Cloning and Expression of a Developmentally Regulated Protein That Induces Mitogenic and Neurite Outgrowth Activity

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A heparin binding mitogenic protein isolated from bovine uterus shares NH_2-terminal amino acid sequence with a protein isolated from newborn rat brain. The cDNA's of the bovine, human, and rat genes have been isolated and encode extraordinarily conserved proteins unrelated to known growth or neurotrophic factors, although identity of nearly 50 percent has been found with the predicted sequence of a retinoic acid induced transcript in differentiating mouse embryonal carcinoma cells. Lysates of COS-7 cells transiently expressing this protein were mitogenic for NRK cells and initiated neurite outgrowth from mixed cultures of embryonic rat brain cells. RNA transcripts encoding this protein were widely distributed in tissues and were developmentally regulated. This protein, previously designated as heparin binding growth factor (HBGF)-8, is now renamed pleiotrophin (PTN) to reflect its diverse activities. PTN may be the first member of a family of developmentally regulated cytokines.

CYTOKINES ACTIVE IN GROWTH AND DEVELOPMENT ONLY recently have been identified. We now report the isolation and sequence of the full-length complementary DNA's (cDNA's) of the bovine, human, and rat genes of a heparin binding protein with mitogenic activity toward rat and mouse fibroblasts (*1*) and neurite outgrowth promoting activity in mixed cultures of embryonic rat brain cells (*2*). The cDNA's predict proteins whose amino acid sequences are (i) unrelated to those of other known mitogens and (ii) indicate extraordinary conservation between bovine, human, and rat species. The gene is highly expressed in brain and in uterus; it is also expressed in gut, muscle, lung, and skin, and is developmentally regulated. The transiently expressed product of the bovine and human cDNA's has both mitogenic and neurite outgrowth promoting activity. This protein, originally described as HBGF-8 (*1*), has no apparent homology with the heparin-binding growth factor family of seven structurally related polypeptides (*3*). We now use the name pleiotrophin (PTN) to replace HBGF-8 to reflect the diverse activities of the protein.

The cDNA clones encoding PTN were isolated as follows. Degenerate oligonucleotide primers were synthesized on the basis of the 25 NH_2-terminal amino acids of PTN and used in combination with AMV (avian myoblastosis virus) reverse transcriptase and the polymerase chain reaction (PCR) (*4*) to selectively amplify PTN cDNA fragments. A cDNA clone of 74 base pairs (bp) that included 33 bp of degenerate primers and 41 bp (289 to 329) of sequence from the NH_2-terminal fragment was obtained from bovine uterine cDNA, and a 330 bp fragment was obtained from rat brain cDNA. The sense primer (A) corresponding to NH_2-terminal protein sequence GKKEKP was 5′-GGIAA(A,G)AA(A,G)GA(A,G)AA-(A,G)CC-3′ and the antisense primer (B) 5′-GGIAC(A,G)CAI-ACI(G,C)(A,T)CCA(T,C)TGCCA-3′ was based on the protein sequence WQWSVCVP (*5*). Primers A and B were used in the PCR reaction to generate the 74-bp bovine PCR product. The sense primer (C) 5′-GA(T,C)TG(T,C)GGIGA(A,G)TGGCA(A,G)TGG-3′ was derived from the protein sequence DCGEWQW; it was combined with the antisense primer (D) 5′-GACTCGAGTCGA-CATCGATGATn (n = 17)-3′ in the PCR reaction to generate the 330-bp rat cDNA fragment, resulting from the annealing of primer D to a poly(A) (polyadenylated) stretch within the coding sequence of PTN (Fig. 1B, amino acid residues 122 to 126). The 41-nucleotide (nt) probe was then used to screen an oligo(dT)- and random-primed cDNA library prepared from bovine uterus poly(A)$^+$ RNA. Two plaque-purified clones of 0.8 kilobase (kb) (clone 40) and of 1.2 kb (clone 30) were obtained and subsequently analyzed. These two cDNA clones overlapped (Fig. 1A) and, when aligned, established a continuous 1204-bp sequence that contained the complete PTN coding sequence (Fig. 1B). The putative initiation codon ATG begins at nucleotide position 178 and established a 504-bp open reading frame that ended at a TAA termination codon at nucleotide position 682. This open reading frame would encode a 168–amino-acid (aa) polypeptide with a molecular mass of 18,902 daltons and an isoelectric point (pI) of 10.3. The sequence flanking the ATG codon does not conform to the proposed GCC($\frac{A}{G}$)CCATGG consensus sequence for optimal initiation by eukaryotic ribosomes (*6*). A 25-aa sequence identical to the experimentally determined NH_2-terminal sequence of purified bovine PTN begins 32 aa downstream of the proposed translation initiation site. The 32-aa sequence (Fig. 1C) is hydrophobic, consistent with a signal peptide whose cleavage site would be the NH_2-terminal

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residue of the mature protein determined by direct amino acid sequencing of bovine (*1*) and rat (*2*) proteins. No other ATG sequence was found upstream, and the conservation of upstream sequences was considerably less than with the coding sequences. No consensus sequences for N-linked glycosylation were identified although the predicted protein is rich in threonine residues which are potential sites for O-linked glycosylation. The 3′ untranslated region is rich in A+T and contains three repeats of the ATTTA sequence that has been observed in many cytokine and oncogene noncoding regions and has been implicated in controlling the stability of the respective mRNA's (*7*). The hydropathy plot (*8*) (Fig. 1C) for the predicted PTN protein established a hydrophobic NH_2-terminal domain of 32-aa residues that could serve as a leader sequence and a hydrophilic COOH-terminal domain. The failure to observe this NH_2-terminus in the sequence obtained from NH_2-terminal amino acid sequencing of the purified mature PTN molecule from bovine uterus and the precise correspondence of the predicted cleavage site for a signal sequence (*9*) with the observed NH_2-terminal amino acid sequence support the potential for this domain to function as a signal peptide.

We also obtained full-length cDNA clones of PTN from human placenta and rat brain cDNA libraries. Comparison of predicted amino acid sequences of PTN from bovine, human, and rat revealed that PTN is conserved across the three species (Fig. 2). Of 168 aa residues of PTN, 163 between bovine and human and 164 between rat and human are identical. The mature forms of bovine, human and rat PTN exhibit overall 98 percent sequence similarity although the 5′ flanking sequences were less well conserved within the regions of DNA sequenced. Searches of protein and nucleic acid sequence data banks (*10*) revealed no significant sequence similarities between PTN and other proteins with neurotrophic activities such as acidic

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                        20                  40
Bovine MQTPQYLQQRRKFAAAFLAFIFILAAVDTAEAGKKEKPEKKVKKSDCGEWQWSVCV
Human  --AQ--Q------------------------------------------------
Rat    -SSQ--Q------------L-------------------------------------
                                                  *       *

          60                  80                  100
Bovine PTSGDCGLGTREGTRTGAECKQTMKTQRCKIPCNWKKQFGAECKYQFQAWGECDLN
Human  ------------------------------------------------------
Rat    ------------------------------------------------------
            *           *     *     *       *

              120                 140                 160
Bovine TALKTRTGSLKRALHNADCQKTVTISKPCGKLTKSKPQAESKKKKKEGKKQEKMLD
Human  ----------------E----------------P---------------------
Rat    ----------------------------------P---------------------
                       *     *
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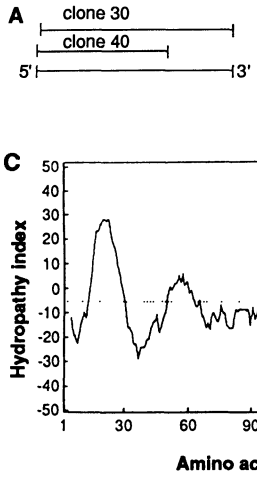
Fig. 2. Comparison of amino acid sequences of bovine, human, and rat PTN. Amino acid numbers are given at the top. Amino acids identical to bovine PTN are indicated by dashes in the sequences of human and rat PTN. Ten cysteines are denoted by asterisks. The human and rat PTN cDNA and deduced amino acid sequence were obtained by screening a human placenta cDNA library (*20*) and a rat embryonic brain cDNA library (Clontech) with bovine clone 40 and the rat 330-bp PCR product, respectively. The arrow indicated the beginning of the mature form of bovine PTN.



B

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1    CAAAGACGGAGTGGAGAGAGTAGAAGAAAGAGAGCAGGGAGTCACCGGGCGTGCGGGGGCGGAGAGCAGCGGCCGCCGCGAGCACCAGCG

91   ACTTGGGTACCTGGACTCAGGGCAGAAAAACCTCTCCCGGATCAACAAAGGCAGCCTGAGCGCGCACCGCTCTTTTGCGACTCCAAAATG
                                                                                          Met
                -30                 -20                 -10
181  CAGACTCCACAGTACCTGCAGCAACGTCGAAAATTTGCAGCTGCCTTTTTGGCATTTATTTTCATCTTGGCAGCTGTGGACACCGCTGAA
     GlnThrProGlnTyrLeuGlnGlnArgArgLysPheAlaAlaAlaPheLeuAlaPheIlePheIleLeuAlaAlaValAspThrAlaGlu

              +1                  10                  20
271  GCAGGAAAGAAAGAGAAACCAGAAAAGAAGGTGAAGAAGTCTGACTGTGGAGAATGGCAGTGGAGTGTGTGTACCCACCAGTGGGGAC
     AlaGlyLysLysGluLysProGluLysLysValLysLysSerAspCysGlyGluTrpGlnTrpSerValCysValProThrSerGlyAsp

              30                  40                  50
361  TGTGGGCTGGGCACCCGCGAGGGCACCCGTACCGGAGCTGAGTGTAAACAAACCATGAAGACCCAGAGATGTAAGATCCCCTGCAACTGG
     CysGlyLeuGlyThrArgGluGlyThrArgThrGlyAlaGluCysLysGlnThrMetLysThrGlnArgCysLysIleProCysAsnTrp

              60                  70                  80
451  AAAAAGCAATTTGGAGCGGAGTGCAAATACCAGTTCCAGGCCTGGGGAGAATGTGATCTGAACACGGCTCTGAAGACCCGAACTGGGAGC
     LysLysGlnPheGlyAlaGluCysLysTyrGlnPheGlnAlaTrpGlyGluCysAspLeuAsnThrAlaLeuLysThrArgThrGlySer

              90                  100                 110
541  CTGAAGCGAGCCCTCCACAACGCCGACTGCCAGAAGACAGTCACCATCTCCAAGCCCTGTGGCAAGCTGACCAAGTCCAAACCTCAAGCA
     LeuLysArgAlaLeuHisAsnAlaAspCysGlnLysThrValThrIleSerLysProCysGlyLysLeuThrLysSerLysProGlnAla

              120                 130
631  GAATCTAAGAAGAAGAAAAAGGAAGGCAAGAAACAGGAGAAGATGCTGGACTAAAAGCCACCACCTTCCGTGGACCATGAAAAGGACATC
     GluSerLysLysLysLysLysGluGlyLysLysGlnGluLysMetLeuAsp *

721  AGCAAACAGGATCAGTTAACTATTGCATTTATATCTACCGTAGGCTTTTTATTCAAAAATTATCTATAGCTTAAGTACACAATAGGCAGA

811  AACAAAATGAAAAGAAAAATTTTGTAGTAGCATTTTTTTTAAATGTATCAATATACCATAGTACCACTAGGGACTTATAATAGAGGACTG

901  TAATCCTATTTAGAATGTTGACTTATAGTACATGTTAAGTGATAGAAAACTGAGGTAAGTTTTTTGAAGTTATGTGATATTTTACATTAC

991  ATTTTTTTTTACATTTTTTTCTTTTGGCAGCAATTTAAATGTTATGACTATGTAAACTACTTCTCTTGTTAGGTAATTTTTTTCACCTAG

1081 ACTTTATTTCCCAATTGAGAAAAATATCTACTAAACAAAGCAGCAATAAAATATGATCATCCTATCTGAGGAAAATATCTCTTTTTCTGC

1171 CAGTGGATTTTTAAAAAATTGTAGTCAACAAAAT
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Fig. 1. Nucleotide sequence and deduced amino acid sequence of bovine PTN cDNA. (**A**) Representation of two bovine PTN cDNA clones, 30 and 40 overlapping. (**B**) Complementary DNA nucleotide sequence encoding the predicted PTN amino acid sequence (*1*). Nucleotides are numbered from the left. The hydrophobic NH_2-terminal domain is designated as amino acids −32 to −1, whereas mature bovine PTN is numbered 1 to 136. The predicted NH_2-terminal sequence from the cDNA clone matched the NH_2-terminal sequence derived from previously purified PTN (*1*) and is underlined. The ATTTA repeat sequences are overlined; the polyadenylation signal is boxed. Total RNA from bovine uterus tissue was extracted (*18*) and poly(A)$^+$ RNA was generated with the use of oligo(dT) columns (Pharmacia). cDNA was synthesized with random primer and oligo(dT) oligonucleotides and constructed in λZAP II vector (Stratagene). Approximately 1×10^6 clones from the cDNA library were screened with a chemically synthesized 41-nt probe representing nucleotides 289 to 329, the precise 41 bp within the 74-bp PCR product when the 5′ and 3′ oligonucleotide degenerate primers were deleted. It hybridized to two clones designated clone 40 and clone 30. The screening procedure was as follows. The recombinant phages were replicated onto nitrocellulose filters and hybridized overnight at 42°C with the 5′ end-labeled probe ([γ-^{32}P]ATP; 6000Ci/mmol, NEN), in 20 percent formamide, 50 mM sodium phosphate (pH 7.0), 5× Denhardt's solution, purified tRNA at 0.1 mg/ml, and 5× standard saline citrate (SSC) (1× SSC is 150 mM sodium chloride, 15 mM sodium citrate). Filters were washed in 1× SSC and 0.1 percent SDS at 55°C and exposed to Kodak XAR film. The λ DNA's were subcloned into Bluescript (Stratagene). The nucleotide sequences were obtained by dideoxy chain termination (*19*) with Sequenase, version 2.0 (U.S. Biochemicals). The sequences obtained were confirmed by sequencing both strands of DNA twice with different primers. (**C**) Hydropathy plot was obtained by using the method of Kyte and Doolittle (*8*). The potential cleavage site between position 32 and 33 was predicted by the PC-GENE program (*9*).

FGF and basic FGF (*11*), ciliary neurotrophic factor (CNTF) (*12*), and the NGF family (*13*). However, the predicted sequence derived from a mouse cDNA of the retinoic acid-induced differentiation factor (*14*) has nearly 50 percent sequence similarity to PTN. This homology of the PTN cDNA to the cDNA derived from transcripts of differentiating mouse embryonic carcinoma cells suggests a gene family of developmentally regulated pleiotrophins.

The 805-bp bovine cDNA clone 40 containing the coding sequence for PTN was used to probe an RNA (Northern) blot of 5 μg of poly(A)$^+$ RNA from adult bovine brain and bovine uterus. A single, ~ 1.5-kb mRNA transcript from both tissues hybridized to the PTN probe (Fig. 3).

The expression pattern of PTN during the postnatal development of the rat was studied with RNA prepared from five kinds of tissues from rats of different ages (Fig. 4). A single 1.5-kb RNA species was detected; however, expression of PTN RNA in each tissue declined as the ages of the rats increased. The PTN was expressed immediately before the animals were born and at the time of birth, and decreased subsequently. Three weeks after the animals were born, there were no detectable transcripts in muscle, lung, or skin; however, they were still present in brain and gut. PTN mRNA was also detected in embryos as early as day 13. These findings are consistent with the previous immunostaining studies (*2*) and indicate that PTN may have a role in early development.

To establish that the protein translated from PTN cDNA elicited the growth factor and neurite outgrowth activities, we cloned the full-length cDNA with the entire coding sequence into an expression vector (pMT2) and transfected it into African monkey kidney (COS-7) cells. Cell lysates from metabolically labeled COS-7 cells transfected with the expression vector pMT2 containing bovine or human PTN cDNA inserts synthesized an 18-kD protein that was identified in immunoprecipitates after incubation with an antiserum (*15*) to residues 1 to 19 of the amino acid sequence of the predicted mature protein (Fig. 5B). The expressed protein was similar in size to that of bovine PTN purified from uterus and was not found in lysates of COS-7 cells transfected with vector alone. PTN transcripts were also detected by Northern blot analysis in transiently expressed COS-7 cells but not detected in blots of RNA (25 μg) from COS-7 cells transfected with vector alone. Lysates of cells into which bovine or human PTN cDNA inserts had been transfected were mitogenically active for NRK cells (Fig. 5A), whereas conditioned media or

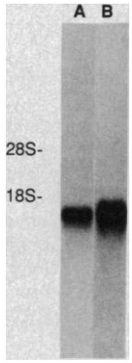


Fig. 3. Northern blot analysis of mRNA from bovine brain and uterus. Poly(A)$^+$ RNA's (*18*) from bovine uterus (lane A, 5 μg) and bovine brain (lane B, 5 μg) was used. Ribosomal RNA's (28*S*, 18*S*) are indicated. RNA's were subjected to electrophoresis on a 1 percent agarose, 6.7 percent formaldehyde gel and transferred to a hybridization transfer membrane (MSI). The 805-bp bovine cDNA clone containing the entire coding sequence was ^{32}P-labeled by random priming (Boehringer Mannheim GmbH) to 1.5 × 10^8 cpm/μg, and then 1 × 10^6 cpm/ml was added and hybridized to the blot for 16 hours at 42°C in 5× SSC containing 50 percent formamide, 5× Denhardt's solution, yeast tRNA (0.1 mg/ml). The blot was washed in 0.2× SSC containing 0.1 percent SDS at 65°C and exposed to Kodak XAR5 film for 48 hours at −70°C with an intensifying screen.

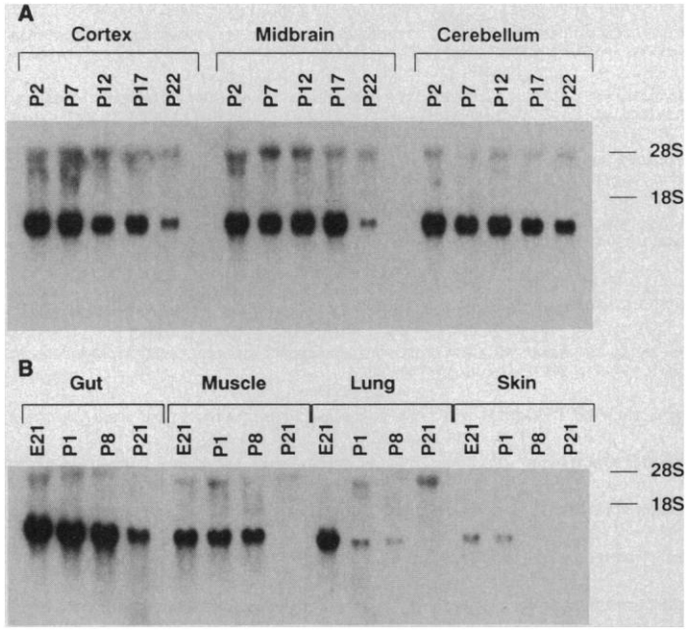


Fig. 4. Expression of PTN in developing rat. Indicated tissues of designated pre- and postnatal ages were isolated. Total RNA was prepared with the use of guanidine thiocyanate (*18*). Each RNA sample (15 μg) was subjected to agarose gel electrophoresis and RNA (Northern) transfer analysis (*21*). A ^{32}P-labeled, antisense RNA probe was prepared with the cloned rat PTN cDNA template and Riboprobe transcription reagents (Promega) according to the manufacturer's protocol. Hybridization was carried out with hybridization fluid (1×10^6 cpm/ml) at 65°C for 18 hours. Filters were washed at 75° with 0.2× SSC (*21*), and exposed to Kodak XAR5 film for 18 hours. (**A**) Expression of rat PTN in postnatally developing brain. (**B**) Expression of rat PTN at embryonic age day 21 (E21) and postnatal age 1, 8, and 21 days (P1, P8, P21) in gut, striated muscle, lung, and skin. For each blot, the location of 28*S* and 18*S* ribosomal RNA's is indicated.

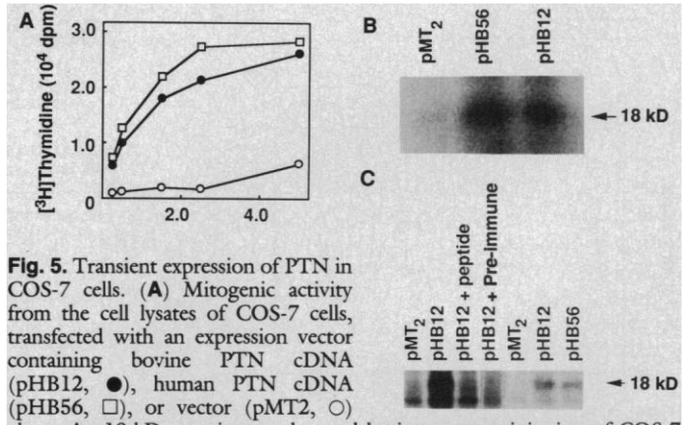
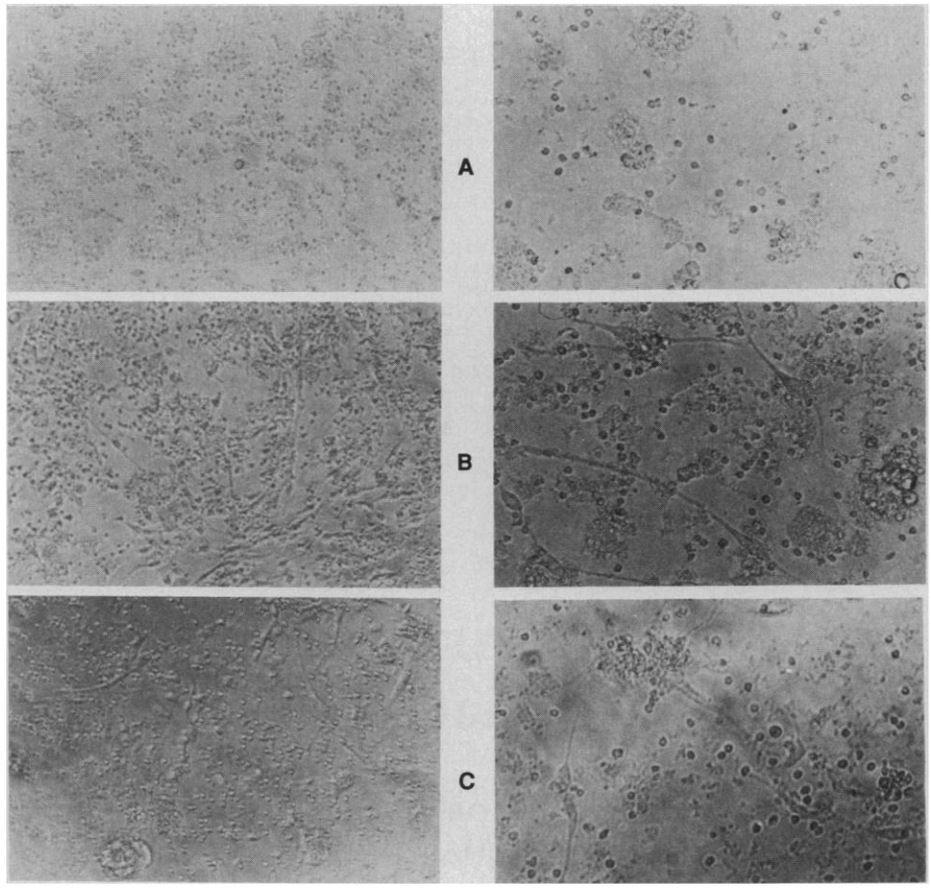


Fig. 5. Transient expression of PTN in COS-7 cells. (**A**) Mitogenic activity from the cell lysates of COS-7 cells, transfected with an expression vector containing bovine PTN cDNA (pHB12, ●), human PTN cDNA (pHB56, □), or vector (pMT2, ○) alone. An 18-kD protein was detected by immunoprecipitation of COS-7 cell lysates (**B**) and conditioned media (**C**) containing heparin at 1 mg/ml (Sigma) from ^{35}S-labeled cells previously transfected with appropriate constructs, using antiserum to PTN peptide (*15*). (A) For transient expression, COS-7 cells were plated at 2 × 10^5 cells into 60-mm plates. On the next day, the plasmid DNA's were introduced into the cells by DEAE-Dextran method with the CellPhect transfection kit (Pharmacia). After transfection, the cells were incubated in Dulbecco's modified Eagle medium (DMEM) containing 10 percent fetal calf serum for 3 days at 37°C. The culture medium was collected, and the cells were washed with cold PBS before resuspending in 1.0 ml of 20 mM Hepes buffer containing 20 mM NaCl (pH 7.4). Thereafter the cells were lysed by three cycles of freezing and thawing and brief sonication. After centrifugation, the supernatants were added to cultured NRK cells containing 200 μl of media (final volume) for mitogenic assay (*1*). (B) For immunoprecipitation of cell lysates, COS-7 cells were labeled for 4 hours with 200 μCi of [^{35}S]methionine and cysteine 60 hours after transfection. The media were collected and the cells were then washed, lysed, and immunoprecipitated as described (*22*). (C) For immunoprecipitations of conditioned media, COS-7 cells were labeled as in (B) except that heparin (1 mg/ml) was added to media. (Lanes 1 and 5) Medium from pMT2 transfected cell; (lane 2) medium from cells transfected with plasmid pHB12; (lane 3) as in lane 2 with 19 residues of NH_2-terminal peptide (2 μg/ml); (lane 4) medium from cells transfected with pHB12 with 5 μl of preimmune serum; (lanes 6 and 7) medium from bovine pHB12 and human pHB56 cDNA transfected COS cells.

Fig. 6. Neurite outgrowth induced by PTN. Phase contrast photomicrographs (10× and 40 ×) of cortical neurons at day 18 from the rat embryo (*2*). These mixed cell populations were cultured for 48 hours in DMEM plus 0.1 percent bovine serum albumin (BSA) in wells on 96-well Falcon plates that had been treated with either (**A**) 200 μl of DMEM and 0.1 percent BSA for 1 hour, (**B**) 30 μl of phosphate-buffered saline (PBS) containing 170 ng of bovine uterus PTN (*1*) for 2 hours, and then by 200 μl of DMEM and 0.1 percent BSA for 1 hour, or (**C**) 30 μl of PBS containing about 150 ng of partially purified recombinant human PTN for 2 hours, and then by 200 μl of DMEM and 0.1 percent BSA for 1 hour. The recombinantly expressed PTN was purified by application of the lysates of COS-7 cells transfected with pHB56 cDNA to a heparin-Sepharose (Pharmacia) column (5 by 50 mm) and eluted with a 0 to 2M NaCl gradient in 40 ml of 50 mM Hepes, pH 7.0. Fractions (1 ml) were collected, and the fractions eluting at 1.1 to 1.4 M NaCl contained an 18-kD protein as the predominant protein after SDS-PAGE and silver staining, were immunoreactive with the antiserum to the synthetic NH_2-terminal peptide of PTN and were mitogenically active. This protein was purified further by application of these samples to a 2.1 × 220 Aquapore BU-300, C_4 column (2.1 by 220 mm; Brownlee) in 0.1 percent TFA (Microbore) high-performance technology (Applied Biosystems). The 18-kD protein eluted off the C_4 column at 55 percent acetonitrile after the application of acetonitrile (0 to 100 percent)–0.1 percent TFA gradient.



lysates from COS-7 cells transfected with the pMT2 vector alone were not. Partially purified lysates of COS-7 cells transfected with pMT2 containing the human PTN insert also were mitogenically active and contained an 18-kD protein that was immunoreactive with a weak rabbit polyclonal antiserum to the peptide (1–19). The mitogenic activity was partially inhibited with this weak antiserum to the NH_2-terminal residues 1 to 19 (*16*). These fractions also promoted neurite outgrowth similar to that of native PTN preparations purified from bovine uterus (Fig. 6). No significant neurite outgrowth activity was observed in the lysate of COS-7 cells transfected with vector alone. Because the cultures of neurons from the newborn rats contained a mixed population of cells, it is not possible to exclude the possibility that PTN acts indirectly through a second cell type to initiate neurite outgrowth from neurons.

These data support the conclusion that the purified, sequenced, and cloned PTN molecule is responsible for the observed mitogenic and neurite outgrowth promoting activity. Despite the identification of a signal sequence, we were unable to find mitogenic activity in conditioned media from PTN transfected COS-7 cells nor could we observe the product in conditioned media by metabolic labeling. However, when heparin was added to the medium, an 18-kD protein with weak mitogenic activity was identified in immunoprecipitates after metabolic labeling (Fig. 5C), suggesting that the protein may be secreted and bind to the extracellular matrix with high affinity. Such binding is suggested by the very tight binding of PTN to heparin-Sepharose columns (*1, 2*) and may be similar to the binding to extracellular matrix reported with the expressed product of the *int-1* gene (*17*). Rough estimates of the intrinsic specific mitogenic activity of the expressed protein suggested that the protein in lysates is of low activity relative to the purified protein. It is possible that transiently expressed PTN may be incompletely processed or poorly folded. In all preparations of PTN tested, the assays were performed to exclude even small quantities of basic FGF. Furthermore, during purification, only fractions on the descending portion of the PTN elution peak were used, to exclude traces of basic FGF which potentially might contaminate the ascending peak fractions (16).

PTN appears to be a member of a family of developmentally regulated cytokines with diverse biological activities.

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5. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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15. Peptide (GKKEKPEKKVKKSDCGEWQ) was synthesized, conjugated to thyroglobulin, and emulsified (1:2) with complete Freund's adjuvant. New Zealand white rabbits were injected subcutaneously (100 μg per rabbit) in multiple dorsal sites. Boosters were given at 2- to 3-week intervals and contained 50 μg of conjugated peptide in incomplete Freund's adjuvant. Rabbit antiserum was passed over protein A Sepharose column (Pharmacia), and bound antibodies were eluted as described by Pharmacia protocol.
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22. For immunoprecipitation, the labeled cells were washed three times with cold PBS and lysed in 0.5 ml of lysis buffer containing 50 mM tris-HCl, pH 7.5, 150 mM NaCl, 0.1 percent SDS, 0.5 percent sodium deoxycholate, 1 percent NP-40, 2 mM PMSF and aprotinin at 2 μg/ml for 30 minutes on ice. The lysate was centrifuged, and the supernatants were diluted with 50 mM tris-HCl, pH 7.5, 150 mM NaCl to 1.0 ml. The immunoprecipitation was done as described by J. Sambrook, E. F. Fritsch, and T. Maniatis [*Molecular Cloning: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, ed. 2, 1989), pp. 18 to 26].
23. The bovine and human PTN sequence data will appear in EMBL nucleotide sequence data bases under the accession numbers X52945 and X52946, respectively. We thank E. Sadler for providing the human placenta cDNA library, R. Kawahara for stimulating discussion, and M. Chu for technical assistance during cloning. Supported by NIH grants HL31102, HL14147, and CA49712; a grant from the Monsanto Company; and a Merck fellowship from the American College of Cardiology (to P.G.M.)

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Targeting the E1 Replication Protein to the Papillomavirus Origin of Replication by Complex Formation with the E2 Transactivator

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The mechanism by which transcription factors stimulate DNA replication in eukaryotes is unknown. Bovine papillomavirus DNA synthesis requires the products of the viral E1 gene and the transcriptional activator protein encoded by the E2 gene. Experimental data showed that the 68-kilodalton (kD) E1 protein formed a complex with the 48-kD E2 transcription factor. This complex bound specifically to the viral origin of replication, which contains multiple binding sites for E2. Repressor proteins encoded by the E2 open reading frame failed to complex with E1 suggesting that the 162–amino acid region of E2 that participates in transactivation contained critical determinants for interaction with E1. The physical association between a replication protein and a transcription factor suggests that transcriptional activator proteins may function in targeting replication initiator proteins to their respective origins of replication.

THE INITIATION OF DNA SYNTHESIS IS A PRECISELY ORchestrated event performed at defined genetic loci termed origins of replication. Such elements have been isolated from bacteria, bacteriophage, yeasts, and viruses that infect eukaryotic cells (*1*). They are cis-acting elements that direct the assembly of specialized nucleoprotein complexes that function in duplicating the genome (*2*). Replication origins frequently contain multiple DNA sequence motifs that are recognized by proteins directly involved in unwinding the origin (*3*). However, several proteins that bind specifically to origins of replication also function in the control of transcription (*4*). The function of transcription factors in the initiation of DNA synthesis is ambiguous.

Much of the information regarding the function of transcription factors in DNA replication stems from analyses of DNA sequences that are required for origin function. In these studies, in vivo replication is measured after the deletion of DNA sequences that bind a specific transcription factor in vitro or stimulate transcription from heterologous promoters in vivo. Thus, the replication of polyoma virus displays a requirement for a transcriptional enhancer sequence (*5*). In addition, a core origin of replication for SV40 is stimulated by auxiliary elements that function in transcriptional control (*6*). A transcriptional enhancer is also a component of the Epstein-Barr virus origin of replication (oriP), and its activity is dependent on the Epstein-Barr nuclear antigen (EBNA) replication protein. The replication and transcription functions of EBNA, which binds to both core origin and enhancer sequences, may involve similar segments of the polypeptide (*7*). Biochemical studies with the cellular proteins CCAAT transcription factor–nuclear factor I (CTF-NFI) and octamer transcription factor 1 (OTF1)–NFIII revealed that they stimulate adenovirus DNA replication in vitro by enhancing the formation of the preterminal protein (deoxycytosine monophosphate) initiation complex (*8*). While NFI DNA binding sites are required for replication in vivo, effects of NFIII binding sites have not been reported (*9*).

The mechanism by which transcription factors function to augment replication is equally obscure. They may participate directly in the initiation of DNA synthesis by bringing replication factors to the template or indirectly by causing localized alterations in chro-

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