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Developmentally Regulated Use of Alternative Promoters Creates a Novel Platelet-Derived Growth Factor Receptor Transcript in Mouse Teratocarcinoma and Embryonic Stem Cells

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Embryonal carcinoma and embryonic stem cells expressed a novel form of platelet-derived growth factor receptor mRNA which was ~1,100 base pairs shorter than the 5.3-kilobase (kb) transcript expressed in fibroblasts and other cell types. The 4.2-kb stem cell transcript was initiated within the genomic region immediately upstream of exon 6 of the 5.3-kb transcript and therefore lacked the first five exons, which encode much of the extracellular domain of the receptor expressed in fibroblasts. In stem cells, the short form was predominant, although both forms were present at low levels. Following differentiation in vitro, expression levels of the long form increased dramatically. These findings suggest that during early embryogenesis, a stem cell-specific promoter is used in a stage- and cell type-specific manner to express a form of the platelet-derived growth factor receptor that lacks much of the extracellular domain and may function independently of ligand.

There is increasing evidence that polypeptide growth factors play an important role in development by regulating the growth and differentiation of embryonic cells (18). The effects of such molecules could in turn be regulated by factors that control their abundance and tissue specificity during development or by alterations in the expression either of the receptors through which they exert their effects or of other elements in the signaling pathway. There is evidence that platelet-derived growth factor (PDGF), a polypeptide mitogen for fibroblasts and smooth muscle cells (10, 25), is expressed in the early mouse embryo (9, 23, 24) and thus may play a role in early mammalian embryogenesis. We have examined the expression of the PDGF receptor in mouse embryonal carcinoma (EC) and embryonic stem (ES) cells. Both of these cell types are derived from and have properties in common with pluripotent stem cells in the early embryo (6, 15, 16). They can differentiate in vitro through processes that mimic the early stages of mouse embryogenesis and can participate in the formation of a normal mouse when injected into blastocysts (3, 21). For these reasons, EC and ES cells offer an in vitro model system for studying regulatory events in embryogenesis.

Two types of PDGF receptors which are encoded by two distinct genes have been identified (17a, 31). In previous studies of the expression of the type β PDGF receptor (PDGF-R) gene, a single 5.3-kilobase (kb) transcript has been detected in a variety of mouse tissues and cell lines (31). However, when we hybridized PDGF-R cDNA sequences to Northern (RNA) blots of poly(A)$^+$ RNA from EC and ES cells, we observed an unusual 4.2-kb transcript (Fig. 1A). In all stem cell lines tested, both the 4.2- and 5.3-kb transcripts were detectable, but their relative abundance varied; in RNA from EC and ES cells grown in the absence of fibroblastic feeder cells, the 4.2-kb transcript constituted >90% of the PDGF-R mRNA (Fig. 1, F9 EC cells; also data not shown), whereas in RNA from stem cells that had been cocultured with feeder cells (Fig. 1A, PSA-1 EC and all ES cells), the 4.2-kb transcript was sometimes found to represent only ~50% of the PDGF-R mRNA. Although precautions were taken to obtain pure stem cell populations, some of the 5.3-kb transcripts detected in these samples were likely to have been derived from residual feeder cells. In all stem cell lines studied, the levels of PDGF-R mRNA were ~100-fold lower than in BALB/c-3T3 fibroblasts (Fig. 1).

Certain stem cell lines differentiate in vitro in a manner that mimics the normal processes of mouse embryogenesis. PSA-1 cells at stages 1, 2, and 3 of differentiation in vitro provide cell cultures that have features in common with mouse embryos at stages prior to (4.5 to 6.5 days of gestation), during (6.5 to 8.5 days), and after (8.5 to 10.5 days) gastrulation, respectively (11, 17). Northern blot analyses of poly(A)$^+$ RNA from PSA-1 cells at these three stages of differentiation showed that the expressions of the two PDGF-R transcripts were developmentally regulated (Fig. 1B). In undifferentiated cells, most of the 5.3-kb transcript could be attributed to expression by the feeder cells (see above) and therefore was expressed at very low levels by stem cells, and no change was observed when the stem cells differentiated to extraembryonic endoderm (PSA-1 cells at stage 1 of differentiation and F9 cells differentiated with retinoic acid [Fig. 1B]). However, the 5.3-kb transcript became the predominant PDGF-R mRNA species when the PSA-1 cells formed a complex mixture of differentiated cell types (stage 3 of differentiation [Fig. 1B]). In some experiments, we observed a predominance of the 5.3-kb transcript in PSA-1 cells at stage 2 of differentiation (data not shown), which suggests that this species of PDGF-R mRNA may first appear during gastrulation, perhaps in the developing mesodermal cells.

Southern blot analyses have indicated that the PDGF-R gene is a single-copy gene and that its structure is the same in EC cells and fibroblasts (data not shown). Therefore, the 5.3- and 4.2-kb transcripts expressed in stem cells must be products of the same gene. They could arise from the use of alternative promoters or from differences in posttranscriptional processing. To determine the structure of the 4.2-kb

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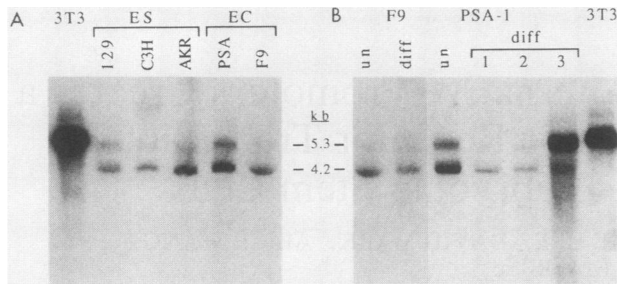

FIG. 1. Expression of PDGF-R in ES and EC cells. (A) Northern blot of poly(A)$^+$ RNAs from undifferentiated ES and EC cell lines and from BALB/c-3T3 fibroblasts. ES cell lines were derived from blastocysts of mouse strains 129, C3H, and AKR (G. R. Martin, unpublished observations). The PSA-1 and F9 EC cell lines were isolated from teratocarcinomas derived from strain 129 embryos as previously described (11, 17). The PSA-1 and ES cells were all maintained in the undifferentiated state by coculture with fibroblastic feeder cells and were separated from the feeder cells prior to RNA isolation by the preplating method (16). F9 cells were maintained in the undifferentiated state by culture on gelatin-coated plates (2). (B) Northern blot of poly(A)$^+$ RNAs from undifferentiated F9 cells (lane un), F9 cells induced to differentiate in monolayer into endodermal cells by treatment with retinoic acid and dibutyryl cAMP (lane diff [29]), undifferentiated PSA-1 cells (lane un), and PSA-1 cells at stages 1, 2, and 3 of differentiation in vitro (lanes diff 1, 2, and 3). PSA-1 cells were induced to differentiate by aggregation and culture in suspension (11, 17). RNA was isolated by precipitation in 3 M LiCl–6 M urea (1), poly(A)$^+$ selected on an oligo(dT) cellulose column (14), separated in a 1% agarose–2.2 M formaldehyde gel, and transferred to nitrocellulose (14). The filters were hybridized under high-stringency conditions to a full-length PDGF-R cDNA probe labeled with ^{32}P by nick translation (31). Lanes contained 20 μg of poly(A)$^+$ RNA except for 3T3 lanes, which contained 2 μg of poly(A)$^+$ RNA.

transcript, we hybridized Northern blots of PSA-1 stem cell poly(A)$^+$ RNA with cDNA probes corresponding to different regions of the 5.3-kb transcript (Fig. 2B). No hybridization to the 4.2-kb transcript was detected with probes 1 and 2, which represented sequences from nucleotides 1 to 1,033 of the 5.3-kb transcript cDNA. By contrast, cDNA probes corresponding to the remainder of the 5.3-kb transcript (probes 3, 4 and 5 [Fig. 2B]), including the 3′ untranslated region, hybridized to the 4.2-kb transcript. This suggests that the 4.2-kb transcript is a truncated version of the 5.3-kb transcript, lacking ~1,100 bases at the 5′ end.

We next compared the extent to which mRNAs from stem cells and 3T3 cells protected radiolabeled RNA probes complementary to different regions of the 5.3-kb transcript from degradation by RNase (Fig. 2C). As expected, RNA probes c and d, complementary to sequences more than 1,400 base pairs downstream of the 5′ end of the 5.3-kb transcript, were similarly protected by mRNAs from stem cells and 3T3 cells. Marked differences were observed with probe a, which contained sequences complementary to the 460 bases at the 5′ end of the 5.3-kb transcript. Protection of a 460-base fragment of probe a was observed with mRNAs from 3T3 cells, but only undigested probe was seen, and no specifically protected fragment was observed when mRNAs from F9 cells were used. This indicates that the 4.2-kb PDGF-R transcript, which is the predominant form of PDGF-R mRNA in F9 cells, does not contain the PDGF-R sequences in probe a. mRNAs from cultures of PSA-1 stem cells were found to protect the 460-base fragment of probe a, consistent with the observation that mRNAs from such cultures may contain significant quantities of the 5.3-kb PDGF-R transcript (Fig. 1). Probe b, which contained sequences complementary to nucleotides 1,033 to 1,361 of the 5.3-kb transcript, gave rise to two protected fragments, an expected 328-base fragment protected by 3T3 mRNA and a ~250-base fragment seen only with stem cell mRNA. These results placed the position at which the two transcripts began to share sequence identity at approximately position 1100 in the cDNA sequence of the 5.3-kb transcript.

To further define the structure of the 4.2-kb transcript, we generated cDNA copies of its 5′ end and sequenced them by the following strategy (8). PDGF-R cDNAs were obtained by reverse transcription of F9 poly(A)$^+$ RNA with a PDGF-R-specific sequence as a primer (nucleotides 2283 to 2300 of the PDGF-R cDNA [31]). Poly(dG) tails were then added to the 3′ ends of the cDNAs by using dGTP and terminal deoxynucleotidyltransferase. These ends of the cDNAs, which correspond to the 5′ ends of the PDGF-R mRNAs, were then amplified by the polymerase chain reaction method (26) by using as primers a 26-nucleotide oligomer containing a stretch of 12 dCs complementary to the poly(dG) tail and a PDGF-R-specific primer (nucleotides 1357 to 1382). To increase specificity, a second polymerase chain reaction was carried out using the same poly(dC) primer and another PDGF-R-specific primer (nucleotides 1268 to 1293). The ~250-base-pair fragment thus amplified was then directly sequenced (4).

When the sequence of this fragment representing the 5′ end of the 4.2-kb PDGF-R transcript was compared with the sequence of the 5.3-kb transcript cDNA (31), nucleotides 21 to 219 of the former were found to be identical to nucleotides 1070 to 1268 of the latter; the sequence of nucleotides 1 to 20 was not found in the 5.3-kb transcript cDNA; however, it was found immediately 5′ to the first nucleotide of exon 6 (cDNA position 1070, [Fig. 3]) in the genomic sequence. These results suggest that transcription of the 4.2-kb PDGF-R mRNA initiates in the genomic region that constitutes an intron for the 5.3-kb-PDGF-R mRNA and that the 4.2- and 5.3-kb transcripts have sequences beginning with exon 6 of the 5.3-kb transcript in common. Consistent with this interpretation is the fact that the distance from the start of exon 6 to the 3′ end of the 5.3-kb PDGF-R mRNA was ~4.2 kb. Moreover, the results from the Northern blot analyses and RNA protection assays discussed above were consistent with the conclusion that the 4.2-kb transcript is identical to the 5.3-kb transcript from exon 6 to its 3′ end.

To confirm this definition of the 5′ end of the 4.2-kb transcript, an RNA probe complementary to sequences spanning the boundary of intron 5-exon 6 of the 5.3-kb transcript (Fig. 3) was used in an RNA protection assay. mRNAs from 3T3 cells protected a single ~170-base fragment, whereas mRNAs from stem cells protected an additional fragment that was larger by approximately 20 bases, which supported our conclusion that the 4.2-kb PDGF-R transcript is initiated ~20 base pairs upstream of the start of exon 6. The sequence of this region was examined for promoter elements (Fig. 3 and data not shown). No TATA box was present, but there was an inverted CAAT box ~35 base pairs upstream of the presumed site of transcription initiation. It is possible that other, unusual promoter elements specific to early embryonic cells were used.

The only long open reading frame in the 4.2-kb transcript was the same as that which encoded the full-length PDGF-R protein. The first AUG codon in this frame was found at positions 119 through 121 of the 4.2-kb transcript (Fig. 3) and specified amino acid residue 313 of the full-length receptor protein (31). However, the nucleotide sequence around this

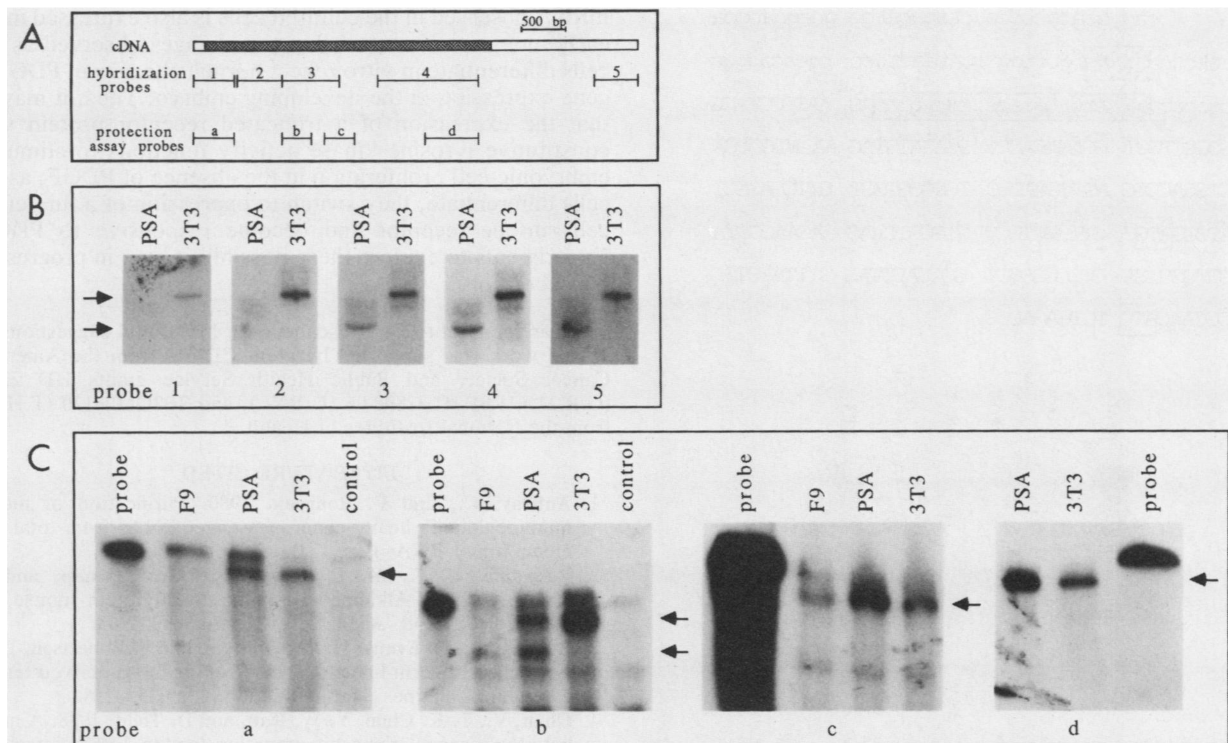

FIG. 2. Analysis of the structure of the 4.2-kb stem cell PDGF-R transcript. (A) The horizontal bar represents the sequence of the 5.3-kb PDGF-R transcript cDNA, with the stippled portion delineating the protein-coding sequences. The portions of the cDNA used as probes for the Northern blot analyses (probes 1 through 5) and RNA protection assays (probes a through d) are represented by the horizontal lines. (B) Northern blots of poly(A)$^+$ RNAs from PSA-1 stem cells (10 μg) and BALB/c-3T3 cells (0.1 μg) hybridized as described in the legend to Fig. 1 to probes 1 through 5 illustrated in panel A. The upper and lower arrows point to the 5.3- and 4.2-kb PDGF-R transcripts, respectively. Here the PSA-1 RNA samples did not contain detectable amounts of the 5.3-kb transcript, which indicates the variability in the contribution of residual feeder cells. (C) Protection from RNase degradation of antisense RNA probes by RNAs from stem cells (PSA-1 and F9) and BALB/c-3T3 cells. Fragments of the PDGF-R cDNA were cloned into pGEM1 or pGEM2 vector (Promega Biotec). The plasmids were linearized at appropriate restriction sites, and ^{32}P-labeled antisense RNA probes were synthesized by transcription from either the SP6 or the T7 phage promoter under the conditions suggested by Promega Biotec. The probes were gel purified, 5×10^5 cpm of each probe was hybridized to RNA from stem cells [20 to 50 μg of poly(A)$^+$ RNA] or 3T3 cells (10 μg of total RNA), and the RNA hybrids were digested with RNase A and T1 under conditions suggested by Promega Biotec. Digested products were separated in a 4% polyacrylamide–8 M urea gel and analyzed by autoradiography. The arrow(s) next to each autoradiograph points to the fragment of the probe protected by either 3T3 mRNA, stem cell mRNA, or both. Since the probes contained sequences from the pGEM vectors, the protected fragments were smaller than the probes in all cases.

AUG codon did not match the consensus sequence thought to be optimal for translation initiation (13). The next AUG codon occurred at positions 200 through 202, which specified amino acid residue 340 of the full-length receptor, and was found in a better context for translation initiation. This suggests that the 4.2-kb transcript can be translated into a truncated PDGF-R protein. This protein would not contain an amino-terminal hydrophobic signal sequence. However, it does contain the hydrophobic transmembrane sequence which was located at amino acid residues 500 to 524 of the full-length receptor. This region could serve as an internal signal for translocation across a membrane (28). In addition, there are examples of secreted proteins that do not contain recognizable signal sequences (19, 20). Therefore, the 4.2-kb transcript could potentially be translated into a cell surface protein. We have not been able to conclusively identify this truncated PDGF-R protein by immunoprecipitation with antisera against receptor peptides. Proteins from stem cell lysates that were immunoprecipitated by one receptor antibody were not recognized by other antibodies (data not shown). However, observations in our laboratory indicated that recognition of the receptor by these antibodies may be sensitive to receptor conformation and to alterations in the primary structure of the protein. Alternatively, the level of expression of this protein may be too low to allow detection by these assays.

The PDGF-R encoded by the 5.3-kb transcript is a 180-kilodalton protein with PDGF-stimulated protein tyrosine kinase activity (5, 7). It consists of three regions: a ligand-binding extracellular region, a transmembrane region, and a split tyrosine kinase domain (Fig. 3) (31). The extracellular region is composed of five immunoglobulinlike domains (J. F. Bazan, R. J. Fletterick, J. A. Escobedo, and L. T. Williams, unpublished observations). The truncated receptor encoded by the short transcript would lack the first three immunoglobulinlike domains of the extracellular region, which may be involved in ligand binding. The function of such a truncated receptor is not known. It is possible that the ligand-binding domains of receptor tyrosine kinases exert an inhibitory effect on receptor activity and that binding of the ligand removes this repression and leads to activation of receptor kinase. Removal of the ligand-binding domain could therefore lead to a constitutively activated receptor. This has been observed in the activation of the insulin receptor kinase

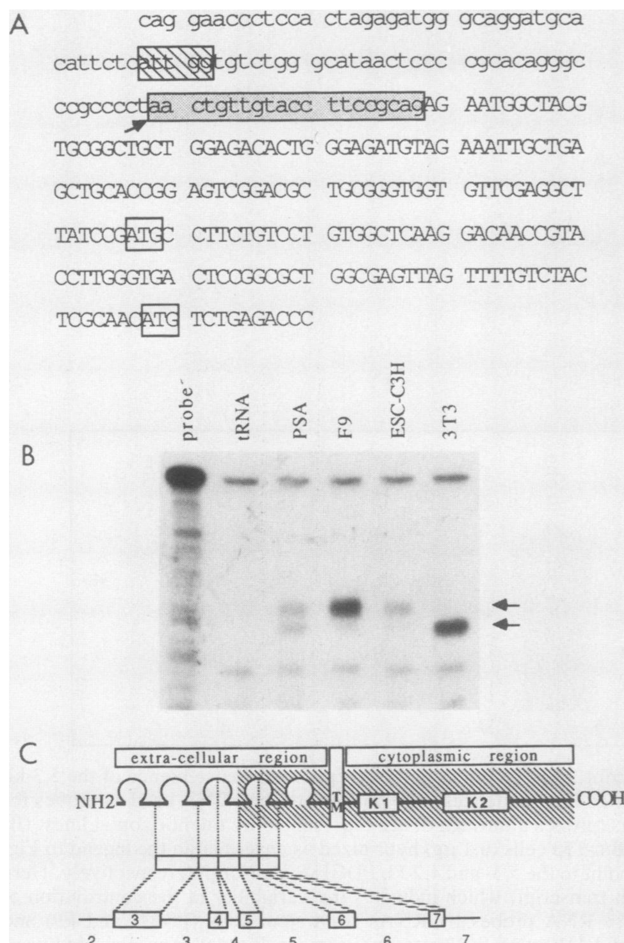

FIG. 3. Relationship of the 4.2- and 5.3-kb PDGF-R transcripts and the proteins they encode. (A) Sequence of the genomic region encoding the 5′ end of the 4.2-kb PDGF-R transcript. The genomic sequence shown spans the junction between intron 5 (lowercase letters) and exon 6 (capital letters) of the 5.3-kb full-length PDGF-R transcript. The arrow indicates the 5′ end of the 4.2-kb stem cell transcript as determined by the polymerase chain reaction method. Also shown are sequences that are not found in the 5.3-kb transcript (▒), the inverted CCAAT box (▧), and the ATG codons that are in the same open reading frame as that which encodes the full-length receptor (□). (B) Map of the initiation site of the 4.2-kb PDGF-R transcript. An RNA protection assay was carried out with a probe complementary to the sequence (except for the last 20 nucleotides) shown in panel A. The lower arrow points to the only fragment protected by 3T3 mRNA. The upper arrow points to the additional larger fragment protected by stem cell mRNA. The method was as described in the legend to Fig. 2. (C) Main structural components of the PDGF-R, including the five immunoglobulinlike domains of the extracellular region, the single transmembrane domain (TM), and the cytoplasmic region with the split kinase domain (K1 and K2). The hatched region denotes the portion of the receptor that is potentially encoded by the 4.2-kb stem cell transcript. Also shown is part of the intron-exon structure of the full-length 5.3-kb PDGF-R transcript and its relation to the various protein domains.

by proteolytic removal of the insulin binding site (27). Moreover, in the case of the epidermal growth factor receptor, truncation of the extracellular domain has been shown to potentiate its growth-promoting properties (12, 22, 30).

Since EC and ES cells closely resemble pluripotent cells in the early embryo, it is likely that the truncated PDGF-R mRNA observed in the cultured cells is also expressed in the early mouse embryo and that the changes observed as EC cells differentiate in vitro reflect normal changes in PDGF-R gene expression in the developing embryo. Thus, it may be that the expression of a truncated receptor protein with constitutive tyrosine kinase activity functions to stimulate embryonic-cell proliferation in the absence of PDGF; as the cells differentiate, they switch to expression of a full-length cell surface receptor and become responsive to PDGF. Experiments to explore these possibilities are in progress in our laboratory.

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