result of restriction fragment length polymorphism.

Expression of the *myl* gene sequence in human leukemia samples. The *myl* cDNA was used to hybridize to the Northern blots of RNA samples of different leukemia patients. As shown in Fig. 7A, mRNA species of 5.5, 4.2, and 3.2 kb were detected. In one APL patient, an additional mRNA of 2.6 kb (lane 6) was also detected. The *myl* cDNA appears to be expressed at high levels in all leukemic cell types. The 3.2-kb mRNA is the predominant band and appeared in all samples tested. No additional bands were observed in the other APL RNA sample (lane 7). Since the additional band in lane 6 did not appear to be a sharp band,

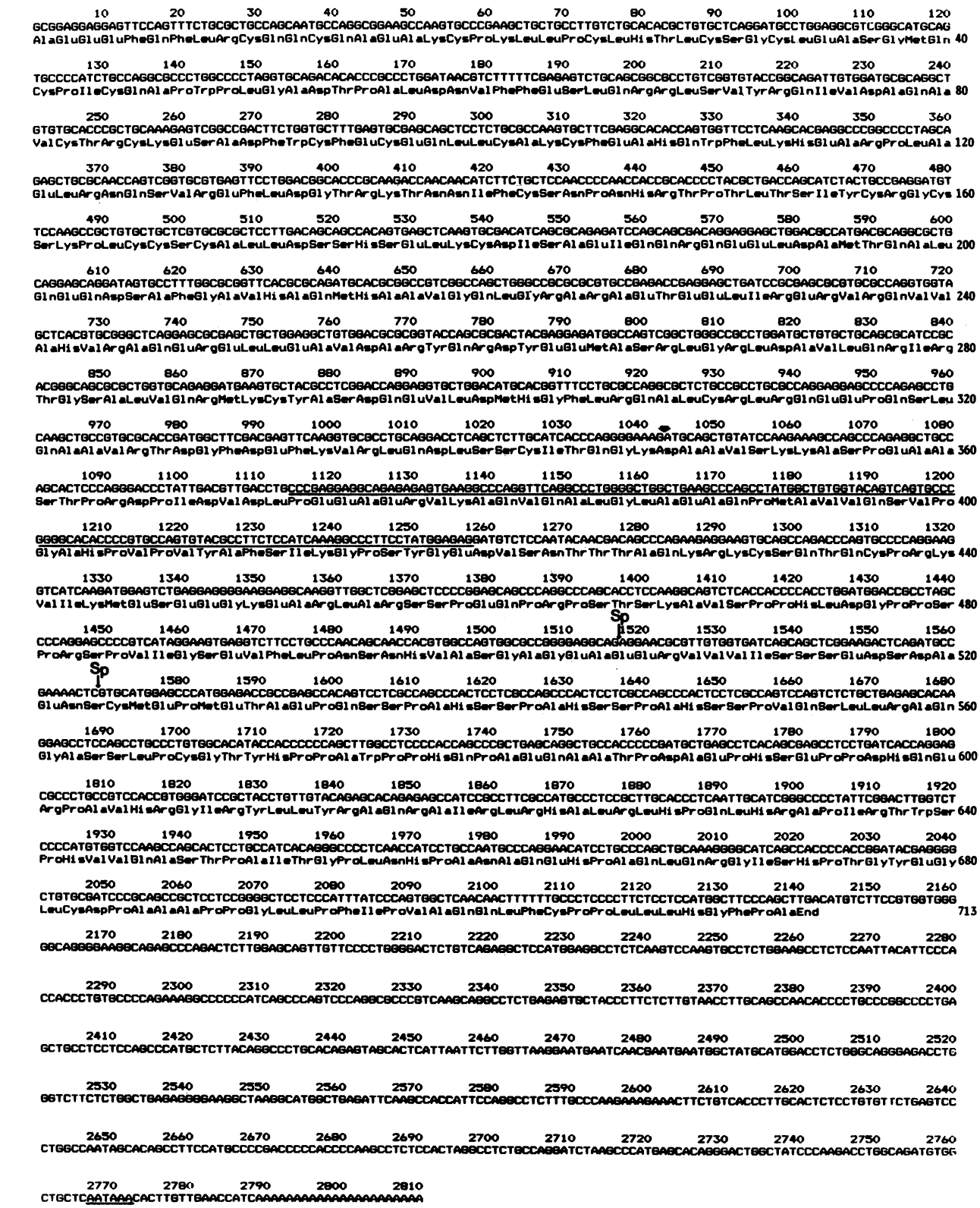

FIG. 3. Nucleotide sequence of the *myl* cDNA pMYL211. Numbers on top of each line indicate nucleotide positions. Numbers on the right indicate positions of the last amino acid on each line. The polyadenylation site AATAAA is underlined. Sp indicates the position of alternative splicing. The underlined region from nucleotides 1114 to 1257 is not found in p1715.

1. GGCGCCGGGGAGGCAGGTAGGGAGAGGAACGCG

2. GGCGCCGGGGAGGCAGAGGAACGCG

3. GGCGCCGGGGAGGCAGGTAGGGAGGTGGGTAGGGCAG---
----1.5KB----CCCCTTCCCCGTTTCAGAGGAACGCG

FIG. 4. The additional 8-bp DNA sequence found in the cDNA of p1715 is a result of alternative splicing. Line 1 shows the DNA sequences of p1715 between nucleotides 817 and 849 (Fig. 2). Line 2 shows the homologous DNA sequence from pMYL211, between nucleotides 1501 and 1525 (Fig. 3). Line 3 shows the genomic DNA sequence of the intron/exon junctions within this region. The 8 bp involved in the alternative splicing are underlined.

the Northern blot was repeated (Fig. 7C). An abnormal band of 2.6 kb was then clearly visible (lane 3). The Northern blot in Fig. 7A was stripped and rehybridized to the ^{32}P-labeled probe of pRA1 (Fig. 7B). The two RARA mRNA species of 2.7 and 3.7 kb were detected in all samples tested. In lane 6, containing a sample from an APL patient, two abnormal mRNA species of 4.2 and 3.2 kb were found. Since the pRA1 cDNA consists of about 520 bp of exon 2 of the RARA gene, a 2.6-kb abnormal mRNA should be detectable. However, failure to identify it may be explained by its comigration with the 2.7-kb normal RARA mRNA. In a second APL patient (lane 7), two abnormal mRNAs of 4.8 and 3.8 kb (slightly overlapped with the 3.7-kb normal band) were found. Samples from both APL patients tested in this Northern blot were analyzed for RARA gene rearrangement. The breakpoint for both patients was localized to intron 2 (data not shown). This observation indicates that the difference in the sizes of the abnormal mRNAs from the two APL patients was the result of different breakpoint sites on chromosome 15.

Since the *myl* cDNA probe isolated from p1715 did not hybridize to any of the abnormal mRNA species detected by the RARA cDNA (Fig. 7A through C), the 4.2- and 3.2-kb mRNAs detected in lane 6 and the 4.8- and 3.8-kb mRNAs detected in lane 7 may represent hybrid *myl*/RARA mRNA rather than RARA/*myl* RNA. In addition, the size difference of the two abnormal mRNAs from the two APL patients was 1 kb, similar to the size difference of the two normal RARA mRNAs (3.7 and 2.7 kb). Our sequence analysis of pRA1 (Fig. 1) at the 3′ end indicates that a second polyadenylation site is used by the RARA gene, in contrast to what was previously reported for cDNA sequences (22, 37). Recently, it was reported that a single transcription start site is used by the RARA gene (5). Therefore, different polyadenylation sites may have resulted in the two different RARA mRNA species.

Detection of the two different fusion transcripts RARA/*myl* and *myl*/RARA in APL. To conclusively prove that both fusion transcripts RARA/*myl* and *myl*/RARA are expressed in the same APL patient, two sets of oligonucleotide primers were designed to amplify the fusion junction of the hybrid mRNA by PCR. RNAs from three APL patients were analyzed. As shown in Fig. 8A, primer set R5/D4 amplified an expected DNA fragment of 760 and 620 bp in three APL samples. Sequence analysis of the amplified DNA fragments confirmed that these DNA fragments were derived from the fusion junction of the RARA/*myl* mRNA (Fig. 8B). The two amplified DNA fragments differed by approximately 140 bp; this is the result of alternative splicing (Fig. 3, nucleotides 1114 to 1257). Sequence analysis of RARA/*myl* mRNA as

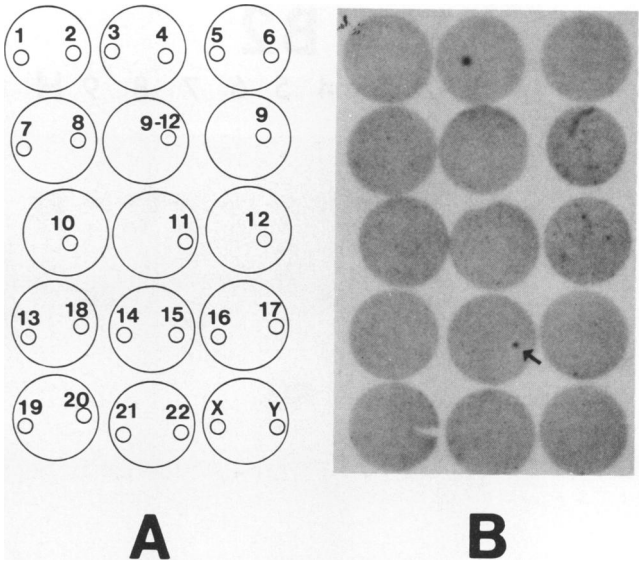

FIG. 5. Chromosome assignment of the *myl* gene sequence of p1715. A 1.2-kb cDNA fragment obtained from PCR amplification (nucleotides 504 to 1704 of p1715) was oligolabeled to 10^9 cpm/μg DNA and hybridized to the chromosome-specific DNA dot blot as described in Materials and Methods. (A) Positions of chromosome spots of each filter disc. (B) X-ray autoradiograph of hybridized filters. Arrow indicates the specific hybridization of the unknown gene sequence to the chromosome 15 spot. An additional signal fell outside the chromosome 3 or 4 spot and is thus not a hybridization signal.

shown in Fig. 8B indicates a clear sequencing tract in all four lanes. However, the sequencing tracts beyond nucleotide 1113 (Fig. 3) become unreadable, presumably due to the overlapping of two different sequences (data not shown). Primer set R2/15U1 amplified a DNA fragment of 1.2 kb in all three APL RNA samples. DNA sequence analysis confirmed that the PCR amplified a DNA fragment derived from the hybrid mRNA of *myl*/RARA (Fig. 8B). Our results conclusively prove that both RARA/*myl* and *myl*/RARA mRNAs were coexpressed in all three APL patients.

It appears that, in APL, translocation breakpoints result in two different hybrid gene structures. A scheme which illustrates the expression of normal and fusion transcripts of the RARA and *myl* genes in APL is outlined in Fig. 9. In one, the RARA gene promoter is upstream of the *myl* gene. Transcriptional control by the RARA promoter element results in a hybrid mRNA consisting of exons 1 and 2 of the RARA gene, which is truncated downstream by the *myl* gene (RARA/*myl* or p1715 in this report). In the other, the *myl* gene promoter is upstream of the RARA gene. Transcriptional control of the *myl* gene results in a hybrid mRNA consisting of the 5′ portion of the *myl* gene truncated downstream by exons 3 to 9 of the RARA gene. In this instance, the use of the two polyadenylation sites should not be affected by the translocation (the 4.2- and 3.2-kb or the 4.8- and 3.8-kb abnormal mRNAs found in Fig. 7, lanes 6 and 7, in this report or the fusion gene as reported by de The et al. [15]). Both the RARA and *myl* genes are expressed at high levels in hematopoietic cells, and both promoter elements apparently remain active after translocation.

DISCUSSION

The translocation breakpoint t(15;17) of APL transcribes two different types of hybrid mRNA. We have identified and

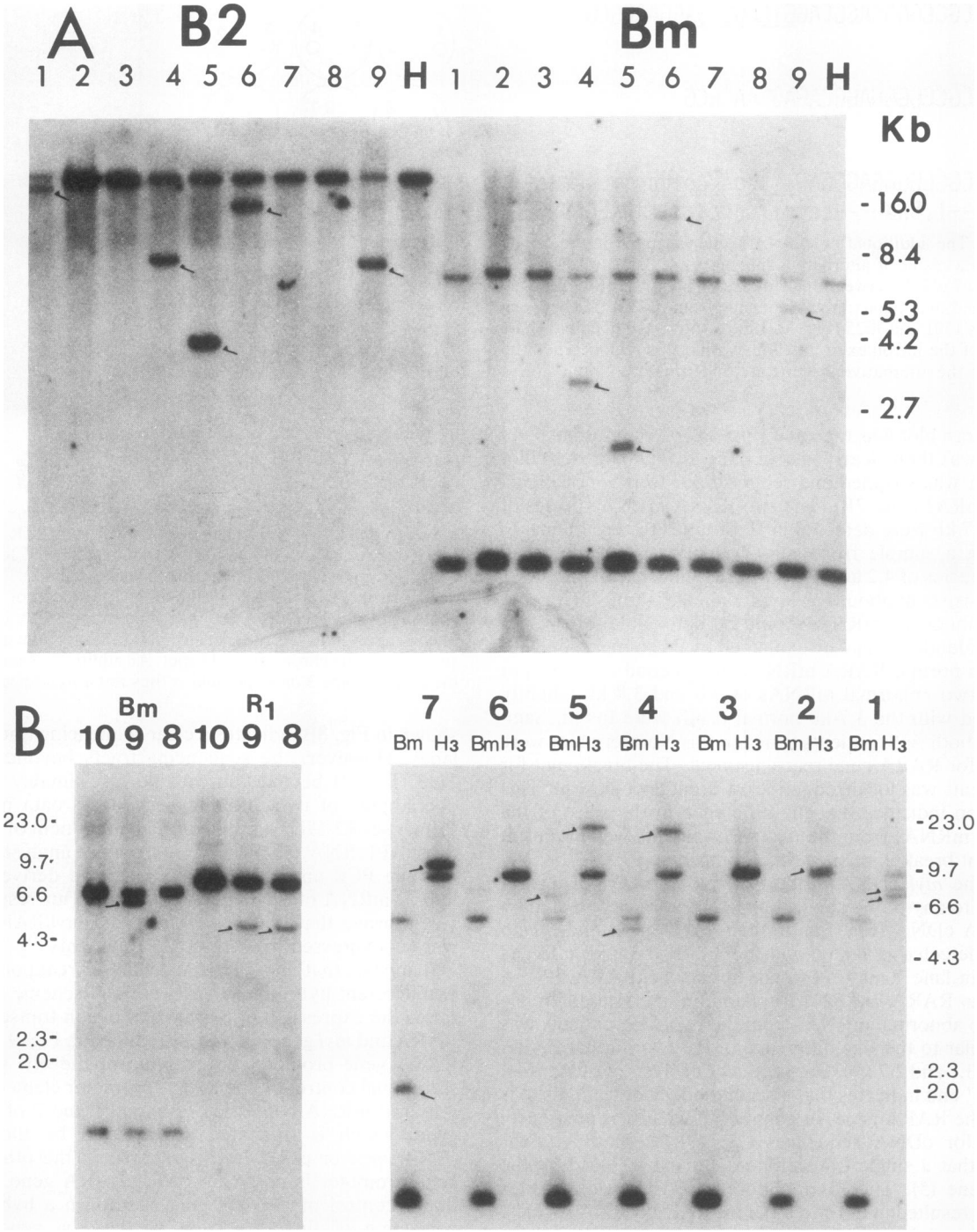

FIG. 6. Southern blot analysis of 17 APL DNA samples with *myl* gene probe. Ten micrograms of each DNA sample was restriction digested with *Bgl*II (B2), *Bam*HI (Bm), *Hin*dIII (H3), or *Eco*RI (R1), electrophoresed on a 0.75% agarose gel, transferred to a Nytran membrane, and hybridized to an oligolabeled 1.2-kb probe as described for Fig. 5. (A) Samples 1 to 7 and 9 are from APL with the t(15;17) breakpoint, and sample 8 is from APL with a $17q^-$ abnormality; H represents DNA isolated from human leukemia cell line HL-60. (B) DNA samples 1 through 9 were obtained from t(15;17)-positive APL. Sample 10 is the remission sample of patient 9. Rearranged bands are indicated by arrows.

characterized a cDNA (RARA/*myl*) encoded from the nonrandom translocation breakpoint t(15;17)(q22;q21) of APL. This cDNA represents the hybrid mRNA regulated by the promoter element of the RARA gene, which is different from the *myl*/RARA fusion gene reported by de The et al. (15). We have demonstrated that both RARA/*myl* and *myl*/RARA fusion transcripts are coexpressed in three different APL patients. This suggests that two different types of hybrid messages are transcribed following the t(15;17) translocation, one from each of the reciprocal translocations. This

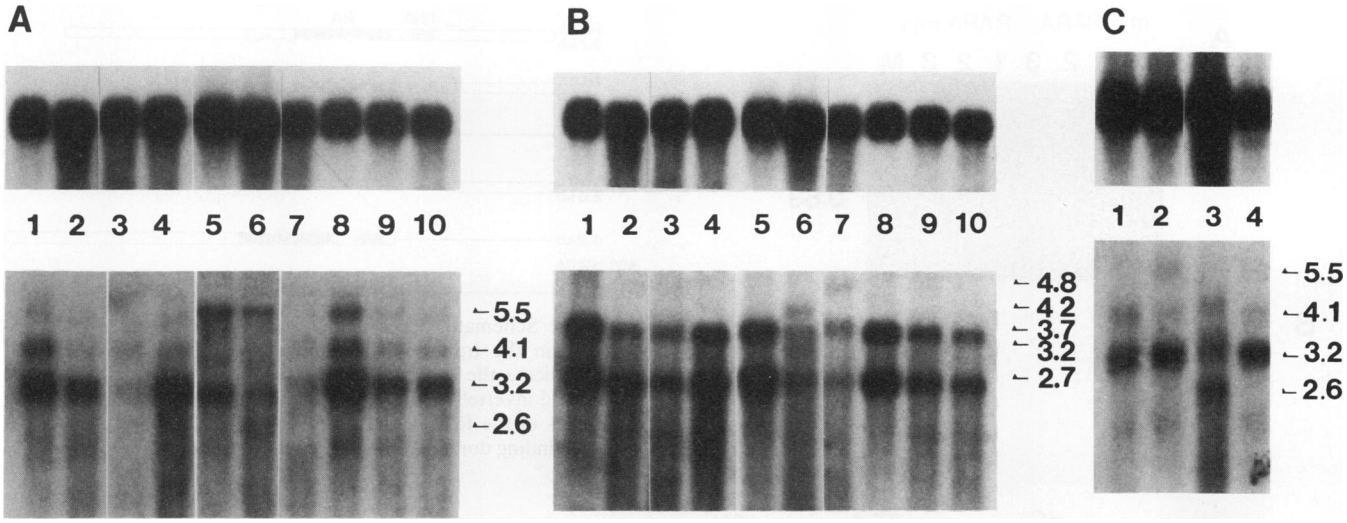

FIG. 7. Northern blot analysis of poly(A) RNA isolated from different leukemia patients. In panels A and B, lanes 2 through 10 contain RNAs isolated from acute myelogenous leukemia (AML) samples. Lanes: 1, HL-60 RNA; 2, chronic lymphocytic leukemia; 3, acute lymphoblastic leukemia; 4, acute monoblastic leukemia (AML-M5); 5, acute myelomonocytic leukemia (AML-M4); 6 and 7, APL (AML-M3); 8, AML with maturation (M2); 9, AML without maturation (M1); 10, acute undifferentiated leukemia (AML-MO). Designations of different AML are according to the FAB (French-American-British) classification (2). Panel A shows hybridization to the ^{32}P-labeled PCR-amplified 1.2-kb probe of *myl* cDNA, and panel B shows hybridization to the pRA1 cDNA probe. Panel C is a repeated Northern blot of panel A. Samples 1, 2, 3, and 4 are the same samples as 10, 9, 6, and 5, respectively. The upper parts of each panel show the same filter hybridized to a β-2 microglobulin cDNA probe (47).

finding differs from those of previous studies on other translocation breakpoints related to hematologic neoplasia, where only one of the translocated genes was transcriptionally active and only one type of hybrid mRNA was found encoded from the breakpoint region (18, 21, 25, 40, 45).

Molecular pathogenesis of t(15;17) translocation breakpoint in APL. RA is a morphogen, as demonstrated in chick limb experiments (7). Since RA can interact directly with RARA (42), RARA may be the key element in regulating RA concentration in the nucleus to achieve differentiation and development. In APL, the translocation breakpoint splits exons I and II from exons III to IX of the RARA gene.

The fusion cDNA RARA/*myl* consists of the first and second exons of the RARA gene. Recently, alternative splicing of the mouse RARA gene has been found, and several different cDNA molecules with different 5′ end (exons I and II) sequences have been isolated (31). This finding indicates that exons I and II of RARA are important for a specific function. The first 59 amino acids of the predicted fusion protein were derived from the RARA exon II. This portion of the protein is believed to be involved in transactivation and target gene specificity (43). The finding of an additional 8-bp sequence in the p1715 cDNA as a result of alternative splicing is particularly interesting. We have shown that RARA/*myl* fusion transcripts with and without the 8-bp sequence exist in the APL RNA sample. Thus, RARA/*myl* with the eight additional nucleotides will be translated into a short fusion protein with a molecular weight of 18,500 due to a switch of the reading frame. RARA/*myl* without the 8-bp sequence would be able to encode a polypeptide of 382 amino acids with a molecular weight of about 40,000. Recently, we have identified two different *myl* cDNAs with additional 29- and 640-bp sequences (unpublished data) at nucleotide 1568 (Fig. 3). These additional sequences also result in an early translation stop of the *myl* mRNA. Alternative splicing of the *myl* gene appears to be a complicated process. We have now identified 20 cDNA clones of *myl*. Characterization of these clones and the *myl* genomic DNA will help us understand the mechanisms of alternative splicing of the *myl* gene. We found several cysteine-rich regions between amino acids 10 and 44, 82 and 105, and 142 and 180 (Fig. 3) which resemble a zinc finger motif found in a new family of DNA-binding proteins (20). In addition, a putative leucine zipper sequence was found between amino acids 164 and 200 (Fig. 3). On the basis of these findings, it was postulated that *myl* may be a transcription factor (16, 27). The biological function of *myl* is currently unknown; however, the extensive alternative splicing mechanisms suggest an important functional gene.

The hybrid *myl*/RARA mRNA detected in this report and by others (13, 15, 34) consists of exons III to IX of the RARA gene and contains the complete DNA- and RA-binding domains (22, 37). The fusion transcript *myl*/RARA can be translated into a polypeptide with a molecular weight of about 83,000. Approximately 200 amino acids on the NH_2-terminal end are derived from the *myl* protein. Since the DNA- and RA-binding domains remained intact, replacing the NH_2 terminal of RARA with a much larger peptide may have altered the properties of RAR function and may result in its becoming oncogenic. The tumorigenic potential of the *myl*/RARA cDNA will be investigated in the future to elucidate this possibility.

The most important issue is the pathogenic events that lead to the leukemogenesis of APL. APL is a clonal proliferation of promyelocytes, and the t(15;17) translocation is a consistent feature of this disease. Recently, all-*trans* RA has proved capable of producing complete remission in a significant number of APL patients (9, 12, 26). The interpretation of this clinical observation could be explained if the t(15;17) translocation breakpoint in APL partially impairs the normal function of RARA, because translocation results in the production of a nonfunctional RARA. When APL patients

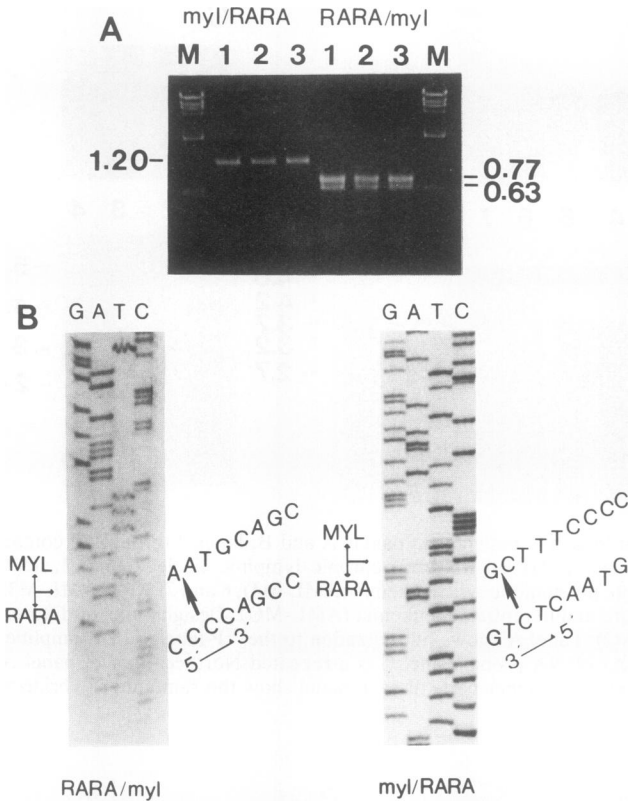

FIG. 8. Detection of fusion transcripts RARA/*myl* and *myl*/RARA in APL. Primers R5 and D4 (nucleotides 258 to 278 and 869 to 887 [Fig. 2]) are designed to preferentially amplify the RARA/*myl* mRNA. Primers 15U1 (nucleotides 894 to 912 [Fig. 3]) and R2 (nucleotides 1288 to 1308 of RARA [22]) are designed to preferentially amplify the *myl*/RARA mRNA. The fusion transcripts were amplified by reverse transcriptase-PCR as described in Materials and Methods. (A) Two DNA fragments of 770 and 630 bp (0.77 and 0.63 bands) were amplified in all three APL samples by using primers set R5/D4. A single band of 1.2 kb (1.20) was amplified in the three APL samples by using primer set 15U1/R2. M, *Hin*dIII-digested lambda DNA size marker. (B) Sequence analysis of the PCR-amplified DNA fragments. Internal primers R1 (nucleotides 443 to 467 [Fig. 2]) and R4 (nucleotides 446 to 465 of RARA [22]) were used to sequence the fusion junctions of RARA/*myl* and *myl*/RARA, respectively. The exact sites of the fusion junctions are indicated by arrows. As indicated, RARA/*myl* was sequenced from 5′→3′ and *myl*/RARA was sequenced from 3′→5′.

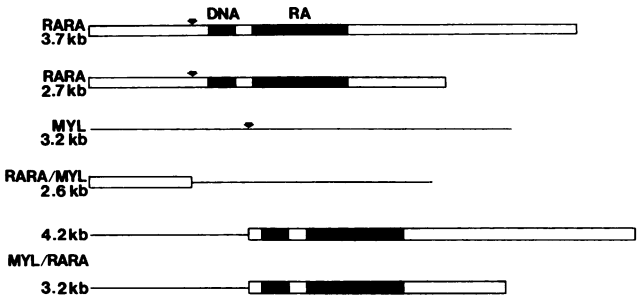

FIG. 9. Schematic representation of the expression of genes involved in the translocation breakpoint t(15;17) in APL. Solid boxes indicate the DNA- and RA-binding domains of the RARA gene. Solid inverted arrowheads indicate the locations of the breakpoint sites of the RARA and *myl* cDNAs. Locations of the DNA- and RA-binding domains were determined as reported by Giguere et al. (22).

are treated with all-*trans* RA, high concentrations of RA may overcome the RARA deficiency, possibly by saturating the RARA-binding sites and enabling the delivery of a higher concentration of RA into the nucleus. Recently it was demonstrated that cellular retinoic acid binding protein (CRABP) plays a role in mediating the RA concentration in cells (4). All-*trans* RA treatment of APL may have abolished the normal function of cellular RA-binding protein allowing an uncontrolled level of RA to be available in the nucleus. Differentiation of the proliferative promyelocytes in APL can then be achieved. Recently it was reported that the *myl*/RARA fusion protein can act as an RA-inducible transcription factor with transactivating properties different from those of RARA (16, 27, 36). From these studies, it was postulated that *myl*/RARA is a dominant negative oncogene product which inhibits expression of RA-responsive genes, thus interfering with promyelocyte differentiation in APL (16, 27). However, experimental data supporting this hypothesis is currently unavailable.

Our results demonstrate that two different types of hybrid mRNA are transcribed from the t(15;17) translocation breakpoint. Although the hybrid mRNAs transcribed from translocation breakpoints have been shown to have oncogenic potential (8, 25), we do not yet have any information on the oncogenic potential of the hybrid messages in APL. We have shown that the *myl* gene is expressed in all leukemia cells tested, and it has been proposed to encode a transcription factor (16, 17). It can be hypothesized that partial inactivation of this gene as a result of the translocation could also have deleterious effects on cell differentiation. Future studies on the functional role and the genomic organization of the *myl* gene along with its involvement with the RARA gene in the t(15;17) translocation breakpoint will contribute to our understanding of APL pathogenesis.

ADDENDUM

After submission of this manuscript, Pandolfi et al. (36), Kakizuka et al. (27), and de The et al. (16) reported the characterization of the *myl*/RARA (or PML/RARA) fusion transcript and of the normal *myl* (or PML) cDNA.

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