Chromosomal localization of the rat erythropoietin receptor gene by fluorescence in situ hybridization

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ABSTRACT

Erythropoietin stimulates proliferation and differentiation of erythroid progenitor cells by binding to a specific membrane receptor, erythropoietin receptor. By using the genomic clone derived from a rat cosmids library, the rat erythropoietin receptor gene was assigned to chromosome 8q24 by fluorescence in situ hybridization.

1. INTRODUCTION

Erythropoietin (Epo), a glycoprotein produced in the mammalian kidney and liver, is a major physiological regulator of erythropoiesis. Epo supports survival of the erythroid precursor cells and stimulates their proliferation and differentiation by binding to the erythropoietin receptor (Epo-R) on the surface of the cell. Epo is also shown to inhibit DNA breakdown, prevent apoptosis of the erythroid progenitor cells (Koury et al., 1990), and stimulate megakaryocytes (Berridge et al., 1988).

The rat Epo-R is a polypeptide of 483 amino acids with a single transmembrane domain (Masuda et al., 1993). The complete cDNA sequence of human (Winkelmann et al., 1990), mouse (D'Andrea et al., 1989) and rat (Masuda et al., 1993) is already given, as well as the genomic gene structure. The extracellular domain of Epo-R is characterized by the presence of four spaced cysteines near the amino terminus and a WS motif which is a conserved feature of the cytokine receptor family. A well conserved region (positions 248-295) exists in the cytoplasmic domain which is located proximal to the transmembrane domain. This region appears to generate growth-stimulating signal. The amino acid sequence of the mature Epo-R protein of the rat has 82% homology to those of human and 94% homology to those of mouse. Epo-R also has a significant homology to the interleukin (IL)-2 receptor beta-chain polypeptide as well as to the receptors for IL-4 and IL-6, which form a new cytokine receptor family.

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The Epo-R also plays an important role in the development of erythroleukemia. In the primary stage of mouse Friend erythroleukemia, the membrane glycoprotein gp55 of the Friend spleen focus-forming virus (SFFV) binds to Epo-R and causes increased erythroblastosis and ultimately leukemia (Li. et al., 1990). Chemically induced leukemias in the rat are frequently derived from erythroid cells (Huggins and Sugiyama, 1966) and associated with various chromosomal rearrangements. It would be extremely important to determine the chromosomal locus of the Epo-R gene for these studies. We, therefore, performed fluorescence in situ hybridization (FISH) and report a chromosomal assignment of the rat Epo-R gene.

2. MATERIALS AND METHODS

The rat genomic library screening for cosmid clones containing the Epo-R

This rat cosmid library was constructed by Oda et al (Oda et al., 1990). DNA fragments (30–50 kb) of the partially EcoRI digested genomic DNA from the liver of the Wistar rat was inserted into the EcoRI site of the pHC79 cosmid vector. This rat cosmid library was kindly provided by A. Ichiyama (Hamamatsu University School of Medicine, Shizuoka, Japan). The multiple cosmid libraries were plated at a low density on LB plates containing ampicillin. After constructing membrane replicas, they were hybridized in situ with a 32P-labeled rat Epo-R cDNA (Masuda et al., 1993). Eight positive colonies were picked up and cultured independently. DNA extracted from four out of eight cosmid clones were digested with EcoRI. The four collected cosmid clones were confirmed to contain the Epo-R sequence by Southern blotting (Fig. 1). DNA from one selected cosmid clone was labeled by incorporation of biotin-14-dATP by nick translation using the Bionick Labeling System (Bethesda Research Lab.) and used as probes.

Preparation of chromosome specimens

Chromosome specimens were prepared from the spleen of a male inbred strain of Wistar rat by ordinary procedures consisting of hypotonic treatment, acetic-methanol (1:3) fixation, and air drying. Chromosomes were identified by R-banding (Takahashi et al., 1990) and G-banding in combination with fluorescence in situ hybridization (FISH) (Inazawa et al., 1993).

FISH

The hybridization protocol followed that of Pinkel et al. (1988), with slight modifications. Briefly, slides carrying metaphase spreads were heated to 65°C for 4 h in air, treated with RNaseA (Sigma) [100 µg/ml in 2×SSC for 1 h at 37°C], dehydrated in an ethanol series, denatured [70% formamide (vol/vol)/2×SSC (final concentration) pH 7, at 72°C for 2 min], and dehydrated in a −20°C ethanol series. They were then treated with proteinase K (0.1 µg/ml) in 20 mM Tris-HCl
Figure 1.  a) Ethidium bromide staining of the EcoRI digested, bands of the collected cosmid clone. Lane 1, HindIII; Lane 2, cosmid clone 1.  b) Southern blot of EcoRI-digested rat genomic clones derived from the cosmid library. Obtained after hybridization with the 1.5 kb Epo-R cDNA probe. It was about 6.6 kb in size. Clone 1(line 1) out of 4 clones obtained was used for FISH.

(pH 7.5), 2 mM CaCl₂ at 37°C for 7.5 min, and dehydrated. The hybridization mixture consisted of 50% formamide/2×SSC, 10% dextran sulfate, 2 mg/ml BSA, 250 μg/ml sonicated salmon sperm DNA, 800 μg/ml sonicated rat liver DNA, and biotin-labeled probes at 5 μg/ml. This mixture was applied to the pretreated metaphase spreads, covered with parafilm, and incubated at 37°C in a humidified chamber overnight. The slides were washed in 50% formamide/2×SSC at 42°C three times (3 min each) and in 2×SSC at room temperature for three times (3 min each). They were removed from the 2×SSC and incubated in 4×SSC/0.05% Tween-20 at room temperature for 5 min. The excess liquid was drained and fluorescein-avidin (5 mg/ml in 4×SSC, 0.05% Tween 20, 1% BSA) was applied. The slides were incubated for 45 min at 37°C. They were then washed three times (5 min each) in 4×SSC, 0.05% Tween-20 at room temperature. The intensity of biotin-linked fluorescence was amplified by the addition of a layer of biotin-labeled goat anti-avidin antibody (5 mg/ml in 4×SSC, 0.05% Tween 20, 1% BSA), and after washing as above, amplified by another layer of fluorescein-avidin DCS (Vector Lab, Burlingame, CA.). After washing and draining the excess liquid from the slide, the DNA was stained with propidium iodide (PI) at 1 μg/ml. The metaphase spreads were observed with a fluorescence microscope and photographed.
3. RESULTS AND DISCUSSION

A genomic clone of the \textit{Epo-R} gene was isolated by screening rat genomic cosmid library from the liver of Wistar rat with a 1.5 kb cDNA \textit{Epo-R} probe (Masuda et al., 1993) (Fig. 1). Cosmid clone 1 was further confirmed to contain the \textit{Epo-R} sequence, using the three independent rat \textit{Epo-R} PCR primers. The inferred bands (136 bp, 117 bp, 126 bp) were obtained when cosmid clone 1 was used as a template for PCR amplification (data not shown). This cosmid clone was used for FISH. In a total of 50 metaphases examined, 130 distinct hybridization signals were identified. One hundreds and fifteen signals were located on chromosomes 8, and 15 signals were located on other chromosomes. The distribution of the signals on rat chromosome 8 is shown in Fig. 2. Thus, we concluded the \textit{Epo-R} gene was mapped on 8q24.

The \textit{Epo-R} gene has been mapped on human chromosome 19 and mouse chromosome 9 (Table 1). According to the linkage analysis in backcross progeny between inbred strains, the mouse \textit{Epo-R} gene has been mapped near the LDLR (low density lipoprotein receptor) on chromosome 9. Our result agrees with the previous report that most loci on rat chromosome 8 have been assigned to mouse chromosome 9 (Yamada et al., 1994). Yeung et al. (1993) assigned the rat \textit{Epo-R}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig2.png}
\caption{Ideogram of rat chromosome 8 with the location of \textit{Epo-R} gene indicated by the distribution of hybridization signals. The signals are localized in 8q24}
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<th>Species</th>
<th>Chromosome</th>
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<tr>
<td>Human</td>
<td>19q13</td>
<td>S</td>
<td>Budarf et al. (1990)</td>
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<tr>
<td>Mouse</td>
<td>9(near centromere)</td>
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<td>Budarf et al. (1990)</td>
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<td>Rat</td>
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Figure 3.  A) Metaphase spreads of rat chromosome hybridization in situ with the Epo-R clone (non-banded).  B) Same on G banded chromosomes.  C) Another metaphase spreads on R banded chromosomes.  Hybridization signals (arrows) on chromosomal band 8q24.
gene to chromosome 8 by using the somatic hybrid panel. More recently, Jacob et al. (1995) reported the construction of the first complete genetic linkage map of the laboratory rat using the 1171 simple sequence length polymorphic markers including a complete single linkage map on chromosome 8 although the loci on chromosome 8 had been classified into the three linkage groups. In order to construct the more detailed map of rat chromosome 8, the combined knowledge of the physical and linkage mapping of each gene is necessary. Since a few loci were identified on chromosome 8, the assignment of the Epo-R gene to 8q24 can be an anchor locus for further analysis.

The role of Epo-R in leukemogenesis has been the focus of great interest. Activation of the Epo-R gene by viral insertion has been observed in the mouse erythroleukemia cell lines (Hino et al., 1991). An activating mutation in the murine Epo-R was observed in erythroleukemia in mice (Longmore et al., 1991). In a human erythroleukemia cell line, UT7, the amplification and rearrangement of the Epo-R gene occurred (Chretien et al., 1994). These results indicate that Epo-R itself has a property of oncogene. The significant biological changes involving the Epo-R gene could also occur, for example, in rat erythroleukemia which exhibits specific chromosomal alterations. Further studies including FISH analysis and other molecular biological analyses to detect gene amplification and rearrangement are required to elucidate the role of the Epo-R gene in rat leukemogenesis.

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REFERENCES


fluorescent in situ hybridization (FISH) and physical ordering of human renin (REN) and cathepsin E (CTSE) gene by multi-color FISH. *Acta Histochem. Cytochem.* 26, 319–324.


