

RETROVIRAL ACTIVATION OF INTERLEUKIN 2 GENE IN A GIBBON APE T CELL LYMPHOMA LINE

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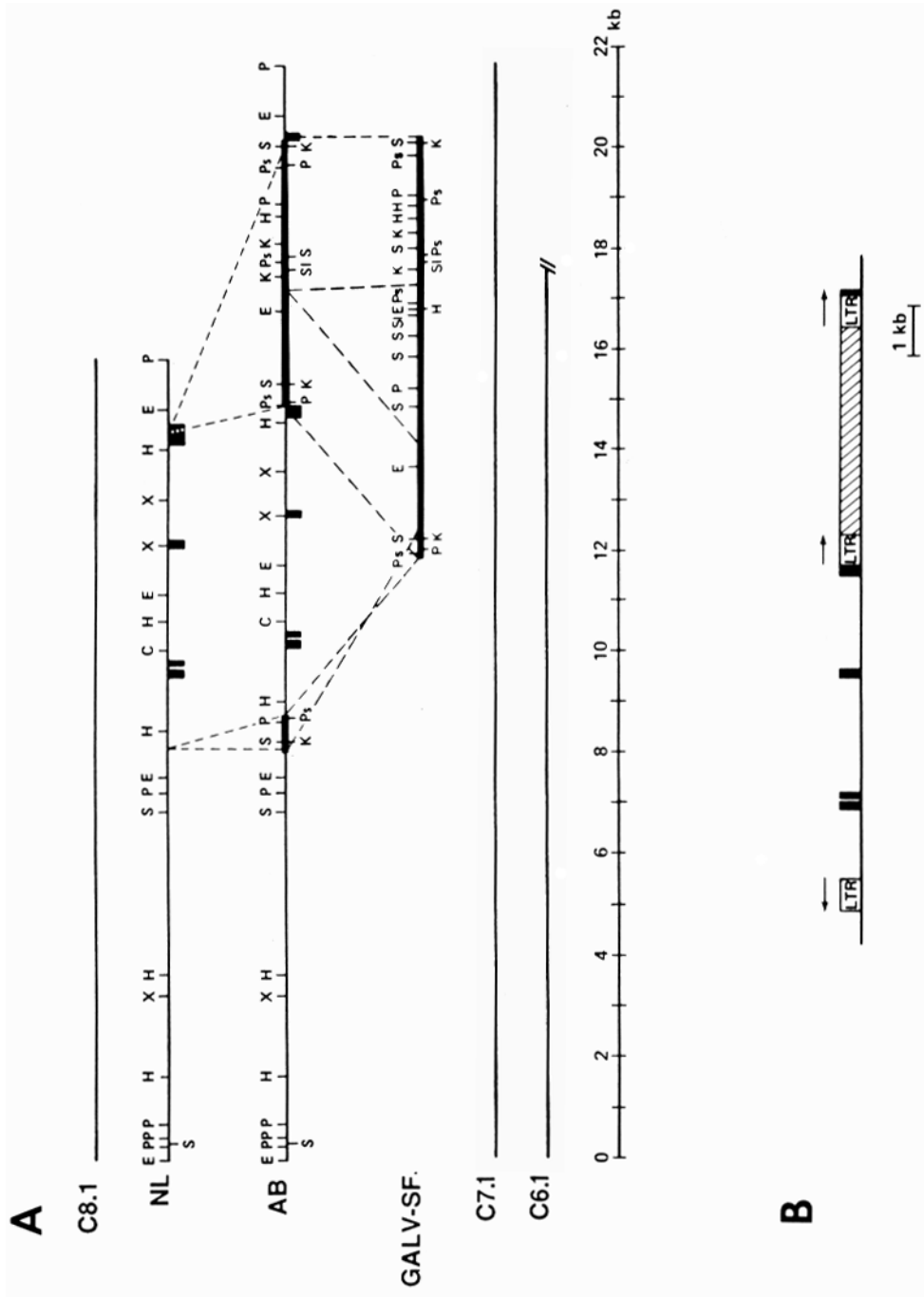
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The MLA 144 cell line was derived from a lymphoma in a gibbon ape (1). At the time of its establishment the line was found to be infected with a leukemia virus (gibbon ape leukemia virus, San Francisco strain [GaLV_{SF}]),¹ which is a C-type retrovirus associated with a T cell lymphoma (1). Although hybridization studies (2, 3) indicate that human T cell leukemia/lymphoma (lymphotropic) virus (HTLV) I and II are not closely related to any GaLV species they may affect the same subset of genes involved with T lymphocyte growth. Specifically, MLA 144 resembles the HTLV-infected cell lines MO and HUT-102 (3, 4) in that it constitutively produces the T cell growth factor IL-2 (5) and IL-2 receptors (6). Although dual constitutive expression of IL-2 and IL-2R genes among GaLV- and HTLV-infected leukemias and lymphomas occurs sporadically (4, 5), in such cases an autocrine mechanism may contribute to malignant cell growth (7).

Recently, Chen et al. (8) established through Southern blotting that there are rearrangements at the 5' and 3' ends of the IL-2 gene in MLA 144 cells. cDNA cloning and sequencing showed that the 3' rearrangement is due to a GaLV_{SF} insertion in the 3' nontranslated region of one IL-2 allele. This insertion leads to the production of a transcript ending in the viral long terminal repeat (LTR) (8). The complete structure of the 3' insertion and whether the 5' rearrangement was also due to GaLV_{SF} insertion remained unclarified. Also unanswered was whether the two rearrangements involved the same IL-2 allele and whether both alleles were transcriptionally active. The present study provides a complete structural characterization of both IL-2 alleles of MLA 144 as well as a determination of the relative transcriptional activity of the two alleles.

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¹ *Abbreviations used in this paper:* GaLV, gibbon ape leukemia virus; HTLV, human T cell leukemia/lymphoma (lymphotropic) virus; LTR, long terminal repeat.



Materials and Methods

MLA 144 Cosmid Library Construction and Screening. Cosmid clones were obtained by a modification of the method of Ish-Horowicz and Burke (9). Briefly, high molecular weight DNA was prepared from MLA 144, and subjected to RNase A treatment and dialysis. Genomic fragments 30–45 kb in size were prepared by partial digestion with *Sau* 3A1. Vector arms were prepared by digesting equal quantities of pGcos4 DNA with *Sst* I or *Sal* I, respectively (10). The aliquots were mixed, treated with calf intestinal alkaline phosphatase, and cut with *Bam* HI. The long vector arms were then isolated from a 1% agarose gel by the freeze-squeeze method (11). 1 μ g of insert DNA was ligated to 2 μ g of arms in a 10- μ l reaction volume in the presence of 66 mM Tris, pH 7.5, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, and 100 μ g/ml BSA for 3 h at 15°C. The ligated DNA was packaged in vitro as described by the manufacturer (Gigapack; Vector Cloning Systems, San Diego, CA), and used to transduce *E. coli* HB101 tested for *recA* negativity (12). High-density screening on tetracycline plates (13) was carried out using a ³²P-labelled human IL-2 cDNA probe (14). 454,000 colonies per microgram of insert DNA were obtained, and of 150,000 colonies screened, 4 reacted strongly with the IL-2 cDNA probe. Of these, three were recovered and mapped by means of single, double, and triple restriction enzyme digests, as well as Southern blotting (15), as described in the text. DNA probes were ³²P-labelled by the random primer method (16). The full-length GaLV probe was a generous gift from M. Scott (17). The farthest 5' Eco RI fragment in clones 6.1, 7.1, and 8.1 was subcloned from the cosmids by using a ³²P-labelled probe made from the 300 bp Eco RI–Pvu II fragment (Fig. 1A; map units 7.2–7.5 kb) obtained from the overlapping Pvu II subclone.

Nucleotide Sequence of 5' GaLV_{sr} Insertion. The Eco RI–Hind III restriction fragment immediately upstream of the first IL-2 exon (map units 7.6–8.5 kb in the normal allele [NL]; Fig. 1A) was sequenced from both IL-2 alleles of MLA 144. The sequencing strategy for the abnormal allele and the number of basepairs sequenced in a given direction were as follows: Eco RI → Pvu II (412), *Sma* I → Eco RI (368), *Sma* I → Hind III (231), Pvu II → Eco RI (342), Pvu II → Hind III (231), and Hind III → *Sma* I (381). The normal MLA IL-2 allele was sequenced from Hind III toward Eco RI (436 bp). The indicated

FIGURE 1. (A) Restriction enzyme maps of the normal (NL) and doubly rearranged (AB) MLA 144 IL-2 alleles. Mapping involved use of single, double, and triple restriction enzyme digests and Southern blotting as described in the text. IL-2 regions were mapped with the enzymes *Cla* I (C), Eco RI (E), Hind III (H), Pvu II (P), *Sma* I (S), and *Xba* I (X). Viral areas were mapped with Eco RI, Hind III, *Kpn* I (K), *Pst* I (Ps), Pvu II, *Sal* I (Sl), and *Sma* I. The transcriptional orientation of the IL-2 gene is 5' to 3' reading left to right. The putative gibbon IL-2 exons are represented by solid blocks, whose placement is based on the close homology between the normal human (14) and normal gibbon IL-2 maps. The borders of the retroviral insertions in the abnormal allele (AB) are indicated by broken lines between the normal and abnormal allele maps. A map of the GaLV_{sr} unintegrated provirus is shown below the abnormal allele map for comparison. The abnormal IL-2 allele contains a single LTR insertion at the 5' end and a deleted provirus at the 3' end. Dotted lines connect the 5' GaLV_{sr} LTR to the 5' GaLV insertion in the abnormal allele. The crossing of the dotted lines indicates the opposing transcriptional orientation of these LTRs. The 3' GaLV insertion contains an internal deletion of 3.25 kb shown by the dotted lines. The region of the three cosmid clones used to map the normal allele (C8.1) and the abnormal allele (C6.1 and C7.1) are indicated by the solid lines above and below the restriction maps. The double hatch mark at the 3' end of the C6.1 clone indicates that the region 3' to this border did not contain further viral sequence and most likely represented DNA recombined from some other part of the MLA genome. (B) A schematic representation of the abnormal IL-2 allele in the gibbon ape MLA 144 cell line. The probable locations of the four IL-2 exons, the fourth split by the 3' retroviral insertion, are indicated by the solid blocks. The retroviral LTRs are shown, with the rest of the 3' viral insertion appearing as a crosshatched bar. Arrows signify the transcriptional orientation of the LTRs. The transcriptional orientation of the 5' inserted LTR opposes that of the IL-2 gene.

restriction fragments were gel purified, cloned into the M13mp18 and M13mp19 vectors, and sequenced by the chain-termination method (18, 19). Sequences were aligned by use of the Bionet (IntelliGenetics, Mountain View, CA) programs.

RNA Preparation and Northern Blotting Analysis. Normal adult gibbon blood was obtained from the Yerkes Regional Primate Research Center. Gibbon PBMC were isolated by the Ficoll-Hypaque method as described (20). Gibbon PBMC were stimulated with PHA-P (20 μ g/ml) (Difco Laboratories, Detroit, MI) and PMA (10 ng/ml) (Sigma Chemical Co., St. Louis, MO) for 18 h before harvest. MLA 144 cells were not stimulated. Jurkat cells, which do not produce IL-2 constitutively (21), were stimulated with the same doses of PHA and PMA for 6 h before harvest. RNA was extracted by the guanidinium thiocyanate method (22), size fractionated (20 μ g/lane) on 1.25% formaldehyde agarose gels, transferred to nitrocellulose filters, and hybridized to a 32 P-labelled human IL-2 cDNA probe (14, 23). The filters were washed four times for 5 min in $2 \times$ SSC, 0.1% SDS at 22°C followed by two 20-min washes in $0.1 \times$ SSC, 0.1% SDS at 65°C. The filters were then dried and exposed to x-ray film for 2.5 d.

Results

Isolation and Characterization of Cosmid Clones Representing the Normal and Doubly Rearranged IL-2 Alleles of MLA 144. Previous Southern blots of Eco RI digests of normal human (14) and normal gibbon DNA (8) hybridized to a full-length 32 P-labelled human IL-2 genomic probe (pIL-2, which includes two 3,700 bp Eco RI fragments containing all four human exons) revealed a band (actually a doublet) at 3,700 bp. In contrast, the Southern blot of MLA 144 DNA revealed a nonrearranged band at 3,700 bp, as well as bands at 4,500 and 6,000 bp, which by use of 5'- and 3'-specific IL-2 probes were shown to represent 5' and 3' rearrangements in the gene, respectively (8).

To characterize the structure of both IL-2 alleles of MLA 144 we made a cosmid library from MLA 144 DNA and isolated three clones, 6.1, 7.1, and 8.1, that hybridized to a 32 P-labelled human IL-2 cDNA probe (14). To map the region containing IL-2 and viral sequences, we subcloned from each of the cosmid clones the overlapping Eco RI and Pvu II fragments that hybridized to 32 P-labelled pIL-2 and/or to a full length 32 P-labelled GaLV probe (pGaLV) (17).

The mapping results indicate that the Southern analysis of Eco RI digests of MLA 144 DNA (described above) is explained by the presence of a normal allele (*NL*, Fig. 1A) represented by cosmid clone 8.1, and a doubly rearranged allele (*AB*, Fig. 1A) represented by cosmid clones 6.1 and 7.1. The normal allele contributes the IL-2-hybridizing 3.7 kb Eco RI fragments seen on the MLA 144 genomic Southern blot, and the doubly rearranged allele contributes the 4.5 and 6.0 kb Eco RI fragments. A 3.8 kb Eco RI fragment at the 3' end of the abnormal allele (described below in greater detail) comigrates with the 3.7 kb Eco RI fragments of the normal allele. Like the normal human and gibbon IL-2 alleles, cosmid 8.1 contains two 3.7 kb Eco RI fragments, which hybridize to pIL-2 but not to pGaLV. The positions of restriction endonuclease sites in the normal gibbon allele show no significant differences from those in the two 3.7 kb Eco RI fragments containing the four exons of the human IL-2 gene (14). For this reason, the exons of the normal gibbon allele are likely to be located similarly to those of the human gene, and are so indicated on the map (*NL*, Fig. 1A).

Cosmids 6.1 and 7.1 represent an IL-2 allele doubly rearranged by the insertion of GaLV sequences in two regions. Each contains Eco RI fragments of 4.2 and

5.1 kb that hybridize to pGaLV and to pIL-2; hence, the two rearranged Eco RI bands seen in the MLA 144 Southern blot are from the same allele and are due to insertions of retroviral sequences (solid lines on map AB, Fig. 1A). Cosmid 7.1 has an additional 3.8 kb Eco RI fragment that hybridizes to pIL-2 and pGaLV and contains the 3'-most portion of the IL-2 gene. Detailed mapping of clones 6.1 and 7.1 defined the sizes and locations of the 5' and 3' retroviral insertions, as shown in Fig. 1A. A small region of inserted GaLV sequence is found ~1.3–2.1 kb upstream of the first IL-2 exon, while a larger insertion (discussed below) interrupts the fourth and final exon. In mapping the abnormal allele, it was essential to establish the locations of the IL-2 sequences displaced by the insertions. To do this we prepared probes from the Eco RI–Hind III fragments at the 5' and 3' ends of human pIL-2, which are equivalent to the Eco RI–Hind III fragments extending from 7.6 to 8.5 kb, and from 14.0 to 14.8 kb on the map of cosmid 8.1 (NL in Fig. 1A). These probes hybridized to the Eco RI–Pvu II fragments of cosmid 6.1 extending from 7.6 to 8.6 kb and 19.7 to 20.7 kb (map AB of Fig. 1) overlapping the ends of the 5' and 3' insertions, respectively. In short, this demonstrates that the Eco RI sites upstream of the 5' insertion and downstream of the 3' insertion (Fig. 1A) are not contributed by GaLV insertions, but instead represent displaced IL-2 sequences.

A comparison of the restriction map of the 3' GaLV insertions in clones 6.1 and 7.1 to a map of the unintegrated GaLV-SF provirus (Fig. 1A) (17) indicates that the 3' insertion in the abnormal allele is a provirus with a 3.25 kb internal deletion sparing the two LTRs (Fig. 1A). Such deletions have been observed in many retroviral insertions (24). The LTRs in the 3' proviral insertion have the same transcriptional orientation as the IL-2 gene. In contrast, the map of the 5' insertion in the abnormal allele suggests that these inserted GaLV sequences represent a single LTR with a transcriptional orientation opposing that of the IL-2 gene. Sequence data presented below confirm this impression. A schematic diagram of the abnormal allele is presented in Fig. 1B.

Sequencing of Normal and Abnormal Alleles in the Region of the 5' GaLV Insertion. To substantiate our conclusions regarding the 5' insertion, we sequenced the Eco RI–Hind III fragment immediately upstream of the first IL-2 exon in both normal and abnormal gibbon IL-2 alleles (Fig. 2). The only discrepancies between the LTR sequence of the 5' insertion and the previously published partial LTR sequences of the 3' insertion (8) were the absence of bases in the latter sequence found at positions 727, 850, 918, 920, 1,023, and 1,025 of our sequence. The last four discrepancies represent a pair of base changes in a direct repeat. A compression of the bands corresponding to this region (CCCTGGCC) on sequencing gels precluded precise reading of this sequence, despite analysis of both strands of DNA. The 5' insertion is an LTR without additional viral sequences represented. Similar single LTR insertions have been described (25–27). A 3 bp duplication of host DNA is seen at the borders of the 5' intergration, similar to the duplications noted for a variety of other retroviruses (24). There is a 96 bp direct repeat in the U3 region of the 5' insertion nearly identical to the 94 bp repeat in the 5' LTR of the 3' insertion (8). Of interest was whether the same direct repeat was present in the previously cloned unintegrated GaLV_{SF} provirus (Fig. 1A), since the published sequence does not contain this repeat

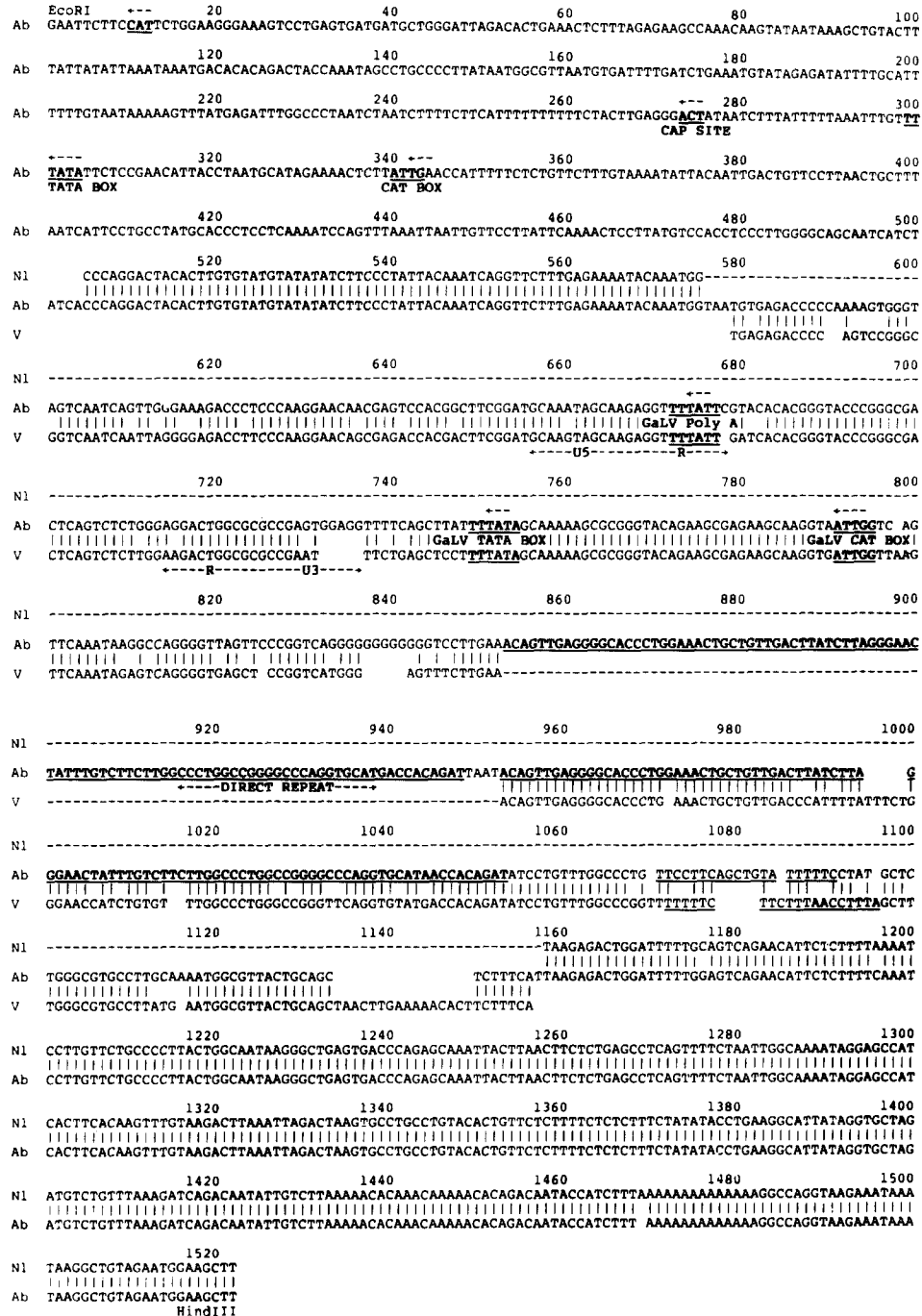


FIGURE 2. Sequences of the GaLV_{sf} LTR (V) (32) and of the normal (NL) and abnormal (AB) MLA 144 IL-2 alleles in the region of the 5' GaLV_{sf} insertion. Vertical bars connect homologous bases. The alignment of the 5' end of the LTRs at positions 1,150-1,156 was made, since the sequence TCTTTCA is in one of the viral inverted terminal repeats. The poor alignment from 1,071 to 1,096 may be due to an inversion of the two underlined se-

(17). On a 2% agarose gel, the three Pst I–Sma I fragments of the LTRs in the 5' and 3' insertions all comigrate at ~420 bp, whereas the two Pst I–Sma I LTR fragments in the GaLV_{SF} clones comigrate at ~320 bp. Comparison of the inserted and unintegrated proviral sequences makes it clear that the observed size difference is most likely due to the presence of the direct repeat in the integrated LTR and the absence of the direct repeat in the unintegrated proviral LTRs. The commonly observed sequencing error of missing direct repeats is therefore excluded, and all three LTRs in the abnormal IL-2 allele of MLA 144 are likely to contain a 96 bp direct repeat.

Further analysis of the sequence upstream from the 5' GaLV_{SF} LTR insertion reveals a set of sequences associated with functional genes, including a CAT box (CAAT) separated from a TATA box (TATAAA) by 35 bp, in turn followed by a potential cap site (AGT) and a potential translation initiation site (ATG) (Fig. 2). These elements are in a transcriptional orientation opposing that of the IL-2 gene. In short, there may be a gene or pseudogene adjacent to the IL-2 gene. The potential implications of this finding are discussed below. As yet we have discovered no significant homology of this sequence to published sequences, and we have begun Northern analysis to determine whether the gene is expressed in MLA or in other tissues.

Northern Analysis of MLA 144, Normal Gibbon, and Human IL-2 Message. To support the notion that one or both retroviral insertions have activated the IL-2 gene in MLA 144, we performed Northern analysis on total RNA extracted from mitogen-stimulated normal gibbon PBMC and unstimulated MLA cells (Fig. 3). RNA derived from the human T cell line Jurkat (21), which can be induced to express large quantities of IL-2 mRNA (28, 29), provided a control. The Jurkat IL-2 mRNA is identical in size (900 bp) to that produced by normal human T cells (28, 29). We found that the predominant MLA 144 mRNA hybridizing to a ³²P-labelled human IL-2 cDNA probe was ~1,125 bp long, 225 bp longer than the IL-2 messages of Jurkat and of PBMC from two normal adult gibbons. When gibbon and MLA 144 RNA samples were mixed and electrophoresed in the same lane, the distribution of hybridizing RNA was the sum of the bands observed when the RNAs were electrophoresed in separate lanes (data not shown); this demonstration excludes a salt concentration effect as an explanation for the difference in migration of the MLA 144 IL-2 message. We conclude that the larger size of the mRNA species produced constitutively by MLA 144 reflects the fact that the GaLV insertion in the fourth exon of the altered IL-2 gene interrupts the 3' nontranslated region of the transcript (8), and that the abnormal allele has been activated by one or both GaLV insertions.

quences. Putative U5-R and R-U3 GaLV_{SF} borders based on homology to simian sarcoma virus (33) as well as the viral CAT box, TATA box, and polyadenylation (*Poly A*) signal are indicated. The direct repeat in the U3 region of the 5' insertion is underlined (positions 853–1,053). The CAT box, TATA box, a potential cap site (AGT), and a potential translation initiation site (ATG) of the putative gene upstream from the 5' LTR insertion lie between positions 9 and 343 in the sequence. Transcriptional orientations are indicated by arrows.

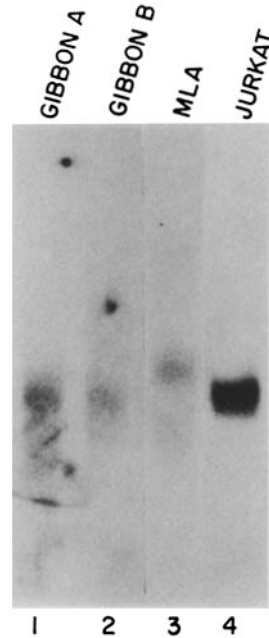


FIGURE 3. Northern analysis of normal gibbon, MLA, and Jurkat RNAs. A Northern blot of total cellular RNA from the PBMC of two normal gibbons (lanes 1 and 2), MLA cells (lane 3), and Jurkat cells (lane 4) is shown. Gibbon PBMC were isolated by the Ficoll-Hypaque method and were then stimulated with PHA-P (1:500 final dilution) and PMA (10 ng/ml) for 18 h before harvest. Jurkat cells were stimulated with the same doses of PHA and PMA for 6 h before harvest, while MLA 144 cells were not stimulated. RNA was extracted by the guanidinium thiocyanate method, size fractionated (20 μ g/lane) on 1.25% formaldehyde/agarose gels, transferred to nitrocellulose filters, and hybridized to a 32 P-labelled human IL-2 cDNA probe. Filters were then washed four times for 5 min in $2 \times$ SSC, 0.1% SDS at 22°C followed by two 20-min washes in $0.1 \times$ SSC, 0.1% SDS at 65°C. Filters were then dried and exposed to x-ray film for 2.5 d.

Discussion

Our studies demonstrate that the MLA 144 cell line has a normal IL-2 allele and a doubly rearranged IL-2 allele with retroviral insertions at its 5' and 3' ends. Since the vast majority of transcripts are from the abnormal allele, one or both retroviral insertions are likely to be responsible for constitutive expression of the allele. Several possible mechanisms could explain the activation of the abnormal allele. Since the transcriptional orientation of the 5' LTR insertion is opposite that of the IL-2 gene, transcription initiated by a viral promoter and extending into the IL-2 gene is unlikely to occur. Nevertheless, there may be a second promoter present in the LTR with an orientation opposite that of the major LTR promoter, as previously shown (30) for the activation of the *c-mos* oncogene by an intracisternal A particle (an endogenous retrovirus) insertion. In this example, both viral and host promoters initiated transcripts containing *c-mos* (30). Preliminary S1 mapping studies indicate that the transcriptional start site of IL-2 message in MLA 144 is identical to that of normal human IL-2 message (data not shown); hence, involvement of a viral promoter in IL-2 gene transcription is unlikely.

Still other mechanisms are possible. By analogy to the *c-myc* oncogene, where it was found that avian leukosis virus insertions either 5' or 3' to *c-myc* in either orientation can activate its expression (31, 32), either of the GaLV insertions may contribute to activation of the altered IL-2 allele in MLA 144 by an enhancer mechanism, perhaps mediated by the 96 bp direct repeat. Recent studies indicate that the inserted LTRs have enhancer activity when linked to the SV40 early region promoter (N. Holbrook, A. Gulino, D. Durand, Y. Lin, and G. Crabtree, manuscript in preparation). Similar duplicated sequences have been observed in the U3 regions of a number of RNA and DNA tumor viruses, including GaLV_{SEATO} (33), the Moloney murine sarcoma virus (34), and SV40 (35–37), the latter two with proven transcriptional enhancer function. Of note is the fact that GaLV_{SEATO} contains a direct repeat of 45 bp located within the 96 bp subunit (33). If the repeats in the MLA 144 LTR insertions are represented by ABC-ABC, then the corresponding region in GaLV_{SEATO} is represented by AB-BC. (A refers to bp 1,020–1,053 B to bp 970–1,019, and C to bp 953–969 in Fig. 2.) This means that GaLV_{SEATO} may have evolved either from GaLV_{SF} or from a common ancestral species by the deletion of a portion of the 96 bp direct repeat. Alternatively, the direct repeats in the LTRs of the MLA 144 insertions and those in the GaLV_{SEATO} LTRs may have resulted from independent duplication events.

The 3' GaLV insertion might lead to constitutive IL-2 expression, either alone or in concert with an enhancer effect, by a different mechanism. The 3' insertion results in deletion of normal IL-2 3' nontranslated sequence from the transcript, as well as inclusion of viral information in (8) and lengthening of the transcript, which might increase IL-2 mRNA stability and contribute toward increased expression of the abnormal IL-2 allele (38).

Recently (39) it was found that the murine cell line WEHI-3B, which constitutively produces IL-3, has an intracisternal A particle insertion 5' to its IL-3 gene. As discussed by the authors, IL-3 is a potential growth factor for this line (39). MLA 144 represents the first primate example of growth factor gene activation due to retroviral insertion and the first example of growth factor gene activation by the insertion of an infectious retrovirus.

Beyond the question of how the abnormal allele is activated, the true cause of the MLA 144 malignancy remains unknown. Clearly, the constitutive expression of IL-2 could have been selected for either in tissue culture or in the host; however, the original tissue specimen is not available to allow examination of this question (T. Kawakami, personal communication). Regardless of the milieu in which it occurred, the second retroviral insertion in the abnormal allele may have been selected for due to its ability to confer a growth advantage on the cell line by further enhancing the activation of IL-2 gene.

Smith (40) derived sublines of MLA 144 whose constitutive expression of IL-2 was apparently abolished by glucocorticoids. The proliferation of these sublines was also substantially reduced though not obliterated by glucocorticoids, an effect reversed by the addition of exogenous IL-2; hence, MLA 144's growth is in part IL-2 dependent and in part IL-2 independent (40). These results imply that, while constitutive IL-2 gene expression may augment the proliferation of MLA 144, other factors must play a role in the cell line's growth. Gene products

other than IL-2 may be implicated, since there are multiple retroviral insertions in the MLA 144 genome (8), and the putative gene upstream of the IL-2 gene may have been activated by the 5' GaLV insertion. If a second independent oncogenic event of this kind is implicated in MLA 144's growth, then the IL-2 autocrine mechanism may represent one of two "hits" (41) required to produce this T cell malignancy. Alternatively, the autocrine mechanism may have been selected for after the oncogenic event(s) occurred.

Summary

The gibbon ape leukemia virus (GaLV_{SF})-infected T cell line, MLA 144, was established from the lymphoma of a gibbon ape (1). The cell line constitutively expresses IL-2 (5) and its receptor (6), implying that an autocrine mechanism could be responsible for or contribute toward its growth.

To explore the mechanism of constitutive IL-2 expression in MLA 144, we have isolated and characterized cosmid clones representing a normal and a doubly inserted IL-2 allele in this cell line. The map of the normal MLA 144 IL-2 allele closely resembles that of the normal human IL-2 gene. The abnormal allele contains a 3' insertion that is a GaLV_{SF} provirus with two long terminal repeats (LTR) and an internal 3.25 kb deletion. At the 5' end of the abnormal allele is a second insertion that DNA sequencing showed to be an isolated GaLV_{SF} LTR with a transcriptional orientation opposing that of the IL-2 gene. We demonstrate by Northern blotting analysis that the vast majority of transcripts are from the abnormal allele, implying that one or both retroviral insertions are responsible for constitutive expression of the allele.

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