JOURNAL OF VIROLOGY, Aug. 1986, p. 292–300
0022-538X/86/080292-09$02.00/0
Copyright © 1986, American Society for Microbiology
Vol. 59, No. 2

Deletions in the N-Terminal Coding Region of the v-*sis* Gene: Determination of the Minimal Transforming Region

MONICA K. SAUER,[1] MARK HANNINK,[2] AND DANIEL J. DONOGHUE[2]*

Departments of Chemistry[2] and Biology,[1] University of California, San Diego, La Jolla, California 92093

Received 20 December 1985/Accepted 21 April 1986

The gene product of the v-*sis* gene is closely related to the B chain of platelet-derived growth factor (PDGF). However, v-*sis* also encodes additional amino acids at its N and C termini, which are not represented in the sequence data of PDGF. We have constructed a series of N-terminal deletion mutants in the v-*sis* gene to define the minimum region required for transformation. These mutants were assayed for biological activity by using retroviral expression vectors which donate a signal sequence, required for translocation across the rough endoplasmic reticulum, to the mutant gene product. The minimal transforming region of the v-*sis* gene product defined by this analysis has 15 residues missing at the N terminus when compared with the PDGF-B chain. There are only two residues separating the closest transforming and nontransforming gene products. Mutant gene products lacking both the basic dipeptide processing site and the N-linked glycosylation site were found to be biologically active, indicating the dispensability of those processing steps. These results delimit the minimal transforming region of the v-*sis* gene product to residues 127 through 214, a total of 21 residues smaller than the PDGF-B chain.

The v-*sis* oncogene is the transforming gene of simian sarcoma virus, an acutely transforming retrovirus which was originally isolated from a woolly monkey sarcoma (4, 30). It arose by recombination between the envelope (*env*) gene of simian sarcoma-associated virus and the cellular gene c-*sis*, resulting in the formation of a fused *env-sis* open reading frame (4). The open reading frame consists of 813 nucleotides, potentially encoding a protein of 271 amino acids. There are six in-frame ATG codons in the open reading frame, the first three being within the *env* region, and it has previously been shown that the first ATG is used in the synthesis of the nascent gene product (11). Between the second and third methionines there is a signal sequence. The signal peptide directs the cotranslational translocation of the nascent protein across the membrane of the rough endoplasmic reticulum and is then cleaved off within the rough endoplasmic reticulum, removing approximately 4 kilodaltons (kDa) from the N terminus of the protein (11, 24). The v-*sis* gene also encodes an N-linked glycosylation site consensus sequence, Asn-(Met)-Thr residues 93 to 95. (Note that amino acid residues are numbered from the N-terminal methionine of the nascent v-*sis* gene product.) Addition of a complex polysaccharide to Asn 93 occurs cotranslationally and, in conjunction with signal sequence cleavage, results in the formation of a glycoprotein of 28 (25) or 32 kDa (11). The glycoprotein, commonly termed $p28^{sis}$, dimerizes in the rough endoplasmic reticulum. During transport through the Golgi complex to the plasma membrane it is proteolytically processed at a basic dipeptide, Lys-Arg residues 110 and 111, to yield a 42-kDa dimer composed of two 20-kDa subunits (25).

The cellular homolog, c-*sis*, is the gene for the B chain of platelet-derived growth factor (PDGF) (5, 18, 32). PDGF is a potent mitogen for cells of mesenchymal origin, including fibroblasts, glial cells, and smooth-muscle cells (27). It is normally synthesized in megakaryocytes and stored in the alpha granules of platelets (19). Binding of PDGF to the PDGF receptor results in the activation of tyrosine protein kinase activity (22) and autophosphorylation of the receptor. PDGF is a dimeric protein of 27 to 35 kDa and is composed of 12- to 18-kDa subunits (17, 32). Peptide sequencing suggests that PDGF consists of two distinct polypeptide chains, A and B. The amino acid sequence of the PDGF-B chain is homologous to residues 112 to 220 of the predicted v-*sis* protein (17, 18, 32).

Both the intracellular and the secreted forms of the v-*sis* protein have been shown to have mitogenic activity for NIH 3T3 cells (3, 6, 15, 23), and the secreted form stimulates autophosphorylation of the PDGF receptor (6, 15, 23). Dimerization is required for the mitogenic activity of both PDGF and the v-*sis* protein (23). The v-*sis* gene encodes 10 cysteine residues, 9 of which are also present in the predicted c-*sis* gene product. Of these, eight cysteines are within the region which is homologous to the PDGF-B chain. There are 51 residues at the C terminus and 111 residues at the N terminus of the v-*sis* gene product which are not present in the PDGF B chain. Previous work indicates that deletion of up to 57 amino acids at the C terminus of the v-*sis* gene product has no effect on biological activity (12). Other reports have demonstrated a requirement for a signal sequence at the N terminus of the v-*sis* gene product (11, 20). The role of the N-terminal region beyond the signal sequence, though, has not been studied extensively. In one report, a deletion mutant with the signal sequence region fused directly to the PDGF-B homologous region was fully transforming (20), but no mutants with deletions extending into the PDGF-B homologous region were studied.

In the present study we have delimited the minimal transforming region of v-*sis*. We have constructed deletions in the N-terminal coding region of the v-*sis* gene and assayed the mutant genes for their biological activities. These results, in conjunction with our earlier analysis of the C-terminal coding region of v-*sis*, delimit the minimal transforming region of the v-*sis* gene product to residues 127 through 214.

* Corresponding author.

TABLE 1. v-*sis* mutants and corresponding expression clones

Mutant[a]	M-MuLV-derived expression clones		SV40-derived expression clones[b]	
	Vector used	Plasmid	Vector used	Plasmid
v-*sis*^{wt}		pDD145	pJC119	pMH29
v-*sis*^{92}	pDD184	pDD188		
v-*sis*^{101}	pDD185	pDD195		
v-*sis*^{102}	pDD184	pDD191	pRB34	pDD205
v-*sis*^{109}	pDD185	pDD199		
v-*sis*^{111}	pDD184	pDD187		
v-*sis*^{112}	pDD184	pDD189		
v-*sis*^{114}	pDD184	pDD193		
v-*sis*^{120}	pDD185	pDD196	pRB34	pDD206
v-*sis*^{121}	pDD186	pDD197	pRB34	pDD208
v-*sis*^{127}	pDD185	pDD194	pRB34	pDD210
v-*sis*^{129}	pDD186	pDD198	pRB34	pDD209
v-*sis*^{132}	pDD186	pDD192	pRB34	pDD211
v-*sis*$^{127/214}$	pDD185	pMS059	pRB34	pMS060

[a] The number indicates the first remaining v-*sis*-encoded residue in the deleted gene product.

[b] Note that not all mutant genes were inserted into SV40-derived clones.

This represents 15 residues fewer at the N terminus and 6 residues fewer at the C terminus than the homologous PDGF-B chain.

MATERIALS AND METHODS

Construction of deletion mutants. The v-*sis* gene which served as the starting clone for BAL 31 mutagenesis was a deleted v-*sis* fragment contained in the plasmid pDD140, in which nucleotides 1 through 271 numbered from the first ATG codon are deleted. This plasmid has the v-*sis* gene flanked by *Xho*I sites and a unique *Sal*I site immediately upstream of the v-*sis* gene. The pDD140 plasmid was linearized by digestion at the *Sal*I site and then treated with Klenow fragment to fill in the 5′ overhang. The DNA was treated with BAL 31 exonuclease at 0.4 U/μg of DNA for times between 20 and 100 s. Following a second treatment of the molecules with Klenow fragment, *Xho*I linkers (CCTCGAGG) were ligated to the DNA, yielding *sis* genes deleted to various distances and flanked by *Xho*I sites. The exact extent of each of the deletions was determined by Maxam-Gilbert sequencing (21). A total of 12 deletion mutants were constructed in this way, with deletion endpoints varying between nucleotides 273 and 392 of the v-*sis* open reading frame (the numbers represent the first remaining v-*sis* nucleotide) (see Fig. 1).

Construction of M-MuLV-derived expression vectors. To allow the expression of the deleted v-*sis* genes in NIH 3T3 cells, the genes were inserted into retroviral expression vectors. However, since the genes do not encode their own signal sequence, which is known to be required for transformation, special vectors were constructed. For details of the construction see Fig. 2. The proviral clone of Moloney murine leukemia virus (M-MuLV), p836 (14), served as the parental plasmid. The plasmid pMS020 was derived from the M-MuLV derivative pDD102 (the construction of which is described by Bold and Donoghue [2]) by the insertion of a unique *Cla*I site immediately downstream of the unique *Xho*I site which serves as the site of insertion for genes to be expressed in the pDD102 retroviral vector. It should be noted that there is a second *Cla*I site in M-MuLV at nucleotide 4980, but this site is methylated in DNA grown in *dam*$^+$ strains of *Escherichia coli* and is resistant to cleavage by *Cla*I. The retroviral vector pMS020 has deletions in the *gag* and *pol* genes and has most of the *env* gene deleted. It does, however, retain the 5′ splice donor site and the 3′ splice acceptor site for the *env* gene. Inserted genes must provide their own ATG codon for initiation of translation.

A *Xho*I-to-*Cla*I fragment encompassing the v-*sis* signal sequence coding region was inserted into pMS020 to yield pDD180. To do this we used a subclone of the v-*sis* gene, pDD179, in which the 5′ *Sal*I site is converted to a *Xho*I site and the *Sst*I site at nucleotide 172 is converted to a *Cla*I site. The *Xho*I-to-*Cla*I fragment of pDD179 was inserted into pMS020 between the unique *Xho*I and *Cla*I sites. The *Xho*I site was destroyed by digestion with *Xho*I, followed by treatment with Klenow fragment and religation. Next, the *Cla*I site at the 3′ end of the signal sequence region was converted to a unique *Xho*I site. To be able to insert deleted genes beginning in all three reading frames, the site conversion was done with *Xho*I linkers of 3 sizes: an 8-mer (CCTCGAGG), a 10-mer (GCCTCGAGGC), and a 12-mer (GCACTCGAGTGC). These reconstructions yielded pDD184, pDD185, and pDD186, respectively (see Fig. 2). The nucleotide sequence across the junctions of all three retroviral vectors was confirmed by Maxam-Gilbert sequencing (21) (data not shown).

The deleted v-*sis* genes were inserted into the appropriate expression vectors as *Xho*I-to-*Xho*I fragments. Colony hybridizations (9) were used to detect the presence of inserts, and restriction mapping was used to confirm the correct orientation of the insert for expression. Some of the plasmids, including pDD188, pDD189, pDD191, and pDD193, were sequenced from a unique restriction site within the deleted v-*sis* gene across the junction to verify that the correct reading frame was maintained from the signal sequence coding region into the v-*sis* coding region. These expression vectors, with the *sis* signal sequence region fused to various deleted v-*sis* genes, were used to transfect NIH 3T3 cells. The expression vectors used for each mutant gene and the resultant plasmids are given in Table 1.

Biological assays. The protocol used for the focus assay was essentially as described previously (1, 8). All cells were grown in Dulbecco modified Eagle medium with 10% calf serum. A 1-μg portion of each DNA, along with 1 μg of DNA of a replication-competent clone of M-MuLV (15) and 20 μg of sheared calf thymus DNA, was transfected as a DNA-calcium phosphate coprecipitate onto a semiconfluent monolayer of NIH 3T3 cells grown in a 60-mm dish. The calcium phosphate coprecipitate was formed by adding the DNA in 400 μl of 0.25 M $CaCl_2$ to 400 μl of 2× HBS (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 280 mM NaCl, 10 mM sodium phosphate [pH 7.0]) and allowing it to precipitate for 45 min. The coprecipitate was added directly to the cells in medium and left for approximately 12 to 18 h. The cells were then trypsinized and split onto four 10-cm dishes. They were refed at 3-day intervals and scored for the appearance of foci at 7 days posttransfection.

The conditioned medium was collected when the assays were scored and titrated onto fresh monolayers of NIH 3T3 cells grown in 60-mm dishes. For the virus titers, 200 μl of the appropriate dilution of conditioned medium, containing 4 μg of polybrene per ml, was allowed to adsorb to the cells at 37°C for 1 h. The cells were then washed and refed and were scored for the presence of foci after 6 days to calculate the titer of rescuable focus-forming virus.

Construction of a clone with the minimal transforming region. A subclone of the v-*sis* gene was constructed which

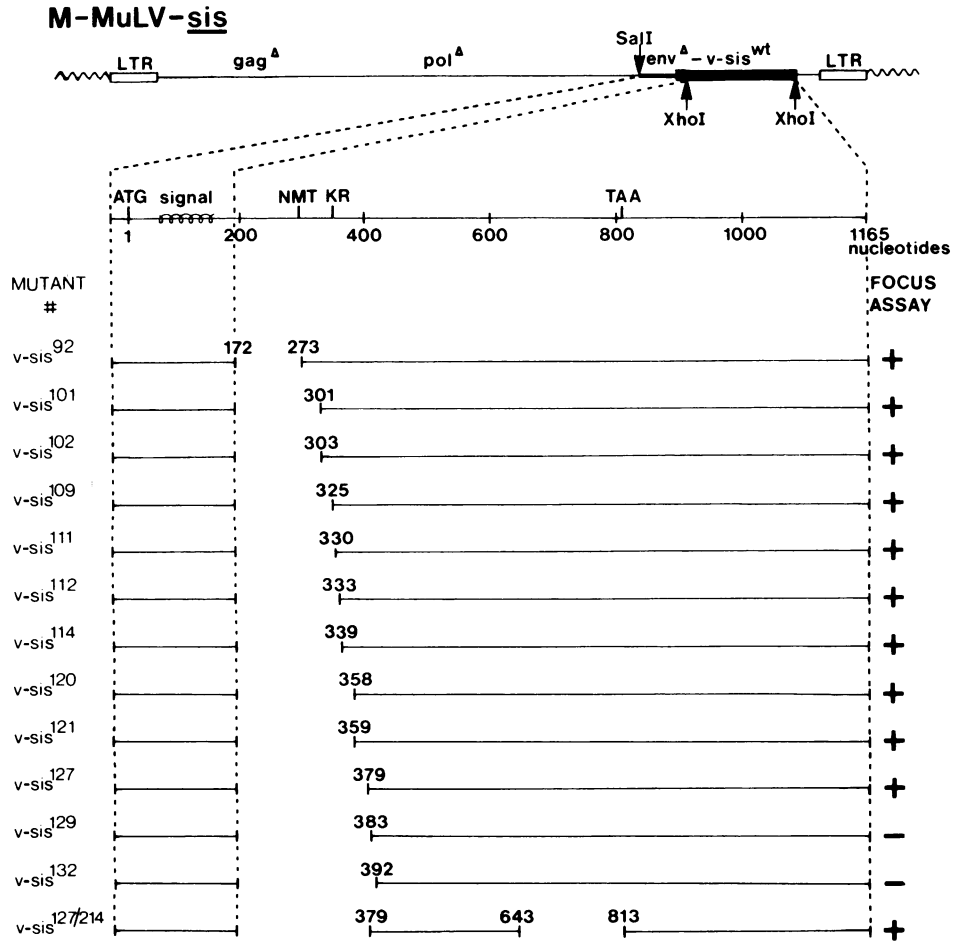

FIG. 1. N-terminal deletion analysis of the v-*sis* gene. The structure of a M-MuLV-derived expression vector with the complete v-*sis*^{wt} gene inserted is displayed at the top of the figure. The *Sal*I and *Xho*I sites bracketing the *sis* signal sequence and the v-*sis* gene are indicated. An expanded map of the nucleotide sequence is shown below, with the positions of the following important landmarks noted: the first ATG; the signal sequence coding region; the region encoding the N-linked glycosylation site, Asn-Met-Thr (NMT); the region encoding the proteolytic cleavage site Lys-Arg (KR); and the translation terminator codon TAA. Nucleotides are numbered from the first ATG codon of the *env-sis* open reading frame and can be converted to the nucleotide numbers of the simian sarcoma virus sequence (4) by adding 3,657. The series of 13 v-*sis* deletion mutants was constructed as described in the text. The nucleotide position at each endpoint is indicated, as determined by Maxam and Gilbert sequencing (21), with the number referring to the first remaining nucleotide of v-*sis* after the *Xho*I linker. In the case of v-*sis*$^{127/214}$, the nucleotide position of the C-terminal deletion endpoint was determined previously (12) and refers to the last remaining nucleotide of v-*sis*. The biological activity of each of the deletion mutants was assayed by its ability to induce foci in NIH 3T3 cells and by the titer of rescuable focus-forming virus. Symbols: +, minimum value of 1.4×10^4 FFU/pmol of transfected DNA; −, less than 1.0×10^1 FFU/pmol. In all cases in which a positive value was obtained in the focus assay the titer of rescuable virus in the conditioned media was at least 2.0×10^5 FFU/ml, and whenever a negative value was obtained the titer was below 5.0 FFU/ml.

combined the most extensive transforming deletions of the N-terminal and C-terminal coding regions. A fragment from pLH11, encoding the N-terminal region of v-*sis*^{127}, was recombined in vitro with a fragment encoding the C-terminal region of v-*sis*^{214} from plasmid pMS008. This yielded a subclone of the v-*sis* gene, pMS058, encoding residues 127 through 214, i.e., with nucleotides 379 through 642. The translation termination codon TAA is present in frame at the end of the mutant gene. The v-*sis* gene is flanked by *Xho*I sites and was inserted as an *Xho*I-to-*Xho*I fragment into the retroviral expression vector pDD185, yielding pMS059 (Table 1).

Transient expression in Cos-1 cells. To allow the expression of the deleted v-*sis* genes in Cos-1 cells, they were inserted into a simian virus 40 (SV40)-derived expression vector. The parental plasmid was pJC119 (29). This vector has a deleted copy of the SV40 genome, with a unique *Xho*I site replacing most of the VP1 open reading frame. It retains, however, the late promoter and the 5′ and 3′ splice sites which are used for the expression of genes inserted at the *Xho*I site. Plasmid pRB34 was derived from pJC119 by conversion of the unique *Xho*I site into a unique *Xba*I site. Seven of the deleted v-*sis* genes were excised from the M-MuLV expression vectors as *Xba*I-to-*Xba*I fragments. These fragments encompassed both the signal sequence coding region, which was donated by the retroviral vector, and the deleted v-*sis* gene. The *Xba*I-to-*Xba*I fragments were inserted into the unique *Xba*I site of pRB34. Colony hybridizations and restriction mapping were again used to verify the presence of the inserted genes in the correct orientation. The plasmids resulting from the insertion of the deleted v-*sis* genes into the SV40-derived expression vector are given in Table 1. The v-*sis*^{wt} gene was inserted into pJC119 as an *Xho*I-to-*Xho*I fragment.

Immunoprecipitations. The SV40 expression plasmid

TABLE 2. Biological activities of v-*sis* deletion mutants

Mutant[a]	Deleted nucleotides[b]	Deleted residues[b]	Junction residues[c]	Mol wt (kDa)[d]	Focus assay (FFU/pmol)[e]
v-*sis*^{wt}				26.4	6.7×10^4
v-*sis*^{92}	173–272	58–91	EASPRGL	23.1	6.7×10^4
v-*sis*^{101}	173–300	58–100	EASASRG	22.1	1.4×10^4
v-*sis*^{102}	173–302	58–101	EASPRGE	22.1	1.4×10^5
v-*sis*^{109}	173–324	58–108	EASASRG	21.3	4.9×10^4
v-*sis*^{111}	173–329	58–110	EASPRGR	21.1	1.9×10^4
v-*sis*^{112}	173–332	58–111	EASPRGS	20.9	8.6×10^4
v-*sis*^{114}	173–338	58–113	EASPRGG	20.7	9.6×10^4
v-*sis*^{120}	173–357	58–119	EASASRE	20.2	2.9×10^4
v-*sis*^{121}	173–358	58–120	EASALEEP	20.2	7.7×10^4
v-*sis*^{127}	173–378	58–126	EASASRC	19.5	2.4×10^4
v-*sis*^{129}	173–382	58–128	EASALEET	19.4	$<1.0 \times 10^1$
v-*sis*^{132}	173–391	58–131	EASALEAE	19.0	$<1.0 \times 10^1$
v-*sis*$^{127/214}$	173–378, 643–813	58–126, 215–271	EASASRC	13.6	2.4×10^4

[a] Number refers to the first remaining v-*sis* encoded residue from the deleted gene.

[b] The v-*sis* signal sequence region inserted into the M-MuLV-derived expression vectors terminates at nucleotide 172, thus encoding amino acid residues 1 through 57. The deletion endpoints refer to the first nucleotide of the deleted v-*sis* gene or to the first residue encoded by the deleted v-*sis* gene. Nucleotides are numbered from the first ATG codon of the *env-sis* open reading frame. The numbers can be converted to the numbers of the published parental virus (simian sarcoma virus) sequence by adding 3,657 (4). Amino acid residues are numbered from the first methionine residue in the full-length v-*sis* gene product.

[c] Owing to the linkers used in the construction of the M-MuLV-derived expression vectors, the junctions between the signal sequence region and the remainder of the deleted v-*sis* gene product contain five or six extra residues, which differ slightly depending on which of the three expression vectors was used. The junction residues are given in the single-letter code. The underlined residues are those encoded by v-*sis*.

[d] Predicted molecular weight, in kilodaltons, of the mutant gene products after signal sequence cleavage and without N-linked glycosylation or proteolytic cleavage.

[e] The biological activity was assayed as described in Materials and Methods.

DNAs were transfected into Cos-1 cells by the DEAE-dextran method. Semiconfluent monolayers of Cos-1 cells grown in 60-mm dishes were washed twice with Tris-saline (10 mM Tris [pH 7.4], 150 mM NaCl), a solution of Tris-saline containing 5 μg of the DNA and 500 μg of DEAE-dextran (molecular weight, 2.0×10^6) per ml was added to the cells, and the mixture was incubated at 37°C for 10 min. After the DNA solution was removed, the cells were shocked with a solution of 10% dimethyl sulfoxide (DMSO) in Tris-saline at room temperature for 1 min. The cells were then washed twice with Tris-saline and refed with fresh medium containing 100 μM chloroquine for 2 h. The cells were refed with fresh medium and allowed to grow for 48 h to begin expressing the transfected genes. They were then metabolically labeled with 100 μCi each of [^{35}S]cysteine and [^{35}S]methionine in Dulbecco modified Eagle medium minus cysteine and methionine for 2 h.

For the immunoprecipitation of intracellular proteins, the cells were washed with versene and then the cell lysates were prepared in 600 μl of RIPA buffer (10 mM sodium phosphate [pH 7.0], 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], and 1% Trasylol, Mobay Chemical Corp.). Cellular debris was pelleted before the supernatant was aliquoted for immunoprecipitation. The antiserum used was raised against bacterially synthesized p28$^{v\text{-}sis}$. Immunoprecipitations were done as described previously (16), with *Staphylococcus* aureus to collect the immunoglobulin G. The immunoprecipitates were suspended in sample buffer (50 mM Tris [pH 6.8], 2% SDS, 20% 2-mercaptoethanol, 10% glycerol), boiled for 2 min, and clarified by pelleting before electrophoresis on 15% SDS-polyacrylamide gels. For the samples which were analyzed under nonreducing conditions, the immunoprecipitates were suspended in sample buffer without 2-mercaptoethanol. For the immunoprecipitation of secreted proteins, the conditioned medium was collected from the labeled cells. The medium was frozen and lyophilized prior to immunoprecipitation. In experiments to assay for the effect of tunicamycin, tunicamycin was present at 4 μg/ml for 2 h prior to labeling and throughout the labeling period. The immunoprecipitations were then performed in the same manner as described above.

RESULTS

N-terminal deletions of v-*sis*. It is known that the v-*sis*^{wt} gene product is homologous to the PDGF-B chain (5, 18, 32). More precisely, the protein encoded by v-*sis* potentially contains 271 amino acids, of which residues 112 through 220 are homologous to the PDGF-B chain. (Note that numbering of the amino acids begins with the first methionine residue of the nascent v-*sis*^{wt} gene product.) The first 52 residues are encoded by the virally derived *env* sequences and include a signal sequence which is required for translocation of the nascent protein across the rough endoplasmic reticulum. Earlier studies involving the use of deletion analysis and site-specific mutations within the signal sequence have shown that the N-terminal signal sequence is required for transformation by v-*sis* (10, 11, 20). However, to delimit the region of the v-*sis* gene beyond the signal sequence which is necessary for transformation, we constructed deletions which extend into the N-terminal portion of the PDGF-homologous domain.

N-terminal deletion mutants of the v-*sis* gene were constructed by BAL 31 mutagenesis. A subclone of the v-*sis* gene, pDD140, was linearized by digestion at a unique *Sal*I site just upstream of the v-*sis* gene and then treated with BAL 31 exonuclease. After treatment with Klenow fragment, *Xho*I linkers were ligated into the molecules to allow for the insertion of the v-*sis* mutant genes, as *Xho*I-to-*Xho*I fragments, into the expression vectors described below. The endpoints of the deletions were determined by nucleotide sequencing and are presented in Fig. 1 and Table 2. In this way, 12 deletion mutants were constructed, terminating at nucleotides between 273 (v-*sis*^{92}) and 392 (v-*sis*^{132}) (Fig. 1). The mutants were named with regard to the number of the first amino acid residue encoded by the v-*sis* gene. The corresponding plasmid numbers are given in Table 1.

Construction of retroviral expression vectors. To express the mutant genes in eucaryotic cells, we constructed retroviral vectors in which the genes are substituted in place of the *env* gene of M-MuLV. The vectors contain 5′ and 3′ long terminal repeats as well as the 5′ and 3′ splice sites of the *env* gene. Since it had previously been shown that a signal sequence is required for a biologically active v-*sis* gene product, we constructed a set of retroviral expression vectors containing the v-*sis* signal sequence coding region (Fig. 2). This was done by inserting a fragment encoding the signal sequence into a M-MuLV derived vector, pMS020. A unique restriction enzyme site was introduced at the end of the signal sequence coding region to allow for insertion of the deleted genes. To accommodate deleted genes beginning in any of the three reading frames, three *Xho*I linkers of different lengths were used, resulting in the construction of

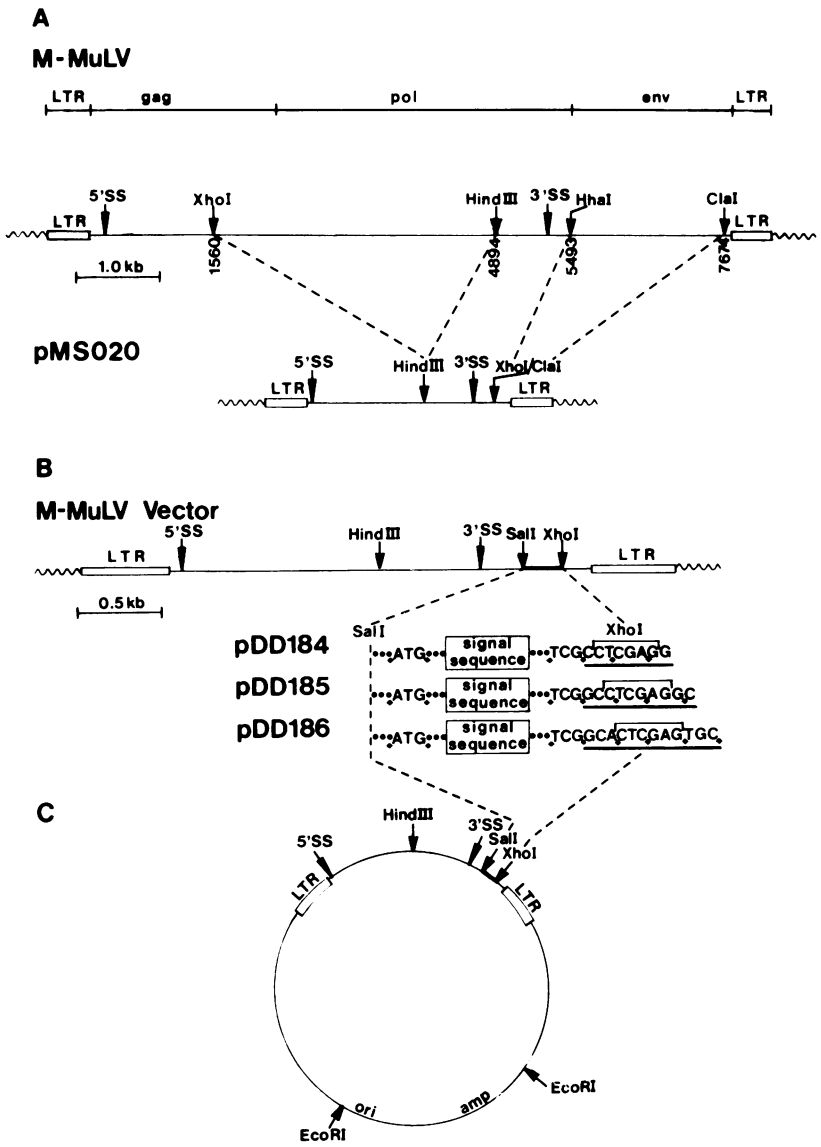

FIG. 2. Construction of retroviral expression vectors. (A) Genome of M-MuLV, with the splice sites and the retroviral genes indicated. The restriction enzyme sites used in the construction of the expression vectors are also shown, with their corresponding nucleotide positions. pMS020 is derived from pDD102, the construction of which is detailed by Bold and Donoghue (2). In pMS020 a unique *Cla*I site has been inserted into the vector adjacent to the unique *Xho*I site. This allows the directed insertion of *Xho*I-to-*Cla*I fragments into the vector. This *Cla*I site is not the same one as is present in the original M-MuLV sequence. (B) M-MuLV-derived expression vectors used in this study. Details of their construction are given in Materials and Methods. A fragment encompassing the v-*sis* signal sequence-coding region was inserted into pMS020 between the *Xho*I and the *Cla*I sites, and then the *Xho*I site was removed and the *Cla*I site was converted to a new, unique *Xho*I site. The indicated *Sal*I site is within the signal sequence fragment. In pDD184 an 8-mer *Xho*I linker was ligated into the vector after the signal sequence region, in pDD185 a 10-mer *Xho*I linker was ligated into the vector, and in pDD186 a 12-mer *Xho*I linker was used. This allowed the insertion of deleted v-*sis* genes beginning in any of the three reading frames. (C) Entire plasmids with the splice sites, long terminal repeats, and important restriction enzyme sites denoted. All three plasmids are about 16 kilobases in size.

the three retroviral expression vectors pDD184, pDD185, and pDD186. These manipulations result in the addition of codons for five or six new residues before the first codon donated by the deleted v-*sis* genes. The codons are shown in Fig. 2, and the residues across the junctions of the deletion mutant proteins are given in Table 2.

Biological assays. The deletion mutants of the v-*sis* gene were assayed for their ability to induce focus formation in NIH 3T3 cells. The M-MuLV-derived expression vectors provide for the expression of inserted genes by using the *env* transcription pathway. The deleted v-*sis* genes were inserted into the retroviral vectors and transfected into NIH 3T3 cells by the DNA-calcium phosphate coprecipitate method to test the effect of each deletion on the transforming activity (1). Since the expression vectors are defective in both their *gag* and *pol* genes and have had their *env* gene replaced, a replication-competent clone of M-MuLV (14) was cotransfected to allow for viral spread and virus rescue. The results of the focus assays are shown in Fig. 1. Biological activities of up to 6.7×10^4 FFU/pmol of transfected DNA were obtained from transfections with the v-sis^{wt} gene (Table 2). Mutants encoding gene products with the *sis* signal sequence

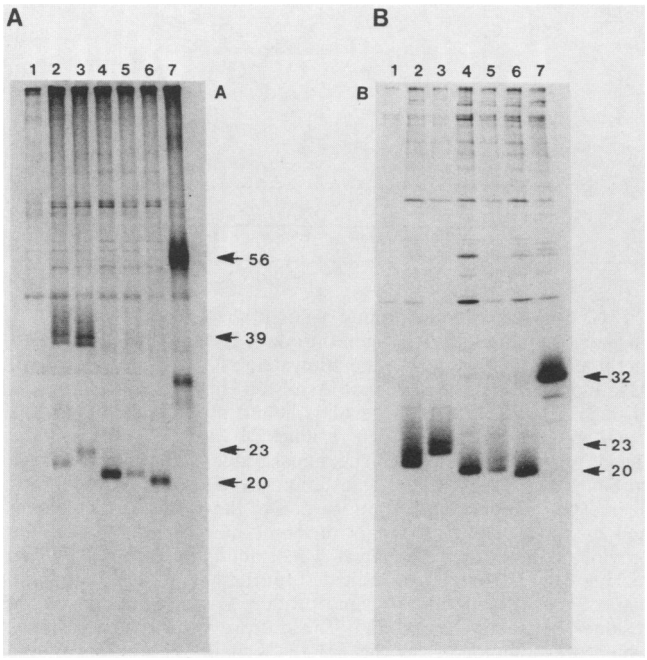

FIG. 3. Immunoprecipitation of mutant v-*sis* gene products with anti-p28^{sis} serum. Cos-1 cells were transfected with pJC119 alone (lane 1), or with the v-*sis*^{120}, v-*sis*^{121}, v-*sis*^{129}, v-*sis*^{127}, v-*sis*^{132}, or v-*sis*^{wt} genes (lanes 2, 3, 4, 5, 6, and 7, respectively), inserted into the SV40 expression vector as described in text. (A) Samples analyzed under nonreducing conditions; (B) identical samples analyzed under reducing conditions. In panel A the arrows indicate the dimeric form of the wild-type v-*sis* protein at 56 kDa, the dimeric form of the three transforming v-*sis* mutant gene products at approximately 39 kDa, and the monomeric forms of the mutant gene products between 23 and 20 kDa. In panel B the arrows indicate the v-*sis*^{wt} protein at 32 kDa and the mutant v-*sis* gene products ranging between 23 and 20 kDa.

fused to residues up to and including residue 127 had biological activities comparable to that of the wild-type gene product. In contrast, mutants encoding gene products with the signal sequence fused to residue 129 or beyond had no detectable biological activity. This sharp decrease in biological activity between v-*sis*^{127} and v-*sis*^{129} allows one to place the N-terminal limit of the transforming region of the v-*sis* gene product, when assayed with the v-*sis* signal sequence, between amino acid residues 127 and 129. It should be noted here that the PDGF-B chain begins with the residue corresponding to residue 112 of the nascent v-*sis*^{wt} protein. Thus it has 15 residues present at the N terminus which are not present in the smallest transforming v-*sis* gene product described here.

We previously described deletions in the C-terminal coding region of v-*sis* which located the C terminus of the minimal transforming region to between residues 204 and 214 (12). The v-*sis*$^{127/214}$ gene was constructed by combining the two genes which delimit the N terminus and the C terminus of the minimal transforming region (Fig. 1). The 5′ end of this gene was donated by v-*sis*^{127}, while the 3′ end was from v-*sis*^{214}. The v-*sis*^{214} gene was previously shown to be the smallest transforming C-terminal deletion mutant gene. The v-*sis*$^{127/214}$ gene extends from nucleotides 379 to 642, thereby encoding residues 127 to 214. The v-*sis*$^{127/214}$ gene was assayed in one of the M-MuLV-derived expression vectors described above (Table 1) and was found to have a biological activity comparable to that of v-*sis*^{wt} (Table 2). Therefore, the minimal transforming region of v-*sis*, when assayed with a signal sequence, can be defined as extending from residue 127 to residue 214. This is considerably smaller than the PDGF-B chain, which corresponds to residues 112 through 220.

As mentioned above, the insertion of the deleted genes into the three different expression vectors results in the formation of different junctions encoding five or six extra amino acids (Table 2). However, these extra amino acids had no effect on transformation, since deletion mutants retaining the same *sis*-encoded amino acids but with different amino acids across their junctions had the same transformation capabilities. Also, the biological activities of the v-*sis* deletion mutants correlated only with the extents of the deletions.

Transient protein expression and dimerization. To study the proteins encoded by the mutant v-*sis* genes, expression assays in Cos-1 cells were performed. Transient expression from SV40 promoters in Cos-1 cells allows higher levels of the desired gene product to be made than are possible in NIH 3T3 cells. Although it is possible to detect expression of mutant v-*sis* gene products in NIH 3T3 cells, the low level of expression precludes their routine use for the experiments described below. In previous studies, including our earlier work with v-*sis*, Cos-1 cells were shown to proteolytically process, glycosylate, and secrete certain proteins properly (7, 11, 26, 29, 31). In experiments done with these deletion mutant genes, a SV40-derived expression vector constructed from pJC119 was used. pJC119 has a deleted copy of the

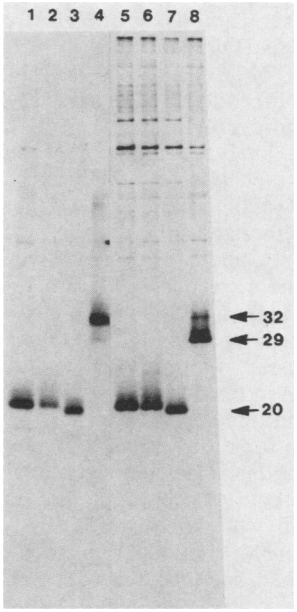

FIG. 4. Immunoprecipitations of v-*sis* proteins from cells with and without tunicamycin treatment. Cos-1 cells were transfected with the v-*sis*^{129} gene (lanes 1 and 5), the v-*sis*^{127} gene (lanes 2 and 6), the v-*sis*^{132} gene (lanes 3 and 7) or the v-*sis*^{wt} gene (lanes 4 and 8), and cell lysates were immunoprecipitated with anti-p28^{sis} serum. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions. Samples in lanes 1 through 4 were from cells which were not treated with tunicamycin, while those in lanes 5 through 7 were from tunicamycin-treated cells. The arrows indicate the glycosylated form of the v-*sis*^{wt} protein at 32 kDa, the unglycosylated form of the wild-type protein at 29 kDa, and the unglycosylated forms of the mutant v-*sis* gene products at approximately 20 kD.

SV40 genome inserted as a *Bam*HI fragment in a pBR322 derivative (29). Most of the VP1 open reading frame, including the first ATG, has been deleted and replaced by a unique *Xho*I site. However, the late promoter and the 5′ and 3′ splice sites remain. Genes inserted at the unique *Xho*I site are expressed by using the late-transcription pathway, with the vector providing for initiation, splicing, and polyadenylation. To insert the v-*sis* signal sequence, donated by the M-MuLV-derived expression vector, along with the deleted v-*sis* genes, the genes were excised with *Xba*I. *Xba*I cuts once in the M-MuLV region upstream of the signal sequence and again in the 3′ long terminal repeat downstream of the deleted genes. Therefore, a new SV40-derived expression vector, pRB34, was constructed, with the *Xho*I site of pJC119 converted to a unique *Xba*I site. This vector was used for insertion of the deleted v-*sis* genes as *Xba*I-to-*Xba*I fragments.

Five of the mutant v-*sis* genes, complete with the vector-donated v-*sis* signal sequence region, were inserted into pRB34 in the correct orientation for expression. These plasmids were transfected into Cos-1 cells and metabolically labelled with [^{35}S]methionine and [^{35}S]cysteine. Cell lysates were immunoprecipitated with antiserum raised against bacterially synthesized p28^{sis}. Immunoprecipitates were analyzed on SDS-polyacrylamide gels under reducing and nonreducing conditions. Cells transfected with the v-*sis*^{wt} gene expressed a 32-kDa reduced protein (Fig. 3B, lane 7), which migrated as a dimer of the expected size when analyzed under nonreducing conditions (Fig. 3A, lane 7). Cells transfected with the five mutant v-*sis* genes yielded immunoprecipitable proteins of approximately the predicted molecular weights (Table 2) when electrophoresed under reducing conditions (Fig. 3B, lanes 2 through 6). The gene product of v-*sis*^{121} (Fig. 3B, lane 3) has an altered mobility when analyzed on reducing SDS-polyacrylamide gels, migrating slightly more slowly than expected. When immunoprecipitates were analyzed under nonreducing conditions, the gene products encoded by the v-*sis*^{120} and the v-*sis*^{121} genes were found to dimerize (Fig. 3A, lanes 2 and 3). Lesser amounts of the dimerized forms of the proteins encoded by the v-*sis*^{129}, v-*sis*^{127}, and v-*sis*^{132} genes were observed (Fig. 3A, lanes 4, 5, and 6, respectively). In all cases, significant amounts of the monomer forms were also detected.

Loss of N-linked glycosylation in the mutant v-*sis* gene products. The v-*sis*^{wt} gene product undergoes N-linked glycosylation at Asn residue 93 (11, 24). Of the mutant v-*sis* genes examined in this study, only the v-*sis*^{92} gene retained the coding region for the consensus glycosylation site Asn-Met-Thr. This region is deleted from all the other mutant v-*sis* genes, and their gene products are not expected to be glycosylated. This was demonstrated by immunoprecipitation analysis of the v-*sis*-encoded proteins from transfected Cos-1 cells that had been treated with tunicamycin. Immunoprecipitates of cells transfected with the v-*sis*^{wt} gene exhibited a protein of 29 kDa when labeling was done in the presence of tunicamycin (Fig. 4, lane 8), as compared with a protein of 32 kDa in the absence of tunicamycin (lane 4). The migration of the gene products encoded by the mutant v-*sis* genes was not affected by treatment with tunicamycin (lanes (1 and 5, 2 and 6, and 3 and 7), confirming that N-linked glycosylation did not occur.

DISCUSSION

We have constructed a series of deletions in the v-*sis* gene which define the N-terminus of the minimal transforming region of v-*sis* (Fig. 5). The deleted genes were assayed for their ability to induce the formation of foci in NIH 3T3 cells by using a M-MuLV-derived expression vector encoding the v-*sis* signal sequence. Mutants encoding a protein in which residues 58 through 126 are deleted are fully transforming, while mutants encoding proteins with deletions extending beyond residue 128 are biologically inactive. Furthermore, a mutant v-*sis* gene encoding deletions at both the N terminus and the C terminus of the PDGF-B homologous region also retains full biological activity. The minimal transforming region of the v-*sis* gene product therefore corresponds to residues 127 through 214. This is 21 residues smaller than the PDGF-B homologous region, which corresponds to residues 112 through 220.

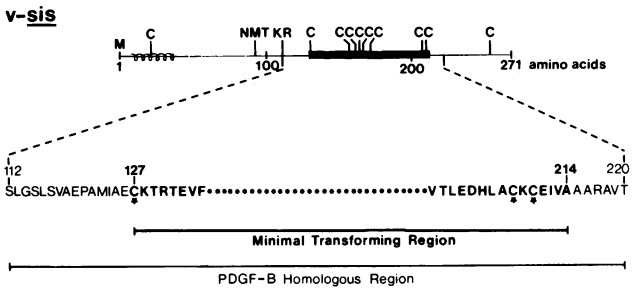

FIG. 5. Minimal transforming region of the v-*sis* gene product. A map of the full-length v-*sis* gene product is shown, with the first methionine (M) residue and the 10 cysteine (C) residues indicated. Also indicated are the sequence Asn-Met-Thr (NMT), residues 93 through 95, which is the site of N-linked glycosylation; and the Lys-Arg basic dipeptide (KR), residues 110 and 111, which is the site of proteolytic cleavage. The signal sequence is indicated by a curlicue. The v-*sis*^{wt} initial translation product contains 271 residues. The minimal transforming region of the v-*sis* gene, as delimited by this analysis of the N terminus in conjunction with the previous data on the C terminus (12), extends from residue 127 to residue 214. This region is indicated by the heavy line. Below the map of the full-length v-*sis* gene product is an expansion of the residues between 112 and 220. These correspond to the residues which are homologous to the PDGF-B chain. The extent of the minimal transforming region, as defined by this study, is indicated below the sequence. The PDGF-B chain homology spans the v-*sis* minimal transforming region and extends beyond it at both the C-terminal and N-terminal ends. The cysteine residues are distinguished by asterisks. However, there are five other cysteines within this region which are not represented in this partial sequence.

Only two residues, Cys^{127} and Lys^{128}, separate the endpoints of the closest transforming and nontransforming deletion mutant gene products, which suggests that those residues are important for transformation by v-*sis*. The full-length v-*sis* gene encodes 10 cysteine residues. Cysteine 28 is encoded by the portion of the viral *env* gene retained in the fused *env-sis* open reading frame and is removed along with the signal sequence. The next eight cysteine residues are within the PDGF-B homologous region, while the last cysteine is outside that region at residue 256. This final cysteine is not required for transformation or dimerization, since C-terminal truncated gene products with endpoints between residues 214 and 246 are still capable of dimerization and transformation (12). However, one or both cysteine residues 208 and 210 are required for dimerization and transformation, since a mutant gene product with an endpoint at residue 214 was able to dimerize and to transform cells, while a mutant gene product with an endpoint at residue 204 was incapable of both processes. The present study indicates that cysteine residue 127 is also required for

transformation. All mutant v-*sis* genes which encode this residue are able to transform NIH 3T3 cells and are capable of dimerization. However, its role in dimerization is not clear, for the v-sis^{127} gene product dimerizes to a lesser degree than the v-sis^{wt} or the other transforming gene products examined in this study. This difference may reflect the contribution of local secondary structure to either the efficiency of formation or the stability of the disulfide bonds. It should be noted that mutant proteins were analyzed with Cos-1 cells rather than NIH 3T3 cells, on the basis of evidence that the processing of secreted proteins occurs indistinguishably in these two cell lines (7, 11, 26, 29, 31). However, there remains the formal possibility that subtle differences between these cell lines may account for the observations reported here concerning dimerization and stability of the mutant gene products in Cos-1 cells, which were correlated with transformation assays in NIH 3T3 cells.

The v-sis^{wt} gene product is proteolytically processed after the basic dipeptide, Lys-Arg residues 110 and 111, to yield a 20-kDa PDGF-related protein as detected under reducing conditions (25). This protein exists as a dimer under nonreducing conditions. Of the mutants studied here, only the four encoding the largest gene products retain the Lys-Arg codons. Other deletion mutants, however, lack the Lys-Arg dipeptide, yet still encode gene products which are capable of dimerization and transformation. The dispensability of N-terminal proteolytic processing is supported by previous work in which the Lys-Arg codons were mutagenized to Asn-Thr with no reduction in the biological activity of the mutant (11).

There is a single consensus site for N-linked glycosylation in the v-*sis* gene product. This is the Asn-(Met)-Thr sequence at residues 93 through 95. Only the v-sis^{92} gene retains the codons for this glycosylation site. All the other mutant v-*sis* gene products studied here, both transforming and nontransforming, lack the N-linked glycosylation site. This was directly demonstrated by the fact that tunicamycin treatment of cells expressing these mutants failed to alter the mobility of the v-*sis* proteins. These results indicate that N-linked glycosylation is dispensable in terms of the biological activity of the v-*sis* gene product.

From the results of this study, in conjunction with our previous work (12), we have delimited the minimal transforming region of the v-*sis* gene product as corresponding to 88 amino acids, residues 127 through 214. This is significantly smaller than the PDGF-B chain, which corresponds to residues 112 through 220 of the v-*sis* gene product. The minimal transforming gene product encoded by the v-$sis^{127/214}$ gene has 6 fewer residues at the C terminus and 15 fewer residues at the N terminus than does the PDGF-B chain. Proteolytic processing and N-linked glycosylation are not required for transformation by v-*sis*, even though these processes do occur in the formation of the mature v-sis^{wt} protein and in the formation of the PDGF-B chain from the nascent c-*sis* gene product. Dimerization, however, is necessary for the biological activity of v-*sis*. Additional studies on the roles of individual cysteine residues are currently being undertaken by means of site-directed mutagenesis. Internal deletions and linker scanning will also be used to further characterize the minimal transforming region of the v-*sis* gene product.

ACKNOWLEDGMENTS

We thank Jay Sweet for his gift of antisera raised against the bacterially synthesized v-*sis* gene product. We thank Linda Haberstroh for her help in the construction of the deletion mutants and Rick Bold and Karen May for excellent technical assistance.

This work was supported by Public Health Service grant CA40573 from the National Cancer Institute. M.K.S. was supported by a postgraduate scholarship from the Natural Sciences and Engineering Research Council of Canada. D.J.D. gratefully acknowledges receipt of support from the Chicago Community Trust/Searle Scholars Program.

LITERATURE CITED

1. **Andersson, P., M. P. Goldfarb, and R. A. Weinberg.** 1979. A defined subgenomic fragment of in vitro synthesized Moloney sarcoma virus DNA can induce cell transformation upon transfection. Cell **16**:63–75.
2. **Bold, R. J., and D. J. Donoghue.** 1985. Biologically active deletion mutants of the v-*mos* oncogene assayed with novel retroviral vectors. Mol. Cell. Biol. **5**:3131–3138.
3. **Deuel, T. F., J. S. Huang, S. S. Huang, P. Stroobant, and M. D. Waterfield.** 1983. Expression of a platelet-derived growth factor-like protein in simian sarcoma virus transformed cells. Science **221**:1348–1350.
4. **Devare, S. G., E. P. Reddy, J. D. Law, K. C. Robbins, S. R. Tronick, and S. A. Aaronson.** 1983. Nucleotide sequence of the simian sarcoma virus genome: demonstration that its acquired cellular sequences encode the transforming protein $p28^{sis}$. Proc. Natl. Acad. Sci. USA **80**:731–735.
5. **Doolittle, R. F., M. W. Hunkapiller, L. E. Hood, S. G. Devare, K. C. Robbins, S. A. Aaronson, and H. N. Antoniades.** 1983. Simian sarcoma virus onc gene, v-*sis*, is derived from the gene (or genes) encoding a platelet-derived growth factor. Science **221**:275–277.
6. **Garrett, J. S., S. R. Coughlin, H. L. Niman, P. M. Tremble, G. M. Giels, and L. T. Williams.** 1984. Blockade of autocrine stimulation in simian sarcoma virus-transformed cells reverses down-regulation of platelet-derived growth factor receptors. Proc. Natl. Acad. Sci. USA **81**:7466–7470.
7. **Gluzman, Y.** 1981. SV40-transformed simian cells support the replication of early SV40 mutants. Cell **23**:175–182.
8. **Graham, F. L., and A. J. Van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
9. **Grunstein, M., and D. S. Hogness.** 1975. Colony hybridization: a method for the isolation of cloned DNA's that contain a specific gene. Proc. Natl. Acad. Sci. USA **72**:3961–3965.
10. **Hannink, M., and D. J. Donoghue.** 1984. Requirement for a signal sequence in the biological expression of the v-*sis* oncogene. Science **226**:1197–1199.
11. **Hannink, M., and D. J. Donoghue.** 1986. Biosynthesis of the v-*sis* gene product: signal sequence cleavage, glycosylation and proteolytic processing. Mol. Cell. Biol. **6**:1343–1348.
12. **Hannink, M., M. K. Sauer, and D. J. Donoghue.** 1986. Deletions in the C-terminal coding region of the v-*sis* gene: dimerization is required for transformation. Mol. Cell. Biol. **6**:1304–1314.
13. **Heldin, C.-H., and B. Westermark.** 1984. Growth factors: mechanism of action and relation to oncogenes. Cell **37**:9–20.
14. **Hoffman, J. W., D. Steffen, J. Gusella, C. Tabin, S. Bird, D. Cowing, and R. A. Weinberg.** 1982. DNA methylation affecting the expression of murine leukemia proviruses. J. Virol. **44**:144–157.
15. **Huang, J. S., S. S. Huang, and T. F. Deuel.** 1984. Transforming protein of simian sarcoma virus stimulates autocrine growth of SSV-transformed cells through PDGF cell-surface receptors. Cell **39**:79–87.
16. **Huang, J. S., S. S. Huang, and T. F. Deuel.** 1983. Human platelet-derived growth factor: radioimmunoassay and discovery of a specific plasma binding protein. J. Cell Biol. **97**:383–388.
17. **Johnsson, A., C.-H. Heldin, A. Wasteson, B. Westermark, T. F. Deuel, J. S. Huang, P. H. Seeburg, A. Gray, A. Ullrich, G. Scrace, P. Stroobant, and M. D. Waterfield.** 1984. The c-*sis* gene encodes a precursor of the B-chain of platelet-derived growth factor. EMBO J. **3**:921–928.
18. **Josephs, S. F., C. Guo, L. Ratner, and F. Wong-Staal.** 1984. Human proto-oncogene nucleotide sequences corresponding to

the transforming region of simian sarcoma virus. Science **223**:487–491.

19. **Kaplan, D. R., F. C. Chao, C. D. Stiles, H. N. Antoniades, and C. D. Scher.** 1979. Platelet alpha granules contain a growth factor for fibroblasts. Blood **53**:1043–1052.
20. **King, C. R., N. A. Giese, K. C. Robbins, and S. A. Aaronson.** 1985. In vitro mutagenesis of the v-*sis* transforming gene defines functional domains of its growth factor-related product. Proc. Natl. Acad. Sci. USA **82**:5295–5299.
21. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
22. **Nishimura, J., J. S. Huang, and T. F. Deuel.** 1982. Platelet-derived growth factor stimulates tyrosine specific protein kinase in swiss mouse 3T3 cell membranes. Proc. Natl. Acad. Sci. USA **79**:4303–4307.
23. **Owen, A. J., P. Pantazis, and H. N. Antoniades.** 1984. Simian sarcoma virus-transformed cells secrete a mitogen identical to platelet-derived growth factor. Science **225**:54–56.
24. **Robbins, K. C., F. Leal, J. H. Pierce, and S. A. Aaronson.** 1985. The v-*sis*/PDGF-2 transforming gene product localizes to cell membranes but is not a secretory protein. EMBO J. **4**:1783–1792.
25. **Robbins, K. C., H. N. Antoniades, S. G. Devare, M. W. Hunkapiller, and S. A. Aaronson.** 1983. Structural similarities between sarcoma virus gene product(s) and human platelet-derived growth factor. Nature (London) **305**:605–608.
26. **Rose, J. K., and J. E. Bergmann.** 1982. Expression from cloned cDNA of cell-surface secreted forms of the glycoprotein of vesicular stomatitis virus in eucaryotic cells. Cell **30**:753–762.
27. **Ross, R., J. Glomset, B. Kariya, and L. Harker.** 1974. A platelet-derived serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro. Proc. Natl. Acad. Sci. USA **71**:1207–1210.
28. **Sporn, M. B., and A. B. Roberts.** 1985. Autocrine growth factors and cancer. Nature (London) **313**:745–747.
29. **Sprague, J., J. H. Condra, H. Arnheiter, and R. A. Lazzarini.** 1983. Expression of a recombinant DNA clone coding for the vesicular stomatitis virus nucleocapsid protein. J. Virol. **45**:773–781.
30. **Theilen, G. H., D. Gould, M. Fowler, and D. L. Dungworth.** 1971. C-type virus in tumor tissue of a woolly monkey (Lagothrix spp.) with fibrosarcoma. J. Natl. Cancer Inst. **47**:881–889.
31. **Warren, T. G., and D. Shields.** 1984. Expression of preprosomatostatin in heterologous cells: biosynthesis, post-translational processing and secretion of mature somatostatin. Cell **39**:547–555.
32. **Waterfield, M. D., G. T. Scrace, N. Whittle, P. Stroobant, A. Johnsson, A. Wasteson, B. Westermark, C.-H. Heldin, J. S. Huang, and T. F. Deuel.** 1983. Platelet-derived growth factor is structurally related to the putative transforming protein $p28^{sis}$ of simian sarcoma virus. Nature (London) **304**:35–39.