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Chromosomal Localization of the Human Proenkephalin and Prodynorphin Genes

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Summary

DNA probes derived from rat and human proenkephalin and prodynorphin genes have been used to localize these two opiate neuropeptide genes on human chromosomes. Hybridization of probes to Southern blots made with DNAs from a rodent-human somatic-cell hybrid panel indicates localization of proenkephalin to human chromosome 8 and of prodynorphin to human chromosome 20. In situ hybridization to metaphase chromosomes confirms these assignments and indicates regional localizations of proenkephalin to 8q23-q24 and of prodynorphin to 20p12-pter. A human genomic prodynorphin clone reveals a frequent two-allele *TaqI* polymorphism.

Introduction

Approximately 25 distinct opioid peptides, isolated from the mammalian brain and from peripheral tissues, are all derived from three distinct genes encoding the precursors for proopiomelanocortin (Nakanishi et al. 1979), proenkephalin (Comb et al. 1982*a*), and prodynorphin (Kakidani et al. 1982). These precursors, which contain multiple biologically active peptides, are produced in numerous sites in the nervous and endocrine systems, where they may be proteolytically processed to form different end products (Douglass et al. 1984). In the brain, opioid peptides may regulate responses to pain and stress (Akil et al. 1984), modulate reproductive functions (Cicero 1980) and seizure activity (Kanamatsu et al. 1986), and may play a role in the establishment of narcotic addiction, tolerance, and withdrawal (Przewlocki et al. 1979). In peripheral tissues, opioid peptides may modulate cardiac (Holaday 1983), reproductive (Douglass et al. 1987), immune (Plotnikoff and Miller 1983), and intestinal (Puig et al. 1977) functions.

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The gene for proopiomelanocortin has previously been localized to human chromosome 2p23-25 (Owerbach et al. 1981; Zabel et al. 1982). In the present paper we report the localization of the two remaining opioid precursor genes, proenkephalin and prodynorphin, to 8q23-q24 and 20p12-pter, respectively. These results allow consideration of proenkephalin and prodynorphin genes as candidate genes for various inherited diseases (Feder et al. 1985; Breakefield and Cambi 1987).

Material and Methods

Probes

PHPE-9 is a 1,255-bp full-length human proenkephalin cDNA cloned into the *PstI* site of pBR322. (Comb et al. 1982*b*). pBgBa is a 900-bp *BglII*-*BamHI* fragment of the main exon of rat prodynorphin cDNA (Civelli et al. 1985) cloned into the *BamHI* site of pUC18. pHDME is a 1.2-kb *EcoRI*-*SacI* fragment of human prodynorphin cDNA containing exon 4 subcloned into pSP65 (J. Douglass, unpublished data). λEnk1 (Comb et al. 1983) is a 16-kb human genomic proenkephalin clone isolated from the Charon 4A library of Lawn et al. (1978). Also isolated from this library, λHDG1 is a 17-kb human genomic prodynorphin clone containing the entire

coding region of prodynorphin (J. Douglass, unpublished data). For blot hybridization, probes were labeled with [^{32}P]dCTP by means of the random-primer method (Feinberg and Vogelstein 1983).

Blot-Transfer Hybridization

DNAs were extracted from cell hybrids or white blood cells, digested with appropriate restriction enzymes, and subjected to blot-transfer hybridization as described elsewhere (Litt and White 1985; Bufton et al. 1986; Buroker et al. 1986). Prior to use in hybridization, radioactive probes λHDG1 and λEnk1 containing sequences homologous to human repeats were prehybridized with a vast excess of nonradioactive, sonicated human placental DNA to prevent hybridization of the repeated sequences with the blots, thereby allowing visualization of low- and single-copy fragments (Litt and White 1985; Sealey et al. 1985). Blots hybridized with human probes were washed at a final stringency of 0.1 × SSC at 65 C. Blots hybridized with the rat dynorphin probe pBgBa were washed at a final stringency of 0.2 × SSC at 50 C.

Somatic-Cell Hybrid Mapping Panel

Six mouse × human and 17 hamster × human somatic-cell hybrid cell lines were used as a mapping panel. These cell lines have been described elsewhere (Bufton et al. 1986; Buroker et al. 1986).

In Situ Hybridization

Plasmids PHPE-9 and pHDME were nick-translated in the presence of [^{3}H]dCTP and TTP to a specific activity of 3×10^7 dpm/μg. In situ hybridization to metaphase chromosomes from a normal male was performed as described elsewhere (Bufton et al. 1986; Buroker et al. 1986).

Results

Hybrid-Cell Panel

Southern transfers of *Hin*dIII-digested DNAs from parental hamster and mouse cell lines and from 22 rodent-human hybrid cell lines were probed with the insert from PHPE-9. Figure 1*A* shows a sample of the resulting autoradiograph. The probe hybridizes to human-specific *Hin*dIII fragments of 2.6 and 3.1 kb in cell lines, such as G35E3, containing human chromosome 8, but not to cell lines, such as G35D2, that lack chromosome 8. These *Hin*dIII fragments

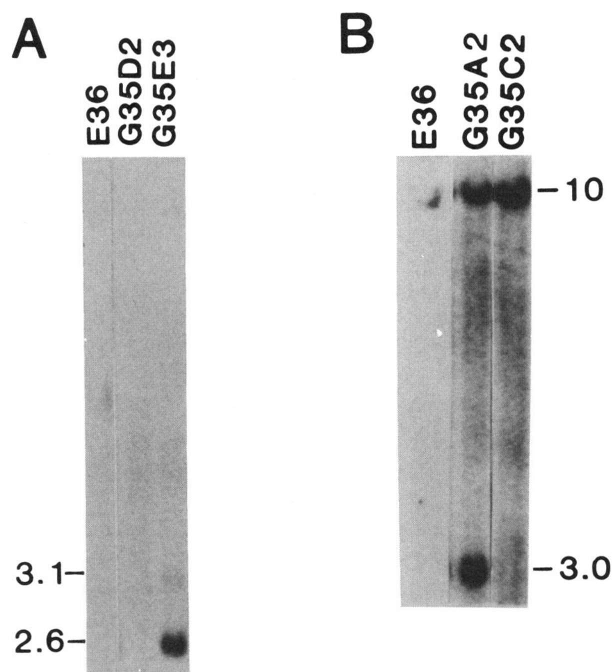

Figure 1 Southern blot of *Hin*dIII digests of DNAs from the hamster parent cell line (E36) and hybrid cell lines probed with (*A*) PHPE-9, a human proenkephalin cDNA clone, and (*B*) pBgBa, a 900-bp *Bgl*II-*Bam*HI fragment of the main exon of the rat prodynorphin gene.

have sizes identical to those observed when PHPE-9 is used to probe Southern transfers of *Hin*dIII-digested human genomic DNA (data not shown). We attribute the high intensity of the 2.6-kb band relative to that of the 3.1-kb band to the fact that 84% of the cDNA probe is homologous to the 2.6-kb band, which contains exon 4, whereas only 16% is homologous to the 3.1-kb band, which contains exon 3. As summarized in table 1, these results are completely concordant only with the presence of human chromosome 8.

The blot shown in figure 1*A* was reprobed with the insert from the rat prodynorphin cDNA probe pBgBa. As shown in figure 1*B*, the probe hybridizes to a human-specific *Hin*dIII fragment of ~3 kb in cell lines, such as G35A2, that contain human chromosome 20. The size of this *Hin*dIII fragment is identical to that observed when pBgBa is used to probe Southern transfers of *Hin*dIII-digested human genomic DNA (data not shown). The 10-kb *Hin*dIII fragment seen in cell lines derived from hamster arises from cross-hybridization of the rat cDNA probe with hamster genomic sequences. As summarized in table

Table 1

Correlation of the Presence of the Proenkephalin Gene and Specific Human Chromosomes in 22 Rodent-Human Hybrids

Human Chromosome	Proenkephalin Gene/ Chromosome Retention				Discordant
	+/+	−/−	+/−	−/+	
1	3	6	6	3	9
2	4	10	6	2	8
3	3	9	6	4	10
4	7	8	3	3	6
5	5	8	5	4	9
6	5	4	5	6	11
7	5	8	5	4	9
8	10	12	0	0	0
9	4	7	6	2	8
10	7	9	3	3	6
11	3	5	6	6	12
12	3	6	6	5	11
13	2	7	6	5	11
14	4	6	3	6	9
15	4	8	6	4	10
16	7	8	2	4	6
17	3	9	6	4	10
18	2	7	5	4	9
19	7	2	1	10	11
20	7	5	3	5	8
21	6	7	4	5	9
22	4	6	6	6	12
X	4	0	0	6	6
Y	1	12	8	0	8

NOTE.—Data are number of hybrid clones. DNA from a panel of hybrid cell lines was analyzed for the presence of the proenkephalin gene as shown in fig. 1*A*. For designation of discordancies, hybrids either having a rearranged chromosome or in which the chromosome was present in <15% of the cells were excluded from analysis.

2, these results are completely concordant only with the presence of human chromosome 20.

In Situ Hybridization

Representative, sequentially stained metaphase spreads from a normal male were probed with the [^{3}H]-labeled human proenkephalin probe PHPE-9 and with the human prodynorphin probe pHDME. The silver grains revealing hybridization are observed with standard staining, and the particular chromosomes to which the probe has hybridized are identified by their fluorescent R-banding pattern. Figures 2 and 3 summarize the results obtained with the proenkephalin and prodynorphin probes, respectively. Each dot in the histograms represents a silver grain observed over a specific chromosomal region. With the proenkephalin probe, 20 of 100 metaphases examined had a grain localized to the region 8q22→q24, with minor clusters of grains on 2q and 20q. We lack sufficient data to indicate whether these minor clusters are reproducible or whether they represent random background fluctuations. If the former is true, it would suggest that under the stringency conditions used for in situ hybridization, proenkephalin cDNA cross-hybridizes with related sequences present at these additional chromosomal locations. However, it should be noted that neither 2q nor 20q corresponds to the localizations of the two other known opioid peptide genes. We also note that the data obtained from probing Southern blots of the hybrid-cell panel (table 1) with the same probe at higher stringency provides no evidence for proenkephalin gene localization to either chromosome 2 or chromosome 20.

Table 2

Correlation of the Presence of the Prodynorphin Gene and Specific Human Chromosomes in 22 Rodent-Human Hybrids

Human Chromosome	Prodynorphin Gene/Chromosome Retention				Discordant
	+/+	−/−	+/−	−/+	
1	3	5	7	3	10
2	2	7	9	2	11
3	5	8	6	2	8
4	5	4	7	5	12
5	6	7	6	3	9
6	7	5	5	4	9
7	5	5	7	4	11
8	6	7	6	3	9
9	5	7	6	1	7
10	5	5	7	5	12
11	5	4	7	4	11
12	5	7	6	2	8
13	3	5	8	4	12
14	7	5	4	5	9
15	3	5	9	5	14
16	8	7	4	3	7
17	4	7	7	2	9
18	4	7	6	1	7
19	10	1	2	7	9
20	11	9	0	0	0
21	6	5	4	4	8
22	7	7	5	3	8
X	5	0	0	5	5
Y	0	9	11	1	12

Note.—Data are number of hybrid clones. DNA from a panel of hybrid cell lines was analyzed for the presence of the prodynorphin gene as shown in fig. 1*B*. For designation of discordancies, hybrids either having a rearranged chromosome or in which the chromosome was present in <15% of the cells were excluded from analysis.

With the prodynorphin probe (fig. 3), 37 (24%) of 150 metaphases had a grain localized to the region 20p12→pter; the remainder of the grains were randomly distributed over the chromosomes.

Search for RFLPs

In the case of proenkephalin, we initially tested the cDNA clone PHPE-9 for its ability to reveal RFLPs. Probing of Southern transfers from panels of six to nine unrelated individuals' DNAs digested with *Bam*HI, *Bgl*II, *Bst*EII, *Eco*RI, *Hin*dIII, *Kpn*I, *Msp*I, *Pst*I, *Pvu*II, *Rsa*I, *Sac*I, and *Taq*I failed to reveal any RFLPs. To extend the search for RFLPs in the proenkephalin region, we tested the ability of a 16-kb genomic clone, λEnk1, to reveal RFLPs. Since λEnk1 contains sequences homologous to human repeats (Comb et al. 1983), we prehybridized the labeled probe with a vast excess of nonradioactive, sonicated human DNA to compete out hybridization of the repeats on the blot and to allow visualization of low- and single-copy sequences. We tested this probe against panels of six to nine unrelated individuals' DNAs digested with 20 restriction enzymes; in addition to the 12 enzymes previously used with the cDNA probe, these included *Bst*NI, *Hae*III, *Dra*I, *Hinf*I, *Bgl*I, *Asp*700, *Bcl*I, *Xba*I, and *Eco*RV. Using these 20 enzymes, we failed to find an RFLP in a total of 704 bp screened.

In our initial screening with the prodynorphin cDNA probe, we also failed to find an RFLP. However, when used as a probe after prehybridization with nonradioactive human DNA, the genomic clone λHDG1 revealed, in addition to several constant fragments, a two-allele *Taq*I RFLP with allelic frag-

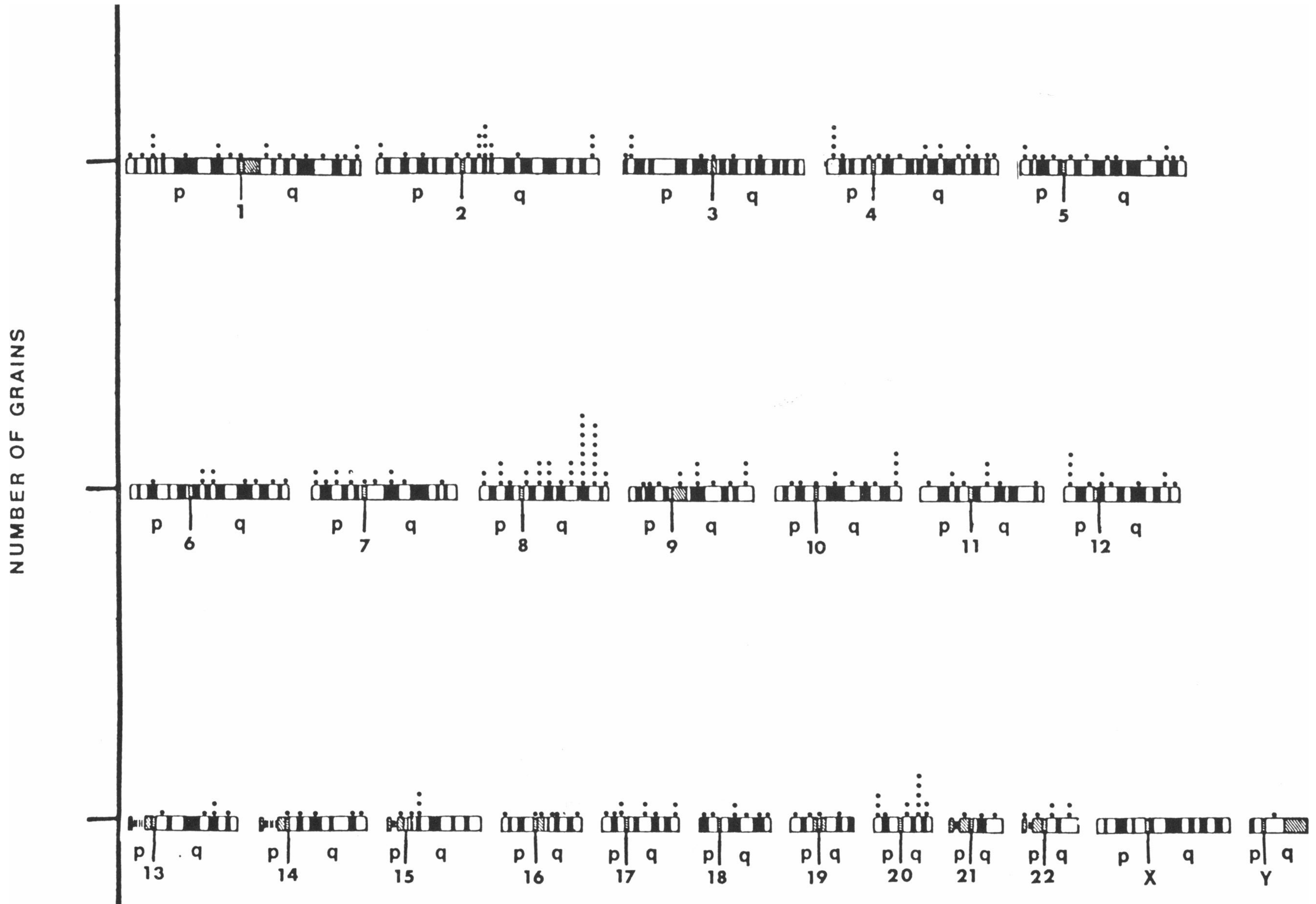

Figure 2 Distribution of silver grains from in situ hybridization of probe pHPE-9 to the chromosomes of a normal male. Each dot represents a grain observed over a specific chromosomal region. Twenty of 100 cells had a grain localized to the region 8q22→qter.

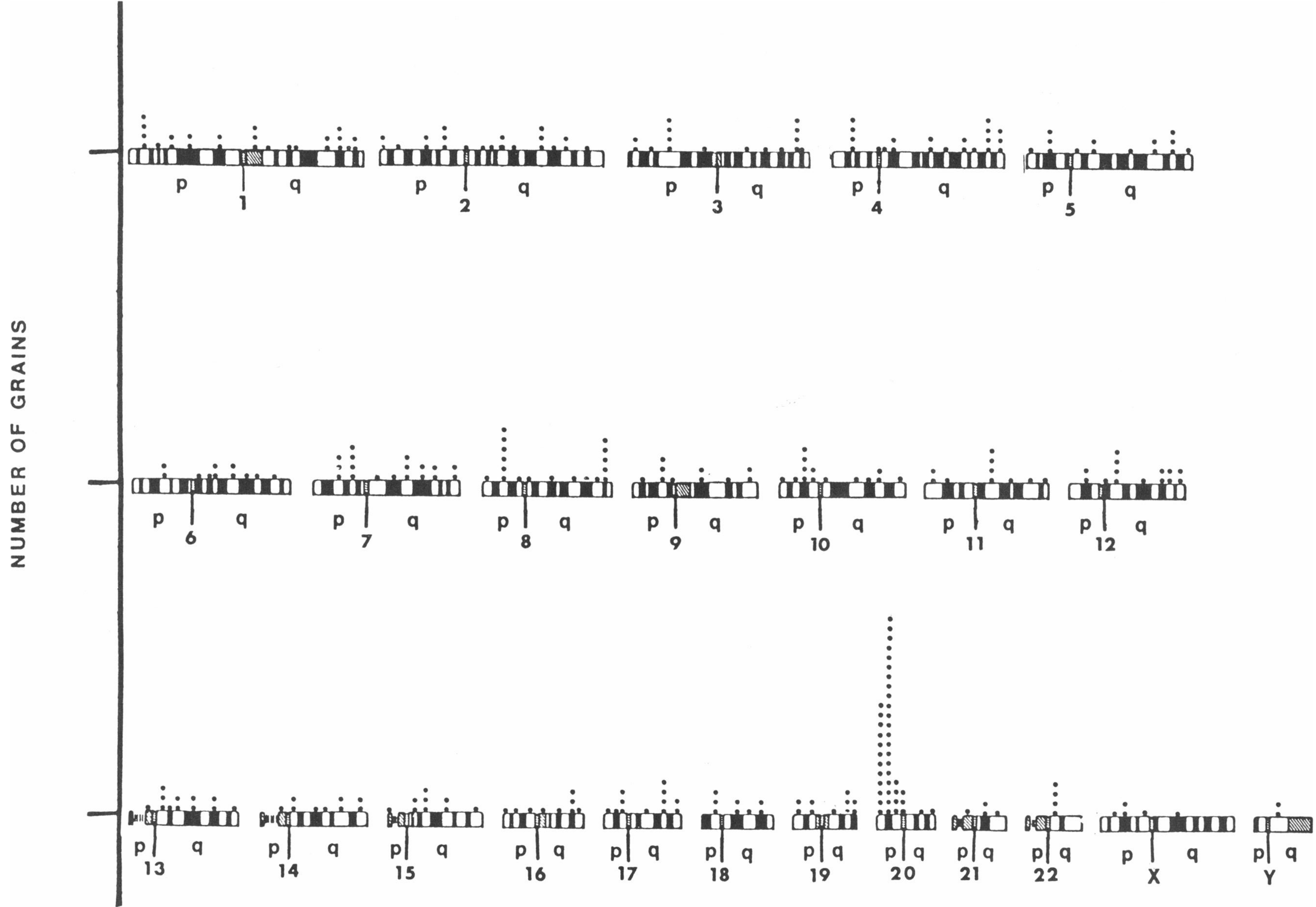

Figure 3 Distribution of silver grains from in situ hybridization of probe pHDME to the chromosomes of a normal male. Each dot represents a grain observed over a specific chromosomal region. Thirty-seven of 150 cells had a grain localized to the region 20p12→pter.

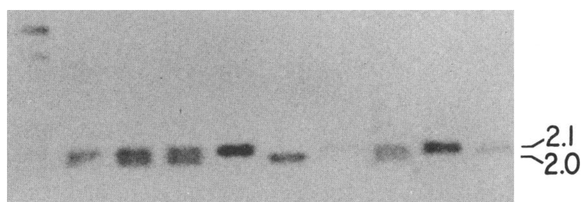

Figure 4 Southern blot of *Taq*I-digested DNAs from nine unrelated individuals probed with a 3-kb *Eco*RI fragment of λHDG-1, illustrating an RFLP with allelic fragments of 2.0 and 2.1 kb. The leftmost lane contains size markers.

ments of 2.1 and 2.0 kb (not shown). In a study of 37 unrelated European Caucasians, the frequency of the minor 2.0-kb allele was .3. The same RFLP, without the constant fragments, was detected with a 3.0-kb *Eco*RI fragment of λHDG1 (fig. 4). The same probes also detect a two-allele *Msp*I RFLP with 2.7- and 2.8-kb allelic fragments. Because the *Taq*I and *Msp*I RFLPs are in complete linkage disequilibrium (data not shown) and because the differences between sizes of allelic fragments are identical, it is likely that these RFLPs reflect an insertion/deletion event rather than a site polymorphism.

Discussion

Of the three known human opioid precursor genes—i.e., proopiomelanocortin, proenkephalin, and prodynorphin—only proopiomelanocortin previously has been mapped to a human chromosome; it resides on the short arm of chromosome 2 (Owerbach et al. 1981; Zabel et al. 1982). Despite the structural similarities between proopiomelanocortin, proenkephalin, and prodynorphin genes (Horikawa et al. 1983), we now find that they reside on different chromosomes. It has been suggested that the opioid precursor genes may have evolved by means of gene duplication (Comb et al. 1983). If this is correct, the dispersed chromosomal locations of these genes in humans indicates that the duplication of the ancestral sequence that gave rise to proenkephalin and proopiomelanocortin predated their dispersion.

Our finding of an RFLP revealed by λHDG1 will allow the placement of the prodynorphin gene on the human genetic linkage map of chromosome 20. Our failure to find an RFLP after screening 704 bp of the proenkephalin gene indicates that placement of that gene on the linkage map of chromosome 8 will require further work. However, when a sufficiently dense genetic-linkage map of the distal half of the long arm of chromosome 8 becomes available, it will be possible to use the RFLPs constituting such a map to search for a disease linkage. Ruling out linkage to such a set of marker loci would be tantamount to ruling out proenkephalin as a candidate gene without requiring a closely linked RFLP. Although a detailed map of the distal half of chromosome 8q is not yet available, several RFLPs have been mapped to this region (Baas et al. 1985; Haluska et al. 1987; Simon et al. 1987) and probes that detect additional RFLPs are being isolated from chromosome 8–specific libraries (Wood et al. 1986). The finding of a linkage between marker loci on 8q22-24 and a disease possibly related to a proenkephalin defect would motivate a more extensive search for RFLPs closely linked to the proenkephalin gene.

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