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Chromosomal Assignments of the Human Endothelin Family Genes: The Endothelin-1 Gene (EDN1) to 6p23-p24, the Endothelin-2 Gene (EDN2) to 1p34, and the Endothelin-3 Gene (EDN3) to 20q13.2-q13.3

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Summary

Chromosomal assignments of the genes for the human endothelin family, the endothelin-1 gene (EDN1), the endothelin-2 gene (EDN2), and the endothelin-3 gene (EDN3), were accomplished by in situ hybridization to human metaphase chromosomes using a ^{3}H-labeled human preproendothelin cDNA probe for each endothelin. The chromosomal assignment of the EDN2 was also performed by Southern blot analysis of somatic cell hybrid DNAs. EDN1, EDN2, and EDN3 were mapped to 6p23-p24, 1p34, and 20q13.2-q13.3, respectively.

Introduction

Endothelin (ET, subsequently classified as endothelin-1 [ET-1]) is a potent and long-acting vasoconstrictor peptide consisting of 21 amino acids originally identified in culture media of porcine aortic endothelial cells (Yanagisawa et al. 1988). The analysis of a human genomic library subsequently led to the identification of this ET as a member of the ET family, comprising three isopeptides: ET-1, endothelin-2 (ET-2), and endothelin-3 (ET-3) (Inoue et al. 1989). Two of 21 amino acids were found to differ between ET-1 and ET-2, and six between ET-1 and ET-3 (Inoue et al. 1989). Tissue distributions of the three ET gene expressions are different from each other: in human, abundant ET-1 mRNA was found in many organs, such as the brain, kidney, lung, uterus, and placenta, as well as vascular endothelial cells; ET-2 mRNA was most abundantly found in renal medulla, followed by the jejunum; and ET-3 mRNA was found most abundantly in the jejunum and adrenal gland, followed by the brain, spleen, and renal medulla (M. Yanagisawa, unpublished observation). In addition to the potent vasoconstrictor and -pressor actions, ET-1 has proved to produce a wide spectrum of biological effects including action as a neuropeptide in the central nervous system (Yoshizawa et al. 1990). On the other hand, the functions of ET-2 and ET-3 have not yet been fully examined except for vasoconstriction. ETs are believed to exert their biological influences by interaction with their specific receptors on the surface of target cells (Masuda et al. 1989).

Each of the ETs is encoded in separate precursor (preproendothelin [PPET]) genes (Inoue et al. 1989) and is produced from its PPET via its intermediate, called "big ET." Big ET-1 is a 38-amino-acid peptide (Yanagisawa et al. 1988), and big ET-2 and big ET-3 are presumed to be 37- and 41-amino-acid peptides, respectively, in human (M. Yanagisawa, unpublished observation). The PPET-1 and PPET-3 genes each contain five exons and the PPET-2 gene contains four exons. In all PPET genes, the mature ET portion is encoded in exon 2 and the tail portion of big ET is encoded in exon 2 and exon 3 (M. Yanagisawa, unpublished observation). The nucleotide sequences of the three genes are highly conserved within the regions

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encoding 21-amino-acid mature ETs (≈80% homology) and, to a lesser degree (≈50%–60% homology), in the regions encoding big ETs and ET-like peptides. ET-like peptides, in which the relative positions of the four cysteine residues are perfectly conserved, are encoded in exon 3 (Yanagisawa et al. 1988). However, little similarity is retained in the sequences of the remaining parts of the PPET genes.

Southern blot analysis indicated that three chromosomal loci of ETs exist and each ET is present in one copy (Inoue et al. 1989). Bloch et al. (1989*a*, 1989*b*) assigned EDN1 to chromosome 6 and EDN3 to chromosome 20 by analysis of genomic DNA from human-mouse somatic cell hybrids. We report here the regional localizations of EDN1 and EDN3 as determined by in situ hybridization using cDNAs coding for human PPET-1 and PPET-3 as probes. In addition, using a cDNA coding for human PPET-2, we have determined the chromosomal localization of EDN2 by in situ hybridization and Southern blot analysis of DNA from human-mouse hybrid cell lines.

Material and Methods

Probes

To avoid possible cross-hybridization between endothelins, parts of the PPET-1 and PPET-3 cDNAs were used as probes for EDN1 and EDN3, respectively. A probe for EDN2 was almost the whole human PPET-2 cDNA.

The EDN1 probes were a 0.45-kb *Eco*RI/*Hin*dIII fragment of the human PPET-1 cDNA clone pHET4-3 (Itoh et al. 1988) and a 0.85-kb *Eco*RI fragment of the human PPET-1 cDNA clone phET9I (Ionue et al. 1989). The clone pHET4-3 contains the whole coding region of the gene but lacks most of the 3′ nontranslated region, and the fragment used as a probe was a 3′-portion of this clone, that is, a portion containing part of exon 4 and part of exon 5 of the gene. The clone phET9I contains the 3′ nontranslated region of the cDNA (Inoue et al. 1989), and the fragment used as a probe consisted of the remaining exon 5 portion of the gene. The EDN2 probe was a 1.3-kb *Eco*RI fragment of the human PPET-2 cDNA clone phJ37-1 and consisted of almost the whole cDNA of the gene (M. Yanagisawa, unpublished data). The EDN3 probe was a 0.9-kb *Bam*HI fragment of the human PPET-3 cDNA clone phJ14-2 (M. Yanagisawa, unpublished data) and consisted of most of the 3′ nontranslated region in exon 5 of the gene.

In Situ Hybridization

In situ hybridization was carried out on chromosome preparations obtained from phytohemagglutinin-stimulated human male lymphocytes cultured by the method of Dutrillaux and Viegas-Pequignot (1985).

Radioactive labeling of the cDNA fragments was performed by the multiprime DNA-labeling system (Amersham, United Kingdom) to a specific activity of 1×10^8 cpm/μg. The radiolabeled probes were hybridized to metaphase spreads at a final concentration of 10–20 ng/ml hybridization solution, essentially as described by Harper and Saunders (1981).

The slides were coated with Konica NM-R2 emulsion and developed after 10 d of exposure at 4°C. Chromosomes were G-banded by the fluorochrome-photolysis-Giemsa (FPG) method.

Somatic Cell Hybrid Analysis

Somatic cell hybrids were generated by fusion (Davidson and Gerald 1976) of human embryo fibroblasts with mouse LMTK-Cl-1D and FM3A cells. The human chromosome content was determined by the method of differential staining of human and mouse chromosomes (Yoshida et al. 1975). DNA (5–10 μg per track) from mouse cells, human-mouse hybrid cell lines, and normal human lymphocytes was digested with *Hin*dIII, electrophoresed through 0.9% agarose, blotted to Hybond N+ membrane (Amersham, United Kingdom) with 0.4 M NaOH, and probed with the phJ37-1 insert DNA radiolabeled with ^{32}P-dCTP by the multiprime DNA-labeling system. After hybridization at 65°C for 18 h the filter was washed twice in 2 × SSPE, 0.1% SDS for 10 min at room temperature, once in 1 × SSPE, 0.1% SDS for 15 min at 65°C, and once in 0.1 × SSPE, 0.1% SDS for 10 min at 65°C. Autoradiography was at −70°C.

Results

Regional Localization of EDN1

Metaphase cells (n = 100) were examined following in situ hybridization with the EDN1 probes. A total of 165 silver grains were associated with chromosomes, 37 of these (23%) being located on chromosome 6 (fig. 1). The distribution of grains on this chromosome was not random: 25 (68%) were on 6p23-p24 (fig. 2*A*, *B*). No other chromosomal sites showed significant labeling.

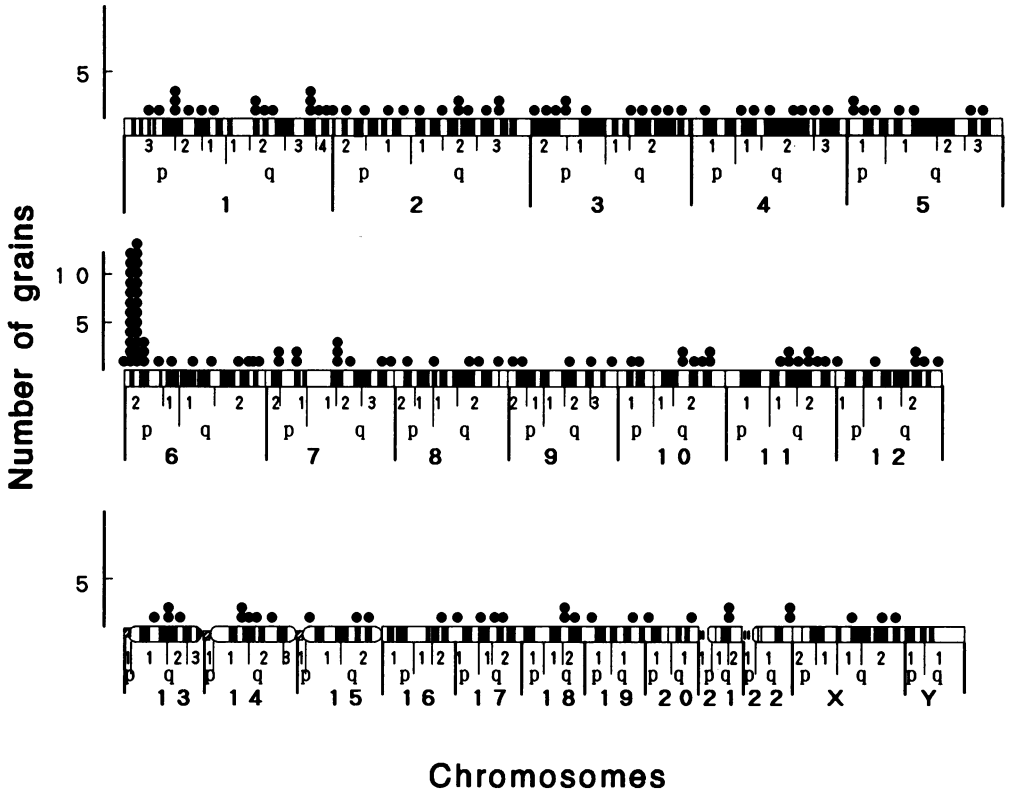
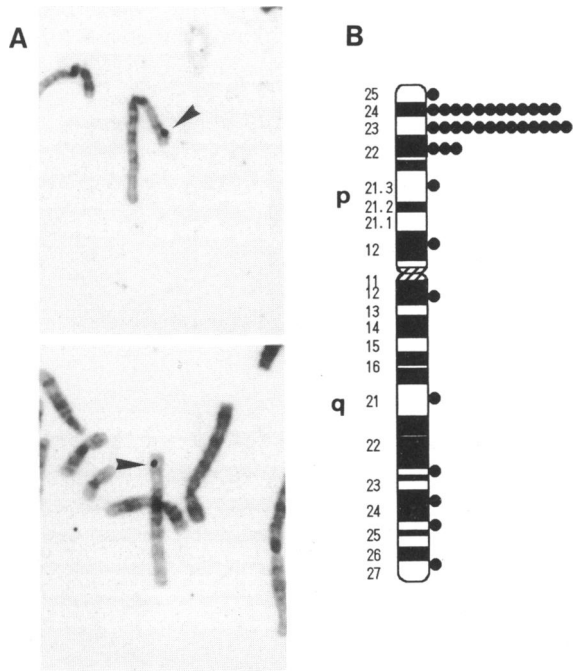


Figure 1 Distribution of 165 grains in 100 metaphase cells hybridized with human PPET-1 probes. A clear concentration of labeling is seen on chromosome 6.



Regional Localization of EDN2

In situ hybridization of the EDN2 probe to human chromosomes resulted in specific labeling at the short arm of chromosome 1 (fig. 3). Of the 120 metaphase cells examined, 45 cells (38%) had grains on the short arm of chromosome 1. Of the 238 grains associated with chromosomes, 51 grains (21%) were on the short arm of chromosome 1, and 35 (69%) of these were on 1p34 with a peak at 1p34.1 (37%) (fig. 4*A*, *B*). No other chromosomal sites, including chromosome 6 and chromosome 20, to which EDN1 and EDN3, respectively, were assigned by Bloch et al. (1989*a*, 1989*b*), showed significant labeling.

The localization of EDN2 to human chromosome 1 was supported by Southern blot analysis of DNA

Figure 2 *A*, Two partial human metaphases showing specific site of hybridization for human PPET-1 cDNA probes in region p23-p24 of chromosome 6; *B*, idiogram of human G-banded chromosome 6 illustrating distribution of labeled site for human PPET-1 cDNA probes.

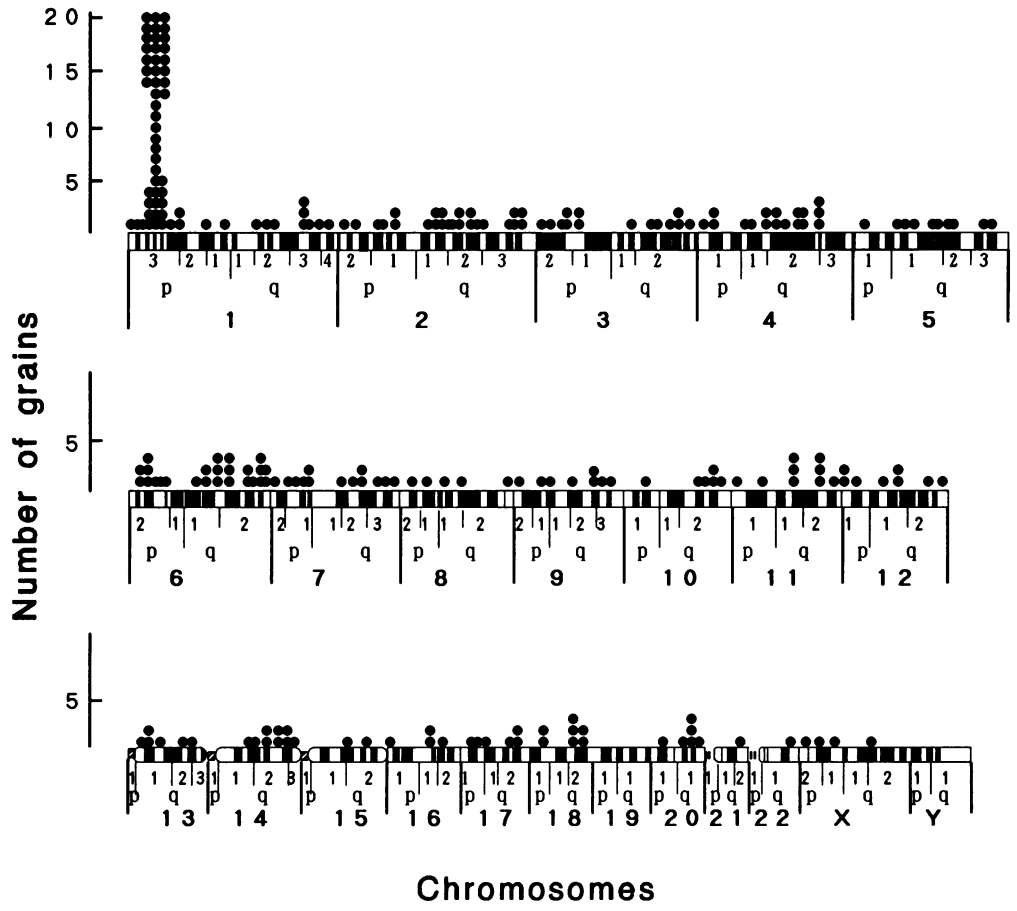


Figure 3 Distribution of 253 grains in 120 metaphase cells hybridized with PPET-2 cDNA probe. A clear concentration of labeling is seen on chromosome 1.

from three human-mouse hybrid cell lines with ^{32}P-labeled phJ37-1 insert as a probe. The three hybrid clones, H/B2-1, H/F4A-1, and A/H-1, contained a chromosome background from parental mouse cells and the following human chromosomes, respectively: 1, 5, 6, 10, 11, 12, 13, 16, 17, 19, (20), 21, and X; 4, 5, 6, 7, 8, 10, (12), 13, 15, 16, 20, 21, 22, and X; and 1 and (17), where chromosomes contained in less than 20% of the cells of the clone are indicated by parentheses. Figure 5 shows the pattern of human and mouse *Hin*dIII restriction fragments that hybridize with the EDN2 probe. In the human control DNA (lane A), hybridization to fragments of 6.4 kb and 2.2 kb was observed. The 6.4-kb and 2.2-kb fragments were also present in hybrids containing a human chromosome 1 (lanes B and E), but not in a hybrid that lacks a human chromosome 1 (lane C).

Cross-hybridization signals with other ET genes were produced in neither in situ nor Southern blot hybridization analyses under the conditions of the present experiment. This would be expected from comparisons between all the PPET cDNA sequences: although the mature ET-2 coding region of the fragment used has about 80% identity with its counterpart in the other ET genes, it consists of only 5% (63 nucleotides) of the total fragment.

Regional Localization of EDN3

In the 80 metaphase cells examined after in situ hybridization, there were 163 silver grains associated with chromosomes, and 33 of these (20%) were located on chromosome 20 (fig. 6). The distribution of grains on this chromosome was not random: 25 (76%) were on the q13.2-q13.3 region of the long arm with a peak at 20q13.2 (52%) (fig. 7*A*,*B*). No other chromosomal sites showed significant labeling.

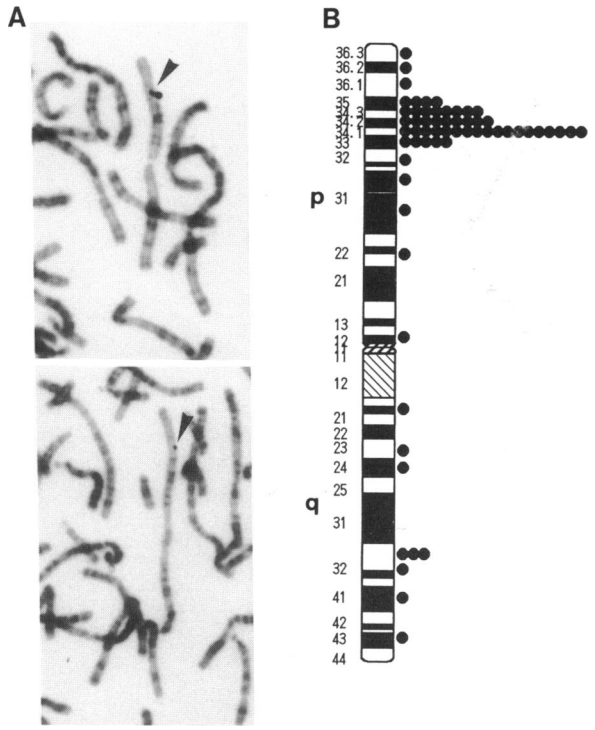


Figure 4 *A*, Two partial human metaphases showing specific site of hybridization for human PPET-2 cDNA probe in region p33-p34 of chromosome 1; *B*, idiogram of human G-banded chromosome 1 illustrating distribution of labeled site for human PPET-2 cDNA probe.

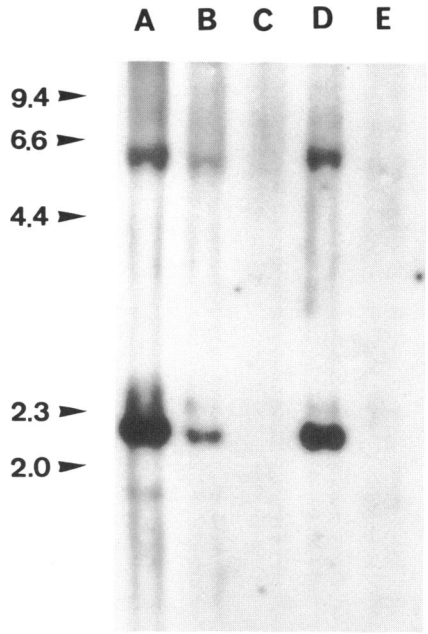


Figure 5 Southern hybridization of human PPET-2 cDNA probe to *Hin*dIII-digested DNA from panel of mouse-human somatic cell hybrid clones. Lane A, human diploid cells; lane E, mouse cells; lanes B–D, human-mouse hybrids containing human chromosome 1 (B, D) and no human chromosome 1 material (C). DNA size markers (kb) are shown at left.

Discussion

The assignments of EDN1 and EDN3 to chromosomes 6 and 20 by Southern blot analysis of DNA from a human-mouse somatic hybrid cell panel using ET-1 and ET-3 cDNAs as probes were previously reported by Bloch et al. (1989*a*, 1989*b*). Our in situ hybridization data for EDN1 and EDN3 were consistent with these previous reports and allowed us to further narrow the bands at which they locate, that is, EDN1 at 6p23-p24 and EDN3 at 20q13.2-q13.3.

The result of in situ hybridization of the EDN2 probe indicated that the most likely localization of EDN2 is 1p34. Southern blot analysis of DNA from three human-mouse hybrid cell lines with the EDN2 probe resulted in no discordancies for chromosome 1 or, possibly, for chromosome 17. However, localization of EDN2 on chromosome 17 is thought to be unlikely, because figure 5 shows more intense 6.4-kb and 2.2-kb bands in lane D (10 μg of DNA from A/H-1 clone containing chromosome 17 in less than 20% of the cells) than in lane B (5 μg of DNA from A/H2-1 clone containing chromosome 17 in all cells). All other chromosomes were excluded by at least one discordance. Somatic cell hybrid analysis therefore supported the localization of EDN2 at 1p34 determined by in situ hybridization.

The data presented in this paper have shown that the three ET genes are not genetically linked to one another in the human genome. The similarity of the structure of the three ETs' precursor genes, however, implies that these ET genes arose during evolution by gene duplications, when big ET and ET-like peptides in the common ancestral gene were already present (Bloch et al. 1989*b*). The poorly conserved sequences other than those encoding big ETs and ET-like peptides suggest that the three genes are evolutionarily relatively distant from each other (Inoue et al. 1989). Other mammals studied so far also have these three ETs (Inoue et al. 1989; Saida et al. 1989), with each mature ET sequence highly conserved (M. Yanagisawa, unpublished observation). These facts suggest that the three ET's are all physiologically indispensable for mammalian species and that the gene duplication events might have occurred before the evolution of mammals. Sarafotoxins S6, snake venom toxins

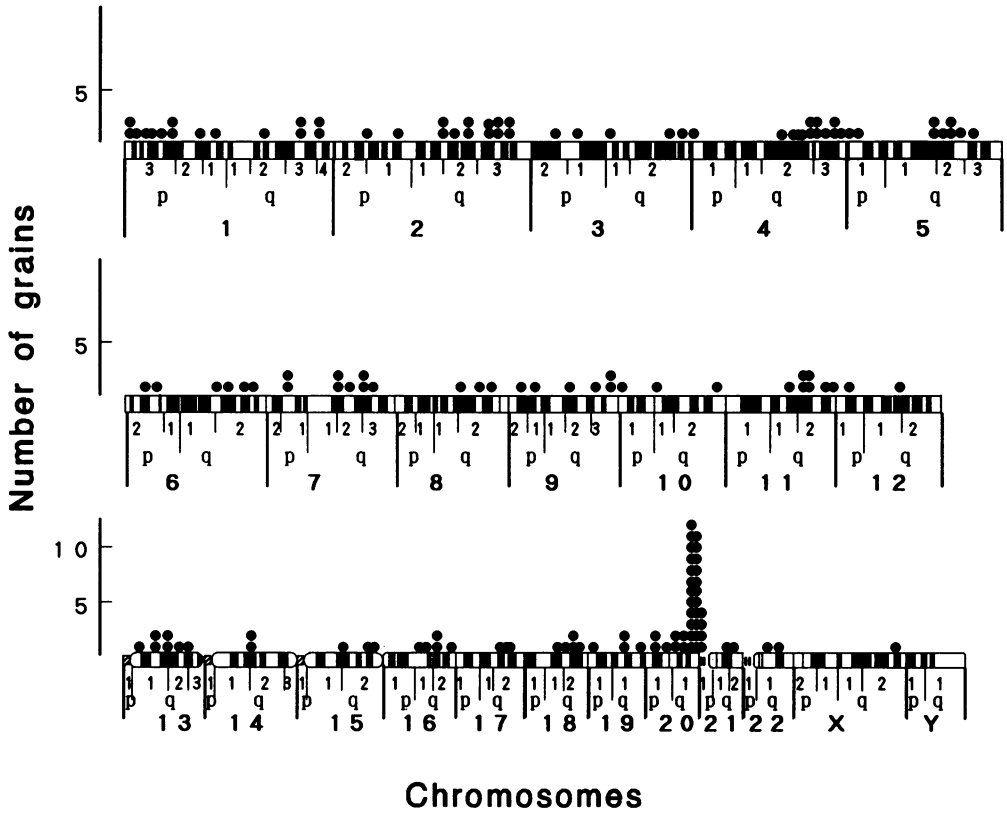


Figure 6 Distribution of 163 grains in 80 metaphase cells hybridized with PPET-3 cDNA probe. A clear concentration of labeling is seen on chromosome 20.

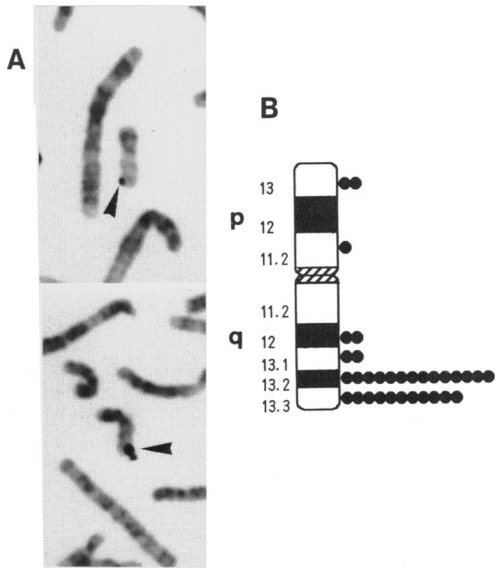


Figure 7 *A*, Two partial human metaphases showing specific site of hybridization for human PPET-3 cDNA probe in region q13.2-q13.3 of chromosome 20; *B*, idiogram of human G-banded chromosome 20 illustrating distribution of labeled site for human PPET-3 cDNA probe.

causing cardiac arrest probably as a direct result of coronary vasospasm, have striking functional and structural similarities with ET (Kloog, et al. 1988; Takasaki et al. 1988). The biochemical and physiological characteristics of sarafotoxins S6 support the speculation that the gene duplications occurred prior to the evolution of mammals. The findings presented in this paper that all three ET genes are located on separate human chromosomes are also compatible with this speculation.

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