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Synthesis and secretion of proteins resembling platelet-derived growth factor by human glioblastoma and fibrosarcoma cells in culture

(c-*sis* oncogene/viral oncogene v-*sis*/immunoprecipitation/RNA blotting)

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ABSTRACT Immunoprecipitation of proteins extracted from metabolically labeled human glioblastoma and fibrosarcoma cells with antiserum to platelet-derived growth factor (PDGF) showed that these cells express and secrete proteins that are recognized specifically by the antiserum. The molecular masses of immunoprecipitated proteins in the lysates of the malignant cells ranged from 16 kDa to 140 kDa. Both cell lines secreted a 31-kDa polypeptide with structural, immunological, and biological properties similar to those of human PDGF. These cell lines were shown to synthesize a 4.4-kb mRNA that contained sequences from all the six currently identified exons of the human c-*sis* gene. These data suggest that the PDGF-like proteins in the two mesenchyme-derived transformed cells are encoded at least in part by the c-*sis* locus.

Platelet-derived growth factor (PDGF), the major mitogen of clotted blood, is required for the growth in culture of mesenchymal cells such as diploid fibroblasts (1), smooth muscle cells (2), and glial cells (3). PDGF consists of two homologous polypeptide chains, PDGF-1 and PDGF-2, linked together by disulfide bonds (4). PDGF-2 is nearly identical to $p28^{sis}$, the transforming gene product of the simian sarcoma virus (SSV) (5). The similarity implies that the two polypeptides are encoded by the same or closely related genes (6, 7). In SSV-transformed cells, $p28^{sis}$ rapidly undergoes a series of discrete processing steps including disulfide-linked dimer formation and proteolytic degradation to yield homodimers structurally and immunologically resembling biologically active PDGF (8). Recently, we have demonstrated that this processed product is secreted by SSV-transformed normal rat kidney (NRK) fibroblasts in a form exhibiting structural, immunologic, and biologic properties, identical to those of active PDGF (9). These findings suggested that the cell transformation induced by the SSV derives from the incorporation of the PDGF/*sis* gene within the retroviral genome. The resulting transforming *onc* gene (v-*sis*) codes for a PDGF-like mitogen and is capable of inducing transformation by the continuous production of this potent mitogen that causes sustained cell proliferation.

The sequence homology of the viral oncogene v-*sis* to its cellular counterpart proto-oncogene c-*sis* (10) suggests that activation of c-*sis* may lead to synthesis of PDGF-like proteins. Polypeptides with PDGF activity and properties have been found in the conditioned media of human glioblastoma and osteosarcoma cells in culture (11–13). As described here and elsewhere (13, 14) these malignant cells synthesize c-*sis* messenger RNAs. Recently, the intracellular production and processing of a PDGF-like mitogen in cultured human osteosarcoma cells has been described (13).

In this report we describe the intracellular synthesis of PDGF-like proteins in cultures of human glioblastoma and fibrosarcoma cells and correlate the presence of these proteins with a c-*sis* mRNA species transcribed by the human c-*sis* locus. The intracellular PDGF-like proteins appear to be precursor and processed molecules of a major secreted protein of 31 kDa that is structurally, immunologically, and functionally similar to human PDGF.

MATERIALS AND METHODS

Cells. Human cells, glioblastoma (A172) and fibrosarcoma (HT-1080), and mouse cells, BALB/c 3T3 (clone A31), were obtained from The American Type Culture Collection. All cells were grown in T75 flasks (Falcon) and were maintained in Dulbecco's modified Eagle's medium containing 10% calf serum supplemented with penicillin (50 units/ml) and streptomycin (50 μg/ml). They were incubated in humidified 5% CO_2 atmosphere at 37°C.

Cell Labeling. Subconfluent or confluent cultures (20–25 $\times 10^6$ cells) were labeled for 3 hr at 37°C in serum-free/cystine-free medium containing [^{35}S]cysteine (specific activity, > 1000 Ci/mmol; 1 Ci = 37 GBq; Amersham) at 200 μCi/ml.

Preparation of PDGF Antiserum. PDGF antiserum was produced in rabbits by the intradermal injection (15) of pure PDGF. PDGF proved to be a poor immunogen and production of antiserum required multiple booster injections of the pure protein given at three month intervals. The antiserum used in the present studies was obtained after nine booster injections over a 27 month period following the initial injection.

Immunoprecipitation of Intracellular Proteins. The culture medium was removed and saved for immunoprecipitation of released proteins (see below). Attached cells were rinsed once with cold P_i/NaCl containing 1 mM phenylmethylsulfonyl fluoride ($PhMeSO_2F$). The cells were removed with a rubber policeman into cold P_i/NaCl/$PhMeSO_2F$ and centrifuged at 800–1000 rpm for 10 min (IEC centrifuge). The labeled cells were resuspended in 1.0–1.5 ml of radioimmunoprecipitation (RIP) buffer consisting of 150 mM NaCl/50 mM Tris Cl, pH 7.4/1% Triton X-100/ 1% sodium deoxycholate/0.1% $NaDodSO_4$/0.2% human serum albumin/1 mM $PhMeSO_2F$, and lysed for 10 min at 4°C under continuous gentle agitation on a rotator. The cell suspension was centrifuged in a microcentrifuge (Fisher) for 5 min to remove insoluble material. Clarified supernatant was precleared with normal rabbit serum (NRS) and protein A bound to Sepharose CL-4 beads (Pharmacia) (protein A-beads). The precleared

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Abbreviations: PDGF, platelet-derived growth factor; $PhMeSO_2F$, phenylmethylsulfonyl fluoride; P_i/NaCl, phosphate-buffered saline; SSV, simian sarcoma virus; SSV-NRK, normal rat kidney cells transformed by SSV; NaCl/Cit, 0.15 N NaCl/0.015 N Na citrate.

lysate was divided into three equal portions and incubated with NRS, antiserum to PDGF, and PDGF antiserum premixed with excess of purified PDGF (1 μg). The mixtures were incubated for 16 to 18 hr at 4°C under continuous gentle agitation. Protein A-beads were added to each mixture and incubation was continued for 90 min at 4°C to allow protein A to bind to the complexes of IgG/PDGF-like proteins. The beads were further pelleted by centrifugation in a microcentrifuge and washed three times in RIP buffer and once in 15 mM Tris Cl, pH 7.4. The pellets of beads were prepared for $NaDodSO_4$ gel electrophoresis as described below.

Immunoprecipitation of Released Proteins. The medium from cultures of metabolically labeled cells was centrifuged at 5000 × *g* (Sorvall) to remove insoluble material. The clarified medium was brought to 0.1% human serum albumin and lyophilized. The dried samples derived from 5 to 10 ml of medium were dissolved in 1.0–1.5 ml RIP buffer and dialyzed against 100 volumes of RIP buffer for 4 hr at 4°C. The dialyzed material was divided into three equal portions and immunoprecipitated as described above.

Gel Electrophoresis. Following immunoprecipitation, pellets of protein A-beads were suspended in 200 μl of 80 mM Tris Cl, pH 6.8/2% $NaDodSO_4$/10% glycerol/0.1% bromophenol blue and placed in boiling water for 3 min. The beads were pelleted by centrifugation in a microcentrifuge and the supernatant fluid was divided into 2 equal volumes; 1 volume received no addition, and to the other was added 2-mercaptoethanol (5% vol/vol). Both were incubated at room temperature for 1 hr. Unreduced and reduced samples were electrophoresed on 14% acrylamide slab $NaDodSO_4$ gels (16). Molecular weight protein markers from Bio-Rad or Pharmacia were used unreduced or reduced. Upon termination of electrophoresis the gels were stained, destained, treated with Amplify (Amersham), and dried under heat and vacuum. Bands of protein markers on the dried gels were marked with ^{14}C-labeled ink and exposed to Kodak XAR film at −80°C for 1–3 weeks.

Stimulation of DNA Synthesis. Incorporation of [^3H]thymidine into quiescent BALB/c 3T3 cells in the presence of media concentrates took place in platelet-poor plasma as described (17).

RNA Hybridization and DNA Probes. Total cellular RNA was extracted by the guanidine isothiocyanate method, poly(A)$^+$-selected, electrophoresed on a formaldehyde-containing agarose gel, transferred to nitrocellulose, and hybridized to [^{32}P]DNA probes according to established procedures (18). After the hybridization was completed, the filters were washed under nonstringent (1× NaCl/Cit, 0.5% $NaDodSO_4$, 60°C) or stringent (0.1× NaCl/Cit, 0.5% $NaDodSO_4$, 60°C) conditions. DNA probes were labeled by nick-translation (18). The presence and the relative amounts of mRNA on the filter were controlled by hybridization with a human β-actin cDNA probe. The DNA probes illustrated in Fig. 3*B* derive from the recombinant phage L33 containing the v-*sis* homologous regions of the human c-*sis* locus (10). Fragments were obtained either from preparative agarose gel electrophoresis of L33 restriction digests or from various L33 subclones in pBR322 plasmid (10, 19).

RESULTS

Intracellular Production of PDGF-Like Proteins. The presence of intracellular PDGF-like proteins in glioblastoma and fibrosarcoma cells was investigated by direct immunoprecipitation. Lysates of metabolically labeled cells were immunoprecipitated by PDGF antiserum and analyzed by $NaDodSO_4$/PAGE. As shown in Fig. 1, nonreduced immunoprecipitates of glioblastoma cell lysates contained PDGF-like polypeptides with molecular masses of 36, 31, and 24 kDa (lane b). These polypeptides disappeared when analyzed under reducing conditions, suggesting that they consisted of disulfide-linked dimers. Immunoprecipitation of these proteins by PDGF antiserum was blocked by the presence of excess unlabeled PDGF (lane c). Nonimmune rabbit serum did not precipitate these polypeptides (lane a). When the immunoprecipitate of the glioblastoma cell lysate was analyzed under reducing conditions, enabling resolution of proteins with high molecular mass, prominent polypeptides of 140, 110, 56, 48, 34, 20, 17, and 16 kDa were observed in samples treated with PDGF antiserum (lane e) but not in samples treated with normal serum (lane d). These polypeptides were not immunoprecipitated when the cell lysate sample was incubated with PDGF antiserum and excess of purified PDGF (lane f).

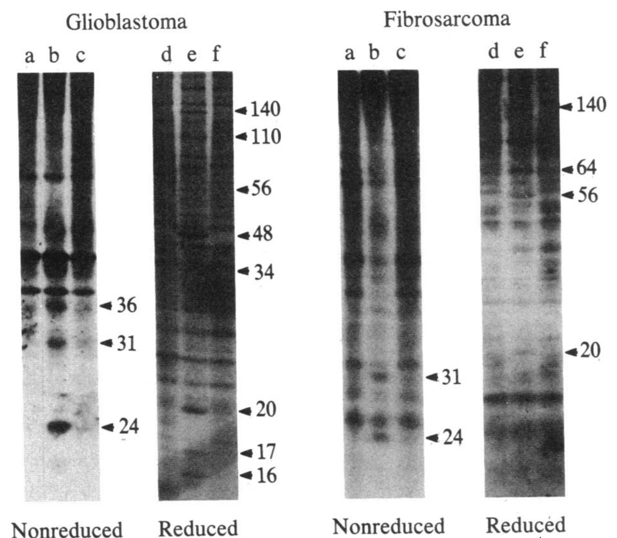

FIG. 1. Gel electrophoresis of proteins immunoprecipitated from lysates of metabolically labeled cells. Lanes a and d, incubation with normal rabbit serum; lanes b and e, incubation with PDGF antiserum; lanes c and f, incubation with PDGF antiserum mixed with excess PDGF (competition experiment).

Similar results were obtained with lysates of fibrosarcoma cells (Fig. 1). PDGF antiserum specifically immunoprecipitated polypeptides of 31 kDa and 24 kDa (lane b) under nonreducing conditions. These polypeptides were not immunoprecipitated by normal serum (lane a) and were competed out by the presence of excess PDGF (lane c). Under reducing conditions, PDGF antiserum specifically immunoprecipitated polypeptides of 140, 64, 56, and 20 kDa in the lysates of fibrosarcoma cells (lanes, d, e, and f).

Recognition of PDGF-Like Proteins in Conditioned Media. We investigated whether PDGF-like proteins of the metabolically labeled cells were released into the culture medium. The proteins were detected by direct immunoprecipitation using PDGF antiserum. The results are shown in Fig. 2. Glioblastoma cells released proteins binding to PDGF antiserum with apparent molecular masses of 16 kDa, 18 kDa, 31 kDa, and 36 kDa under nonreducing conditions (lane b). Under these conditions, the predominant immunoprecipitated protein was 31 kDa. These proteins were not immunoprecipitated by normal serum (lane a) or PDGF antiserum neutralized in the presence of excess PDGF (lane c). When the samples electrophoresed under reducing conditions, the predominant 31-kDa and the less prominent 36-kDa components disappeared and instead the major component exhibited a molecular weight of about 16 kDa that apparently represented the monomeric reduced chains of PDGF (lane b). Under reducing conditions it was also possible to detect 140-kDa and 120-kDa protein species. Similar high molecular weight proteins have been immunoprecipitated by PDGF antiserum

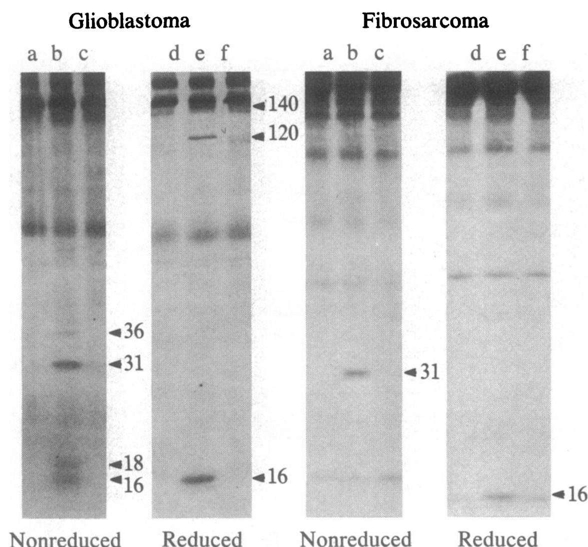

FIG. 2. Gel electrophoresis of proteins immunoprecipitated from concentrates of conditioned medium from labeled cells. Lanes a and d, incubation with normal rabbit serum; lanes b and e, incubation with PDGF antiserum; lanes c and f, incubation with PDGF antiserum mixed with excess PDGF (competition experiment).

from medium removed from cultures of human osteosarcoma cells (13).

Human fibrosarcoma cells also release proteins immunoprecipitated by PDGF antiserum (Fig. 2). Under nonreducing conditions there was only a single PDGF-like protein of 31 kDa that reacted with PDGF antiserum (lanes a–c). This protein was converted to a 16-kDa molecule under reducing conditions (lanes d–f). There was no visible band of 36 kDa in the fibrosarcoma media, although a trace amount not visible under the present conditions is possible.

Stimulation of DNA Synthesis by Released Proteins. Media derived from A172 and HT-1080 cells were assayed for their ability to stimulate DNA synthesis in quiescent BALB/c 3T3 cells. The results are summarized in Table 1. It can be seen that increasing volumes of concentrated medium resulted in increasing incorporation of radiolabeled thymidine into the DNA of BALB/c 3T3 cells. Stimulation of thymidine incorporation remained essentially unaltered even when the conditioned media were heated at 100°C for 5 min. However, conditioned medium treated with 2-mercaptoethanol lost its stimulatory activity. These properties, heat stability and inactivation by 2-mercaptoethanol, are identical to those described previously for human PDGF (20).

Expression of c-*sis* mRNA. The presence of PDGF-related polypeptides in A172 and HT-1080 cells prompted us to characterize the c-*sis* mRNA present in these cells. The presence of v-*sis*-related mRNA species has already been detected in these and in other human tumor cell lines (14). However, except in one case (21), the organization of this mRNA has not been studied in relation to the known human c-*sis* locus and to its intron–exon arrangement (10, 22). This characterization is relevant since different lines have been shown to produce different sizes of PDGF-related molecules. In the present studies, c-*sis* transcripts in A172 and HT-1080 cells were studied by isolating poly(A)$^+$ RNAs and analyzing them by RNA blotting (18) using the set of c-*sis*-specific probes illustrated in Fig. 3*B*. These probes have been selected as being representative of each of the known human c-*sis* exons defined either by their homology with v-*sis* (10) or by their presence in a cloned c-*sis* cDNA copy from a human tumor lymphoid cell line (21). Fig. 3*A* illustrates the results of these experiments. A 4.4-kb hybridization band is detectable in HT-1080 (lane 1) and A172 (lane 2) RNAs but not in the human peripheral blood lymphocyte poly(A)$^+$ RNA (lane 3) used as a negative control. The same band of virtually the same intensity was detectable with all the indicated probes (only one representative experiment is illustrated in

Table 1. Stimulation of [^{3}H]thymidine incorporation in Balb/c 3T3 cells by serum-free conditioned medium derived from cultured human fibrosarcoma and glioblastoma cells

Serum-free medium	Incorporation of [^{3}H]thymidine, cpm × 10^{-3}
Fibrosarcoma cells	
0 (control)	12
10 µl	47
25 µl	84
10 µl (heated at 100°C for 5 min)	44
25 µl (heated at 100°C for 5 min)	92
25 µl (reduced with 2-mercaptoethanol)	11
Glioblastoma cells	
0 (control)	14
10 µl	68
50 µl	139
10 µl (heated at 100°C for 5 min)	78
50 µl (heated at 100°C for 5 min)	140
10 µl (reduced with 2-mercaptoethanol)	16
50 µl (reduced with 2-mercaptoethanol)	20

Serum-free cell-conditioned medium containing <50 µg of protein per ml was concentrated 5- to 10-fold, dialyzed against 0.15 M NaCl, and tested for ability to stimulate incorporation of [^{3}H]thymidine into quiescent BALB/c 3T3 cells (17). Heat-treated cells were placed in boiling water for 5 min. Reduced samples were prepared by addition of 2-mercaptoethanol (5%, vol/vol) followed by incubation at room temperature for 1 hr. Mercaptoethanol-containing and control samples were dialyzed separately against 0.15 M NaCl.

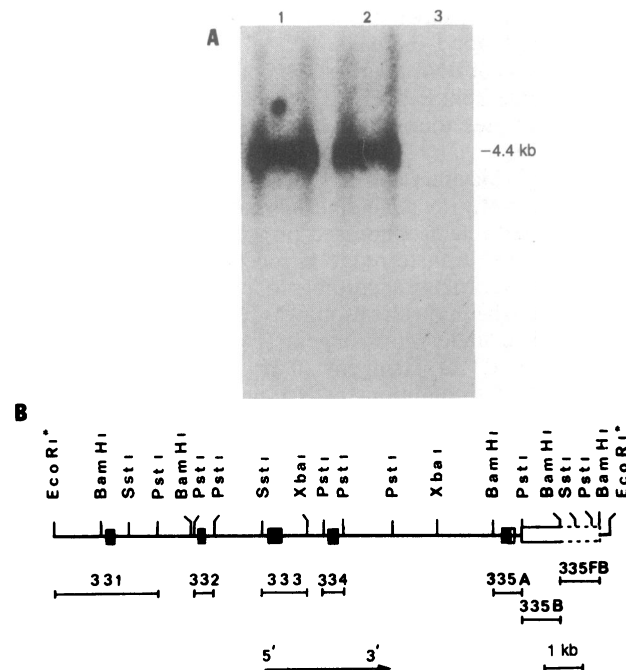

FIG. 3. c-*sis* mRNA in fibrosarcoma (HT-1080) and glioblastoma (A172) cells. (*A*) Hybridization of a DNA probe (shown in *B*) to poly(A)$^+$ RNA HT-1080 (lane 1), A172 (lane 2), and normal human peripheral blood lymphocytes (lane 3). (*B*) Restriction enzyme map of the human c-*sis* locus in the recombinant phage—L33. DNA fragments used as probes are indicated. The regions spanning the six known c-*sis* exons (19) are boxed. Solid boxes represent the translated regions, and open boxes represent the 3′ untranslated region (19). The broken line limiting the 3′ portion of the sixth exon refers to the still undetermined borders of this exon (19).

Fig. 3*A*). The intensity of this band remained unaltered using hybridization washing conditions which allowed the detection of only completely homologous sequences (see *Materials and Methods*). We believe that this 4.4-kb RNA corresponds to the 4.0–4.2 kb v-*sis*-related species previously reported (13, 14) since we detected the same band (i.e., 4.4 kb) using a v-*sis* probe (not shown). The slight difference in size is likely due to the different molecular weight markers (i.e., RNA versus DNA) used in the various studies. No hybridization was obtained when DNA fragments mapping between the ones indicated in Fig. 3*B* were used as probes.

Taken together, these results indicate that the 4.4-kb RNA species is likely to originate from the described c-*sis* locus and to contain the same genetic information predicted by nucleotide sequencing of this locus (10, 22) and of a corresponding cloned cDNA copy (21).

DISCUSSION

The present studies describe the synthesis and release of PDGF-like proteins by human glioblastoma (A172) and fibrosarcoma (HT-1080) cells whose normal counterparts are targets of PDGF action. Two major PDGF-like polypeptides of 31 kDa and 36 kDa were shown by NaDodSO$_4$/PAGE to be present in the lysates of A172 and HT-1080 cells under nonreducing conditions. These molecular masses are within the reported molecular mass range of unreduced PDGF (6, 7). These polypeptides were not present when the immunoprecipitates of the lysates were analyzed under reducing conditions, suggesting the presence of disulfide-linked dimers, which is consistent with the dimeric nature of PDGF. A 24-kDa polypeptide was also immunoprecipitated specifically by the PDGF antiserum from the lysates of both cell lines and was detectable by NaDodSO$_4$/PAGE only under nonreducing conditions. This 24-kDa polypeptide has previously been detected in the immunoprecipitates of lysates of SSV-transformed cells under nonreducing conditions (14). It was recognized by PDGF antiserum but not by antiserum raised against a synthetic polypeptide corresponding to the COOH-terminal region of the v-*sis* product (14). This suggests that the 24-kDa PDGF-like polypeptide was partially processed at the arginine and lysine-rich COOH-terminal region of PDGF. It is possible that the 24-kDa polypeptide recognized in the lysates of A172 and HT-1080 cells is similar to that observed in the SSV-transformed cell lysates. Recently, we have detected a similar 24-kDa polypeptide that is specifically immunoprecipitated with PDGF antiserum from lysates of human megakaryoblast-like cells (23).

Examination of the immunoprecipitates of the cell lysates by NaDodSO$_4$/PAGE under reducing conditions revealed the presence of polypeptides with molecular masses ranging from 16 kDa to 140 kDa that were specifically immunoprecipitated by PDGF antiserum. The 16-kDa–56-kDa components may represent precursor PDGF-like molecules and processed products. The nature of the higher molecular mass polypeptides (110 kDa, 140 kDa) is unknown. These molecular masses exceed the coding capacity of the c-*sis* precursor for PDGF-2 chain, which is about 30 kDa for the monomeric chain. It is conceivable that the 110-kDa and 140-kDa components represent higher molecular mass precursors of PDGF-1 chain, but evidence for the presence of such precursors in these cell lines is lacking. Another possibility is that the high molecular weight components represent complexes of PDGF-like polypeptides with other intracellular proteins that co-immunoprecipitate in the presence of PDGF antiserum. As reported recently by Graves *et al.* (13), high molecular weight components were detected with PDGF antiserum in the lysates of a human osteosarcoma cell line (U-2 OS).

The major PDGF-like polypeptide detected in conditioned medium from the glioblastoma and fibrosarcoma cultures had a molecular mass of about 31 kDa under nonreducing conditions (Fig. 2). After reduction, the 31-kDa component disappeared from both cell lines and was replaced by a 16-kDa component. These data suggest that the 31-kDa molecule detected in the unreduced state represents a disulfide-linked dimer that upon reduction is resolved into single 16-kDa polypeptide chains. This is consistent with the properties of human PDGF (20) and the PDGF-like polypeptide secreted by the SSV-transformed NRK cells (9). The latter was identified in culture medium from SSV-transformed cells as a 34-kDa polypeptide that upon reduction appeared as a 17-kDa monomer (9). Similar findings were reported by Graves *et al.* (13) for cultured human osteosarcoma cells. The 140-kDa and 120-kDa proteins detected in lysates of culture medium of A172 cells may represent the same components.

These findings are consistent with the PDGF-like activity exhibited by the conditioned medium of the glioblastoma and fibrosarcoma cell lines. As shown in Table 2, the ability of the medium to stimulate [^{3}H]thymidine uptake by 3T3 cells was abolished by reduction but not by heating at 100°C for 5 min. These properties were previously demonstrated for human PDGF (6) and for the activity of medium derived from SSV-transformed NRK cells (9). These data are also consistent with the recent findings of Nister *et al.* (12), who demonstrated PDGF-like activity in the culture medium from human glioblastoma cells. Purified PDGF-like polypeptides obtained from the culture medium of these cells had a molecular mass of 31 kDa under nonreducing conditions (12), a mass that is identical to the immunoprecipitated 31-kDa component demonstrated by us to be present in the culture medium from both the glioblastoma and fibrosarcoma cells.

The production of PDGF-like mitogen by the A172 and HT-1080 cells appears to result from activation of the c-*sis* oncogene. The present results provide a preliminary characterization of the c-*sis* transcripts present in these cells (Fig. 3) confirming and extending previous results on the presence of v-*sis*-related RNA in some human mesenchymal tumor cell lines (13, 14). Hybridization to probes specific for the coding region of the human c-*sis* gene under stringent hybridization conditions suggests that the mRNA species detected in the A172 and HT-1080 cells are likely to be encoded by the described c-*sis* locus and not by related genes. Within the limits of accuracy of hybridization analysis the mRNA species detected in both cell lines appear to contain the same exons as those present in a cDNA clone from a human lymphoma cell line (21) and possibly in the v-*sis*-related mRNA of similar size reported in a human osteosarcoma cell line (13). However, by our analysis we cannot exclude small differences in the initiation or termination sites of RNA synthesis, RNA splicing, or somatic mutations in one or both of the alleles. These differences or different post-translational processing of the primary protein product may account for the differences in molecular masses of the PDGF-like proteins detectable in the cell lines studied here and in other PDGF-producing cell lines (ref. 13; unpublished data).

Qualitative or quantitative alterations in the expression of the *sis* oncogene in these cells may lead to abnormal levels of endogenous PDGF production and/or production of a structurally altered PDGF. These alterations may be involved in the process leading these cells toward neoplastic transformation.

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