論文の内容の要旨

論文題目　フレンドウイルスLTR(long terminal repeat)挿入によりエリスロポエチン受容体の過剰発現が認められたマウス赤白血病細胞株の解析

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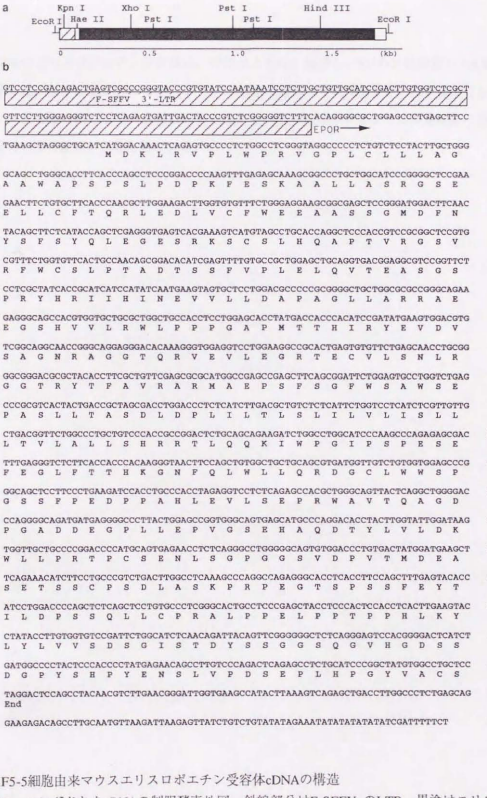
エリスロポエチンは、分子量34,000の糖蛋白で赤芽球前駆細胞に作用し、赤芽球への分化を促進する．その受容体はcDNAクローニングの結果、他のサイトカイン（インターロイキン2、3、4、5、6、7、G-CSF、GM-CSF、プロラクチン、成長ホルモン）受容体と構造上相同性を持ち、これらと共に、サイトカイン受容体スーパーファミリーを形成している．従来、エリスロポエチン受容体については、赤芽球前駆細胞、フレンドウイルス感染細胞、赤白血病細胞株などが用いられ、解析されてきたが、それぞれ細胞表面に発現している受容体は、1,000個以下であった．著者は、マウス赤白血病細胞株F5-5において、細胞表面に約10,000個のエリスロポエチン受容体が発現していることを見いだし、解析を行った．リコンビナントエリスロポエチンをクロラミンT法を用いて、ヨード標識し、F5-5細胞における結合能を調べ、Scatchard解析を行ったところ、解離定数は5.1×10^{-10}Mと従来の報告と同様であったが、細胞あたりの受容体数は10,000と過剰発現していた．架橋剤を用いた架橋実験では、従来の報告同様、SDS-PAGEで分子量150,000、130,000、100,000の位置にリガンド－受容体複合物のバンドがみとめられた．F5-5細胞及びフェニルヒドラジン

投与DDDマウス脾臓よりRNAを抽出し、エリスロポエチン受容体cDNAをプローブとし、Northern解析を行ったところ、F5-5細胞では、エリスロポエチン受容体mRNAの過剰発現が認められた．さらに、DDDマウス腎臓及びF5-5細胞より、DNAを抽出し、Southern解析を行ったところ、F5-5細胞では、BamHI、PstI、HindIII制限酵素処理すべてにおいて、一方のalleleの再構成が認められた．この再構成は、エリスロポエチン受容体cDNAの5'側をプローブとして解析した場合に認められることから、エリスロポエチン受容体遺伝子の上流になんらかの遺伝子の挿入が予想された。F5-5細胞cDNAライブラリーよりエリスロポエチン受容体をクローニングを行ったところ、F5-5細胞に発現しているエリスロポエチン受容体には、開始点の46塩基上流にフレンド脾限局巣誘発ウイルス多血症株(F-SFFV$_p$)のLTRの挿入が認められた．RNaseプロテクション解析から、これらの細胞株で発現しているエリスロポエチン受容体は、主にLTRの挿入により再構成された遺伝子より発現していた．以上より、F5-5細胞におけるエリスロポエチン受容体の過剰発現は、挿入されたウイルスLTRがプロモーターとして作用しているためと考えられた．F5-5細胞の親株であるTSFAT-3細胞及び兄弟株であるT3Cl2O細胞、T3K-1細胞においても、エリスロポエチン受容体の過剰発現及び遺伝子再構成が認められたため、LTR部分とエリスロポエチン受容体のＥｘｏｎ1部分でプライマーを作成し、各々の細胞から抽出したＤＮＡを用いてPCR(polymerase chain reaction)を行ったところ、これら全ての細胞株で、LTRとエリスロポエチン受容体の融合遺伝子が認められた．以上より、F-SFFV$_p$のLTRの挿入は、本細胞株樹立時に起こっている可能性がある。最近、エリスロポエチン受容体がgp55と結合し、活性化され、エリスロポエチン非依存性の増殖をおこすとの報告がある。これらの報告を考慮すると、本細胞株においては、エリスロポエチンの過剰発現が、腫瘍化に有利に働いた可能性が考えられる。

F5-5細胞の分化にともなうエリスロポエチン受容体の変化を調べるため、ヘモグロビン産生細胞に分化誘導する生理活性物質EDF(erythroid differentiation factor) / activin Aを用いて、F5-5細胞を分化誘導し、Northern解析した。SCF(stem cell factor)の受容体であるc-kitは分化にともない発現が減少し、βグロビンは、発現が増加した。一方、エリスロポエチン受容体のmRNAの発現は、ほとんど変化はみられなく、ウイルスプロモーターにより構成的に発現されているため分化にともなう調節機構から逸脱しているものと考えらた。しかし、

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End
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図1 .F5-5細胞由来マウスエリスロポエチン受容体cDNAの構造

(a)クローニングされたcDNAの制限酵素地図、斜線部分はF-SFV$_P$のLTR、黒塗はエリスロポエチン受容体遺伝子のORF(open reading frame)を示す。

(b)エリスロポエチン受容体cDNAの塩基配列及びアミノ酸配列

細胞表面のエリスロポエチン受容体は、分化にともない減少し、5日めには細胞当り4,000に減少した。このRNAレベルと蛋白レベルの解離の理由は不明であるが、EDFはエリスロポエチン受容体の転写以後の過程に影響を与えている可能性や分布または代謝経路に影響を与えている可能性があると思われる。

Unregulated Expression of Erythropoietin Receptor Gene in a Murine Erythroleukemia Cell Line Caused by Insertion of Spleen Focus-Forming Virus Long Terminal Repeat

SUMMAY

A murine erythroleukemia (MEL) cell line F5-5 expressed 10,000 binding sites for erythropoietin (EPO) per cell, ten-fold as many as other MEL cell lines and normal erythroid progenitors. Northern and Southern blot analysis revealed overexpression of mRNA for the EPO receptor (EPOR) and rearrangement of one of the EPOR gene alleles in F5-5 cells, respectively. Molecular cloning of F5-5-derived cDNA encoding EPOR revealed that 5' noncoding region of the EPOR cDNA corresponds to the long terminal repeat (LTR) sequence of the polycythemic strain of Friend spleen focus-forming virus. Induction of erythroid differentiation of F5-5 cells resulted in a time-dependent decrease in the number of cell surface EPOR, while expression of EPOR mRNA remained nearly constant. These results suggest that the promoter insertion accounts for the unregulated expression of EPOR mRNA in F5-5 cells, although the cell surface EPOR expression seems to be down-regulated during maturation by unknown post-transcriptional mechanisms.

INTRODUCTION

All blood cells are derived from a few common pluripotent stem cells, probably requiring mechanism for achieving controlled commitment of progenitor cells into myeloid (erythroid, granulocytic, megakaryocytic) and lymphoid lineages. The regulation of hematopoiesis is achieved by two control systems. Stromal cells in bone marrow control some of the cellular events in hematopoiesis by cell contact processes or by the production of short-range local regulatory factors. A second control system involves the coordinated interaction of hematopoietic growth factors that stimulate the proliferation and differentiation of progenitor cells.

In erythropoiesis, two separate classes of erythroid committed progenitor cells were recognized using colony assay. The early erythroid progenitor cell was termed a burst forming unit-erythroid (BFU-E), which responds to erythropoietin (EPO) and burst-promoting activities (interleukin-3, granulocyte-macrophage colony-stimulating factor, and interleukin-4). The more differentiated progenitor cell was termed colony forming unit-erythroid (CFU-E), which respond to EPO and

differentiate into proerythroblast. EPO is a 34,000-dalton glycoprotein produced in the kidney which stimulates both proliferation and differentiation of erythroid progenitor cells. The effects of EPO, like other peptide hormones, are initiated through the interaction of the ligand with its specific cell surface receptor. The EPO receptor (EPOR) has been identified and characterized on erythroid precursor cells, including spleen cells from mice treated with phenylhydrazine (1,2) or infected with Friend virus (3-5), hematopoietic cell lines (6-11), and fetal liver cells (12,13). EPOR was also reported to be aberrantly expressed on a myeloma cell line (14), and was detected on endothelial cells (15); the significance of these findings is unknown.

The molecular mechanism by which EPO and the EPOR complex induces erythropoiesis is unknown. However, cDNAs for the murine (16) and human (17,18) EPOR have been recently cloned, which will facilitate the analysis of the early events in erythroid differentiation. The EPOR is a member of the cytokine receptor superfamily recently defined, including the interleukin-2 receptor β subunit (19), gp130 (20), the receptors for interleukin-3 (21), interleukin-4 (22,23), interleukin-5 (24), interleukin-6 (25), interleukin-7 (26), granulocyte-macrophage colony-stimulating factor (27), granulocyte colony-stimulating factor (28), leukemia inhibitory factor (29), growth hormone (30), prolactin (31), and a common β subunit of granulocyte colony stimulating factor receptor (32), interleukin-3 receptor (33), and interleukin-5 receptor (34).

The number of cytokine receptors is quite low, 100-fold less than that for other families of growth factor receptors. The same applies for the EPOR. In the previous reports (6,9-11), almost all murine erythroleukemia (MEL) cells had limited numbers of the EPOR (several hundred sites/cell). However, author found a MEL cell line F5-5 which had high level of cell surface EPOR (10,000 binding sites/cell). In order to clarify the mechanism of overexpression of EPOR in F5-5 cells, the following experiments were performed. Moreover, changes of EPOR expression in F5-5 cells during erythroid differentiation were examined. .

MATERIALS and METHODS

Growth Factors and Probes

Recombinant human EPO (37) was provided by Kirin-Amgen Inc. Recombinant erythroid differentiation factor (EDF; also named activin A) (38) was provided by Ajinomoto Co. Inc. EPO was iodinated by the chloramine-T method with a minor modification as described elsewhere (12). Briefly, 0.15μg of chloramine-T (Sigma Chemical Co., St. Louis, MD) and 0.5mCi of $Na^{125}I$ (ICN Biochemicals Inc., Irvine, CA) were added to EPO (3μg) in 40μl of sodium phosphate buffer (0.3M, pH 7.4). After incubation for 10min at room temperature, the reaction was quenched by the addition of sodium metabisulfite (Sigma Chemical Co., St. Louis, MD). Labeled EPO was separated from reactants by passing the mixture through a Sephadex G-25 column equilibrated with 0.03M phosphate buffer, pH 7.4 containing 0.1% bovine serum albumin. Specific activities of iodinated EPO (^{125}I-EPO) were 38 to 55μCi/μg without the loss of biological activity as described previously (7). Murine EPOR (provided by Dr. A. D. D'Andrea), c-myc (39), β-globin (40), β-actin (41), uroporphyrinogen decarboxylase (UPD) (42), and c-kit (43; provided by Dr. A. Tojo) cDNA fragments were electrophoretically purified from agarose gels and labeled by using hexadeoxynucleotide random primers with [α-^{32}P]dCTP (ICN Biochemicals Inc.).

Culture of MEL cells

A MEL cell line TSFAT-3 cell, and the subclones F5-5, T3Cl2O, and T3K-1 cells (44) were maintained in Ham's F-12 medium containing 10% fetal calf serum, penicillin (100units/ml), and streptomycin (100μg/ml) at 37°C in a humidified atmosphere containing 95% air, 5% CO_2. The biological characters of these cell lines were described in reference 44. TSFAT-3, F5-5, and T3Cl2O cell lines were differentiation inducible, whereas T3K-1 cell line was differentiation resistant.

Assay for Binding of ^{125}I-EDF to F5-5 Cells

F5-5 cells were washed twice and resuspended in α-medium containing 0.1%

bovine serum albumin, 20mM 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid (HEPES), and 0.01% bacitracin with 0.02% sodium azide, pH7.4 (binding buffer). Cells ($1x10^6$) were incubated with various concentrations of ^{125}I-EPO in 200µl of binding buffer at 15°C for 180 min. At the end of incubation, duplicate 80µl alliquots of the incubation mixture were transferred onto a cushion of di-n-butyl phthalate in 400 µl polyethylene centrifuge tubes. The cells were centrifuged at 10,000xg for 2 min. The supernatant was aspirated, and the tubes were cut off just above the cell pellet and assayed for cell-associated radioactivity. Nonspecific binding measured in the presence of a 100-fold excess of unlabeled EPO was subtracted from total binding.

Affinity Labeling Protocol

F5-5 cells ($5x10^6$) were incubated with 10nM ^{125}I-EPO in 500µl of binding buffer, in the presence or absence of unlabeled EPO at 15°C for 180 min. After washing twice with ice-cold phosphate-buffered saline (PBS), the cells were resuspended in 500µl of PBS. To cross-link the bound ^{125}I-EPO, disuccinimidyl suberate (DSS) freshly prepared in acetonitrile was added to final concentration of 0.2mM and incubated at 4°C for 15 min with frequent agitation. The reaction was quenched with 3 volumes of 10mM Tris buffer containing 1mM EDTA and 150mM NaCl (pH7.4). After 5 min, the cell were pelleted and solubilized in 50mM HEPES buffer (pH7.4) containing 1%(v/v) Triton X-100, 1mM phenylmethylsulfonyl fluoride, and 1,000 trypsin inhibitor units of aprotinin per ml. The cell suspensions were gently stirred at 4°C for 15 min and centrifuged at 15,000xg at 4°C for 10 min to remove nuclei and cellular debris. The supernatants were mixed with 1/3 volume of a 3-fold concentrated electrophoresis sample buffer of Laemmli (45) in the presence of 50mM dithiothreitol and boiled for 3 min Electrophoresis of the affinity labeled samples were performed on sodium dodecyl sulfate(SDS)-polyacrylamide gel, using 8% polyacrylamide gels in a discontinuous buffer system as described previously (45). After electrophoresis, the gels were fixed, stained with 0.25% Coomassie blue, destained, dried, and subjected to autoradiography using Fuji RX

X-ray film at -70°C with intensifying screen. Molecular weight markers used were ovalbumin (45kDa), bovine serum albumin (66kDa), phosphorylase b (93kDa), α-galactosidase (116kDa), and myosin (200kDa) (Bio Rad Laboratories, Richmond, CA).

Northern Blot Analysis

Total cellular RNA was prepared from MEL cells and phenylhydrazine treated DDD mice spleens by a guanidine/cesium chloride centrifugation method (46). Poly(A)$^+$ RNA was obtained using oligo(dT)-latex (Takara Shuzo Co. Ltd.) and electrophoresed in a 1% agarose gel containing formaldehyde. RNA was transferred to a nylon filter (Schleicher & Schuell Inc.) and hybridized with ^{32}P-labeled cDNA probes in 50% formamide, 3xSSC (1xSSC is 0.15M NaCl and 0.015M sodium citrate, pH7.4), 50mM Tris HCl (pH7.5), 0.1% SDS, 20μg of tRNA per ml, 20μg of boiled salmon sperm DNA per ml, 1mM EDTA, 0.02% bovine serum albumin, 0.02% polyvinylpyrolidone, 0.02% Ficoll for 40hr at 37°C.

Southern Blot Analysis

High molecular-weight DNAs from cells and tissues were prepared by standard methods (46) and digested to completion with BamHI, EcoRI, or HindIII. After separation of the DNA by electrophoresis on 0.8% agarose gels, the DNA was transferred to nylon filter and hybridized with ^{32}P-labeled full length murine EPOR cDNA and 5' KpnI-XhoI EPOR fragment as the probes.

Screening of the cDNA Library and DNA sequencing

cDNA library constructed by using λ phage gt10 from poly(A)$^+$ RNA isolated from F5-5 cells was provided by Dr. Y. Misawa (42). Plaques were blotted on a nylon filter and hybridized with ^{32}P-labeled murine EPOR probe. The phage-inserted cDNAs from positive plaques were cloned into pUC119 plasmid vector and sequenced by using Sequenase (United States Biochemical Co.).

RNase Protection Assay

RNase protection assay was performed by the previously described method (46). Briefly, a ^{32}P-labeled RNA probe was prepared by in vitro transcription of an EcoRI-XhoI fragment (431bp) containing 143bp of the 3' LTR sequence and 288bp of 5' EPOR sequence (EcoRI-XhoI fragment) derived from F5-5 cells, cloned into pBluescript SK(+) plasmid vector. Poly(A)$^+$ RNA (10μg) of MEL cell lines and phenylhydrazine treated DDD mice spleens were hybridized with the RNA probe at 45°C for 12h and digested with RNase T1 and RNase A at 30°C for 60 min. The samples were then analyzed by electrophoresis on a 6% polyacrylamide gel containing 42% urea.

PCR (polymerase chain reaction) Analysis

PCR amplification was performed by a previously described method (47). Briefly, sample DNA was amplified in 1xPCR buffer (50mM KCl, 10mM Tris-HCl [pH 8.4], 1.5mM $MgCl_2$, 0.1mg/ml gelatin), 0.2mM each deoxynucleoside triphosphate, 0.5μM of each primer, and 25U of Taq polymerase (Takara Shuzo CO., LTD.) per ml. Amplification was carried out for 30 cycles of 60 sec at 90°C, 60 sec at 55°C and 90 sec at 72°C. Primers for the reaction were derived from the 3'-LTR sequence of F-SFFV$_P$ (5'-CGGGTACCCGTGTATCCAA-3') and from the EPOR sequence in exon I (5'-CTTTGCTCTCAAACTTGGGGT-3'). The PCR product was electrophoresed in a 3% NuSieve-1% agarose gel.

RESULTS

Characterization of the EPOR

(1) Affinity and Number of EPOR : Binding of ^{125}I-EPO to F5-5 cells is time- and temperature-dependent (Fig. 1). Fig. 2 shows the typical saturation-binding data for EPO at 15°C. Scatchard analysis (48) of ^{125}I-EPO binding to F5-5 cells results in a rectilinear plot, suggesting a single class of binding sites. The Kd value deduced from the slopes of Scatchard plots are 5.1×10^{-10}M for F5-5 cells. The abscissa intercept in the Scatchard plots indicate the presence of about 10,000 receptor sites per cell in F5-5 cells (repeated experiments with different batches of

^{125}I-EPO gave results similar to those described above).

(2) Cross-Linking Studies of ^{125}I-EPO to F5-5 cells : ^{125}I-EPO was cross-linked to F5-5 cells by using disuccinimidyl suberate. Fig. 3 shows an autoradiogram from SDS-polyacrylamide gel electrophoresis of F5-5 cell lysates cross-linked with ^{125}I-EPO. There appear to be specifically labeled bands corresponding to molecular weights of approximately 150,000, 130,000, and 100,000. The lowest band would represent the partially degraded product of the ^{125}I-EPO-EPOR complex. These results are compatible with those obtained for other MEL cells (13).

Northern Blot Analysis

Poly(A)$^+$ and/or total RNA from various MEL cells and phenylhydrazine-treated DDD mice spleens were examined by Northern blot hybridization. As shown in Fig. 4A, much higher expression of EPOR mRNA was observed in F5-5 cells than in spleen cells of anemic mice. Overexpression of the EPOR mRNA was also detected in a parental TSFAT-3 cell line and in other subclones, such as T3Cl2O and T3K-1 cell lines, but the level was slightly lower than in F5-5 cell (Fig. 4B).

Rearrangement of the EPOR Gene in MEL Cell Lines

Southern blot analysis of the digests of genomic DNA from DDD mouse kidney cells or from F5-5, T3Cl2O, T3K-1, and TSFAT-3 cells were performed with the murine EPOR cDNA probe. The normal EPOR gene in a DDD mouse was detected as three BamHI fragments, three EcoRI fragments, and three HindIII fragments. In F5-5 cells, additional bands but no amplification were observed after BamHI, EcoRI and HindIII restriction analysis (Fig. 5A). These additional bands were detected only with the 5' KpnI-BamHI fragment of the EPOR cDNA as a probe, suggesting that a DNA rearrangement occurred in the 5'-region of the EPOR gene (Fig. 5B). The same rearrangement was observed in the parental cell line TSFAT-3 and other subclones (T3K-1 and T3Cl2O).

Cloning and Sequencing of EPOR cDNA from F5-5 Cells

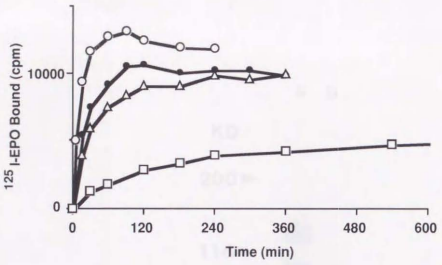


Fig. 1. **Time course of ^{125}I-EPO binding to F5-5 cells.** The cells ($1x10^7$) were incubated with 1.2nM ^{125}I-EPO in the presence or absence of excess unlabeled EPO at 37°C (○), 22°C (●),15°C (△), and 4°C (□). Specific binding per $5x10^5$ cells was determined as described under " Materials and Methods". Each data point is the mean of duplicate determinations.

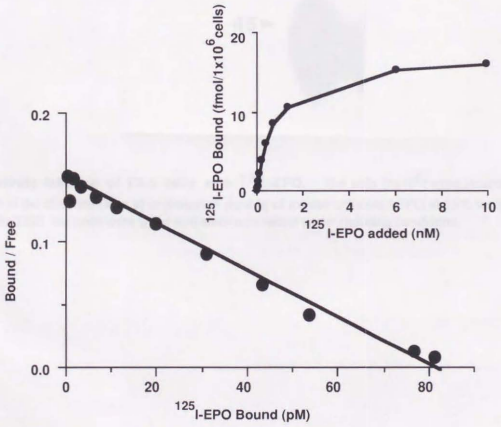


Fig. 2. **Saturation analysis of ^{125}I-EPO binding to F5-5 cells (A) and Scatchard analysis of the binding data (B).** The cells ($1x10^6$) were incubated with increased concentrations of ^{125}I-EPO in the presence or absence of excess unlabeled EPO at 15°C for 3h. Specific binding was determined as described under "Materials and Methods". Each data point is the mean of duplicate determinations.

Fig. 3. **Affinity labeling of F5-5 cells with ^{125}I-EPO.** The cells ($5x10^6$) were incubated with 10nM ^{125}I-EPO in the absence (lane a) or presence (lane b) of excess unlabeled EPO at 15°C for 3h. After cross-linking with DSS, the cells were lysed and electrophoresed under reducing conditions.

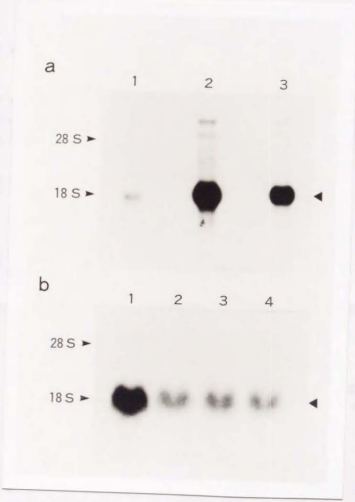


Fig. 4. **Northern blot analysis of the murine EPOR gene.** (a) Poly(A)$^+$ RNAs (2μg) prepared from phenylhydrazine-treated DDD mouse spleens (lane 1), MEL cell lines F5-5 (lane 2), and T3Cl2O (lane 3) were electrophoresed, transferred, and hybridized with murine EPOR cDNA as the probe. (b) Total RNAs (20μg) prepared from MEL cell lines F5-5 (lane 1), T3Cl2O (lane 2), T3K-1 (lane 3), and TSFAT-3 (lane 4) were electrophoresed, transferred, and hybridized with murine EPOR cDNA as the probe.

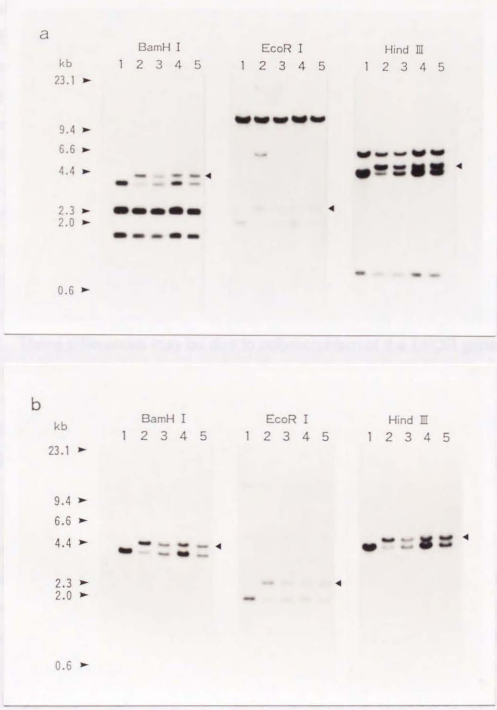


Fig. 5. **Southern blot analysis of the murine EPOR gene.** Genomic DNAs (10μg) prepared from normal DDD mouse kidney (lane 1), MEL cell lines F5-5 (lane 2), and T3CI2O (lane 3), T3K-1 (lane 4), and TSFAT-3 (lane 5) were digested with the indicated restriction enzymes, electrophoresed, transferred, and hybridized with full-length murine EPOR cDNA (a) or 5' fragment (b) as the probe.

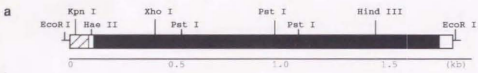
To facilitate a detailed analysis of the sequence content of EPOR mRNA from F5-5 cells, a cDNA library was screened for recombinant phages hybridizing to full-length EPOR cDNA as the probe. Several overlapping clones were obtained, and a single clone containing the 1.8 kb insert was further analyzed. As shown in Fig. 6A, the transcript defined by this clone contains a single open reading frame corresponding to that of the published sequence (16). The nucleotide sequence of this clone, however, extends to another 154 bp to the 5' end of the reported cDNA (16,49). Inspection of this additional sequence indicated that the 5' sequence from the F5-5 EPOR cDNA is identical to that of the 3' LTR of F-SFFV$_P$ (50). The nucleotide sequence of the EPOR coding region was identical to that of the cDNA reported by D'Andrea et al. (16), except for the conservative amino acid changes at positions 195 (G→A), 968 (C→T), 1152 (G→A), and 1200 (G→T) of the cDNA (Fig.6B). These differences may be due to polymorphism of the EPOR gene among different strains of mouse (Fig.7). Furthermore, an RNase protection assay of poly(A)$^+$ RNA from the MEL cells revealed that the aberrant EPOR transcripts containing the 3' LTR sequence were strongly expressed in F5-5, T3Cl2O, T3K-1, and TSFAT-3 cells, whereas the normal EPOR alleles were weakly transcribed (Fig.8).

PCR analysis

Genomic DNAs isolated from F5-5, T3Cl2O, T3K-1 and TSFAT-3 cells were analyzed by the PCR method. As shown in Fig.9, LTR-EPOR fragments (272bp) from the 3'-LTR sequence of F-SFFV$_P$ and from the EPOR gene. Amplified 272bp PCR products were confirmed by sequencing. These results indicated the insertion of F-SFFV$_P$ LTR just upstream of the EPOR gene in F5-5, T3Cl2O, T3K-1, and TSFAT-3 cells.

Differentiation-related change of EPOR expression in F5-5 cells

Fig. 10 shows the change in the binding of ^{125}EPO to F5-5 cells and benzidine positive cells during the erythroid differentiation induced by EDF.



b

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GTCCTCCGACAGACTGAGTCGCCCGGGTACCCGTGTATCCAATAAATCCTCTTGCTGTTGCATCCGACTTGTGGTCTCGCT      81
                F-SFFV  3'-LTR
GTTCCTTGGGAGGGTCTCCTCAGAGTGATTGACTACCCGTCTCGGGGGTCTTTCACAGGGGCGCTGGAGCCCTGAGCTTCC     162
                                                                    EPOR
TGAAGCTAGGGCTGCATCATGGACAAACTCAGAGTGCCCCTCTGGCCTCGGGTAGGCCCCCTCTGTCTCCTACTTGCTGGG     243
                  M  D  K  L  R  V  P  L  W  P  R  V  G  P  L  C  L  L  A  G

GCAGCCTGGGCACCTTCACCCAGCCTCCCGGACCCCAAGTTTGAGAGCAAAGCGGCCCTGCTGGCATCCCGGGGCTCCGAA     324
A  A  W  A  P  S  P  S  L  P  D  P  K  F  E  S  K  A  A  L  L  A  S  R  G  S  E

GAACTTCTGTGCTTCACCCAACGCTTGGAAGACTTGGTGTGTTTCTGGGAGGCGAGCCGGCGAGCTCCGGGATGGACTTCAAC     405
E  L  L  C  F  T  Q  R  L  E  D  L  V  C  F  W  E  E  A  A  S  S  G  M  D  F  N

TACAGCTTCTCATACCAGCTCGAGGGTGAGTCACGAAAGTCATGTAGCCTGCACCAGGCTCCCACCGTCCGCGGCTCCGTG     486
Y  S  F  S  Y  Q  L  E  G  E  S  R  K  S  C  S  L  H  Q  A  P  T  V  R  G  S  V

CGTTTCTGGTGTTCACTGCCAACAGCGGACACATCGAGTTTTGTGCCGCTGGAGCTGCAGGTGACGGAGGCGTCCGGTTCT     567
R  F  W  C  S  L  P  T  A  D  T  S  S  F  V  P  L  E  L  Q  V  T  E  A  S  G  S

CCTCGCTATCACCGCATCATCCATATCAATGAAGTAGTGCTCCTGGACGCCCCCGCGGGGCTGCTGGCGCGCCGGGCAGAA     648
P  R  Y  H  R  I  I  H  I  N  E  V  V  L  L  D  A  P  A  G  L  L  A  R  R  A  E

GAGGGCAGCCACGTGGTGCTGCGCTGGCTGCCACCTCCTGGAGCACCTATGACCACCCACATCCGATATGAAGTGGACGTG     729
E  G  S  H  V  V  L  R  W  L  P  P  P  G  A  P  M  T  T  H  I  R  Y  E  V  D  V

TCGGCAGGCAACCGGCAGGAGGGACACAAAGGGTGGAGGTCCTGGAAGGCCGCACTGAGTGTGTTCTGAGCAACCTGCGG     810
S  A  G  N  R  A  G  G  T  Q  R  V  E  V  L  E  G  R  T  E  C  V  L  S  N  L  R

GGCGGGACGCGCTACACCTTCGCTGTTCGAGCGCGCATGGCCGAGCCTGAGCTTCAGCGGATTCTGGAGTGCCTGGTCTGAG     891
G  G  T  R  Y  T  F  A  V  R  A  R  M  A  E  P  S  F  S  G  F  W  S  A  W  S  E

CCCGCGTCACTACTGACCGCTAGCGACCTGGACCCTCTCATCTTGACGCTGTCTCTCATTCTGGTCCTCATCTCGTTGTTG     972
P  A  S  L  L  T  A  S  D  L  D  P  L  I  L  T  L  S  L  I  L  V  L  I  S  L  L

CTGACGGTTCTGGCCCTGCTGTCCCACCGCCGGACTCTGCAGCAGAAGATCTGGCCTGGCATCCCAAGCCCAGAGAGCGAC    1053
L  T  V  L  A  L  L  S  H  R  R  T  L  Q  Q  K  I  W  P  G  I  P  S  P  E  S  E

TTTGAGGGTCTCTTCACCACCCACAAGGGTAACTTCCAGCTGTGGCTGCTGCAGCGTGATGGTTGTCTGTGGTGGAGCCCG    1134
F  E  G  L  F  T  T  H  K  G  N  F  Q  L  W  L  L  Q  R  D  G  C  L  W  W  S  P

GGCAGCTCCTTCCCTGAAGATCCACCTGCCCACCTAGAGGTCCTCTACAGAGCCACGCTGGGCAGTTACTCAGGCTGGGGAC    1215
G  S  S  F  P  E  D  P  P  A  H  L  E  V  L  S  E  P  R  W  A  V  T  Q  A  G  D

CCAGGGGCAGATGATGAGGGGCCCTTACTGGAGCCGGTGGGCAGTGAGCATGCCCAGGACACCTACTTGGTATTGGATAAG    1296
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TGGTTGCTGCCCCGGACCCCATGCAGTGAGAACCTCTCAGGGCCTGGGGGCAGTGTGGACCCTGTGACTATGGATGAAGCT    1377
W  L  L  P  R  T  P  C  S  E  N  L  S  G  P  G  G  S  V  D  P  V  T  M  D  E  A

TCAGAAACATCTTCCTGCCCGTCTGACTTGGCCTCAAAGCCCAGGCCAGAGGGCACCTCACCTTCCAGCTTTGAGTACACC    1458
S  E  T  S  S  C  P  S  D  L  A  S  K  P  R  P  E  G  T  S  P  S  S  F  E  Y  T

ATCCTGGACCCCAGCTCTCAGCTCCTGTGCCCTCGGGCACTGCCTCCCGAGCTACCTCCCACTCCACCTCACTTGAAGTAC    1539
I  L  D  P  S  S  Q  L  L  C  P  R  A  L  P  P  E  L  P  P  T  P  P  H  L  K  Y

CTATACCTTGTGGTGTCCGATTCTGGCATCTCAACAGATTACAGTTCGGGGGGCTCTCAGGGAGTCCACGGGGACTCATCT    1620
L  Y  L  V  V  S  D  S  G  I  S  T  D  Y  S  S  G  G  S  Q  G  V  H  G  D  S  S

GATGGCCCCTACTCCCACCCCTATGAGAACAGCCTTGTCCCAGACTCAGAGCCTCTGCATCCCGGCTATGTGGCCTGCTCC    1701
D  G  P  Y  S  H  P  Y  E  N  S  L  V  P  D  S  E  P  L  H  P  G  Y  V  A  C  S

TAGGACTCCAGCCTACAACGTCTTGAACGGGATTGGTGAAGCCATACTTAAAGTCAGAGCTGACCTTGGCCCTCTGAGCAG    1782
End

GAAGAGACAGCCTTGCAATGTTAAGATTAAGAGTTATCTGTCTGTATATAGAAATATATATATATATCGATTTTTCT        1859
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Fig. 6. **Structure of murine EPOR cDNA derived from F5-5.** (a) Schematic representation and restriction map of the cloned cDNA. Open and black boxes represent the sequences of the noncoding and coding regions of the murine EPOR, respectively. Hatched box represents the sequence corresponding to the 3' LTR of F-SFFV$_P$. (b) Murine EPOR cDNA nucleotide derived from F5-5 cells with predicted amino acid sequence.

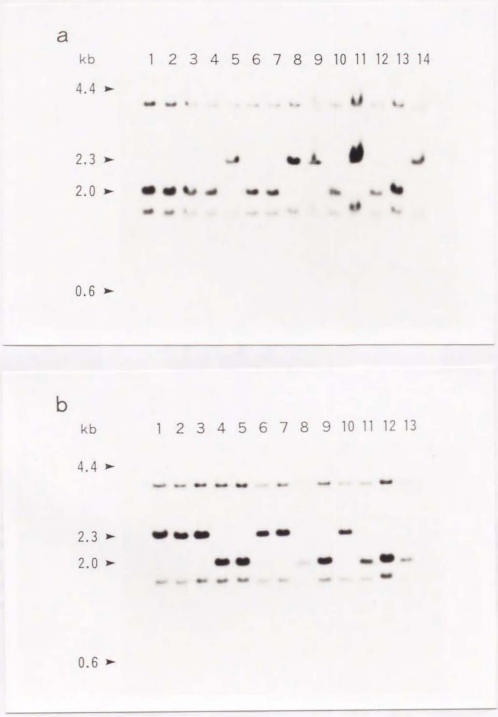

Fig. 7. **Southern blot analysis of BamHI-digested mouse kidney DNA for EPOR.** Genomic DNAs (10μg) prepared from various laboratory mouse kidney were digested with BamHI restriction enzymes, electrophoresed, transferred, and hybridized with murine EPOR cDNA. The samples are: (a) 1, DBA/2 cr; 2,DBA/2 J; 3,DBA/2 N; 4,C57BL/b; 5,DDD/1; 6,BALB/C; 7,C3H/He; 8,NZB/San; 9,GR; 10,C57BL/10sn; 11,WHT/Ht; 12,A/J; 13,STR/N; 14,FM; (b) 1,NZW/N; 2,NZB/KI; 3,NC; 4,I; 5,CBA/KI; 6,SWR/S; 7,SS; 8,CF#1; 9,AKR; 10,C58/J; 11,STL/J; 12,RR; 13,DBA/1 J.

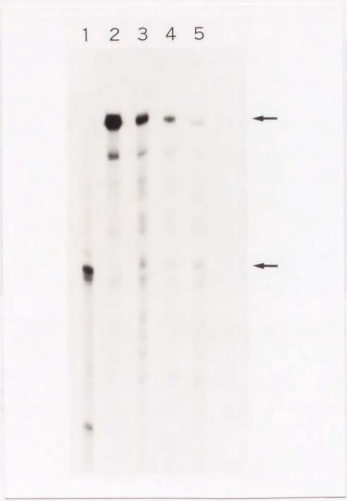

Fig. 8. **RNase protection assay of murine EPOR mRNA.** Poly(A)$^+$ RNAs (10μg) prepared from phenylhydrazine-treated DDD mouse spleens (lane 1), MEL cell lines F5-5 (lane 2), T3CI2O (lane 3), T3K-1 (lane 4), and TSFAT-3 (lane 5) were hybridized with ^{32}P-labeled RNA probe containing F-SFFV$_P$ LTR sequence and murine EPOR sequence. The hybridized samples were treated with RNase A and RNase T1, and electrophoresed. Arrows indicate the protected RNA fragments. The arrows indicate protected bands.

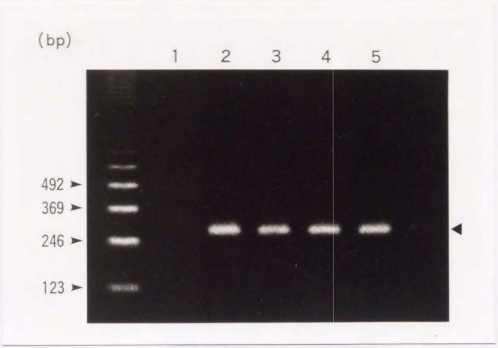

Fig. 9. **PCR amplification of EPOR LTR fragments.** Genomic DNAs (100ng) prepared from DDD mouse spleen (lane 1), F5-5 (lane 2), T3Cl2O (lane 3), T3K-1 (lane 4), and TSFAT-3 (lane 5) were amplified. Primers for the reaction were derived from 3' LTR sequence of F-SFFVP and from the EPOR gene (descrived in "Materials and Methods"). The markers on the left are from a 123-bp DNA ladder (BRL).

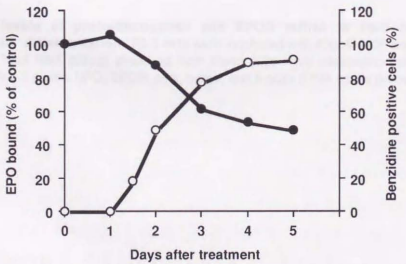

Fig. 10. **Change in specific ^{125}I-EPO binding to F5-5 cells and ratio of benzidine-positive cells during EDF-induced erythroid differentiation.** F5-5 cells were incubated with 400pM EDF and harvested after the indicated period. A portion of the cells was stained with benzidine dye, and the ratio of positive cells (○) was scored as the percentage of the total. Specific binding (●) was determined as described in 'Materials and Methods".

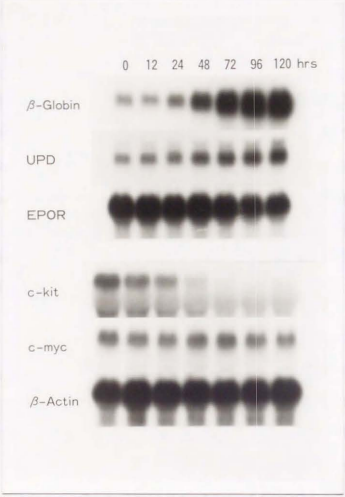

Fig. 11. **Relative levels of proto-oncogenes and EPOR mRNA in F5-5 cells during EDF-induced erythroid differentiation.** F5-5 cells were incubated with 400pM EDF and harvested after the indicated period. Total RNA (20μg) prepared from these cells were electrophoresed, transferred, and hybridized with murine β-globin, UPD, EPOR, c-kit, c-myc, and β-actin cDNA as the probes.

Specific binding of EPO to F5-5 cells decreased gradually during erythroid differentiation. Scatchard analysis indicates Kd=5.2x10^{-10}M for differentiated cells cultured with 400pM EDF for 5 days, the number of binding sites per cell being 4,000. Cross-linking studies of ^{125}EPO indicated that F5-5 cells had the same three bands as undifferentiated cells.

Total RNA prepared from F5-5 cells after incubation with 400pM EDF for up to 120 h, were hybridized with the murine EPOR, c-kit, c-myc, UPD, β-globin, and β-actin cDNA as probes (Fig. 11.). Expression of β-globin and UPD mRNA significantly increased during erythroid differentiation. Expression of c-kit mRNA rapidly decreased during erythroid differentiation. However, the expression of EPOR mRNA decreased slightly, in spite of the decrease in number of cell surface EPO binding sites per cell.

DISCUSSION

This paper demonstrates the overexpression of EPOR mRNA associated with the rearrangement of its gene in MEL cell clone F5-5. The EPOR transcript from F5-5 cells initiates in the 3' LTR sequence of F-SFFV$_P$. The PCR and sequence analyses indicated that the F-SFFV$_P$ LTR integrated upstream of the EPOR gene. An RNase protection assay indicated that the aberrant EPOR transcripts containing the LTR sequence were strongly expressed. These results indicate that the overexpression of the EPOR mRNA was caused by the F-SFFV$_P$ viral promoter insertion just upstream of the EPOR genes. The same integration upstream of the EPOR gene were also observed in other subclones of the MEL cell lines (T3Cl2O, T3K-1 and TSFAT-3). Recently Lacombe et al. (51) reported the same result for the T3Cl-2 cell line. Southern blot analysis revealed that the rearranged fragments were increased in length by the same amount (about 500 bp) in all digests. This result suggests that a single isolated LTR is inserted upstream of EPOR gene, as Lacombe et al. reported (51). In F5-5 cells, the EPOR expression is higher than the other clones and the rearranged alleles are approximately twice as numerous as the normal alleles. It may be that the F5-5 cell line has evolved to a subclone

which has additional rearranged alleles by duplication, or that it contains another subclone which has only rearranged alleles.

The promoter activity of the F-SFFV$_P$ LTR is very strong during the various stages of erythroid differentiation and thus expression of the EPOR mRNA was not reduced during terminal differentiation of F5-5 cells by EDF (52). Curiously, expression of the cell surface EPORs was down-modulated after EDF treatment. EDF didn't inhibit the binding of ^{125}I-EPO to F5-5 cells. Several possibilities can be raised against this discrepancy. [1] EDF may be affect on post-transcriptional pathway of EPOR synthesis. [2] EDF may have some effects on distribution or metabolism of cell surface EPOR. [3] Decrease of EPOR binding site may be related to the decrease in cell size during erythroid differentiation as Mayeux et al. reported (9). [4] It may be possible that EPO receptor has a second subunit like the receptors for interleukin-2, interleukin-3, interleukin-5, interleukin-6, and granulocyte-macrophage colony-stimulating factor and EDF may have some effects on a second subunit of EPOR. While the expression of c-kit mRNA, which is receptor for stem cell factor (SCF) (53-57), decreased during EDF-induced erythroid differentiation. This results suggest that c-kit products are expressed only in early progenitor cells and down-regulated with differentiation. It may be possible that c-kit and SCF system play an important role in stem cell proliferation and EDF may act as a natural regulator of erythroid differentiation in bone marrow (58,59).

Dysregulated expression of the receptor genes because of insertion of viral elements was recently reported for two members of the interleukin receptor family. The interleukin-2 receptor β-chain was constitutively expressed in the T-lymphoma cell line EL-4 (60). The interleukin-6 receptor with a truncated cytoplasmic domain was overexpressed in a plasmacytoma cell line P3U1 (61). The promoter or enhancer activity of the LTR sequence of the intracisternal A particle (IAP) gene was responsible in both of these cases. Furthermore, in avian leukosis virus (ALV)-induced erythroblastosis, the integrated provirus generated an activated version of epidermal growth factor (EGF) receptor fused to a viral gag and env protein (62). Previous studies have suggested that the aberrant expression of growth factor receptors might contribute to the development of neoplasms.

F-SFFV$_P$-induced erythroleukemia is known as a model of multistep leukemogenesis. In the preleukemic stage of the disease, the modified env product termed gp55 is only responsible for EPO-independent growth of nonclonal erythroblasts. Malignant evolution and clonal selection of these cells appears to be caused by a series of genetic changes in cellular genes. This step involves disruption of the p53 gene (63-65) and/or activation of the Spi-1 gene (66), which is is likely to be caused by the insertion of the F-SFFV$_P$ provirus. F5-5 cells, the parental cell line TSFAT-3 cells, and other subclones (T3Cl2O cells and T3K-1 cells) were characterized as follows. [1] EPOR was overexpressed by insertion of the viral promoter. [2] Gp55 was expressed (67). [3] Expression of EPO mRNA was not detected by PCR prosedure using murine EPO primers (data not shown). [4] Rearrangement of Spi-1 gene was not detected (Dr. Misawa, personal communication). [5] CD43/melF gene, which enhaned the antigen-specific T cell activation (68), was amplified and rearranged (69). Recent studies have shown that gp55 is capable of inducing neoplastic proliferation in EPO-dependent permanent cell lines (36). One suggestion about the possible mechanism by which gp55 induces EPO-independent growth is that the direct binding of gp55 to the EPOR provokes signal transduction through the receptor in a manner similar to that of EPO (35,70). More recently, it has been reported that an activating point mutation in EPOR induces erythroleukemia in mice (71,72). It is possible that overexpression of the EPOR will confer growth advantage to a F-SFFV$_P$-infected erythroid progenitor cell, leading to positive clonal selection through further leukemogenic steps. The role of CD43/melF in leukemogenesis is unknown. More examinations are required to decide this issue.

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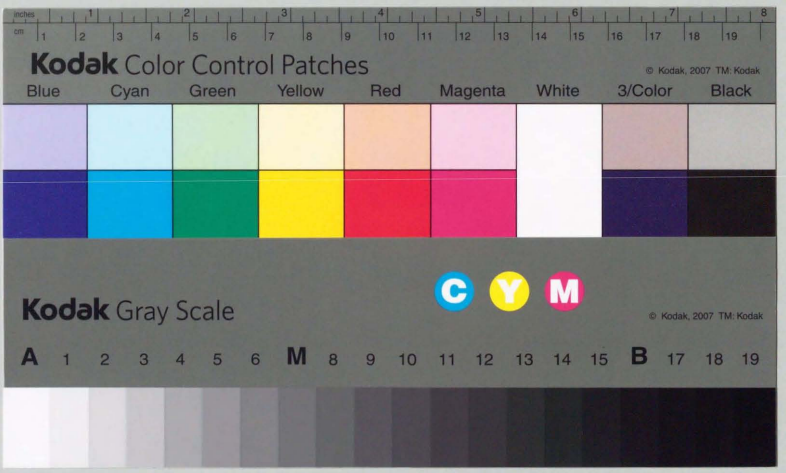
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