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Localization of the Gene for Pigment Epithelium-Derived Factor (PEDF) to Chromosome 17p13.1 and Expression in Cultured Human Retinoblastoma Cells

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The gene for pigment epithelium-derived factor (PEDF) was localized to chromosome 17 by the analysis of three independent somatic cell hybrid panels. Fluorescence *in situ* hybridization shows a specific hybridization signal at the terminal portion of the short arm of chromosome 17. PCR analysis of somatic cell hybrids containing specific regions of 17 was subsequently used to sublocalize PEDF to 17p13.1–pter. PEDF thus maps to a region containing a number of cancer-related loci and thus must be considered a candidate gene for these cancers. Preliminary studies with cultured human Y79 retinoblastoma cells indicate that expression of PEDF is associated with relatively undifferentiated, proliferating cells rather than their differentiated, slow-growing counterparts. This and the fact that the PEDF protein can act as a potent neurotrophic differentiating agent suggest that PEDF is linked to proliferative events that terminate in final phenotypic determination within specific cell lineages. © 1994 Academic Press, Inc.

INTRODUCTION

Pigment epithelium-derived factor (PEDF), originally identified in conditioned medium of cultured human fetal retinal pigment epithelial (RPE) cells (Tombran-Tink and Johnson, 1989), induces extensive neuronal differentiation in human Y79 retinoblastoma cells, a neoplastic counterpart of normal retinoblasts (Tombran-Tink *et al.*, 1991). These changes include the extension of a complex meshwork of neurites and expression of neuronal marker molecules such as neuron-specific enolase and neurofilament proteins. Since purified PEDF, RPE-conditioned medium, and soluble components of bovine retina interphotoreceptor matrix (IPM) (Tombran-Tink *et al.*, 1992) all contain similar neurotrophic activity, we have suggested that PEDF is synthesized by RPE cells and secreted into the IPM, where it could influence development/differentiation of the neural retina (Steele *et al.*, 1993).

We have recently cloned the human PEDF gene and found it to be a member of the serpin gene family (Steele *et al.*, 1993). Serpins are a group of serine protease inhibitors (Carrell and Travis, 1985), some of which have also been reported to exhibit neurotrophic activity (Monard, 1988). We now report that the PEDF gene maps to 17p13.1–pter, a region of a cluster of cancer genes. Provocatively, we also have found PEDF to be highly expressed in undifferentiated human Y79 retinoblastoma cells but absent or markedly downregulated in their differentiated counterparts.

MATERIALS AND METHODS

Chromosomal localization: Southern hybridization. A panel of 25 well-characterized human–hamster somatic cell hybrid DNAs was purchased from Bios Laboratories (New Haven, CT). Hybrids were characterized by karyotype analysis (Carlock *et al.*, 1986), and the chromosome content of current passages of each cell line was determined by Giemsa banding analysis of 20 metaphases. DNA samples from 4 individuals were subjected to restriction fragment length analysis using a number of restriction enzymes to establish a polymorphism within the PEDF gene (data not shown). Only *Rsa*I was found to be positive in this regard. For Southern hybridization, DNA samples were digested with *Rsa*I, and 8 μg of each digested DNA sample was fractionated by electrophoresis in a 1% agarose gel and transferred onto neutral nylon membranes (Biodyne A; Pall Corp.).

Two 20-mer PCR primers were synthesized from the middle of the translated region of the previously described PEDF cDNA sequence (Steele *et al.*, 1993). The primer pair PEDF 353 (sense, 5'-CTGGGA-GCGGACGAGCGAAC-3') and PEDF 354 (antisense, 5'-TGGGA-CAGTGAGGACCGCC-3') amplifies a 667-bp product from the PEDF cDNA. PCR amplification was carried out for 30 cycles (each cycle: 94°C, 1 min; 55°C, 1 min; and 72°C, 1 min). Fifty nanograms of the amplified product was labeled by random priming using a random Prime-it kit purchased from Stratagene (La Jolla, CA). Unincorporated nucleotides were removed using Stratagene Cloning Systems Nuctrap Push columns. The nylon filters were prehybridized for 30 min at 65°C in QuikHyb solution (Stratagene, LaJolla, CA) followed by hybridization for 1 h at 65°C in the same solution supplemented with 1×10^6 cpm/ml radiolabeled PEDF probe. Posthybridization washes were performed for 30 min at 25°C using 2× SSC/0.1% SDS

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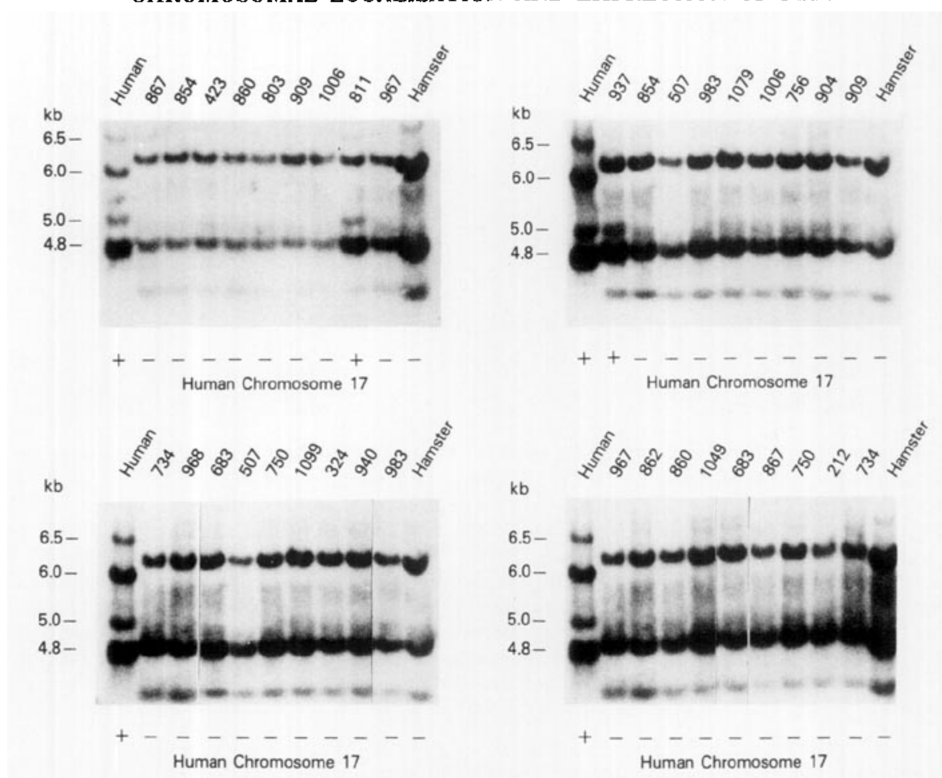

FIG. 1. Southern blot analysis of DNAs from 25 different human–hamster somatic cell hybrids. Each lane contains 8 μg of DNA digested with *Rsa*I. The blot was hybridized with a 667-bp human PEDF cDNA PCR fragment. Human parental cell DNA and hamster cell DNA are indicated. The molecular sizes indicated on the left correspond to the positions of the hybridization bands from human genomic DNA. Numbers at the top refer to different hybrid cell lines. The presence (+) or absence (−) of human chromosome 17 is indicated at the bottom.

and 30 min at 65°C in 2× SSC/0.1% SDS. Filters were exposed to Hyperfilm-MP (Amersham, IL) with an intensifying screen.

Chromosomal localization: PCR assay. DNAs from human–rodent somatic cell hybrid panels (1 and 2) were purchased from the NIGMS Human Genetic Mutant Cell Repository at the Coriell Institute for Medical Research (Camden, NJ). PCR and chromosomal localization studies (Sambrook *et al.*, 1989) were performed using PEDF primers 1 and 2 and hybrid DNAs from NIGMS panel 1. PEDF primers 1 and 2 were designed from the 3′ translated region of the PEDF cDNA using OLIGO primer analysis software (National Biosciences, Plymouth, MN). PEDF 1 (sense, 5′-CACCTTAACCAGCCTTTCATCTTC-3′) and PEDF 2 (antisense, 5′-AACCTTACAGGGGCAGCCTTCG-3′) amplified a 179-bp product from the PEDF cDNA and human genomic DNA. The PCR reaction contained 10 m*M* Tris–HCl (pH 8.3), 50 m*M* KCl, 80 μ*M* of each dNTP, 1 m*M* $MgCl_2$, 200 ng of each primer, 100 ng of genomic DNA, and 1.25 units of Amplitaq (Perkin Elmer Cetus). Amplification was carried out for 40 cycles (each cycle consisting of 94°C, 1 min; 57°C, 1 min; and 72°C for 2 min).

For panel 2, two additional 24-mer PCR primers, PEDF 498 and 499, from the 3′-untranslated region of the PEDF cDNA were designed. Partial purification and desalting of this primer pair were accomplished by passing the oligonucleotide through a Sephadex G-25 column. The primer pair PEDF 498 (sense, 5′-TATCCCAGTTTA-ATATTCCAATAC-3′) and PEDF 499 (antisense, 5′-TTGTATGCA-TTGAAACCTTACAGG-3′) defines a 99-bp domain in the 3′ noncoding region of the human PEDF cDNA sequence. PCR was performed in a 100-μl reaction containing 10 m*M* Tris–HCl (pH 8.3), 50 m*M* KCl, 100 μ*M* of each dNTP, 1.5 m*M* $MgCl_2$, 200 ng of genomic DNA from each hybrid, 200 ng of PEDF primers (498, 499) and 1.25 units of Amplitaq (Perkin Elmer Cetus). DNA was subjected to preamplification heating at 94°C for 10 min, after which amplification was carried out for 20 cycles. Each cycle consisted of 1 min denaturation at 94°C, 1 min annealing at 47°C, and 2 min extension at 72°C. A final extension was carried out at 72°C for 7 min. One microliter of the amplified product was reamplified using the above procedure.

Fluorescence in situ hybridization (FISH). A 7-kb fragment of the PEDF gene from a cosmid clone (jt101) was miniprepped, the plasmid was purified (Quiagen plasmid protocol; Quiagen Inc., Chatsworth, CA) and digested with *Not*I (Gibco BRL, Gaithersburg, MD), and the insert size was determined on a 0.5% agarose gel. FISH was performed at Bios Laboratories after the PEDF probe was labeled by nick translation with dUTP digoxigenin (Lichter *et al.*, 1990). A chromosome 17 centromeric-specific probe, D17Z1 (Oncor, Gaithersburg, MD), which hybridizes to a 171-bp α-satellite tandem repeat, was used as a marker in a cohybridization study. The D17Z1 probe was nick translated and labeled with biotin dATP. The PEDF genomic DNA probe was detected using antidigoxigenin–fluorescein-conjugated FAB fragments; D17Z1 was detected with avidin-conjugated fluorescein–isothiocyanate (FITC). Chromosomes were counterstained with propidium iodide. Eight separate hybridizations were performed for each probe.

Subchromosomal localization: PCR assay. DNAs from a deletion mapping panel of eight somatic cell hybrids containing different regions of human chromosome 17 (Guzzetta *et al.*, 1992) were used to further sublocalize the PEDF gene. PCR conditions for panel 1 were used in this assay, including primers 1 and 2. Up to 600 ng of DNA for the chromosome 17 HO-11 hybrid was used in the PCR assay.

Expression analysis: Northern hybridization. For RNA extraction, human Y79 retinoblastoma cells were grown in suspension culture under the following three conditions: (A) medium (MEM, Mediatech) containing 15% fetal bovine serum (Y79-control); (B) serum-free, defined medium (MEM, Y79-SF); (C) serum-free, defined medium containing 5% bovine soluble interphotoreceptor matrix components (Y79-IPM) as previously described (Tombran-Tink *et al.*, 1992). Under a fourth condition (D), the cells were stimulated with 5% bovine IPM for 1 week in suspension culture and then attached and allowed to differentiate for 10 days (differentiated Y79) as previously described (Tombran-Tink *et al.*, 1992). Approximately 95% of these cells differentiate into a neuronal phenotype under this condition. Cells maintained under conditions A–C are morphologically undifferentiated clusters that remain in suspension. Cells under all four condi-

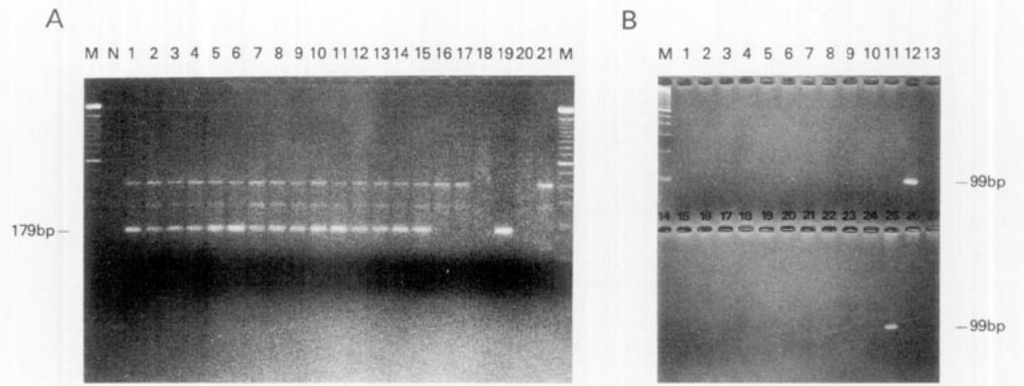

FIG. 2. (**A**) PCR analysis of a panel of somatic cell hybrids (NIGMS Mapping Panel 1) carrying various human chromosomes. The assay detects a single amplification product of 179 bp using primers 1 and 2 from the 3′ region of the PEDF cDNA sequence. **M,** size marker, 100-bp BRL DNA ladder; **N,** negative control without genomic DNA. Lanes **1–18,** cell hybrids—sequentially these are GM/NA 09925, 09926, 09927, 09928, 09929, 09930A, 09931, 09932, 09933, 09934, 09935A, 09936, 09937, 09938, 09940, 10324, 10567, and 10611. Lane **19,** human genomic DNA; lane **20,** hamster genomic DNA; lane **21,** mouse genomic DNA. Only hybrids 1–15 and the human genomic DNA containing human chromosome 17 yield the 179-bp PCR amplification product. (**B**) PCR analysis of a human rodent somatic cell hybrid panel (NIGMS Mapping Panel 2) using PEDF primers 498 and 499 from the 3′ noncoding region of PEDF cDNA sequence; detection is of a single product of 99 bp. **M,** 100-bp BRL DNA ladder. Lanes **1–24,** genomic DNA from 24 different human–rodent hybrid cell lines—sequentially these are NA 06317, 06318B, 07299, 10114, 10115, 10156B, 10253, 10323, 10449, 10478, 10479, 10498, 10567, 10611, 10629, 10791, 10826B, 10868, 10888, 10898, 10926B, 10927A, 11010, and 11418. Lane **25,** human genomic DNA; lane **26,** mouse genomic DNA; lane **27,** hamster genomic DNA. Only the GM/NA 10498 hybrid (lane **12**) and the human genomic DNA (lane **25**) yield the 99-bp PCR amplification product.

tions were harvested at a total of 17 days in culture and poly(A)$^+$ RNA extracted by the method of Sambrook *et al.* (1989).

Human fetal RPE cells were kindly provided by Dr. Dean Bok (Jules Stein Institute, UCLA). These cells were obtained from 20- to 21-week-old donors and were cultured in SF-defined medium for 6 months prior to isolation of poly(A)$^+$ RNA (Sambrook *et al.,* 1989). Two micrograms of poly(A)$^+$ RNA from each sample was electrophoresed on a 1% formaldehyde denaturing gel, transferred onto neutral nylon membrane, UV cross-linked, and hybridized with the PCR-amplified 667-bp PEDF cDNA probe. Hybridization procedures were similar to those used for Southern hybridization of somatic cell hybrid panels.

RESULTS

Chromosomal localization: Southern hybridization. On Southern blots, somatic cell hybrid DNAs were scored for the presence or absence of human-specific hybridization with the PEDF 667-bp PCR fragment. Figure 1 shows blots of *Rsa*I-digested DNA from 25 different human–CHO hybrid cell lines hybridized to the PEDF cDNA probe. This probe identifies 6.5-, 6.0-, 5.0-, and 4.8-kb human restriction fragments, as can be seen in the human genomic DNA control lanes in each blot. Of the 25 somatic cell hybrids examined, only 2 contained a PEDF-specific band of an approximate 5.0-kb molecular size. These hybrids, 811 and 937, are the only ones containing human chromosome 17. A positive correlation therefore is observed between the presence of this band and human chromosome 17.

Chromosomal localization: PCR assay. Using PEDF primers 1 and 2, a 179-bp product is amplified from human genomic DNA (Fig. 2A, lane 19) but is clearly absent from hamster and mouse genomic DNA (lanes 20 and 21). In a human–rodent somatic cell hybrid panel (NIGMS MAP 1), the 179-bp product is observed in somatic hybrids 1–15, which contain a number of human chromosomes, including chromosome 17, but not in hybrids 16–18, which do not contain chromosome 17 (Fig. 2A). A second panel, NIGMS MAP 2, also was investigated using PEDF primers 498 and 499, which yield a 99-bp product (Fig. 2B). Each hybrid of this panel contains only one human chromosome. Of the 24 hybrids tested, only GM/NA 10498 (Fig. 2B, lane 12) was positive and was the only one to contain human chromosome 17. Amplification was seen with human genomic DNA (lane 25) but not with mouse and hamster DNAs (lanes 26 and 27, respectively).

Subchromosomal localization: FISH. Fluorescence *in situ* hybridization analyses of eight metaphase chromosome spreads using a digoxigenin-labeled PEDF probe revealed that 50% of the cells demonstrated specific fluorescent signals on chromosome 17 (Fig. 3). The identity of the chromosome and its specificity were confirmed since the PEDF probe clearly cohybridized with D17Z1, a probe specific to α-satellite tandem repeats in the centromere of chromosome 17. Of importance as well is that, in each case, the genomic probe gave a specific hybridization signal at the terminal end of the short arm of chromosome 17 (region 13.1).

Subchromosomal localization: PCR assay. The use of human–rodent cell hybrids containing defined regions of chromosome 17 (Guzzetta *et al.,* 1991; 1992) allows accurate subchromosomal localization of the PEDF gene (Figs. 4A–4C). An idiogram of the hybrids used is given in Fig. 4D. PCR primers 1 and 2, which yield a 179-bp product, again were used in this study. In Fig. 4A, the PCR amplification product was seen with four of these hybrids: MH22-6 contains the entire chromosome;

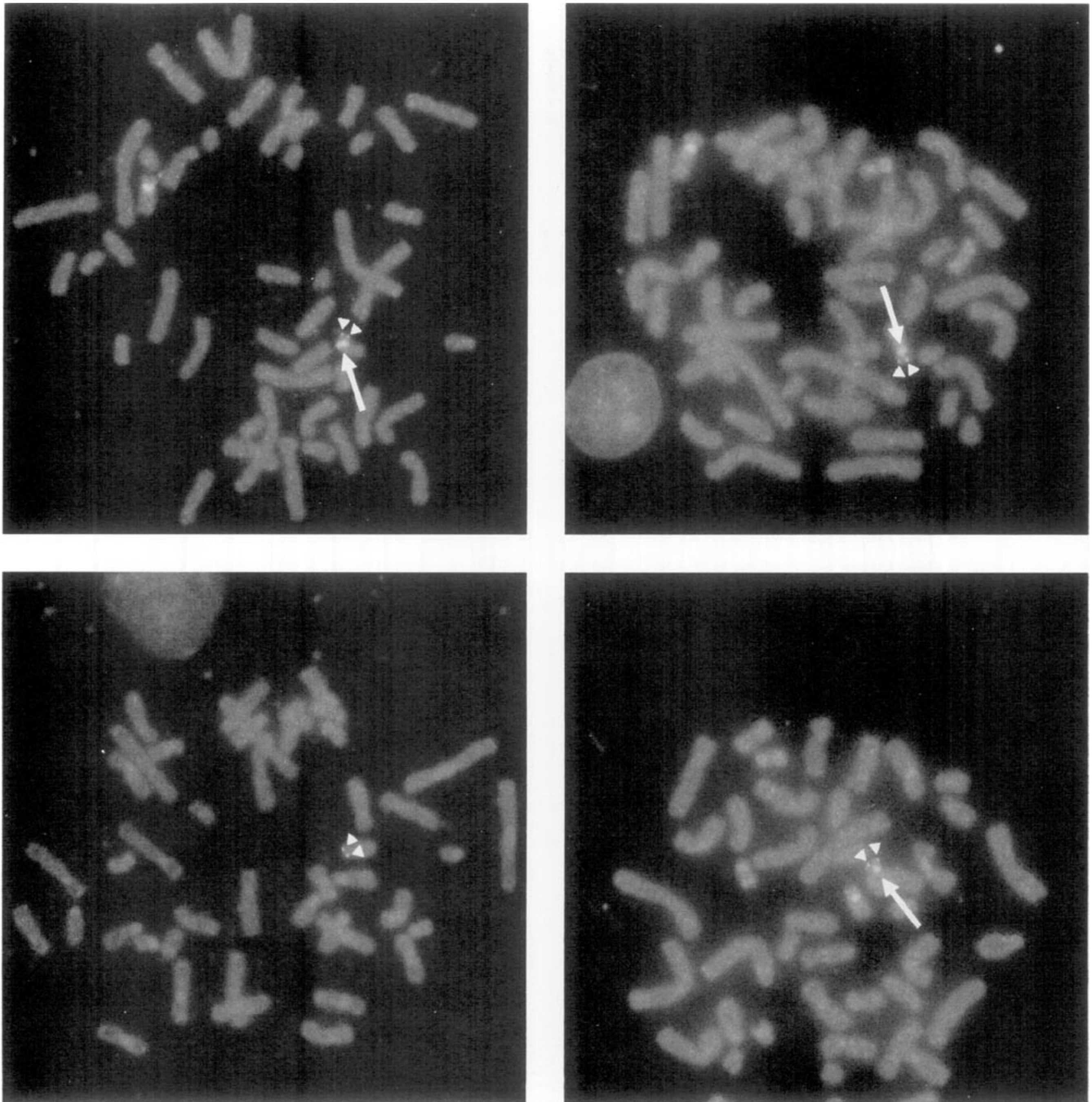

FIG. 3. Localization of the PEDF gene by *in situ* fluorescence hybridization using human metaphase chromosomes and dUTP digoxigenin-labeled PEDF genomic DNA. Arrows indicate hybridization of a biotinylated chromosome 17 centromere-specific probe (D17Z1). Arrowheads indicate localization of the PEDF genomic DNA to the terminal portion of the short arm of chromosome 17.

DHA-4 has a deletion in the p11.2 region; 88-H5 and 357-2D retain much of the p arm, including the 13.1 region (see Fig. 4D). In Fig. 4B, two hybrids are seen to be negative: L(17n)C, which contains only 17q; and MH41, which has only the distal q arm. The hybrid 12.3B, however, which retains 17p and the proximal q region, is positive. Finally and of greatest significance, Fig. 4C shows that the hybrid HO-11, which is missing only the 17p13.1 to 17pter region, is negative. Together, these data localize the PEDF gene to 17p13.1–pter.

Expression analysis: Northern hybridization. PEDF was originally detected as a secreted protein in the medium of human fetal retinal pigment epithelial (RPE) cells (Tombran-Tink and Johnson, 1989). These cells demonstrate a single 1.5-kb mRNA on Northern blots (Fig. 5, lane 1). Because of the marked neurotrophic effect of PEDF on cultured human Y79 retinoblastoma cells, we also examined the cells for the presence of PEDF mRNA. Surprisingly, undifferentiated Y79 retinoblastoma cells demonstrate a 1.5-kb message even

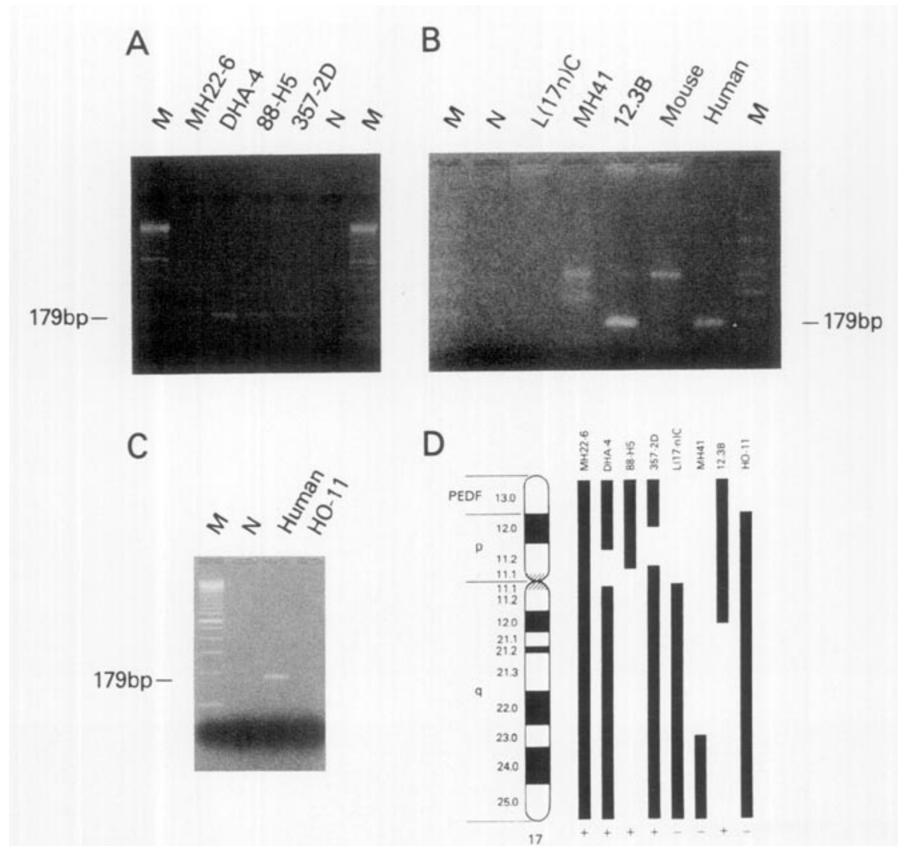

FIG. 4. Regional mapping of the PEDF gene by PCR amplification using somatic cell hybrid panels containing specific regions of chromosome 17. PCR assay detects a single amplification of 179 bp in human genomic DNA using primers 1 and 2 of PEDF. In all panels, "M" refers to the BRL 100-bp DNA ladder; "N" refers to the negative control lacking DNA; mouse or human genomic DNA was used as control as indicated. (A) MH22-6, hybrid retaining the entire chromosome 17; DHA-4, hybrid with p11.2 deletion; 88-H5, hybrid with a translocation retaining 17pter–17p11.2; 357-2D, hybrid with deletion within 17p11.2 extending into 17p12. (B) L(17n)c, hybrid containing only 17q; MH41, hybrid containing only distal q arm; 12.3B, contains p arm and proximal q region. (C) HO-11, hybrid containing a deletion from 17p13.1 to 17pter. (D) Idiogram of human chromosome 17 showing mapping areas of the specific hybrids used. Solid bars indicate the retained area(s) of chromosome 17 in the somatic cell hybrids. The presence (+) or absence (−) of the 179-bp amplified human-specific PEDF PCR fragment in each hybrid cell line is indicated at the bottom.

though little if any PEDF protein can be detected in the medium (unpublished observations). Cells grown in suspension culture for 17 days with 15% fetal bovine serum (Fig. 5, lane 2), SF-defined medium (lane 3), and 5% soluble components of the bovine interphotoreceptor matrix (IPM) (lane 4) all express the PEDF message at substantial but varying levels and all remain morphologically undifferentiated (see Fig. 5, bottom, A). In contrast, induction of a marked neuronal phenotype (bottom, B) and total inhibition of PEDF message (lane 5) are seen in Y79 cells that were treated with soluble components of the bovine IPM for 1 week and then maintained in attachment culture for 10 additional days.

DISCUSSION

Analysis of three independent human–rodent cell hybrid panels and fluorescence *in situ* hybridization were used in the present study to localize the PEDF gene to 17p13.1. PCR analyses of both multichromosomal and monochromosomal somatic cell hybrid panels with two separate pairs of PEDF primers all identified chromosome 17 as the location of the PEDF gene. Fluorescence *in situ* hybridization studies of human metaphase chromosomes confirmed this gross assignment and further localized the gene to the terminal portion of the short arm of 17. Subsequent PCR analyses using a third panel of somatic cell hybrids containing specific deletions of chromosome 17 definitively mapped PEDF to 17p13.1–pter. This is close to the tumor suppressor p53 gene that is involved in the Li-Fraumeni grouping of cancers (Isobe *et al.*, 1986; Levine and Monard, 1990; Malkin *et al.*, 1990). More pertinent to the present study, however, is that deletion mapping studies on 17p provide evidence for the existence of a tumor suppressor gene close to p53 that could be involved in breast cancer (Sato *et al.*, 1990) and several embryologically unrelated neoplasms (Cogen *et al.*, 1990). Further studies with PEDF are especially relevant to tumors such as medulloblastomas (Saylors *et al.*, 1991; Ohgaki *et al.*, 1991) and pediatric primitive neuroectodermal tumors (PNET) (Biegel *et al.*, 1992), which map to 17p but do not seem to involve p53 mutations.

The close chromosomal juxtaposition of the PEDF gene and a number of cancer genes and the fact that PEDF itself is involved in events controlling differentia-

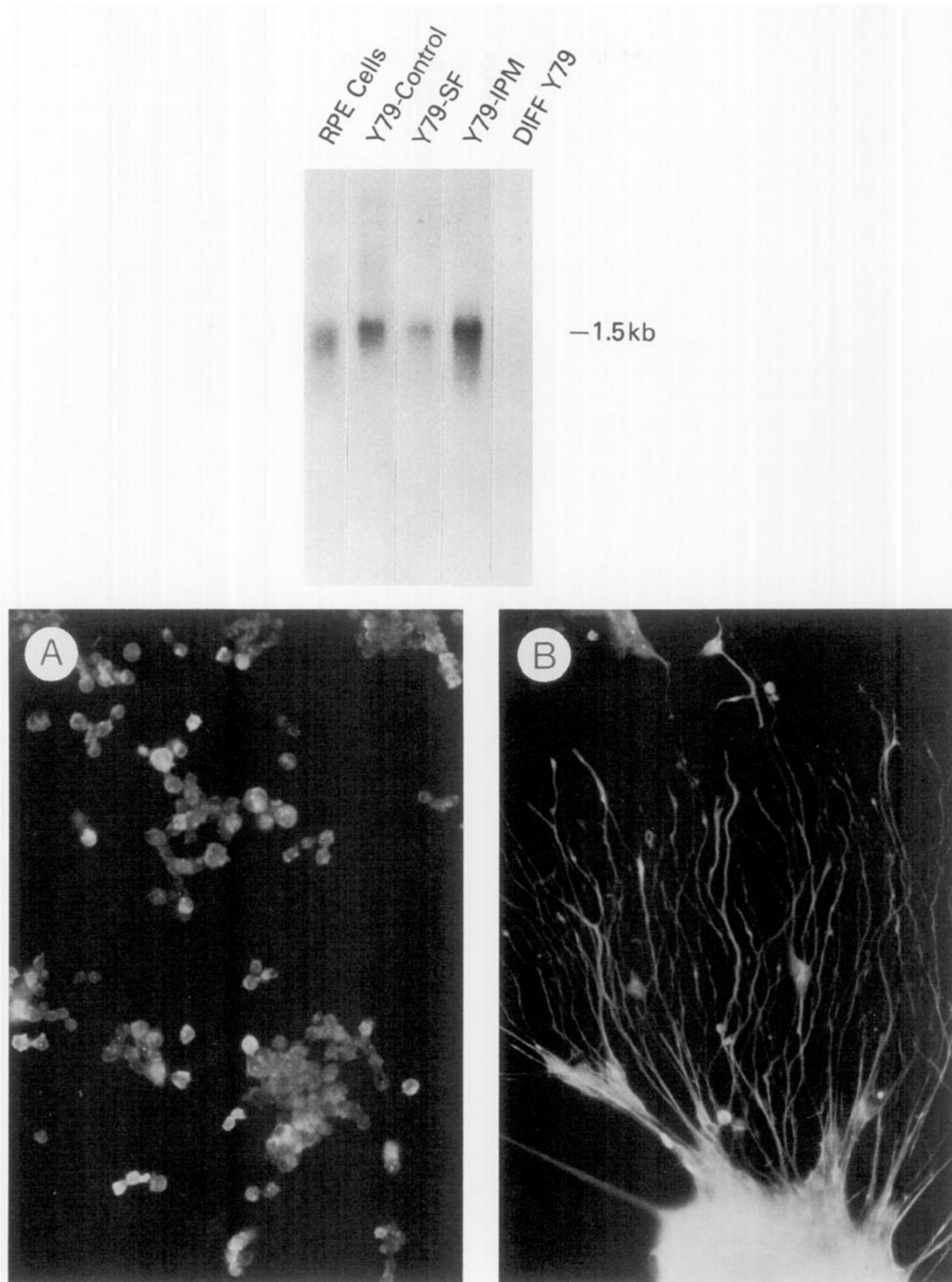

FIG. 5. (**Top**) Northern blot analyses of mRNA samples from cultured human Y79 retinoblastoma cells using a PCR-amplified 667 bp PEDF probe. Two micrograms of poly$(A)^+$ mRNA was applied to each lane as follows: lane **1**, human fetal pigment epithelial cells; lane **2**, undifferentiated Y79 cells in suspension culture grown in serum-containing medium for 17 days; lane **3**, undifferentiated Y79 cells in suspension culture in serum-free medium for 17 days; lane **4**, undifferentiated Y79 cells in suspension culture treated with bovine IPM for 17 days; lane **5**, differentiated Y79 cells grown in suspension culture for 7 days with IPM and an additional 10 days in attachment culture without IPM. (**Bottom, A**) Proliferating, undifferentiated Y79 cells. (**Bottom, B**) Nonproliferating, differentiated cells.

tion (Steele *et al.,* 1993) made it of interest to analyze PEDF expression (i.e., mRNA abundance) in cultured human Y79 retinoblastoma cells, especially under conditions in which a differentiated, neuronal phenotype was either suppressed or highly expressed (Tombran-Tink and Johnson, 1989; Tombran-Tink *et al.,* 1991, 1992; Steele *et al.,* 1993). PEDF mRNA was readily detectable in morphologically undifferentiated Y79 human retinoblastoma cells in suspension culture, where there is relatively rapid growth. On the other hand, mRNA was undetectable in attached, nonproliferating, differentiated cells. This finding appears to be paradoxical if one considers that the PEDF protein induces a highly neuronal phenotype when added to Y79 cell cultures (Tombran-Tink and Johnson, 1989; Tombran-Tink *et al.,* 1991, 1992; Steele *et al.,* 1993). Why do the cells produce PEDF mRNA yet remain morphologically undistinguished and why is PEDF mRNA downregulated in the differentiated cells? Preliminary evidence from Western blot analysis indicates that, although the message is readily detected in the Y79 cells, the PEDF protein is very low in the culture medium. Thus, exogenous PEDF protein would be necessary for expression of a neuronal phenotype. Also, we have previously found that PEDF is maximally effective as a neuronal-inducing agent when administered to rapidly proliferating Y79 cells in suspension culture prior to attachment and differentiation (Tombran-Tink and Johnson, 1989). Because they have been "instructed" by PEDF in suspension, attachment of the cells may give the signal for cessation of proliferation and final differentiation. This scenario actually parallels the well-known phenomenon found in the embryonic retina *in vivo* in which retinoblasts first move to the RPE–retina interface, where they appear to be "instructed" by an unknown factor(s), perhaps PEDF produced and secreted by the fetal RPE cells. After such instruction, the cells can subsequently migrate back to specific strata, where they "attach" and terminally differentiate.

In general, then, by virtue of its map location, PEDF now must be considered a new candidate gene for the constellation of cancer-related loci at 17p. Moreover, its expression/lack of expression in undifferentiated/differentiated human retinoblastoma cells and its potent neurotrophic effect indicate that PEDF may be a normal cell cycle factor involved in a programmed series of events that includes both proliferation and commitment to a final, differentiated phenotype.

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