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Structural organization and expression of human DNA sequences related to the transforming gene of avian myeloblastosis virus

(recombinant DNA/human *onc* gene/heteroduplex analysis/human hematopoietic cells)

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ABSTRACT **Bacteriophage libraries of human DNA were screened for sequences homologous to the transforming gene (v-*myb*) of avian myeloblastosis virus. The three overlapping clones isolated were shown to contain a total of 1.0 kilobase pair (kbp) of sequence related to v-*myb* distributed over 6.2 kbp. Restriction enzyme mapping and heteroduplex analysis revealed the presence of five *myb*-related domains interrupted by four stretches of nonhomology. To study the extent of human DNA coding sequences that constitute the *myb* gene homologue, c-*myb* (human), probes spanning about 30 kbp were prepared from the clones and used to study transcription in a human hematopoietic cell line (MOLT-4). Each of the probes hybridized a 4.5-kilobase transcript, which suggests that either the c-*myb* (human) gene encompasses 30 kbp or it contains two or more transcription units that each give rise to a mRNA of 4.5 kilobases.**

Acute retroviruses, which transform cells in culture and rapidly induce tumors in animals, contain oncogenic sequences, designated v-*onc* (1), that are required for the induction and maintenance of viral transformation (for a recent review, see ref. 2, chapts. 8 and 9). Such sequences appear to be derived from normal cellular homologues (c-*onc* genes) that are highly conserved genes usually present in single or low copy number (2). Some molecularly cloned c-*onc* genes, if linked to terminal regions of the viral genome that include promoter sequences, can transform mouse fibroblasts *in vitro* (3–5). Evidence for potential involvement of these cellular homologues in animal tumorigenesis has been provided by the demonstration of enhanced expression of two avian c-*onc* genes, c-*myc* and c-*erb*, respectively, in the lymphomas and erythroblastosis induced in chickens by avian leukosis virus, which itself contains no *onc* gene (6, 7). Furthermore, transforming genes isolated from some human tumor cell lines appear to be modified cellular homologues of the *ras* family of viral *onc* genes (8–10).

Workers in our laboratories have been investigating the structure and expression of c-*onc* genes in normal and neoplastic human tissues. When a large number of cultured human cells was screened for the presence of transcripts homologous to the *onc* gene (v-*myb*) of avian myeloblastosis virus (AMV), only immature hematopoietic cells were found to contain mRNA related to v-*myb* (11–13). The expression of the c-*myb* gene appears to be inversely linked to differentiation: (*i*) The level of mRNA decreases in the promyelocytes of the human neoplastic cell line HL-60 when the cells undergo retinoic acid or dimethyl sulfoxide-induced differentiation and (*ii*) immature T-lymphocytes express the *myb* gene, whereas the mature peripheral blood T-lymphocytes do not (13). To characterize the human cellular sequences that are related to v-*myb* we have isolated recombinant clones encompassing 35 kilobase pairs (kbp) of human DNA containing sequences homologous to v-*myb*. An analysis of the structure and expression of these sequences is presented in this report.

MATERIALS AND METHODS

Isolation of c-*myb* (Human) Clones. A Charon 4A library of an *Eco*RI partial digest of human adult liver DNA and a Charon 28 library of human placenta DNA (partial *Mbo* I digest) were gifts from T. Maniatis (14) and P. Leder, respectively. The bacteriophage were plated at a density of 2×10^4 plaque-forming units per dish using *Escherichia coli* LE392 and C600 kindly provided by L. Enquist. The method of Benton and Davis (15) was used to prepare nitrocellulose filter replicas of the infected bacterial lawns. Filters were hybridized with a ^{32}P-labeled probe (2×10^8 cpm/μg, 2×10^6 pmol/ml) (designated H3.9) that contained sequences representing both the *pol* and *myb* genes of AMV (16). Hybridizations were carried out in 6× standard saline citrate (NaCl/Cit; 1× NaCl/Cit = 0.15 M NaCl/0.015 M sodium citrate)/5× Denhardt's solution (1× Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/sheared salmon sperm DNA (100 μg/ml) at 62°C for 24 hr, and the filters were washed at 45°C in 0.1× NaCl/Cit/0.1% $NaDodSO_4$ (17) and autoradiographed at −70°C for 24 hr using Kodak XAR film and Dupont Cronex Lightning Plus screens.

Analysis of Cloned DNAs. Restriction enzyme digests of cloned DNAs were subjected to agarose gel electrophoresis and Southern blotting (18). Filter hybridization was carried out using the conditions described above or under the following less stringent conditions: hybridization at 37°C, 50% formamide/5× NaCl/Cit/10% dextran sulfate (19)/salmon sperm DNA (100 μg/ml)/5× Denhardt's solution, washing at 22°C, 50% formamide/3× NaCl/Cit. Autoradiography was performed as described above.

Analysis of Cell RNA. RNA was extracted from a human T-cell line, MOLT-4 (20), by a modification (21) of the method of Cox (22). RNA (10 μg) was denatured in a solution containing 2.2 M formaldehyde. Electrophoresis was performed at 30 V overnight. RNA was electrophoretically transferred to a GeneScreen membrane (New England Nuclear) by using a Bio-Rad Trans-Blot cell at 10 V for 2 hr in 25 mM sodium phosphate buffer at pH 6.5. Hybridization of filters was carried out according to the method of Thomas (23).

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Abbreviations: AMV, avian myeloblastosis virus; NaCl/Cit, 0.15 M NaCl/0.015 M sodium citrate; bp, base pair(s); kbp, kilobase pair(s); kb, kilobase(s).

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RESULTS

Isolation of v-*myb*-Related Clones from Phage Libraries of Human DNA. An AMV subgenomic probe (3.9-kbp *Hin*dIII fragment, clone H3.9), containing the entire v-*myb* gene (24), previously shown to detect homologous sequences in human DNA, was used to screen two bacteriophage libraries of human DNA. From 5×10^5 plaques of the Charon 28 library, one homologous clone designated λ-1 was isolated. The v-*myb*-related sequences were found by molecular hybridization to reside within a 3.0-kbp *Eco*RI fragment (Fig. 1). From 2×10^6 plaques of the Charon 4A library (14) two clones homologous to v-*myb* were isolated. One clone (λ-2) contained two *Eco*RI fragments of 2.0 and 2.6 kbp and the other clone (λ-3) contained a single 2.0-kbp *Eco*RI fragment that hybridized to the v-*myb* probe (Fig. 1). When hybridization reactions were carried out under more relaxed conditions (20% formamide instead of 50%), 1.0- and 9.0-kbp *Eco*RI fragments in λ-2 were also detected. However, the significance of these results is unclear, because under these hybridization conditions, wild-type λ *Hin*dIII fragments also hybridized. Restriction enzyme maps of each clone were determined and the results indicated that clones λ-1 and λ-3 overlapped with the two ends of clone λ-2 (Fig. 1). In comparing these maps it became evident that λ-2 lacked two *Bam*HI sites present in λ-3 but contained an *Eco*RI site absent in λ-3 (Fig. 1). These differences may represent allelic variation because the clones were isolated from the same library. To further confirm that the clones overlapped, fragments of the clones that lacked *Alu* family sequences (25) were hybridized to appropriate restriction enzyme digests of each other (data not shown), giving the restriction map shown in Fig. 1. Together the clones cover a total of almost 35 kbp of nonoverlapping sequence. The 2.6- and 2.0-kbp *Eco*RI *myb*-containing fragments from clone λ-2 located between 13 and 19 kbp on the map were used as probes to determine the complexity of c-*myb* (human) in normal placental DNA. Each probe detected only a single *Eco*RI band at 2.6 and 2.0 kbp, respectively (data not shown).

Organization of *myb*-Related Sequences Within the Human c-*myb* Domain. The position of sequences homologous to v-*myb* in c-*myb* (human) was determined by hybridizing, with the v-*myb* probe, appropriate restriction digests of the three recombinant clones and their various pBR322 subclones. These sequences were found to be localized within a 6.2-kbp region flanked on the 5′ side by ≈12 kbp and on the 3′ side by 17 kbp of *myb*-unrelated sequences. It was also possible to show the presence of a large nonhybridizing sequence between the *myb* domains in λ-1 and λ-2, which is located between the *Bam*HI and *Sst* I sites at ≈12 and 15 kbp, respectively (Fig. 1). This sequence may be composed of either exons or introns not present in v-*myb*.

To orient the human sequences with respect to the v-*myb* gene, the *myb*-related *Eco*RI fragments of 3.0, 2.6, and 2.0 kbp were used as probes and hybridized with various restriction digests of the AMV clone H3.9. Representative data are shown in Fig. 2. The 3.0-kbp *Eco*RI fragment of clone λ-1 hybridized to the *Hin*dIII/*Eco*RI fragment of AMV (Fig. 2*A*), the 2.6-kbp *Eco*RI fragment of λ-2 hybridized to the *Eco*RI/*Sal* I v-*myb* sequence (Fig. 2*B*), and the 2.0-kbp *Eco*RI segment from clones λ-2 and λ-3 hybridized to the *Sal* I/*Hin*dIII region (Fig. 2*C*). The 3′ terminus of v-*myb* between *Bal* I and *Hin*dIII could not be detected in c-*myb* (human) even under nonstringent hybridization conditions. Based on these data, the regions of homology between c-*myb* (human) and v-*myb* could be localized within their respective maps (Figs. 1 and 2). Also, the c-*myb* (human) map could be oriented with respect to the v-*myb* sequences (Fig. 1).

Heteroduplex analyses further defined the organization of

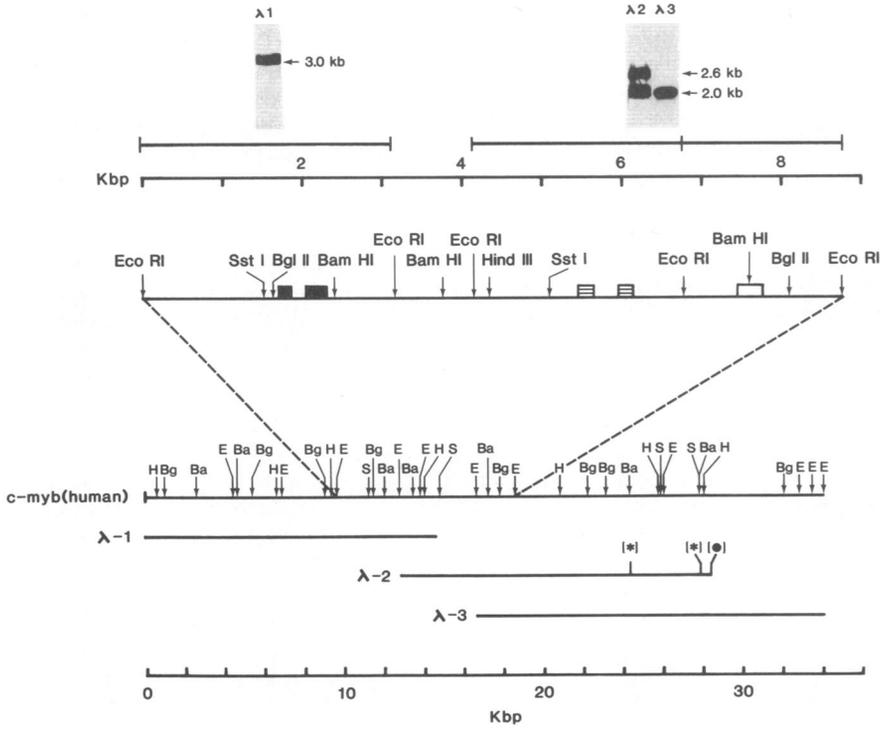

FIG. 1. Restriction enzyme map of c-*myb* (human). The extents of the three phage clones isolated from human libraries are indicated by the lines at the bottom. *Bam*HI sites present in clone λ-3, but absent in clone λ-2, are indicated by (*) and a unique *Eco*RI site present in clone λ-2, but absent in clone λ-3, is indicated by (●). A composite map of the three clones is shown and an 8.0-kbp region containing the *myb*-homologous regions is shown in expanded form above. *myb*-related regions are indicated by boxes (see Fig. 2). The positions of the homologous regions were deduced by restriction enzyme and heteroduplex analyses (Figs. 2 and 3). H, *Hin*dIII; Bg, *Bgl* II; Ba, *Bam*HI; E, *Eco*RI; S, *Sst* I.

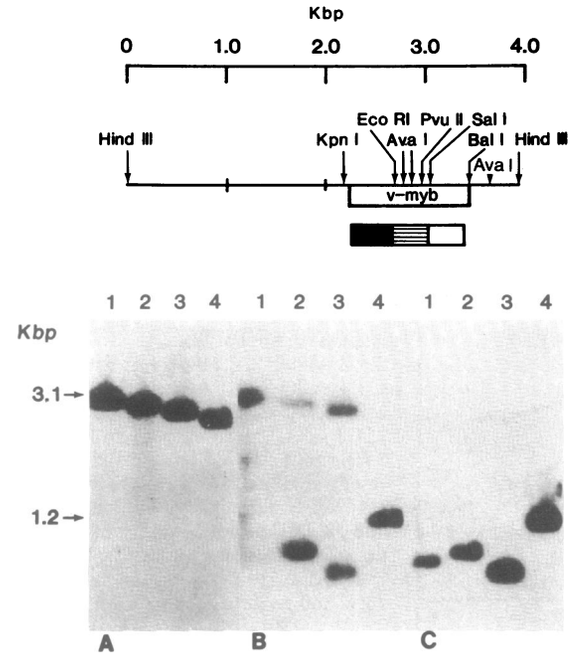

FIG. 2. Localization of regions of homology between v-*myb* and c-*myb* (human) by restriction enzyme analysis. The 3.9-kbp AMV *Hin*dIII fragment (clone H3.9) was cleaved with *Sal* I (lanes 1), *Pvu* II (lanes 2), *Ava* I (lanes 3), and *Eco*RI (lanes 4), subjected to agarose gel electrophoresis and Southern blotting and hybridized to probes prepared from clone λ-1 3.0-kbp *Eco*RI fragment (*A*) and 2.6-kbp (*B*) and 2.0-kbp (*C*) *Eco*RI fragments from clone λ-2 described in Fig. 1. A restriction map of AMV clone H3.9 is shown and the region corresponding to the cellular insert is indicated. The boxes represent the v-*myb* regions related to c-*myb* (human) as deduced from this experiment and the heteroduplex data (Fig. 3). The fragments that hybridized to the 3.0-, 2.6-, and 2.0-kbp probes are indicated by the closed, striped, and open boxes, respectively.

the *myb*-related sequences in human DNA (Fig. 3). For these experiments, subcloned fragments of clone λ-1 (3.0-kbp *Eco*RI) and clone λ-2 (7.0-kbp *Hin*dIII) were hybridized to the AMV clone H3.9. The 3.0-kbp *Eco*RI fragment contained two stretches of homologous sequences ≈100 and 275 base pairs (bp) long interrupted by a 200-bp unrelated sequence (regions 4, 5, and 6 in Fig. 3 *A–C*). From the lengths of the flanking unrelated sequences (regions 2, 3, 7, and 8), it was deduced that clone λ-1 represents the 5′ portion of the v-*myb* gene. The region of homology commences about 100 bp to the right of the *Kpn* I site in AMV (Fig. 2), indicating conservation of the 5′ end of v-*myb* in human DNA. The presence of additional nonhomologous DNA regions in c-*myb* (human) is shown in Fig. 3 *D–F*. Thus, the sequence bounded by the *Hin*dIII sites at 14 and 21 kbp on the c-*myb* (human) map contained three *myb*-homologous regions of 150, 150, and 300 bp, respectively, interrupted by 350- and 1,500-bp stretches of unrelated DNA (regions 4–8). The lengths of the flanking single-stranded arms (regions 2, 3, 9, and 10) indicated that the 2.6-kbp *Eco*RI fragment represents the middle portion of v-*myb* (*Eco*RI/*Sal* I sites) and that the 2.0-kbp *Eco*RI fragment to its right on the map represents the 3′ portion of v-*myb* (*Sal* I/*Bal* I sites) (Fig. 2). The latter result was confirmed by heteroduplex analysis of the subcloned 2.0-kbp *Eco*RI fragment (data not shown). It was apparent that about 100 bp at the 3′ end of v-*myb* located upstream of the *myb*-helper virus 3′ junction were not detected by this technique. Examination of the sequences to the right of the *Hin*dIII site at 21 kbp on the c-*myb* (human) map did not reveal any other homologous region (data not shown). Thus, ≈1,000 bp of sequence related to v-*myb* are present within a 6-kbp region of human DNA.

Expression of c-*myb* (Human) in an Immature Human T-Cell Line. It has been shown previously that immature human hematopoietic cells express a poly(A)-containing 4.5-kilobase (kb) RNA homologous to v-*myb* (13). Because the complexity of this RNA molecule is much greater than that of the 1.2-kbp v-*myb* gene, it was of interest to determine what other sequences within c-*myb* (human) were transcribed. For these studies the terminal deoxyribonucleotide transferase positive immature T-cell line, MOLT-4 (20) was used. Probes representing about 30 kbp of c-*myb* (human) sequence were prepared and hybridized to MOLT-4 RNA (Fig. 4). The probes included fragments containing 5′ flanking sequences (lanes a, b, and c), *myb*-related *Eco*RI fragments (lanes d, f, and g), the 1-kbp *Eco*RI fragment (lane e) located between the 3.0 and 2.6 kbp *myb*-containing fragments, and 3′ flanking regions (lanes h, i, and L). The major RNA species detected by all the probes was 4.5 kbp, the same size as that detected with a v-*myb* probe (lane 1) but with various intensities, suggesting differences in the length of coding sequences within each fragment. Half of the human DNA probes also detected a minor, smaller RNA species with a M_r that varied with the different probes. These bands could not be detected with the v-*myb* probe even after prolonged exposure using nonstringent hybridization and washing conditions. The significance of these minor RNA species remains to be determined. These results suggest that the c-*myb* (human) gene extends over at least 30 kbp.

DISCUSSION

The majority of cellular *onc* genes including c-*abl*, c-*erb*, c-*fes*, c-*myc*, c-*ras*, c-*sis*, and c-*src*, either in their species of origin or in human cells (4, 10, 26–37), consists of several *onc*-related domains interrupted by unrelated sequences. Comparison of the structures of c-*myb* (human) and c-*myb* (chicken) reveals a similar overall organization in that c-*myb* (chicken) contains at least six v-*myb*-related segments within an 8- to 9-kbp-long region (38, 39). In contrast, c-*mos* and c-*ras*-2 are present as single uninterrupted segments in both murine and human DNAs (4, 40–42). The c-*myb* (human) locus contains a region of ≈6 kbp in which at least five *myb*-homologous domains, totaling 1 kbp, are separated by four *myb*-unrelated regions. By the hybridization techniques used, we were not able to show the presence in c-*myb* (human) of the last 100 nucleotides at the 3′ end of v-*myb* cell-derived sequences. Because ≈15 kbp of 3′ flanking *myb*-unrelated sequences were analyzed, it is likely that these 3′ *myb* sequences are not well conserved in human DNA. A similar finding was made for c-*mos* (human) (40, 41).

The transcription of c-*myb* appears to be restricted to immature hematopoietic cells (12), and expression of *myb* decreased in a promyelocytic cell line, HL 60, after it was induced to differentiate (13). The normal transcript size of 4.5 kb is substantially more complex than v-*myb* (43) but is similar in size to c-*myb* mRNA in normal chicken hematopoietic tissue (ref. 44; unpublished results). The isolation of a 35-kbp stretch of human DNA in our studies made it possible to determine the origin of *myb*-unrelated sequences in the transcript present in MOLT-4 cells. The data indicated that the 4.5-kb *myb* transcript contains sequences distributed over at least 30 kbp.

The v-*myb* product in leukemic chicken myeloblasts is a M_r 48,000 protein presumably synthesized from a spliced subgenomic viral mRNA starting with 5′ terminal helper viral sequences and terminating with the 3′ end of the viral *env* gene (45). Thus, the AMV oncogene appears to contain helper viral sequences at both ends. Our findings with the human c-*myb*

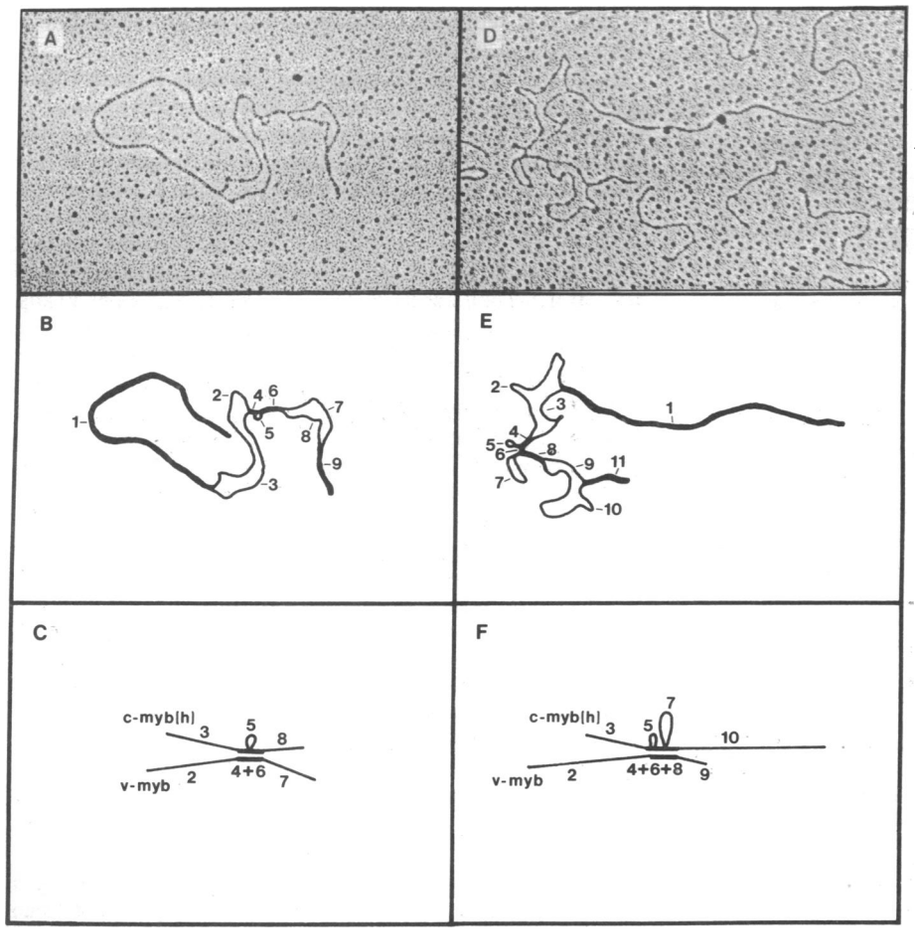

FIG. 3. Heteroduplex analysis of c-*myb* (human). (*A–C*) Heteroduplex formed between a pBR322 subclone of a 3.0-kbp *Eco*RI fragment isolated from clone λ-1 and a 3.9-kbp *Hin*dIII fragment of AMV (clone H3.9), which contains the entire v-*myb* gene. Contour-lengths (in kbp) of features are as follows: 1, 3,694 ± 208; 2, 2,247 ± 159; 3, 1,637 ± 118; 4, 100 ± 30; 5, 223 ± 33; 6, 275 ± 50; 7, 1,292 ± 91; 8, 985 ± 160; and 9, 520 ± 35. Features 2, 4, 6, and 7 represent v-*myb*; 3, 4, 5, 6, and 8 represent c-*myb* (human); and 1 and 9 represent pBR322. (*D–F*) Heteroduplex formed between a subcloned 7.0-kbp fragment of λ-2 and H3.9. Contour lengths (in kbp) of features are as follows: 1, 3,836 ± 240; 2, 1,342 ± 250; 3, 2,610 ± 259; 4, 150 ± 50; 5, 342 ± 76; 6, 150 ± 30; 7, 1,333 ± 174; 8, 296 ± 33; 9, 691 ± 130; 10, 3,285 ± 328; 11, 634 ± 62. Features 2, 4, 6, 8, and 9 represent v-*myb*; 3, 4, 5, 6, 7, 8, and 10 represent c-*myb* (human); and 1 and 11 represent pBR322. Double-stranded regions in the tracings (*B, E*) and in the interpretive sketches (*C, F*) are indicated by heavy lines, and single strands, by thin lines. Plasmid DNAs were cleaved with *Pvu* I, which did not cleave the inserts. Double- and single-stranded forms of φX174 DNA were used as length standards. Twenty molecules of each heteroduplex were measured using a Tektronix graphics tablet and computer.

locus are consistent with transcription of cellular sequences on both sides of the v-*myb*-related sequences. Of interest is the fact that the 1-kbp DNA fragment located between v-*myb*-related sequences also detected, albeit faintly, the 4.5-kb transcript. This faint hybridization could have resulted from the presence of a *myb*-related sequence either very short or A+T rich. Our results also suggest that the human cellular *myb* product should be much larger than the viral oncogene product, as is the case in the chicken (45).

Specific chromosomal rearrangements have been described in several human tumors (46, 47), and it has been speculated that chromosomal alteration can lead to activation of cellular genes that are otherwise inactive (48). Evidence for this hypothesis has been provided by studies on the specific chromosomal translocations in Burkitt lymphoma involving the c-*myc* gene (49–51). The availability of molecular clones of human c-*myb* allowed us to assign the gene to human chromosome 6 (ref. 52; unpublished results) and localize it to region q22–q24 (53). Deletions in the long arm of chromosome 6 have been found in several cases of acute lymphocytic leukemia and T-cell lym-

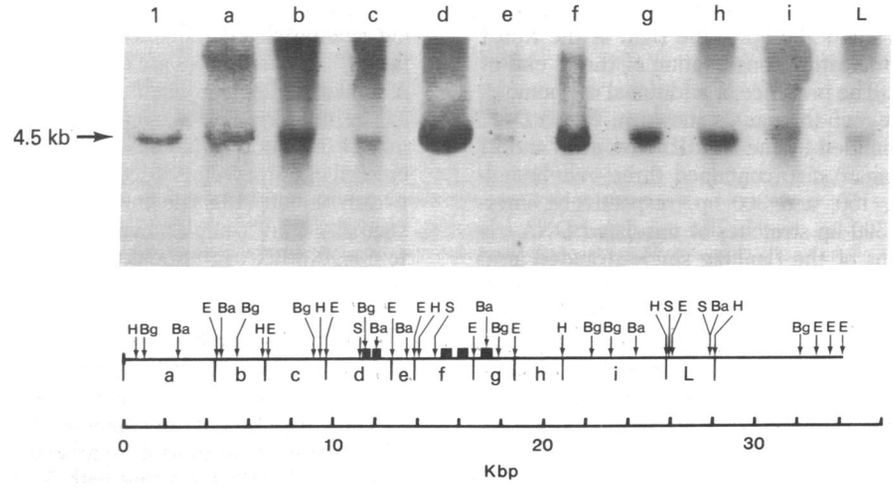

FIG. 4. Analysis of transcripts in MOLT-4 cells. Probes were prepared from the regions of c-*myb* (human) designated a–L and hybridized to MOLT-4 cellular RNA (lanes a–L). A v-*myb* probe (clone H3.9) was used in lane 1. Filters were exposed so that bands <4.5 kb could be visualized in most cases without excessively overexposing the major 4.5-kb band. Abbreviations are as in Fig. 1.

phomas in humans (54). In the MOLT-4 cell line the long arm of chromosome 6 apparently translocates to chromosome 7 (54). Interestingly, the MOLT-4 cell line, other immature T-cell lines, and T cells obtained directly from leukemia patients contain increased amounts of *myb* mRNA (12, 55). Recently, both c-*myb* gene rearrangements and alterations in c-*myb* transcription in some mouse plasmacytomas have been described (56). Thus, all of these observations suggest a role for the *myb* gene in hematopoietic cell neoplasms.

Note Added in Proof. Leprince *et al.* have also recently reported the structure of the human c-*myb* locus (57).

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