Synergy Between AUUUA Motif Disruption and Enhancer Insertion Results in Autocrine Transformation of Interleukin-3–Dependent Hematopoietic Cells

By Marty W. Mayo, Xiaoyang Wang, Paul A. Algate, Guillermo F. Arana, Paul E. Hoyle, Linda S. Steelman, and James A. McCubrey

Previously, we characterized the transposition of an intracisternal type A particle (IAP) to the 3′ untranslated region (UTR) of the interleukin-3 (IL-3) gene, which displaced two of the six AUUUA motifs associated with mRNA stability in an IL-3–secreting clone. To determine whether this repositioning was involved in the autocrine transformation of the parental IL-3–dependent FL5.12 cell line, the germline (gL-3) and rearranged IL-3 (rIL-3) genes were isolated and subcloned into a gene transfer vector. Moreover, the IAP-long terminal repeat (LTR) and the IL-3 3′ UTR AUUUA motifs were deleted (rIL-3 + ΔLTR and ggL-3 + ΔAUUUA) in some IL-3 constructs to ascertain their role in the transformation process. The IAP-LTR was also added to these constructs (rIL-3 + ΔLTR + IAP-LTR, ggL-3 + ΔAUUUA + IAP-LTR, and ggL-3 + IAP-LTR), to determine whether it was necessary for autocrine transformation. The ability of the modified IL-3 genes to abrogate the IL-3 dependency of FL5.12 cells had the following rank order: rIL-3 was greater than rIL-3 + ΔLTR + IAP-LTR, which was greater than ggL-3 + ΔAUUUA + IAP-LTR, which was greater than ggL-3 + ΔAUUUA, which was equal to rIL-3 + ΔLTR, which was greater than ggL-3. The half-life of IL-3 mRNA was 20-fold longer in cells containing a mutated as opposed to a wild-type AUUUA region. All of the factor-independent cells that expressed the IL-3 transgenes secreted IL-3 and were tumorigenic after injection into BALB/c nude mice. These results indicated that two events could synergize in the autocrine transformation of hematopoietic cells: (1) addition of a transcriptional enhancer present in a retroviral LTR, and (2) disruption of an mRNA stability region.

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EUKEMIC CELLS THAT proliferate in response to a cytokine that they abnormally synthesize exhibit autocrine growth. This type of transformation has been well documented in many different neoplasms.1,3 Aberrant regulation of early-acting hematopoietic growth factors, such as like interleukin-3 (IL-3), can promote an autocrine-transformed phenotype of hematopoietic cells.9-12 IL-3 is a 28-kD glycosylated protein normally secreted by activated T cells and natural killer cells.13-15 This multiclonal-stimulating factor exerts its biologic activity by binding to the interleukin-3 receptor (IL-3R).16 The IL-3R is composed of an α-chain that associates with the ligand17,18 and a β-chain, which is essential for signal transduction.19 The binding of IL-3 to its cognate receptor activates a Janus kinase (Jak), which leads to the phosphorylation and dimerization of signal transducers and activators of transcription (STATs) factors that in turn regulate the expression of key genes involved in growth.20 Because of the delicate balance in the hematopoietic system, IL-3 synthesis is highly regulated at the both the transcriptional and posttranscriptional levels.10-12,21

To investigate the mechanisms involved in growth autonomy of hematopoietic cells, we focused on a murine cell line that contains an intracisternal A particle (IAP) provirus integrated within the IL-3 gene. IAPs are retroviral-like elements that are harbored in the cisternae of the endoplasmic reticulum and the Golgi complex due to defects in envelope protein synthesis.22 It has been estimated that in mice and other species of rodents, there are approximately 1.000 IAP elements per haploid genome.22 Expression of IAP proviral elements has been found to be active during early mouse development and in normal tissues, including thymus, liver, and pancreatic β cells.22 Like other retroviruses, integrated IAP proviruses contain long terminal repeats (LTRs) that control viral transcription of the gag, pol, and env coding regions, although IAPs have deleted portions of their env gene.22-28 IAPs act as endogenous mutagens, as they are known to transpose12,27 and alter the expression of oncogenes,28,29 developmental genes,30,31 growth factor genes,9,10,12,32-35 and receptor genes.36,37 Interestingly, IAP expression is controlled genetically, as only a family of highly related elements displays tissue-specific transcription.25,26

IAP transposition resulting in the autocrine secretion of a growth factor typically occurs within the 5′ untranslated region (UTR) of lymphokine genes.10,32,34 The activation of cellular genes can occur through a number of mechanisms including the displacement of negative cis-acting repressors,9,10,12,32-34 increased transcription resulting from elevated enhancer activity,10,28,29,34 and alternative promoter sites provided by the IAP-LTR.35 However, little is known about the mechanisms that govern gene activation after IAP proviral transposition within the 3′ UTR. Stocking et al10 described a rearrangement in which an IAP transposed within the 3′ UTR region of the granulocyte macrophage-colony stimulating factor (GM-CSF) gene. They proposed that this event abrogated growth factor dependence by displacing sequences required for mRNA turnover and polyadenylation.36 Both of these regions have profound implications on the mRNA stability of short-lived messages such as lymphokines and immediate-early genes, and the functions of these sequences have been reviewed extensively.24-42 Aberrant regulation of
mRNA stability is likely to be very important in the etiology of many hematopoietic neoplasms.45,46

Previously, it was shown that the AU-rich elements (ARE) located within the 3' UTR of the GM-CSF gene were in part responsible for mRNA destabilization and could promote enhanced decay of the β-globin message when inserted into the 3' UTR.47 Stability of mRNA is regulated both in cis and in trans. The consensus sequence for the ARE is loosely defined as an AUUUA motif repeated once or several times within the 3' UTR. Point mutations have shown that three AUUUA motifs are the minimum requirement for the destabilizing effect; two motifs are insufficient to affect stability in the case of GM-CSF mRNA.48 Interestingly, ARE-stimulated decay seems to require translation in some circumstances.49 A model for mRNA degradation is the decay pathway and is initiated by the shortening of the poly A tail, followed by decapping and 5' → 3' or 5' → 3' exonuclease degradation of the transcripts. Alternatively, mRNA decay can also initiate before the shortening of the poly (A) tail by decapping, followed by 5' → 3' exonuclease degradation and endonuclease degradation of the transcripts. The degradation of immediate-early gene and cytokine mRNA may occur through a deadenylation-dependent pathway, as c-fos and some other mammalian early-response genes carrying functional AREs display a biphasic decay, in which rapid shortening of the poly (A) tail leads to the decay of the mRNA body.50

Examination of the rearranged IL-3 gene present in FL-IL3-R2 cells revealed that the transposition occurred within the 3' UTR and displaced four of the six AUUUA motifs, as well as the normal IL-3 transcriptional termination and polyadenylation regions. Messages transcribed from the rearranged IL-3 locus in FL-IL3-R2 cells use the polyadenylation signal provided by the proximal IAP-LTR, which caused the transcripts to be approximately 140 nucleotides larger than IL-3 transcripts derived from the WEHI-3B cell line, which constitutively expresses IL-3 as a result of an IAP insertion within the promoter region.51 Studies of mRNA half-life documented that the rearranged gene in FL-IL3-R2 cells encodes an IL-3 message that has a much slower decay rate than transcripts derived from the WEHI-3B cell line,52 suggesting alteration of mRNA stability as a possible mechanism by which the IAP transposition resulted in the autocrine transformation.

In this study, we sought to determine the mechanism of autocrine transformation in the FL-IL3-R2 cell line. Here, we present evidence that the transposition of the IAP in the 3' UTR resulted in transformation by enhanced IL-3 transcription and increased message stabilization due to partial displacement of the ARE region. These results indicate that stabilization of the IL-3 transcript was not sufficient to initiate autocrine transformation unless accompanied by elevated transcription in the strictly regulated hematopoietic System.

**MATERIALS AND METHODS**

**Cell culture.** The E-3-dependent lymphoid line FL5.1251 was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% iron-supplemented, defined bovine calf serum (CS; Hyclone, Logan, UT) and 20% supernatant prepared from the WEHI-3B cell line (WEHI-conditioned medium, WCM), which served as a source of IL-3. The IL-3 rearranged line FL-IL3-R211 and transfected lines that produced IL-3 and did not require exogenous IL-3 for growth were cultured in DMEM plus 5% CS. In indicated experiments, cells were treated with 50 × 10^-5 mol/L phorbol 12-myristate 13-acetate (PMA; Sigma, St Louis, MO) or 10 × 10^-5 mol/L calcium ionophore (A23187; Boehringer Mannheim, Indianapolis, IN). Cellular proliferation was measured by incubating cultures in the presence of 5'-thymidine (6.7 Ci/mmol/L; NEN, Boston, MA), and uptake was measured by scintillation counting as previously described.52 Recombinant murine IL-3 and GM-CSF were purchased from Genzyme (Boston, MA). In some experiments, supernatants were incubated with either α-IL-3 monoclonal antibody (MoAb; Genzyme) or αGM-CSF MoAb (Oncogene Sciences, Uniondale, NY) for 1 h at 37°C before the addition of FDC-P1 cells as described.53

**Plasmid construction.** Standard recombinant DNA procedures were used for plasmid constructions.46 Restriction enzymes were purchased from GibcoBRL-Life Technologies (Gaithersburg, MD), Promega (Madison, WI), or New England Biolabs (Beverly, MA). The gIL-3 3' UTR and rearranged (rIL-3) alleles were cloned by a two-step process into the BamHI-EcoRI sites of the pSV2neo expression vector53 using the 3' end unique EcoRI site and the BamHI sites present at the 5' end and the third exon of the IL-3 gene. To facilitate further manipulation, the 5' end EcoRI site was destroyed by a fill-in reaction, and a 5' end unique EcoRI site was created by inserting a BamHI internal EcoRI linker (GATC GA-ATTCC) into BamHI partially digested gIL-3-neo. Subsequently, the Mo-MuLV56 and IAP LTRs were inserted into both the 5' and 3' ends of the gIL-3 construct (gIL-3 + Mo-MuLV-LTR, gIL-3 + IAP-LTR) using the 5' and 3' end EcoRI sites. The IAP-LTR constructs described in these studies contain the lymphocyte-specific IAP-LTR isolated from the rearranged IL-3 gene present in FL-IL3-R2 cells.57 After creation of these IL-3 gene vectors, the LTRs were inserted in both orientations. There did not appear to be a significant difference in the frequency of factor-independent cells when the LTR was in either the 5' → 3' or 3' → 5' orientation (data not presented). Therefore, the results of transfections performed with a given LTR in different orientations were combined. The IL-3 + ΔLTR construct was made by deletion of an EcoI fragment, which removed most of the 5'IAP-LTR sequences without disrupting the virally encoded polyadenylation element. Subsequently, an IAP-LTR was added back to this construct at the 3' end unique EcoRI site (Fig 1). The gIL3 + ΔAUUUA nomenclature means the deletion of the entire AUUUA cassette, ie, (AUUUA), and was created by closing a Hae III-Kas I fragment into a Kas I-bunted NeoI fragment of gIL-3-neo. Blunt-end ligations were performed as described.57 The gIL3 + ΔAUUUA + 3'IAP-LTR constructs were made by subcloning the IAP-LTR in the 3' end EcoRI site of the gIL3 + ΔAUUUA construct. The gIL3 + PCR DNA vector was constructed by inserting a 360-bp EcoRI-HindIII cut polymerase chain reaction (PCR) product containing bases -251 to +105 of the IL-3 gene into the gIL3-neo construct after removing the IL-3 gene fragment from the 5' end EcoRI site to the +105-bp HindIII site. The PCR was performed by using upstream primer 5'-GGCCGAATTCCTTCCCAACACGTGTT-3' and downstream primer 5'-CTGATTCTTCCCAACACCTGGT-3'. Subsequently, the IAP-LTR was inserted into the -251 EcoRI site of this vector. A 450-bp piece of bacteria DNA (from Bacteroides fragilis) was also inserted at the -251 EcoRI site to serve as a negative control. Plasmids containing positive constructs were identified by restriction endonuclease digestions and were confirmed by Southern blot hybridization. The DNA sequences of the PCR product and the regions surrounding the deletions in the ΔLTR and ΔAUUUA constructs were verified by the

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Fig 1. Structure of germline and modified IL-3 genes. (A) The germline and rearranged genes were isolated from the FL-IL3-R2 cell line,\textsuperscript{5} which was derived from the IL-3-dependent FL5.12 line.\textsuperscript{5} The IAP transposition occurred within the ARE at +2053 as indicated by the arrow within the germline configuration.\textsuperscript{5} The transcribed region is in bold and includes exons 1 through 5. (B) Both germline and rearranged IL-3 genes were inserted into the pSV2neo vector\textsuperscript{36} as described in Materials and Methods. Subsequent manipulations were performed with the LTRs from either Mo-MuLV (hatched rectangles)\textsuperscript{36} or the IAP-LTR (solid rectangles) contained in the rearranged IL-3 gene in FL-IL3-R2. Bacterial DNA was also inserted at -25 kb (stippled rectangle) relative to the IL-3 promoter region as a control. B, BarnHI; E, EcoRI; H, Hae III; K, Kas I; and N, Neo I. The polyadenylation sites (pA) in both the germline gene and IAP-LTR are shown.
method of Sanger et al.8 The base pair numbers indicate above were relative to the IL-3 transcription initiation site (Fig 1).

Transfection and isolation of G418-resistant transfecants. Logarithmic stage FL5.12 cells were washed (2×) in phosphate-buffered saline (PBS) and resuspended in DMEM plus 20% CS at a concentration of 2 × 10⁶/mL. The restriction enzyme PvuI was used to linearize the constructs because it cleaves within the amp" gene and does not interrupt either the IL-3 or neo sequences. Linearized constructs (10 to 20 μg) were resuspended with a 500-μL cell aliquot (1 × 10⁶ cells total) and incubated on ice for 10 minutes. Cells were electroporated using a capacitance extender at 350 V and 950 microfarads (μF; BioRad gene pulser and capacitance extender, BioRad, Richmond, CA). Following electroporation, cells were plated in 100-mm culture dishes containing DMEM plus 20% CS supplemented with 20% WCM. After 48 hours, the cells were collected, washed with PBS, and cultured in 96-well, round-bottom plates in either DMEM plus 5% CS containing the antibiotic G418 (2 mg/mL; Geneticin, Sigma) only, or under the same conditions supplemented with 20% WCM. The 96-well plates were then fed every 3 days. After 2 weeks in culture, the number of wells containing G418-resistant, stably transfected cells was enumerated.

Southern and Northern analysis. Cellular RNAs were isolated, and Southern blot analyses were performed as previously described.11 Most of the transfecants contained one to two copies of the introduced construct, and all of the subsequent analyses were performed on clones that contained one to two copies of the introduced IL-3 gene.

Cellular RNAs were isolated by CsCl gradient purification as previously described12,20 or by Trizol (Life Technologies). Total cellular RNAs (20 μg) were loaded onto a 1.2% agarose gel containing 18.5% formaldehyde and electrophoresed at 5 V/cm. RNA gels were blotted onto Hybond-N nylon membranes (Amersham, Arlington Heights, IL) and immobilized in a vacuum oven for 2 hours at 80°C. Filters were prehybridized and subsequently hybridized with a random-primed, labeled cDNA-specific probe, as described previously.20 For mRNA half-life determinations, RNAs were isolated from cells cultured in the presence of 10 μg/mL of actinomycin-D (Sigma). The levels of hybridization were quantified by densitometric scanning23 of autoradiographs and were normalized to the levels of β-actin mRNA detected in the samples. Half-lives of mRNA were then calculated using linear regression analysis.

RNA detection using the PCR. Total cytoplasmic RNA was prepared as above and included in a 20-μL cDNA synthesis reaction containing: 1 μg of total RNA, reverse transcriptase buffer, 1 mmol/L of each deoxyxynucleotide triphosphate (dNTP), 2.5 μmol/L oligo-dT, and 50 U Mu-MuLV reverse transcriptase (Promega). After incubation at 42°C for 15 minutes, the reverse transcriptase was inactivated by heating at 99°C for 5 minutes. Subsequently, 2.5 U of Taq DNA polymerase (Gibco/BRL), PCR reaction buffer, and 1 μmol/L of each upstream and downstream primers were added to the reaction mixture to make a 100-μL volume total. PCR was performed as follows: 1 minute at 95°C for denaturing, 1 minute at 60°C for annealing, and 1 minute at 72°C for the polymerase reaction. The primers for IL-3 were 5'-AATCACGGCAGGGATACCC-3' and 5'-GGAAATCACCTGAGATCTCG-3', defining a 200-bp cDNA fragment that could readily be distinguished from a genomic IL-3 DNA fragment by size (greater than 1 kb). The primers for β2-microglobulin were 5'-TCTTCTCACTTAGCGCCCGT-3' and 5'-CACGATACGTTGGTGCCT-3', defining a 308-bp fragment. To detect IL-3 cDNAs, 35 to 50 cycles of PCR were performed, and additional nucleotides and TAQ polymerase were added after 35 cycles. The PCR products were electrophoresed on 2% agarose gels and visualized after ethidium bromide staining of the gel. To confirm the results observed by ethidium bromide staining, the PCR gel was also transferred to nitrocellulose and hybridized with an IL-3-specific probe, and similar results were observed.

Nuclear run-on transcription assays. Nuclei isolation and transcription reactions were performed as described previously.13,14 IL-3 transcription levels were determined by densitometric scanning of autoradiographs, and signal intensities were normalized to genomic DNA controls.

Nucleic acid probes. The IL-3-specific DNA probe was acquired from Dr Ian Young (Australian National University, Canberra, Australia). The IL-3α and IL-3β probes were obtained from Dr Atsushi Miyajima (DNAx, Palo Alto, CA). The IL-4 probe was provided by Dr William Paul (National Institutes of Health [NIH] Bethesda, MD). The IL-4R probe was obtained from Dr Steven Gillis (Immunex, Seattle, WA). The IL-6 probe was procured from Dr Jacques Van Snick (Ludwig Institute, Brussels, Belgium). The c-myc exon 2 and 3-specific probe was a gift from Dr Michael Cole (Princeton University, Princeton, NJ). The murine mast cell growth factor probe (Mu-MGFP) was provided by Dr Stewart Lyman (Immunex).

Tumorigenicity of cell lines. Cells (1 × 10⁶) were injected intra-peritoneally into BALB/c nude mice. The abdominal area of the mice was palpated every 3 days to monitor for the presence of tumor formation. Mice with tumors (≥1 cm in diameter) were killed. To ensure that the injected cells were responsible for the malignant growth, cells were aseptically removed from the spleen, bone marrow, and the axillary, inguinal, and sacral lymph nodes. Transformed cell lines recovered from mice maintained transgene expression and were easily recovered under selective G418 conditions.

RESULTS

Construction of vectors containing the germline, rearranged, and modified IL-3 genes and transfection of IL-3-dependent cells. To determine whether the IAP transposition within the 3' UTR region of the IL-3 gene was sufficient to promote factor-independent growth, the gIL-3 and rIL-3 alleles were cloned from FL-IL3-R2 cells and inserted into the pSV2neo expression vector, as described in Materials and Methods (Fig 1). The parental IL-3-dependent FL5.12 cells were transfected by electroporation and subsequently selected in the neomycin analogue, G418, either in the presence or absence of exogenous IL-3 (Table 1). No neo' cells were retrieved from mock-transfected cells, indicating that the G418 concentration was sufficient to eliminate non-neo'-transfected cells. The neo'-IL-3-dependent clones were isolated from the gIL3 and pSV2neo electroporated cells, indicating that some cells had stably inherited the constructs. However, factor-independent cells (ie, cells that grew in DMEM plus G418 in the absence of exogenous IL-3) were not recovered from cells transfected with either the gIL-3 construct (n = 5 times) or empty vector control pSV2neo (n = 2). In contrast, factor-independent cells were readily recovered from the cells transfected with the rIL-3 gene (Table 1). Therefore, unlike the gIL-3 or pSV2neo vector, inheritance of the rIL-3 allowed approximately 50% of the stably transfected cells to grow in the absence of exogenous IL-3 (Table 1).

Mechanism by which the IAP transposition supports factor-independent growth. The fact that the rIL-3 contained only the proximal 5' LTR and was capable of promoting factor-independent growth suggested that the transposition altered the structure of the IL-3 locus either by supplying
elements that enhance transcription and/or by removing potentially important 3’ sequences critical for mRNA stability. To determine which of the IAP transposition-modified sequences were necessary for the expression of the IL-3 gene in the autocrine-transformed cells, three different approaches were used: (1) deletion of the IAP-LTR from rIL-3, (2) removal of the AUUUA motifs from gIL-3, and (3) addition of the retroviral LTR sequences to either the complete germline IL-3 gene or pSV2neo vector. Approximately twofold more autocrine-transformed cells were recovered when the IAP-LTR was inserted 5’ as opposed to 3’ of the IL-3 promoter region.

To determine the effects of insertion of an IAP-LTR closer to the IL-3 promoter region, an IAP-LTR was inserted at -25 kb. There was a modest 1.6-fold increase when the IAP-LTR was inserted at -25 kb as compared with the germline IL-3 gene, which contained the IAP-LTR at -1.5 kb. As controls, bacterial DNA (bDNA) was inserted at the -25 kb site (gIL-3 + bDNA), and the modified vector used to generate these two constructs (gIL-3 + PCR-DNA) was transfected into FL5.12 cells. No factor-independent clones were recovered (PCR-DNA refers to the construction of the deleted germline IL-3 gene; see Materials and Methods).

These results indicated that the IAP-LTR increased the recovery of factor-independent cells but not by a high frequency. Therefore, the IAP-LTR was relatively weak in stimulating the frequency of factor-independent cells, and the recovery increased when the mRNA stability sequences were deleted and the IAP-LTR was close to the IL-3 promoter region.

The effects of a stronger enhancer on IL-3 gene expression were determined. The Moloney murine leukemia virus (Mo-MuLV) LTR was inserted into the gIL-3 construct at the -1.5 and +4.5 kb positions relative to the IL-3 promoter. More IL-3–independent cells (6-fold to 11-fold) were observed with the Mo-MuLV versus IAP LTRs (Table 1). In contrast with the results observed with the IAP-LTR, the Mo-MuLV–LTR stimulated equally the frequency of factor-independent cells when it was positioned either 5’ or 3’ of the IL-3 promoter region.

**IL-3 transcription in the factor-dependent FL5.12 cell line.** There are two central questions in determining how the IAP transposition resulted in the expression of the IL-3 gene: (1) Is IL-3 mRNA transcribed in the IL-3–independent parental FL5.12 cells? (2) Is this mRNA stabilized after the disruption of the AUUUA motifs? To determine whether a low level of IL-3 transcripts were present in FL5.12 cells, the reverse transcription (RT)-PCR technique of mRNA detection was used. IL-3 cDNAs were not detected in FL5.12

### Table 1. Abrogation of IL-3 Dependency After Transfection With Modified IL-3 Genes

<table>
<thead>
<tr>
<th>Construct</th>
<th>LTR Distance From Promoter* (kb)</th>
<th>DMEM + G418</th>
<th>IL-3 + G418</th>
<th>DMEM/IL-3†</th>
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<td>Mock</td>
<td></td>
<td>0/576</td>
<td>0/576</td>
<td>0</td>
</tr>
<tr>
<td>pSV2neo</td>
<td></td>
<td>0/192</td>
<td>19/192</td>
<td>0</td>
</tr>
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<td>91/480</td>
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<td>87/576</td>
<td>48</td>
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<td>64/384</td>
<td>3</td>
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<td>5/192</td>
<td>12/192</td>
<td>42</td>
</tr>
<tr>
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<td>71/384</td>
<td>3</td>
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<tr>
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<td>7</td>
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<td>1.5</td>
<td>9/192</td>
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*Distance between the LTR and the IL-3 promoter region.
† Division of number of wells by 96 equals the number of times the transfection with a particular DNA was performed.
‡ Percentage of growth in the absence versus presence of IL-3.

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cells after RT-PCR analysis (Fig 2, lane 1). Similar results were observed in unstimulated FDC-P1 cells, another IL-3-dependent cell line (lane 5). In contrast, IL-3 cDNAs were readily detected in WEHI-3B cells (lane 9), which constitutively transcribed the IL-3 gene.

To determine whether FL5.12 cells could be stimulated to express IL-3 transcripts, the cells were treated with agents known to promote gene expression. Calcium ionophores and phorbol esters can induce cytokine gene transcription and increase the stabilization of labile transcripts.\(^{12,45,64-66}\) Treatment of FL5.12 and FDC-P1 cells with both the phorbol ester phorbol myristate acetate (PMA) and the calcium ionophore A23187 resulted in the detection of IL-3 cDNAs (lanes 3 and 7), whereas culture with either PMA (lanes 2 and 6) or calcium ionophore (lanes 4 and 8) alone did not result in the appearance of these cDNAs. Neither additional PCR cycles (approximately 50 total) nor Southern blotting and hybridization of the filter with a radiolabeled IL-3 probe resulted in the detection of IL-3 cDNAs in unstimulated cells (data not presented). These results indicated that in these unstimulated IL-3-dependent cells, the mRNA transcripts for the IL-3 gene did not accumulate to levels that were readily detected by RT-PCR (Fig 2).

**Increased IL-3 transcription in the autocrine-transformed cells.** To determine whether the rate of IL-3 transcription was increased in factor-independent cells transfected with the LTR-containing constructs, nuclear run-on experiments were performed. Nuclei from each transfected clone were incubated in vitro with \([\alpha\text{-}\text{32P}]-\text{UTP}\) to allow elongation of already initiated RNA transcripts. After isolation, the labeled RNAs were hybridized to immobilized, single-stranded DNA probes specific for transcribed RNAs, as shown in Fig 3. Because no IL-3 nascent transcription was detected in the parental IL-3-dependent line FL5.12, the amount of hybridization measured by the densitometer was considered the baseline. The level of IL-3 nascent transcription was ninefold higher in the cell line that inherited the rIL-3 or the gIL-3 + MO-MuLV-LTR, and both of these constructs contained a retroviral LTR (Fig 3).

**Disruption of the 3' ARE sequences is sufficient for message stabilization.** To determine whether the IAP transpo-
sition, which deranged the mRNA stability sequences, resulted in IL-3 transcripts with a long half-life, mRNAs were isolated from cells transfected with the various constructs that had been incubated with the transcriptional inhibitor actinomycin-D. IL-3 mRNAs isolated from FL5.12 cells transfected with the rIL-3, gIL-3 + ΔAUUUA + IAP-LTR, and rIL-3 + ΔLTR + IAP-LTR constructs had long half-lives (t1/2, greater than 12 hours; Fig 4). In contrast, the half-lives of IL-3 mRNAs isolated from the FL5.12 cells transfected with the gIL-3 gene and either the Mo-MuLV-LTR or IAP-LTRs were short (t1/2, 0.6 hours) and approximately 20-fold more labile (Fig 4).

To ensure that the actinomycin-D treatment was effective, the Northern blots were also hybridized with probes specific for the c-myc and β-actin genes, which encode mRNAs with short and long half-lives, respectively. The mRNA half-lives for c-myc (t1/2, 0.5 hours) and β-actin (t1/2, greater than 12 hours) in the cells transfected with the rIL-3 gene or the gIL-3 + ΔAUUUA + IAP-LTR construct were similar to those observed in other cells.71 Thus, there was not a global change in mRNA stability in cells containing mutant IL-3 genes. The IL-3 AUUUA motifs were determining the degradation rate of the IL-3 mRNA, because mutation or deletion of these sequences resulted in the accumulation of only IL-3 mRNA with a prolonged half-life (Fig 4).

Secretion of IL-3 by the autocrine-transformed cells. To determine whether the mRNA expression was biologically significant, proliferation assays were performed. These experiments were performed to determine whether the IL-3-transfected cells were secreting IL-3, because FL5.12 cells can be transformed with oncogenes (either v-abl or v-fms) and not secrete IL-3.11 Moreover, cells could become factor-independent by additional means unrelated to the inheritance and expression of the cytokine gene. Because FL5.12 cells incorporate 3H-thymidine poorly, supernatants were tested on the more sensitive FDC-P1 line in the presence or absence of an IL-3-specific MoAb. The results presented in Fig 5 are representative, as IL-3 was observed in the supernatants of all factor-independent transformants that received the IL-3 gene. The level of IL-3 secreted from the transformants that inherited the rIL-3 gene and the other IL-3 constructs was sufficient to support the continued proliferation of the parental IL-3-dependent cell line (data not presented). Higher levels of IL-3 bioactivity were detected in the supernatants prepared from cells containing the gIL-3 + Mo-MuLV-LTR construct, whereas the lowest levels were observed in the cells transfected with either

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**Fig 4.** Half-life of IL-3 mRNA in transfected lines. The half-life of IL-3 mRNA was determined by treatment of the cells with actinomycin-D and extraction of the mRNA; followed by Northern blot analysis. Representative Northern blots are presented. RNA half-lives were determined after densitometric scanning of the autoradiograms and linear regression analysis. The second row under (A), (B), and (C) was hybridized with a c-myc probe. The second row under (D), (E), and (F) was hybridized with a β-actin probe. The number of times samples from cell lines transfected with the indicated constructs was included in the calculations for the IL-3 mRNA half-lives is indicated in parentheses. (A) FL-rIL-3–T1 cells. (B) FL–gIL-3 + ΔAUUUA + 3′IAP-LTR-T2 cells. (C) FL–gIL-3 + 5′Mo-MuLV-LTR-T3. (D) FL–rIL3 + ΔLTR + IAP-LTR-T2. (E) FL–gIL-3 + 3′IAP-LTR-T1. (F) FL–gIL-3 + 3′Mo-MuLV-LTR-T4. (G) Linear regression analysis of mRNA half-life estimation: ■, FL–rIL-3–T cells (n = 10); ▲, FL–gIL-3 + ΔAUUUA + 3′IAP-LTR-T cells (n = 5); ●, FL–gIL-3 + Mo-MuLV-LTR-T (n = 12);
the gIL-3 + ΔAUUUA or gIL-3 + IAP-LTR constructs (data not presented).

**Tumorigenicity of autocrine-transformed cells.** To determine the oncologic consequences of inheritance of the rearranged and modified IL-3 constructs, the cells were injected into immunocompromised mice. The tumorigenicity of the cell lines was determined, because not all factor-independent cells will form tumors,\(^1\) and the latency period was examined to determine whether certain cell lines that secreted higher levels of IL-3 would form tumors more rapidly than cells that secreted lower levels of IL-3. Three to five mice were injected with each cloned cell line. Groups of mice that were injected with the same recombinant IL-3 construct are combined in Table 2. Tumors were not observed in mice injected with cells containing the gIL-3 or pSV2neo control vectors, which were IL-3-dependent (Table 2). However, inheritance of the rIL-3 gene resulted in the cells becoming tumorigenic. When the mRNA stability region was deleted, the cells were also tumorigenic. In addition, factor-independent cells that contained the gIL-3 gene and either the Mo-MuLV or IAP LTRs also induced tumors after injection into nude mice, whereas tumors were not observed in the mice that received the IAP-LTR-transfected cells that were factor-dependent (Table 2). Interestingly, factor-independent cells that secreted less IL-3 (eg, FL-gIL3 + 3'IAP-LTR) had a longer latency period than cells that secreted more IL-3 (eg, FL-gIL3 + MoMuLV-LTR). Factor-independent G418\(^*\) cells could be recovered from the spleen, bone marrow, and draining lymph nodes of mice with tumors. The cells isolated from these tissues contained the transfected IL-3 genes and grew in an autocrine fashion (data not presented). In summary, once the cells had lost their IL-3 dependency, they became tumorigenic and homed to hematopoietic organs after injection into mice (Table 2).

**DISCUSSION**

These studies have shown that inheritance of a rearranged IL-3 gene containing an IAP inserted into the 3' UTR was sufficient to transform IL-3–dependent cells to grow in the absence of exogenous IL-3 and render the cells tumorigenic. Although IAP transposition has been observed to occur in many cytokine,\(^{32,35}\) oncogene,\(^{28}\) homeo-box,\(^{30,31}\) and cytokine receptor and immunoglobulin genes,\(^{27,36,37}\) the fundamental importance of the transpositions in the tumorigenic process has not been clearly documented for two basic reasons: (1) the cell lines examined were established tumors that have deviated during years in culture from the original transformed cell and may have undergone many IAP transposi-

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**Table 2. Tumorigenicity of Transfected Cell Lines**

<table>
<thead>
<tr>
<th>Cell Line (n(^*))</th>
<th>IL-3 Dependency</th>
<th>No. of Mice</th>
<th>Latency (wk)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tumors/Total</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FL5.12</td>
<td>+</td>
<td>0/8</td>
<td>&gt;30</td>
</tr>
<tr>
<td>FL-pSV2neo (1)</td>
<td>+</td>
<td>0/4</td>
<td>&gt;30</td>
</tr>
<tr>
<td>FL-gIL-3 (2)</td>
<td>+</td>
<td>0/0/0</td>
<td>&gt;30</td>
</tr>
<tr>
<td>FL−rIL-3 (3)</td>
<td>−</td>
<td>26/26</td>
<td>2-4</td>
</tr>
<tr>
<td>FL−rIL-3 + ΔLTR (1)</td>
<td>−</td>
<td>4/4</td>
<td>2-3</td>
</tr>
<tr>
<td>FL−gIL-3 + ΔAUUUA (1)</td>
<td>−</td>
<td>5/5</td>
<td>2-3</td>
</tr>
<tr>
<td>FL−gIL-3 + 5'IAP-LTR (2)(^\dagger)</td>
<td>+</td>
<td>8/8</td>
<td>2-3</td>
</tr>
<tr>
<td>FL−gIL-3 + 5'IAP-LTR (5)(^\dagger)</td>
<td>+</td>
<td>0/20</td>
<td>&gt;30</td>
</tr>
<tr>
<td>FL−gIL-3 + 3'IAP-LTR (3)</td>
<td>−</td>
<td>12/12</td>
<td>3-5</td>
</tr>
<tr>
<td>FL−gIL-3 + 5'Mo-MuLV-LTR (1)</td>
<td>−</td>
<td>3/3</td>
<td>2-3</td>
</tr>
<tr>
<td>FL−gIL-3 + 3'Mo-MuLV-LTR (2)</td>
<td>−</td>
<td>8/8</td>
<td>2-3</td>
</tr>
</tbody>
</table>

\(^*\) Number of cloned cell lines examined. Three to five mice were injected with each cell line that was a clone. The results from the clones transfected with the same DNA construct have been combined with the following exception: transfection with FL−gIL-3 + 5'IAP-LTR resulted in factor-independent and factor-dependent clones.

\(^\dagger\) Mice were injected with two different clones derived after transfection with the 5'IAP-LTR–gIL-3 construct. The cells were IL-3–independent and did form tumors.

\(^\dagger\) Mice were injected with five different clones derived after transfection with the 5'IAP-LTR–gIL-3 construct. The cells were IL-3–dependent and did not form tumors.
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...tions that were not responsible for the malignant phenotype; and (2) there have been no investigations that determined the ability of the affected gene containing the IAP transposition-modified sequences to transform cells that would demonstrate that it was involved in the oncogenic process. Our results document that cis-acting mutations due to IAP transposition can directly result in the abrogation of growth factor independence without the need for additional mutations to occur in the cell. The IAP transposition was responsible for the alteration of the IL-3 mRNA stability, as the half-life was elongated in transfected cells that received either the rearranged or ARE-deleted IL-3 genes, but not the germine IL-3 genes. The contribution of mRNA destabilization in the induction of factor independence is indicated, because either the disruption or the deletion of the ARE region resulted in constructs that would abrogate the parental cell line’s dependency on the addition of exogenous IL-3, whereas without disruption of the ARE region resulted in constructs that would abrogate the parental cell line’s dependency. The remaining two motifs clustered together is required for the destabilizing effect induced by the IL-3 ARE sequence. Moreover, the investigators determined that the more critical region of the AUUUA repeats requires the presence of three of these repeats for efficient binding, which results in mRNA instability. This binding site would not be present in the rIL-3 gene examined in our studies, which results in protracted mRNA stability. The presence of several reiterated AUUUA motifs in the 3' UTR of cytokine mRNAs might provide a safe mechanism to ensure complete removal of cytokine transcripts when the cells no longer require their expression, and disruption of these motifs can, under certain circumstances, lead to malignant transformation.

IL-3 gene transcripts do not accumulate to significant levels in FL5.12 cells; however, IL-3 cDNAs were detected after the simultaneous addition of phorbol esters and calcium ionophores. Therefore, mRNA stabilization by itself should not normally be adequate for autocrine transformation. Indeed, only low frequencies of factor-independent cells were observed after transfection with gIL-3 constructs that lacked the AUUUA region (rIL-3 + \( \Delta LTR \) and gIL-3 + \( \Delta UUUUA \))—frequencies that were not as high as those observed with the rearranged construct. The mechanism by which these factor-independent cells arose is under investigation. Southern blot analysis indicates that they contain one to two copies of the introduced IL-3 genes, suggesting that inheritance of multiple copies of the IL-3 transgenes did not appear to be the cause of IL-3 independence. These constructs could have integrated in areas of transcriptionally active chromatin, which promoted transgene expression as IL-3 transcripts were detected in these cells that had long half-lives (data not presented). There was a low level of IL-3 transcripts detected by RT-PCR in the gIL-3-transfected cells, they were not detected by Northern blot analysis. Furthermore, the frequency of spontaneous factor-independent cells from different clones of gIL-3-transfected cells was low (less than 1 in 10^6 cells), and mRNA isolated from these spontaneous factor-independent gIL-3 transcripts had short half-lives (data not presented). The means by which these cells became factor-independent is under investigation. There could be additional mutations present in these cells (eg, mutations at either the IL-3Ra or IL-3Rβ genes) or other genes involved in signal transduction. In contrast, mRNA isolated from the rIL-3 + \( \Delta LTR \) and gIL-3\( \Delta A U T T T A \) factor-independent cells had a long half-life similar to that observed with the rIL-3-transfected cells (Fig 4 and data not presented). These low-level stable transcripts present in the gIL-3 + \( \Delta A U T T T A \) and rIL-3 + \( \Delta L T R \) transfected cells were most likely sufficient for growth in the absence of exogenous IL-3 in these factor-independent cells.

The IAP-LTR was necessary for autocrine transformation. After deletion of the IAP-LTR, a 16-fold lower frequency of factor-independent cells was observed. Moreover, insertion of an IAP-LTR into the rIL-3 + \( \Delta L T R \) construct restored the ability of the construct to induce autocrine transformation. After addition of an IAP-LTR to the gIL-3 + \( \Delta U U U U U A \) construct, a modest twofold increase in the retrieval of factor-independent cells was observed. This IAP-
LTR was approximately 4.5 kb away from the IL-3 promoter region, whereas in either the rIL-3 or rIL-3 + ΔLTR + IAP-LTR constructs, the LTR was roughly 2 kb distal. These results suggest two possibilities: (1) The IAP-LTR is a weak stimulator of IL-3 gene expression, and its ability is further weakened when it is positioned farther away from the promoter; or (2) the chimeric sequences of the IL-3 UTR and the IAP-LTR influence the secondary structure of the mRNA instability region, which interacts with RNA-binding proteins and alters their affinities, leading to a higher recovery of autocrine-transformed cells.

These studies have shown that the transposition of an IAP-LTR to the IL-3 3' UTR was a complex mutation that resulted in factor independence. Addition of the IAP-LTR either 5' or 3' to the IL-3 gene (outside of the AUUUA motifs) did not result in an IL-3 gene that transformed IL-3-dependent cells as efficiently as the rearranged IL-3 gene. In contrast, a more effectively transcribed IL-3 gene was obtained after addition of a MuMLV-LTR at the same genetic positions, either 5' or 3' to the promoter region. This difference in strengths of the retroviral enhancers is not unexpected and has been detected in transient transfection assays with the LTRs inserted in chloramphenicol acetyl transferase (CAT) vectors.23,24 Mu-MuLV is not an endogenous murine retrovirus and contains a strong LTR that is often used in retroviral vectors to promote the elevated expression of introduced cDNAs. In contrast, IAPs are replication-defective endogenous murine retroviruses. These results indicate that IAP-LTRs by themselves are poor transformers of hematopoietic cells and that they must integrate (transpose) into sequences that normally serve to regulate IL-3 expression for recovery of factor-independent cells. Because the mouse genome contains approximately 1,000 IAP genomes,22 it is not surprising that IAPs do not possess strong enhancers in their regulatory LTR molecules, which could otherwise lead to lethal mutations if they transposed at significant frequencies.

The gIL-3 promoter region was modified to determine whether insertion of an IAP-LTR closer to the start of transcription would increase the effectiveness of transformation. The WEHI-3B cell line contains an IAP transposition at a similar location.21 Also, these studies investigated whether deletion of upstream sequences would result in an IL-3 gene that transformed cells, as a sequence (NIP) that has been shown to repress transcription was identified at a similar position in the human IL-3 promoter.68 Removal of the upstream IL-3 promoter sequences did not increase the recovery of IL-3-independent cells as compared with the gIL-3 construct. Addition of an IAP-LTR at this position increased the recovery of IL-3-independent cells only 1.6-fold relative to when the IAP-LTR was placed at −1.5 kb upstream. These results suggested that deletion of the upstream IL-3 promoter sequences did not result in a strongly activated IL-3 gene. Alternatively, there may be additional sequences present in the upstream IL-3 promoter region that were necessary for efficient expression of the IL-3 gene which were removed in these constructs. It should be noted that all of the IL-3 constructs examined in this study contained at most 1.5 kb of upstream IL-3 gene sequences and could lack DNA sequences that are involved in regulation of IL-3 gene expression. Indeed, in the constructs that contain the IAP-LTR inserted closer to the IL-3 promoter region, it is likely that important regulatory sequences that control IL-3 gene expression were deleted. Recombinant constructs containing portions of the upstream IL-3 promoter region are being added back to these IL-3 promoter-deleted constructs (containing an IAP-LTR at −25) to determine whether the upstream IL-3 promoter sequences increase the frequency of factor-independent cells. Recently, a common enhancer region has been located between the human IL-3 and GM-CSF genes.80 The corresponding region in the murine IL-3 and GM-CSF genes is not present in these constructs. Therefore, the contribution of these important enhancer sequences could not be addressed by these studies.

The mechanisms of cellular transformation induced by IAPs are complex. In addition to the IAP transposition in the IL-3 promoter region in WEHI-3B cells, there is another IAP insertion in the hox-2.4 gene.30,31 It has been shown that these two genes can synergize and lead to the transformation of primary myeloid cells.81 It has been demonstrated that IAP expression can become activated by hypomethylation,82 which may lead to additional transpositions resulting in the aberrant regulation of genes involved in growth control. Whether there are other transpositions and mutations present in WEHI-3B and IL-3-R cells that promote enhanced IL-3 synthesis is unknown.

These results provide an interesting model for carcinogenesis, because two biochemical effects that alter gene expression were changed in one genetic event. Cancer may, in some circumstances, be the accumulation of subtle mutations that by themselves are insufficient to transform cells. However, when these mutations are contained in either the same gene or cell they may result in an activated oncogene or a malignant cell.

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REFERENCES

SYNERGY IN AUTOCRINE TRANSFORMATION


28. Young DC, Griffin JD: Nucleotide sequence of the intracisternal A-particle genome inserted 5' to the interleukin-3 gene of the leukemia cell line WEHI-3B. Nucleic Acids Res 14:5901, 1986


From www.bloodjournal.org by guest on November 11, 2017. For personal use only.
70. Algaze PA, Steelman LS, Mayo MW, Miyajima A, McCubrey JA: Regulation of the interleukin-3 (IL-3) receptor by IL-3 in the fetal liver-derived FL5.12 cell line. Blood 83:2459, 1994
77. Lagano CA, Brown CY, Goodall GJ: AUUUA is not sufficient to promote poly(A) shortening and degradation of an mRNA: The functional sequence within AU-rich elements may be UUAUUUAUU is the key AU-rich sequence motif that mediates mRNA degradation. Mol Cell Biol 15:2219, 1995
Synergy between AUUUA motif disruption and enhancer insertion results in autocrine transformation of interleukin-3-dependent hematopoietic cells

MW Mayo, XY Wang, PA Algate, GF Arana, PE Hoyle, LS Steelman and JA McCubrey