Spleen Focus-forming Virus Long Terminal Repeat Insertional Activation of the Murine Erythropoietin Receptor Gene in the T3Cl-2 Friend Leukemia Cell Line*

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We have characterized the structure of the erythropoietin receptor gene promoter in normal murine erythroid tissues and in Friend-induced tumor cells. Using primer extension analysis, we identified two distinct transcriptional start sites, which were located 2 base pairs apart in anemic spleens, fetal liver, Friend-induced tumoral spleens, and mouse erythroblastoma cells. In contrast, transcription was initiated 37 base pairs upstream of the normal cap sites in T3Cl-2, a Friend virus-induced murine erythroleukemia cell line. Also, the erythropoietin receptor mRNA in T3Cl-2 was overexpressed when compared with other erythroleukemia cell lines. We found that abnormal transcription occurring in T3Cl-2 cells resulted from an erythropoietin receptor gene alteration. Indeed, one erythropoietin receptor allele was rearranged by insertion of a spleen focus-forming virus long terminal repeat within the noncoding region of the first exon, 45 bases upstream of the ATG initiation codon and in the same 5' → 3' orientation. The transcription of the rearranged allele was shown to be directed from the long terminal repeat promoter, leading to a long terminal repeat-erythropoietin receptor fusion transcript, whereas the normal erythropoietin receptor allele was weakly transcribed. Such altered receptor gene activation may provide a positive pressure in the development of tumorigenic erythroleukemia.

Erythropoiesis is regulated mainly by the glycoprotein hormone erythropoietin (Epo), which binds with high affinity to a specific receptor (1). The murine erythroid-specific receptor (Epo-R) cDNA has been cloned recently (2). It encodes a 507-amino acid polypeptide chain with a single membrane-spanning domain and shares significant structural homologies with several hematopoietin receptors, defining a new receptor gene superfamily (3). The Epo-R messenger RNA has only been found in cells of the erythroid lineage (2), and the promoter sequence of the murine Epo-R gene has seemed to be active only in erythroid cells (4).

The Friend spleen focus-forming virus (SFFV) is a replication-defective murine type C leukemia virus that causes rapid splenomegaly and erythroleukemia in susceptible adult mice. Erythroid cells infected with the polycythemia-inducing variant of SFFV (SFFVp) can proliferate in the absence of added Epo (for review see Ref. 5). The SFFVp env gene-related glycoprotein (gp55) is responsible for the induction of erythroid proliferation and Epo independence (6), and it has been suggested that the intracellular form of gp55 directly interacts with the Epo-R, thereby bypassing the usual Epo requirement for signal transduction and promoting prolonged proliferation of these cells (7, 8).

In this report, we have analyzed the Epo-R expression and the Epo-R gene structure of a murine erythroleukemia cell line, T3Cl-2. This Friend cell line originated from a polycythemic Friend virus-induced leukemia in a DDD mouse (9) and exhibited a single class of Epo-R receptors with a Kd of 620–660 pm (10, 11). We found an abnormal Epo-R mRNA transcription initiation site in this cell line and studied the DNA rearrangement that accounted for this abnormal initiation. We showed that a SFFV LTR was inserted in the first exon of the Epo-R gene.

EXPERIMENTAL PROCEDURES

Cell Cultures and Tissues—Different cell lines were used in this study: T3Cl-2 (a generous gift from E. Goldwasser, University of Chicago) and MEL cells are Epo-unresponsive Friend cells, IW 32 is a helper F-MuLV-induced erythroleukemia cell line (12), and Red is a Rauscher erythroleukemia cell line which differentiates under Epo action. These cell lines were grown in suspension in (5% CO2, 95% air) at 37°C. Spleens from adult ICFW mice injected with either the anemic (FVA) or the polycythemic (FVP) strain of Friend virus were removed 4 weeks after inoculation. Spleens from adult ICFW mice rendered anemic by bleeding and mouse fetal liver (day 15 of gestation) were also used. These organs were immediately frozen, then crushed to a powder and homogenized in 4 M guanidine thiocyanate.

Oligonucleotides and Primers—Oligonucleotides were synthesized on a Milligen Biosearch 8700 DNA synthesizer and purified on a 20% acrylamide-urea gel. INTS-310 (AATCTGCTGTCTGCCCAC) is a primer upstream Epo-R exon 1. Epo-R exon 1 primers were TR8 (CAGGCTGCACCCAGACTAGGA) and INTS-190 (CCAGGCTCCCCAGCAAGTA). The LTR primers selected because of their conservation in SFFV and F-MuLV (13) were: INTS-

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Isolation of Epo-R cDNA and Genomic Clones—Total cellular RNA from fetal mouse liver was extracted according to Chirgwin et al. (14). Ten µg of fetal mouse liver RNA were used to prepare cDNA (40 units of RNasin, 100 mM Tris, pH 8.3, 140 mM KCl, 10 mM MgCl₂, 28 mM β-mercaptoethanol, 1 mM dNTPs, and 10 units of avian DNA polymerase I'CH supplied by Perkin-Elmer Cetus) and 1 mM each of the oligonucleotides relative to the A of the translational initiation codon. Primers used for PCR amplification of the Epo-R cDNA with one pair of oligonucleotide primers, INTS-110 (TTCCTGA-CTTTAA) deduced from the previously published sequence (2), and INTS-370 (ACAACCCCTCACTCGGCGCG) for the other region.

Among three positive clones, the clone λEpo was purified and digested by NcoI. The 3.4-kb restriction fragment containing the 5' region of the Epo-R gene (exon 1 and promoter) was subcloned in pUC18 and sequenced.

Primer Extension Analysis—Primer extension analysis was performed using oligonucleotide TR8 derived from exon 1, extension products of 220 bases were detected on a few bases, our sequence agreed perfectly with the previously described one (4).

Transcription of the Mouse Epo-R Gene—Primer extension analysis was used to determine the transcriptional start sites in normal erythroid tissues, erythroleukemia cell lines, and Friend-induced spleen tumors (Fig. 1A). Using the oligonucleotide TR8 derived from exon 1, extension products of 220 and 218 bases defined two cap sites at -149 and -147 nucleotides relative to the A of the translational initiation codon. In contrast, the major T3CL-2 cap site was located 37 bases upstream from the distal cap site. The minor 215-nucleotide extension product might correspond to an elongation stop due to possible secondary structures. Transcription from the normal cap sites was much lower than from the former one and was detected only after longer exposure. In addition, the amount of Epo-R mRNA was enhanced in T3CL-2 cells. This was confirmed by Northern blot analysis, since a stronger signal was detected in T3CL-2 with an Epo-R cDNA probe, when compared with the other cell lines tested, provided the same amount of total RNA was loaded (Fig. 1B). The Epo-R transcript appeared of normal size in T3CL-2 cells, since the 37-base difference between the normal and altered mRNA could not be resolved by this technique.

Epo-R Gene Rearrangement in T3CL-2 Line—In order to investigate whether abnormal transcription of the Epo-R in

RESULTS

Isolation and Characterization of the Epo-R Promoter—Among the positive clones isolated from the genomic mouse liver, one was extensively studied. The insert was 11 kb long, encompassing 9 kb of the Epo-R promoter region and exons 1–4 of the Epo-R gene. The nucleotide sequence of part of exon 1 and of the promoter region (932 bp 5' from the initiation site) was determined (data not shown). Except for a few bases, our sequence agreed perfectly with the previously described one (4).

The expression of Epo-R mRNA was determined by Northern blot analysis using full-length Epo-R cDNA probe and with actin probe. Lane 1, T3CL-2 cells; lane 2, MEL cells; lane 3, 1W 32 cells; and lane 4, Red cells.
T3CI-2 was associated with genomic alterations, Southern blot analysis was performed with genomic DNA isolated either from T3CI-2 or from normal mouse DDD liver. The probe used for characterization of the Epo-R gene was a 1.3-kb genomic HindIII-EcoRI fragment located at the 5' terminus of the gene (Fig. 2B). A rearrangement could be seen in T3CI-2 when the genomic DNA was cleaved by the restriction enzymes BamHI, EcoRI, and HindIII (Fig. 2A). In addition to germ line fragments (BamHI, 3.5 kb; EcoRI, 2.2 kb; and HindIII, 4.2 kb), new fragments of higher mobility and of equal intensity (BamHI, 4.0 kb; EcoRI, 2.7 kb; and HindIII, 4.7 kb) were observed in T3CI-2 cells, suggesting that these cells had one rearranged Epo-R allele (Fig. 2B). The size of the rearranged fragment, which was 0.5 kb longer than the germ line allele in all digests, was consistent with the hypothesis of an isolated viral LTR insertion. This was strengthened by the presence of a new KpnI restriction site in the rearranged allele (data not shown). To test this hypothesis, we amplified the 5' end of the T3CI-2 Epo-R gene using the polymerase chain reaction.

Cloning and Sequencing of the Rearranged T3CI-2 Allele—The abnormal initiation sites and the high level of mRNA expression observed by primer extension analysis of T3CI-2 mRNA strongly suggested the use of an LTR initiation site for transcription of the rearranged T3CI-2 allele. Therefore, we performed PCR on T3CI-2 cDNA with two oligonucleotides, INTS-370 and INTS-371, located 5' and 3' from the LTR transcriptional initiation site, respectively, and coupled to the INTS-190 primer. We obtained a PCR product (see Fig. 3) with the INTS-371 primer but not with the INTS-370 primer. Furthermore, the direct sequencing of the primer extension product and of the PCR amplification product (obtained with primers INTS-190 and -371) indicated that the 5' region of the Epo-R mRNA found in T3CI-2 was in fact derived from an LTR sequence and that the abnormal T3CI-2 initiation site was located precisely at the beginning of the R region of a viral LTR (Fig. 3A). To investigate this LTR insertion at the genomic level, we amplified T3CI-2 genomic DNA and normal mouse DDD DNA by PCR, using the INTS-371, -370, -381, and -382 LTR oligonucleotides combined with either the INTS-190 or INTS-310 Epo-R oligonucleotides (Fig. 3B). We reproducibly obtained amplified products of the expected size, leading to the conclusion that a single isolated LTR was inserted in the first exon of one Epo-R allele of T3CI-2. None of these combinations gave an amplification product with the mouse DDD DNA used as control. Finally, using a combination of two Epo-R-specific oligonucleotide primers (INTS-310 and INTS-190), we obtained one amplified fragment on DDD DNA (354 bp) and two fragments on T3CI-2 DNA, one corresponding to the normal allele (354 bp) and one 515 bp larger (=869 bp) corresponding to the rearranged allele. These different fragments were recovered from the gel and ligated into appropriate vectors for cloning and sequencing. Nucleotide sequence analysis revealed that an LTR in the same transcriptional orientation as the Epo-R gene was inserted 45 bp upstream of the translational initiation ATG. The LTR insertion was flanked by a duplication of 5 bp of the Epo-R gene (CAGGG) at positions 143-147 (Fig. 4).

**Fig. 3.** Schematic representation of the LTR insertion in the T3CI-2 cell line. A, study of the transcription start site on T3CI-2 cDNA by PCR amplification. B, localization of SFFV-LTR insertion in T3CI-2 DNA by PCR amplification. On these diagrams, the different oligonucleotides (single head arrows) used for PCR amplifications and the PCR amplification products (thick double head arrows) are positioned. The CAGGG sequence indicated on the T3CI-2 Epo-R normal allele corresponds to the LTR insertion site. Multiple head arrows, retroviral transcription initiation site.

**DISCUSSION**

In the present study we report on the characterization for the first time of a rearrangement of the murine Epo-R gene in a Friend virus-induced erythroleukemia cell line. Our results strongly suggest that the SFFV, already known to interact with the Epo-R by the gp55 env gene-related glycoprotein,
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It was found that the Epo-R, which is probably the same as the LTR insertion did not interrupt the reading frame, the T3C1-2 Epo-R protein was expected to be normal. The number of binding sites expressed on the four cell lines was determined by equilibrium binding experiments as described (21) and found to be 972 (T3C1-2 cells), 500 (MEL cells), 380 (IW32 cells), and 870 (Red cells) per cell. For the IW32 cell line, the exact determination of the receptor number was hampered by Epo secretion (22). Thus, there is no correlation between the overexpression of mRNA and the number of Epo binding sites displayed at the T3C1-2 cell surface. Since transcription from the normal T3C1-2 allele is very weak, we assume that most of the Epo binding sites originate from the rearranged allele, indicating that the receptor protein does bind Epo and is functionally normal. It is possible, as recently suggested by Yoshimura et al. (8), that the Epo-R, which is probably multimeric, requires the presence of a second receptor subunit for cell membrane expression. If such a subunit is expressed at a low level in the T3C1-2 cell line, then, only a few Epo binding sites would be present at the cell surface. The T3C1-2 cell line also expresses SFFV gp55, and it is known that SFFV-infected Epo-independent cells had fewer receptors than their factor-dependent counterpart (23). In addition, gp55 is retained in the rough endoplasmic reticulum, and a minor percentage (5%) is transported via the Golgi apparatus to the cell surface (for review, see Ref. 24). Since gp55 interacts with Epo-R in the rough endoplasmic reticulum, resulting in intracellular retention and decrease of cell surface expression of the Epo-R (8, 23), this may also account for the reduced number of Epo binding sites on the T3C1-2 cell surface. We are currently producing anti-Epo-R antibodies to address this issue.

The LTR sequence inserted in T3C1-2 was aligned with other LTRs. As described for SFFV LTR, there was a deletion of the second direct repeat in the U3 region of the T3C1-2 inserted LTR, strongly suggesting that this LTR derived from the SFFV component of the Friend virus complex rather than from a replication competent helper virus (25). Comparison with other SFFV LTRs revealed a few mismatches in the U3 region, making it difficult to assess the precise origin of this SFFV LTR.

Many cases of retroviral insertional mutagenesis have been reported as a mechanism to increase expression of oncoproteins in several murine models, including Friend virus-induced erythroleukemia (15), myeloid leukemia (26-28), and T-cell lymphoma (29-31). The inserted virus can be either a complete provirus or solely a LTR. Similarly, hematopoietic cell lines have been reported to become growth factor-independent due to the integration of either a provirus genome or intracisternal A particle LTR in the vicinity of a growth factor gene leading to constitutive gene activation (32-34). More recently, constitutive IL2-R gene expression due to the insertion of an intracisternal A particle-derived LTR sequence has been described in the murine lymphoma line El-4 (35). Replacement of the murine IL6-R intracytoplasmic domain with a part of LTR of an intracisternal A particle gene has also been re-
ported in a plasmacytoma cell line (36). In these cases, the viral insertion element up-regulated the receptor expression through promoter or enhancer activity. It has been shown recently that the SFFV gp55 glycoprotein associates with the Epo-R and promotes growth activation in the absence of Epo (8). In this report, we provided evidence that SFFV LTR could also interfere with the Epo-R gene expression by promoter-type insertion activation. This insertion resulted in an overexpression of the Epo-R mRNA in the T3C1-2 cell line. However, the number of binding sites on this cell line was similar to that of the other erythroblastoid cell lines tested. According to Yoshimura et al. (8), Epo-R and SFFV gp55 interact intracellularly. Since T3C1-2 cells have been shown to express large amounts of gp55 (37), we suggest that this type of intracellular interaction occurred in T3C1-2 cells and provided a positive pressure in the development of transplantable and tumorigenic erythroblastoid.

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