Detection of c-*sis* Transcripts and Synthesis of PDGF-Like Proteins by Human Osteosarcoma Cells

Abstract. *Platelet-derived growth factor (PDGF) has been previously shown to be homologous to the transforming gene of simian sarcoma virus (v-*sis*), and inappropriate expression of the cellular counterpart of the v-*sis *gene (c-*sis*) has been implicated in the generation of mesenchymal tumors. The U-2 OS human osteosarcoma line was shown to contain multiple c-*sis *transcripts. Immunoprecipitation experiments with antiserum to PDGF identified a variety of polypeptides ranging in size from 18,000 to 165,000 daltons that were immunoprecipitated specifically from U-2 OS cell extracts. The osteosarcoma also was shown to secrete a 29,000-dalton protein having the serological and structural characteristics of PDGF.*

A variety of transformed cells have been reported to produce mitogenic factors (*1*), suggesting that the ability to produce such factors is important in the establishment and maintenance of transformation (*2*). The human osteosarcoma-derived cell line U-2 OS elaborates a mitogen similar to platelet-derived growth factor (PDGF) (*3*) and does not require exogenous PDGF for growth in vitro (*4*). PDGF represents the major mitogen of human serum for cells of mesenchymal origin (*5*). It is stored in the α-granules of platelets (*6*) and has been purified from human serum (*7*), clinically outdated blood platelets (*8*), and platelet-rich plasma (*9*). Elucidation of its amino-terminal amino acid sequence suggested that it consists of two homologous polypeptide chains (PDGF-1 and PDGF-2) linked by disulfide bonds (*10*). PDGF shares extensive sequence homology with $p28^{sis}$, the oncogene product of the simian sarcoma virus (SSV), an acute transforming primate retrovirus (*11*). In SSV-transformed cells, $p28^{sis}$ is processed into a disulfide-linked dimer structurally similar to the PDGF dimer, which is secreted by the cells and is specifically immunoprecipitated with antiserum to PDGF (*12*). In addition, a number of cell lines derived from mesenchymal tumors have been reported to contain RNA (c-*sis*), which hybridizes to a v-*sis* DNA probe (*13*).

We used a DNA probe to v-*sis* and antiserum to PDGF to investigate the synthesis of PDGF-like products by U-2 OS cells. To determine whether c-*sis* transcripts are synthesized in U-2 OS cells, we isolated total polyadenylated [poly(A)$^+$] RNA from the cells and subjected it to RNA blot analysis with a ^{32}P-labeled v-*sis* DNA probe (*14*). Two bands corresponding to RNA species of 4.0 and 3.7 kilobases (kb) in length were detected (Fig. 1A). Occasionally a smaller, more diffuse band (~2.6 kb) was also observed. We believe that the 4.0-kb RNA corresponds to the 4.2-kb c-*sis* RNA species identified earlier in a number of human sarcomas and glioblastomas (*13*). Comparison of the intensity of each c-*sis* RNA band to a dilution series of a standard RNA indicated that each cell contains 10 to 20 copies of the c-*sis* transcript (data not shown). The different sizes of the c-*sis* transcripts may be due to differences in the initiation or termination sites of RNA synthesis, differential RNA splicing, or the expression of different but related genes, or they may reflect somatic mutation of one or both alleles. The size of the transcript and the lack of integrated proviral DNA in the U-2 OS genome (data not shown) make it unlikely that the hybridizing species are derived from an SSV v-*sis* gene. No c-*sis* RNA was detected in total poly(A)$^+$ RNA isolated from MG-63 osteosarcoma cells (Fig. 1B), which do not produce PDGF (*15*).

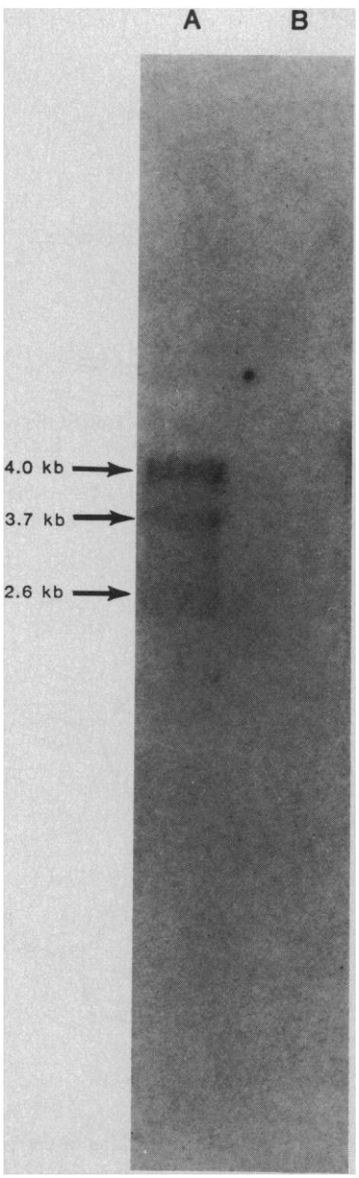

Fig. 1. RNA blot analysis of poly(A)$^+$ RNA from U-2 OS and MG-63 human osteosarcoma cells with the use of a v-*sis* DNA probe (*14*). Poly(A)$^+$ RNA was isolated by extraction in 5*M* guanidinium thiocyanate and subsequent oligo(dT) cellulose chromatography. Poly(A)$^+$ RNA (5 μg) was subjected to electrophoresis on a 1.2 percent agarose gel and transferred to nitrocellulose. The filter was then hybridized with a nick-translated Pst I restriction fragment of simian sarcoma proviral DNA, which contains the v-*sis* region. (Lane A) U-2 OS; (lane B) MG-63.

The 4.0- and 3.7-kb RNA species have a potential coding capacity of 130 to 150 kilodaltons (kD), which is approximately four times the molecular size of mature PDGF (*10*). Thus, it is possible that the mature form of the PDGF-like factor is derived from a very large precursor. To investigate these possibilities, we labeled U-2 OS cells with [^{35}S]cysteine, immunoprecipitated cytoplasmic extracts with antiserum to purified PDGF (*16*), and analyzed the precipitate by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography (*17*). Proteins with molecular sizes of 56, 34, 32, and 18 to 20 kD were detected with antiserum to PDGF but not with control serum on a 16 percent polyacrylamide gel run under nonreducing conditions (Fig. 2A, lanes 1 and 2). This experiment was repeated with 8 percent polyacrylamide gels to detect the large forms (Fig. 2A, lanes 3 to 5). Proteins with molecular sizes of 220, 210, and 54 to 60 kD were precipitated by antiserum to PDGF but not in the presence of excess PDGF (1 μg) or with control serum.

Under reducing conditions, the amounts of 210- to 220-kD species were much lower. Instead, a prominent 165-kD protein was precipitated in addition to minor species of 130, 115, and 90 kD. Precipitated material migrating in the 56- to 62-kD range was also detected and probably corresponded to the 54- to 60-kD protein identified under nonreducing conditions. Precipitation of all these species was inhibited by the addition of excess PDGF. The apparent shift in the mobility of the reduced 165-kD protein to proteins of 210- to 220-kD under non-

reducing conditions suggests that it is covalently associated with one or more other proteins in its native state.

Experiments were undertaken to analyze the forms of PDGF-like polypeptides secreted by U-2 OS cells. Cysteine-free minimum essential medium (MEM) was conditioned by U-2 OS cells in the presence of [^{35}S]cysteine (*18*), concentrated, immunoprecipitated as described (*17*), and analyzed on a 16 percent gel (Fig. 2B). Under nonreducing conditions, a protein with a molecular size of approximately 200 kD and one of 29 kD were precipitated by antiserum to PDGF but not by control serum or in the presence of excess PDGF (Fig. 2B, lanes 1 to 3). Reduced precipitates had molecular sizes of 110, 16, and 15 kD (lanes 4 to 6). The 200- and 29-kD proteins present under nonreducing conditions disappeared upon reduction, suggesting that they were derived from the 110-, 16-, and 15-kD proteins, respectively. A 29-kd protein that can be reduced to a 16- and 15-kD polypeptide is consistent with the structure of PDGF.

To establish the specificity of the immunoprecipitation assay, we repeated the immunoprecipitation experiments with isotopically labeled MG-63 cells and GM-10 human diploid fibroblasts (Fig. 2C). No difference was detected when the precipitation was carried out with antiserum to PDGF or control serum.

That antiserum to PDGF cross-reacts with biologically active factors secreted by U-2 OS cells was confirmed by partially purifying U-2 OS medium as described (*19*) and assaying it for stimulation of [^{3}H]thymidine incorporation in 3T3 cells (Table 1). The amount of [^{3}H]thymidine incorporated into 3T3 cells in partially purified conditioned medium was five times greater than that in control. Antiserum to PDGF blocked 81 percent of the mitogenic activity of this conditioned medium.

The results indicate that the mitogenic factor secreted by U-2 OS is PDGF. First, U-2 OS cells were shown to produce RNA transcripts that are homologous to the c-*sis* proto-oncogene. The c-*sis* gene has recently been shown to encode the PDGF-2 chain (*20*). Also, DNA sequence analysis of complementary DNA (cDNA) clones constructed from U-2 OS poly(A)$^{+}$ RNA confirms that the osteosarcoma contains transcripts derived from the c-*sis* gene (*21*). Second, immunoprecipitation of conditioned medium from U-2 OS cells revealed that they secrete a 29-kD protein that shares antigenic determinants with PDGF. This 29-kD protein is a disulfide-linked dimer of 15- and 16-kD proteins,

Table 1. Effect of antiserum to PDGF on partially purified osteosarcoma-derived growth factor.

Sample	[^{3}H]Thymidine incorporation (count/min)	Inhibition (percent)
CM-Sephadex preparation	127,561	
+ antiserum to PDGF (1:1000)	84,155	34
+ antiserum to PDGF (1:333)	24,028	81
Control	19,462	

a structural feature characteristic of PDGF. Third, the same antiserum used in the experiments described above blocked the mitogenic activity of medium conditioned with U-2 OS.

Precipitation of U-2 OS cell extracts revealed several cross-reacting proteins ranging from 18 to 20 kD up to 165 kD. The smallest proteins (18 to 20 kD) have an apparent size that agrees well with the size of the reduced PDGF chains. The 165-kD protein was slightly larger than the total coding capacity of the largest c-*sis* transcript detected (approximately 150 kD), but this could be accounted for by the presence of carbohydrate groups on the 165-kD species. The simplest interpretation of our results is that the 18- to 20-kD polypeptides are mature forms of PDGF chains, the 165-kD polypeptide is a PDGF precursor, and the remaining species represent processing intermediates. We have, however, no direct proof that this interpretation is correct, and alternative explanations are possible. For example, one or more of the polypeptides we have identified may not have PDGF antigenic determinants but might be tightly associated with peptides that do react with antiserum to PDGF and, consequently, are precipitated together with them. Recently, it has been shown that cell extracts treated with antiserum to p21ras coprecipitate the transferrin receptor with p21ras protein (*22*). Alternatively, the different sizes of the proteins reactive with antiserum to PDGF may reflect the multiple RNA

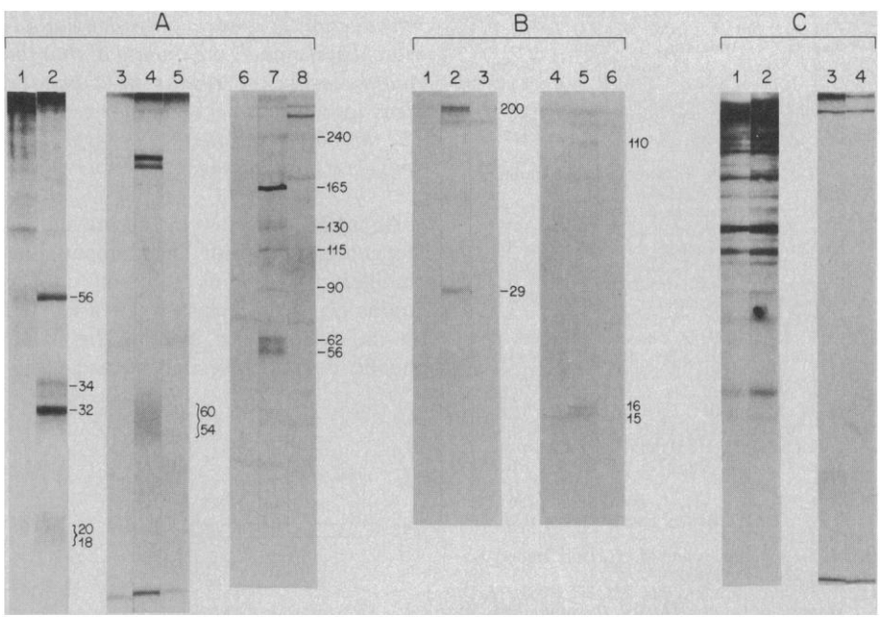

Fig. 2. Immunoprecipitation of PDGF-like peptides from U-2 OS human osteosarcoma cells. Numbers to the right of bands denote kilodaltons. U-2 OS cells were labeled and immunoprecipitated as described (*16*). (A) Cell extracts analyzed on 16 percent gels under nonreducing conditions after treatment with normal rabbit serum (lane 1) or antiserum to PDGF (lane 2); on an 8 percent gel under nonreducing conditions after treatment with normal rabbit serum (lane 3), antiserum to PDGF (lane 4), or antiserum to PDGF plus excess PDGF (lane 5); and on 8 percent gels under reducing conditions after treatment with normal rabbit serum (lane 6), antiserum to PDGF (lane 7), or antiserum to PDGF plus excess PDGF (lane 8). (B) Labeled medium was prepared as described (*18*) and analyzed on 16 percent gels under nonreducing conditions after exposure to normal serum (lane 1), antiserum to PDGF (lane 2), or antiserum to PDGF plus excess PDGF (lane 3). Samples analyzed under reducing conditions were treated with normal serum (lane 4), antiserum to PDGF (lane 5), or antiserum to PDGF plus excess PDGF (lane 6). (C) Labeled MG-63 cell extracts treated with normal serum (lane 1) or antiserum to PDGF (lane 2) and analyzed on a 16 percent gel under nonreducing conditions. GM-10 cells, similarly prepared, were treated with normal serum (lane 3) or antiserum to PDGF (lane 4) and analyzed on an 8 percent gel under reducing conditions.

species found in these cells. The complete characterization of c-*sis* cDNA clones, as well as experiments designed to elucidate the precursor-product relations of the various proteins, should help to determine the relevance of these proteins to the synthesis, assembly, and function of PDGF in U-2 OS cells.

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References and Notes

1. M. A. Anzano *et al.*, *Cancer Res.* **42**, 4776 (1982); J. E. DeLarco and G. J. Todaro, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4001 (1978); B. Ozanne, R. J. Fulton, P. L. Kaplan, *J. Cell Physiol.* **110**, 81 (1982); A. B. Roberts *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 3494 (1980).
2. H. L. Moses and R. A. Robinson, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41**, 3008 (1982); P. L. Kaplan, M. Anderson, B. Ozanne, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 485 (1982); M. B. Sporn, G. J. Todaro, *N. Engl. J. Med.* **303**, 878 (1980).
3. C.-H. Heldin, B. Westermark, A. Wasteson, *J. Cell Physiol.* **105**, 235 (1980); C. Betsholtz *et al.*, *Biochem. Biophys. Res. Commun.* **117**, 176 (1983).
4. D. T. Graves, A. J. Owen, H. N. Antoniades, *Cancer Res.* **43**, 83 (1983).
5. H. N. Antoniades, D. Stathakos, C. D. Scher, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 675 (1973); N. Kohler and A. Lipton, *Exp. Cell Res.* **87**, 297 (1974); R. Ross, J. Glomset, B. Kariya, L. Harker, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1207 (1974); B. Westermark, A. Wasteson, K. Uthne, *Exp. Cell Res.* **96**, 58 (1975).
6. D. R. Kaplan, F. C. Chao, C. D. Stiles, H. N. Antoniades, C. D. Scher, *Blood* **53**, 1043 (1979).
7. H. N. Antoniades, D. Stathakos, C. D. Scher, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2635 (1975).
8. H. N. Antoniades, C. D. Scher, C. S. Stiles, *ibid.* **76**, 1809 (1979); H. N. Antoniades, *ibid.* **78**, 7314 (1981); C.-H. Heldin, B. Westermark, A. Wasteson, *ibid.* **76**, 3722 (1979); *ibid.* **78**, 3664 (1981).
9. T. F. Deuel *et al.*, *J. Biol. Chem.* **256**, 8896 (1981); E. W. Raines and R. Ross, *ibid.* **257**, 5154 (1982).
10. H. N. Antoniades and M. W. Hunkapiller, *Science* **220**, 963 (1983).
11. R. F. Doolittle *et al.*, *ibid.* **221**, 275 (1983); M. D. Waterfield *et al.*, *Nature (London)* **304**, 35 (1983).
12. K. C. Robbins, H. N. Antoniades, S. G. Devare, M. W. Hunkapiller, S. Aaronson, *Nature (London)* **305**, 605 (1983). A. J. Owen, P. Pantazis, H. N. Antoniades, *Science* **225**, 54 (1984).
13. A. Eva *et al.*, *Nature (London)* **295**, 116 (1982).
14. J. M. Chirgwin, A. E. Przybyla, R. J. MacDonald, W. J. Rutter, *Biochemistry* **18**, 5294 (1979); H. Aviv and P. Leder, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1408 (1972); P. S. Thomas, *ibid.* **77**, 5201 (1980); P. W. J. Rigby, M. Dieckmann, C. Rhodes, P. Berg, *J. Mol. Biol.* **113**, 237 (1977); K. C. Robbins, S. G. Devare, E. P. Reddy, S. A. Aaronson, *Science* **218**, 1131 (1982).
15. D. T. Graves, S. R. Williams, H. N. Antoniades, A. J. Owen, *Cancer Res.* **44**, in press.
16. Antiserum to PDGF was produced in rabbits as described by A. J. Owen *et al.* [*Proc. Natl. Acad. Sci. U.S.A.* **79**, 3203 (1982)]. Confluent U-2 OS cells were incubated for 2 hours in cysteine-free MEM containing 300 μCi of carrier-free [^{35}S]cysteine per milliliter. The cells were collected (~ 10^7 per batch) and lysed with immunoprecipitation buffer [0.3*M* NaCl, 0.02*M* tris-HCl (*p*H 7.5), 0.001*M* EDTA, 0.01*M* NaI, 0.002*M* phenylmethylsulfonyl fluoride (PMSF), 0.3 percent Triton X-100, and 1 percent human serum albumin (HSA). The extracts were cleared with 50 μg of Protein A–Sepharose. Normal rabbit serum or antiserum to PDGF (2 μl) was then added to equal portions of the cell extract and allowed to incubate for 18 hours at 4°C. Immune complexes were recovered by the addition of Protein A–Sepharose (40 μg).
17. The washed Protein A–Sepharose beads were resuspended in SDS sample buffer [50 mM tris-HCl (*p*H 6.8), 2 percent SDS, 1 m*M* PMSF, and 12 percent glycerol] and boiled for 3 minutes. The beads were pelleted, and the supernatants were collected and divided into two equal portions. Dithiothreitol (100 m*M* final concentration) was added to one of the samples and left for 90 minutes at 37°C. 2-Mercaptoethanol (10 percent final volume) was added to the reduced sample 15 minutes before applying the sample to gels (30 by 20 cm by 0.8 mm SDS-PAGE). Resolving gels (8 percent) were used to analyze the large peptides and 16 percent gels for the small peptides. The gels were run as described [P. Pantazis and W. M. Bonner, *J. Biol. Chem.* **256**, 4669 (1981)]. Gels were dried and autoradiographed as described [W. M. Bonner, M. H. P. West, J. D. Stedman, *Eur. J. Biochem.* **109**, 17 (1980)].
18. Labeled conditioned medium from U-2 OS cells was prepared and immunoprecipitated as described by A. J. Owen *et al.* [in (*12*)].
19. CM-Sephadex that had been swollen was added to MEM conditioned with U-2 OS cells (100 ml), dialyzed overnight against water, poured on a column, and eluted with 1*M* NaCl. The eluted fractions were dialyzed exhaustively against acetic acid, lyophilized, and resuspended in HSA (1 percent).
20. S. F. Joseph, C. Guo, L. Ratner, F. Wong-Staal, *Science* **223**, 487 (1984); I.-M. Chiu *et al.*, *Cell* **37**, 123 (1984).
21. P. Tempst *et al.*, in preparation.
22. T. Finkel and G. M. Cooper, *Cell* **36**, 115 (1984).
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Functional Insertion of an Alu Type 2 (B2 SINE) Repetitive Sequence in Murine Class I Genes

Abstract. *The regulation of expression of the family of MHC (major histocompatibility complex) class I genes is complex. Sequence analysis has revealed that class I genes from the H-2D subregion of the MHC (which includes the D and L genes) differ from the class I gene from the H-2K subregion (the K gene) by the insertion of a type 2 Alu-like repetitive element (the murine B2 sequence) within the 3′ noncoding region of the D and L genes. The consequence of this insertion in the D and L genes is the introduction of a novel polyadenylation signal, which is preferentially used over the more distal signal, the analog of that found in the K gene. The insertion of the type 2 Alu-like sequence results in a change in the preferred site for endonucleolytic cleavage which is necessary for generating a correct 3′ terminus for polyadenylation. The data demonstrate that the type 2 Alu-like sequence has a function; the data also suggest a possible regulatory role of this sequence in the expression of class I genes.*

About 25 to 35 class I genes have been mapped to the major histocompatibility complex (MHC) of different mouse strains (*1*). Those class I genes located on the centromeric side of the MHC encode polymorphic cell-surface transplantation antigens (designated K, D, and L) which play a critical role in inducing graft rejection and in permitting cytotoxic T cells to distinguish self from nonself (*2*). Other class I genes located in the Qa2,3 and Tla regions on the telomeric side of the MHC encode structurally related antigens that differ in their tissue distribution and possibly in their function (*3*).

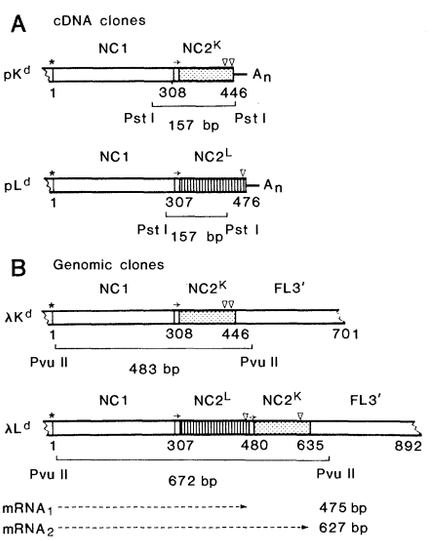

Fig. 1. Diagram of the organization at the 3′ ends of class I cDNA and genomic clones. The cDNA structures (designated p) for K^d and L^d were derived from $pH2^d$-4 and $pH2^d$-3(5), respectively. The genomic structures (designated λ) for K^d and L^d were from λ2.14 (*23*) and λL^d-D5. The noncoding region 1 is designated NC1, the noncoding region 2 as NC2, the poly(A) (polyadenylated) tract as A_n, and the 3′ flanking sequences as FL3′. Analogous but nonhomologous regions are indicated by stipples and stripes. The asterisks denote the TGA translational termination codons, the arrows indicate the direct repeats, and the triangles identify the polyadenylation signals. Sequences are numbered with reference to the nucleotide immediately following the TGA termination codon.