Localization of the Gene Encoding Human Erythroid-Potentiating Activity to Chromosome Region Xp11.1→Xp11.4

KAY HUEBNER,[1] MASAHARU ISOBE,[1] JUDITH C. GASSON,[2] DAVID W. GOLDE,[2] AND CARLO M. CROCE[1]

SUMMARY

We have localized the human gene for erythroid potentiating activity (EPA) to the X chromosome by analysis of its segregation pattern in mouse-human somatic cell hybrids. The EPA gene has been further localized to human chromosome region Xp11.1→Xp11.4 by in situ hybridization of a molecularly cloned EPA genomic fragment to metaphase chromosomes.

INTRODUCTION

Erythroid potentiating activity (EPA) [1] is a glycoprotein of molecular mass (M_r) 28,000 that was purified from medium conditioned by the HTLV-II infected Mo-T lymphoblast cell line. Purified EPA stimulates colony formation by primitive burst-forming units erythroid (BFU-E), erythroid precursor cells, as well as more mature erythroid progenitors colony forming units, erythroid (CFU-E) [2, 3]. Purified EPA specifically stimulates human and murine cells of the erythroid lineage, unlike murine interleukin-3 which stimulates precursors from all hematopoietic lineages [4]. Based on primary amino acid sequence obtained from the purified protein, oligonucleotide probes were used to isolate complementary DNA (cDNA) molecular clones encoding EPA from a Mo cDNA library [5]. The full-length EPA cDNA clone has been expressed in Cos monkey cells and Chinese hamster ovary cells. The purified biosynthetic (recombinant) EPA protein is biologically active ([5] and J. C. G. and D. W. G., unpublished results, 1985). A human recombinant λ phage library was screened

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with the full-length EPA cDNA clone, and overlapping genomic clones were used to obtain a restriction map. EPA appears to be encoded by a single gene that is approximately 3 kilobase pairs (kbp) in length and is interrupted by at least two intervening sequences [5].

The physiological functions of EPA in vivo are unknown but the availability of purified EPA and a knowledge of the molecular organization and location of this modulator should facilitate further functional studies. To determine the chromosomal location of the gene encoding human EPA, we have used the EPA cDNA clone to screen a panel of mouse-human hybrids retaining different complements of human chromosomes. A 5.2-kb genomic clone that contains the entire coding region for EPA (pEH 5.2 [5]) was used to regionally localize the EPA locus by in situ hybridization to human metaphase chromosomes.

MATERIALS AND METHODS

Molecular Probes

The EPA cDNA clone, EPA 57, is a full-length cDNA of ~ 900 bp inserted in pBR322 at the *Pst*I site [5]. The entire plasmid was nick-translated using all four [^{32}P]dNTPs and used to screen the panel of mouse-human hybrid DNAs for presence of the EPA gene by Southern blot hybridization. The 5.2-kbp *Hind*III fragment of the EPA genomic clone, pEH 5.2 [5], was nick-translated [6] using all four [^{3}H]dNTPs and used for in situ hybridization to human metaphase chromosomes.

Cells

Isolation, propagation, and characterization of parental cells and somatic cell hybrids used in this study have been described [7–11]. Hybrids were characterized for expression of enzyme markers assigned to each of the human chromosomes [7]. Some hybrid clones were karyotyped by trypsin/Giemsa and/or G-11 banding methods as described [7]. In addition, the presence of human chromosomes in many of the mouse-human hybrids have been confirmed by DNA hybridization using probes for genes assigned to specific human chromosomes [7–11].

For regional localization of the EPA gene, two additional hybrid DNAs were used: DNA from a mouse-human hybrid retaining only the human X chromosome, designated hybrid 91B HAT, was provided by B. Migeon; and DNA from a rat-human hybrid [10] retaining a translocation chromosome t(14:X)(14pter→14q32.1::Xq13.1→Xqter), which includes most of human chromosome 14 plus most of the long arm of the human X chromosome. The human parent of this second hybrid with the t(14:X) chromosome was from a patient with Klinefelter syndrome in which the 14:X translocation was also observed. This human cell line (GM 0074) was obtained from the Human Genetic Mutant Cell Repository (Institute for Medical Research, Camden, N.J.).

Southern Blot Analysis

DNAs from human peripheral blood lymphocytes (PBL) or leukemia cells, mouse cell lines, and rodent-human hybrid cell lines were extracted by cell lysis, proteinase K digestion, phenol extraction, and ethanol precipitation. Cellular DNAs were digested with an excess of appropriate restriction enzymes, sized in 0.8% agarose gels, and transferred to nitrocellulose or nylon filters as described by Southern [12]. Hybridization was carried out in 50% formamide, 4 × NaCl/Cit (1 × NaCl/Cit is 0.15 M NaCl/ 0.015 M sodium citrate, pH 7.0), 0.2 mg of sonicated salmon sperm DNA per ml, 1× Denhardt's solution (0.02% bovine serum albumin/0.02% Ficoll/0.02% poly-vinylpyrrolidone) at 42°C for 15 hrs. Some hybridizations were performed at 68°C

without formamide. After hybridization, filters were washed and exposed to Kodak XAR-5 film with intensifying screens.

In Situ Hybridization

Metaphase spreads were prepared with normal human female phytohemagglutinin-stimulated (for 72 hrs) in vitro lymphocyte cultures. EPA genomic subclone pEH 5.2 was nick-translated with [^{32}H]dCTP (62 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear, Boston, Mass.), [^{3}H]dGTP (39.9 Ci/mmol), [^{3}H]dTTP (100.1 Ci/mmol), and [^{3}H]dATP (51.9 Ci/mmol). The techniques used for in situ hybridization were essentially as described by Harper and Saunders [13]. Chromosome preparations were treated with pancreatic RNase A (Sigma, St. Louis, Mo.) and then denatured in 70% formamide in 2 × NaCl/Cit, pH 7.0, at 70°C for 2 min. The chromosome preparations were then hybridized with ^{3}H-labeled EPA probe pEH 5.2 (specific activity 3 × 10^{7} cpm/µg) at a concentration of 140–280 ng/ml in 50% formamide, 2 × NaCl/Cit, 10% dextran sulfate (Pharmacia, Piscataway, N.J.), pH 7.0, for 20 hrs at 37°C. A 300-fold excess of sonicated salmon sperm DNA was included as carrier. Slides were thoroughly rinsed in 50% formamide, 2 × NaCl/Cit, at 39°C, exposed to Kodak NTB2 nuclear track emulsion for 16 days at 4°C, and developed with Kodak Dektol at 15°C. The chromosomes were then G-banded essentially as described by Cannizzaro and Emanuel [14] with a mixture of 6 parts of borate buffer (50 mM Na_2SO_4/2.5 mM $Na_2B_4O_7$, pH 9.2) to 1 part of Wright's/Giemsa stain solution (2.4 g of Wright's stain per liter/1.4 g of Giemsa stain per liter in methanol).

RESULTS

The EPA Gene Is on the Human X Chromosome

In order to localize the human EPA gene to a specific chromosome, cellular DNA from a panel of well-characterized mouse-human hybrids [7–11] and a rat-human hybrid [10, 15] retaining defined subsets of human chromosomes was cleaved with various restriction enzymes, electrophoresed, transferred to nitrocellulose or nylon membranes, and hybridized to the ^{32}P-labeled EPA cDNA in pBR322. Human DNA and positive hybrid DNAs exhibited the following fragments that hybridize to EPA cDNA: for BamHI digestion ~ 37 kbp, for HindIII ~ 5.2 kbp, and for EcoRI ~ 13 kbp; hybridization of the EPA probe to mouse DNA was not detected under the conditions used (fig. 1, lane 1). More than 26 hybrid DNAs were tested by Southern blotting analysis for presence or absence of the human EPA locus; an example of such a blot of HindIII-digested mouse, human, and rodent-human hybrid DNAs after hybridization to ^{32}P-labeled EPA cDNA is shown in figure 1. Human PBL-derived DNA (fig. 1, lane 2) and five hybrid DNAs (fig. 1, lanes 5–7, 9, 10) exhibited the expected 5.2-kbp HindIII fragment of the EPA locus; mouse DNA (fig. 1, lane 1) and three hybrid DNAs did not show hybridization to the EPA cDNA. All other hybrids in the panel were similarly tested for presence of the EPA gene. In all mouse-human hybrids tested, hybridization to the EPA probe correlated with the presence of the human X chromosome (see table 1). Table 1 summarizes these data and shows that no other human chromosome is correlated with presence of the EPA gene in hybrids. Two other hybrid DNAs (not included in table 1) were then tested to confirm and refine localization of the EPA gene to the X chromosome. DNA from a mouse-human hybrid containing only human
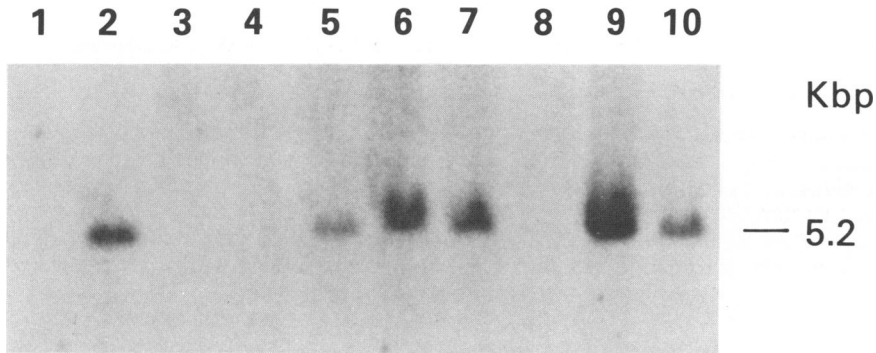
Fig. 1.—Segregation of the EPA gene in mouse-human hybrids. DNA (10 μg) from (*1*) mouse cell line; (*2*) human PBL, and rodent-human hybrids (15 μg) retaining human chromosomes; (*3*) 9, 12, 13, 14, 17, 22; (*4*) 9, 12, 13, 14, 17, 21, 22; (*5*) 1, 2, 3, 4, 6, 7, 9, 11, 12, 14, 15, 18, 20, 21, 22, X; (*6*) 3, 8, 11, 14, 21, 22, X; (*7*) 1, 3, 4, 5, 6, 10, 11, 14, 17, 18, X; (*8*) translocation chromosome t(14pter→14q32.1::Xq13→Xqter; (*9*) 4, 18, X; (*10*) 6, translocation chromosome t(14q11→14qter::20pter→20q13), X was cleaved with an excess of restriction enzyme *Hind*III, fractionated on an agarose gel, transferred to a nitrocellulose filter, and hybridized to ^{32}P-labeled EPA cDNA plasmid. Molecular weight of the EPA *Hind*III fragment is shown on the right of the figure.

chromosome X (B. Migeon, personal communication, 1985) also retained the EPA 13-kbp *Eco*RI fragment (data not shown); DNA from a hybrid cell containing, as its only human chromosome, a translocated chromosome, t(14pter→14q32.1::Xq13.1→Xqter) [15], which thus retains most of the long arm of the human X chromosome, is negative for the EPA hybridizing sequences (see fig. 1, lane 8), thus regionally localizing the EPA gene to the Xpter→Xq13.1 region of the human X chromosome.

The EPA Gene Is at Human Chromosome Region Xp11.1→Xp11.4

Localization of the EPA gene to the short arm of the human X chromosome has been further refined by in situ hybridization of ^{3}H-labeled EPA genomic DNA (pEH 5.2) to metaphase chromosomes from PBL of a normal female. In three separate attempts to localize the EPA gene by in situ hybridization using the EPA cDNA clone, we failed to find significant grain localization to specific chromosome regions, perhaps because the cDNA insert, at ~ 900 bp, is somewhat short for chromosomal in situ hybridization. Thus, the pEH 5.2 genomic fragment, which is not entirely repeat free but does specifically detect EPA gene fragments on Southern blots [5], was employed. After autoradiography, metaphase spreads were analyzed for grain localization. An example of one such spread is shown in figure 2 (upper). About 23% of all grains were located on the short arm of the X chromosome. Over 80% of the Xp grains were between Xp11.1 and Xp11.4, with most grains at Xp11.2. A histogram depicting the silver grain distribution along the human chromosomes is shown in figure 2 (lower). The short arm of the X chromosome represents approximately 2.1% of the haploid genome, and our observation that more than 18% of the human EPA probe hybridization was localized to the proximal half of this region is highly significant (P < .001). Thus, cytological hybridization localizes the hu-

TABLE 1

CORRELATION OF PRESENCE OF EPA GENE AND SPECIFIC HUMAN CHROMOSOMES
IN 26 MOUSE-HUMAN HYBRIDS

HUMAN CHROMOSOMES	No. HYBRID CLONES EPA GENE/CHROMOSOME RETENTION				No. DISCORDANT
	+/+	−/−	+/−	−/+	
1	5	10	11	0	11
2	1	8	15	2	17
3	10	8	6	2	8
4	13	9	3	1	4
5	8	7	8	3	11
6	11	10	5	0	5
7	5	8	11	2	13
8	5	5	11	5	16
9	4	7	12	3	15
10	5	10	11	0	11
11	6	10	10	0	10
12	3	6	13	4	17
13	6	6	10	4	14
14	12	6	4	4	8
15	5	9	11	1	12
16	2	9	14	1	15
17	9	2	7	8	15
18	8	9	8	1	9
19	1	10	15	0	15
20	9	8	7	2	9
21	4	7	12	3	15
22	7	7	9	3	12
X	16	10	0	0	0

NOTE: DNA from a panel of hybrid cells characterized for the presence of specific human chromosomes by isozyme analysis and, in some cases, by karyotypic analysis; and DNA:DNA hybridization using DNA probes for genes assigned to specific chromosomes was analyzed for the presence of the human EPA gene as shown in figure 1.

man EPA gene to the region between Xp11.1 and Xp11.4 with most grains at Xp11.2.

DISCUSSION

The region of the X chromosome to which the EPA gene has been localized, Xp11.1→Xp11.4, is thus far surprisingly free of assigned genes, considering the number of biochemical markers and genetic diseases that have been regionally mapped on the human X chromosome. Only the testicular feminization syndrome (TFM) [16] and Menkes syndrome, a recessive disorder characterized by early retardation in growth, peculiar hair, and focal cerebral and cerebellar degeneration [17], which is closely linked to tfm in mouse [16, 18], have been localized in or near this region, Xp11→Xq13 [19–21], although there are many X-linked traits that have not been regionally localized. Specific rearrangements of the X chromosome have not been associated with specific types of malignancies except for monosomy X in endometrial carcinoma in estrogen-treated Turner syndrome patients and extra X chromosome in breast carcinoma and mediastinal teratoma in Klinefelter syndrome [21].
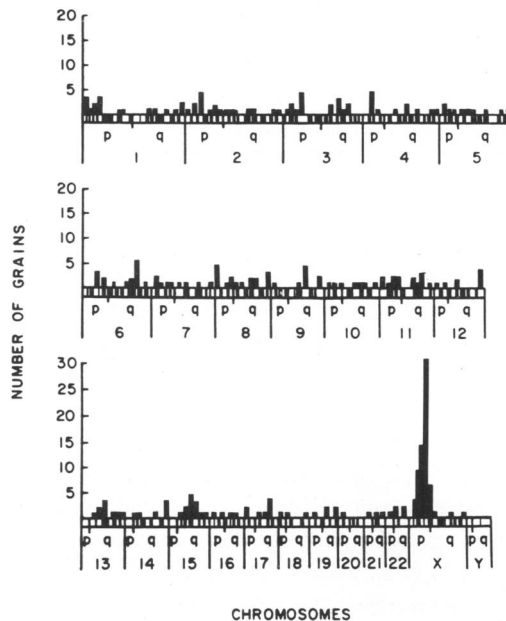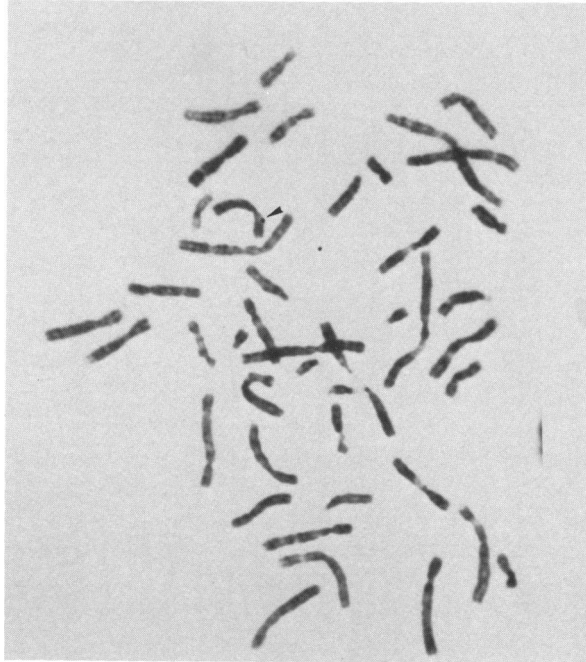
FIG. 2.—Localization of EPA gene in the human genome by in situ hybridization analysis (*upper*). Photograph of a G-banded lymphocyte metaphase spread hybridized with the EPA genomic subclone pEH 5.2. *An arrow* indicates a grain found over the X chromosome (*lower*). Diagram showing the grain distribution in 75 metaphases. *The abscissa* represents the chromosomes in their relative size proportion; *the ordinate* shows the no. silver grains. The distribution of 270 grains on 75 spreads was scored; 50 were found over Xp11.1→Xp11.4.

Assignment of the EPA gene to the X chromosome is of interest from several perspectives: it is the first growth factor found to be X-linked and must therefore undergo dosage compensation since genes on the X chromosome are functionally haploid, except for genes at the distal end of the short arm that escape inactivation; it is in a region of the X chromosome in which an additional biochemical marker should prove useful; and there are many X-linked traits and genetic diseases for which DNA samples could be tested for changes in the EPA gene at the genomic level. It is particularly interesting that, after completion of this study, the sequence of a cDNA clone for a human TIMP (tissue inhibitor of metalloproteinases) gene [22] was shown to be identical to the EPA cDNA sequence. Since destruction of collagen fibers, perhaps mediated by collagenase, a metalloproteinase, is a hallmark of various pathological conditions [22], a mutation in the EPA gene (in the guise of TIMP) could interfere with physiologically necessary inhibition of extracellular metalloproteinases, resulting in tissue destruction. Such a scenario could conceivably account for the defects reminiscent of connective tissue disorders [18, 23], characteristic of Menkes syndrome, which maps tantalizingly near the EPA gene [24]. When more is learned about the in vivo effects and functions of the EPA-TIMP gene, it should be possible to determine if the EPA gene is involved in any of the various X-linked disorders in man.

ACKNOWLEDGMENTS

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