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Two major findings with respect to the role and regulation of the growth factor pleiotrophin (PTN) were contributed by my laboratory: (1) We expanded our discovery that in the human PTN gene a tissue-specific promoter was generated by the germ line insertion of an endogenous retrovirus 25 million years ago. This is the first report of a retroviral insertion contributing a tissue-specific promoter in a human gene and only the second human gene that was reported to be altered in its expression pattern by retroviral elements. We now report that this element is also inserted in the BRCA-1 locus. (2) In additon, we analyzed the signal transduction pattern of PTN and showed that it works through MAP kinase / PI 3-kinase. Two proteins close to 200 kDa are phosphorylated in response to PTN and are likely candidate receptors for this growth factor.

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INTRODUCTION

Under our proposal we study the role of the growth factor pleiotrophin (PTN). We hypothesize that PTN is an essential, rate-limiting growth factor for PTN-positive breast cancers. Our experiments are designed to address this hypothesis. We were the first laboratory to purify PTN from human cancer cells (MDA-MB 231 breast cancer cell line) [1]. We were also the first laboratory to generate biologically active recombinant PTN [2] and to demonstrate its potential role as a tumor growth and angiogenesis factor [2]. Furthermore, we showed its distinct expression in human breast cancer samples [2] and published the genomic structure of the human PTN gene first [3].

We propose that the secreted polypeptide growth factor pleiotrophin (PTN) plays a major role in the growth and metastasis of breast cancer. This hypothesis is based on the high levels of PTN expression in 60% of tumor samples from breast cancer patients but not in normal tissues and on the biological effects of PTN in selected tumors models. In particular, the activity of PTN on endothelial cells indicates that PTN can serve as a tumor angiogenesis factor and its expression can thus enhance the ability of breast cancer to metastasize.In summary, our studies quoted above as well as the data published by others show:

a. PTN is a secreted growth factor expressed in a number of human breast cancer cell lines and in the majority of human breast cancer samples.

b. PTN stimulates endothelial cells and can act as a tumor angiogenesis factor that promotes tumor invasion and metastasis [4-6].

OVERVIEW OF THE GOALS:
In our studies, we elucidate the role of PTN and the hormonal regulation of its activity, with the ultimate goal to develop novel therapeutic strategies.

In particular,

(1) we study hormonal regulation of PTN
(2) investigate whether PTN expression can support tumor growth
(3) generate mutated PTN protein for functional studies and
(4) target PTN mRNA to repress production of PTN and thus inhibit tumor growth

OVERVIEW OF MAJOR FINDINGS IN THIS REPORT:
In this report cycle, I am pleased to present significant new data of our own on two of the above goals, i.e. goal (1) regulation studies and goal (3) functional studies. Under goal (2) I will discuss experimental data that were published recently by a competing laboratory and use the same design that was proposed under this goal.

goal (1) regulation studies: In our expanded series of experiments which were prompted by the surprise discovery some two years ago that an endogenous human retrovirus (HERV) had inserted itself into the human PTN gene and directly controls tissue-specific expression of this gene. The original data were published in Dec. of 1996 in PNAS and a copy of the paper is included in the appendix [6]).

Due to this exciting finding of a functional retroviral regulator in a human gene, we focussed further efforts in this area and moved into two distinct directions: (i) we elucidated the structure of the insert further and report on this here. Our present data indicate that the HERV-element in the PTN gene inserted in parallel into the BRCA-1 gene locus and resides in the intron downstream of BRCA-1 exon 2. We speculate that this insertion will confer additional regulation of human BRCA-1 as was observed by us with human PTN. Studies on this were initiated. A paper with that data was accepted in J. Virology (pending some revisions) and the manuscript is included in the appendix. (ii) with a series of promoter/reporter constructs we recently initiated the mutational analysis of the promoter and its hormone sensitivity.

goal (3) functional studies: Here I present data on the signal transduction mechanisms of PTN that we were able to elucidate and publish in the Journal of Biological Chemistry [7]. A copy of the paper is included in the appendix. Furthermore, I discuss data from a competing laboratory that was successful in generating a dominant-negative mutant PTN molecule along the design that we had applied in our experiments [8].

OVERVIEW OF METHODS:

For goals (1) we used gene cloning of the discovered retroviral promoter in the human PTN gene to lay the basis for mechanistic studies of hormonal regulation. The methods are described at the end of the section

For goal (3) we set up signal transduction assays mostly with immunoblotting to detect posttranslational modifications of proteins in the signal transduction cascade.

BODY

Goal (1):
Regulation of pleiotrophin (PTN)

Background:
Hormones and growth factors define the capacity of human breast cancer to grow and metastasize. One of the essential requirements for the development of breast cancer are circulating steroid hormones and one of the most widely used drug therapies of breast cancer with the anti-estrogen tamoxifen is based on this fact. Furthermore, growth factor gene expression can supplement for hormone stimulation and thus contribute to hormone-independent cancer growth as well as to resistance to anti-hormone therapy (reviewed e.g. in [9]).

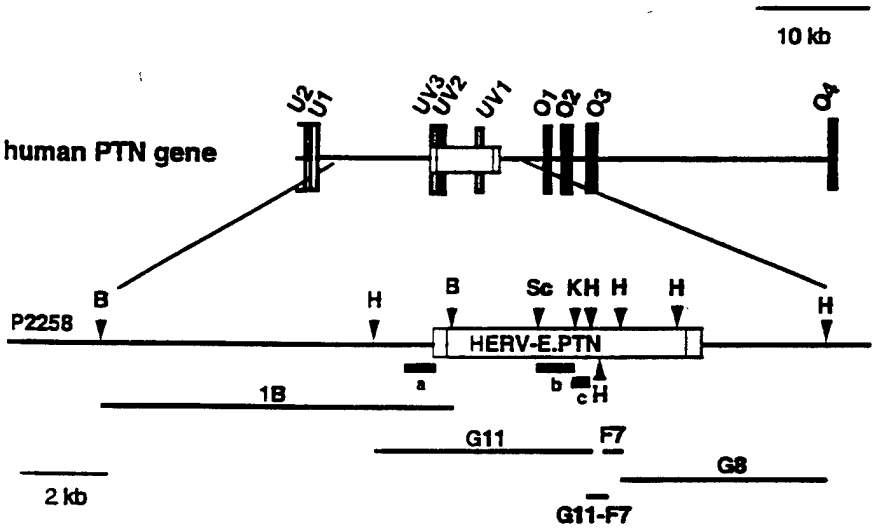
Work accomplished:
Summary: We discovered that in the human PTN gene a tissue-specific promoter was generated by the germ line insertion of a human endogenous retrovirus (HERV) some 25 million years ago. This is the first report of a retroviral insertion contributing a tissue-specific promoter in a human gene and only the second human gene that was reported to be altered in its expression pattern by retroviral elements. In the last report cycle, I described this discovery in detail. A paper published since then is included in the appendix [6]). In this report cycle, the laboratory focused on the evaluation of the precise structure of this insert, its phylogenesis and parallel insertions in other genes. Surprisingly, the same HERV inserted into the PTN gene was also found in the BRCA-1 gene locus in the intron downstream of exon 2. The potential implications for the regulation of human versus murine BRCA-1 prompted us to plan some preliminary studies and will most likely open up a new area of research interest in the laboratory.

Discovery of a retroviral insertion in the human PTN gene:
To elucidate the mechanisms that regulate expression of the human PTN gene, we examined the 5'regions of mRNAs isolated from different tissues by 5'RACE PCR. To our surprise, 5'RACE PCR clones with mRNA from placenta contained novel 5'UTR that are distinct from the previously described 5'UTR in human placental and brain cDNAs. Sequence comparisons revealed that the novel 5'exons contained in the PTN mRNA from placenta are highly homologous to different regions of human endogenous retrovirus (HERV) type C [10-12]. Based on its Glu-tRNA primer binding site specificity and the location within the PTN gene, we named this element **HERV-E.PTN.**

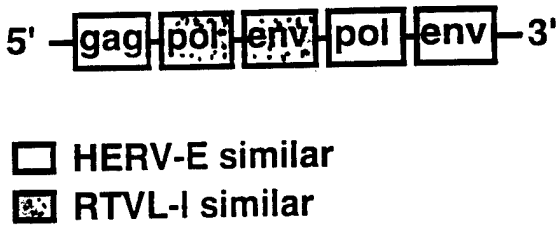
Detailled analysis of the HERV fragment:
Analysis of human genomic DNA revealed that the HERV-E fragment is inserted in sense orientation into the intron region immediately upstream of the ORF of the human PTN gene expanding this region relative to the murine gene (Fig. 1A).

HERV-E.PTN appears to be a recombined viral element based on its high homology (70 to 86 %) in distinct areas to members of two distantly related HERV type C families, HERV-E and RTVL-I. Furthermore, its pseudogene region is organized from 5' to 3' into gag-, pol-, env-, pol, env- similar sequences (Fig. 1B). Interestingly, full length and partial HERV-E.PTN homologous sequences were found in the human X chromosome, the human hereditary haemochromatosis region and the BRCA1 pseudogene (Fig. 2).

Fig. 1. Organization of the human PTN gene and location of the HERV-E.PTN
(A) Mapping and partial restriction map of the HERV-E.PTN.
The PTN ORF exons (O1 to O4), 5'-UTR exons (U1, U2), HERV-derived exons (UV1 to UV3) and the HERV element are boxed. The characterized region of the P1 clone P2258, the BamHI- (1B) and HindIII- (G11, F7, G8) subclones and the PCR clone (G11-F7) is enlarged. Genomic Southern blot probes (a,b,c) and restriction sites are shown (B=BamHI, H=HindIII, Sc=ScaI, K=KpnI).



(B) Cartoon of the structure of HERV-E.PTN based on retroviral pseudogenes.



Similarity of HERV-E.PTN to the human BRCA1 and other gene loci.

Despite some deleted and inserted areas, the entire HERV-E.PTN element is ~80 % homologous to a 9.7 kb region in the human X chromosome (GenBank no. Z83820; PAC clone 215K18) and to a 6.5 kb area in the human hereditary haemochromatosis (hHH) region (GenBank no. U91328). Interestingly, the sequence comparison revealed that the respective regions in the X chromosome and the hHH-region are more homologous between each other than they are to the HERV E.PTN sequence and their similarity is extended upstream and downstream of their HERV-E.PTN homology. This suggests to us that the recombinant HERV-E.PTN element inserted into the human PTN gene en bloc and that the insertion happened more recently. Surprisingly, we found also an 80% homology between the 5'-end of HERV-E.PTN and a newly published 1.3 kb stretch of intronic sequence immediately downstream of exon 2 in the BRCA1 pseudogene (GenBank no. U77841; Fig. 2). Furthermore, dot plot matrix analysis show a higher homology to the 5' end of HERV-E.PTN than to the corresponding region in HERV-E clone 4-1 (Fig. 2). The similar organization of the BRCA1 gene and pseudogene around exons 1A, 1B, and 2 indicates that the HERV-E.PTN-like sequence is inserted into both copies of BRCA1 (Fig. 3).

Fig. 2. Comparison of HERV-E. PTN and the BRCA-1 gene locus

Dot plot matrix analysis of the BRCA1 pseudogene relative to HERV-E.PTN and HERV-E clone 4-1. x axis = BRCA1 pseudogene (GenBank U77841, position 2500 to 4098), y axes are HERV-E.PTN (position 1 to 2000) and HERV-E clone 4-1 (position 1 to 2000). Window size =30; hash value = 2; homology = 90 %

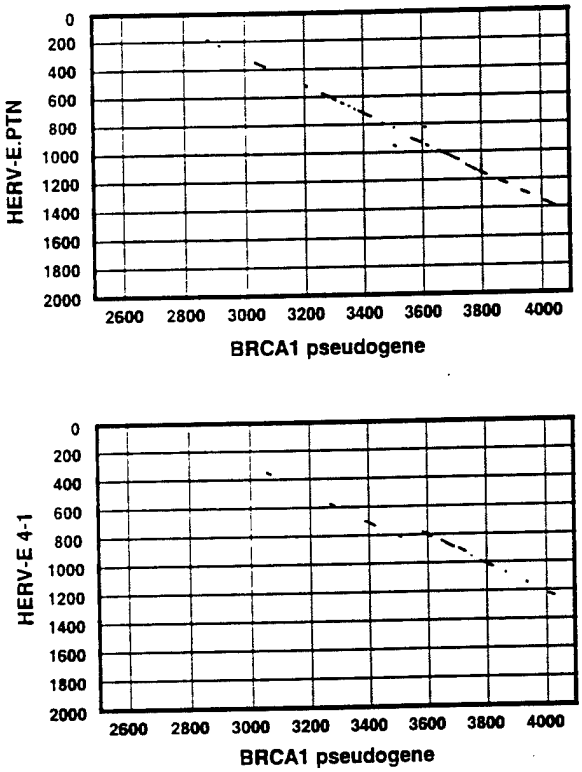
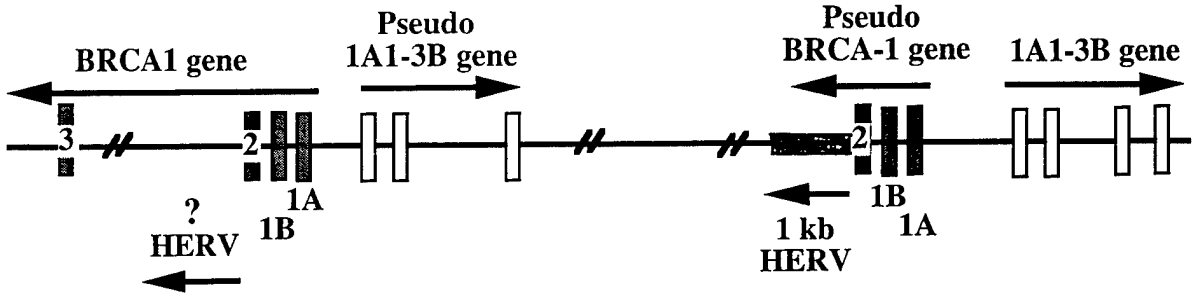


Fig. 3. The HERV-E.PTN homologous region in the BRCA1 locus.
Arrows represent the direction of transcription, grey boxes represent exons from BRCA1 and pseudo BRCA1 genes (1A, 1B, 2 and 3), white boxes symbolize the NBR2 (Next to BRCA1 gene 2; formerly known as pseudogene 1A1-3B; ~30 kb) and NBR1 genes (Next to BRCA1 gene 1, formerly known as gene 1A1-3B). The black box represents the recently sequenced HERV-E.PTN homologous region (1243 nt) in the pseudo BRCA1 gene. The genomic organization, restriction enzyme sites (E= EcoRI, H=HindIII, P= PstI) and the size (kb) of restriction fragments are adapted from the GenBank data base and the published literature[13,14]

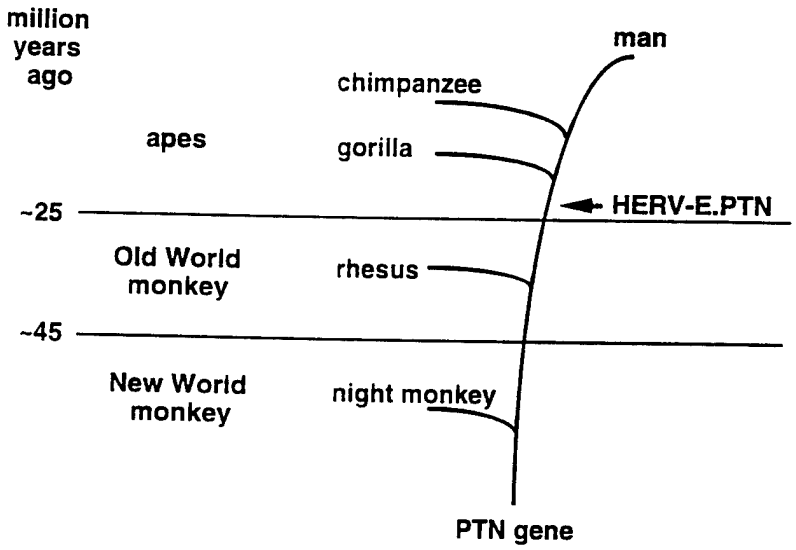


Finally, Southern analyses indicate that the HERV-E.PTN element is present in the PTN gene of human, chimpanzee and gorilla, but not of rhesus monkey suggesting that genomic insertion occurred after the separation of monkeys and apes about 25 million years ago (Fig. 4; see next page).

Analysis of transcription factor binding sites (cis-elements) and transacting factors on the HERV-E.PTN promoter; interaction with hormones.

With a series of promoter/reporter constructs we recently initiated the mutational analysis of the promoter and its hormone sensitivity. These preliminary data are currently being analyzed and compiled.

Fig. 4. Evolutionary tree and proposed time point of the HERV-E.PTN insertion into the PTN gene (most likely also the BRCA-1 locus).



Methods

Human DNA clones, sequencing and data analysis

Clones G-11, G-8 and F-7 were obtained from a HindIII restriction library of the human pleiotrophin (PTN) P1 clone P2258 (Genome Systems, St. Louis, MO) by screening with oligonucleotides deduced from the HERV-derived 5'untranslated exons UV3 (G11 and G8; 5'CTTCACTATCTCGGTGTCTC3') and UV1 (F7; 5'CCCCCCATGCTGTGGTAACTTTA ATAAATACC3') of the human PTN gene (GenBank nos. U71455 and U71456; 32). Clone G11-F7 was generated by PCR with P1 clone P2258 and oligonucleotides deduced from clone G11 (sense; 5'TTACTGGGCACTCTGCC3') and F7 (antisense; 5'TTGTCAGGTCAGG TGGC3') and subcloned into a TA-cloning vector (Invitrogen). Clone 1B was obtained from a BamHI restriction library of clone P2258. After uni- and partial bidirectional cycle sequencing with dye labelled terminator and AmpliTaq DNA polymerase FS (Perkin Elmer, Foster City, CA), BLAST and FastA GenBank searches were performed. The program DNA Strider was used to define open reading frames and restriction sites, Mac Vector to generate dot plot matrix analysis.

DNA isolation, Southern analysis, probes and polymerase chain reaction (PCR)

Genomic DNA from whole blood, tissues or cultured cells was isolated with the QIAamp Blood Kit (Qiagen Inc, Chatsworth, CA), digested with BamHI or HindIII, separated in a 0.7 % agarose gel (10 or 20 µg), transferred to a nylon membrane (Magnacharge; MSI, Westboro, MA) and hybridized in 10 % dextran sulphate supplemented formamid standard solution (17) at 42°C. After hybridization, blots were washed for low stringency hybridization twice with 2xSSC/0.1%SDS, twice with SSC/0.1% SDS for 15 min at 42°C, and once at 62°C for 15 min. For high stringency hybridization two wash steps with 0.1xSSC/0.1% SDS for 25 min at 65°C were added. Hybridizations were performed with various random-primed labelled DNA probes (rediprime, Amersham, Arlington Heigths, Il): probe a: 632 nt long PCR fragment, containing 602 nt cellular sequence upstream of the HERV-E.PTN plus 30 nt 5'LTR sequence (Fig 1A; corresponding to region -11869 to -11238 in (32); probe b: 826 nt long ScaI/KpnI restriction fragment isolated from the P2258 subclone G11 (Fig. 1A position 2439 to 3262).; probe c: 406 nt long KpnI/HindIII restriction fragment isolated from P2258 subclone G11 (Fig. 1A; position 3262 to 3667). PCRs were performed with recombinant Taq polymerase from Life Technologies (Gaithersburg, MD) as recommended by the vendor.

Nucleotide sequence accession number

The HERV-E.PTN sequence reported has been deposited in the GenBank data base (accession number Bankit 172816).

Problems & solutions and Next steps:

Analysis of the transcriptional regulation of PTN by hormones using the newly discovered promoter in the human gene is ongoing. In particular, we hope to define next, where regulatory elements are located and what defines response in certain contexts. For this experiments we generated a series of mutant and deleted promoter/reporter constructs that are being tested in hormone-dependent and independent breast cancer cell lines.

Goal (2):
To study the effect of expression of PTN on the malignant phenotype of PTN-negative breast cancer cells.

Background:
Hormones and growth factors define the capacity of human breast cancer to grow and ultimately to metastasize. One of the essential requirements for the development of breast cancer are circulating steroid hormones and one of the most widely used drug therapies of breast cancer with the anti-estrogen tamoxifen is based on this fact. Furthermore, growth factor gene expression can supplement for hormone stimulation and thus contribute to hormone-independent cancer growth as well as to resistance to anti-hormone therapy (reviewed e.g. in [9]).

Work accomplished:

Transfection of PTN into PTN-negative breast cancer cells:
We have transfected PTN-negative, estrogen-dependent MCF-7 wild-type and T-47D wild-type human breast cancer cells with an expression construct for PTN (see [2] and have generated a series of different cell lines (mass-transfected and some clonal cell lines) expressing PTN. We have tested the cells *in vitro* for their proliferation and colony forming abilities as well as for expression of PTN mRNA and the secretion of protein.

A wide range of expression levels of PTN was achieved in different MCF-7-derived and T-47D-derived cell lines. No gross difference in the *in vitro* phenotype of the cells was observed. No significantly different proliferation on plastic surface or colony forming ability was found. Based on the current data we conclude that PTN is not utilized by T-47D or by MCF-7 cells as an autocrine growth factor. While this work in my laboratory was ongoing, an abstract was published by Dr. Roy Bicknell's and Adrian Harris's laboratory at Oxford University in the UK [15] suggesting that this group had independently followed the same course of study with the same cell lines and design as proposed by me under this goal.

The data were published in 1997 in Cancer Research [16] and show an angiogenic role for pleiotrophin in tumorigenesis of MCF-7 cells. PTN was overexpressed in MCF-7 breast carcinoma cells. The group found that expression of PTN had no effect on in vitro growth but conferred a tumor growth advantage in animals. They also showed that enhanced tumor growth correlated with increased vascular density in the tumors. Furthermore, they showed that endothelial proliferation was induced by supernatants from the transfected MCF-7 cells implicating an angiogenic role for PTN.

I was very pleased that another laboratory confirmed the potential implications of the angiogenic activity of PTN for breast cancer using the model that I had also planned to use in the proposal. I decided that it would not be appropriate to duplicate the animal study that was proposed and planned for 1997. Instead I decided to rather quote the published work of the Harris/Bicknell group for futher reference on this point.

On a more general note, I was disappointed that the Harris/Bicknell laboratory had beaten us with this series of experiments but I felt relieved that we had found out early enough to avoid unnecessary duplication of effort. We have since established a close collaboration with the laboratory exchanging in particular our ribozyme constructs for their use in some of their cell lines found to be spontaneous PTN expressors.

Goal (3):
The function of the different domains of the PTN protein

Background:
The secreted PTN protein contains two distinct cysteine-rich domains (on two separate exons) that contains three and two disulfide bridges respectively. Disulfide bridge formation is required for biological activity of the protein. We hypothesize that defined mutations will generate a protein that can still bind to the receptor but will fail to activate the receptor and can thus serve as an antagonist.

Work accomplished earlier:
We have generated point mutant PTNs that have the N-terminal or the C-terminal cysteine changed to a serine and thus disrupted disulfide bridge formation. We tested the effects of the mutant proteins in transfection assays using expression vectors for the mutant and for wild-type PTN in PTN-responsive SW-13 cells as indicator cells of activity. To our astonishment we found that the N- or C-terminal cysteine mutations affect the activity in transient transfection assays only very little. We concluded from this finding that the N-terminal and the C-terminal disulfide bridge are not essential for stability and activity of the protein when only one of them is destroyed.

A dominant-negative PTN protein:
Late in 1997 the laboratory of Dr. Tom Deuel at Harvard University published a paper [8] that human breast cancer growth is inhibited by a dominant negative pleiotrophin mutant. Dr. Deuel, one of our competitors in this field had told me of this finding earlier on during one of many phone conversations on the progress in the PTN area without revealing the details of the construct. They generated a mutant cDNA that encodes one half of the PTN (a truncated PTN) which they showed to heterodimerize with the endogenous PTN protein. They showed that the mutant gene product blocked PTN-induced transformation of NIH 3T3 cells. Most interestingly, mutant PTN expressed in human breast cancer MDA-MB-231 cells blocked their transformed phenotype. This very aggressive cell line no longer formed tumors in mice. Dr. Deutel concludes in the paper [8] that ".... This finding establishes an important role of PTN in the dysregulated growth of human breast cancer cells and suggests that constitutive expression of PTN may be essential to the malignant phenotype of human breast cancers in vivo.. "

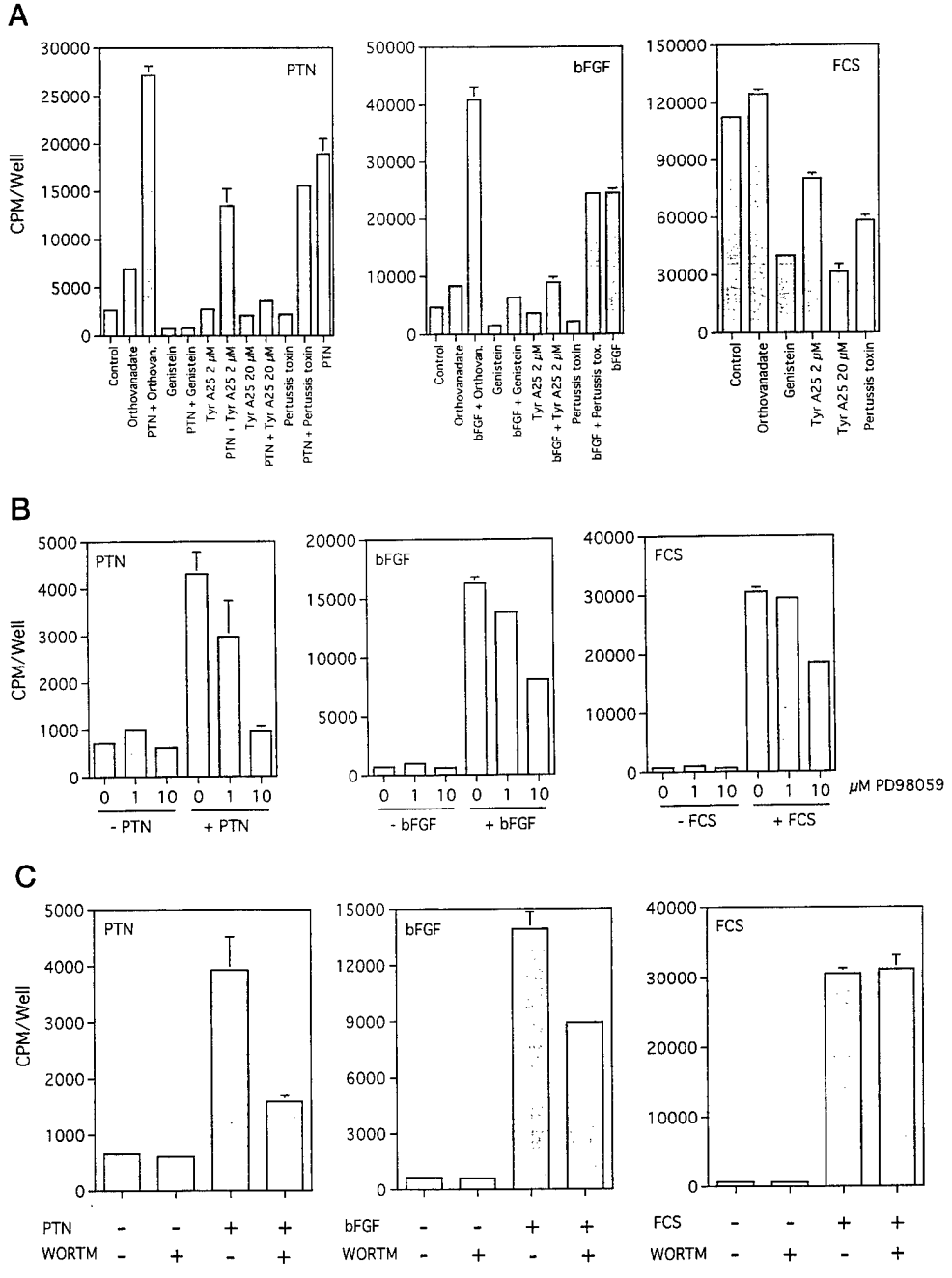
In our studies, we had also included a design for the same constructs Dr. Deuel's laboratory used. However, it was opted to go for the point-mutations first and later investigate the truncated PTN forms in a systematic way. Since the Deuel laboratory was successful in finding an inhibitory protein, we will now obtain the truncated constructs from Dr. Deuel and test them in our assays to further elucidate their inhibitory role.

Signal transduction pathway of PTN: (These data was published in JBC in 1997 [7]; see appendix).
To assess the activity of the differently mutated forms of PTN more easily and more directly, we set up a short-term assay that would detect the induction of phosphorylation of proteins in the PTN signal-transduction pathway. We characterized by immunoprecipitation and by Western-blotting with anti-phosphotyrosine antibodies a 190 kDa protein as well as MAP-Kinase proteins

that are tyrosine-phosphorylated within a few minutes (1 to 5 min.) after stimulation of PTN-responsive cells in culture. Amongst other cell lines, we used primary BEL (bovine epithelial lens) cells to investigate the signal transduction pathways involved in the mitogenic activity of recombinant PTN. PTN was purified from conditioned media of SW-13 cells transfected with the human PTN cDNA. We found that inhibitors of tyrosine kinase, MAP kinase or PI3 kinase inhibit DNA synthesis stimulated by PTN (Fig. 5)

Fig. 5. Effect of signal transduction inhibitors on the mitogenic activity of PTN as assessed by tritiated thymidine incorporation.

Serum-starved BEL (bovine epithelial lens) cells were treated with the indicated drugs starting 1 hr before addition of PTN (10 ng/ml), bFGF (1 ng/ml) or 5 % FCS for another 18 hrs. Inhibitors used: **A,** orthovanadate (12.5 μM), genistein (2 μg/ml), pertussis toxin (1 μg/ml) or tyrphostin A25. The background for the right panel (without FCS) was 4192 +392 cpm. **B,** MEK-1 inhibitor PD98059; **C,** PI3 kinase inhibitor wortmannin (10 nM).



Analysis of tyrosine-phosphorylated proteins following PTN stimulation showed phosphorylation of two novel 190 kDa and 215 kDa proteins in addition to SHC, ERK1 and ERK2 (Fig.6, below and Fig. 7, next page).

Fig. 6.PTN-induced tyrosine phosphorylation of 190 and 215 kDa proteins.
Top panel: Cell lysates from control (lanes 1 and 3) or PTN-stimulated cells (10 ng/ml for 10 min; lanes 2 and 4) were subjected to direct electrophoresis and Western blotting (WB PY; lanes 1 and 2; 25 µg of lysate per lane) or to immunoprecipitation and subsequent Western blotting (IP PY, WB PY; lanes 3 and 4; 500 µg of lysate) with the anti-phosphotyrosine antibody 4G10. For the immunoprecipitations, agarose-coupled 4G10 antibody was used. Details in Materials and Methods.

Bottom panel: In a parallel experiment, cell lysates (25 µg) from control cells (lane 1), PTN stimulated cells (10 ng/ml, 10 min; lane 2) or bFGF-stimulated cells (100 ng/ml, 10 min; lane 3) were subjected to electrophoresis and Western blotting with the 4G10 antibody.

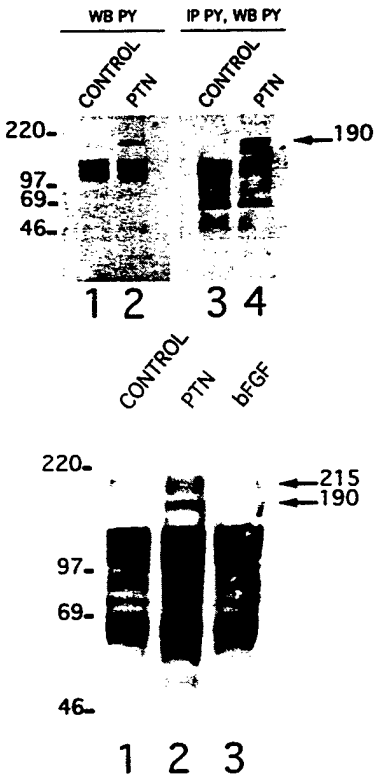


Fig. 7. PTN-induced tyrosine phosphorylation of SHC, ERK1 and ERK2.
Cell lysate (25 μg) from control cells (lane 1) or from cells stimulated with PTN (10 ng/ml; 10 min) without (lane 2) and with pretreatment with the PI3 kinase inhibitor wortmannin (10 nM; 1 hr) were separated by SDS-PAGE (10% gel) and tyrosine-phosphorylated proteins present in the lysates were detected by Western blotting with the 4G10 anti-phosphotyrosine antibody (WB PY).

Both panels represent blots from the same gel transfered for 2 hrs (bottom) and then overnight (top) using a second nitrocellulose membrane. SHC proteins on the membrane in the bottom panel were identified after stripping and reprobing with an anti-SHC antibody (WB SHC).

A mobility shift of phosphorylated ERK1 and ERK2 was detected with a panERK antibody confirming the phosphorylation of the two ERKs (Fig. 8, below). Furthermore, in vitro immunocomplex kinase assay with Akt1, a natural substrate of PI3 kinase, showed an activation of the kinase following PTN stimulation and a reversal by the PI3 kinase inhibitor wortmannin (Fig. 9). We conclude that the mitogenic activity of PTN is dependent on tyrosine kinase activation and utilizes the MAP kinase and the PI3 kinase pathways to transduce a mitogenic signal.

Fig. 8.PTN-induced mobility shift of ERK1 and ERK2.
Cell lysate (25 µg) from control cells (lane 1) or from cells stimulated with PTN (10 ng/ml; 10 min) without (lane 2) and with pretreatment with the PI3 kinase inhibitor wortmannin (10 nM; 1 hr) were separated by SDS-PAGE (10% gel) and ERKs present in the lysates were detected by Western blotting with a panERK antibody (Details in Materials and Methods). Phosphorylated (pERK) and non-phosphorylated (ERK) ERKs are indicated.

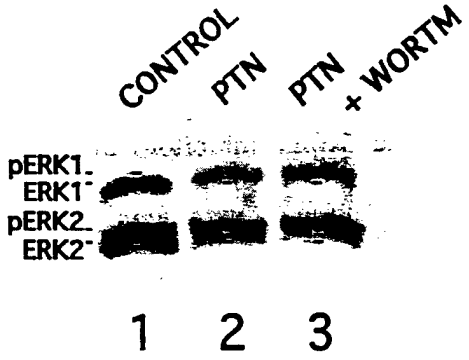
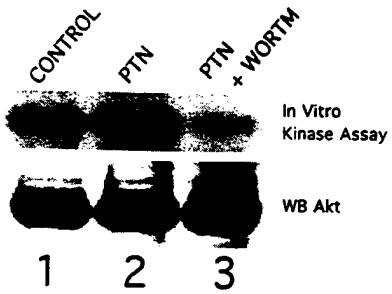


Fig. 9. Activation of Akt1 after stimulation of BEL cells with PTN.
Cell lysates (500 µg) from control cells (lane 1) or from cells stimulated with PTN (10 ng/ml; 10 min) without (lane 2) and with pretreatment with the PI3 kinase inhibitor wortmannin (10 nM; 1 hr) were immunoprecipitated with an anti-Akt1 antibody and immunocomplexes were subjected to an in vitro kinase assay with MBP as a substrate (top) or separated by SDS-PAGE and Akt present in the complexes was detected by Western blotting (WB Akt). Details in Materials and Methods.



Methods

Reagents

Orthovanadate, genistein, tyrphostin A25, pertussis toxin were purchased from LC laboratories (Woburn, MA). Wortmannin was from Sigma Chemicals (St Louis, MO). PD98059 was obtained from Biolabs (Beverly, MA). [^{3}H]thymidine and gamma [^{32}P]ATP were purchased from Amersham (Buckinghamshire, England). Myelin basic protein was obtained from Sigma. Bovine basic fibroblast growth factor (bFGF) was from Collaborative Research (Bedford, MA). Anti-PTN antibody was a generous gift of Dr. A. Seddon (American Cyanamide Company, NY). Anti-phosphotyrosine antibody 4G10 was purchased from UBI (Lake Placid, NY). Anti-panERK and anti-SHC antibodies were from Transduction Laboratories (Lexington, KY). Anti-Akt1 antibodies were from Santa Cruz (Santa Cruz, CA) and UBI.

Cell Culture

Bovine Epithelial Lens (BEL) cells were a generous gift of Dr. J. Courty (Universite Paris 12, France) and routinely grown in DMEM supplemented with 10% FCS, 2.2 g/l sodium bicarbonate (Life Technologies, Gaithersburg, MD) and 1 ng/ml bFGF. PTN-overexpressing SW13 cells (SW-13/PTN cells clone $W_2$8), produced by stable transfection with the pRcCMV vector containing the PTN cDNA, were maintained in IMEM supplemented with 10% FCS (Life Technologies).

Thymidine Incorporation Assay

This assay was carried out essentially as described in Ref. (12). BEL cells were seeded in 24-well plates for 2 to 3 days in DMEM supplemented with 10% FCS and 2.2 g sodium bicarbonate (Life Technologies). The cells were then serum-starved for 24 hr after which test samples were added. In the experiments involving inhibitors of signal transduction, the cells were pretreated with the drugs for 1 hr prior to adding the growth factors. The cells were then incubated for 18 hrs at 37° C and 5% CO_2 and then [^{3}H]thymidine was added. After an additional incubation period of 6 hrs the cells were fixed with 10% trichloro acetic acid, washed with water and lyzed overnight with 0.3 N NaOH. Total radioactivity incorporated was counted using a Beckman scintillation counter. Each experiment included buffer or vehicle as controls.

Purification of PTN from Conditioned Media

Conditioned media from approximately 10^9 SW-13/PTN cells grown for 4 to 5 days in 1.5 l of DMEM/2% FCS was adjusted to 50 mM TrisHCl pH 7.5/0.5 M NaCl and was passed through a 2 ml heparin-Sepharose column (Pharmacia, Piscataway). The column was then washed with 40 ml of 50 mM TrisHCl pH 7.5/0.5 M NaCl and heparin-bound proteins were eluted with 10 ml of 50 mM TrisHCl pH 7.5/1 M NaCl. The eluate was diluted to 50 mM TrisHCl pH 7.5/0.25 M NaCl and passed through a Mono S column using an FPLC system (Pharmacia). The column was washed extensively in the same buffer containing 0.45 M NaCl and the bound proteins eluted using a gradient from 0.45 to 2 M NaCl. Fractions of 1 ml were collected, quickly aliquoted and stored at - 80 °C.

Western Blot

After separation in SDS-PAGE gels, proteins were transferred to a nitrocellulose membrane (Biorad, Hercules, CA) for 2 hrs at 150 mAmps/gel unless indicated otherwise in 25 mM Tris pH8.3/200 mM glycine/20% methanol. The membrane was blocked in PBS (phospate-buffered saline)/0.1% Tween 20/5% powdered milk and probed with the antibodies at appropriate dilutions

for 1 hr at room temperature. The blots were then washed in PBS/0.1% Tween 20 and incubated with the appropriate secondary antibody coupled to horseradish peroxidase (Amersham) for 1 hr. After additional washing in PBS/0.1% Tween 20, bound antibody was visualized using the enhanced chemiluminescence reagents system from Amersham.

Immunoprecipitations

Cells were grown to 80% confluence in 15 cm dishes, serum starved for 48 hrs and then stimulated with PTN or bFGF for 10 minutes. Cell lysates were prepared by scrapping the cells in immunoprecipitation (i.p.) buffer (50 mM TrisHCl pH 8, 150 mM NaCl, 0.5% deoxycholic acid, 1% NP40, 10% glycerol, 1 mM sodium orthovanadate, 1 μM okadaic acid, 50 mM sodium fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin A) and incubating them for 15 min at 4 °C in a rotating rack. The lysates were cleared by centrifugation and protein content was measured with the Biorad protein assay kit. 0.5 to 1 mg of protein were incubated overnight at 4 °C with 20 μl of 4G10 anti-phosphotyrosine antibody coupled to agarose beads (UBI). The beads were then washed in the i.p. Buffer and proteins were eluted by boiling in SDS-PAGE sample buffer and subjected to electrophoresis and Western blotting.

Immunocomplex Kinase Assay

Cell lysates, prepared as described above and precleared with protein G-Sepharose, were incubated for 4 hrs at 4 °C with 3 μg sheep anti-Akt1 antibody (UBI). The immunocomplexes were captured with protein G-Sepharose at 4 °C for 1 hr. The beads were then washed with 50 mM TrisHCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT and the kinase assay was carried out as described in Ref. (18) with a slight modification: The beads were resuspended in a kinase buffer (50 mM TrisHCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 5 μM ATP, 1 μM protein kinase A inhibitor peptide, 25 μg/ml myelin basic protein and 2 μCi gamma ^{32}P-ATP) and incubated for 30 min at 30 °C. The reaction was stopped by addition of 5X sample buffer and boiling. Samples were then electrophoresed, transferred to a nitrocellulose membrane and the membrane processed for autoradiography.

Statistical analysis

Unless stated otherwise data points were run in triplicate and experiments repeated at least twice. Typically the mean ±SE from a representative experiment is presented. As appropriate, Student's t-test or ANOVA was used to assess the statistical significance of differences between measurements (Statview 4.02 program; Abacus Concepts Inc.; Berkeley, CA). The respective p-values are given in the text. $p<0.05$ was considered significant.

Goal 4:
To inhibit production of PTN

Background
We planned to use three independent approaches to target PTN mRNA and thus reduce the amount of PTN produced by PTN-positive breast cancer cells:

1. antisense oligonucleotides
2. antisense constructs
3. ribozyme constructs

Data from these approaches were shown in the last report. No additional data on this goal were generated in the meantime.

CONCLUSIONS

We discovered a novel retrovirally-derived promoter in the human PTN gene (published in reference [6] and under revision for J. Virology) and have now embarkeded on the regulatory elements in this promoter. Furthermore, we have expanded signal transduction studies that were published in [7]. As suggested in the last review, the respective papers are now included in the appendix.

REFERENCES

1) Wellstein A, Fang WJ, Khatri A, Lu Y, Swain SS, Dickson RB, et al A heparin-binding growth factor secreted from breast cancer cells homologous to a developmentally regulated cytokine. J Biol Chem 1992; 267:2582-2587.

2) Fang WJ, Hartmann N, Chow D, Riegel AT, Wellstein A Pleiotrophin stimulates fibroblasts, endothelial and epithelial cells, and is expressed in human cancer. J Biol Chem 1992; 267:25889-25897.

3) Lai S, Czubayko F, Riegel AT, Wellstein A Structure of the human heparin-binding growth factor gene pleiotrophin. Biochem Biophys Res Commun 1992; 187:1113-1122.

4) Courty J, Dauchel MC, Caruelle D, Nguyen TT, Barritault D Purification and characterization of a new endothelial cell growth factor named HARP (Heparin Affin Regulatory Peptide). J Cell Biochem 1991; 15F:Abstr. 221.(Abstract)

5) Czubayko F, Schulte AM, Berchem GJ, Wellstein A Melanoma angiogenesis and metastasis modulated by ribozyme targeting of the secreted growth factor pleiotrophin. Proc Natl Acad Sci USA 1996; 93:14753-14758.

6) Schulte AM, Lai S, Kurtz A, Czubayko F, Riegel AT, Wellstein A Human trophoblast and choriocarcinoma expression of the growth factor pleiotrophin attributable to germ line insertion of an endogenous retrovirus. Proc Natl Acad Sci USA 1996; 93:14759-14764.

7) Souttou B, Ahmad S, Riegel AT, Wellstein A Signal transduction pathways involved in the mitogenic activity of pleiotrophin: Implication of MAP kinase and PI3 kinase pathways. J Biol Chem 1997; 272:19588-19593.

8) Zhang N, Zhong R, Wang ZY, Deuel TF Human breast cancer growth inhibited in vivo by a dominant negative pleiotrophin mutant. J Biol Chem 1997; 272:16733-16736.

9) Dickson R, Lippman ME Estrogenic regulation of growth and polypeptide growth factor secretion in human breast carcinoma. Endocr Rev 1987; 8:29-43.

10) Repaske R, Steele PE, O'Neill RR, Rabson AB, Martin MA Nucleotide sequence of a full-length human endogenous retroviral segment. J Virol 1985; 54:764-772.

11) Steele PE, Rabson AB, Bryan T, Martin MA Distinctive termini characterize two families of human endogenous retroviral sequences. Science 1984; 225:943-947.

12) Tomita N, Horii A, Doi S, Yikouchi H, Ogawa M, Mori T, et al Transcription of human endogenous retroviral long terminal repeat (LTR) sequence in a lung cancer cell line. Biochem Biophys Res Comm 1990; 166:1-10.

13) Brown MA, Chun-Fang X, Nicolai H, Griffiths B, Chambers JA, Black D, et al The 5'-end of the BRCA1 gene lies within a duplicated region of human chromosome 17q21. Oncogene 1996; 12:2507-2513.

14) Xu CF, Brown MA, Nicolai H, Chambers JA, Griffiths BL, Solomon E Isolation and characterisation of the *NBR2* gene which lies head to head with the human *BRCA*1 gene. Hum Mol Genet 1997; 6:1057-1062.

15) Choudhuri R, Zhang HT, Ziche M, Bicknell R The neurotrophins midkine and pleitrophin are angiogenic and promote tumor growth. Proc Am Assoc Cancer Res 1996; 38:400.(Abstract)

16) Choudhuri R, Zhang HT, Donnini S, Ziche M, Bicknell R An angiogenic role for the neurokines midkine and pleiotrophin in tumorigenesis. Cancer Res 1997; 57:1814-1819.

Appendix Materials

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Structure and phylogenetic analysis of an endogenous retrovirus inserted into the human growth factor gene pleiotrophin

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Abstract

A human endogenous retrovirus-like element (HERV), flanked by LTRs of 502 and 495 nt, is inserted into the human pleiotrophin (PTN) gene upstream of the open reading frame. Based on its Glu-tRNA primer binding site specificity and the location within the PTN gene, we named this element HERV-E.PTN. HERV-E.PTN appears to be a recombined viral element based on its high homology (70 to 86 %) in distinct areas to members of two distantly related HERV type C families, HERV-E and RTVL-I. Furthermore, its pseudogene region is organized from 5' to 3' into gag-, pol-, env-, pol, env- similar sequences. Interestingly, full length and partial HERV-E.PTN homologous sequences were found in the human X chromosome, the human hereditary haemochromatosis region and the BRCA1 pseudogene. Finally, Southern analyses indicate that the HERV-E.PTN element is present in the PTN gene of human, chimpanzee and gorilla, but not of rhesus monkey suggesting that genomic insertion occurred after the separation of monkeys and apes about 25 million years ago.

Introduction

Pleiotrophin (PTN) is a secreted heparin-binding polypeptide growth factor (16) with an apparent molecular mass of 18 kDa (42) and a restricted time- and tissue-dependent expression pattern during development. PTN is expressed in rodents at the highest level in the central nervous system during the perinatal period, markedly decreased thereafter and present in only few adult rodent tissues (2,26,29,40). Similar patterns of expression were seen in normal human adult tissues (32), however, in various human tumor specimens and tumor cell lines PTN is expressed at high levels (9,33). Regarding its biological activity, PTN has growth promoting and transforming activity on fibroblasts (4,16) and epithelial cells (9,42), mitogenic activity on endothelial cells (6,9,15), and induces tube formation of endothelial cells and angiogenesis *in vitro* (15). Furthermore, PTN induces proteolytic enzyme activity from endothelial cells (14), and we recently showed that PTN plays an essential role for the growth, angiogenesis and metastasis of human melanoma and the growth of human trophoblast-derived choriocarcinoma *in vivo* (7,32).

Regarding its genomic organization, we recently reported that a type C human endogenous retrovirus-like (HERV) element is inserted into the human PTN gene in the intron between the 5' untranslated and coding region (32). This insert in the human genome expands the region relative to the murine gene and we showed that insertion of the HERV element generated a phylogenetically new promoter within the human PTN gene. Due to this promoter function, fusion transcripts between HERV-derived 5' untranslated exons (so-called UV3, UV2 and UV1) and the intact open reading frame (ORF) of PTN are expressed in the human trophoblast as early as 9 weeks after implantation, in term placenta and in trophoblast-derived human choriocarcinoma cell lines (JEG-3 and JAR) (32) and tissues (unpublished data).

HERV elements are assumed to be prehistoric sequences from infective retroviruses that inserted into the germ-line of human progenitors millions of years ago, mostly before the divergence of apes and Old World monkeys. Up to 1 % of the human genome consists of solitary LTRs, partial and complete proviral sequences. Along their homology with animal retroviruses they are classified into mammalian type C (class I),

type B and D as well as avian type C (class II) similar ERV (20,27,44). These ERVs are named and organized into families based on their putative tRNA-primer binding site specificity e.g. HERV-E for Glu-tRNA, HERV-I for Ile-tRNA, HERV-K for Lys-tRNA. The human genome contains ERVs from different families which differ in their copy numbers from 1 to 10,000 (44). These ERVs are non-infective, replication-defective retroviral fossils transmitted as stable Mendelian genes, mostly incapable of coding for the retroviral proteins *gag*, *pol* or *env* due to the accumulation of various mutations. Exceptions to this rule are e.g. the HTDV/HERV-K and ERV-3 (HERV-R) that are capable of expression of retroviral proteins (19,41). Numerous authors have described retroviral transcripts in different human tissues and cell lines, preferentially of placenta, embryonic or neoplastic origin (11-13,25,28,43), and some investigators reported the identification of HERV/cellular gene fusion transcripts (8,10,18). However, as far as we know, only two reports of an HERV germ-line insertion that induces changes in the expression pattern of a functional human gene product have been published to date. Expression of human amylase in the salivary gland is generated through the insertion of an HERV-E element in reverse orientation upstream of the human amylase genes. This retroviral element functions as a tissue-specific enhancer (31,38). We showed that expression of the human growth factor pleiotrophin in the trophoblast and in choriocarcinoma cell lines is attributable to the insertion of an HERV-E element immediately upstream of the coding region of the gene. This insertion generates a phylogenetically new promoter, driving the expression of HERV-PTN fusion transcripts and generates full-length, biologically active PTN protein (32).

Here we report the complete structure and phylogenetic analysis of the HERV insertion into the PTN gene. We present evidence that it is a recombinant element between HERV-E and RTVL-I family members and demonstrate that closely-related sequences are localized in the human X chromosome, the human hereditary haemochromatosis region and in the BRCA1 pseudogene.

Material and Methods

Tissue and blood samples

Human term placenta was a gift from Dr. Simons (Georgetown University, Washington, DC), rhesus monkey term placenta and whole blood samples were a gift from Dr. Wolf (Oregon Regional Primate Center, Beaverton, OR), whole blood samples from night monkey, gorilla and chimpanzee were kindly provided by Dr. Davidson (Georgetown University, Washington, D.C.), Lisa Stevens (National Zoological Park, Smithsonian Institution, Washington, D.C.) and Dr. M. Jenning (New York University LEMSIP Primate Lab., Tuxedo, NY), respectively.

Northern Blot Analysis and reverse transcriptase (RT)-polymerase chain reaction (PCR)

Total RNA from placenta tissues were isolated with the RNA STAT-60 method (Tel-Test, Friendswood, TX), poly(A)+ RNA by using oligo(dT) cellulose as recommended by the vendor (Boehringer Mannheim, Germany). RNA samples were separated and blotted as reported earlier (17). After hybridization with a probe specific for the open reading frame of PTN (9), the membrane was washed and autoradiographed (9). As a loading control, the membrane was reprobed with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For the reverse-transcriptase (RT)-PCR, random primed cDNA was generated from poly(A)+ RNA from placenta of rhesus monkey using the avian myeloblastosis virus (AMV) reverse transcriptase (Boehringer Mannheim, Germany) and oligonucleotide hexamers as recommended by the vendor. PCR was performed with an antisense oligonucleotide specific for the second translated exon O2 (5'CTGGGTCTTCATGGTTTGC3') of human pleiotrophin in combination with sense primers specific for the 5'untranslated exon U1 (5'CAGGGCGTAATTGAGTC3') or the HERV-derived 5'untranslated exon UV3 (5'CCTGACTTGCTCAGTCGATC3'). Recombinant *Taq* polymerase from Life Technologies (Gaithersburg, MD) was used as recommended by the vendor.

Human DNA clones, sequencing and data analysis

Clones G-11, G-8 and F-7 were obtained from a *Hind*III restriction library of the human pleiotrophin (PTN) P1 clone P2258 (Genome Systems, St. Louis, MO) by screening with oligonucleotides deduced from the HERV-derived 5'untranslated exons UV3 (G11 and G8; 5'CTTCACTATCTCGGTGTCTC3') and UV1 (F7; 5'CCCCCCATGCTGTGGT-AACTTTAATAAATACC3') of the human PTN gene (GenBank nos. U71455 and U71456; 32). Clone G11-F7 was generated by PCR with P1 clone P2258 and oligo-nucleotides deduced from clone G11 (sense; 5'TTACTGGGCACTCTGCC3') and F7 (antisense; 5'TTGTCAGGTCAGG TGGC3') and subcloned into a TA-cloning vector (Invitrogen). Clone 1B was obtained from a *BamH*I restriction library of clone P2258 as reported earlier (32). After uni- and partial bidirectional cycle sequencing with dye-labelled terminator and AmpliTaq DNA polymerase FS (Perkin Elmer, Foster City, CA), BLAST and FastA GenBank searches were performed. The program DNA Strider was used to define open reading frames and restriction sites, Mac Vector to generate dot plot matrix analysis.

DNA isolation, Southern analysis, probes and polymerase chain reaction (PCR)

Genomic DNA from whole blood, tissues or cultured cells was isolated with the QIAamp Blood Kit (Qiagen Inc, Chatsworth, CA), digested with *BamH*I or *Hind*III, separated in a 0.7 % agarose gel (10 or 20 µg), transferred to a nylon membrane (Magnacharge; MSI, Westboro, MA) and hybridized in 10 % dextran sulphate-supplemented formamid standard solution (17) at 42°C. After hybridization, blots were washed for low stringency hybridization twice with 2xSSC/0.1%SDS, twice with 0.1xSSC/0.1% SDS for 15 min at 42°C, and once at 62°C for 15 min. For high stringency hybridization two wash steps with 0.1xSSC/0.1% SDS for 25 min at 65°C were added. Hybridizations were performed with various random-primed labelled DNA probes (rediprime, Amersham, Arlington Heigths, Il): probe **a**: 632 nt long PCR fragment, containing 602 nt cellular sequence upstream of the HERV-E.PTN plus 30 nt 5'LTR sequence (Fig 1A; corresponding to region -11869 to -11238 in (32); probe **b**:

826 nt long *ScaI*/*Kpn*I restriction fragment isolated from the P2258 subclone G11 (Fig. 1A and 1B position 2439 to 3262).; probe **c**: 406 nt long *Kpn*I/*Hind*III restriction fragment isolated from P2258 subclone G11 (Fig. 1A and 1B position 3262 to 3667). PCRs were performed with recombinant *Taq* polymerase from Life Technologies (Gaithersburg, MD) as recommended by the vendor.

Nucleotide sequence accession number

The HERV-E.PTN sequence reported in this paper has been deposited in the GenBank data base (accession number Bankit 172816).

Results

Sequence and structure of the retroviral-like element HERV-E.PTN

To complete the analysis of the structure and sequence of the endogenous retrovirus-like (ERV) element integrated into the human pleiotrophin (PTN) gene, we used P1 clone P2258. Initially, a *Hind*III restriction library of the P1 clone was screened with oligonucleotides specific for the HERV-derived 5'untranslated exons UV3 and UV1 (Fig. 1A). Using the UV3-specific probe, two clones, G11 (4925 nt) and G8 (4932 nt) were picked due to the hybridization with the 5' and 3' LTR. Clone G11 overlaps with the previously reported 1.9 kb *Hind*III,*BamH*I (H,B) fragment (32) and extends this by 3043 nt downstream. Clone G8 extends the virus-like sequence downstream of the *Hind*III site in exon UV1 including the 3'LTR (2209 nt) and additional 2723 nt intronic sequence. Using the UV1-specific probe, clone F7 (356 nt) was picked which extends the sequence upstream of the *Hind*III site in exon UV1. To bridge the gap between clone G11 and F7 PCR was used (clone G11-F7, 105 nt).

Figure 1B shows the nucleotide sequence and structural analysis derived therefrom. The 6337 nt long ERV element with LTRs of 502 and 495 nt length, is integrated into the human PTN gene. Based on its similarity to other ERVs it belongs to the class I group (see below). Its tRNA primer binding site suggests that it is a new member of the HERV-E family (17-of-18 nt match to the 3' end of rat glutamic acid tRNA, see Fig. 1B, (32,34). The sequence TTTCT separates the prehistoric primer binding site from the 5'LTR, as was observed in the HERV-E members 4-1 and 4-14 (30). Accordingly, we named this element HERV-E.PTN.

In this HERV-E.PTN insert exon UV3 covers the complete 5' LTR plus 2 nt of upstream and 28 nt of downstream sequence. Exon UV2 (239 nt) starts 6 nt downstream from exon UV3 and exon UV1 (488 nt) is localized 3271 nt downstream from exon UV2 (Fig. 1B), considerably closer than the 4.5 kb we had estimated from gel electrophoresis of various long-range PCR products (32).

Similarity to ERVs from different families

The HERV-E.PTN is separated into areas with high similarity to members of the HERV-

E and HERV-I or RTVL-I (= *retro*virus-*like*) family. The first 1516 and the last 2576 nt are up to 82% homologous to members of the HERV-E family (boxes I in Fig. 1B; GenBank no. M32220 and M32219, (39); K02168, (30)). Compared to the provirus sequence of clone 4-1, the HERV-E.PTN underwent two major deletions in its 4-1 similar region, 1619 nt and 651 nt in the *pol* and *env* pseudogene region, respectively. The middle part of the HERV-E.PTN contains 65 and 741 nt sequence stretches with 86 % and 70 % homology to the *pol* and *env* pseudogene regions of a RTVL-I member, respectively (boxes II, Fig. 1B; GenBank no. M92068; (22)). Despite the shortness of the 65 nt sequence, its high homology to a region in the RTVL-Ib *pol*- pseudogene supports its RTVL-Ib origin. Interestingly, a 65 nt sequence of the human X chromosome also showes a similar (87 %) homology (see below) and nucleotide alignment studies, suggest that an HERV-E.PTN similar element is also inserted into this locus (see below). Figure 2 shows the respective dot plot matrices comparing the HERV-E.PTN with HERV-E clone 4-1 and RTVL-Ib (panel A), as well as a cartoon of the organization of the HERV-E.PTN pseudogene region (panel E).

Similarity to sequences in the human X chromosome, the human hereditary haemochromatosis (hHH) region and the BRCA1 pseudogene

Despite some deleted and inserted areas, the entire HERV-E.PTN element is ~80 % homologous to a 9.7 kb region in the human X chromosome (GenBank no. Z83820; PAC clone 215K18) and to a 6.5 kb area in the human hereditary haemochromatosis (hHH) region (GenBank no. U91328; see Fig. 1B, Box III and Fig. 2A). Interestingly, the sequence comparison revealed that the respective regions in the X chromosome and the hHH-region are more homologous between each other than they are to the HERV-E.PTN sequence and their similarity is extended upstream and downstream of their HERV-E.PTN homology (data not shown). This suggests to us that the recombinant HERV-E.PTN element inserted into the human PTN gene en bloc and that the insertion happened more recently. Surprisingly, we found also an 80% homology between the 5'-end of HERV-E.PTN and a newly published 1.3 kb stretch of intronic sequence immediately downstream of exon 2 in the BRCA1 pseudogene (GenBank no. U77841;

Fig. 2A (3)). Furthermore, dot plot matrix analysis show a higher homology to the 5'-end of HERV-E.PTN than to the corresponding region in HERV-E clone 4-1 (Fig. 2C). The similar organization of the BRCA1 gene and pseudogene around exons 1A, 1B, and 2 indicates that the HERV-E.PTN-like sequence is inserted into both copies of BRCA1 (Fig. 2D).

The long terminal repeats (LTRs) and integration site

The HERV-E.PTN 5'LTR encompasses the first 502 nt, and the 3'LTR (495 nt) starts 5341 nt downstream. The 3'LTR shows 56 nt differences to the 5' LTR (17 nt deletions, 10 nt insertions and 29 nt exchanges) which results in a homology of 89%. Based on the U3-R-U5 organization of retroviral LTRs (37), the HERV-E.PTN LTRs are organized in putative 440 or 426 nt U3, 23 nt R and 39 or 46 nt U5 sequence in the 5' and 3'LTR, respectively (Fig. 1C). The highest aberration between both LTRs is localized in the U5 region (12 nt), and sequence comparison to members of the HERV-E family showed a lower homology for the 5'LTR U5 region (e.g. 59 % versus 89 % when compared to the 5'LTR U5 region of HERV-E clone 4-1). Compared with a solitary LTR of the HERV-E family, LTR22 is 60 % and 65 % homologous to regions in the HERV-E.PTN 5' and 3'LTR, respectively (GenBank nos. M32220 (39)). Furthermore, the last 205 and 211 nt of the 5' or 3' LTR of HERV-E.PTN have a 61 % and 69 % homology to the 5' LTR of HERV-E clone 4-1, respectively. Overall, the HERV-E.PTN LTRs have a scattered homology to different regions of different HERV-E LTRs (see also (32)).

Regarding the integration site, the direct repeated DNA adjacent to the HERV-E.PTN differs from provirus integration sites. Whereas the number of nucleotides in direct repeats is 4 to 6 for different provirus species (1,5), a 123 nt sequence upstream of the 5'LTR is directly repeated downstream of the 3'LTR (Fig. 1B).

Open reading frames

Relevant open reading frames coding for the viral proteins *gag*, *pol* or *env* are not present in this fossil of a retrovirus. Starting 671 and 3916 nt downstream from the 5'LTR, retroviral *gag* and *env* protein-like reading frames extend only for 267 and 123

nt respectively (Fig. 1B).

Phylogenetic analysis

Based on the similarity of HERV-E.PTN to different ERVs and the organization of its pseudogene coding region, we assume that this element is a product of a recombination between a member of the HERV-E and the RTVL-I family.

Southern analysis of *BamH*I-digested genomic DNA from human, gorilla, rhesus monkey (Old World monkey), night monkey (New World monkey) and mouse probed with a fragment containing the RTVL-I env-similar sequence (probe **b**, Fig. 1A) showed a similar pattern of bands (~20) in human, gorilla and rhesus monkey (Fig. 3B). As expected, no hybridization signal was detected in mouse, but New World monkey DNA showed three bands at low stringency (Fig. 3A). Southern analysis with a probe corresponding to a different RTVL-I env-similar region (probe **c**, Fig. 1A) confirmed the hybridization signal in DNA from New World monkey (data not shown). We conclude from this that RTVL-I related elements are inserted into the germ-line of New World monkeys and that the genomes of apes and Old World monkeys harbor RTVL-I env-like elements similar to humans.

To define if the HERV-E.PTN is present in PTN genes of non-human primates, we performed Southern analysis of *BamH*I- and/or *Hind*III-digested genomic DNA from human, chimpanzee, gorilla and rhesus monkey. Based on the human PTN gene structure and restriction map (Fig. 1A) we expected a 8.5 kb *BamH*I and a 4.9 kb *Hind*III hybridization signal using a probe specific for the human intronic sequence immediately upstream of the HERV-E.PTN element (probe **a**, Fig. 1A). Indeed, a single ~8.5-kb *BamH*I- or 4.9-kb *Hind*III-fragment was detected in human as well as in gorilla DNA (Fig. 4). Chimpanzee DNA, tested after *Hind*III digestion also showed the 4.9 kb band in addition to a ~7.5 kb fragment of similar intensity (Fig. 4B). In contrast to that, the rhesus monkey analysis showed a single ~12 kb *BamH*I- and a single ~2.2 kb *Hind*III-fragment (Fig. 4). Since both enzymes cut inside as well as outside of the HERV-E.PTN insertion, we conclude from this dramatic change in the restriction map of rhesus monkey versus apes that the HERV is not inserted into the rhesus PTN gene.

In addition, Northern analysis performed with mRNA from rhesus placenta failed to detect the PTN transcript in contrast to the strong PTN signal obtained with total RNA from human placenta (Fig 4C). RT-PCR analysis of rhesus placenta mRNA showed that low levels of PTN mRNA are transcribed from the promoter upstream of the 5'untranslated exon U1. However, this analysis failed to show the PTN fusion transcript expected from the HERV-E.PTN insertion. In conjunction with our data on human placenta where we found a 10 : 1 ratio of HERV-PTN : U1-PTN mRNA (32) these data also support the notion that HERV-E.PTN is not inserted into the rhesus PTN gene.

Discussion

Here we report the complete structure and evolutionary history of the endogenous retrovirus-like element (HERV) inserted into the human pleiotrophin (PTN) gene (32). Based on its tRNA primer binding site specificity and its location between the 5'-untranslated and coding region of the human PTN gene, we named this element HERV-E.PTN. It is flanked by LTRs of 502 and 495 nt length and shows no relevant open reading frames for the retroviral proteins *gag, pol* or *env*. The HERV-E.PTN represents a non-infective, replication-defective retroviral element with homology of 70 to 86 % to members of the HERV-E or the RTVL-I family; two distantly related ERV families of the MLV (murine leukemia virus) genus. Furthermore, it is found in the human genome in connection with at least four different genes or genetic loci, i.e. PTN, the X chromosome (PAC clone 215K18), the hereditary haemochromatosis region and the BRCA1 pseudogene (Fig. 2).

Two members of the HERV-E family (clone 4-1, 4-14) were originally isolated by two successive cross-species genomic library screenings under low-stringency hybridization conditions with probes from the murine leukemia virus (MLV) and the African green monkey endogenous retrovirus (24). The human genome is estimated to contain up to 50 copies of full length and truncated members of this family (36). The RTVL-I family was identified by chance during the analysis of the haptoglobin-related gene locus. Its members are less homologous to mammalian type-C retroviruses (21). Members of the HERV-E and RTVL-I families have been detected in the genomes of Old World monkeys, apes and humans (22,30), and PCR analysis revealed that the HERV-E (4-14) and RTVL-Ia elements are present in at least one identical location in these primates (35). Therefore, these retroviruses inserted into the primate germ-line before the divergence of apes and Old World monkeys, an event estimated to have occurred some 25 million years ago.

Our findings are consistent with the notion that the HERV-E.PTN and its closely-related elements on the human X chromosome and in the hereditary haemochromatosis region represent recombined elements generated between HERV-E and RTVL-I family members. The organization of HERV-E.PTN does not match with the typical retrovirus

genome organization of *gag*, *pol*, *env*, but has *pol*- and *env*-like sequences from RTVL-I inserted between *gag*- and *pol*-like sequences of HERV-E (Fig. 2). The possibility, that the human genome harbors recombinant elements containing the RTVL-I *env*-like region was suggested earlier by Wilkinson et al., (1994) (44). Southern analysis and screening of a genomic library showed an up to 3-fold higher copy number for the 3'-part of RTVL-I elements in the human genome compared to the 5'-part (25:8 copies; (22)). Using a probe from the RTVL-I env-similar sequence (3'-part) of HERV-E.PTN, our genomic Southern blots revealed a hybridization pattern in ape (gorilla) and Old World monkey (rhesus) similar to that in human (Fig. 3). We conclude from this that the genomes of gorilla and rhesus harbor recombinant elements that contain the RTVL-I *env*-like region as does the human genome, and it is tempting to speculate that HERV-E.PTN recombinant precursors may already be present in the rhesus genome. In addition, we show that RTVL-I-related retroviruses are already inserted in the germ-line of New World monkeys, at least 45 million years ago. Recently, Martin et al., (1997) (23) reported the identification of RTVL-I-related retroviruses even in lower vertebrates like reptiles and fish.

It is difficult to formally prove that the HERV-E.PTN element inserted into the human PTN gene *en bloc*, versus a recombination of an already inserted HERV-E element with a RTVL-I member *in loco*. However, sequence comparisons and structural similarity between HERV-E.PTN and its related elements on the human X chromosome and in the human hereditary haemochromatosis (hHH) region make parallel and independent germ line recombination events between HERV-E and RTVL-I members in these different loci rather unlikely. Furthermore, the HERV-E.PTN similar elements on the X chromosome and the hHH-region are more similar to each other than they are to the HERV-E.PTN sequence. This suggests to us that the insertion of HERV-E.PTN into the PTN gene is a more recent event.

Surprisingly, in the HERV-E.PTN flanking sequences, a 123 nt region upstream of the 5' LTR is directly repeated downstream of the 3' LTR (Fig. 1D). This is in contrast with other proviral DNA which is typically flanked by direct repeats of only 4 to 6 nt length (1,5). We can not explain the origin of the 123 nt long direct repeat, but we find it rather

unlikely that the sequence repetition was generated through integrase-mediated integration of retroviral DNA.

From the Southern analysis and the fact that chimpanzees are phylogenetically closer to humans than gorillas we conclude, that the HERV-E.PTN is present in the PTN gene of human, chimpanzee and gorilla. However, in the *Hind*III digested genomic DNA from chimpanzee an additional band of ~ 7.5 kb hybridized to the probe corresponding to the human 5' upstream sequence of the HERV-E.PTN (probe **a**, see Fig 1A; Fig. 4B). Hybridization with a probe specific for the coding region of the human PTN gene showed a single band, excluding an amplification of the entire PTN locus in this chimpanzee (data not shown). We conclude from this that most likely a duplication of unknown size, containing the PTN intronic sequence upstream of the corresponding chimpanzee HERV-E.PTN but excluding the PTN coding region is present in this chimpanzee genome.

Finally, a comparison of hybridization signals between human and ape versus rhesus monkey shows a different hybridization pattern after *BamH*I or *Hind*III digestion (Fig. 4). Furthermore, Northern analysis as well as RT-PCR studies with poly-(A)+ RNA from rhesus placenta tissue failed to show HERV-PTN fusion transcripts (Fig. 4C). Our studies thus indicate, that the HERV-E.PTN entered into the PTN gene of non-human primates after the divergence of apes and Old World monkeys (Fig. 5)

In conclusion, we report herein the complete structure and phylogenetic analysis of the human endogenous retrovirus HERV-E.PTN inserted into the human growth factor gene pleiotrophin. We show evidence that the HERV-E.PTN is a recombined element of two distantly related HERV type C families, HERV-E and RTVL-I, and that closely-related elements are localized in the human X chromosome, in the human hereditary haemochromatosis region and in a recently sequenced region of the BRCA1 pseudogene.

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Abbreviations

breast and ovarian cancer susceptibility gene 1 = BRCA1; human endogenous retrovirus = HERV; human hereditary haemochromatosis = hHH; long terminal repeat = LTR; murine leukemia virus = MLV; Next to BRCA1 gene 1 or gene 2 = NBR1 or NBR2; nucleotide(s) = nt(s); pleiotrophin = PTN; position = pos.; retrovirus-like = RTVL; reverse transcriptase = RT; untranslated region = UTR.

References

1. **Andrake, M. D. and A. M. Skalka.** 1996. Retroviral Integrase, putting the pieces together. J. Biol. Chem. **271**:19633-19636.
2. **Bloch, B., E. Normand, I. Kovesdi, and P. Böhlen.** 1992. Expression of the HBNF (heparin-binding neurite-promoting factor) gene in the brain of fetal, neonatal and adult rat: an in situ hybridization study. Brain Res Dev Brain Res **70**:267-278.
3. **Brown, M. A., X. Chun-Fang, H. Nicolai, B. Griffiths, J. A. Chambers, D. Black, and E. Solomon.** 1996. The 5'-end of the BRCA1 gene lies within a duplicated region of human chromosome 17q21. Oncogene **12**:2507-2513.
4. **Chauhan, A. K., Y. S. Li, and T. F. Deuel.** 1993. Pleiotrophin transforms NIH3T3 cells and induces tumors in nude mice. Proc. Natl. Acad. Sci. USA **90**:679-682.
5. **Chen, H. R. and W. C. Barker.** 1984. Nucleotide sequences of the retroviral long terminal repeats and their adjacent regions. Nucleic Acids Research **12**:1767-1778.
6. **Courty, J., M. C. Dauchel, D. Caruelle, M. Perderiset, and D. Barritault.** 1991. Mitogenic properties of a new endothelial cell growth factor related to pleiotrophin. Biochem. Biophys. Res. Commun. **180**:145-151.
7. **Czubayko, F., A. M. Schulte, G. J. Berchem, and A. Wellstein.** 1996. Melanoma angiogenesis and metastasis modulated by ribozyme targeting of the secreted growth factor pleiotrophin. Proc. Natl. Acad. Sci. USA **93**:14753-14758.
8. **Di Cristofano, A., M. Strazullo, L. Longo, and G. La Mantia.** 1995. Characterization and genomic mapping of the ZNF80 locus: expression of this zinc-finger gene is driven by a solitary LTR of ERV9 endogenous retroviral family. Nucleic Acids Research **23**:2823-2830.
9. **Fang, W., N. Hartmann, D. T. Chow, A. T. Riegel, and A. Wellstein.** 1992. Pleiotrophin stimulates fibroblasts and endothelial and epithelial cells and is expressed in human cancer. J. Biol. Chem. **267**:25889-25897.

10. **Feuchter-Murthy, A. E., J. D. Freeman, and D. L. Mager.** 1993. Splicing of a human endogenous retrovirus to a novel phospholipase A2 related gene. Nucl Acid Res **21**:135-143.

11. **Gattoni-Celli, S., K. Kirsch, S. Kalled, and K. J. Isselbacher.** 1986. Expression of type C-related endogenous retroviral sequences in human colon tumors and colon cancer cell lines. Proc. Natl. Acad. Sci. USA. **83**:6127-6131.

12. **Herbst, H., M. Sauter, and N. Mueller-Lantzsch.** 1996. Expression of human endogenous retrovirus K elements in germ cell and trophoblastic tumors. Am J Path **149**:1727-1735.

13. **Johansen, T., T. Holm, and E. Bjorklid.** 1989. Members of the RTVL-H family of human endogenous retrovirus-likw elements are expressed in placenta. Gene. **79**:259-267.

14. **Kojima, S., T. Inui, H. Muramatsu, H. Kimuara, S. Sakakibara, and T. Muramatsu.** 1995. Midkine is a heat and acid stable polypeptide capable of enhancing plasminogen activator activity and neurite outgrowth extension. Biochem. Biophys. Res. Commun. **216**:574-581.

15. **Laaroubi, K., J. Delbe, F. Vacherot, P. Desgranges, M. Tardieu, M. Jaye, D. Barritault, and J. Courty.** 1994. Mitogenic and in vitro angiogenic activity of human recombinant heparin affin regulatory peptide. Growth Factors. **10**:89-98.

16. **Li, Y. S., P. G. Milner, A. K. Chauhan, M. A. Watson, R. M. Hoffman, C. M. Kodner, J. Milbrandt, and T. F. Deuel.** 1990. Cloning and expression of a developmentally regulated protein that induces mitogenic and neurite outgrowth activity. Science **250**:1690-1694.

17. **Liaudet-Coopman, E. D. E., A. M. Schulte, M. Cardillo, and A. Wellstein.** 1996. A tetracycline-responsive promoter system reveals the role of a secreted binding protein for FGFs during the early phase of tumor growth. Biochem. Biophys. Res. Commun. **229**:930-937.

18. **Liu, A. Y. and B. A. Abraham.** 1991. Subtractive cloning of a human endogenous retrovirus and calbindin gene in the prostate cell line PC3. Cancer Research **51**:4107-4110.

19. **Loewer, R., K. Boller, B. Hasenmaier, C. Korbmacher, N. Mueller-Lantzsch, J. Loewer, and R. Kurth.** 1993. Identification of human endogenous retroviruses with complex mRNA expression and particle formation. Proc. Natl. Acad. Sci. USA **90**:4480-4484.

20. **Loewer, R., J. Loewer, and R. Kurth.** 1996. The viruses in all of us: Characteristics and biological significance of human endogenous retrovirus sequences. Proc. Natl. Acad. Sci. USA. **93**:5177-5184.

21. **Maeda, N.** 1985. Nucleotide sequence of the haptoglobin and haptoglobin-related gene pair. J. Biol. Chem. **260**:6698-6709.

22. **Maeda, N. and H. S. Kim.** 1990. Three independent insertions of retrovirus-like sequences in the haptoglobin gene cluster of primates. Genomics **8**:671-683.

23. **Martin, J., E. Herniou, J. Cook, R. Waugh O'Neill, and M. Tristem.** 1997. Human endogenous retrovirus type I-related viruses have an apparently widespread distribution within vertebrates. J. Virol. **71**:437-443.

24. **Martin, M. A., T. Bryan, T. F. McCutchan, and H. W. Chan.** 1981. Detection and cloning of murine leukemia virus-related sequences from African Green monkey liver DNA. J. Virol. **39**:835-844.

25. **Medstrand, P. and J. Blomberg.** 1993. Characterization of novel reverse transcriptase encoding human endogenous retroviral sequences similar to type A and type B retroviruses: Differential transcription in normal human tissues. J. Virol. **6**:6778-6787.

26. **Merenmies, J. and H. Rauvala.** 1990. Molecular cloning of the 18-kDa growth-associated protein of developing brain. J. Biol. Chem. **265**:16721-16724.

27. **Patience, C., D. A. Wilkinson, and R. A. Weiss.** 1997. Our retroviral heritage. Trends in Genetics **13**:116-120.

28. **Rabson, A. B., P. E. Steele, C. F. Garon, and M. A. Martin.** 1983. mRNA transcripts related to full-length endogenous retroviral DNA in human cells. Nature **306**:604-607.

29. **Rauvala, H.** 1989. An 18-kd heparin-binding protein of developing brain that is distinct from fibroblast growth factors. EMBO J. **8**:2933-2941.

30. **Repaske, R., P. E. Steele, R. R. O'Neill, A. B Rabson, and M. A. Martin.** 1985. Nucleotide sequence of a full-length human endogenous retroviral segment. J Virol. **54**:764-772.

31. **Samuelson, L. C., K. Wiebauer, C. M. Snow, and M. H. Meisler.** 1990. Retroviral and pseudogene insertion sites reveal the lineage of human salivary and pancreatic amylase genes from a single gene during primate evolution. Mol Cell Biol **10**:2513-2520.

32. **Schulte, A. M., S. Lai, A. Kurtz, F. Czubayko, A. T. Riegel, and A. Wellstein.** 1996. Human trophoblast and choriocarcinoma expression of the growth factor pleiotrophin attributable to germ-line insertion of an endogenous retrovirus. Proc. Natl. Acad. Sci. U. S. A. **93**:14759-14764.

33. **Schulte, A. M. and A. Wellstein.** 1997. Pleiotrophin and related molecules, p. 273-289. In R. Bicknell, C. M. Lewis, and N. Ferrara (eds.), Tumour Angiogenesis. Oxford University Press, Oxford, New York, Tokyo.

34. **Sekiya, T., Y. Kuchino, and S. Nishimura.** 1981. Mammalian tRNA genes: nucleotide sequences of rat genes for $tRNA^{Asp}$, $tRNA^{Gly}$ and $tRNA^{Glu}$. Nucleic Acids Research **9**:2239-2250.

35. **Shih, A., E. E. Coutavas, and M. G. Rush.** 1991. Evolutionary implications of primate endogenous retroviruses. Virology **182**:495-502.

36. **Steele, P. E., A. B. Rabson, T. Bryan, and M. A. Martin.** 1984. Distinctive termini characterize two families of human endogenous retroviral sequences. Science **225**:943-947.

37. **Temin, H. M.** 1981. Structure, variation and synthesis of retrovirus long terminal repeat. Cell **27**:1-3.

38. **Ting, C. N., M. P. Rosenberg, C. M. Snow, L. C. Samuelson, and M. H. Meisler.** 1992. Endogenous retroviral sequences are required for tissue-specific expression of a human salivary amylase gene. Genes Dev. **6**:1457-1465.

39. **Tomita, N., A. Horii, S. Doi, H. Yikouchi, M. Ogawa, T. Mori, and K. Matsubara.** 1990. Transcription of human endogenous retroviral long terminal repeat (LTR) sequence in a lung cancer cell line. Biochem. Biophys. Res. Comm. **166**:1-10.

40. **Vanderwinden, J. M., P. Mailleux, S. N. Schiffmann, and J. J. Vanderhaeghen.** 1992. Cellular distribution of the new growth factor pleiotrophin (HB- GAM) mRNA in developing and adult rat tissues. Anat Embryol (Berl) **186**:387-406.

41. **Venables, P. J. W., S. M. Brookes, D. Griffiths, R. A. Weiss, and M. T. Boyd.** 1995. Abundance of an endogenous retroviral envelope protein in placental trophoblasts suggests a biological function. Virology **211**:589-592.

42. **Wellstein, A., W. J. Fang, A. Khatri, Y. Lu, S. S. Swain, R. B. Dickson, J. Sasse, A. T. Riegel, and M. E. Lippman.** 1992. A heparin-binding growth factor secreted from breast cancer cells homologous to a developmentally regulated cytokine. J. Biol. Chem. **267**:2582-2587.

43. **Wilkinson, D. A., D. Freeman, N. L. Goodchild, C. A. Kelleher, and D. L. Mager.** 1990. Automomous expression of RTVL-H endogenous retroviruslike elements in human cells. J. Virol. **64**:2157-2167.

44. **Wilkinson, D. A., D. L. Mager, and J. A. C. Leong.** 1994. Endogenous human retroviruses, p. 465-535. In J. A. Levy (ed.), The Retroviridiae. Plenum Press, New York.

45. **Xu, C. F., M. A. Brown, H. Nicolai, J. A. Chambers, B. L. Griffiths, and E. Solomon.** 1997. Isolation and characterisation of the *NBR2* gene which lies head to head with the human *BRCA*1 gene. Hum. Mol. Genet. **6**:1057-1062.

Figure 1

Organization of the human PTN gene, location of the HERV-E.PTN, its complete sequence and homologies.

(A) Mapping and partial restriction map of the HERV-E.PTN. The PTN ORF exons (O1 to O4), 5'-UTR exons (U1, U2), HERV-derived exons (UV1 to UV3) and the HERV element are boxed. The characterized region of the P1 clone P2258, the *BamH*I- (1B) and *Hind*III- (G11, F7, G8) subclones and the PCR clone (G11-F7) is enlarged. The genomic Southern blot probes (**a**,**b**,**c**) and restriction sites are shown (B=*BamH*I, H=*Hin*dIII, Sc=*Sca*I, K=*Kpn*I).

(B) Nucleotide sequence of HERV-E.PTN and its homology to other ERVs. The 6337 nt long HERV-E.PTN is numbered continuously from 5' to 3', the flanking sequences are shown in small letters, the LTRs are labelled and marked in their borders (/..\) as well as the putative $tRNA^{Glu}$ primer binding site. The short translated amino acid sequences similar to gag and env fragments are shown beneath the nucleotide sequence (stop codons=***). The HERV-derived exons of the human PTN gene are boxed (UV3, UV2, UV1). The boxed regions I, II and III are similar to other Genbank entries (I=HERV-E, II=RTVL-I, III= X-chromosome PAC clone 215K18 and human hereditary haemochromatosis region) as discussed in the text. The 5'end of the HERV-E.PTN sequence corresponds to position -11267 in (32).

(C) Comparison of the LTR sequences. Dots represent identity, dashes indicate spacing introduced for optimal alignment and differing nucleotides are indicated. Putative U3, R and U5 regions (boundaries = ‡), the putative retroviral TATA box (ATTTTAA) and polyadenylation signal (ATTAAA) as well as the $tRNA^{Glu}$ primer binding sites localized downstream of the 5'LTR are shown.

(D) HERV-E.PTN flanking sequences. The underlined nucleotides indicate the predicted TATA boxes of the HERV-derived promoter of the human PTN gene and the defined transcription start site of the HERV-PTN fusion transcript.

Figure 2

(A) Dot plot matrix analysis of HERV-E.PTN relative to sequences from the HERV-E clone 4-1 and RTVL-Ib. x axis = HERV-E.PTN; y axes are HERV-E clone 4-1 (GenBank no. K02168) and RTVL-Ib (GenBank no. M92068). Window size = 30; hash value = 2; homology = 70 %. The organization of the pseudogene region of the HERV-E clone 4-1 and the RTVL-Ib are indicated on the right ordinate.

(B) Dot plot matrix analysis of HERV-E.PTN relative to sequences from the human X-chromosome PAC clone 215K18, the human hereditary haemochromatosis (hHH) region, the BRCA1 pseudogene and dot plot matrix analysis of HERV-E clone 4-1 relative to the sequence from the human X chromosome PAC clone 215K18. x axis = HERV-E.PTN or HERV-E clone 4-1 (Genbank no. K02168); y axes are HERV-E clone 4-1; RTVL-Ib (GenBank no. M92068); PAC 215K18 (GenBank no. Z83820); hHH-region (GenBank no. U91328); BRCA1 pseudogene (GenBank no. U77841). Window size = 30; hash value = 2; homology = 70 %. The organization of the BRCA1 pseudogene is indicated on the right ordinate.

(C) Dot plot matrix analysis of the BRCA1 pseudogene relative to HERV-E.PTN and HERV-E clone 4-1. x axis = BRCA1 pseudogene (GenBank U77841, position 2500 to 4098), y axes are HERV-E.PTN (position 1 to 2000) and HERV-E clone 4-1 (position 1 to 2000). Window size =30; hash value = 2; homology = 90 %

(D) The HERV-E.PTN homologous region in the BRCA1 locus. Arrows represent the direction of transcription, grey boxes represent exons from BRCA1 and pseudo BRCA1 genes (1A, 1B, 2 and 3), white boxes symbolize the NBR2 (Next to BRCA1 gene 2; formerly known as pseudogene 1A1-3B; ~30 kb) and NBR1 genes (Next to BRCA1 gene 1, formerly known as gene 1A1-3B). The black box represents the recently sequenced HERV-E.PTN homologous region (1243 nt) in the pseudo BRCA1 gene. The genomic organization, restriction enzyme sites (E= *Eco*RI, H=*Hind*III, P= *Pst*I) and the size (kb) of restriction fragments are adapted from Brown et al., (1996) and Xu et al., (1997) (3,45). Note that this diagram is not drawn to scale.

(E) Cartoon of the structure of HERV-E.PTN based on retroviral pseudogenes .

Figure 3

Southern blot hybridization of *BamHI*-digested genomic DNA with a probe corresponding to the RTVL-I env-similar region (probe **b,** Fig.1A).

(A) Low stringency wash (20 μg DNA).

(B) High stringency wash. (10 μg DNA from human and gorilla; 20 μg DNA from rhesus, night monkey (NWM) and mouse)

Figure 4

HERV-E.PTN is localized in the PTN gene of human, chimpanzee and gorilla. Southern analysis with *BamH*I- **(A)** or *Hind*III- **(B)** digested genomic DNA using a probe corresponding to the sequence upstream of the HERV-E.PTN plus 30 nt 5'LTR sequence (probe **a**, Fig. 1A). Loading: 10 µg human DNA in A, 20 µg in B; 10 µg gorilla DNA in A, 20 µg in B; 20 µg rhesus DNA; 20 µg chimpanzee DNA; 20 µg rat DNA. **(C)** Northern analysis with 20 µg of total RNA from human placenta and 10 µg of poly(A)+ RNA from rhesus monkey placenta.

Figure 5

Evolutionary tree and proposed time point of the HERV-E.PTN insertion into the PTN gene.

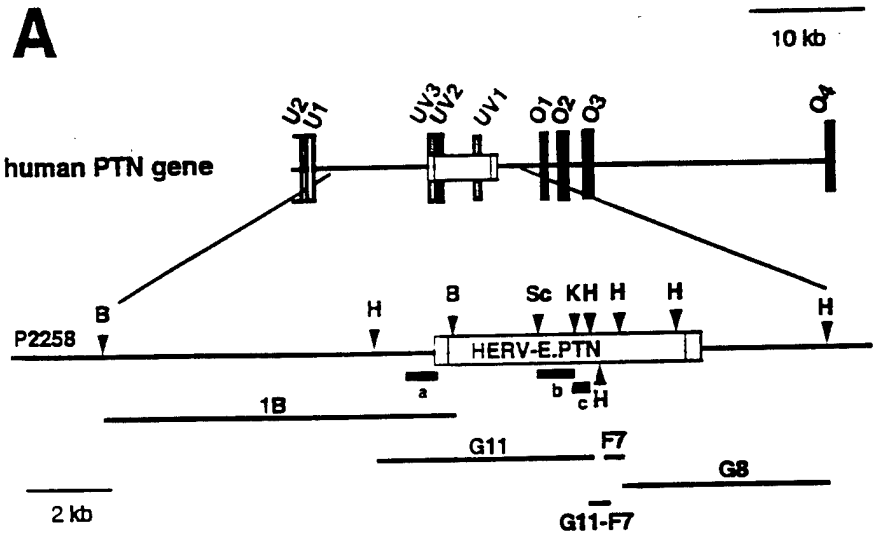

A
10 kb
human PTN gene
U2
U1
UV3
UV2
UV1
O1
O2
O3
O4
B
H
B
Sc
KH
H
H
H
P2258
HERV-E.PTN
a
b
c
H
1B
G11
F7
G8
2 kb
G11-F7

Fig. 1

B

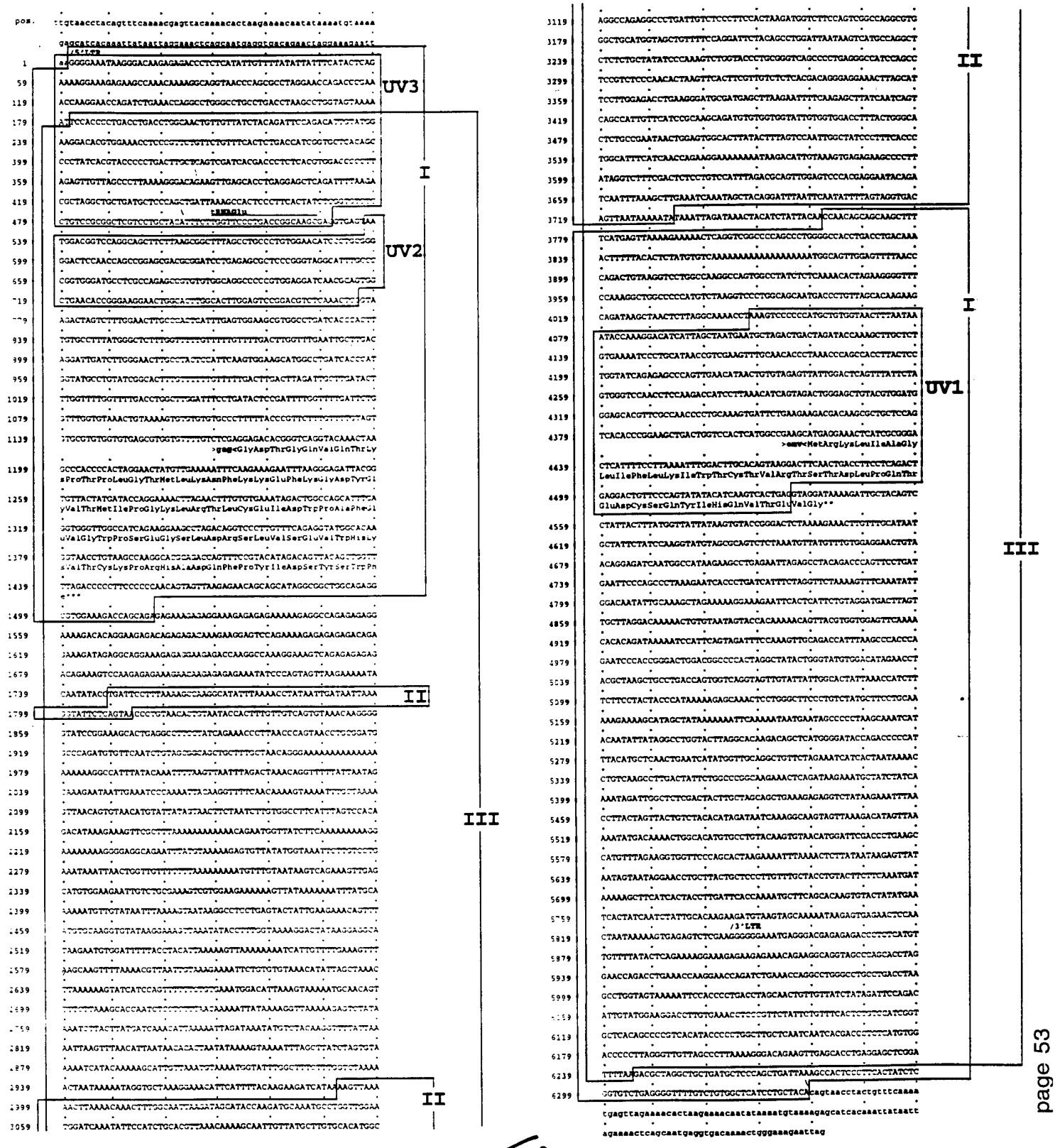
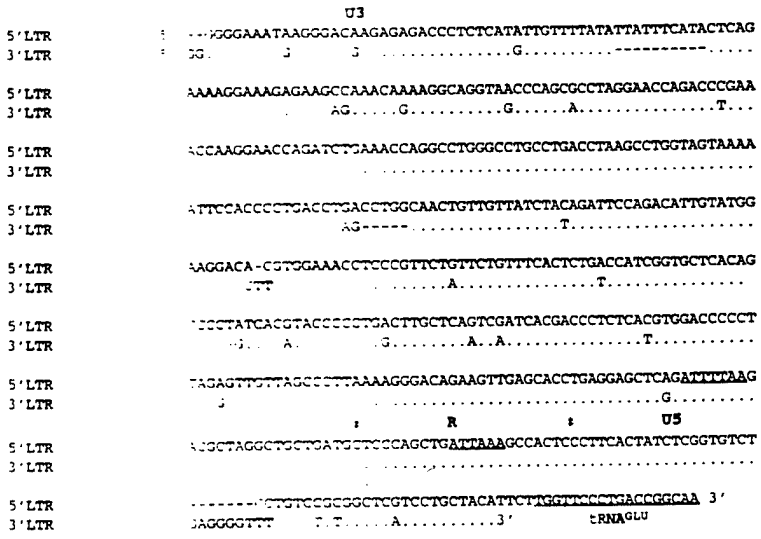


Fig. 1

C


U3
5'LTR
3'LTR
R
U5
tRNA GLU

D

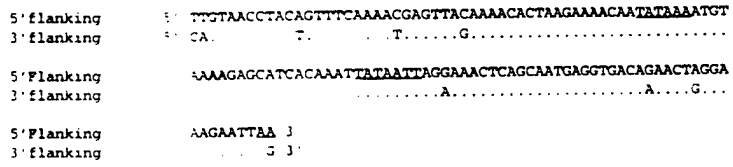

5'flanking
3'flanking
5'Flanking
3'flanking

Fig. 1

A

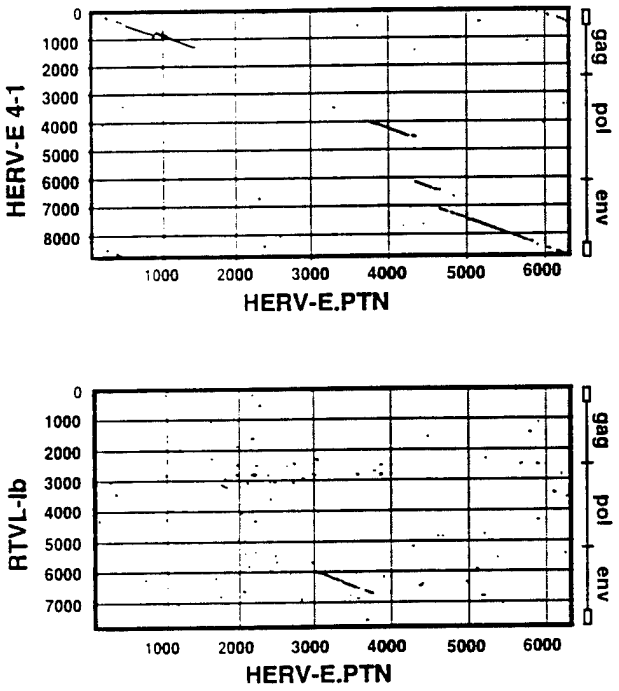

HERV-E 4-1
0
1000
2000
3000
4000
5000
6000
7000
8000
1000
2000
3000
4000
5000
6000
HERV-E.PTN
gag
pol
env
RTVL-Ib
0
1000
2000
3000
4000
5000
6000
7000
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Fig. 2

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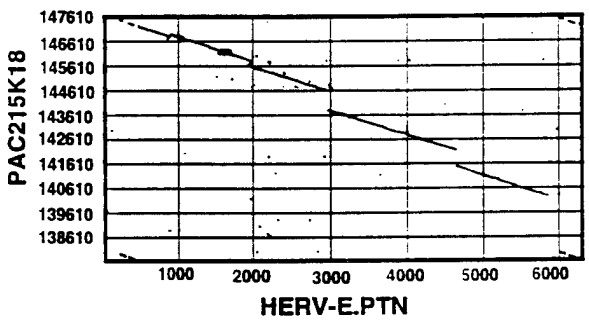
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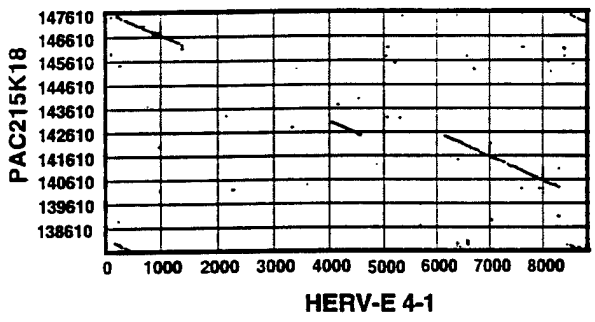
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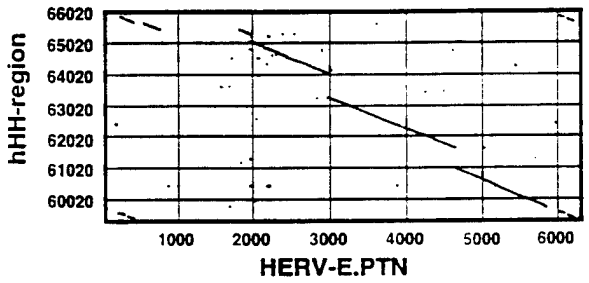
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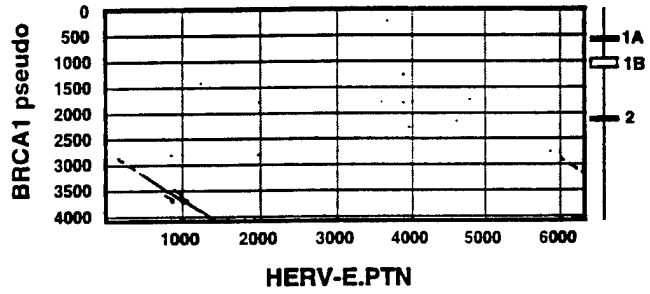
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Fig. 2

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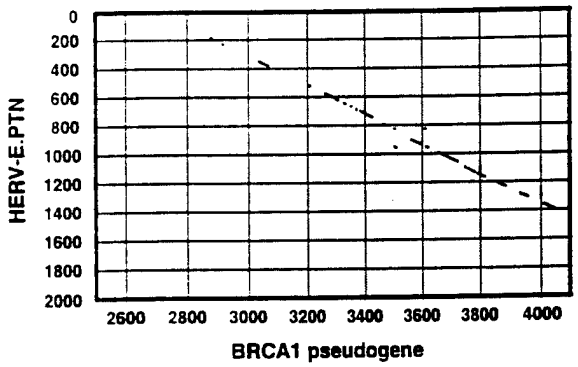
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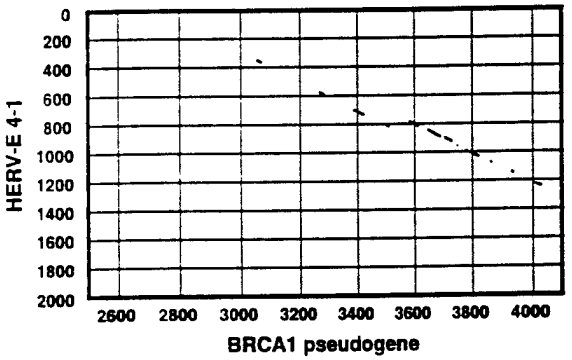
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Fig. 2

D

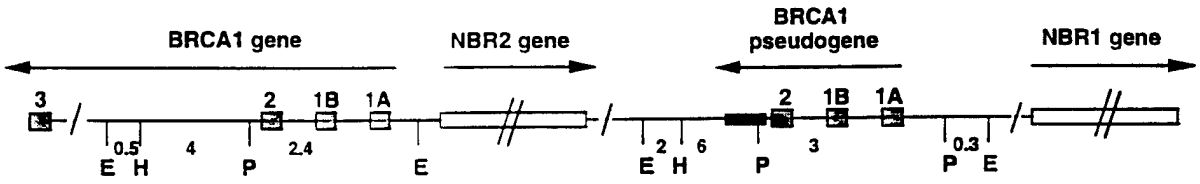
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Fig. 2

E

HERV-E similar
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Fig. 2

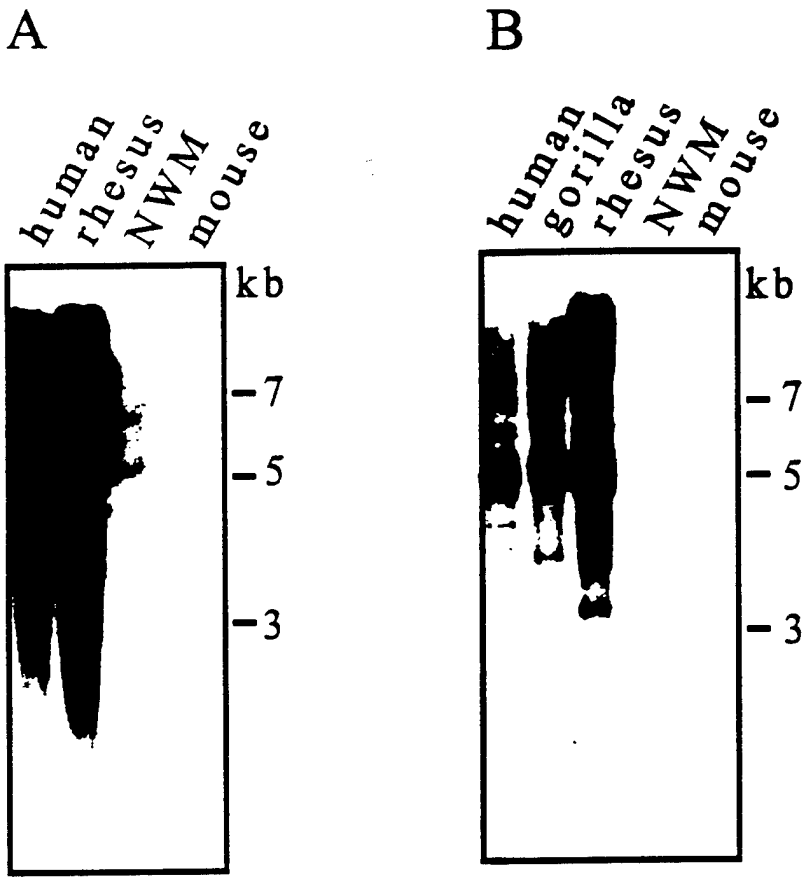
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Fig. 3

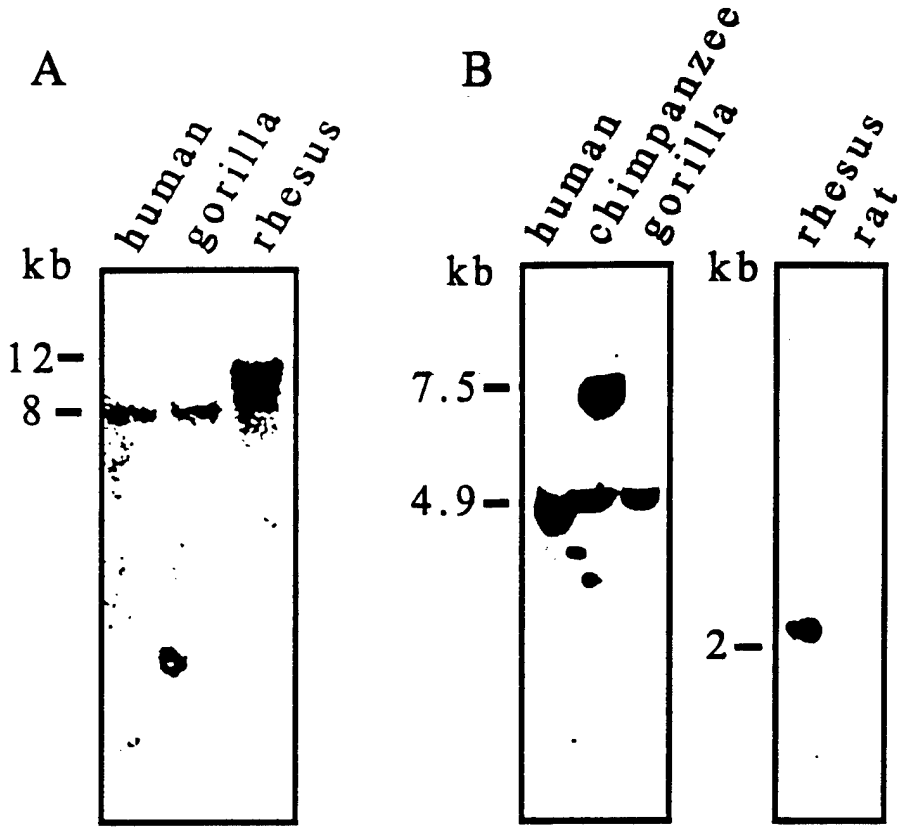
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Fig. 4

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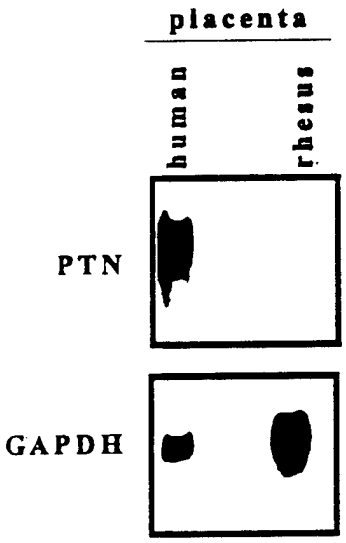
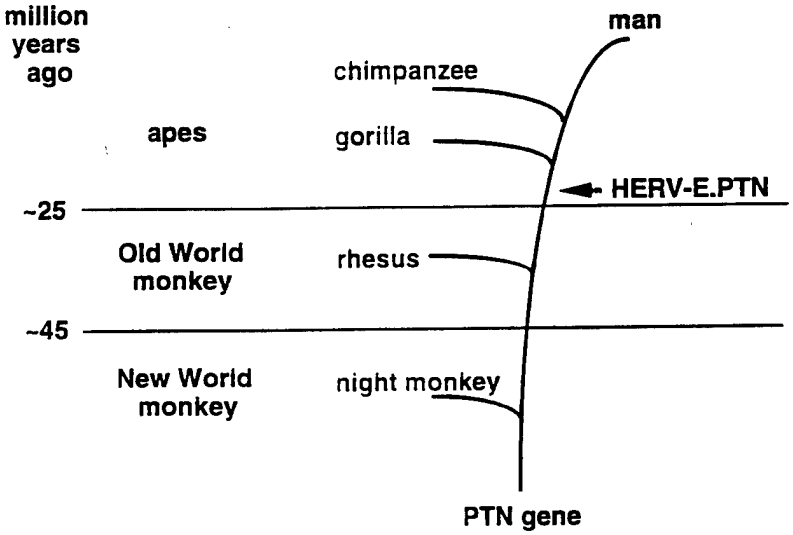


Fig. 4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~45
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PTN gene

Fig. 5

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Signal Transduction Pathways Involved in the Mitogenic Activity of Pleiotrophin

IMPLICATION OF MITOGEN-ACTIVATED PROTEIN KINASE AND PHOSPHOINOSITIDE 3-KINASE PATHWAYS*

(Received for publication, March 21, 1997, and in revised form, May 23, 1997)

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Pleiotrophin (PTN) is a developmentally regulated protein which exhibits neurite-outgrowth, mitogenic, and angiogenic properties. It has also been shown to be involved in tumor growth and metastasis. Here we used primary BEL (bovine epithelial lens) cells to investigate the signal transduction pathways involved in the mitogenic activity of recombinant PTN. PTN was purified from conditioned media of SW-13 cells transfected with the human PTN cDNA. We show that inhibitors of tyrosine kinase, mitogen-activated protein kinase, or phosphoinositide (PI) 3-kinase inhibit DNA synthesis stimulated by PTN. Analysis of tyrosine-phosphorylated proteins following PTN stimulation showed phosphorylation of two novel 190- and 215-kDa proteins in addition to SHC, ERK1, and ERK2. A mobility shift of phosphorylated ERK1 and ERK2 was detected with a panERK antibody confirming the phosphorylation of the two ERKs. Furthermore, *in vitro* immunocomplex kinase assay with Akt1, a natural substrate of PI 3-kinase, showed an activation of the kinase following PTN stimulation and a reversal by the PI 3-kinase inhibitor wortmannin. We conclude that the mitogenic activity of PTN is dependent on tyrosine kinase activation and utilizes the mitogen-activated protein kinase and the PI 3-kinase pathways to transduce a mitogenic signal.

Pleiotrophin (PTN)[1] (1) is an 18-kDa secreted protein belonging to a new family of heparin-binding factors which includes midkine (2) and the chicken retinoic-inducible heparin-binding protein (3). Both, PTN and midkine are developmentally regulated proteins which exibit neurite outgrowth, angiogenic and mitogenic properties (reviewed in Ref. 4), and which enhance plasminogen activator activity (5). PTN and midkine share 50% of sequence homology, but they have no homology with the fibroblast growth factor family of heparin-binding growth factors which display several overlapping biological properties (reviewed in Ref. 6).

The mitogenic activity of PTN is a subject of controversy as conflicting results were reported by different laboratories. On one hand, PTN purified from tissues or expressed in bacteria and in insect cells is devoid of mitogenic activity but still promotes neurite outgrowth (7–9); however, Courty *et al.* (10) reported mitogenic activity after purification of PTN from bovine brain. On the other hand when expressed in eukaryotic cells, PTN is capable of stimulating thymidine incorporation and cell growth in different cell systems including fibroblasts (11), epithelial cells (11, 12), and endothelial cells (11, 13). The failure of purifying mitogenically active PTN in the former systems listed above may be explained by either loss of mitogenic activity during the purification or erroneous folding of the protein when highly expressed. This latter explanation would be in agreement with the findings of Laaroubi *et al.* (13) who demonstrated that PTN expressed in NIH3T3 cells is produced in two forms distinct in their structure and properties. These two forms can be separated in two peaks on a Mono S column; the first peak containing mitogenically active PTN and the second peak containing non-mitogenic PTN. Thus the mitogenic activity of PTN would depend on the balance between the active form(s) and the non active form(s). Further support of the role of PTN in cell growth is provided by experiments showing that transfection of the PTN cDNA in SW13 cells and NIH3T3 cells conferred a growth advantage in soft agar and tumorigenesis in nude mice (11, 14). In addition, recent results from our laboratory show that depletion of PTN from human melanoma and choriocarcinoma cells with ribozymes inhibits tumor growth, invasion, angiogenesis, and metastasis (15–17). Taken together these results point to an important role of PTN in cell proliferation and differentiation as well as in tumor progression. In this report we study the signal transduction pathways involved in the mitogenic activity of PTN and show an implication of the MAP kinase and PI 3-kinase pathways.

MATERIALS AND METHODS

Reagents—Orthovanadate, genistein, tyrphostin A25, and pertussis toxin were purchased from LC Laboratories (Woburn, MA). Wortmannin was from Sigma. PD98059 was obtained from Biolabs (Beverly, MA). [^{3}H]Thymidine and [γ-^{32}P]ATP were purchased from Amersham (Buckinghamshire, United Kingdom). Myelin basic protein was obtained from Sigma. Bovine basic fibroblast growth factor (bFGF) was from Collaborative Research (Bedford, MA). Anti-PTN antibody was a generous gift of Dr. A. Seddon (American Cyanamide Co.). Anti-phosphotyrosine antibody 4G10 was purchased from UBI (Lake Placid, NY). Anti-panERK and anti-SHC antibodies were from Transduction Laboratories (Lexington, KY). Anti-Akt1 antibodies were from Santa Cruz (Santa Cruz, CA) and UBI.

Cell Culture—Bovine epithelial lens (BEL) cells were a generous gift of Dr. J. Courty (Universite Paris 12, France) and routinely grown in

* This work was supported in part by a SPORE grant from the National Cancer Institute, National Institutes of Health (CA58185), an investigator-initiated grant from the U. S. Army Medical Research Materiel Command Breast Cancer Program (DAMD 17-94-J-4445) (to A. W.), and a Career Development Award from the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health (to A. T. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 734 solely to indicate this fact.

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[1] The abbreviations used are: PTN, pleiotrophin; BEL, bovine epithelial lens cells; bFGF, basic fibroblast growth factor; ERK, extracellular signal regulated kinase; FCS, fetal calf serum; MEK, MAP/ERK kinase; PI 3-kinase, phosphoinositide 3-kinase; MAP, mitogen-activated protein; PAGE, polyacrylamide gel electrophoresis; PY, phosphorylated tyrosine.

This paper is available on line at http://www.jbc.org

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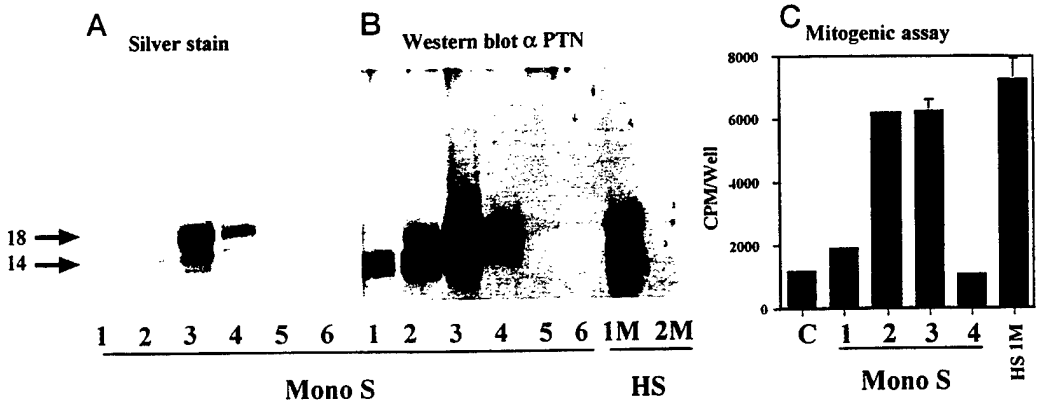

FIG. 1. **Purification and mitogenic activity of PTN.** Recombinant PTN was purified from SW-13/PTN cell conditioned media by initial heparin-Sepharose and subsequent Mono S chromatography. *A*, SDS-PAGE (15% gel) and silver staining of 20-μl aliquots from the Mono S fractions containing PTN. *Numbers* with *arrows*, 14-kDa and 18-kDa PTN. *B*, Western blot for PTN of the fractions from *panel A* in addition to the 1 and 2 M NaCl eluates from heparin-Sepharose. 20-μl aliquots from each fraction were resolved by SDS-PAGE (15% gel), transferred to a nitrocellulose membrane, and blotted with an anti-PTN antibody. *C*, stimulation of [^{3}H]thymidine incorporation in BEL cells by 10-μl aliquots from the heparin-Sepharose and Mono S fractions in *panels A* and *B*. For details see "Materials and Methods."

Dulbecco's modified Eagle's medium supplemented with 10% FCS, 2.2 g/liter sodium bicarbonate (Life Technologies, Gaithersburg, MD), and 1 ng/ml bFGF. PTN overexpressing SW13 cells (SW-13/PTN cells clone W_28), produced by stable transfection with the pRcCMV vector containing the PTN cDNA (11), were maintained in Iscove's modified Dulbecco's medium supplemented with 10% FCS (Life Technologies).

Thymidine Incorporation Assay—This assay was carried out essentially as described in Ref. 12. BEL cells were seeded in 24-well plates for 2–3 days in Dulbecco's modified Eagle's medium supplemented with 10% FCS and 2.2 g of sodium bicarbonate (Life Technologies). The cells were then serum starved for 24 h after which test samples were added. In the experiments involving inhibitors of signal transduction, the cells were pretreated with the drugs for 1 h prior to adding the growth factors. The cells were then incubated for 18 h at 37 °C and 5% CO_2 and then [^{3}H]thymidine was added. After an additional incubation period of 6 h the cells were fixed with 10% trichloroacetic acid, washed with water, and lyzed overnight with 0.3 N NaOH. Total radioactivity incorporated was counted using a Beckman scintillation counter. Each experiment included buffer or vehicle as controls.

Purification of PTN from Conditioned Media—Conditioned media from approximately 10^9 SW-13/PTN cells grown for 4 to 5 days in 1.5 liters of Dulbecco's modified Eagle's medium, 2% FCS was adjusted to 50 mM Tris-HCl, pH 7.5, 0.5 M NaCl and was passed through a 2-ml heparin-Sepharose column (Pharmacia, Piscataway). The column was then washed with 40 ml of 50 mM Tris-HCl, pH 7.5, 0.5 M NaCl and heparin-bound proteins were eluted with 10 ml of 50 mM Tris-HCl, pH 7.5, 1 M NaCl. The eluate was diluted to 50 mM Tris-HCl, pH 7.5, 0.25 M NaCl and passed through a Mono S column using an fast protein liquid chromatography system (Pharmacia). The column was washed extensively in the same buffer containing 0.45 M NaCl and the bound proteins eluted using a gradient from 0.45 to 2 M NaCl. Fractions of 1 ml were collected, quickly aliquoted, and stored at −80 °C.

Western Blot—After separation in SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Bio-Rad) for 2 h at 150 mAmps/gel unless indicated otherwise in 25 mM Tris, pH 8.3, 200 mM glycine, 20% methanol. The membrane was blocked in phosphate-buffered saline, 0.1% Tween 20, 5% powdered milk and probed with the antibodies at appropriate dilutions for 1 h at room temperature. The blots were then washed in phosphate-buffered saline, 0.1% Tween 20 and incubated with the appropriate secondary antibody coupled to horseradish peroxidase (Amersham) for 1 h. After additional washing in phosphate-buffered saline, 0.1% Tween 20, bound antibody was visualized using the enhanced chemiluminescence reagents system from Amersham.

Immunoprecipitations—Cells were grown to 80% confluence in 15-cm dishes, serum starved for 48 h, and then stimulated with PTN or bFGF for 10 min. Cell lysates were prepared by scrapping the cells in immunoprecipitation (IP) buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.5% deoxycholic acid, 1% Nonidet P-40, 10% glycerol, 1 mM sodium orthovanadate, 1 μM okadaic acid, 50 mM sodium fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin A) and incubating for 15 min at 4 °C in a rotating rack. The lysates were cleared by centrifugation and protein content was measured with the Bio-Rad protein assay kit. 0.5 to 1 mg of protein were incubated overnight at 4 ° C with 20 μl of 4G10 anti-phosphotyrosine antibody coupled to agarose beads (UBI). The beads were then washed in IP buffer and proteins were eluted by boiling in SDS-PAGE sample buffer and subjected to electrophoresis and Western blotting.

Immunocomplex Kinase Assay—Cell lysates, prepared as described above and precleared with protein G-Sepharose, were incubated for 4 h at 4 °C with 3 μg of sheep anti-Akt1 antibody (UBI). The immunocomplexes were captured with protein G-Sepharose at 4 °C for 1 h. The beads were then washed with 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol and the kinase assay was carried out as described in Ref. 18 with a slight modification: the beads were resuspended in a kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 5 μM ATP, 1 μM protein kinase A inhibitor peptide, 25 μg/ml myelin basic protein, and 2 μCi [γ-^{32}P]ATP) and incubated for 30 min at 30 °C. The reaction was stopped by addition of 5 × sample buffer and boiling. Samples were then electrophoresed, transferred to a nitrocellulose membrane, and the membrane processed for autoradiography.

Statistical Analysis—Unless stated otherwise data points were run in triplicate and experiments repeated at least twice. Typically the mean ± S.E. from a representative experiment is presented. As appropriate, Student's *t* test or ANOVA was used to assess the statistical significance of differences between measurements (Statview 4.02 program; Abacus Concepts Inc.; Berkeley, CA). The respective *p* values are given in the text. $p < 0.05$ was considered significant.

RESULTS

Purification and Mitogenic Activity of PTN—PTN was purified from 1.5 liters of SW-13/PTN conditioned media by a two-step procedure. The first step involved heparin-Sepharose chromatography and elution with a buffer containing 1 M NaCl. For the second step of purification, the 1 M NaCl eluate was diluted to 0.25 M NaCl and applied to a Mono S column on fast protein liquid chromatography. PTN was then eluted from the column with a gradient of NaCl. PTN eluted from this column at 0.75 M NaCl. The fractions containing the peak were analyzed by SDS-PAGE and silver staining for purity and by Western blot to identify PTN. As shown in Fig. 1*A*, a >95% purification was achieved. Two major bands of apparent molecular mass of 14 and 18 kDa were detected by silver staining and were identified as PTN by Western blotting (Fig. 1, *A* and *B*). Although elution of the two PTN forms appears to overlap, it is obvious from Fig. 1*A* that the 14-kDa protein is eluted first and thus has a slightly lower affinity for the column. This result is consistent with a truncation of positively charged amino acids. As the C terminus of the protein is rich in lysines, it is likely that the 14-kDa PTN protein represents a C-terminally truncated form of PTN as demonstrated using an anti-PTN antiserum raised against a 10-mer peptide from the N terminus of PTN.[2]

[2] A. Wellstein, unpublished data.

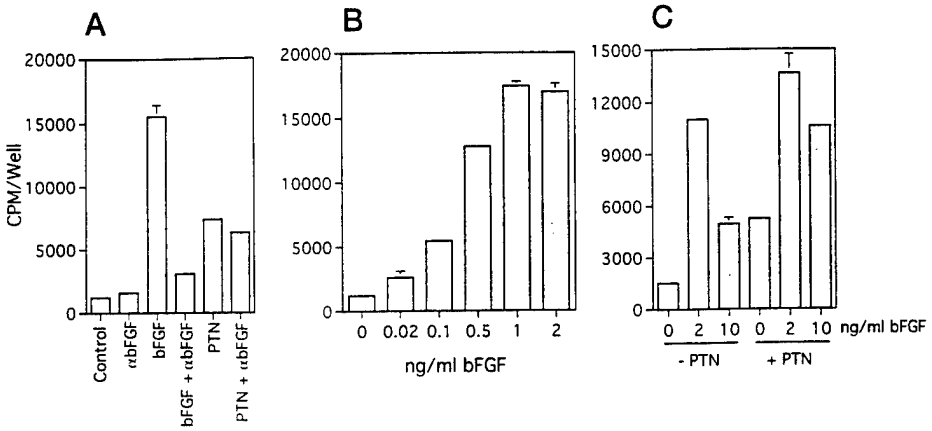

FIG. 2. **Comparison of PTN and bFGF mitogenic activities.** Serum-starved BEL cells were stimulated for 18 h and the thymidine incorporation was detected as described under "Materials and Methods." *A*, neutralization of bFGF but not of PTN mitogenic activities by an anti-bFGF antibody. PTN (10 ng/ml) or bFGF (1 ng/ml) were incubated for 1 h at 4 °C without or with 80 μg/ml anti-bFGF antibody (R&D systems) prior to the addition to serum-starved BEL cells. *B*, dose-response curve of bFGF. *C*, effects of a combination of bFGF (2 or 10 ng/ml) and PTN (10 ng/ml).

The mitogenic activity of PTN was assayed on serum-starved and growth-arrested BEL cells which have been shown to be stimulated by this growth factor (12). Fig. 1*C* shows that the heparin-Sepharose 1 M NaCl fraction and Mono S fractions 2 and 3 induced thymidine incorporation by 5–6-fold. Fraction 1 contained only weakly active material and fraction 4 showed no activity. Conditioned medium from cells transfected with the empty pRc/CMV vector processed for purification under the same conditions showed no mitogenic activity (data not shown). These results indicate that the purified PTN was a mixture of mitogenic and non-mitogenic proteins. Since the mitogenically active form eluted first from the column, it is conceivable that mitogenic activity is mostly due to the truncated protein. The Mono S fraction number 3 was used in the following experiments.

The Mitogenic Activity of PTN Is Independent from the Mitogenic Activity of bFGF—It has been demonstrated previously that bFGF is a potent mitogen for BEL cells (12). To rule out the possibility of contamination of the PTN fractions by bFGF produced endogenously by SW-13 cells (19) and to demonstrate that the mitogenic activity of PTN is independent from that of bFGF, we carried out the experiments shown in Fig. 2. A dose-response curve of bFGF is shown in Fig. 2*B*. The plateau of stimulation was reached at 1 ng/ml bFGF. When BEL cells were stimulated with bFGF or PTN in the presence of anti-bFGF neutralizing antibodies, only the bFGF activity was reduced (Fig. 2*A*) demonstrating that PTN is not contaminated by bFGF. Finally, when BEL cells were stimulated with very high concentrations of bFGF (2 and 10 ng/ml), addition of PTN still stimulated the cells above the stimulation achieved by bFGF alone (Fig. 2*C*). This result indicates that the effects of PTN and bFGF on DNA synthesis are mutually independent and suggests that the PTN receptor is different from the FGF receptor.

Effects of Inhibitors of Tyrosine Kinase, MEK-1, and PI 3-Kinase on the Mitogenic Signal of PTN—To determine which signal transduction pathway(s) is(are) used for mitogenic signaling by PTN, we initially utilized different inhibitors of signal transduction and bFGF, as well as serum, as positive controls. bFGF is known to signal through membrane receptors with tyrosine kinase activity (20) and serum contains a mixture of factors that use different signal transduction pathways.

Pertussis toxin had no effect on DNA synthesis stimulated by PTN and bFGF, but markedly inhibited DNA synthesis induced by serum (Fig. 3*A*). This result indicates that the mitogenic signals of PTN and bFGF are not mediated via pertussis toxin-sensitive G proteins. The tyrosine kinase inhibitors genistein and tyrphostin A25 strongly inhibited DNA synthesis induced by PTN, bFGF, and serum, whereas the tyrosine phosphatase inhibitor orthovanadate did not (Fig. 3*A*). Rather, orthovanadate slightly enhanced PTN-, bFGF-, and serum-induced thymidine incorporation. These results indicate that PTN like bFGF stimulates DNA synthesis through tyrosine kinase pathways and that probably the PTN receptor is not a tyrosine phosphatase. Interestingly, tyrphostin A25 was less potent against PTN than against bFGF since 2 μM of this inhibitor inhibited the bFGF effect by more than 60% and the PTN effect by less than 30% ($p < 0.01$ for inhibition of PTN *versus* inhibition of bFGF). This suggests that some of the tyrosine kinases involved in the signaling of both growth factors are distinct.

The effects of inhibitors of the MAP kinase and the PI 3-kinase pathways on PTN activity were also studied. The MEK-1 inhibitor PD98059 inhibited PTN-, bFGF-, and serum-induced DNA synthesis in a dose-dependent manner (Fig. 3*B*). Interestingly, PTN-stimulated mitogenesis was more sensitive than bFGF- and serum-stimulated mitogenesis. The PTN effect was completely inhibited at 10 μM of the inhibitor whereas a less than 50% inhibition was seen for the other two factors. Similarly, the PI 3-kinase inhibitor wortmannin strongly inhibited the PTN effect (by 75%; $p < 0.01$ for PTN *versus* control) and less efficiently the bFGF effect (by 35%; $p < 0.01$ for bFGF *versus* control; Fig. 3*C*). Serum-induced DNA synthesis was not affected by wortmannin at 10 nM (Fig. 3*C*). Taken together, these results indicate that the MAP kinase and the PI 3-kinase pathways are involved in the mitogenic signal of PTN as well as of bFGF. The apparent selectivity of different inhibitors signifies that the coupling of the signaling steps in the PTN and bFGF pathways are distinct and/or that additional pathways may be used by bFGF and not by PTN. Furthermore, the incomplete inhibition of DNA synthesis by wortmannin indicates that mitogenesis is only partly dependent on a functional PI 3-kinase pathway.

PTN Stimulates Protein Tyrosine Phosphorylation—In BEL cells PTN stimulates tyrosine phosphorylation of a predominant 190-kDa protein as assessed by Western blotting of total cell lysate or after immunoprecipitation and Western blotting with an anti-phosphotyrosine antibody (Fig. 4, *top*). A less prominent 215-kDa protein is tyrosine phosphorylated in addition to the 190-kDa species (Fig. 4, *bottom*, and Fig. 5, *top*). The phosphorylation of p190 and p215 was specific for PTN since both proteins were not phosphorylated after bFGF stim-

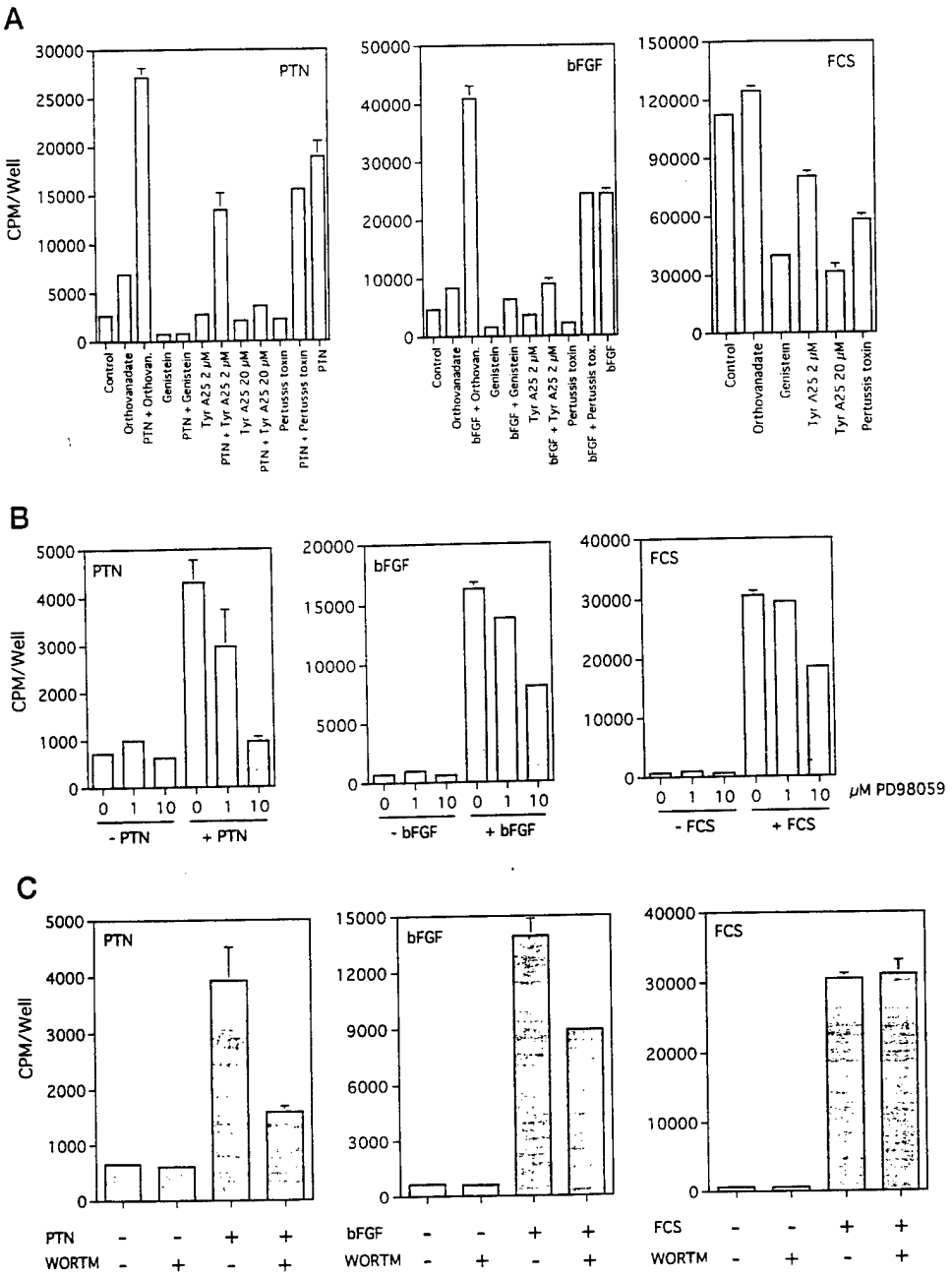

FIG. 3. **Effect of signal transduction inhibitors on the mitogenic activity of PTN.** Serum-starved BEL cells were treated with the indicated drugs starting 1 h before addition of PTN (10 ng/ml), bFGF (1 ng/ml), or 5% FCS for another 18 h. Inhibitors used: *A*, orthovanadate (12.5 μM), genistein (2 μg/ml), pertussis toxin (1 μg/ml), or tyrphostin A25. The background for the *right panel* (without FCS) was 4192 ± 392 cpm. *B*, MEK-1 inhibitor PD98059. *C*, PI 3-kinase inhibitor wortmannin (10 nM). For details see "Materials and Methods."

ulation (Fig. 4, *bottom*) although ERK1 and ERK2 were found tyrosine phosphorylated in response to bFGF (data not shown). In addition, several low molecular weight proteins are tyrosine phosphorylated following PTN stimulation. These proteins have apparent molecular masses of 66, 52, 46, 44, and 42 kDa corresponding to the 3 molecular forms of SHC and to ERK1 and ERK2, respectively (Fig. 5, *bottom*). A Western blot for SHC (Fig. 5, *bottom*) and for ERK (see Fig. 6) confirmed that indeed SHC and ERK1/ERK2 were phosphorylated after PTN stimulation of the cells. Pretreatment of the cells with the PI 3-kinase inhibitor wortmannin did not influence phosphorylation of any of the above proteins as expected from the separate signaling pathways.

To independently confirm phosphorylation of ERK1 and ERK2 following PTN stimulation, cell extracts from unstimulated and stimulated cells were analyzed by Western blotting using a panERK antibody. After PTN stimulation a discrete increase in upward-shifted forms of ERK1 and ERK2 were observed in the Western blots (Fig. 6). This mobility shift is typical of phosphorylated forms of the ERKs. Upon closer inspection a single band of ERK1 was detected in the control cells corresponding to the non-phosphorylated form. Following PTN stimulation, all of the ERK1 was shifted to an apparently higher molecular mass suggesting complete phosphorylation of ERK1 (Fig. 6). For ERK2, a doublet was detected in the control cells corresponding to both non-phosphorylated and phosphorylated forms. Upon PTN stimulation, all of the non-phosphorylated ERK2 shifted to the higher mobility form as expected

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FIG. 4. **PTN-induced tyrosine phosphorylation of 190- and 215-kDa proteins.** *Top panel*, cell lysates from control (*lanes 1* and *3*) or PTN-stimulated cells (10 ng/ml for 10 min; *lanes 2* and *4*) were subjected to direct electrophoresis and Western blotting (*WB PY; lanes 1* and *2*; 25 μg of lysate per lane) or to immunoprecipitation and subsequent Western blotting (*IP PY, WB PY; lanes 3* and *4*; 500 μg of lysate) with the anti-phosphotyrosine antibody 4G10. For immunoprecipitations, agarose-coupled 4G10 antibody was used. Details under "Materials and Methods." *Bottom panel*, in a parallel experiment, cell lysates (25 μg) from control cells (*lane 1*), PTN-stimulated cells (10 ng/ml, 10 min; *lane 2*), or bFGF-stimulated cells (100 ng/ml, 10 min; *lane 3*) were subjected to electrophoresis and Western blotting with the 4G10 antibody.

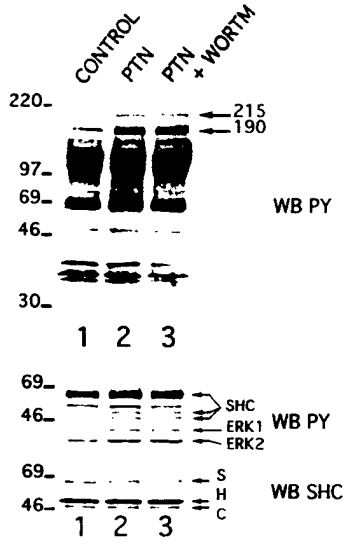

FIG. 5. **PTN-induced tyrosine phosphorylation of SHC, ERK1, and ERK2.** Cell lysate (25 μg) from control cells (*lane 1*) or from cells stimulated with PTN (10 ng/ml; 10 min) without (*lane 2*) and with pretreatment with the PI 3-kinase inhibitor wortmannin (10 nM; 1 h) were separated by SDS-PAGE (10% gel) and tyrosine-phosphorylated proteins present in the lysates were detected by Western blotting with the 4G10 anti-phosphotyrosine antibody (*WB PY*). Both panels represent blots from the same gel transferred for 2 h (*bottom*) and then overnight (*top*) using a second nitrocellulose membrane. SHC proteins on the membrane in the *bottom panel* were identified after stripping and reprobing with an anti-SHC antibody (*WB SHC*).

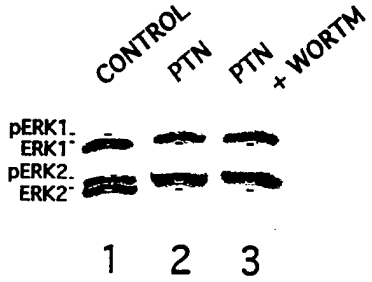

FIG. 6. **PTN-induced mobility shift of ERK1 and ERK2.** Cell lysate (25 μg) from control cells (*lane 1*) or from cells stimulated with PTN (10 ng/ml; 10 min) without (*lane 2*) and with pretreatment with the PI 3-kinase inhibitor wortmannin (10 nM; 1 h) were separated by SDS-PAGE (10% gel) and ERKs present in the lysates were detected by Western blotting with a panERK antibody (see "Materials and Methods"). Phosphorylated (pERK) and non-phosphorylated (ERK) ERKs are indicated.

from pERK2. These results are corroborated by the anti-phosphotyrosine blots in Fig. 5 (*bottom*). As expected from the separate signaling pathways, pretreatment of the cells with wortmannin did not affect phosphorylation of either ERK1 or ERK2.

Activation of the PI 3-Kinase Pathway following PTN Stimulation—Since wortmannin inhibited PTN-induced mitogenesis, we decided to also assay for PI 3-kinase activation and used an *in vitro* assay of the Akt1 kinase to address that. Akt1 (protein kinase B) has been previously shown to be a substrate of PI 3-kinase and to be activated by phosphorylation (21). For our studies, Akt1 was immunoprecipitated from lysates of unstimulated or stimulated cells and immunoprecipitates were used for *in vitro* phosphorylation of myelin basic protein. The data presented in Fig. 7 show an increase of phosphorylation of myelin basic protein following PTN stimulation and a reversal by pretreatment with 10 nM wortmannin. These results indicate that Akt1 activation by PTN was mediated by PI 3-kinase. We conclude from this that PTN also activates the PI 3-kinase pathway to exert its mitogenic effect.

DISCUSSION

PTN is a heparin-binding growth factor that is involved in cell growth and differentiation processes. It stimulates neurite outgrowth, fibroblast, endothelial, and epithelial cell growth (reviewed in Ref. 4). We report here a role of MAP kinase and PI 3-kinase pathways in the mitogenic activity of PTN. Experiments carried out in the presence of signal transduction inhibitors showed that drugs interfering with the tyrosine kinase pathway, genistein and tyrphostin A25, inhibited the mitogenic effect of PTN whereas an inhibitor of the G-protein coupled pathway, pertussis toxin, did not. This result demonstrates that PTN stimulates cell division through a tyrosine kinase pathway and that the PTN receptor is not a G protein-coupled receptor. Interestingly, the different inhibitors showed a different sensitivity of the PTN- *versus* the bFGF-induced DNA synthesis which indicates to us a differential sensitivity either of the respective receptor tyrosine kinases or of their coupling to the pathway downstream. Furthermore, we also found two novel, yet unidentified 190- and 215-kDa phosphoproteins in response to the PTN stimulus and not after bFGF stimulation. The apparent molecular mass of the p190 is consistent with that of a protein described to be phosphorylated after PTN stimulation of NIH3T3 cells (22). In our opinion these proteins are most likely receptor molecules for PTN.

We utilized several well characterized inhibitors to dissect the downstream signaling pathways of PTN in more detail and found that the MEK1 inhibitor PD98059 as well as the PI 3-kinase inhibitor wortmannin inhibited the mitogenic activity of PTN (Fig. 3). This demonstrates that the growth factor

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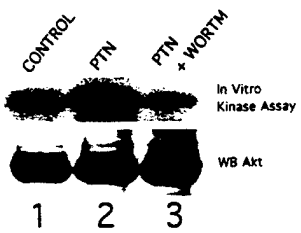

FIG. 7. **Activation of Akt1 after stimulation of BEL cells with PTN.** Cell lysates (500 μg) from control cells (*lane 1*) or from cells stimulated with PTN (10 ng/ml; 10 min) without (*lane 2*) and with pretreatment with the PI 3-kinase inhibitor wortmannin (10 nM; 1 h) were immunoprecipitated with an anti-Akt1 antibody and immunocomplexes were subjected to an *in vitro* kinase assay with myelin basic protein as a substrate (*top*) or separated by SDS-PAGE and Akt present in the complexes was detected by Western blotting (*WB Akt*). Details under "Materials and Methods."

exerts its mitogenic effect through both the MAP kinase and the PI 3-kinase pathways. MEK1 is a component of the MAP kinase pathway which is downstream of activated receptor tyrosine kinases (23, 24). Intermediary steps involve the docking and phosphorylation of adaptor proteins (25) and we demonstrate here that the adaptor protein SHC becomes phosphorylated upon stimulation of BEL cells by PTN (Fig. 5, *bottom panel*). Activated SHC has been shown to bind the complex Grb2 (growth factor receptor-bound 2) and SOS (son of sevenless) which activates Ras by GTP exchange. Ras activates Raf, which in turn activates MEK1. The next step in the cascade is the activation by phosphorylation on tyrosine, serine, and threonine of the MAP kinases ERK1 and ERK2 by MEK1 which culminates in activation of several transcription factors (26). Following stimulation by PTN, ERK1 and ERK2 were indeed phosphorylated as demonstrated by Western blotting and the mobility shift of the proteins (Figs. 5 and 6). Furthermore, tyrosine kinase and MEK1 inhibitors blocked PTN-induced mitogenesis (Fig. 3, *A* and *B*), suggesting that the activation of the above signaling molecules was crucial for the growth factor activity of PTN.

Finally, we studied the participation of PI 3-kinase in the PTN signaling. PI 3-kinase is a heterodimeric protein kinase formed by two subunits of 85 and 110 kDa (27). The p85 subunit is a regulatory protein that mediates, via its SH2 domains, binding of PI 3-kinase to activated receptor tyrosine kinases. The p110 subunit possesses the catalytic domain of the protein. Following binding to a receptor tyrosine kinase, PI 3-kinase phosphorylates phosphoinositides which serve as second messengers in the activation of downstream kinases. Two pathways diverge from PI 3-kinase: one leads to phosphorylation of the $p70^{S6K}$ ribosomal subunit and the other one to activation of the Akt1 and Akt2 proteins (21, 28). Recent studies show that antisense Akt2 RNA inhibited tumor growth of transfected pancreatic cancer cells linking the PI 3-kinase pathway to tumor growth (29). Furthermore, PI 3-kinase and Akt were implicated in the prevention of apoptosis (reviewed in Ref. 30). Our results show that stimulation of cells with PTN leads to activation of Akt1 (Fig. 7) and this activation as well as mitogenesis of PTN was inhibited by the PI 3-kinase inhibitor wortmannin (Figs. 3*C* and 7). Taken together, these results show that the PI 3-kinase pathway is important for the mitogenic signaling of PTN. It is important to note that pretreatment of cells with wortmannin did not affect tyrosine phosphorylation of proteins involved in the Ras/Raf/MAP kinase pathway, indicating that it functions independently from the PI 3-kinase pathway and that both pathways are essential for a full mitogenic signal.

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REFERENCES

1. Li Y. S., Milner, P. G., Chauhan, A. K., Watson, M. A., Hoffman. R. M., Kodner, C. M., Milbrandt, J., and Deuel, T. F. (1990) *Science* **250,** 1690–1694
2. Kadomatsu, K., Tomomura, M., and Muramatsu, T. (1988) *Biochem. Biophys. Res. Commun.* **151,** 1312–1318
3. Raulais, D., Lagente-Chevallier, O., Guettet, C., Duprez, D., Courtois, Y., and Vigny, M. (1991) *Biochem. Biophys. Res. Commun.* **174,** 708–715
4. Schulte, A. M., and Wellstein, A. (1997) in *Tumour Angiogenesis* (Lewis, C. M., Bicknell, R., and Ferrara N., ed) pp. 273–289, Oxford University Press, Oxford
5. Kojima, S., Inui, T., Muramatsu, H., Kimura, T., Sakakibara, S., and Muramatsu, T. (1995) *Biochem. Biophys. Res. Commun.* **216,** 574–581
6. Basilico, C., and Moscatelli, D. (1992) *Adv. Cancer Res.* **59,** 115–164
7. Hampton, B. S., Marshak, D. R., and Burgess, W. S. (1992) *Mol. Biol. Cell* **3,** 85–93
8. Takamatsu, H., Itoh, M., Kimura, M., Gospodarowicz, D., and Amann, E. (1992) *Biochem. Biophys. Res. Commun.* **185,** 224–230
9. Raulo, E., Julkunen, I., Merenmies, J., Pihlaskari, R., and Rauvala, H. (1992) *J. Biol. Chem.* **267,** 11408–11416
10. Courty, J., Dauchel, M. C., Caruelle, D., Perderiset, M., and Barritault, D. (1991) *Biochem Biophys. Res. Commun.* **180,** 145–151
11. Fang, W., Hartmann, N., Chow, D. T., Riegel, A. T., and Wellstein, A. (1992) *J. Biol. Chem.* **267,** 25889–25897
12. Delbe, J., Vacherot, F., Laaroubi, K., Barritault, D., and Courty, J. (1995) *J. Cell. Physiol.* **164,** 47–54
13. Laaroubi, K., Delbe, J., Vacherot, F., Desgranges, P., Tardieu, M., Jaye, M., Barritault, D., and Courty, J. (1994) *Growth Factors* **10,** 89–98
14. Chauhan, A. K., Li, Y. C., and Deuel, T. F. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 679–682
15. Czubayko, F., Riegel, A. T., and Wellstein, A. (1994) *J. Biol. Chem.* **269,** 21358–21363
16. Czubayko, F., Schulte, A. M., Berchem, G. J., and Wellstein, A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 14753–14758
17. Schulte, A. M., Lai, S., Kurtz, A., Czubayko, F., Riegel, A. T., and Wellstein, A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 14759–14764
18. Kohn, A. D., Kovacina, K. S., and Roth, R. A. (1995) *EMBO J.* **14,** 4288–4295
19. Wellstein, A., Lupu, R., Zugmaier, G., Flamm, S. L., Cheville, A. L., Delli Bovi, P., Basilico, C., Lippman, M. E., and Kern, F. G. (1990) *Cell Growth Differ.* **1,** 63–71
20. Schlessinger, J., and Ullrich, A. (1992) *Neuron* **9,** 383–391
21. Franke, T. F., Yang, S., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tchilis, P. N. (1995) *Cell* **81,** 727–736
22. Li, Y. S., and Deuel, T. F. (1993) *Biochem. Biophys. Res. Commun.* **195,** 1089–1095
23. Seger, R., and Krebs, E. G. (1995) *FASEB J.* **9,** 726–735
24. Heldin, C. H. (1995) *Cell* **80,** 213–223
25. Pawson, T. (1995) *Nature London* **373,** 573–580
26. Hill, C. S., and Treisman, R. (1995) *Cell* **80,** 199–211
27. Dhand, R., Hara, K., Hiles, I., Bax, B., Gout, I., Panayotou, G., Fry, M. J., Yonezawa, K., Kasuga, M., and Waterfield, M. D. (1994) *EMBO J.* **13,** 511–521
28. Burgering, B. M. T., and Coffer, P. J. (1995) *Nature* **376,** 599–602
29. Cheng, J. Q., Ruggeri, B., Klein. W. M., Sonoda, G., Altomare, D. A., Watson, D. K., and Testa, J. R. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 3636–3641
30. Franke, T. F., Kaplan, D. R., and Cantley, L. C. (1997) *Cell* **88,** 435–437

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Medical Sciences

Human trophoblast and choriocarcinoma expression of the growth factor pleiotrophin attributable to germ-line insertion of an endogenous retrovirus

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ABSTRACT **Retroviral elements are found in abundance throughout the human genome but only rarely have alterations of endogenous genes by retroviral insertions been described. Herein we report that a human endogenous retrovirus (HERV) type C is inserted in the human growth factor gene pleiotrophin (PTN) between the 5′ untranslated and the coding region. This insert in the human genome expands the region relative to the murine gene. Studies with promoter-reporter constructs show that the HERV insert in the human PTN gene generates an additional promoter with trophoblast-specific activity. Due to this promoter function, fusion transcripts between HERV and the open reading frame of PTN (HERV-PTN) were detected in all normal human trophoblast cell cultures as early as 9 weeks after gestation ($n = 7$) and in all term placenta tissues ($n = 5$) but not in other normal adult tissues. Furthermore, only trophoblast-derived choriocarcinoma cell lines expressed HERV-PTN mRNA whereas tumor cell lines derived from the embryoblast (teratocarcinoma) or from other lineages failed to do so. We investigated the significance of HERV-PTN mRNA in a choriocarcinoma model by targeting this transcript with ribozymes and found that the depletion of HERV-PTN mRNA prevents human choriocarcinoma growth, invasion, and angiogenesis in mice. This suggests that the tissue-specific expression of PTN due to the HERV insertion in the human genome supports the highly aggressive growth of human choriocarcinoma and possibly of the human trophoblast.**

Pleiotrophin (PTN) is a secreted heparin-binding polypeptide growth factor (1) with mitogenic (1–3) and transforming effects (3) on fibroblasts and growth factor activity on epithelial (2, 4, 5) and endothelial (2, 5, 6) cells. Furthermore, PTN induces the release of proteolytic enzymes from endothelial cells (7) and stimulates neurite outgrowth (8) and tube formation by endothelial cells *in vitro* (5) as well as angiogenesis in the rabbit corneal pocket assay (6). PTN gene expression is regulated in a time- and tissue-specific manner during rodent development and PTN mRNA is found at high levels in the central nervous system during the perinatal period, is down-regulated thereafter, and is present at low levels in a few adult tissues (1, 9–11). On the other hand, the PTN gene is up-regulated in several human tumor tissues and tumor cell lines (2) but little is known about the regulatory elements in this gene (12).

To understand the mechanisms that regulate expression of the human PTN gene, we studied 5′ ends of PTN mRNA isolated from various human tissues that express the PTN gene. To our surprise we found that all placenta samples, in contrast to brain, expressed PTN mRNA with 5′ exons that are homologous to a human endogenous retrovirus (HERV) and are spliced onto the intact open reading frame (ORF) of PTN. Upon analysis of human genomic DNA, we located the insertion of an HERV fragment into the intron region upstream of the ORF of the human PTN gene expanding this region relative to the ancestral PTN gene shared with other nonprimate species.

HERV-like particles were identified more than two decades ago in human oocytes, teratocarcinoma cells, and mammary carcinoma tissues, as well as in placenta, and retroviral transcripts were detected in a number of tissues and cell lines (13–17). These findings reflect the high number of HERV fragments found integrated throughout the human genome (several thousand copies). However, only one example of HERV germ-line insertion that induces changes of the expression pattern of a functional human gene product has been reported to date (18, 19): a C-type HERV was found integrated in reverse orientation into the 5′ flanking region of human amylase genes and was shown to function as an enhancer that confers additional salivary gland expression of amylase.

In the present paper, we demonstrate that germ-line insertion of an HERV fragment generates a phylogenetically new promoter within the human PTN gene. This insertion confers trophoblast-specific expression of functional PTN gene products. We evaluate the significance of this finding for the growth phenotype of human trophoblast-derived choriocarcinoma and discuss potential implications for the growth of the normal human trophoblast.

MATERIALS AND METHODS

Cell Lines and Growth Assays. Human choriocarcinoma (JEG-3 and JAR), teratocarcinoma (PA-1), and adrenal carcinoma (SW-13) cells were from the American Type Culture Collection and were grown in Iscove's modified medium (IMEM) with 10% fetal calf serum (FCS; Life Technologies, Gaithersburg, MD); human melanoma cells (1205LU; gift from M. Herlyn, Wistar Institute, Philadelphia) in KSFM/L15 medium mixed at a ratio of 3:1 (Life Technologies) and supplemented with 5% FCS. Primary cells grown from chorionic villus samples obtained for prenatal diagnostics were a gift of J. Simon (Georgetown University) and were kept in IMEM with 20% FCS. To determine the proliferation rates of differently modified JEG-3 cells, 2×10^4 cells were plated in triplicates into six-well plates and the number of cells was counted at different time intervals.

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Abbreviations: HERV, human endogenous retrovirus; PTN, pleiotrophin; 5′-UTR, 5′ untranslated region; RACE, rapid amplification of cDNA ends.

Data deposition: The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U71455 and U71456).

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Gene Structure Analysis. Nonoverlapping phagemid P1 clones (Genome Systems, St. Louis) were used to complete the structural analysis of the 5′ untranslated region (5′-UTR) of the human PTN gene reported earlier (20, 21). P1 clones containing the upstream untranslated exon U1 (P203) or the first exon of the ORF, O1 (P2258), were obtained by PCR screening with specific primers. Long-range PCR (Expand long template PCR, Boehringer Mannheim) with P1 clone 2258 (containing O1) or with human genomic DNA and subcloning and sequencing of inserts was used to compile the structure of the human PTN gene. The nucleic acid sequence of the inserted promoter region and of HERV-derived exons UV3 and in part of UV2 were obtained from a genomic DNA fragment subcloned from a *Bam*HI restriction library of P1 clone P2258 (containing O1), which was screened for positive clones with a UV3-specific probe. Furthermore, the 5′ rapid amplification of cDNA ends (RACE) PCR products of placenta cDNA were sequenced for comparison and to complement data from the genomic cloning.

5′-RACE PCR. Two 5′-RACE cDNA libraries generated from human placenta and human brain mRNA (CLONTECH) served to generate PCR fragments of the 5′ ends of different PTN cDNAs. Nested primers derived from exon O1 were used as 3′ primers in the PCR, and an antisense oligonucleotide to the anchor sequence was used as a 5′ primer. PCR fragments were subcloned into TA-cloning vectors (Invitrogen) and inserts were sequenced.

Mapping of the Transcription Start Site by Primer Extension. The avian myeloblastosis virus (AMV) reverse transcriptase primer extension system (Promega) was used with poly(A)$^+$ RNA (9 μg) or total RNA (50 μg) as a template. RNA from JEG-3 and JAR choriocarcinoma cells (PTN-positive) or SW-13 cells (PTN-negative) was incubated with two UV3-specific nested primers designed to hybridize to the sequence stretch between positions −11154 and −11170 and positions −11168 and −11184, respectively (see Fig. 1*B*). After denaturation of the RNA for 30 min at 65°C, the primer hybridization reaction was incubated for 1 h at 52°C followed by a 1-h incubation at 42°C with AMV reverse transcriptase. The samples were then heated for 10 min at 90°C in formamide loading buffer and analyzed on a 6% sequencing gel. Sequencing reactions with each of the primers were used to read the position of the extended product.

Transcriptional Activity. A 1.9-kb *Hin*dIII–*Bam*HI (H, B) genomic fragment from P1 clone P2258 was used for these studies. This fragment starts upstream of the *Alu* region (position −12,534) and contains the TATA box and transcription start site of the HERV-PTN fusion transcripts and ends in exon UV2 (positions −10,640) (see Fig. 1). The fragment was cloned in both orientations into the pXP-1 promoterless luciferase reporter gene vector (25) and then used in transient transfection assays in different cell lines. For this, cells were plated overnight at 60–70% confluence in six-well plates and then transfected in Optimem (Life Technologies) with 1 μg of DNA per well using 7 μl of LipofectAmine (Life Technologies) for the JEG-3, JAR, and SW-13 cells and 2.5 μl of Transfectam (Pharmacia) for the 1205LU cells. After 5 h, transfection medium was replaced by fresh culture medium and the cells were incubated for another 24–36 h. Thereafter cells were harvested, washed, lysed in 0.25 M Tris·HCl (pH 7.8), and freeze–thawed three times, and 5–20 μl of the lysate was mixed with 350 μl of 0.1 M potassium phosphate/15 mM $MgCl_2$/5 mM ATP at pH 7.8 and assayed for luciferase activity using 1 mM D-luciferin as a substrate. The promoter activity is shown as fold induction relative to the parent vector pXP-1. A cytomegalovirus-driven luciferase expression vector was used to control for transfection efficacy.

Depletion of PTN mRNA by Using Ribozyme Targeting. The PTN-targeted ribozyme Rz*261* (26) was expressed under the control of the tTA/heptameric operator binding site and a cytomegalovirus minimal promoter (27). For this purpose, the major portion of the luciferase gene and the simian virus 40 polyadenylylation site in the pUHC13-3 plasmid (27) were deleted by *Hin*dIII/*Hpa*I digestion and replaced with the Rz*261*/bovine growth hormone polyadenylylation *Hin*dIII–*Pvu*II fragment from the pRc/Rz*261* expression vector (26). The remaining luciferase start codon was replaced by a *Sal*I/*Cla*I/*Hin*dIII cassette to yield the construct pTET/Rz*261*. This ribozyme is designed to cleave PTN mRNA 3′ of nt 261 of the ORF (26). In JEG-3 cells, the ribozyme expression vector (pTET/Rz*261*, 0.5 μg), was cotransfected with the tTA expression vector [pUHG15-1 (27), 0.5 μg] and pRc/CMV (0.1 μg) to provide G418 resistance. After selection for stable integrants in the presence of G418 at 1 mg/ml, the cells were tested for PTN expression by Northern blot analysis.

Northern Blot Analysis. Total RNA from cell lines or tissues was isolated with the RNA STAT-60 method (Tel-Test, Friendswood, TX), separated, and blotted as reported (2). In addition, a human multiple tissue Northern blot (CLONTECH) was used. PTN cDNA probes specific for the ORF (2) or 5′ untranslated exon U1 (287-nt fragment) or HERV-derived exon UV3 (257-nt fragment) were hybridized, washed, and autoradiographed for 48 h as described (2). After exposure, blots were stripped and reprobed. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control.

RESULTS

Insertion of Retroviral Elements into the Human PTN Gene. To elucidate the mechanisms that regulate expression of the human PTN gene, we examined the 5′ regions of mRNAs isolated from placenta and adult brain by 5′-RACE PCR. In particular, PTN expressed in placental tissues appeared of interest to us, since placenta is mostly derived from fetal tissues and a human cDNA clone was originally reported from the screening of a placental library (1). Furthermore, *in situ* hybridization (9) as well as Northern blot analysis (unpublished data) with rodent trophoblast tissues had failed to detect a signal for PTN in contrast to a strong signal in Northern blots with human placenta (see below). To our surprise, 10 of 11 5′-RACE PCR clones with mRNA from placenta contained novel 5′-UTRs that are distinct from the previously described 5′-UTR in human placental and brain cDNAs.

Sequence comparisons revealed that the novel 5′ exons contained in the PTN mRNA from placenta are highly homologous to different regions of HERV type C (22, 23, 28). Analysis of human genomic DNA revealed that the HERV fragment is inserted in sense orientation into the intron region immediately upstream of the ORF of the human PTN gene expanding this region relative to the murine gene (Fig. 1*A*). Low-stringency Southern blot analysis confirmed insertion of HERV also in the rhesus monkey genome and showed the lack thereof in murine genomic DNA (unpublished data). The most 5′ HERV-derived PTN exon (UV3) is homologous to the viral 5′ long terminal repeat region and the downstream UV2 and UV1 exons are homologous to regions of the HERV *gag*, *pol*, and *env* pseudogenes (70, 85, and 80% identity, respectively; Fig. 1*B*) (22, 23). Unlike infectious C-type viruses encountered today, all of which contain a $tRNA^{Pro}$ primer binding site in their DNA (22), this prehistoric virus contains a $tRNA^{Glu}$ primer binding site as its signature (Fig. 1*B*).

Expression of HERV-PTN Fusion Transcripts and of PTN Protein. The three HERV-derived exons are directly spliced to the first exon in the ORF of the PTN gene (O1) using splice donor sites distinct from the site reported in the retrovirus (Fig. 1*B*) (22). The HERV-PTN fusion transcripts were present in placental cDNA at a ratio of 8:1:1 for UV3, UV2, and UV1, respectively, based on the number of clones obtained from the 5′-RACE PCR analysis. Exon U1 spliced to O1 was found in only 1 of 11 placental 5′-RACE PCR clones. This

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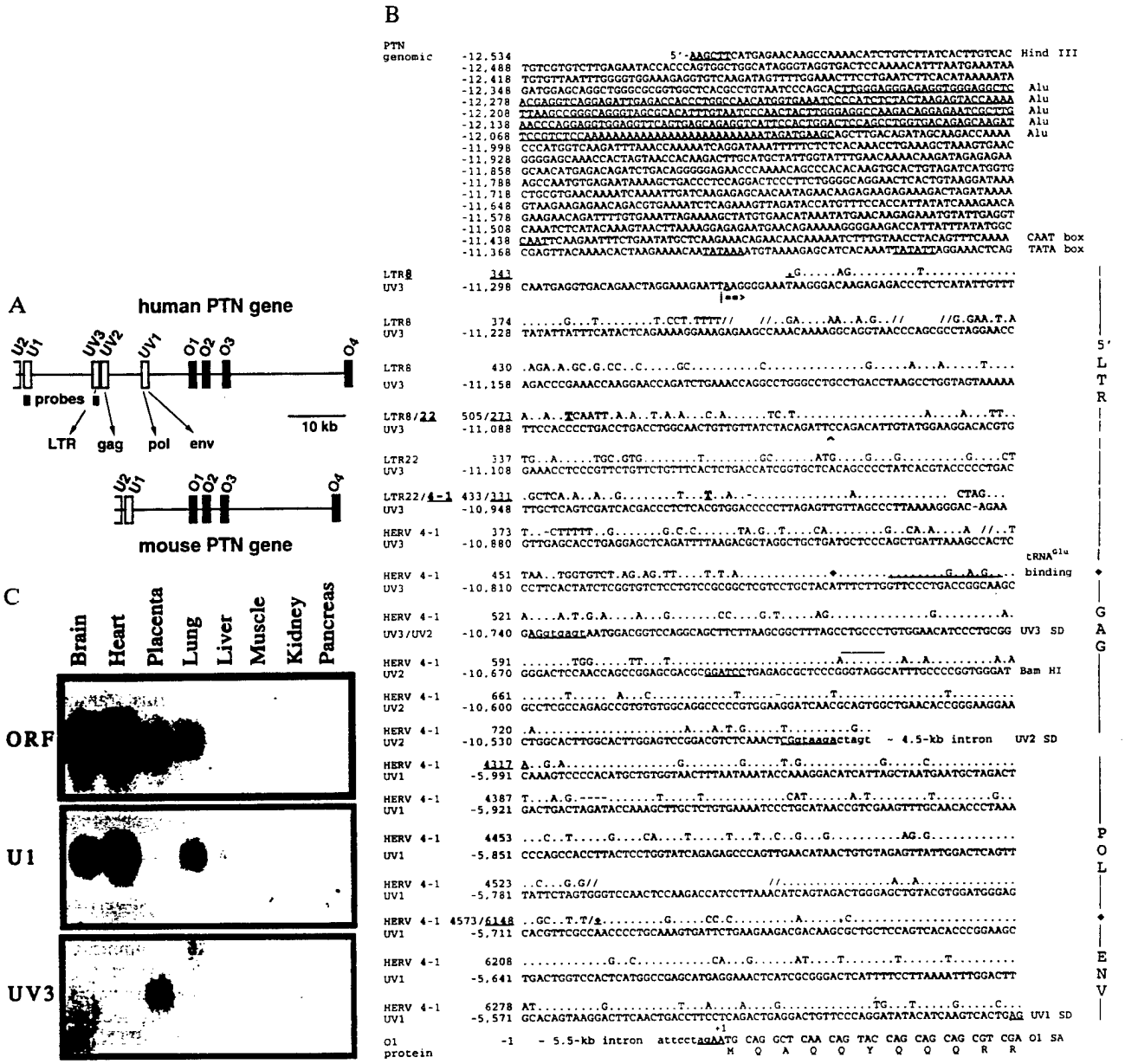

B

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PTN
genomic    -12,534                    5'-AAGCTTCATGAGAACAAGCCAAAACATCTGTCTTATCACTTGTCAC  Hind III
           -12,488 TGTCGTGTCTTGAGAATACCACCCAGTGGCTGGCATAGGGTAGGTGACTCCAAAACATTTAATGAAATAA
           -12,418 TGTGTTAATTTGGGGTGGAAAGAGGTCTCAAGATAGTTTTGAAACTTCCTGAATCTTCACATAAAAATA
           -12,348 GATGGAGCAGGCTGGGCGGCGGTGGCTCACGCCTGTAATCCCAGCACTTGGGAGGGAGAGGTGGGAGGCTC  Alu
           -12,278 ACCAGGTCAGGAGATTGAGACCACCCTGGCCAACATGGTGAAATCCCCATCTCTACTAAAGATACCAAAA  Alu
           -12,208 TTAAGCCGGGCAGGGTAGCGCACATTTGTAATCCCAACTACTTGGGAGGCCAAGACAGGAGAATCGCTTG  Alu
           -12,138 AACCCAGGAGGTGGAGGTTCAGTGAGCAGAGGTCATTCCACTGGACTCCAGCCTGGTGACAGAGCAAGAT  Alu
           -12,068 TCCGTCTCCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  Alu
           -11,998 CCCATGGTCAAGATTTAAACCAAAAATCAGGATAAATTTTTCTCTCACAAACCTGAAAGCTAAAGTGAAC
           -11,928 GGGGAGCAAACCACTAGTAACCACAAGACTTGCATGCTATTGGTATTTGAACAAAACAAGATAGAGAGAA
           -11,858 GCAACATGAGACAGATCTGACAGGGGGAGAACCCAAAACAGCCCACACAAGTGCACTGTAGATCATGGTG
           -11,788 AGCCAATGTGAGAATAAAAGCTGACCCTCCAGGACTCCCTTCTGGGGCAGGAACTCACTGTAAGGATAAA
           -11,718 CTGCGTGAACAAAATCAAAATTGATCAAGAGAGCAACAATAGAACAAGAGAAGAGAAAGACTAGATAAAA
           -11,648 GTAAGAAGAGAACAGACGTGAAAATCTCAGAAAGTTAGATACCATGTTTCCACCATTATATCAAAGAACA
           -11,578 GAAGAACAGATTTTGTGAAATTAGAAAAGCTATGTGAACATAAATATGAACAAGAGAAATGTATTGAGGT
           -11,508 CAAATCTCATACAAAGTAACTTAAAAGGAGAGAATGAACAGAAAAAGGGGAAGACCATTATTTATATGGC
           -11,438 CAATTCAAGAATTTCTGAATATGCTCAAGAAACAGAACAACAAAAATCTTTGTAACCTACAGTTTCAAAA  CAAT box
           -11,368 CGAGTTACAAAACACTAAGAAAACAATATAAAAATGTAAAAGAGCATCACAAATTATATTAGGAAACTCAG  TATA box

LTR8         141                                    .G.....AG.........T.............
UV3        -11,298 CAATGAGGTGACAGAACTAGGAAAGAATTAAGGGGAAATAAGGGACAAGAGAGACCCTCTCATATTGTTT
                                                    |==>

LTR8         374 ......G...T........T.CCT.TTTT//    //..GA....AA..A.G../.     //G.GAA.T.A
UV3        -11,228 TATATTATTTCATACTCAGAAAAGGAAAGAGAAGCCAAACAAAAGGCAGGTAACCCAGCGCCTAGGAACC

LTR8         430 .AGA.A.GC.G.CC..C.....GC..........C..............G.....A...A.....T....
UV3        -11,158 AGACCCGAAACCAAGGAACCAGATCTGAAACCAGGCCTGGGCCTGCCTGACCTAAGCCTGGTAGTAAAAA

LTR8/22    505/271 A..A..TCAATT.A.A..T.A.A...C.A......TC.T...................A....A...TT..
UV3        -11,088 TTCCACCCCTGACCTGACCTGGCAACTGTTGTTATCTACAGATTCCAGACATTGTATGGAAGGACACGTG
                                                                ^

LTR22        337 TG..A.....TGC.GTG.........T.........GC......ATG....G...G.........G....CT
UV3        -11,108 GAAACCTCCCGTTCTGTTCTGTTTCACTCTGACCATCGGTGCTCACAGCCCCTATCACGTACCCCCTGAC

LTR22/4-1  433/111 .GCTCA.A..A..G........T...T..A..-..............A.............. CTAG...
UV3        -10,948 TTGCTCAGTCGATCACGACCCTCTCACGTGGACCCCCTTAGAGTTGTTAGCCCTTAAAAGGGAC-AGAA

HERV 4-1     373 T..-CTTTTT..G.......G.C.C......TA.G..T....CA........G..CA.A....A //..T
UV3        -10,880 GTTGAGCACCTGAGGAGCTCAGATTTTAAGACGCTAGGCTGCTGATGCTCCCAGCTGATTAAAGCCACTC
                                                                                  tRNA^Glu
HERV 4-1     451 TAA..TGGTGTCT.AG.AG.TT....T.T.A.............*.........G..A.G... binding  *
UV3        -10,810 CCTTCACTATCTCGGTGTCTCCTGTCCGCGGCTCGTCCTGCTACATTTCTTGGTTCCCTGACCGGCAAGC

HERV 4-1     521 A....A.T.G.A....A...G.......CC....G.T.....AG..............G.........A.
UV3/UV2    -10,740 GAGgtgagtAATGGACGGTCCAGGCAGCTTCTTAAGCGGCTTTAGCCTGCCCTGTGGAACATCCCTGCGG  UV3 SD
                                                                                  ------
HERV 4-1     591 .......TGG.....TT...T.........................A.........A..A.........A.A
UV2        -10,670 GGGACTCCAACCAGCCGGAGCGACGCGGATCCTGAGAGCGCTCCCGGGTAGGCATTTGCCCCGGTGGGAT  Bam HI

HERV 4-1     661 .......T.....  A...C.............T.....-.....T.....T.....T.....
UV2        -10,600 GCCTCGCCAGAGCCGTGTGTGGCAGGCCCCCGTGGAAGGATCAACGCAGTGGCTGAACACCGGGAAGGAA

HERV 4-1     720 .A.....................A...A.T.G.....T.........G..
UV2        -10,530 CTGGCACTTGGCACTTGGAGTCCGGACGTCTCAAACTCgtgaaagactagt  ~ 4.5-kb intron   UV2 SD

HERV 4-1    4117 A..G.A.................G.......G.....T.G...........G.....C.....
UV1         -5,991 CAAAGTCCCCACATGCTGTGGTAACTTTAATAAATACCAAAGGACATCATTAGCTAATGAATGCTAGACT

HERV 4-1    4387 T...A.G.----.......T.....T.............CAT......A.T.........T.........G..
UV1         -5,921 GACTGACTAGATACCAAAGCTTGCTCTGTGAAAATCCCTGCATAACCGTCGAAGTTTGCAACACCCTAAA

HERV 4-1    4453 ...C..T.....G....CA....T.....T...T..C..G...G...........AG.G.............
UV1         -5,851 CCCAGCCACCTTACTCCTGGTATCAGAGAGCCCAGTTGAACATAACTGTGTAGAGTTATTGGACTCAGTT

HERV 4-1    4523 ..C...G.G//                        //.................A..A..............
UV1         -5,781 TATTCTAGTGGGTCCAACTCCAAGACCATCCTTAAACATCAGTAGACTGGGAGCTGTACGTGGATGGGAG

HERV 4-1 4573/6148 ..GC..T.T/*.........G.....CC.C..........A.....C.....................
UV1         -5,711 CACGTTCGCCAACCCCTGCAAAGTGATTCTGAAGAAGACGACAAGCGCTGCTCCAGTCACACCCGGAAGC

HERV 4-1    6208 ............G..C............CA...G......AT....T.......T......T.........
UV1         -5,641 TGACTGGTCCACTCATGGCCGAGCATGAGGAAACTCATCGCGGGACTCATTTTCCTTAAAATTTGGACTT

HERV 4-1    6278 AT.........G..........T...A....A....G.............TG...T.....G.....C...
UV1         -5,571 GCACAGTAAGGACTTCAACTGACCTTCCTCAGACTGAGGACTGTTCCCAGGATATACATCAAGTCACTGAG  UV1 SD
                                                                +1
O1             -1   - 5.5-kb intron  attcctagAATG CAG GCT CAA CAG TAC CAG CAG CAG CGT CGA  O1 SA
protein                              M  Q  A  Q  Q  Y  Q  Q  Q  R  R
```

FIG. 1. Insertion of HERV into the human PTN gene and tissue-specific distribution of HERV-PTN fusion transcripts. (*A*) Comparison of the genomic organization of the human and murine PTN genes. Homologous exons corresponding to the ORF (O1 to O4) or 5′-UTR (U2, U1) of the murine (24) and the human gene (20, 21), as well as the HERV-derived exons (UV3, UV2, and UV1) and the position of exon-specific probes, are shown. (*B*) Sequence comparison between the HERV insert in the human PTN gene and type C HERV fragments LTR8, LTR22, and HERV4-1 (22, 23) using the GCG program. Gaps (///) were introduced for optimal alignment. The PTN gene numbering system (GenBank accession nos. U71455 and U71456) on the left refers to the first nucleotide of the translation start codon as +1. The viral sequence positions were taken from the respective GenBank entries for LTR8, LTR22, and HERV4-1 (accession nos. M32219, M32220, and K02168, respectively). A 280-nt *Alu* sequence in the promoter region and CAAT and TATA boxes are underlined and intronic sequence is denoted by lowercase type. The transcription start site (|==>) was mapped by primer extension. Similarity to retroviral elements is indicated on the right and the respective nucleotide positions separating the different elements are highlighted in the sequence. The tRNA^{Glu} primer binding site, UV3 cDNA end (^), restriction sites used for generation of the promoter-reporter constructs, and the N-terminal sequence of the PTN protein are shown. The splice donor (SD) sites of UV3, UV2, and UV1, as well as the splice acceptor (SA) in exon O1, are underlined and the retroviral SD of HERV 4–1 is indicated in addition (by dots above the sequence). (*C*) Northern blot analysis of mRNA from various human tissues using exon-specific probes.

predominant expression of the HERV-derived exons in placenta contrasted with the lack of expression of these exons in brain; all seven 5′-RACE PCR clones obtained from brain contained only exon U1 directly spliced to O1. Northern blot analysis with exon-specific probes confirmed this striking difference between placenta and brain (Fig. 1*C*). Other retroviral transcripts reported from placental tissues (17) did not show cross-hybridization signals.

We detected HERV-PTN fusion transcripts not only in human term placenta tissue ($n = 5$) but also in the trophoblast of a fetus stillborn after 15 weeks of gestation, in primary cultures of cells grown from trophoblast biopsies obtained for prenatal diagnostics (9–12 weeks of gestation; $n = 7$), and in trophoblast-derived human choriocarcinoma cell lines (Fig. 2*A*). In addition to Northern blot analysis, RNase protection studies and reverse transcription-coupled PCR confirmed the presence of HERV-PTN fusion transcripts and the lack of U1 exon usage in JEG-3 and JAR choriocarcinoma cells (data not shown). In contrast to the use of HERV-derived 5′ exons in the trophoblast and choriocarcinoma, PTN mRNA from other

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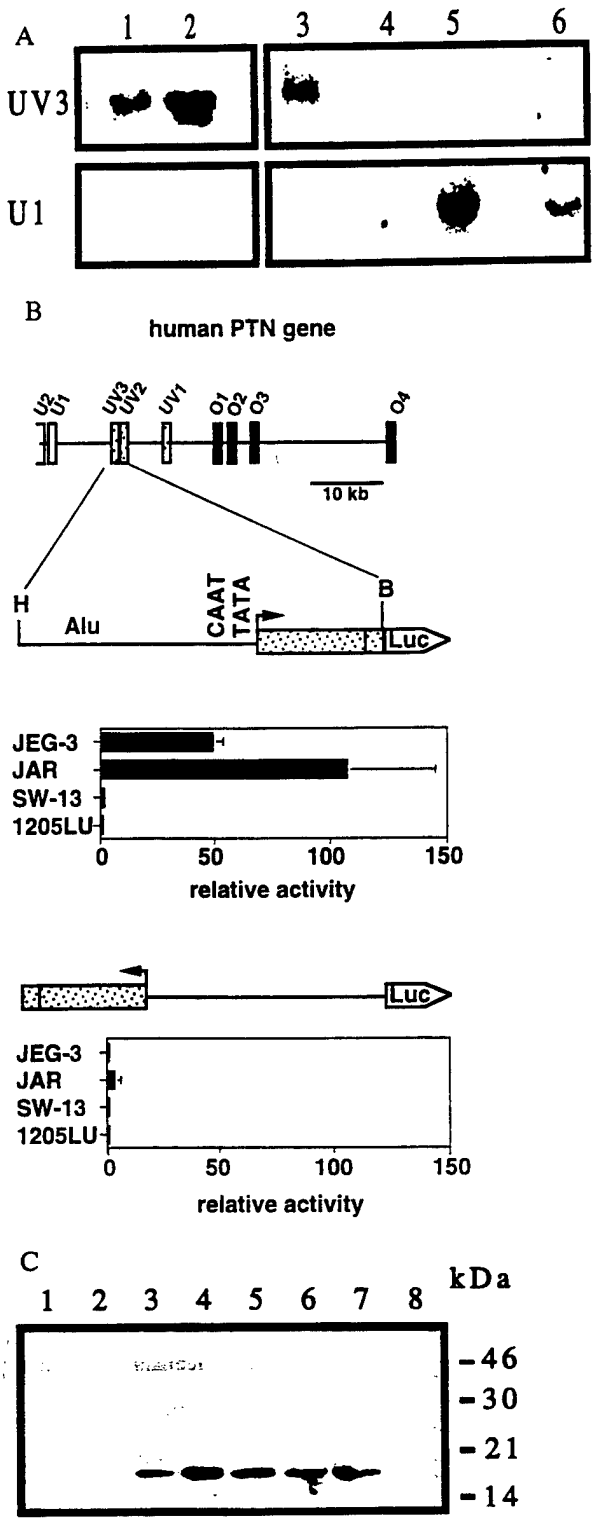

FIG. 2. Expression of the PTN gene in various human cell lines and promoter activity of the HERV insert. (*A*) Northern blot analysis of total RNA from choriocarcinoma cells JEG-3 and JAR (lanes 1 and 2), a chorion biopsy from a stillborn fetus (lane 3), adrenal carcinoma SW-13 (lane 4), melanoma 1205LU (lane 5), and teratocarcinoma PA-1 cells (lane 6) using exon-specific probes. (*B*) Transcriptional activity of a *Hin*dIII–*Bam*HI (H. B) genomic fragment inserted into the promoterless pXP-1 reporter vector (25) in sense and antisense orientation. Data are the mean ± SD of triplicate determinations and are representative of at least two transient transfection experiments for each cell line. Luciferase activity, normalized to the protein content, is expressed relative to that obtained with the pXP-1 vector. (*C*) Western blot for PTN present in the culture medium of JEG-3 cells. Proteins present in medium conditioned by JEG-3 cells were concentrated and partially purified by heparin-affinity chromatography using a NaCl step gradient of 0.9 to 1.5 M (lanes 1–8) and analyzed as described (4).

embryonic or adult tissues and tumor cells contained U1 as their first 5′-UTR exon (Figs. 1*C* and 2*A*), implying that these transcripts originate from the promoter upstream of U1 (12).

Western blot analysis showed that the PTN protein is produced and released into the culture medium by choriocarcinoma cells (Fig. 2*C*). This protein was mitogenically active and stimulated colony formation of an indicator cell line, SW-13 cells (2, 4) (data not shown).

Promoter Function Due to the HERV Insertion. Primer extension with primers targeted within the most 5′ UV3 exon of the HERV insert mapped the start site of the HERV-PTN transcripts to an adenosine residue 39 nt downstream of putative CAAT and TATA boxes (see Fig. 1*B*). Presence of these elements at the transcription start site suggested that the HERV insertion might have generated an additional promoter in the intron immediately upstream of the coding region of the human PTN gene. To investigate whether this putative promoter was responsible for the trophoblast-specific expression of HERV-PTN, we performed transient transfection assays with promoter-reporter constructs. Upstream of a luciferase reporter gene, we inserted a genomic fragment that starts 1.5 kb upstream of the transcription start site, contains the CAAT and TATA boxes, and extends downstream of the start site into exon UV2 (Figs. 1*B* and 2*B*). Transcriptional activity of the resulting construct was observed exclusively in human choriocarcinoma cells (JEG-3 and JAR cells) and only when the HERV insert was oriented as *in vivo*. Luciferase activity in JEG-3 and JAR cells transfected with the HERV promoter-reporter construct in sense orientation was 50- to 100-fold that of cells transfected with the promoterless vector alone. Deletion of the *Alu* element did not affect this transcriptional activity (data not shown). Only background activity was detected in PTN-positive human melanoma (1205LU) or in PTN-negative adrenal carcinoma (SW-13) cells (Fig. 2*B*). We conclude from these data that the HERV insertion in the human PTN gene generates a functional promoter that confers high tissue-specific expression.

Depletion of HERV-PTN with Ribozymes to Evaluate its Biological Role. We investigated the biological significance of the expression of HERV-PTN in trophoblast-derived tissues using JEG-3 choriocarcinoma cells as a model system. Tumor growth of these cells in experimental animals mimics the highly invasive and angiogenic growth phenotype of the normal human trophoblast (29) and clinical choriocarcinoma, and we hypothesized that one of the contributing factors to this phenotype could be the expression of HERV-PTN. To address this hypothesis, we examined the effects of reducing the abundance of HERV-PTN transcripts in JEG-3 cells by stable expression of a PTN-targeted ribozyme (26). A vector (pTET/Rz261) with high transcriptional activity in these cells (unpublished data) was used to express the ribozyme.

Northern blot analysis revealed that ribozyme expression reduced the amount of HERV-PTN mRNA in JEG-3 cells to background levels (Fig. 3*A*). No difference in the proliferation rate of PTN-depleted versus control cells was apparent *in vitro* (Fig. 3*B*), suggesting that the cells do not require PTN as an autocrine growth factor even though they secrete the protein in a biologically active form (see above). However, a marked difference in the growth phenotype of PTN-depleted versus control cells was observed after xenografting tumor cells into athymic nude mice. In an initial study, we implanted the tumor cells into their "natural" intraabdominal environment to observe their orthotopic growth behavior. The control cells formed large tumor masses that invaded the abdominal organs

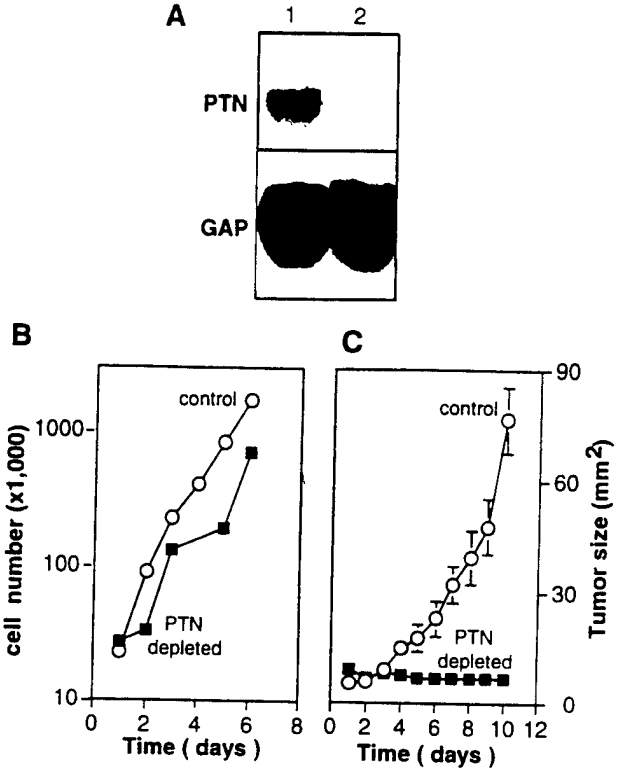

FIG. 3. Effect of the depletion of HERV-PTN mRNA from JEG-3 choriocarcinoma cells. (*A*) Northern blot analysis of total RNA from control JEG-3 cells (lane 1) and JEG-3 cells expressing a ribozyme targeted to PTN mRNA (lane 2) is shown. (*B*) Proliferation *in vitro* of control and PTN mRNA-depleted JEG-3 cells. (*C*) Growth curves of subcutaneous tumors in athymic nude mice. Two million control or PTN mRNA-depleted JEG-3 cells were inoculated. The data represent tumor sizes (mean ± SD).

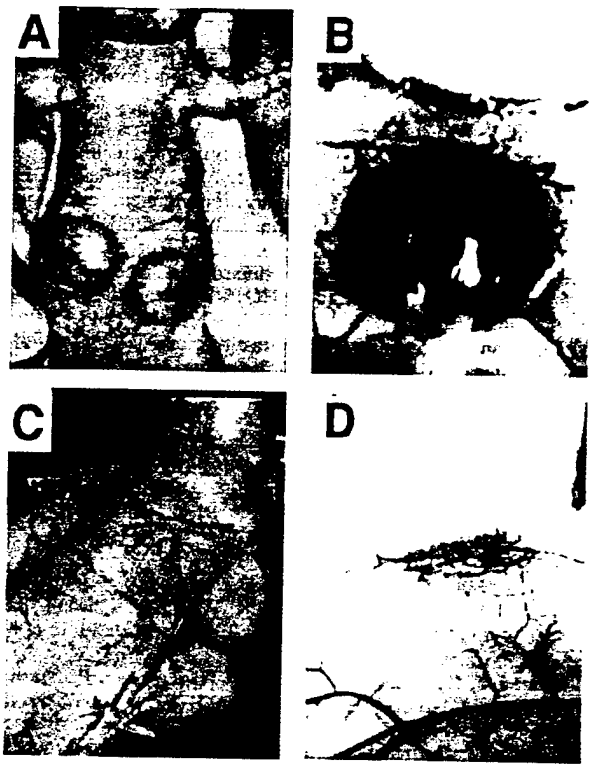

FIG. 4. Representative subcutaneous tumors grown from two million control (*A* and *B*) or PTN mRNA-depleted (*C* and *D*) JEG-3 cells 2 weeks after injection of cells into athymic nude mice.

within 2–3 weeks, whereas only a few small seedings of PTN-depleted tumor cells were detected in the abdomen at the end of the study ($n = 5$ and $n = 4$ animals, respectively). Parallel results were obtained after subcutaneous injection of tumor cells. In contrast to control cells, which grew rapidly into highly angiogenic tumors ($n = 7$; Figs. 3*C* and 4 *A* and *B*), no tumor growth was observed with PTN-depleted cells ($n = 9$; Figs. 3*C* and 4 *C* and *D*). These observations indicate that PTN is an essential and rate-limiting factor for choriocarcinoma growth, invasion, and angiogenesis *in vivo*.

DISCUSSION

We report herein that integration of an HERV element into the human PTN gene generates a novel tissue-specific promoter not present in the common ancestral gene from which the primate and murine PTN genes descended. Transcriptional activity of this promoter results in the expression of HERV-PTN fusion transcripts specifically in human trophoblast-derived normal and tumor cells. The integration site of the HERV fragment shows several features indicative of inserted retroelements, i.e., location at the 5′ region of a gene (30), presence of an *Alu* sequence of 280 nt and of a stretch of 26 adenosine residues at its 5′ end (16) (Fig. 1*B*).

The HERV found in the human PTN gene represents a noninfective replication-defective prototype of a retrovirus (22) that integrated into ancestral DNA before the divergence of apes (including human predecessors) and Old World monkeys more than 25 million years ago (31). It is quite surprising that altered expression of human genes due to the insertion of retroviral elements appears to be an extremely rare event (see Introduction), bearing in mind the high number of HERV fragments found integrated throughout the human genome (several thousand copies) (16, 18, 19). On the other hand, an alteration of a gene expression pattern will only penetrate during phylogenesis, if a better survival chance is associated. Our functional studies show that ribozyme-mediated depletion of HERV-PTN mRNA in human choriocarcinoma cells reverses their highly aggressive growth phenotype in an *in vivo* model. From this it is tempting to speculate that the expression of HERV-PTN could be one of the factors that enhances the invasive growth phenotype also of the normal human trophoblast and that this was advantageous during phylogenesis. In line with this speculation, the superficial less-invasive implantation of the murine trophoblast (29) coincides with the lack of trophoblast-specific PTN gene expression.

Formation of the trophoblast is one of the early differentiation events occurring in the developing embryo after it organizes into an inner and outer cell mass; the trophoblast evolves during the first weeks from the outer cell mass. Interestingly, teratocarcinoma (PA-1) cells that stem from the undifferentiated embryoblast (32), as well as adult tissues and tumor cells derived from different germ layers, utilize the PTN promoter that is located in a region in common with the murine gene (i.e., upstream of U1: Figs. 1*A* and *C* and 2*A*; ref. 12). In contrast, the phylogenetically novel HERV-PTN transcription unit is active in human trophoblast-derived normal and tumor cells and tissues, and our data suggest that activation of this promoter occurs early during the formation of the trophoblast.

In conclusion, a distinct role of retroviral integration into somatic cells for the development of solid tumors or leukemia was demonstrated in rodents (mouse mammary tumor virus and Moloney murine leukemia virus), birds (avian leukosis virus), and cats (feline leukemia virus) (33). We show herein that trophoblast-specific expression of the human PTN gene is due to the germ-line insertion of a retroviral fragment that generates a phylogenetically novel promoter in the human

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gene. The resulting gene products appear to be responsible for the aggressive and invasive growth phenotype of human choriocarcinoma and possibly also of the human trophoblast.

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1. Li, Y. S., Milner, P. G., Chauhan, A. K., Watson, M. A., Hoffman, R. M., Kodner, C. M., Milbrandt, J. & Deuel, T. F. (1990) *Science* **250,** 1690–1694.
2. Fang, W. J., Hartmann, N., Chow, D., Riegel, A. T. & Wellstein, A. (1992) *J. Biol. Chem.* **267,** 25889–25897.
3. Chauhan, A. K., Li, Y. S. & Deuel, T. F. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 679–682.
4. Wellstein, A., Fang, W. J., Khatri, A., Lu, Y., Swain, S. S., Dickson, R. B., Sasse, J., Riegel, A. T. & Lippman, M. E. (1992) *J. Biol. Chem.* **267,** 2582–2587.
5. Laaroubi, K., Delbe, J., Vacherot, F., Desgranges, P., Tardieu, M., Jaye, M., Barritault, D. & Courty, J. (1994) *Growth Factors* **10,** 89–98.
6. Courty, J., Dauchel, M. C., Caruelle, D., Perderiset, M. & Barritault, D. (1991) *Biochem. Biophys. Res. Commun.* **180,** 145–151.
7. Kojima, S., Inui, T., Muramatsu, H., Kimuara, H., Sakakibara, S. & Muramatsu, T. (1995) *Biochem. Biophys. Res. Commun.* **216,** 574–581.
8. Rauvala, H. (1989) *EMBO J.* **8,** 2933–2941.
9. Vanderwinden, J. M., Mailleux, P., Schiffmann, S. N. & Vanderhaeghen, J. J. (1992) *Anat. Embryol.* **186,** 387–406.
10. Böhlen, P. & Kovesdi, I. (1991) *Prog. Growth Factor Res.* **3,** 143–157.
11. Kurtz, A., Schulte, A. M. & Wellstein, A. (1995) *Crit. Rev. Oncog.* **6,** 151–177.
12. Li, Y. S., Hoffman, R. M., Le Beau, M. M., Espinosa, R., Jenkins, N. A., Gilbert, D. J., Copeland, N. G. & Deuel, T. F. (1992) *J. Biol. Chem.* **267,** 26011–26016.
13. Kalter, S. S., Helmke, R. J., Heberling, R. L., Panigel, M., Fowler, A. K., Strickland, J. E. & Hellman, A. (1973) *J. Natl. Cancer Inst.* **50,** 1081–1084.
14. Kalter, S. S., Helmke, R. J., Panigel, M., Heberling, R. L., Felsburg, P. J. & Axelrod, L. R. (1973) *Science* **179,** 1332–1333.
15. Kalter, S. S., Heberling, R. L., Smith, G. C. & Helmke, R. J. (1975) *J. Natl. Cancer Inst.* **55,** 735–736.
16. Wilkinson, D. A., Mager, D. L. & Leong, J. A. C. (1994) in *The Retroviridiae*, ed. Levy, J. A. (Plenum, New York), pp. 465–535.
17. Loewer, R., Loewer, J. & Kurth, R. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 5177–5184.
18. Samuelson, L. C., Wiebauer, K., Snow, C. M. & Meisler, M. H. (1990) *Mol. Cell. Biol.* **10,** 2513–2520.
19. Ting, C. N., Rosenberg, M. P., Snow, C. M., Samuelson, L. C. & Meisler, M. H. (1992) *Genes Dev.* **6,** 1457–1465.
20. Lai, S., Schulte, A. M., Wellstein, A. & Riegel, A. T. (1995) *Gene* **153,** 301–302.
21. Lai, S., Czubayko, F., Riegel, A. T. & Wellstein, A. (1992) *Biochem. Biophys. Res. Commun.* **187,** 1113–1122.
22. Repaske, R., Steele, P. E., O'Neill, R. R., Rabson, A. B. & Martin, M. A. (1985) *J. Virol.* **54,** 764–772.
23. Tomita, N., Horii, A., Doi, S., Yikouchi, H., Ogawa, M., Mori, T. & Matsubara, K. (1990) *Biochem. Biophys. Res. Commun.* **166,** 1–10.
24. Katoh, K., Takeshita, S., Sato, M., Ito, T. & Amann, E. (1992) *DNA Cell Biol.* **11,** 735–743.
25. Nordeen, S. K. (1988) *BioTechniques* **6,** 454–457.
26. Czubayko, F., Riegel, A. T. & Wellstein, A. (1994) *J. Biol. Chem.* **269,** 21358–21363.
27. Gossen, M. & Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 5547–5551.
28. Steele, P. E., Rabson, A. B., Bryan, T. & Martin, M. A. (1984) *Science* **225,** 943–947.
29. Cross, J. C., Werb, Z. & Fisher, S. J. (1994) *Science* **266,** 1508–1518.
30. Rohdewohld, H., Weiher, H., Reik, W., Jaenisch, R. & Breindl, M. (1987) *J. Virol.* **61,** 336–343.
31. Shih, A., Coutavas, E. E. & Rush, M. G. (1991) *Virology* **182,** 495–502.
32. Stewart, T. A. & Mintz, B. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 6314–6318.
33. Fan, H. (1994) in *The Retroviridiae*, ed. Levy, J. A. (Plenum, New York), pp. 313–353.