Deletions in the C-Terminal Coding Region of the *v-sis* Gene: Dimerization Is Required for Transformation

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The *v-sis* gene encodes chain B of platelet-derived growth factor. However, this gene codes for additional amino acids at both the N terminus and the C terminus of its gene product which are not present in the amino acid sequence of platelet-derived growth factor. We constructed a series of deletion mutants with deletions in the *v-sis* gene in order to define the C-terminal limit of the *v-sis* gene product which is required for transformation. Deletion mutants of the *v-sis* gene which encoded truncated gene products up to 57 residues shorter than the *v-sis*^{wt} gene product were still able to transform cells. The minimal transforming region of the *v-sis* gene product contained six residues fewer than were present in chain B of platelet-derived growth factor. Only 10 residues, including the sequence Cys-Lys-Cys, separated the smallest transforming gene product from the largest nontransforming gene product. These cysteine residues were also important for dimerization of the *v-sis* gene product, since all of the nontransforming *v-sis* deletions were unable to form dimers when they were analyzed under nonreducing conditions. Our results suggest that there is a strong connection between transformation and dimerization.

The *v-sis* oncogene was isolated as the oncogene of simian sarcoma virus (SSV) (3, 27). SSV acquires cellular genetic information at the expense of a portion of its *env* gene, resulting in the formation of an *env-sis* fused open reading frame (3). This 813-nucleotide open reading frame is potentially capable of encoding a 271-amino acid protein. There are a total of six ATG codons in the *env-sis* open reading frame, and previous work has shown that the first ATG codon is used in the synthesis of the initial translation product (submitted for publication). Located between the second and third ATG codons is a coding region for a signal peptide sequence which directs the cotranslational translocation of the nascent polypeptide chain across the membrane of the rough endoplasmic reticulum (10). Signal sequence cleavage and the addition of an N-linked oligosaccharide to asparagine residue 93 (counting from the N terminus of the predicted initial translation product) occur during translation (22; submitted for publication). (Note that throughout this paper the amino acid number of a residue is its distance from the N terminus of the predicted initial translation product.) Approximately 4 kilodaltons (kDa) is removed from the N terminus of the initial translation product as a result of signal sequence cleavage. The resulting glycoprotein, which is detected as a 28-kDa protein (21) or a 32-kDa protein (submitted for publication), dimerizes soon after crossing the rough endoplasmic reticulum membrane. During transport through the Golgi apparatus to the cell surface, the *v-sis* gene product is proteolytically processed at a basic dipeptide (Lys-Arg at residues 110 and 111), resulting in the formation of a 42-kDa dimer composed of two 20-kDa subunits (21). Further processing of the *v-sis* gene product has been reported to occur at the C terminus, resulting in the formation of a platelet-derived growth factor (PDGF)-related 24-kDa dimer (21). Secretion of a *v-sis*-encoded 17-kDa protein (20) or 20-kDa protein (6) from SSV-transformed cells has also been reported. The secreted form(s) of the *v-sis* gene product also exists as dimers when preparations are analyzed under nonreducing conditions.

The *v-sis* gene encodes chain B of PDGF (4, 16, 29). PDGF consists of a dimer of two subunits (16, 29), although it is not known whether PDGF is a pair of related homodimers or an A-B heterodimer. PDGF is a potent mitogen for cells of mesenchymal origin (24) and stimulates the autophosphorylation of its cell surface receptor (19). Both the intracellular and secreted forms of the *v-sis* gene product possess mitogenic activity for NIH 3T3 cells (2, 6, 14, 20), and the secreted form stimulates the autophosphorylation of the PDGF receptor (6, 14, 20). Dimerization of both the *v-sis* gene product and PDGF is required for mitogenic activity (20). Consistent with this requirement for dimerization is the presence of 10 cysteine residues in the *v-sis* gene product, 9 of which are conserved in the *c-sis* gene product. A comparison of the amino acid sequence data for PDGF with the predicted amino acid sequence of the *v-sis* gene product has shown that the PDGF-homologous region of the *v-sis* gene product extends from residue 112 to residue 220, a region containing 8 of the 10 cysteine residues (15). Therefore, the *v-sis* gene product contains 51 residues at its C terminus which are not present in PDGF. These residues are also present in the predicted amino acid sequence of the full-length *c-sis* gene product. The importance of these C-terminal residues for transformation is not known. A previous report indicated that a truncated 239-amino acid *v-sis* gene product retains biological activity (17). However, this was only a single endpoint and still retained 19 amino acids beyond the PDGF-homologous region.

In this study we determined the C-terminal limit of the biologically active *v-sis* gene product. We constructed a series of mutants with deletions in the *v-sis* gene and assayed the mutant *v-sis* genes for their ability to induce focus formation in NIH 3T3 cells. We found that up to 57 amino acid residues could be removed from the C terminus of the *v-sis* gene product without affecting its transformation potential. An analysis of the gene products encoded by the transforming *v-sis* deletions by immunoprecipitation with anti-PDGF serum suggested that C-terminal processing does not occur. However, a truncated *v-sis* gene product which lacked the C-terminal 67 residues was completely unable to

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induce focus formation in NIH 3T3 cells. Likewise, additional C-terminal truncations or an internal deletion in the *v-sis* gene product was unable to transform cells. Our results delimited the minimal transforming region of the *v-sis* gene product to 103 residues, extending from residue 112 to residue 214 (six residues shorter than the PDGF-homologous portion of the *v-sis* gene product). Located within the 10 residues which separated the smallest transforming *v-sis* gene product from the largest nontransforming *v-sis* gene product were two cysteine residues, residues 208 and 210. The inability of the nontransforming *v-sis* gene products to form dimers when preparations were analyzed under nonreducing conditions indicated that these cysteine residues are involved in dimer formation of the *v-sis* gene product. Our results demonstrated that dimer formation is required for transforming ability, as well as the mitogenic activity of the *v-sis* gene product.

MATERIALS AND METHODS

Construction of deletion mutants of the *v-sis* gene. The *v-sis* gene contains a TGA codon in a +1 reading frame located within the *Bst*EII site at nucleotide 711 (counting from the first ATG codon of the *env-sis* open reading frame). This TGA codon was inserted into the correct reading frame of the *v-sis* gene by the manipulations described below. To construct the deleted *v-sis* gene that encoded 239 amino acids (*v-sis*²³⁹), a subclone of the *v-sis* gene in pBR322 (pDD119) was linearized by restriction digestion with *Bst*EII. The 5' overhang was filled in by using the Klenow fragment of *Escherichia coli* DNA polymerase I in the presence of the four deoxynucleoside triphosphates. The blunt-ended molecules were ligated by using T4 DNA ligase in the presence of ATP and were used to transform competent *E. coli* C600 cells. The resulting plasmid contained a -1 frameshift at nucleotide 717, putting the TGA codon in the reading frame of the *v-sis* gene. To construct the *v-sis*²²³ gene, a portion of the blunt-ended molecules described above was further digested with *Sma*I before ligation and transformation. This deletion of 43 nucleotides caused a -1 frameshift, which puts the TGA codon in frame at nucleotide 669. To construct the *v-sis*^{177/239} gene, a 1,500-base pair *Pvu*II-to-*Hind*III fragment containing the 5' portion of the *v-sis* gene was isolated. This fragment was ligated to a 3,500-base pair filled-in *Bst*EII-to-*Hind*III fragment containing the 3' end of the *v-sis* gene and the vector portion of pBR322. This resulted in an internal deletion of 183 nucleotides, and the correct reading frame was maintained across the restored *Bst*EII site. This plasmid was used in the construction of the *v-sis*¹⁷⁷ gene. Digestion with *Bst*EII followed by fill-in of the 5' overhang and ligation of the blunt-ended molecules resulted in a -1 frameshift, putting the TGA codon in the *v-sis* reading frame. To construct the *v-sis*¹³⁶ gene, pDD119 was linearized at the *Bgl*II site at nucleotide 403 by digestion with *Bgl*II. The 5' overhang was filled in, and an 8-mer *Xba*I linker, CTCTAGAG, was ligated into the molecules. The ligation mixture was transfected into competent *E. coli* cells, and recombinant molecules containing an *Xba*I site in place of the *Bgl*II site were selected. This resulted in the TAG codon from the linker being placed into the reading frame of the *v-sis* gene. The nucleotide sequences of these deletion mutants of the *v-sis* gene were confirmed by nucleotide sequencing (18).

For the *Bal* 31 deletions, the manipulations described below were performed to obtain a subclone of the *v-sis* gene which had the following characteristics: (i) flanking *Xho*I (5') and *Cla*I (3') sites to facilitate insertion of the deleted *v-sis*

genes into a retroviral vector, and (ii) a unique restriction site at or near the TAA codon of the *v-sis* gene. The 1,190-base pair *Sal*I (5')-to-*Xba*I (3') fragment of SSV containing the entire *env-sis* coding region was previously subcloned into a pBR322 derivative, pDD161. The desired flanking sites were introduced by linker insertion, resulting in plasmid pDD164. A 429-base pair 5' fragment of the *v-sis* gene was removed from pDD164 by digestion with *Bgl*II and *Xho*I, followed by fill-in and ligation; this resulted in the restoration of both sites. The 429-base pair fragment which was removed contained one of the two *Mst*II sites which are present in the *v-sis* gene. The resulting plasmid, pDD165, contained only one *Mst*II site, which spanned the TAA termination codon of the *v-sis* gene. This *Mst*II site was converted to a *Bam*HI site in the following way. The plasmid was linearized with *Mst*II, the 5' overhang was removed by S1 nuclease treatment and treated with the Klenow fragment of *E. coli* DNA polymerase I in the presence of the four deoxynucleoside triphosphates, and a *Bam*HI linker (CCG GATCCGG) was ligated into the molecules. The resulting plasmid, pDD175, lacked the TAA terminator codon of the *v-sis* gene and contained a unique *Bam*HI site in place of the *Mst*II site at nucleotide 813. The 5' end of the *v-sis* gene was then inserted into pDD175 as a *Xho*I-to-*Bgl*II fragment, resulting in pMS001. This plasmid contained the entire *v-sis* gene flanked by a 5' *Xho*I site and a 3' *Cla*I site and had a unique *Bam*HI site in place of the TAA terminator codon of the *v-sis* gene. Plasmid pMS001 was linearized by digestion with *Bam*HI, and the 5' overhang was filled in by using the Klenow fragment and deoxynucleoside triphosphates. *Bal* 31 digestions were carried out according to the instructions of the suppliers by using a *Bal* 31 concentration of 0.3 U/μg of DNA. Digestions were carried out at 30°C for time periods ranging from 20 s to 2 min. *Bal* 31 digestion was stopped by phenol extraction and ethanol precipitation. Following treatment of the molecules with the Klenow fragment, a self-complementary 26-mer oligonucleotide, TCAATCAG TCAAGCTTGACTGAT TGA, was ligated into the molecules. The molecules were screened for the presence of the *Hind*III site present in the termination oligonucleotide and for the presence of the *Cla*I site at the 3' end of the *v-sis* insertion. A total of 17 deletion mutants of the *v-sis* gene were constructed in this fashion. The extent of each deletion was determined by nucleotide sequencing into the *v-sis* gene from the *Hind*III site, using the method of Maxam and Gilbert (18).

In order to allow expression of the deleted *v-sis* genes in NIH 3T3 cells, the deletions of the *v-sis* gene were inserted into pDD102, a Moloney murine leukemia virus (M-MuLV)-derived expression vector (1a, 9). The subclones containing the deleted *v-sis* genes consisted of two classes. One class contained the *v-sis* gene as an *Xho*I-to-*Xho*I insertion, while the other contained the *v-sis* gene as an *Xho*I-to-*Cla*I insertion. The *v-sis* genes which were flanked by *Xho*I sites were inserted into pDD102 as *Xho*I-to-*Xho*I fragments. We constructed a variant of pDD102, pMS020, in which a *Cla*I site was inserted immediately after the *Xho*I site. This allowed insertion of the *v-sis* genes which were flanked by *Xho*I and *Cla*I sites. The advantage of the latter method is that the correct orientation of the insertion was achieved in more than 95% of the cases, while with the other method at best 50% of the recombinant clones contained the *v-sis* insertion in the correct orientation for expression. In all cases, the correct orientation of the inserted *v-sis* gene was confirmed by restriction mapping of the plasmid. In order to allow expression of the *v-sis* genes in COS-1 cells, the *v-sis* genes

were inserted into the unique *Xho*I site of pJC119 (26). This resulted in the *v-sis* genes being put under the transcriptional control of the simian virus 40 late promoter.

Focus assays and virus titers. Focus assays and viral titrations were performed essentially as described previously (1, 8). Briefly, semiconfluent monolayers of NIH 3T3 cells grown in 60-mm dishes were transfected with a calcium phosphate precipitate containing 1 μ g of a *v-sis* DNA clone, 1 μ g of a DNA clone of replication-competent M-MuLV (12), and 20 μ g of sheared calf thymus DNA. A calcium phosphate precipitate was formed by adding 400 μ l of 0.25 M CaCl_2 to the DNA in 400 μ l of 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–280 mM NaCl–10 mM sodium phosphate (pH 7.0). The solution was allowed to stand for 45 min before it was added directly to the medium containing cells. The precipitate was left on the cells overnight before we fed the preparations and split them into dishes (4 by 10 cm) the next day. The cells were fed at 3-day intervals and scored at 7 to 10 days posttransfection. The conditioned medium was collected when the assays were scored and titrated onto fresh monolayers of NIH 3T3 cells for the presence of focus-forming virus. Typically, 200 μ l of an appropriate dilution of conditioned medium containing 4 μ g of Polybrene per ml was allowed to adsorb to the cells for 1 h. The cells were washed, fed, and scored after 4 to 5 days. All cells were grown in Dulbecco modified minimal essential medium supplemented with 10% calf serum.

Immunoprecipitation. Semiconfluent monolayers of COS-1 cells grown in 60-mm dishes were transfected with DNAs by using the DEAE-dextran method. The cells were washed twice with Tris-saline (10 mM Tris, pH 7.4, 150 mM NaCl), a solution of Tris-saline containing 5 μ g of DNA per ml and 50 μ g of DEAE-dextran (molecular weight, 2.0×10^6) per ml was added to the cells, and the preparation was incubated at 37°C for 10 min. The DNA solution was removed, and the cells were shocked with a solution of 10% dimethyl sulfoxide in Tris-saline at room temperature for 1 min. The cells were washed twice with Tris-saline and fed with fresh medium containing 100 μ M chloroquine for 2 h. The cells were then fed with fresh medium for 48 h before they were labeled. The cells were labeled with 100 μ Ci of [35 S]cysteine and 100 μ Ci of [35 S]methionine in Dulbecco modified Eagle medium lacking cysteine and methionine for 2 h. For immunoprecipitation of intracellular proteins, cell lysates were prepared in 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], 1% Trasyolol), and cellular debris was pelleted before the sample was divided for immunoprecipitation. Immunoprecipitation was performed as described previously (13) by using *Staphylococcus* protein A to collect the immunoglobulin G. The immunoprecipitates were suspended in sample buffer (50 mM Tris, pH 6.8, 2% SDS, 20% β -mercaptoethanol, 10% glycerol), boiled for 2 min, and clarified before electrophoresis on 15% SDS–polyacrylamide gels. For the samples that were analyzed under nonreducing conditions, the immunoprecipitates were suspended in sample buffer lacking β -mercaptoethanol. For the immunoprecipitates that were treated with endoglycosidase F, the immunoprecipitates were suspended in Endo F reaction buffer (5) and boiled for 2 min. The *Staphylococcus* protein A was pelleted, and 1- μ l portions of Endo F were added to the samples before overnight incubation at 25°C. Then 2 $\times$ sample buffer was added, and the samples were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE). For immu-

noprecipitation of conditioned medium, the conditioned medium was collected, lyophilized, and suspended in 200 μ l of RIPA buffer before immunoprecipitation.

RESULTS

The predicted amino acid sequence of the *v-sis* gene product is homologous to the amino acid sequence of PDGF from residue 112 to 220 (15, 16, 29), with the result that the *v-sis* gene product contains 51 more residues at its C terminus than PDGF does. In order to understand how much of the C terminus of the wild-type *v-sis* (*v-sis*^{wt}) gene product is dispensable for transformation, we constructed a series of mutants with deletions in the *v-sis* gene (Fig. 1).

Two methods were used to ensure that the resulting deletions of the *v-sis* gene encoded proteins with defined C termini. In one approach, the out-of-frame TGA codon located at nucleotides 712 to 714 was inserted into the correct reading frame to provide for translation termination. When this method was used, deleted *v-sis* genes that were expected to encode truncated gene products 239, 223, 177, and 136 amino acids long were constructed. An additional mutant with an internal deletion of the *v-sis* gene in which the correct reading frame was maintained was also constructed. This *v-sis* deletion resulted in the removal of residues 178 to 238 from the *v-sis*^{wt} gene product.

Additional deletion mutants with mutations in the *v-sis* gene were constructed by *Bal* 31 mutagenesis. The *v-sis* gene was then linearized at its 3' end and treated with *Bal* 31 exonuclease for varying amounts of time. The DNA ends were treated with the Klenow fragment of *E. coli* DNA polymerase I, and this was followed by ligation of a termination oligonucleotide into the molecules. This oligonucleotide, TCAATCAGTCAAGCTTGACTGATTGA, contained TGA codons in all possible reading frames, thereby ensuring that the deleted *v-sis* genes encoded truncated gene products with defined termini. The exact positions of the deletions were determined by nucleotide sequencing, and the predicted amino acid sequences were deduced for these mutants (Table 1). Using this method, we constructed an additional 17 C-terminal deletion mutants of the *v-sis* gene that encoded truncated gene products which were 121 to 246 amino acids long.

The deletion mutants with mutations in the *v-sis* gene were assayed for their ability to induce focus formation in NIH 3T3 cells. For these experiments we used a M-MuLV-derived expression vector which provided for the expression of inserted genes by using the M-MuLV *env* transcription pathway (1a, 10). The *v-sis* genes were inserted into the retroviral vector, introduced into NIH 3T3 cells by the calcium phosphate coprecipitation method, and assayed for their ability to induce the formation of foci (Fig. 1). Since the expression vector was defective in both the *gag* and *pol* genes, a replication-competent DNA clone of M-MuLV (12) was cotransfected in order to provide for viral spread and rescue. Biological activities of up to 5.9×10^4 foci per pmol were obtained from transfection experiments with the *v-sis*^{wt} gene (Table 1). Mutants in which up to 57 residues were deleted from the C terminus of the *v-sis*^{wt} gene product (for example, *v-sis*²¹⁴) possessed biological activity equal to that of the *v-sis*^{wt} gene. However, *v-sis*²⁰⁴, which encoded a protein lacking the 67 C-terminal residues, was inactive when it was assayed for focus formation. Other mutant *v-sis* genes that encoded proteins in which up to 150 C-terminal or 62 internal amino acids had been removed were also biologically inactive. The conditioned media from the focus assays were collected and titrated onto fresh monolayers of NIH

FIG. 1. Deletions in the *v-sis* gene. A series of 22 mutants with deletions in the *v-sis* gene were constructed as described in the text. The endpoint of each deletion was determined by nucleotide sequencing, and the superscript of each deletion designation indicates the number of amino acids encoded by the deleted *v-sis* gene. The number of amino acids was determined by counting from the N terminus of the predicted initial translation product of the *v-sis* gene. As shown in Table 1, the deletions had slightly different termini, depending on the exact reading frame into the termination oligonucleotide. For example, three independent isolates of the *v-sis*²³⁴ gene were isolated, each possessing a different C terminus; these were designated *v-sis*²³⁴_a, *v-sis*²³⁴_b, and *v-sis*²³⁴_c. A map of the *v-sis*^{wt} gene is shown at the top. The *v-sis* deletions were inserted into the M-MuLV-derived expression vector shown at the top. The plus and minus signs indicate the biological activities of the individual *v-sis* deletions as determined by their ability to induce the formation of foci in NIH 3T3 cells and by the titer of rescuable focus-forming virus. The values obtained in these experiments are shown in Table 1. LTR, Long terminal repeat.

3T3 cells in order to assay for the presence of rescuable focus-forming virus. The transforming *v-sis* genes had high levels of rescuable focus-forming virus in the conditioned media from the focus assays, while the *v-sis* genes which

gave no detectable foci in the focus assays also lacked detectable focus-forming virus in the conditioned media. The sharp difference in biological activity between the *v-sis*²¹⁴ and *v-sis*²⁰⁴ genes allowed the C-terminal limit of the biolog-

TABLE 1. Biological activities of the deletion mutants of the *v-sis* gene

Deletion mutant	Plasmid ^a	Exact endpoint of mutant		No. of residues deleted ^d	Results of biological assays	
		Nucleotide ^b	C-terminal sequence class ^c		Focus assay (FFU/pmol) ^e	Titer (FFU/ml)/
<i>v-sis</i> ²⁷¹	pDD145	813	wt	0	5.9×10^4	2.5×10^6
<i>v-sis</i> ²⁴⁶	pMS042	737	C	25	4.8×10^3	3.0×10^5
<i>v-sis</i> ²³⁹	pMH75	716	D	32	4.4×10^3	8.0×10^3
<i>v-sis</i> ^{234a}	pMS030	704	C	37	3.4×10^4	1.0×10^7
<i>v-sis</i> ^{234b}	pMS036	702	A	37	1.9×10^4	6.0×10^6
<i>v-sis</i> ^{234c}	pMS034	701	C	37	1.4×10^4	1.0×10^7
<i>v-sis</i> ²³¹	pMS027	693	A	40	3.5×10^4	5.0×10^5
<i>v-sis</i> ²²⁷	pMS040	681	A	44	9.6×10^3	1.7×10^6
<i>v-sis</i> ^{223a}	pMS034	668	C	48	3.4×10^4	5.0×10^6
<i>v-sis</i> ^{223b}	pMH67	668	D	48	2.4×10^4	1.5×10^5
<i>v-sis</i> ²²⁰	pMS044	660	A	51	2.9×10^4	2.0×10^6
<i>v-sis</i> ²¹⁶	pMS029	647	C	55	5.8×10^4	1.0×10^7
<i>v-sis</i> ²¹⁴	pMS033	642	A	57	4.3×10^4	3.7×10^6
<i>v-sis</i> ²⁰⁴	pMS052	613	B	67	$<1.0 \times 10^1$	$<5.0 \times 10^0$
<i>v-sis</i> ²⁰⁰	pMS039	599	C	71	$<1.0 \times 10^1$	$<5.0 \times 10^0$
<i>v-sis</i> ¹⁹⁸	pMS037	594	A	73	$<1.0 \times 10^1$	$<5.0 \times 10^0$
<i>v-sis</i> ¹⁸¹	pMS038	544	B	90	$<1.0 \times 10^1$	$<5.0 \times 10^0$
<i>v-sis</i> ¹⁷⁷	pMH76	531	E	94	$<1.0 \times 10^1$	$<5.0 \times 10^0$
<i>v-sis</i> ¹⁴²	pMS028	427	B	129	$<1.0 \times 10^1$	$<5.0 \times 10^0$
<i>v-sis</i> ¹³⁶	pMH68	408	F	135	$<1.0 \times 10^1$	$<5.0 \times 10^0$
<i>v-sis</i> ¹³¹	pMS032	394	B	140	$<1.0 \times 10^1$	$<5.0 \times 10^0$
<i>v-sis</i> ¹²¹	pMS041	362	C	150	$<1.0 \times 10^1$	$<5.0 \times 10^0$
<i>v-sis</i> ^{177/239}	pMH66	813	wt	62	$<1.0 \times 10^1$	$<5.0 \times 10^0$

^a Plasmids which contained the deletion mutants in the M-MuLV-derived expression vector.

^b Numbers of the last nucleotides derived from the *v-sis* gene, counting from the first ATG codon of the *env-sis*-open reading frame; these numbers can be converted to the nucleotide numbers of the SSV sequence (3) by adding 3,657.

^c C-terminal amino acid sequences of the deletion mutants. The letters indicate in which class the C-terminal amino acid sequence of a deletion mutant belongs, depending on the reading frame into the termination oligonucleotide. The classes were defined as follows: class A deletion mutants contained the amino acid sequence Ser-Ile-Ser-Gln-Ala at their C termini; class B mutants contained the sequence X-Asn-Gln-Ser-Ser-Leu-Thr-Asp, where X was Leu (except for the *v-sis*¹³¹ gene product, which had a Val at this position); class C mutants had the amino acid sequence Z-Gln-Ser-Val-Lys-Leu-Asp at their C termini, where Z was the amino acid sequence of the *v-sis*^{wt} gene product at this position (except for the *v-sis*^{234c} gene product, which had a His in place of the Gln found in the *v-sis*^{wt} gene product); class D mutants had the amino acid sequence of the *v-sis*^{wt} gene product at the position indicated; class E mutants ended in Val-Thr; and class F mutants ended in Leu. wt, Wild type.

^d Numbers of amino acids which were deleted from the *v-sis*^{wt} gene product.

^e FFU, Focus-forming units.

^f Titers of rescuable focus-forming virus present in the conditioned media of the focus assays.

ically active *v-sis* gene product to be placed between residues 214 and 204.

In order to obtain high levels of the gene products coded for by the deleted *v-sis* genes, we used a transient expression assay. Transient expression in COS-1 cells is a widely used method which allows high levels of expression of a desired gene (7, 23, 26, 28). Furthermore, the ability of COS-1 cells to proteolytically process and secrete hormones in a biologically active form has been well established (28). For these experiments we used simian virus 40-derived expression vector pJC119, which allowed for the expression of genes inserted at a unique *Xho*I site by using the late transcription pathway (26). Two mutant *v-sis* genes which encoded transforming gene products were inserted into pJC119 in the correct orientation for expression and then introduced into COS-1 cells. Cell lysates prepared from metabolically labeled cells were subjected to immunoprecipitation with antiserum directed against a peptide corresponding to residues 52 to 61 of the predicted *v-sis*^{wt} gene product. Cells transfected with the *v-sis*^{wt} gene yielded a 32-kDa protein when they were analyzed under reducing SDS-PAGE conditions (Fig. 2a, lane 1), while cells transfected with the *v-sis*²²³ or *v-sis*²³⁹ gene contained 26- and 28-kDa proteins (Fig. 2a, lanes 3 and 5). When the immunoprecipitates were analyzed under nonreducing conditions, the transforming gene products dimerized, yielding proteins of the expected sizes (Fig. 2b, lanes 1, 3, and 5).

The *v-sis*^{wt} gene product is proteolytically processed at a

basic dipeptide, Lys-Arg at residues 110 and 111, resulting in the formation of a PDGF-related 20-kDa protein (21). In order to determine whether the truncated *v-sis* gene products encoded by the mutant genes are proteolytically processed, cell lysates from COS-1 cells transfected with the *v-sis*²³⁹ and *v-sis*²²³ genes were immunoprecipitated with anti-PDGF serum. We found that the *v-sis*²³⁹ and *v-sis*²²³ genes encoded PDGF-related protein doublets (16.5 and 16 kDa and 14.5 and 14 kDa, respectively) in addition to the 28- and 26-kDa proteins also detected with the N-terminal peptide serum (Fig. 3, lanes 1 and 3). When the immunoprecipitates were analyzed under nonreducing conditions, PDGF-related dimers of the appropriate sizes were found (Fig. 3, lanes 5 and 7). The sizes of the smaller PDGF-related proteins are consistent with proteolytic processing at the Lys-Arg dipeptide. No smaller PDGF-related proteins were specifically immunoprecipitated from COS-1 cells that expressed the *v-sis* genes.

The conditioned medium of the transfected COS-1 cells was also examined for the presence of PDGF-related proteins. We found that PDGF-related 16.5- and 16-kDa proteins and 14.5- and 14-kDa proteins were secreted into the media from COS-1 cells transfected with the *v-sis*²³⁹ and *v-sis*²²³ genes, respectively (Fig. 3, lanes 4 and 2). These proteins dimerized when they were analyzed under nonreducing conditions (Fig. 3, lanes 6 and 8) and comigrated with the intracellular processed forms of the mutant *v-sis* gene products, indicating that no further processing

FIG. 2. Expression of the transforming *v-sis* genes in COS-1 cells. The *v-sis*^{wt} gene (lanes 1 and 2), the *v-sis*²²³ gene (lanes 3 and 4), and the *v-sis*²³⁹ gene (lanes 5 and 6) were expressed in COS-1 cells under the transcriptional control of the simian virus 40 late promoter. Lysates from cells metabolically labeled with [³⁵S]cysteine were immunoprecipitated with antiserum raised against a peptide corresponding to residues 52 through 61 of the predicted amino acid sequence of the *v-sis* gene product. (A) Samples analyzed under reducing conditions. (B) Identical samples analyzed under nonreducing conditions. Lanes 1, 3, and 5 contained immunoprecipitates with the anti-peptide serum, while in lanes 2, 4, and 6 the anti-peptide serum was preincubated with an excess of the peptide before immunoprecipitation. The arrows indicate the positions of 32-, 26-, and 28-kDa proteins in panel A and the positions of the dimer forms of these proteins in panel B.

occurred during secretion. Further evidence that the *v-sis* gene products are secreted from COS-1 cells was obtained by testing the conditioned media of transfected COS-1 cells for the presence of mitogenic activity. Cells transfected with the transforming *v-sis* genes secreted a mitogenic activity into the media, while cells transfected with either nontransforming *v-sis* genes or vector DNA alone contained no mitogenic activity in their conditioned media (data not shown).

We also expressed the *v-sis*²¹⁴ and the *v-sis*²⁰⁴ genes in COS-1 cells, since these genes defined the limit of the C terminus of the *v-sis* gene product which is required for transformation. When COS-1 cell lysates were immunoprecipitated with antiserum raised against the bacteri-

ally expressed gene product and analyzed under reducing SDS-PAGE conditions, we found that the *v-sis*²¹⁴ and *v-sis*²⁰⁴ genes encoded 25- and 24-kDa proteins (Fig. 4a, lanes 3 and 4). The 1-kDa difference between the proteins is consistent with the eight-amino acid difference between the two mutant *v-sis* genes. However, only the *v-sis*²¹⁴ gene encoded a protein that was capable of dimerization when preparations were analyzed under nonreducing SDS-PAGE conditions (Fig. 4b, lanes 3 and 4). Smaller PDGF-related 13.5- and 13-kDa proteins were detected in lysates of cells transfected with the *v-sis*²¹⁴ gene, but they were not detected in lysates from cells transfected with the *v-sis*²⁰⁴ gene, indicating that only the *v-sis*²¹⁴ gene product was proteolytically processed at the Lys-Arg dipeptide. Several other

FIG. 3. Immunoprecipitation of the transforming *v-sis* gene products with anti-PDGF serum. COS-1 cells were transfected with the *v-sis*²²³ gene (lanes 1, 2, 5, and 6) and the *v-sis*²³⁹ gene (lanes 3, 4, 7, and 8) and metabolically labeled with [³⁵S]cysteine. Lysates (lanes 1, 3, 5, and 7) and conditioned media (lanes 2, 4, 6, and 8) were immunoprecipitated with anti-PDGF serum and analyzed under reducing (lanes 1 through 4) or nonreducing (lanes 5 through 8) SDS-PAGE conditions. The arrows indicate the positions of full-length PDGF-related 26- and 28-kDa proteins in lanes 1 through 4. The positions of the doublets of PDGF-related proteins (14.5 and 14 kDa and 16.5 and 16 kDa) resulting from proteolytic processing are also indicated. The positions of the dimers are indicated in lanes 5 through 8.

nontransforming mutants of the *v-sis* gene were analyzed in a similar manner, with the result that none of the nontransforming genes was found to encode a gene product which was capable of dimerization under nonreducing conditions (data not shown).

The *v-sis* gene product is modified by the addition of a high-mannose oligosaccharide to Asn residue 93 during its translocation across the membrane of the rough endoplasmic reticulum (22; submitted for publication). Although the deletion mutants retained the signal sequence encoded by the *v-sis* gene and could be localized to the Golgi apparatus by using indirect immunofluorescence (data not shown), we wanted to determine whether the mutant *v-sis* gene products were modified by glycosylation. Therefore, immunoprecipitates of several transforming and nontransforming *v-sis* gene products were treated with endoglycosidase F and analyzed by SDS-PAGE. The mobilities of both the transforming and nontransforming *v-sis* gene products were altered in a man-

ner consistent with the presence of an N-linked oligosaccharide (Fig. 5). As in the case of the *v-sis*^{wt} gene product, the oligosaccharide added to the mutant *v-sis* gene products was of the high-mannose type, as indicated by its sensitivity to endoglycosidase H (data not shown).

DISCUSSION

We constructed a series of mutants with deletions in the *v-sis* gene which defined the C terminus of the biologically active *v-sis* gene product. Deleted *v-sis* genes which encode truncated proteins that are 214 amino acids long or longer are fully active when they are assayed for focus formation in NIH 3T3 cells. However, the removal of only 10 more residues from the C terminus of the *v-sis* gene product results in the loss of biological activity, suggesting that these residues possess an important feature for transformation.

The principal difference between the gene products encoded by the transforming and nontransforming mutants of

FIG. 4. Immunoprecipitation of the *v-sis*²¹⁴ and *v-sis*²⁰⁴ gene products. COS-1 cells were transfected with vector DNA alone (lane 1), with the *v-sis*^{wt} gene (lane 2), with the *v-sis*²¹⁴ gene (lane 3), or with the *v-sis*²⁰⁴ gene (lane 4). Lysates were labeled with [³⁵S]cysteine and [³⁵S]methionine and immunoprecipitated with antiserum raised against the bacterially synthesized *v-sis* gene product, and the immunoprecipitates were analyzed under reducing (A) or nonreducing (B) SDS-PAGE conditions. The arrows indicate the positions of the PDGF-related 32-, 25-, and 24-kDa proteins in panel A and the respective dimers in panel B.

the *v-sis* gene is the failure of the nontransforming gene products to dimerize. In most other respects, including translocation across the rough endoplasmic reticulum and glycosylation, the nontransforming *v-sis* gene products are indistinguishable from the transforming *v-sis* gene products. An additional difference between the transforming and nontransforming gene products is the lack of proteolytic processing of the nontransforming gene products. However, a mutant of the *v-sis*^{wt} gene which lacks the basic dipeptide at residues 110 and 111 retains full biological activity (submitted for publication), indicating that the lack of proteolytic

processing by the nontransforming deletion mutants reported here does not explain their lack of biological activity. Our results strongly suggest that the loss of biological activity is solely due to the failure to dimerize. This conclusion is supported by the previous demonstration that both PDGF and the *v-sis*^{wt} gene product lose their mitogenic activity after reduction (20).

The *v-sis* gene product contains a total of 10 cysteine residues. Of these 10, cysteine residue 28 is encoded by the *env*-derived sequences of the *v-sis* gene. This residue is removed during signal sequence cleavage and probably does not contribute to the dimerization of the *v-sis* gene product. The next eight cysteine residues are located within the PDGF-homologous portion of the *v-sis* gene product, extending from residue 112 to residue 220. The tenth cysteine residue is located outside this region at residue 256. Our results indicate that this C-terminal cysteine residue is not required for dimerization, since all of the mutant *v-sis* gene products which have C termini between residue 246 and 214 are capable of forming dimers under nonreducing conditions. These mutant *v-sis* gene products are also fully active in transformation. However, cysteine residues 208 and 210, which are the eighth and ninth cysteine residues (counting from the N terminus of the predicted full-length gene product), are located within the 10 residues which separate the transforming *v-sis*²¹⁴ gene product from the nontransforming *v-sis*²⁰⁴ gene product as the sequence Cys-Lys-Cys (Fig. 6). The failure of the *v-sis*²⁰⁴ gene product to dimerize suggests that one or both of these cysteine residues are involved in the formation of interchain disulfide bonds. The mere presence of a cysteine residue in the vicinity of the C terminus of the *v-sis* gene product is not sufficient for dimerization, since the *v-sis*^{177/239} gene product, which contains an internal deletion of 62 amino acids, also fails to dimerize. This gene product contains all of the cysteine residues found in the *v-sis*^{wt} gene product except cysteine residues 208 and 210. These cysteine residues clearly are crucial for the formation of proper disulfide bonds in the *v-sis* gene product. Several questions regarding the importance of these cysteine residues for proper folding remain. Are both cysteine residues 208 and 210 involved in the formation of interchain disulfide bonds, and are both required for dimerization, or is one sufficient? With which other cysteine residues do these residues form disulfide bonds? What is the importance of local secondary structure for the formation and stability of the proper disulfide bonds? Site-directed mutagenesis of these cysteine residues should allow these and other questions to be answered.

The *v-sis* gene product is proteolytically processed at a Lys-Arg dipeptide (residues 110 and 111), resulting in the formation of a PDGF-related 20-kDa protein (21). This protein contains 8 of the 10 cysteine residues and is capable of dimerization under nonreducing conditions. We found that the gene products encoded by the deletion mutants of the *v-sis* gene are also proteolytically processed at this dipeptide. It is interesting that only the transforming gene products are proteolytically processed at this dibasic residue. Although processing at this dipeptide is not required for transformation, the smaller PDGF-related forms of the *v-sis* gene product resulting from this processing event are presumably biologically active in transformation, since they retain the mitogenic activity of the *v-sis* gene product. This allows definition of the minimal transforming region of the *v-sis* gene, which consists of 103 amino acids extending from residue 112 to residue 214 (Fig. 6). A total of six residues can be removed from the C terminus of the PDGF-homologous

FIG. 5. Glycosylation of the mutant *v-sis* gene products. COS-1 cells were transfected with vector DNA alone (lanes 1 and 2), the *v-sis*^{wt} gene (lanes 3 and 4), the *v-sis*²³⁹ gene (lanes 5 and 6), the *v-sis*²²³ gene (lanes 7 and 8), the *v-sis*¹⁷⁷ gene (lanes 9 and 10), or the *v-sis*^{177/239} gene (lanes 11 and 12). Lysates were labeled with [³⁵S]cysteine and [³⁵S]methionine, immunoprecipitated with antipeptide serum, and not treated (lanes 1, 3, 5, 7, 9, and 11) or treated (2, 4, 6, 8, 10, and 12) with endoglycosidase F. Immunoprecipitates were analyzed by SDS-PAGE under reducing conditions. The arrows indicate the positions of the glycosylated forms of the *v-sis*-encoded 28-, 26-, 19-, and 23-kDa proteins which are present in lanes 3, 5, 7, 9, and 11.

region of the *v-sis* gene product with no loss of biological activity.

The *v-sis* gene product has been reported to undergo proteolytic processing at its C terminus, resulting in the formation of a PDGF-related protein which is detected under nonreducing conditions as a 24-kDa dimer (21). The site of C-terminal processing and its importance for transformation are not known. Furthermore, the nature of the subunits of this dimer is not known, since they have not been detected under reducing conditions. The size of the fully processed *v-sis* gene product, which presumably is composed of two 12-kDa subunits, is approximately the same size as the gene product encoded by the minimal transforming region defined by the *v-sis*²¹⁴ gene. If this is the case, it suggests that cysteine residues 208 and 210 are retained after C-terminal processing. This would agree with the detection of this form

of the *v-sis* gene product under nonreducing conditions. However, the failure to detect the subunits which compose the 24-kDa dimer under reducing conditions raises the possibility that this protein is not derived from C-terminal processing of the *v-sis* gene product, but is a result of some other type of modification or is unrelated to the *v-sis* gene product altogether. The nature of the proposed proteolytic event which results in the formation of the 24-kDa dimer is also not known. There are no basic dipeptide residues in the vicinity of cysteine residues 208 and 210 that could be a possible site(s) of proteolytic processing. Our failure to detect a C-terminal processed *v-sis* gene product in COS-1 cells is further evidence that this processing event does not occur at basic dipeptide residues, since COS-1 cells do possess the enzymes required for proteolysis at basic dipeptides (28). However, it is possible that the C-terminal proc-

M L L T S S L H H P R H Q M S P G S W K K L I I L L S ^{*}C V P 30
 G G G G T S L Q N K N P H Q P M T L T W Q G D P I P E E L Y 60
 K M L S G H S I R S F D D L Q R L L Q G D S G K E D G A E L 90
 D L N M T R S H S G G E L E S L A R G K R S L G S L S V A E 120
 P A M I A E ^{*}C K T R T E V G E I S R R L I D R T N A N F L V 150
 W P P ^{*}C V E V Q R ^{*}C S G ^{*}C ^{*}C N N R N V Q ^{*}C R P T Q V Q L R P 180
 V Q V R K I E I V R K K P I F K K A T V T L E D G L A ^{*}C ^{*}C 210
 E I V A A A R A V ⁺T R S P G T S Q E Q R A K T T Q S R V T I 240
 R T V R V R R P P K G K H R K ^{*}C K H T H D K T A L K E T L G A 271

FIG. 6. Minimal transforming region of the *v-sis* gene product. The entire amino acid sequence of the *v-sis* gene product is given by using the single-letter code. The signal sequence residues of the *v-sis* gene product are also indicated with italics. The minimal transforming region of the *v-sis* gene product, defined in this study as amino acid residues 112 to 214, is shown in large letters; the rest of the sequence is shown in small letters. The cysteine residues are indicated by asterisks. The C terminus of chain B of PDGF from amino acid sequence data (15) is indicated by the arrow above residue 220. The single-letter amino acid code used is: A, alanine; R, arginine; N, asparagine; D, aspartic acid; C, cysteine; Q, glutamine; E, glutamic acid; G, glycine; H, histidine; I, isoleucine; L, leucine; K, lysine; M, methionine; F, phenylalanine; P, proline; S, serine; T, threonine; W, tryptophan; Y, tyrosine; V, valine.

essing of the *v-sis* gene product represents a novel proteolytic event. The nature and importance of the putative C-terminal processed form of the *v-sis* gene product will require further investigation.

The C-terminal amino acid sequences of some of the mutant *v-sis* gene products are different from the predicted amino acid sequence of the *v-sis*^{wt} gene product as a result of different reading frames in the termination oligonucleotide (Table 1). However, two lines of evidence indicate that these extra C-terminal amino acids have little or no effect on the biological activity of the deleted *v-sis* genes. First, some deletions in the *v-sis* gene which retained the same number of *v-sis*-encoded amino acids but utilized different TGA codons of the linker were found to have the same biological activity. Second, the biological activity of the *v-sis* deletions followed a clear pattern which correlated only with the extent of the deletion itself.

A secreted form of the *v-sis* gene product has been identified in conditioned media of SSV-transformed cells as a 20-kDa protein (6) or a 17-kDa protein (20). The transforming *v-sis* deletion mutants described here also secreted a PDGF-related protein into the media. The secreted form most likely results from N-terminal processing at residues 110 and 111, but is not processed at the C terminus. The

secretion of a PDGF-related protein from the transforming *v-sis* deletions is supported by our finding of a mitogenic activity in the conditioned media of COS-1 cells that express the transforming *v-sis* genes.

The best correlation that we have found between the biological properties of the *v-sis* deletion mutants and the physical properties of the encoded proteins is between transformation and dimerization. The connection between dimerization and transformation has recently been described on the basis of two mutants with mutations in the *v-sis* gene which encode truncated gene products that differ in size by 50 amino acids (17). Our data support previous work with both PDGF and the *v-sis* gene product, which showed that mitogenic activity was lost upon reduction (20). These results strengthen the relationship between the two biological properties of the *v-sis* gene product, transformation and mitogenesis. However, why the mitogenic activity of the *v-sis* gene product results in transformation while the mitogenic activity of PDGF does not is not known. Both the *v-sis* gene product and PDGF are able to stimulate autophosphorylation of the cell surface PDGF receptor (13, 19, 20, 25). However, it has recently been proposed that the *v-sis* gene product is able to interact with the PDGF receptor inside the cell, perhaps during transport through the Golgi

apparatus and exocytotic vesicles (11, 14). Alternatively, it is possible that the *v-sis* gene product, since it lacks subunit A of PDGF, has a different effect on the PDGF receptor than PDGF does. In either case, it is clear from our results that dimerization of the *v-sis* gene product as a B-B homodimer is required for transformation.

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