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Genetics

Long-distance activation of the *Myc* protooncogene by provirus insertion in *Mlvi-1* or *Mlvi-4* in rat T-cell lymphomas

(provirus insertion/oncogenesis)

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ABSTRACT **T-cell lymphomas induced by Moloney murine leukemia virus frequently have proviruses integrated at the *Mlvi-4* and *Mlvi-1* loci, which map approximately 30 and 270 kilobases 3′ of the promoter region of the *Myc* protooncogene, respectively. Provirus insertion in these loci is responsible for the activation of adjacent genes. To determine whether *Myc* expression was also affected by these provirus insertions, we constructed T-cell hybrids between two rat thymic lymphomas containing a provirus in *Mlvi-4* or *Mlvi-1* and the murine T-cell lymphoma line BW5147. These hybrids segregated the provirus-containing rearranged alleles from the normal nonrearranged alleles of *Mlvi-4* and *Mlvi-1*, and they carried an intact copy of rat *Myc*. Using an S1 nuclease protection assay, we observed that the expression of the rat *Myc* cosegregated with the rearranged *Mlvi-4* or *Mlvi-1* locus. However, provirus insertion in these loci had no effect on promoter utilization or on the expression of the murine *Myc* locus. We conclude that provirus insertion exerts a long-range cis effect on the expression of *Myc*. Therefore, provirus integration in a single locus may affect the expression of multiple genes, some of which may be located a long distance from the site of integration.**

Oncogenesis by retroviruses that lack a transforming gene depends on mutations that are caused by provirus integration into the cellular genome (1, 2). The loci targeted by provirus insertion were identified either as known cellular protooncogenes or as regions of integration that were common among tumors (1, 2). Many common regions of integration identify cellular genes that map in the immediate vicinity of the integrated provirus. The genes flanking the provirus in these cases are activated by mechanisms such as promoter insertion, enhancer insertion, and/or interruption of the normal gene that leads to the synthesis of truncated or hybrid RNA transcripts (3, 4). In all these cases the integrated provirus is known to activate a single gene located in its immediate vicinity.

Moloney murine leukemia virus (Mo-MuLV), a retrovirus that lacks a transforming gene, induces thymic lymphomas in rats (5–7) and mice (8–10). The rat thymomas contain DNA rearrangements mediated by provirus insertion in multiple common regions of integration. These regions include *Mlvi-1* (5, 6), which was shown to be identical with the *Mis-1/Pvt-1* locus (8–14), *Myc* (mouse or rat c-*myc* protooncogene) (8–10, 15, 16), *Mlvi-2* (5, 6), *Mlvi-3* (17), *Mlvi-4* (unpublished work), *Pim-1* (18, 19), *Dsi-1* (20), *Gin-1* (21), and *Tpl-1* (22). Two of these loci, *Mlvi-4* and *Mlvi-1*, were localized in the same chromosomal region as the c-*myc* protooncogene on rat chromosome 7, on mouse chromosome 15, and on human chromosome 8 (refs. 11–13, 17, and 23; unpublished work). Further studies have shown that *Mlvi-4* maps at about 30 kilobases (kb) 3′ of *Myc* (unpublished work) and *Mlvi-1* at about 270 kb 3′ of this protooncogene (24, 25). All the proviruses in both loci examined to date integrate in the same transcriptional orientation, and provirus insertions in these loci activate two flanking cellular genes, *Mlvi-1* and *Mlvi-4* (ref. 26; unpublished work). The experiments described here were undertaken to determine whether, in addition to the activation of *Mlvi-1* and *Mlvi-4*, these provirus insertions also activate *Myc*. This possibility was suggested by earlier studies showing activation of the human c-*myc* gene by chromosomal translocation at sites relatively distant from this protooncogene in Burkitt lymphomas (27–30). The results we present here indeed show that provirus insertions in *Mlvi-1* and *Mlvi-4* enhance *Myc* expression. Therefore, provirus integration in a single locus may affect the expression of multiple genes, some of which may be located at a long distance from the site of integration.

MATERIALS AND METHODS

T-Cell Lymphomas. The origin of the T-cell lymphomas used here has been reported (5–7, 26). They were induced by intraperitoneal inoculation of newborn Osborn Mendel and Long Evans rats with 50,000 XC plaque-forming units of Mo-MuLV. Cell lines established from these tumors will be described in a separate report (P.A.L., A. Klein-Szanto, and P.N.T., unpublished work).

T-Cell Hybridomas. Two tumors, 6889 and 2780, containing a provirus in the *Mlvi-4* and *Mlvi-1* loci, respectively, were adapted to grow intraperitoneally in nude mice, while they lacked the ability to grow in culture. These cells were fused with cells of the hypoxanthine/aminopterin/thymidine (HAT)-sensitive murine T-cell lymphoma line BW5147 by using polyethylene glycol (31). The hybrids derived from these fusions were selected in culture in the presence of HAT medium. Since the parental cells 6889 and 2780 lacked the ability to grow in culture and BW5147 was HAT-sensitive, this procedure selected for hybrid cells. Hybrid clones were screened by dot blot analysis and hybridization to rat *Myc* probes to determine whether they contained rat *Myc*. Following four or five passages to allow for the segregation of rat chromosomes, the hybrids were examined by Southern blot analysis and hybridization to *Mlvi-1*, *Mlvi-4*, and *Myc* probes.

Preparation of Cellular DNA and Southern Blotting. High molecular weight DNA was prepared as described (5). After cleavage with restriction endonucleases the DNA was fractionated in 0.8% agarose gels and transferred to nylon membranes (Hybond-N from Amersham). Hybridizations to *Mlvi-1*, *Mlvi-4*, and *Myc* probes were performed as described (5).

Isolation of Polyadenylylated RNA and Northern Blotting. Total cell RNA was isolated by the guanidinium thiocyanate/cesium chloride method (32), and polyadenylylated RNA was selected by affinity chromatography on oligo(dT) cellulose (33). Five micrograms of polyadenylylated RNA was elec-

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Abbreviation: Mo-MuLV, Moloney murine leukemia virus.

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trophoresed in denaturing formaldehyde/agarose gels and, after transfer to nylon membranes (34), hybridized to a 0.7-kb *Bgl* II–*Xho* I fragment derived from the rat *Myc* and containing exon III sequences (16). Following removal of the probe the RNA immobilized in the filters was hybridized to a murine actin probe (35).

S1 Nuclease Protection Assay. Total RNA was prepared by the guanidinium thiocyanate/cesium chloride method (32). Thirty micrograms of total RNA was hybridized in solution to single-stranded, uniformly labeled DNA probes specific for *Myc* exon I or III. The exon I single-stranded probe was prepared by subcloning the insert of plasmid pR*myc*1.4 (provided by M. Cole, Princeton University) in M13 mp18 phage. The probe is a 530-nucleotide *Bgl* II–*Bam*HI fragment. Transcripts originating in the P_2 promoter protect a fragment of 430 nucleotides. The exon III probe, a 0.7-kb *Bgl* II–*Xho* I DNA fragment (16), was also subcloned in the M13 mp18 vector. Labeled probe (200,000 cpm) was hybridized for 16 hr at 55°C for exon I and 59°C for exon III in a final volume of 20 μl containing 80% (vol/vol) formamide, 0.4 M NaCl, 40 mM Pipes, and 1 mM EDTA at pH 6.4. Digestion with nuclease S1 was done at 37°C for 30 min in a final volume of 100 μl containing 0.3 M NaCl, 30 mM sodium acetate (pH 4.8), 3 mM $ZnSO_4$, and 100 units of S1 nuclease (Amersham). The reaction products were analyzed in a 4% polyacrylamide/7 M urea gel.

RESULTS

The effects of provirus integration in *Mlvi-1* and *Mlvi-4* on the expression of *Myc* were first examined by Northern blot analysis of polyadenylylated RNA from normal and tumor tissues and hybridization to the rat *Myc* exon III probe. The results (Fig. 1) revealed an increase of *Myc* RNA in tumors with a provirus in *Mlvi-1* (B_8L, B_2, D_3, and C_3), *Mlvi-4* (6889), or 5′ of *Myc* (D_1). These findings, therefore, supported the hypothesis that deregulation of *Myc* may be due to provirus integration in distant loci.

To test this hypothesis further, we constructed T-cell hybrids by fusing cells from the murine T-cell lymphoma line BW5147 with either of two rat thymomas, 2780 and 6889, which contain a provirus in *Mlvi-1* and *Mlvi-4*, respectively. The purpose of this experiment was to segregate the rearranged from the nonrearranged rat chromosome 7 and to ask whether the expression of the *Myc* protooncogene observed in rat thymomas cosegregates with the allele containing the integrated provirus.

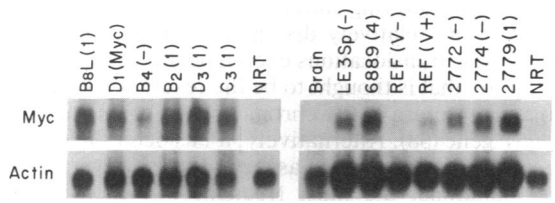

FIG. 1. Provirus integration in *Myc*, *Mlvi-1*, and *Mlvi-4* correlates with *Myc* expression. Normal and tumor cell polyadenylylated RNA was analyzed by Northern blotting with a 0.7-kb *Bgl* II–*Xho* I rat *Myc* probe that spans exon III (16). After removal of the probe the same filters were hybridized to a murine actin probe (35) to determine the approximate amount of RNA per lane. The extent of hybridization was determined by autoradiography and by quantitation of the radioactivity on the filter with the AMBIS radioactivity-imaging system (AMBIS Systems Inc., San Diego; data not shown). The AMBIS tracing confirmed the data presented here, showing that the amount of RNA was similar in all the tumor lanes. The two panels (*Left* and *Right*) represent independent experiments. Lanes are identified by the name of the normal or tumor tissue from which the RNA was derived. For each tumor, the locus rearranged by provirus insertion is indicated in parentheses (1; *Mlvi-1*; 4, *Mlvi-4*, a minus sign indicates that no proviruses were detected in this region). REF (V−) and REF (V+), uninfected and MoMuLV-infected rat embryo fibroblasts, respectively. NRT, normal rat thymus.

The segregation of the two allelic rat chromosomes 7 from tumor 2780 in the cell hybrids was monitored by using the *Mlvi-1* probe pBS8.5. The rat germ-line *Mlvi-1* was detected as a 9-kb band in *Pst* I digests, whereas the rearranged allele was detected as a strong band at 12 kb and a very weak band at 7.5 kb (Fig. 2*A*). The *Mlvi-1* rearrangement generated two *Pst* I fragments because the Mo-MuLV provirus, which is cleaved by *Pst* I, had integrated within the germ-line *Pst* I fragment detected by the probe. The homologous mouse region was detected as an 8.3-kb band. Four cell hybrids lost one of the *Mlvi-1* alleles. Cell hybrids H1-8, H1-9, and H1-19 retained the rearranged allele and cell hybrid H1-7 retained the germ-line allele. Each of the hybrids retained a copy of the rat *Myc* locus in germ-line configuration. This was evidenced by hybridization of *Hin*dIII-digested DNA from the cell hybrids and the parental cell lines with a rat *Myc* exon III probe. The 10-kb fragment seen in the hybrids was due to the unrearranged rat *Myc* locus, whereas the 4-kb band was generated by the mouse *Myc* (Fig. 2*C*). These results demonstrated that all cell hybrids contained both the rat and the mouse *Myc* gene. Digestion of genomic DNA with *Kpn* I, *Xba* I, *Hin*dIII, and *Bam*HI (16) revealed that the rat *Myc* allele in the hybrids was unrearranged (data not shown).

The segregation of the two allelic rat chromosomes 7 from tumor 6889 in the hybridomas was monitored by using the *Mlvi-4* probe pLE18. The rat germ-line and rearranged *Mlvi-4* alleles were represented by 3.5-kb and 13-kb *Eco*RI bands, respectively. The homologous mouse sequences were detected as a 3.8-kb *Eco*RI band (Fig. 3*A*). Genomic DNA from these hybridomas was digested with *Hin*dIII (Fig. 3*C*) or with *Kpn* I, *Xba* I, or *Bam*HI (data not shown) and subjected to Southern blot analysis with the rat *Myc* exon III probe,

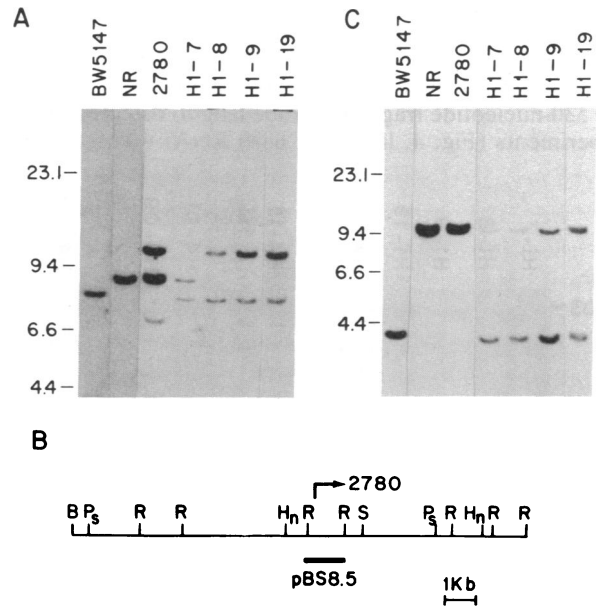

FIG. 2. (*A*) Southern blot analysis of genomic DNA from cell hybrids (H1-7, -8, -9, and -19) derived from the fusion of the rat thymoma 2780, containing a provirus in one allele of the *Mlvi-1* locus, with the murine T-cell lymphoma line BW5147. Genomic DNA was digested with *Pst* I and hybridized to the *Mlvi-1* probe pBS8.5. NR, DNA from normal rat. Markers at left indicate length in kilobases. (*B*) Restriction map of the rat *Mlvi-1* locus. Arrow indicates the location and transcriptional orientation of the integrated provirus. B, *Bam*HI; P_s, *Pst* I; R, *Eco*RI; H_n, *Hin*cII; S, *Sal* I. (*C*). Southern blot analysis of DNA from cell hybrids as in *A*, except that the DNA was digested with *Hin*dIII and hybridized to a rat *Myc* exon III probe (16).

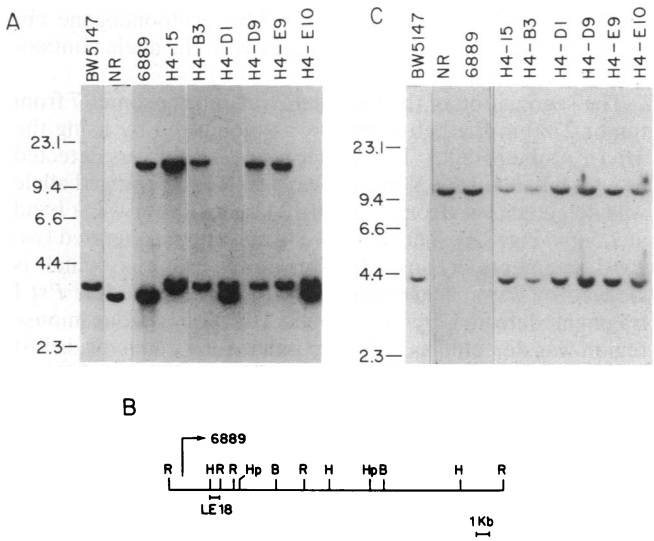

FIG. 3. (*A*) Southern blot analysis of genomic DNA from cell hybrids (H4 lanes) derived from the fusion of the rat thymoma cell line 6889, containing a provirus in one allele of the *Mlvi-4* locus, with the murine T-cell lymphoma line BW5147. DNA was digested with *Eco*RI and hybridized to the *Mlvi-4* probe pLE18. NR, DNA from normal rat. (*B*) Restriction map of the *Mlvi-4* locus showing the location and transcriptional orientation (arrow) of the provirus in the rearranged allele. B, *Bam*HI; H, *Hin*dIII; R, *Eco*RI; H_p, *Hpa* I (*C*) Southern blot analysis as in *A*, except that the DNA was digested with *Hin*dIII and hybridized to a rat *Myc* exon III probe.

revealing that all of them contained intact and unrearranged murine and rat *Myc* loci.

Expression of *Myc* RNA in these hybrids was determined by an S1 nuclease protection assay using the rat *Myc* exon I probe. This analysis detected the rat *Myc* RNA initiated at the P_2 promoter as a protected fragment of the expected size of 430 nucleotides (Fig. 4). Since the 5′ end of the probe maps between the P_1 and P_2 promoters, this experiment could not map the start site of the transcript originating in P_1. However, the 530-nucleotide fragment (probe length) detected in these experiments (Fig. 4, lane with 6889 RNA) was most likely

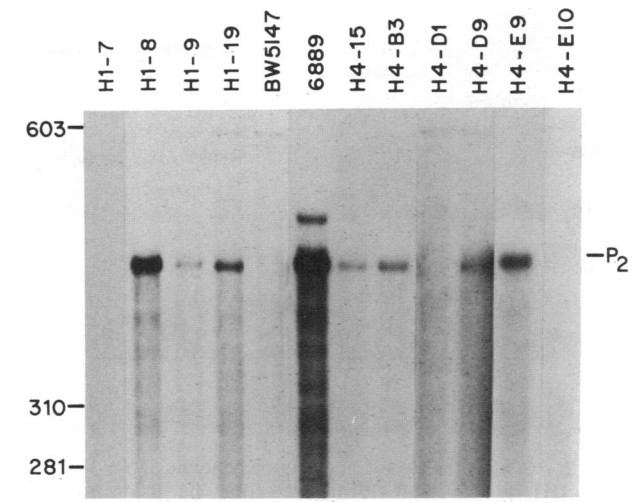

FIG. 4. S1 nuclease protection of rat *Myc* by RNA from cell hybrids with germ-line or rearranged *Mlvi-1* or *Mlvi-4* alleles. Thirty micrograms of total RNA was hybridized to a single-stranded uniformly labeled rat *Myc* exon I probe. After S1 nuclease digestion, the reaction products were analyzed in a 4% polyacrylamide/7 M urea gel. Size markers at left indicate length in nucleotides. Marker at right indicates the fragment protected by *Myc* transcript initiated at the P_2 promoter.

protected by an RNA that was transcribed from the P_1 promoter. These results showed that all cell hybrids containing a provirus in *Mlvi-1* or *Mlvi-4* expressed the rat *Myc* protooncogene, whereas those containing the germ-line allele from two different thymomas did not express detectable levels of the rat *Myc* transcript. This finding demonstrates that the expression of rat *Myc* is linked to the provirus-mediated rearrangement at the *Mlvi-4* or *Mlvi-1* locus. The promoter utilization in both the parental rat tumors and the hybrids favored the P_2 promoter. This is contrary to what is observed in translocations involving *Myc*, where there is preferential utilization of the P_1 promoter (36).

The effect of the distant proviruses on the expression of *Myc* could be transmitted either in cis, by an unknown mechanism, or in trans, by the protein product of a gene adjacent to the integrated provirus. To distinguish between these possibilities, we examined the level of *Myc* RNA in the T-cell hybrids described above, using a probe derived from exon III of rat *Myc* in an S1 nuclease protection assay. Given the high degree of structural conservation of exon III of c-*myc* genes, this assay detected both rat and mouse *Myc* messages. The results (Fig. 5) revealed that while the rat *Myc* was expressed only in hybrids with a rearrangement in the *Mlvi-4* locus, the mouse *Myc* was expressed in all the hybrids and that its level of expression did not correlate with the rearranged rat *Mlvi-4* allele. Assuming that the trans-acting factors regulating the expression of *Myc* function across species barriers, these findings indicate that the effect of provirus integration on *Myc* expression is mediated by a cis-acting mechanism. Furthermore, if the product of *Myc* regulates its own expression (37), the lack of correlation between the relative levels of expression of the rat and mouse *Myc* genes suggests that such a regulation may be indirect.

DISCUSSION

The purpose of the experiments presented in this report was to determine whether *Myc* expression in MoMuLV-induced rat thymic lymphomas was influenced by provirus integration in the *Mlvi-1* and *Mlvi-4* loci. Our findings provide evidence that, indeed, provirus integration in these loci affects the expression of *Myc* from a long distance. The same findings suggest that the observed long-range effect of provirus integration on *Myc* expression is transmitted in cis. A similar long-range effect on c-*myc* expression was observed previously in tumors carrying chromosomal translocations involving breakpoints relatively distant from this protooncogene (27–30). These translocations exert a positive effect on c-*myc* transcription that is thought to be the result of changes in the chromatin structure in the chromosomal region surrounding the c-*myc* gene (38). Alternatively, it has been suggested that these translocations may be associated with several types of c-*myc* mutations, the most frequent of which affect the nontranslated exon I (30, 39). The detection of a consensus heptamer–nonamer recognition sequence in exon I of *Myc* has been interpreted to suggest that mutations in this exon,

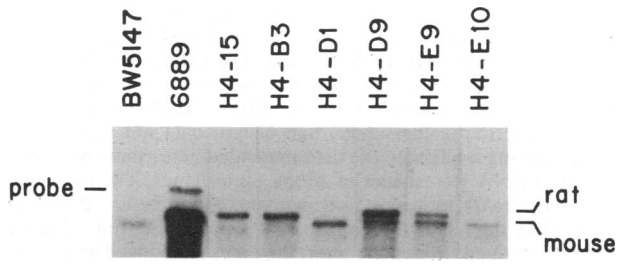

FIG. 5. S1 nuclease protection of *Myc* by RNA from cell hybrids with normal or rearranged *Mlvi-4* alleles. The *Myc* probe was derived from rat exon III and detects both rat and mouse *Myc* transcripts.

following translocation, were due to the operation of mechanisms normally associated with the occurrence of somatic mutations in the immunoglobulin heavy-chain (*Igh*) locus (40). The findings presented here are unique in that provirus integration generates a subtle chromosomal rearrangement compared to a chromosomal translocation. Furthermore, it appears unlikely that somatic mutations may play a role in the activation of the *Myc* allele on the chromosome targeted by the provirus because (*i*) the S1 nuclease protection assay detects an exon I DNA fragment of the expected size, (*ii*) provirus insertion in *Mlvi-1* or *Mlvi-4* is not associated with gross rearrangements of *Myc*, and (*iii*) provirus integration is not known to be associated with mutations in neighboring genes (41). Therefore the long-distance activation of *Myc* described in this report is unlikely to be caused by superimposed somatic mutations.

Provirus integration in *Mlvi-4* and *Mlvi-1* occurs in DNA regions that are conserved among species. These integration domains are surrounded by long stretches of nonconserved DNA (26). We speculate that the conserved sequences may represent important regulatory elements that influence c-*myc* activity from a long distance. Provirus integration may exert its effect by interrupting these regulatory elements. Such sequences have been described previously in at least two instances. In the human β-globin gene, regulatory sequences detected by DNase I hypersensitivity were localized 50 kb 5′ and 20 kb 3′ of the gene (42–44). These sequences were shown to be essential for the proper tissue-specific expression of β-globin in transgenic mice (42–45). A similar long-distance effect has been reported for the *Igh* enhancer, which activates variable gene (V_H) promoters located at least 17.5 kb upstream (46). It is not clear why provirus integration in these regions may exert the long-range effects on *Myc* expression we have described in this report. It is possible, however, that because of the three-dimensional organization of the chromosomal DNA, *Mlvi-1* and *Mlvi-4* are juxtaposed to regulatory regions in the immediate vicinity of *Myc*. Alternatively, large chromosomal regions located between matrix attachment sites (47, 48) may represent domains of coordinately regulated transcriptional units. Perturbations within these domains induced by provirus integration or other mechanisms may affect the expression of nearby as well as relatively distant genes. Any attempts to understand this phenomenon should take into account the finding that all the *Mlvi-4* and *Mlvi-1* proviruses integrate in a single transcriptional orientation. This suggests that the orientation of transcription of the provirus may be an important component of the long-distance *Myc* activation we have described here.

Independent of the mechanism by which provirus integration affects the expression of distant genes, the data presented in this report will have important implications for our understanding of oncogenesis by nontransforming retroviruses. It has been widely held until now that provirus integration may be responsible for the transcriptional enhancement of a single gene in the immediate vicinity of the integrated provirus. Our data suggest that this may not be correct and that a single provirus may affect the expression of multiple genes, some of which may be located a long distance from the site of integration.

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