

Intracisternal A-Type Particle-Mediated Activations of Cytokine Genes in a Murine Myelomonocytic Leukemia: Generation of Functional Cytokine mRNAs by Retroviral Splicing Events

KEVIN B. LESLIE,* FRANCES LEE, AND JOHN W. SCHRADER

The Biomedical Research Centre, 2222 Health Sciences Mall, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada

Received 21 February 1991/Accepted 7 August 1991

Previously we have described the derivation of three distinct classes of leukemic cell clones from a single in vivo-passaged myelomonocytic leukemia, WEHI-274, that arose in a mouse infected with the Abelson leukemia virus/Moloney leukemia virus complex (K. B. Leslie and J. W. Schrader, *Mol. Cell. Biol.* 9:2414-2423, 1989). The three classes of cell clones were characterized by distinct patterns of growth in vitro, the production of cytokines, and the presence of cytokine gene rearrangements. However, all three classes of WEHI-274 clones bore a common rearrangement of the *c-myb* gene, suggesting that all were derived from the one ancestral cell and that at least three distinct and independent autostimulatory events were involved in the progression of a single myeloid leukemic disease. In this article, we demonstrate that the autocrine growth factor production by the WEHI-274 leukemic clones resulted from cytokine gene activations mediated by the insertion of an intracisternal A-type particle (IAP) sequence 5' to the interleukin-3 (IL-3) gene, in the case of the class I clone, or 5' to the gene for granulocyte-macrophage colony-stimulating factor (GM-CSF), in the case of the class II clones. IAPs are defective murine retroviruses encoded by endogenous genetic elements which may undergo transpositions and act as endogenous mutagens. The functional IL-3 and GM-CSF mRNAs were generated by mechanisms in which the splice donor apparatus of the IAP sequence has been used in IAP *gag*-to-IL-3 or -GM-CSF splicing events.

There are numerous reports in the literature of instances in which the dependence of an immortal cell line on exogenous growth factors has been abrogated by autocrine or nonautocrine mechanisms, with a coincident onset of malignant behavior in vivo (27). Experiments in which retroviral constructs directing the synthesis of interleukin-2 (IL-2), IL-3, IL-5, or granulocyte-macrophage colony-stimulating factor (GM-CSF) have been introduced into factor-dependent hemopoietic cell lines have demonstrated elegantly that the aberrant constitutive production of a hemopoietin by an immortalized hemopoietic progenitor cell can be a critical step in myeloid leukemogenesis. The pathological, constitutive activation of growth factor genes resulting in autocrine growth in vitro has been described in a number of myeloid leukemias derived in vivo (2, 26, 27).

Many examples can be cited in which proto-oncogenes have been pathologically activated by the insertion of retroviral sequences (4, 6, 9, 17, 18, 22, 39, 40, 42, 47, 48). In studies of murine leukemogenesis, this mechanism of retrovirus-mediated activation of cellular genes has also been described for the pathological activation both of cytokine genes (3, 10, 44, 46, 49, 50) and of genes encoding cytokine receptors (23, 45). While examples of such retrovirus-mediated activations have been described for typical retroviral proviruses (44), the vast majority of activations appear to be mediated by the transposition of intracisternal A-type particle (IAP) proviral sequences (3, 10, 44, 46, 49, 50). IAPs are defective murine retroviruses encoded by endogenous genetic elements which are present at a copy number of about 1,000 per haploid genome of *Mus musculus* (7, 30). While differing from transmissible retroviruses primarily in lacking

an extracellular phase (24, 25, 34), nonetheless IAPs may undergo transpositions and act as endogenous mutagens.

Previously we have described the derivation of three distinct classes of leukemic cell clones from an in vivo-passaged myelomonocytic leukemia, WEHI-274, that arose in a mouse infected with the Abelson leukemia virus/Moloney murine leukemia virus (MoMLV) complex (27) but in which there was no evidence of Abelson leukemia proviral insertions. The three classes of cell clones could be distinguished by their patterns of growth in vitro, the production of cytokines, and the presence of cytokine gene rearrangements. Class I comprised a single clone, bearing a rearrangement of the IL-3 gene, resulting in autocrine growth behavior in vitro mediated by IL-3. Class II comprised multiple clones, all bearing identical rearrangements of the GM-CSF gene but with no alterations in the IL-3 gene, resulting in autocrine growth behaviors mediated by GM-CSF. The cell clones of class III bore rearrangements of neither the GM-CSF nor the IL-3 gene and did not exhibit direct or indirect patterns of autocrine growth behaviors. However, a paracrine mode of growth stimulation was found to be operating both in vitro and in vivo, and it appeared to be mediated by IL-1 α which was constitutively released by this leukemic line and was able to stimulate the release of GM-CSF from fibroblasts and other cells (28). Although the three classes of WEHI-274 clones were heterogeneous with respect to the production of growth factors, all clones bore a common rearrangement of the *c-myb* gene (27), suggesting that all were derived from one ancestral cell in the original animal. Thus, these experiments demonstrated that at least three distinct and independent autostimulatory events were involved in the progression of a single myeloid leukemic disease.

In this study, we demonstrate that the cytokine gene

* Corresponding author.

activations in the WEHI-274 leukemic clones have been mediated by the insertion of an IAP sequence 5' to the IL-3 gene, in the case of the class I clone, or 5' to the GM-CSF gene, in the case of the class II clones. We describe the generation of functional cytokine mRNAs from these rearranged genes by splicing mechanisms from IAP to cytokine gene.

MATERIALS AND METHODS

Cells and cell culture conditions. Cells were routinely grown in RPMI 1640 supplemented with newborn calf serum (10%), 2-mercaptoethanol (5×10^{-5} M), glutamine (2.8×10^{-3} M), penicillin (100 U/ml), and streptomycin (100 U/ml) and with WEHI-3B-conditioned medium (2% of a 10-fold concentrate) as indicated. The cell lines WEHI-274.14 and WEHI-274.28 have been described elsewhere (27). The murine T-cell line used was the p41.1 line described by Ziltener et al. (51).

Genomic DNA preparation, Southern blotting, and preparation of genomic libraries. Genomic DNA was isolated according to standard methods (33), and 7- μ g aliquots were digested with restriction endonucleases according to the recommendations of the manufacturer. Digests were separated by 0.7% agarose gel electrophoresis then blotted according to standard methods (33, 43). In the preparation of genomic libraries from the WEHI-274.14 cell line, *EcoRI*-digested genomic DNA was size fractionated by 0.6% agarose gel electrophoresis, and 4.5- to 11-kb DNAs were ligated into the vector λ ong C (Stratagene). Recombinant phage were screened with an IL-3 genomic probe.

RNA preparation, Northern (RNA) blotting, and preparation of cDNA. The isolation of whole-cell polyadenylated RNA and its analysis by 1.0% agarose-formaldehyde gel electrophoresis was performed according to the method of Gonda et al. (18). For all gels, 2 μ g of RNA was loaded per lane except for the RNA derived from concanavalin A-activated T cells, for which aliquots were 0.2 μ g. The approximate sizes of specific bands were estimated by extrapolation using the 18S and 28S ribosomal bands as molecular size markers. First-strand cDNA was prepared from poly(A)-selected, whole-cell RNA by standard methods (33) using MoMLV reverse transcriptase (Bethesda Research Laboratories).

PCR and oligonucleotide primers. Second-strand synthesis of cDNAs and their subsequent amplifications, or amplifications of specific genomic DNA fragments, were performed by polymerase chain reaction (PCR) in a DNA Thermal Cycler (Perkin Elmer Cetus), using *TaqI* DNA polymerase (Bethesda Research Laboratories) under conditions recommended by the supplier. Annealing temperatures used were 58°C in the generation of clone p6B4, 53°C in the generation of clones p115 and p132, 54°C in the generation of clone p323a.1, and 62°C in the performance of nested PCR reactions and the generation of clones p1271.4 and p1328. Reactions with single pairs of oligonucleotide primers were subjected to 30 rounds of amplification, while nested amplifications were performed in two successive rounds of 15 cycles.

The oligonucleotide primers used in the preparation of cDNA and in PCR reactions of cDNA or genomic DNA contained the following regions: sense IAP long terminal repeat (LTR) R-region primer, 5'-GTTTTCTCTCTCTTGCTTCTTG-3'; antisense IL-3 exon 5 primer, 5'-TTAATATCCACGGTTCCACGGTTA-3'; antisense GM-CSF exon 4, 5'-TCATTTTTGGCTTGGTTTTTGCAT-3'; sense

IAP *gag* region nested primers, 5'-ACTGGCGCAAGGAA GATCCCTCAT-3' and 5'-AGAAGTGCAGGGTCCGCGGTAA TAAA-3'; antisense IL-3 exons 2 and 3 nested primers, 5'-GACAGGTTTACTCTCCGAAAGCTC-3' and 5'-TCCTCAGAGAGGGACCTTCATCAT-3'; and antisense GM-CSF exons 2 and 3 nested primers, 5'-GGGTCTGCACACATGTTA GCTTCT-3' and 5'-CTCGTTAGAGACGACTTCTACCTC-3'.

DNA sequencing. Genomic and cDNA clones were subcloned into M13 vectors and plasmid pUC12. Single- and double-stranded sequencing were performed with [α - 32 P] dATP by dideoxynucleotide chain termination. All sequences were read from both strands.

Hybridization conditions and probes. DNA fragments were labeled by random priming with random hexamers according to the method of Feinberg and Vogelstein (15). Hybridization with Northern and Southern filters was performed in 50% formamide-0.1% Ficoll-0.1% polyvinylpyrrolidone-0.1% bovine serum albumin-6 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-1% sodium dodecyl sulfate (SDS)-15 mM EDTA-10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) for 16 h at 42°C. Filters were washed at 55°C for 1 h in 0.1% SDS-2 \times SSC with three changes of buffer and then at 55°C for 15 min in 0.1% SDS-0.1 \times SSC prior to autoradiography.

The genomic IL-3 probe was a 3-kb *BamHI-BamHI* fragment, which included about 1.7 kb of sequence 5' to the IL-3 TATA box (courtesy of A. Dunn [37]). The GM-CSF probe was a *PstI-to-EcoRI* fragment of a pUC8 subclone of GM-CSF cDNA clone 3.2 (courtesy of N. Gough [19]). Other probes are as described in the text.

RESULTS

Rearrangement and activation of IL-3 and GM-CSF genes in distinct clones of WEHI-274. Previous studies have shown that one allele of the IL-3 gene is rearranged in the class I clone WEHI-274.14 (27). Using an IL-3 genomic probe (a 3-kb *BamHI-BamHI* fragment which included exons 1 and 2 and about 1.7 kb of sequence 5' to the IL-3 coding region), Southern blot analysis of *EcoRI*-digested DNA from WEHI-274.14 revealed 9.1- and 5.6-kb bands in addition to the germ line 8.5-kb band. Detailed restriction endonuclease mapping using multiple genomic probes revealed that the genomic rearrangement involved the 5' end of the IL-3 gene, with the junction between rearranged and germ line DNA being less than 1,000 bp upstream of the site of the normal IL-3 coding region (27). Northern blot analysis using whole-cell polyadenylated RNA from the class I clone (WEHI-274.14) revealed grossly abnormal IL-3 gene transcripts of 8, 4.5, and 1.3 kb (Fig. 1A), significantly larger than the 1.1-kb RNA species from an activated T-cell clone.

Southern blot analysis of a class II clone, WEHI-274.28, revealed a rearrangement of one allele of the GM-CSF gene but a germ line IL-3 gene (27). This rearrangement of the GM-CSF gene was present and identical in all of the clones of class II. Restriction endonuclease mapping indicated that the junction between rearranged and germ line DNA in the class II clones occurred in a region between 3.5 and 6.0 kb upstream of the GM-CSF coding region (27). Northern blot analysis of mRNAs from the clones of class II showed no evidence of RNA species hybridizing with an IL-3 probe (Fig. 1A) but did demonstrate grossly abnormal GM-CSF gene transcripts of 10 and 1.5-kb (Fig. 1B). These mRNA species are significantly larger than the 1.3-kb GM-CSF gene transcripts in activated T cells. The two additional species

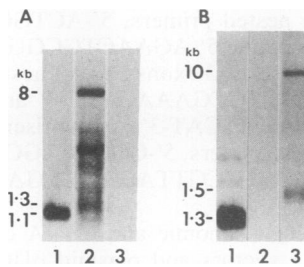


FIG. 1. Northern blot analysis of WEHI-274 clones for IL-3- and GM-CSF-specific RNAs. Whole-cell polyadenylated RNA was fractionated on denaturing gels and analyzed by Northern blotting, using an IL-3 genomic probe (A) and a GM-CSF cDNA probe (B). Lanes: 1, conA-activated T-cell line; 2, class I clone (274.14); 3, class II clone (274.28).

seen by Northern blot analysis of T-cell-derived RNA (Fig. 1B, lane 1) are assumed to be nuclear precursors of the mature 1.3-kb GM-CSF cytoplasmic message, since the sample used included both nuclear and cytoplasmic polyadenylated RNA.

Characterization of the IL-3 gene rearrangement in WEHI-274.14. To characterize the nature of the IL-3 gene rearrangement in the class I clone (WEHI-274.14), and perhaps obtain clues as to the mechanism of generation of the abnormal IL-3 gene transcripts, lambda clones of genomic DNA were generated in parallel with experiments amplifying genomic DNA by PCR.

From a genomic library constructed from WEHI-274.14 DNA, a recombinant lambda clone (pDR) was isolated bearing a 5.6-kb insert that hybridized with an IL-3 genomic probe (3-kb *Bam*HI-*Bam*HI fragment including about 1.7 kb of sequence 5' to the IL-3 coding region). This 5.6-kb insert was assumed to represent the 5.6-kb *Eco*RI band seen on Southern blot analysis of DNA from the class I clone. Sequence analysis of the pDR clone revealed that the IL-3 gene has been disrupted by the insertion of an IAP proviral sequence (Fig. 2) in the same transcriptional orientation as the IL-3 gene. The DNA sequence of the 5' IAP LTR (as determined from clone pDR) bore a high homology with

other IAP LTRs described in the literature (Fig. 3). Thus, the 5.6-kb fragment comprised approximately 5.2 kb of 5' genomic IL-3 sequence and 0.4 kb of IAP sequence.

The 3' junction of the IAP proviral insertion was cloned by amplification of genomic DNA by PCR using a sense oligonucleotide primer derived from the R region of the IAP LTR (as determined from DNA sequence analysis of the 5' LTR of the IAP) and an antisense oligonucleotide primer derived from exon 5 of the IL-3 gene. The PCR-generated products were digested with *Hind*III and ligated into pUC12, and the recombinant plasmid was screened with the IL-3 genomic probe. DNA sequence analysis of clone p6B4 (containing a 460-bp fragment) confirmed its derivation from the 3' junction of the IAP proviral insertion, 5' to the IL-3 coding region (Fig. 2). DNA sequencing of this 3' LTR revealed 100% homology with the 5' IAP LTR in the R and U5 regions (data not shown).

Thus, in the WEHI-274.14 cell line, an IAP provirus has inserted 206 bp 5' to the initiator methionine codon of the IL-3 gene. The IAP sequence is flanked by a typical 4-bp inverted repeat 5'-TGTT/AACA-3', with a typical 6-bp duplication at the site of insertion (5'-GCAGTC-3') (Fig. 2). Using the knowledge of restriction sites predicted from the sequence analysis of clones pDR and p6B4, together with detailed restriction mapping of WEHI-274.14 genomic DNA by Southern analysis, it is evident that the inserted IAP sequence is about 6.2 kb in length (Fig. 2).

Splicing of IAP mRNAs from germ line proviral sequences. Previous studies have shown that the abnormal 8-kb IL-3 gene transcript seen in WEHI-274.14 (Fig. 1A) is initiated from a site upstream of the normal IL-3 transcription initiation site (27). Since the IL-3 gene was disrupted by the insertion of an IAP proviral sequence 5' to the gene (Fig. 2), and since the pattern of IL-3 mRNA species seen by Northern analysis of WEHI-274.14-derived mRNAs (Fig. 1A) suggested the existence of presumed alternatively spliced forms of the IL-3-bearing transcripts, we were interested in two questions. First, do RNAs from IAP proviral sequences splice in a similar manner to that of the transcripts of retroviral proviruses? Second, is the pattern of IL-3 mRNA species seen in WEHI-274.14 due to splicing events involving the IAP inserted upstream of the IL-3 gene? To

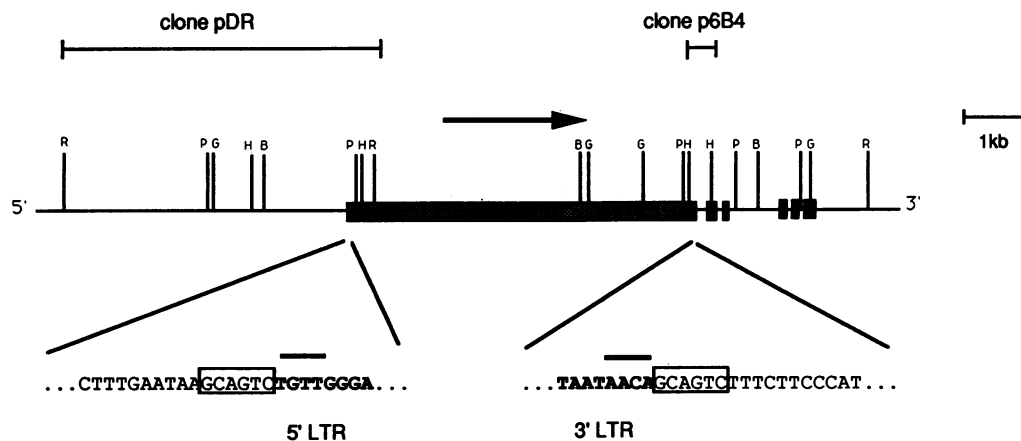


FIG. 2. Insertion of an IAP proviral sequence 5' to the IL-3 gene in WEHI-274.14. Shown is the restriction map and position of an IAP proviral sequence (hatched boxes; dark hatch, LTR regions) relative to the IL-3 gene (solid boxes). The transcriptional orientation of the IAP sequence is indicated by an arrow. Beneath the genomic map are sequence data describing the point of insertion of the IAP sequence (heavy lines above the nucleotide sequence indicate flanking 4-bp inverted repeats; boxed sequences indicate the 6-bp duplication at the site of insertion). Abbreviations: R, *Eco*RI; P, *Pst*I; G, *Bgl*II; H, *Hind*III; B, *Bam*HI.

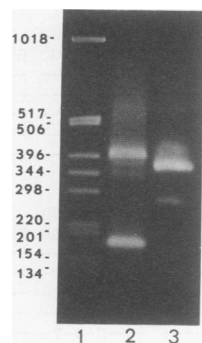


FIG. 5. Analysis of IAP *gag*-to-cytokine splicing events by PCR. Whole-cell polyadenylated mRNAs from the class I clone WEHI-274.14 (lane 2) and the class II clone WEHI-274.28 (lane 3) were reverse transcribed and subjected to PCR using nested IAP *gag* region oligonucleotide primers and nested IL-3 (exons 2 and 3) oligonucleotide primers (lane 2) or GM-CSF (exons 2 and 3) oligonucleotide primers (lane 3). Amplified DNAs were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide. The sizes of molecular weight markers (lane 1) are shown in base pairs.

gag sequence of the unspliced p115 clone occurs in a region encoding a functional splice donor site, according to guidelines established by Shapiro and Senapathy (41) (Fig. 4A). This splice donor site is conserved in several other published IAP sequences, including both germ line (27, 34) and transposed (49, 50) IAP sequences. Interestingly, another splice donor consensus is evident only 19 bp downstream of the site used in the p132 clone, theoretically as functional as its more 5' neighbor. This additional splice donor consensus is similarly conserved in other published IAP sequences (Fig. 4A). The point at which the p132 sequence becomes homologous with the *env* regions of published IAP sequences occurs in a region encoding a functional splice acceptor site according to the guidelines published by Shapiro and Senapathy (41) (Fig. 4B). Similarly, this splice acceptor site is conserved in several other published germ line and transposed IAP sequences. Thus, primed with the information that IAP mRNAs do indeed undergo splicing events, we sought to investigate the mechanism underlying the generation of the abnormal IL-3 gene transcripts in the class I clone (WEHI-274.14) and the abnormal GM-CSF gene transcripts in the class II clone (WEHI-274.28).

Characterization of the abnormal IL-3 transcripts in the class I clone WEHI-274.14. On the assumption that transcription initiating 5' to a gene activated by a head-to-tail IAP insertion (i.e., an IAP provirus inserted in the same transcriptional orientation as the activated gene) most probably will initiate from one or another IAP LTR, we attempted to amplify by PCR cDNAs bearing both IAP LTR and IL-3 sequences by using nested oligonucleotide primers. The sense oligonucleotide primers were designed from a region approximately 100 bp 5' to the *gag* splice donor site demonstrated in Fig. 4, while the antisense oligonucleotide primers were derived from IL-3 coding regions spanning exons 2 and 3. PCR-generated cDNAs from the two major bands (Fig. 5, lane 2) were ligated into pUC12, and clones were screened with an IAP *gag* probe (derived from p115) and an IL-3 cDNA probe.

DNA sequence analysis of a double-positive clone, p1271.4, derived from the larger, approximately 400-bp PCR-generated cDNA, revealed a presumed IAP-IL-3 splic-

ing event (Fig. 6A). In the case of this IAP-IL-3 splicing event, the splice donor site used was the more 3' of the two potential splice donor sites demonstrated earlier (Fig. 4A) rather than the site used in generation of the spliced mRNA from the nontransposed IAP element discussed earlier (clone p132). The splice acceptor site used is a cryptic acceptor located 24 bp 5' to the transcription initiation site of the germ line IL-3 gene (Fig. 6A). Thus, the p1271.4 clone was derived from an mRNA, apparently initiating in the 5' LTR of an IAP inserted 5' to the IL-3 gene, which had spliced from a functional splice donor site in the IAP *gag* region to a cryptic splice acceptor found a short distance upstream of the IL-3 coding region (Fig. 6B). The size of this PCR-generated cDNA is very close to the calculation of 398 bp predicted for such a spliced mRNA. Moreover, the additional 351 bases expected at the 5' end of such a spliced mRNA which has initiated from the 5' LTR of the IAP (as compared with a transcript initiating at the normal initiator of the IL-3 gene) (Fig. 6A) is in reasonable agreement with the Northern analysis of WEHI-274.14 mRNAs (Fig. 1). In such Northern analyses (Fig. 1), the smaller cytoplasmic IL-3 message from WEHI-274.14 was shown to be slightly larger than the normal T-cell-derived IL-3 message.

DNA sequence analysis of another double-positive clone, p1328, derived from the smaller, approximately 180-bp PCR-generated cDNA, revealed a further IAP-IL-3 splicing event. In the case of this IAP-IL-3 splicing event, again the splice donor site used was the more 3' of the two potential splice donor sites demonstrated earlier (Fig. 4A). However, the splice acceptor site used is the normal splice acceptor of exon 2 of the germ line IL-3 gene (Fig. 6A). Thus, the p1328 clone was derived from an mRNA, apparently initiating in the 5' LTR of the IAP inserted 5' to the IL-3 gene, which had spliced from a functional splice donor site in the IAP *gag* region to the functional splice acceptor of exon 2 of the IL-3 coding region. The two additional faint bands at approximately 370 and 340 bp in Fig. 5, lane 2 are assumed to represent rare splicing events from the IAP *gag* splice donor to splice acceptor consensus sequences observed at positions 696 and 737 of the published murine genomic IL-3 sequence (37).

While the spliced mRNA represented by the p1271.4 cDNA would be expected to be productive of a functional IL-3 protein, that represented by the p1328 cDNA would not. Thus, the IAP-IL-3 splice demonstrated in the p1328 clone has resulted in (i) a coding region with no initiator methionine codon and (ii) a predicted amino acid sequence truncated of the first 29 residues of the mature IL-3 protein with no hydrophobic leader peptide.

Characterization of the abnormal GM-CSF transcripts in the class II clone WEHI-274.28. Comparison of the Northern blot analyses of the class I and class II clones (Fig. 1) revealed similarities in the size distribution of IL-3 mRNAs from the class I clone and GM-CSF mRNAs from the class II clone, namely, the existence of a large dominant species of 8 or 10 kb and a less intense, smaller species of 1.3 or 1.5 kb. Experiments were devised to explore the possibility that both the abnormal IL-3 and GM-CSF gene transcripts were generated by similar IAP-mediated mechanisms.

Again, on the assumption that transcription initiating 5' to a gene activated by a head-to-tail IAP proviral insertion most probably will initiate from one or another IAP LTR, we attempted to amplify by PCR cDNAs bearing both IAP LTR and GM-CSF sequences. The sense oligonucleotide primer encoded the first 25 bases of the LTR R region from clone pDR, while the antisense oligonucleotide primer was derived

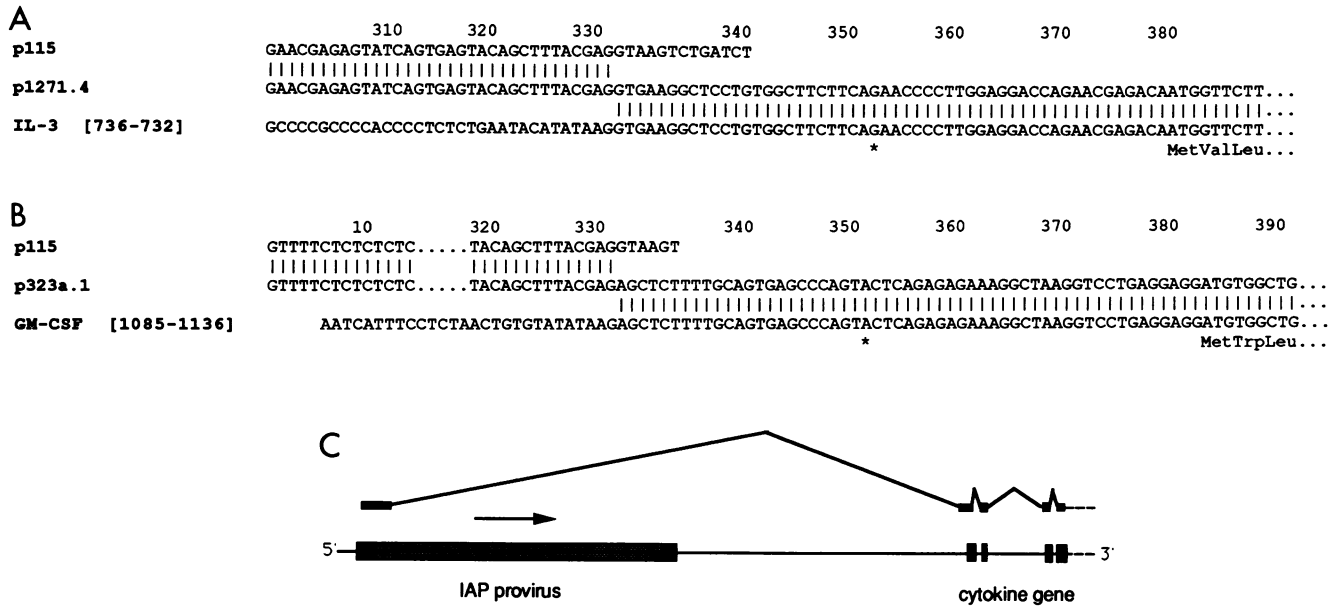


FIG. 6. IL-3 transcripts in WEHI-274.14 and GM-CSF transcripts in WEHI-274.28 processed by splicing from IAP-to-cytokine coding sequences. (A) Selected regions of the nucleotide sequence of cDNA clone p1271.4 demonstrating its origin from a spliced IAP-IL-3 mRNA. Vertical lines indicate homology with an unspliced IAP proviral transcript (p115) or the 5' genomic sequence of IL-3 (IL-3 [37]). Numbers indicate distance in base pairs from the postulated 5' terminus of the clone p1271.4; triple-letter amino acid coding represents the predicted translation products of clone p1271.4 and IL-3; dots indicate artificial breaks in the sequence introduced to aid clarity. The asterisk indicates the transcription initiation site of the germ line IL-3 gene. (B) Selected regions of the nucleotide sequence of cDNA clone p323a.1 demonstrating its origin from a spliced IAP/GM-CSF mRNA. Vertical lines indicate homology with an unspliced IAP proviral transcript (p115) or the 5' genomic sequence of GM-CSF (GM-CSF [36]). Numbers indicate distance in base pairs from the postulated 5' terminus of clone p323a.1; triple-letter amino acid coding represents the predicted translation products of clone p323a.1 and GM-CSF; dots indicate artificial breaks in the sequence introduced to aid clarity. The asterisk indicates the transcription initiation site of the germ line GM-CSF gene. The generation of these spliced IAP-cytokine transcripts are represented (top line) diagrammatically in panel C (not to scale). Thick lines represent exonic sequences; thin lines represent intronic sequences.

from the 3' region of GM-CSF exon 4. PCR-generated cDNAs were ligated into pUC12, and clones were screened with an IAP LTR probe (derived from pDR) and a GM-CSF cDNA probe. DNA sequence analysis of a double-positive clone, clone p323a.1, revealed a presumed IAP-to-GM-CSF splicing event (Fig. 6B). This finding suggests the strong possibility that the GM-CSF gene rearrangement observed in the class II clones (27) was indeed secondary to a head-to-tail insertion of an IAP provirus 5' to the GM-CSF gene.

In the case of this IAP-GM-CSF splicing event, again the splice donor site used was the more 3' of the two potential splice donor sites demonstrated earlier (Fig. 4A and 6A). The splice acceptor site used is a cryptic acceptor located 23 bp 5' to the transcription initiation site of the germ line GM-CSF gene (Fig. 6B). Thus, the p323a.1 clone was derived from an mRNA, presumably initiating in the 5' LTR of an IAP inserted 5' to the GM-CSF gene, which had spliced from a functional splice donor site in the IAP *gag* region to a cryptic splice acceptor found a short distance upstream of the GM-CSF coding region (Fig. 5B). The size of this PCR-generated cDNA is very close to the calculation of 373 bp predicted from such a spliced mRNA, and again the additional 351 bases expected at the 5' end of such a spliced mRNA which has initiated from the 5' LTR of the IAP (as compared with a transcript initiating at the normal initiator of the GM-CSF gene) (Fig. 6B) is in reasonable agreement with the Northern analysis of WEHI-274.28 mRNAs (Fig. 1). Thus, the smaller cytoplasmic GM-CSF message from WEHI-274.28 was shown to be slightly larger than the normal T-cell-derived GM-CSF message (Fig. 1).

To investigate the existence of alternative IAP-to-GM-CSF splicing events, we attempted to amplify by PCR cDNAs bearing both IAP *gag* and GM-CSF sequences, using nested oligonucleotide primers. Again, the sense oligonucleotide primers were designed from the region approximately 100 bp 5' to the *gag* splice donor site demonstrated in Fig. 4, while the antisense oligonucleotide primers were derived from GM-CSF coding regions spanning exons 2 and 3. DNA sequence analysis of the two major PCR-generated cDNAs (Fig. 5, lane 3) revealed that the larger, approximately 370-bp band was derived from the same spliced mRNA species as the p323a.1 clone, while the smaller, approximately 270-bp band was derived from a distinct IAP-to-GM-CSF spliced mRNA. This latter mRNA again utilized the more 3' of the two *gag*-encoded splice donor sites (Fig. 4A) but spliced to an acceptor site in a region encoding the signal peptide of the GM-CSF protein (position 1211 of the published murine genomic GM-CSF sequence [36]). Thus, while such an mRNA bears the full coding sequence of the mature GM-CSF protein, including a *gag*-derived initiator methionine codon, there is no sequence encoding a hydrophobic leader peptide.

DISCUSSION

In this study, we have described the independent, pathological, IAP-mediated activations of cytokine genes (GM-CSF and IL-3) in a single in vivo-derived murine myeloid leukemia. Both rearrangements resulted in autocrine growth behaviors by the respective cells. In both cytokine gene

activations, functional mRNAs have been generated by a mechanism in which the splice donor apparatus of the IAP sequence has been used in an IAP *gag*-to-IL-3 or -GM-CSF splicing event.

Transposition of IAP sequences are well documented in the literature, such sequences ranging in length from isolated LTRs (10) to full-length 7.2-kb IAP proviral sequences (20). Such examples may involve stable alterations to the germ line DNA, transmitted vertically in the context of activated (5) or disrupted and inactivated (20, 32) cellular genes. Non-germ line IAP transpositions may be detected by virtue of subsequent alterations of cell growth and neoplastic transformation. Such IAP transpositions in hematological malignancies have been described in the pathological activation of genes for IL-3 (10, 49, 50), IL-5 (46), IL-6 (3), and GM-CSF (10, 44) and of the genes of the receptors for IL-2 (23) and IL-6 (45). In at least two cases, more than one IAP transposition may have been involved in the progression of a single myeloid leukemia. Thus, in addition to the current study in which two distinct IAP insertions have activated the GM-CSF and the IL-3 genes in a single leukemic disease, the WEHI-3B cell line bears IAP-mediated activations of both the IL-3 gene (37, 49, 50) and the HOX-2.4 gene (4, 22).

The transcriptional activation of IAP sequences appears to be under the control of a complex series of regulatory proteins (11–13, 31) and is influenced by such variables as DNA methylation (14, 29, 35) and the state of cellular differentiation (21) or transformation (1). Activation of cellular genes by local insertion of IAP sequences has been postulated to be due to one of at least three general mechanisms: the introduction of a *cis*-acting IAP LTR enhancer either 5' (37, 49, 50) or 3' (44, 45) to the gene; the more theoretical possibility of separating the promoter of a cellular gene from 5' inhibitory sequences by the intervening insertion of an IAP sequence; and the introduction of an alternative, LTR-encoded promoter 5' to the cellular gene (9, 23, 37, 46, 49, 50).

A new promoter may be introduced when an IAP sequence is inserted 5' to a cellular gene, irrespective of the orientation of the proviral or LTR sequences relative to the transcriptional orientation of the cellular gene. This *in vitro* demonstration of the bidirectionality of LTR-mediated promoter activity (8, 31) has recently been given pathological significance. Thus, the head-to-head IAP insertion into the 5' end of the *c-mos* coding region in the XRPC24 myeloma resulted in the transcriptional activation of that gene from a cryptic promoter in the 5' LTR of the IAP (9). Existing reports of transcripts generated from cellular genes activated by the insertion of an IAP promoter 5' to that gene have described only two mechanisms, neither of which involves substantial RNA splicing events. First, the IAP sequence may be inserted within the coding region of the cellular gene, such that transcription will proceed directly from the LTR into exonic sequence (9). Second, the IAP sequence may be inserted such that transcription must proceed from the LTR through sequences 5' to the cellular gene before encountering the coding region of the cellular gene. In the IAP-mediated activation of the HOX-2.4 gene in WEHI-3B, it is assumed that a small sequence of nonintronic RNA has been spliced out of the IAP/HOX-2.4 transcript, based on the observation that the genomic sequence (4) differs significantly from the cDNA sequence (22). This splicing event, however, appears to be mediated by cryptic splice donor and acceptor sites lying between the site of IAP insertion and the HOX-2.4 coding region, that is, sites not related to the inserted IAP proviral sequence itself.

In this study, we have characterized the independent, pathological rearrangements of cytokine genes (GM-CSF and IL-3) by IAP proviral insertions, which have resulted in activations of those cytokine genes and autocrine growth behaviors by those cells bearing the gene activations. Functional IL-3 and GM-CSF mRNAs have been generated by subjugating the splice donor apparatus of the IAP sequence, resulting in IAP *gag*-to-IL-3 or -GM-CSF splicing events. The larger size of the smaller abnormal IL-3 mRNA species (about 1.3 kb) in the class II clone WEHI-274.14 relative to that of the T-cell-derived message (about 1.1 kb) is close to the predicted size increase from the addition of the IAP-encoded LTR and *gag* sequences (Fig. 1A and 6A). Similarly, the larger size of the smaller abnormal GM-CSF mRNA species (about 1.5 kb) in the class II clone WEHI-274.28 relative to that of the T-cell-derived message (about 1.3 kb) is close to the predicted size increase from the addition of the IAP-encoded LTR and *gag* sequences (Fig. 1B and 5A).

The large 8-kb IL-3 mRNA species observed on Northern analysis of WEHI-274.14 (class I) mRNAs (Fig. 1A) is consistent in size with a transcript initiating in the 5' LTR of the IAP inserted 5' to the IL-3 gene, but unspliced save the removal of IL-3 intronic sequences. This prediction is in agreement with the earlier finding that this large 8-kb IL-3 mRNA hybridizes with a probe prepared from the region now demonstrated to lie between the insertion of the IAP provirus and the transcription initiation site of the germ line IL-3 gene (27). Similarly, the large 10-kb GM-CSF mRNA species observed on Northern analysis of WEHI-274.28 (class II) mRNAs (Fig. 1B) probably represents the analogous primary IAP-to-GM-CSF read-through transcript. On Northern analysis of IL-3 mRNAs from WEHI-274.14 (Fig. 1A), in addition to the fully spliced 1.3-kb transcript and the 8-kb partially spliced read-through transcript, a 4.5-kb species is evident. The size of this 4.5-kb transcript is consistent with it representing a spliced product of the 8-kb transcript truncated by the *gag*-to-*env* splicing event demonstrated in this study for mRNAs from germ line IAP proviral sequences (Fig. 4). We are currently exploring this possibility.

A similar phenomenon of transcriptional read-through from an inserted proviral sequence into a 3' gene has been described in the case of a *B2m* gene rearranged by the insertion of an Abelson murine leukemia proviral sequence into intron 1 of that gene (16). In that study, Northern blot analysis revealed a large mRNA species presumed to be the unspliced (or partially spliced) read-through transcript, which gave rise to a smaller, spliced mRNA. Thus, while we are not aware of any examples in the literature of splicing events from IAP to cellular sequences in the generation of functional cytokine or other mRNAs, certainly such a mechanism is not without precedent for retrovirus-mediated gene activations (18, 38, 42, 47). Shen-Ong et al. (42) have described spliced *gag-onc* transcripts derived from the insertion of MoMLV proviral DNA 5' to the murine *c-myc* gene. In this case, the splice donor is a cryptic consensus present in the MoMLV *gag* region, splicing to a cryptic splice acceptor in exon one of the *c-myc* gene and resulting in a N-terminal deletion the *c-myc* protein. A similar splicing mechanism was seen by Gonda et al. (18) in the WEHI-274 tumor studied in this work, in which a MoMLV provirus has inserted into the second intron of the *c-myc* gene. While the same *gag*-encoded cryptic splice donor used in the example of Shen-Ong et al. is used in the WEHI-274 tumor, the splice acceptor site is the physiological acceptor of exon 3 of the *c-myc* gene in that example. Again, the resultant mRNA

encodes a protein truncated at its N terminus. In a further example (47), analysis of transcripts of a *c-Ki-ras* gene, rearranged by the 5' insertion of Friend proviral DNA, again revealed a chimeric mRNA species processed by a splicing event associated with the fortuitous creation of a cryptic splice donor site at the junction between the proviral and cellular DNA sequences. Analogous to the situation in the current study, the splice acceptor site is a cryptic site lying just 5' to exon one of the *c-Ki-ras* gene, giving rise to a presumably normal translation product. However, save the *B2m* gene rearrangement (16), in none of these examples are chimeric mRNAs produced by subjugation of the normal splice donor apparatus of the retrovirus as has been demonstrated in the current study.

While functional cellular gene transcripts may arise from RNA processing of primary transcripts with splicing from 5' retrovirus to 3' cellular gene, the converse may occur when a cellular gene has been activated by the insertional of a retroviral enhancer 3' to the cellular gene (38). In such situations, chimeric mRNAs can be demonstrated which have been processed by a splicing event from a normal splice donor of the activated cellular gene, to a splice acceptor in the 3' retroviral sequence.

From this and earlier studies, it is evident that a significant number of potentially transforming genetic lesions in murine hematological malignancies are due to IAP transposition with resultant pathological gene activations. While there is little evidence for a role for endogenous transposable elements in human neoplasias, analysis of the molecular mechanisms of IAP-mediated murine gene activations will continue to provide insights into the myriad mechanisms through which retroviruses contribute to pathology in specific mammalian and avian diseases.

ACKNOWLEDGMENTS

We are indebted to A. Dunn and N. Gough for the IL-3 genomic and GM-CSF cDNA probes. We thank Scott Pownall for technical assistance in the production of oligonucleotide primers and Kira Lueders for critical review of the manuscript.

This work was supported by The National Cancer Institute of Canada (grant 5-5681) and The Biomedical Research Centre.

REFERENCES

- Augenlicht, L. H., D. Kobrin, A. Pavlovec, and M. E. Royston. 1984. Elevated expression of an endogenous retroviral long terminal repeat in a mouse colon tumor. *J. Biol. Chem.* **259**: 1842-1847.
- Baumbach, W. R., E. R. Stanley, and M. D. Cole. 1987. Induction of clonal monocyte-macrophage tumors in vivo by a mouse *c-myc* retrovirus: rearrangement of the CSF-1 gene as a secondary transforming event. *Mol. Cell. Biol.* **7**:664-671.
- Blankenstein, T., Z. Qin, W. Li, and T. Diamantstein. 1990. DNA rearrangement and constitutive expression of the interleukin-6 gene in a mouse plasmacytoma. *J. Exp. Med.* **171**:965-970.
- Blatt, C., D. Aberdam, R. Schwartz, and L. Sachs. 1988. DNA rearrangements of a homeobox gene in myeloid leukaemic cells. *EMBO J.* **7**:4283-4290.
- Burt, D. W., A. D. Reith, and W. J. Brammer. 1984. A retroviral provirus closely associated with the Ren-2 gene of DBA/2 mice. *Nucleic Acids Res.* **12**:8579-8593.
- Canaani, E., O. Drazan, A. Klar, G. Rechavi, D. Ram, J. B. Cohen, and D. Givol. 1983. Activation of the *c-mos* oncogene in a mouse plasmacytoma by insertion of an endogenous intracisternal A-particle genome. *Proc. Natl. Acad. Sci. USA* **80**:7118-7122.
- Christy, R. J., A. R. Brown, B. C. Gourlie, and R.-C. C. Huang. 1985. Nucleotide sequences of murine intracisternal A-particle gene LTRs have extensive variability within the R region. *Nucleic Acids Res.* **13**:289-302.
- Christy, R. J., and C. C. Huang. 1988. Functional analysis of the long terminal repeats of intracisternal A-particle genes: sequences within the U3 region determine both the efficiency and direction of promoter activity. *Mol. Cell Biol.* **8**:1093-1102.
- Cohen, J. B., T. Unger, G. Rechavi, E. Canaani, and D. Givol. 1983. Rearrangement of the oncogene *c-mos* in mouse myeloma NSI and hybridomas. *Nature (London)* **306**:797-799.
- Dührsen, U., J. Stahl, and N. M. Gough. 1990. In vivo transformation of factor-dependent hemopoietic cells: role of intracisternal A-particle transposition for growth factor gene activation. *EMBO J.* **9**:1087-1096.
- Falzon, M., and E. L. Kuff. 1988. Multiple protein-binding sites in an intracisternal A particle long terminal repeat. *J. Virol.* **62**:4070-4077.
- Falzon, M., and E. L. Kuff. 1989. Isolation and characterization of a protein fraction that binds to enhancer core sequences in intracisternal A-particle long terminal repeats. *J. Biol. Chem.* **264**:21915-21922.
- Falzon, M., and E. L. Kuff. 1990. A variant binding sequence for transcription factor EBP-80 confers increased promoter activity on a retroviral long terminal repeat. *J. Biol. Chem.* **265**:13084-13090.
- Feenstra, A., J. Fewell, K. Lueders, and E. Kuff. 1986. In vitro methylation inhibits the promoter activity of a cloned intracisternal A-particle LTR. *Nucleic Acids Res.* **14**:4343-4352.
- Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**:6-13.
- Frankel, W. N., T. A. Potter, and T. V. Rajan. 1989. Effect of proviral insertion on transcription of the murine B2m gene. *J. Virol.* **63**:2623-2628.
- Gattoni-Celli, S., W. L. Hsiao, and I. B. Weinstein. 1983. Rearranged *c-mos* in a MOPC 21 murine myeloma cell line and its persistence in hybridomas. *Nature (London)* **306**:795-796.
- Gonda, T. J., S. Cory, P. Sobieszczuk, D. Holtzman, and J. Adams. 1987. Generation of altered transcripts by retroviral insertion within the *c-myc* gene in two murine monocytic leukemias. *J. Virol.* **61**:2754-2763.
- Gough, N. M., D. Metcalf, J. Gough, D. Grail, and A. R. Dunn. 1985. Structure and expression of the mRNA for murine granulocyte-macrophage colony stimulating factor. *EMBO J.* **4**:645-653.
- Hawley, R. G., M. J. Shul, and N. Hozumi. 1984. Transposition of two different intracisternal A particle elements into an immunoglobulin kappa-chain gene. *Mol. Cell. Biol.* **4**:2565-2572.
- Howe, C. C., and G. C. Overton. 1986. Expression of the intracisternal A-particle is elevated during differentiation of embryonal carcinoma cells. *Mol. Cell. Biol.* **6**:150-157.
- Kongsuwan, K., J. Allen, and J. M. Adams. 1989. Expression of Hox-2.4 homeobox gene directed by proviral insertion in a myeloid leukemia. *Nucleic Acids Res.* **17**:1881-1892.
- Kono, T., T. Doi, G. Yamada, M. Hatakeyama, S. Minamoto, M. Tsudo, M. Miyasaka, T. Miyata, and T. Taniguchi. 1990. Murine interleukin-2 receptor β chain: dysregulated gene expression in lymphoma line EL-4 caused by a promoter insertion. *Proc. Natl. Acad. Sci. USA* **87**:1806-1810.
- Kuff, E. L., A. Feenstra, K. Lueders, L. Smith, R. Hawley, N. Hozumi, and M. Shulman. 1983. Intracisternal A-particle genes as movable elements in the mouse genome. *Proc. Natl. Acad. Sci. USA* **80**:1992-1996.
- Kuff, E. L., and K. K. Lueders. 1988. The intracisternal A-particle family: structure and functional aspects. *Adv. Cancer Res.* **51**:183-276.
- Laker, C., C. Stocking, U. Bergholz, N. Hess, J. F. De Lamarter, and W. Ostertag. 1987. Autocrine stimulation after transfer of the granulocyte/macrophage colony-stimulating factor gene and autonomous growth are distinct but interdependent steps in the oncogenic pathway. *Proc. Natl. Acad. Sci. USA* **84**:8458-8462.
- Leslie, K. B., and J. W. Schrader. 1989. Growth factor gene activation and clonal heterogeneity in an autostimulatory myeloid leukemia. *Mol. Cell. Biol.* **9**:2414-2423.

28. Leslie, K. B., H. J. Ziltener, and J. W. Schrader. The role of IL-1 and GM-CSF in the paracrine stimulation of an in vivo-derived murine myeloid leukemia. *Blood*, in press.
29. Lesser, J., J. Lasneret, M. Canivet, R. Emanoil-Ravier, and J. Peries. 1986. Simultaneous activation by 5-azacytidine of intracisternal R particles and murine intracisternal-A particle related sequences in Syrian hamster cells. *J. Virol.* **155**:249-256.
30. Lueders, K. K., and E. L. Kuff. 1980. Intracisternal A-particle genes: identification in the genome of *Mus musculus* and comparison of multiple isolates from a mouse gene library. *Proc. Natl. Acad. Sci. USA* **77**:3571-3575.
31. Luria, S., and M. Horowitz. 1986. The long terminal repeat of the intracisternal A particle as a target for transactivation by oncogene products. *J. Virol.* **57**:998-1003.
32. Man, Y. M., H. Delius, and D. P. Leader. 1987. Molecular analysis of elements inserted into mouse γ -actin processed pseudogenes. *Nucleic Acids Res.* **15**:3291-3304.
33. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. Mietz, J. A., Z. Grossman, K. K. Lueders, and E. L. Kuff. 1987. Nucleotide sequence of a complete mouse intracisternal A-particle genome: relationship to known aspects of particle assembly and function. *J. Virol.* **61**:3020-3029.
35. Mietz, J. A., and E. L. Kuff. 1990. Tissue and strain-specific patterns of endogenous proviral hypomethylation analysed by two-dimensional gel electrophoresis. *Proc. Natl. Acad. Sci. USA* **87**:2269-2273.
36. Miyatake, S., T. Otsuka, T. Yokota, F. Lee, and K. Arai. 1985. Structure of the chromosomal gene for granulocyte-macrophage colony stimulating factor: comparison of the mouse and human genes. *EMBO J.* **4**:2561-2568.
37. Miyatake, S., T. Yokota, F. Lee, and K. Arai. 1986. Structure of the chromosomal gene for murine interleukin 3. *Proc. Natl. Acad. Sci. USA* **82**:316-320.
38. Peckham, I., S. Sobel, J. Comer, R. Jaenisch, and E. Barklis. 1989. Retrovirus activation in embryonal carcinoma cells by cellular promoters. *Genes Dev.* **3**:2062-2071.
39. Rechavi, G., D. Givol, and E. Canaani. 1982. Activation of a cellular oncogene by DNA rearrangement: possible involvement of an IS-like element. *Nature (London)* **300**:607-611.
40. Setoguchi, M., Y. Higuchi, S. Yoshida, N. Nasu, Y. Miyazaki, S. Akizuki, and S. Yamamoto. 1989. Insertional activation of N-myc by endogenous Moloney-like murine retrovirus sequences in macrophage cell lines derived from myeloma cell line-macrophage hybrids. *Mol. Cell. Biol.* **9**:4515-4522.
41. Shapiro, M. B., and P. Senapathy. 1987. RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. *Nucleic Acids Res.* **15**:7155-7175.
42. Shen-Ong, G. L. C., H. C. Morse III, M. Potter, and J. F. Mushinski. 1986. Two modes of *c-myc* activation in virus-induced mouse myeloid tumors. *Mol. Cell. Biol.* **6**:380-392.
43. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**:503-517.
44. Stocking, C., C. Löliger, M. Kawai, S. Suci, N. Gough, and W. Ostertag. 1988. Identification of genes involved in growth autonomy of hematopoietic cells by analysis of factor-independent mutants. *Cell* **53**:869-879.
45. Sugita, T., T. Totsuka, M. Saito, K. Yamasaki, T. Taga, T. Hirano, and T. Kishimoto. 1990. Functional murine interleukin-6 receptor with intracisternal A particle gene product at its cytoplasmic domain. *J. Exp. Med.* **171**:2001-2009.
46. Tohyama, K., K. H. Lee, K. Tashiro, T. Kinashi, and T. Honjo. 1990. Establishment of an interleukin-5-dependent murine hemopoietic progenitor cell line, LyD9, and its malignant transformation by autocrine secretion of interleukin-5. *EMBO J.* **9**:1823-1830.
47. Trusko, S. P., E. K. Hoffman, and D. L. George. 1989. Transcriptional activation of cKi-ras proto-oncogene resulting from retroviral promoter insertion. *Nucleic Acids Res.* **17**:9259-9265.
48. Weinstein, Y., J. L. Cleveland, D. S. Askew, U. R. Rapp, and J. N. Ihle. 1987. Insertion and truncation of *c-myc* by murine leukemia virus in myeloid cell line derived from cultures of normal hematopoietic cells. *J. Virol.* **61**:2339-2343.
49. Ymer, S., W. Q. J. Tucker, H. D. Campbell, and I. G. Young. 1986. Nucleotide sequence of the intracisternal A-particle genome inserted 5' to the interleukin-3 gene of the leukaemia cell line WEHI-3B. *Nucleic Acids Res.* **14**:5901-5918.
50. Ymer, S., W. Q. J. Tucker, C. J. Sanderson, A. J. Hapel, H. D. Campbell, and I. G. Yung. 1985. Constitutive synthesis of interleukin-3 by leukaemia cell line WEHI-3B is due to retroviral insertion near the gene. *Nature (London)* **317**:255-258.
51. Ziltener, H. J., I. Clark-Lewis, B. Fazekas de St. Groth, L. E. Hood, S. B. H. Kent, and J. W. Schrader. 1987. Antipeptide antibodies define the N-terminal structure of the pan-specific hemopoietin interleukin-3. *J. Immunol.* **138**:1099-1044.