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Identification of a Signal for Nuclear Targeting in Platelet-Derived-Growth-Factor-Related Molecules

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The v-*sis* gene encodes p28^{sis}, the transforming protein of simian sarcoma virus. This gene resulted from a fusion of the *env* gene of simian sarcoma-associated virus and the woolly monkey gene for the B chain of platelet-derived growth factor (PDGF). Previous work has shown that the v-*sis* gene product undergoes signal sequence cleavage, glycosylation, dimerization, and proteolytic processing to yield a secreted form of the protein. If transport across the endoplasmic reticulum is blocked by the introduction of a charged amino acid residue within the signal sequence, the protein does not dimerize, is not secreted, and is no longer transforming as assayed by focus-forming ability in NIH 3T3 cells. Instead, this mutant protein localizes to the nucleus as demonstrated by both indirect immunofluorescence and cell fractionation. Using a series of deletion mutations, we delimited an amino acid sequence within this protein which is responsible for nuclear localization. This region is completely conserved in the predicted human c-*sis* protein, although it lies outside of regions required for transformation by the v-*sis* gene product. This nuclear transport signal is contained within amino acid residues 237 to 255, RVTIRTVRVRRPPKGKHRK. An amino acid sequence containing these residues is capable of directing cytoplasmic v-*sis* mutant proteins to the nucleus. This sequence is also capable of directing less efficient nuclear transport of a normally cytoplasmic protein, pyruvate kinase. Pulse-chase experiments indicate that the half-lives of nuclear and cytoplasmic v-*sis* mutant proteins are approximately 35 min. Using the heat-inducible *hsp70* promoter from *Drosophila melanogaster*, we showed that the nuclear v-*sis* protein accumulates in the nucleus within 30 min of induction. The identification of a nuclear transport signal in the v-*sis* gene product raises interesting questions regarding the possibility of some function for PDGF or PDGF-related molecules in the nucleus.

The v-*sis* oncogene codes for the transforming protein of simian sarcoma virus (SSV), an acutely transforming retrovirus which was originally isolated from a woolly monkey fibrosarcoma (9, 56). SSV acquired this gene as the result of a recombination event involving sequences of simian sarcoma-associated virus and the monkey genome. These sequences were acquired at the expense of a portion of the viral *env* gene, resulting in the formation of an *env-sis* open reading frame of 813 nucleotides, encoding a protein of 271 amino acids (9). Nucleotide sequence analysis of the v-*sis* gene has shown that the predicted protein is homologous to the B chain of platelet-derived growth factor (PDGF) (12, 28, 57). The v-*sis* protein produced in SSV-transformed cells is immunologically related to PDGF and has comparable mitogenic activity, thus indicating a functional similarity between the two proteins (8, 47). The nascent v-*sis* gene product contains a signal peptide sequence located near its amino terminus. This signal sequence, which is derived from the *env* gene of the parental retrovirus, is required for the transforming activity of the v-*sis* protein (19). The signal sequence directs the cotranslational translocation of the nascent polypeptide across the membrane of the endoplasmic reticulum (ER). After signal sequence cleavage occurs, glycosylation and dimerization proceed within the ER (20, 48). This is followed by a proteolytic processing event at a basic dipeptide, Lys-Arg, residues 110 and 111 (47). This processing event, however, is not required for the activity of the v-*sis* protein. Mutations encoding proteins lacking this processing site are as biologically active as the wild-type protein (20, 49). Further processing of the v-*sis* protein has also been reported to occur at the C terminus (47).

It is presumed that the v-*sis* protein transforms cells via an autocrine mechanism by which cells produce a growth factor, as well as express the receptor for that growth factor (23, 54). Autocrine stimulation would lead to chronic activation of PDGF receptors and continuous production of growth signals. PDGF-related proteins have been found in conditioned medium of SSV-transformed cells, indicating that the v-*sis* protein is secreted from these cells (8, 16, 25, 40). Conditioned medium from these cells is able to initiate autophosphorylation of the PDGF receptor (16, 25, 40), thus supporting an autocrine mechanism of transformation. Both the intracellular and secreted forms of the v-*sis* protein are mitogenic for NIH 3T3 cells (8, 16, 25, 40). Thus, stimulation of PDGF receptors may occur at the cell surface or within cells expressing the v-*sis* protein. The interaction of v-*sis* protein with the PDGF receptor most likely occurs extracellularly, since transport to the cell surface has been correlated with transformation by membrane-anchored v-*sis* proteins (21). Also, antibodies against PDGF can inhibit acute transformation by SSV, suggesting that cell surface stimulation of the receptor is required for transformation (26).

We have previously reported that sequences encoding a functional signal peptide are required for the biological activity of v-*sis* (19). When sequences encoding the signal peptide are deleted or altered by the introduction of codons for charged amino acid residues, the resulting v-*sis* mutations no longer transform as assayed by focus formation in NIH 3T3 cells (20). Proteins encoded by these mutations do not enter the lumen of the ER, do not dimerize, and are not secreted from cells. When the subcellular localization of these proteins is examined by indirect immunofluorescence, the proteins are shown to accumulate in the nucleus.

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It has become apparent that many karyophilic proteins contain discrete amino acid sequences responsible for nuclear localization (6, 10, 11, 17, 18, 30, 31, 34, 38, 46, 51, 52). These sequences are sufficient to transport proteins to the nucleus at rates in excess of that expected from diffusion (10, 41, 42), thus suggesting the existence of some specific transport mechanism. Several nuclear transport signals (NTSs) have been characterized. These include the 13 N-terminal amino acids of *Saccharomyces cerevisiae MATα2* (18), the 74 N-terminal amino acids of *S. cerevisiae GAL4* (51), and the 21 N-terminal amino acids of *S. cerevisiae* ribosomal protein L3 (38). These NTSs are capable of directing β-galactosidase to the nucleus when NTS–β-galactosidase fusion proteins are produced. The simian virus 40 (SV40) large-T antigen contains an NTS composed of a short stretch of basic amino acids: PKKKRKV (31). This amino acid sequence can efficiently transport a pyruvate kinase (PK) fusion protein to the nucleus. The polyomavirus large-T antigen has been reported to contain two NTSs (46). Only one of these (VSRKRPA) is sufficient to transport a fusion protein to the nucleus, while the other (PKKARED) is only functional in the context of polyomavirus large-T antigen lacking the first NTS. Recently, short synthetic peptides containing the SV40 large-T antigen NTS have been cross-linked to cytoplasmic proteins of various sizes and shown to cause specific nuclear accumulation of these proteins in a dose-dependent manner (17, 34). These reports suggest that some type of receptor-mediated phenomenon is involved in nuclear transport.

In the present study, the nuclear form of the v-*sis* protein was examined for the presence of an NTS. Several deletions were constructed in the v-*sis* gene, and the encoded proteins were examined for subcellular localization in COS-1 cells by indirect immunofluorescence and cell fractionation. The data presented here demonstrate that specific nuclear transport is due to the presence of an NTS in the C-terminal region of the v-*sis* protein. This region is completely conserved between the v-*sis* protein and the predicted human c-*sis* protein. A sequence which encompasses this NTS (amino acid residues 237 to 256) is capable of targeting cytoplasmic v-*sis* mutant proteins to the nucleus. This sequence can also direct less efficient nuclear transport of a known cytoplasmic protein, PK. Experiments in which synthesis of the nuclear v-*sis* protein was rapidly induced demonstrated that this nuclear accumulation occurs quickly, thus suggesting the use of a nuclear transport system. That the v-*sis* gene product contains an NTS suggests the possibility of some function for v-*sis* or related molecules in the nucleus.

MATERIALS AND METHODS

Construction of plasmids. The v-*sis* mutations described in this paper were transiently expressed in COS-1 cells by using the SV40-derived expression vector pJC119 (55). This vector provides high levels of expression from the SV40 late promoter. pJC119-derived plasmids containing deletions in the region encoding the C terminus of the v-*sis* protein have been described previously (22). Plasmids pMH74 and pMH101, which contain point mutations in the sequence encoding the signal peptide of the v-*sis* protein, have also been described (20). To construct plasmids encoding truncated proteins containing both mutated signal sequences and truncated C termini, the large *Cla*I-*Bgl*II fragments of pMH74 or pMH101 were ligated with the small *Bgl*II-*Cla*I fragments from pJC119-derived clones containing the C-terminal deletions described previously. This resulted in plasmids pDM01, pDM02, pDM05, pAL00, pAL01, and pAL42.

TABLE 1. Signal sequence mutant classes and amino acid sequences at C termini of deletion mutations

Plasmid	Signal sequence mutation[a]	v-*sis* protein amino acid endpoint[b]	C-terminal amino acid sequence[c]
pMH74	E	271	wt
pDM07	E	271	wt
pDM06	E	271	wt
pAL104	E	271	wt
pAL28	E	264	QSVKLD
pAL23	E	260	QSVKLD
pAL25	E	256	INQSSLTD
pAL30	E	256	SISQA
pAL35	E	255	SISQA
pAL27	E	249	QSVKLD
pAL42	E	246	QSVKLD
pAL26	E	240	QSVKLD
pAL01	E	239	wt
pAL00	E	234	SISQA
pDM05	E	223	wt
pDM02	R	200	QSVKLD
pDM01	R	181	LNQSSLTD

[a] Mutation-encoded proteins contain either a valine-to-glutamic-acid substitution (E) within the v-*sis* protein signal peptide at amino acid position 29 or a leucine-to-arginine substitution (R) at amino acid position 25.

[b] Indicated is the position of the last v-*sis*-encoded amino acid for each deletion mutation.

[c] The amino acid sequence at the C terminus of each deletion mutation is given. The amino acids shown are those contributed by the termination linker described in Materials and Methods. wt, No termination linker contributed amino acids.

Additional C-terminal deletions were constructed by using BAL 31 exonuclease as previously described (22). Briefly, pMS001 (22) was digested at its unique *Bam*HI site and blunted with the Klenow fragment of *Escherichia coli* DNA polymerase I in the presence of deoxynucleoside triphosphates, and BAL 31 exonuclease was used to create deletions in the 3′ portion of the v-*sis* coding region. The BAL 31 digestion was then halted by phenol extraction, and the DNA ends were again blunted with the Klenow fragment, after which a self-complementary 26-mer *Hin*dIII linker (TCAATCAGTCAAGCTTGACTGATTGA) encoding termination codons in three reading frames (2) was added by ligation. Clones were sequenced by the method of Maxam and Gilbert to determine exact nucleotide endpoints (37). The small *Bgl*II-*Apa*I fragments containing the deleted 3′ regions of these clones were ligated into the large *Bgl*II-*Apa*I fragment from pMH74 to provide sequences encoding a mutant signal sequence. This resulted in plasmids, pAL23, pAL25, pAL26, pAL27, pAL28, pAL30, and pAL35. The proteins encoded by these C-terminal deletions have different C termini, depending on the reading frame into the termination linker. The different C termini, as well as the signal sequence mutations, used are summarized in Table 1.

The internal deletions pDM06, pDM07, and pAL104 were constructed as follows. pDM06 was created by ligating the small *Bgl*II-*Cla*I fragment of pMH123 to the large *Cla*I-*Bgl*II fragment of pMH74. pMH123 is a pJC119 derivative containing v-*sis* sequences derived from pMH66, previously described (22). pDM07 was created by digesting pMH74 with *Bgl*II and *Bst*EII, blunting the ends with Klenow fragment, and ligating the blunted ends together. pAL104 was created by partial *Eco*RI digestion of pMH74, followed by digestion with *Bgl*II, blunting of the ends with Klenow fragment, and

ligation of the blunted ends together. These constructs preserve the reading frame across their junctions.

To isolate DNA sequences coding for amino acids contained in region 2, an intermediate plasmid, pAL37, was created by digesting pAL12 at its unique *Bst*EII site, blunting the ends with Klenow fragment, and ligating in a *Bam*HI 10-mer linker (CCGGATCCGG). Plasmid pAL12 is a pBR322 subclone of the deletion contained in pAL25 (see Fig. 1). Plasmid pAL37 was then digested with *Hin*dIII and filled in with Klenow fragment, and three different *Bam*HI linkers were ligated in, yielding pAL38 (resulting from insertion of a CGGATCCG linker), pAL39 (CCGGATCCGG linker), and pAL40 (CGCGGATCCGCG linker). These plasmids contain the putative NTS coding region (encoding amino acids 237 to 256) flanked at the 3' end by *Bam*HI sites in three different reading frames. To construct the v-*sis* clones which were targeted to the nucleus, pAL26 (which encodes a cytoplasmic form of the v-*sis* protein) was digested with *Bgl*II, and the *Bam*HI fragment containing sequences encoding the NTS from pAL40 was ligated into the *Bgl*II site, giving pAL46. Plasmid pAL26 was also digested at the *Bal*I site, and a *Bam*HI linker (CCGGATC CGG) was ligated in, yielding pAL47. A *Bam*HI partial digest was then performed on pAL47, and the *Bam*HI fragment containing sequences encoding the NTS from pAL39 was inserted at the newly created *Bam*HI site in pAL47, yielding pAL91. Plasmid pDM57 was created in a manner identical to that used for pAL46, except that a further deleted v-*sis* gene contained in plasmid pDM01 was used as the recipient plasmid instead of pAL26.

The v-*sis* NTS-PK fusions were constructed by using the parent plasmid XR30PK (31). The fusions were constructed by digestion of XR30PK with *Bam*HI, blunting of the ends with Klenow fragment, and religation to destroy the unique *Bam*HI site in XR30PK. This plasmid was then digested with *Eco*RI to remove sequences encoding the SV40 large-T antigen NTS and blunted with Klenow fragment, and a *Bam*HI 10-mer linker (CCGGATCCGG) was added. Sequences encoding the NTS flanked by *Bam*HI sites were isolated from pAL40 and inserted into this new *Bam*HI site to produce NTS-PK fusions with one (pAL103) and two (pAL101) copies of the NTS. A plasmid encoding PK without an NTS (pAL79) was created by digesting XR30PK with *Eco*RI, blunting the ends with Klenow fragment, and adding an *Xho*I 10-mer linker (GCCTCGAGGC) to preserve the reading frame across the junction. In all cases, preservation of the reading frame was confirmed by nucleotide sequencing.

To place the gene encoding the nuclear form of the v-*sis* protein under control of the *Drosophila melanogaster* heat shock promoter, the *hsp70* promoter was obtained as a *Sal*I-to-*Hin*dIII fragment in plasmid pHAP (a gift from Hugh R. B. Pelham [44]). The *Sal*I site was changed to a *Cla*I site by ligation of a *Cla*I 8-mer linker (CATCGATG) to a filled-in *Sal*I site, yielding pMH118. A *Cla*I (sticky)-*Hin*dIII (blunt) fragment from pMH118 (containing the *hsp70* promoter) was ligated to the *Cla*I-*Bst*EII fragment from pMH29 (containing the 3' region of the v-*sis* gene) and a *Bst*EII (sticky)-BamHI (blunt) fragment from pAL20 (containing the 5' region of the v-*sis* gene with sequences encoding the mutated signal peptide originally obtained from pMH74), resulting in pAL66. Plasmid pAL66 thus contains the v-*sis* gene from pMH74 under control of the *hsp70* promoter. Plasmid pMH141 contains the wild-type v-*sis* protein under control of the *hsp70* promoter on a plasmid containing the SV40 origin of replication. The small *Xba*I-*Sst*I fragment from pAL66 was ligated with the large *Xba*I-*Sst*I fragment of pMH141, resulting in pAL68. Plasmid pAL68 thus contains the v-*sis* gene from pMH74 under control of the *hsp70* promoter and additionally contains the SV40 origin to allow replication in COS-1 cells.

Biosynthetic labeling, cell fractionation, and pulse-chase analysis. Semiconfluent monolayers of COS-1 cells were grown at 37°C on 60-mm (diameter) dishes in Dulbecco modified Eagle medium containing 10% calf serum, 100 U each of penicillin G and streptomycin per ml, and 2.5 μg of amphotericin B (Fungizone) per ml. DNA was introduced into these cells by the DEAE-dextran method as previously described (22). At 48 h posttransfection, cells were labeled with 100 μCi each of [^{35}S]cysteine and [^{35}S]methionine (>1,000 Ci/mmol) in Dulbecco modified Eagle medium lacking cysteine and methionine.

For cell fractionation experiments, cells were labeled for 2 h, washed three times with VE (0.5 mM EDTA, 140 mM NaCl, 2.7 mM KCl, 5.1 mM Na_2HPO_4, 1.3 mM KH_2PO_4 [pH 7.4]) and incubated with lysis buffer (10 mM Tris [pH 7.6], 150 mM NaCl, 5 mM $MgCl_2$, 1% Aprotinin, 1% Nonidet P-40) for 5 min on ice. Nuclei were pelleted at 300 × *g* for 7 min, and the supernatant was removed. Sodium deoxycholate to 1% and sodium dodecyl sulfate (SDS) to 0.2% were added to the supernatant, which was then centrifuged for 5 min in a microcentrifuge to clarify the cytoplasmic fraction. The pelleted nuclei were washed three times with lysis buffer and then suspended in 50 μl of 0.5% SDS to disperse the nuclear pellet. To this suspension was added 0.5 ml of RIPA buffer (10 mM sodium phosphate [pH 7.0], 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 1% Aprotinin). The cytosolic and nuclear fractions were then immunoprecipitated with antisera raised in rabbits against the bacterially synthesized v-*sis* gene product as previously described (24). Immunoprecipitated proteins were then subjected to SDS-(15%) polyacrylamide gel electrophoresis (SDS-PAGE), followed by fluorography and autoradiography to visualize radioactively labeled proteins.

For pulse-chase experiment, cells were labeled for 30 min with [^{35}S]cysteine and [^{35}S]methionine as described above and then chased with cold medium for 0, 1, or 2 h, followed by lysis in RIPA buffer. Clarified lysates were then immunoprecipitated and analyzed by using SDS-PAGE as described above for cell fractionation.

Indirect immunofluorescence. COS-1 cells were grown on cover slips and transfected with appropriate plasmids encoding various proteins. At 48 h posttransfection, cells were fixed for 10 min at room temperature with 3% paraformaldehyde in phosphate-buffered saline (10 mM sodium phosphate, 150 mM NaCl [pH 7.4]), washed with 100 mM glycine in phosphate-buffered saline, and then permeabilized with 0.5% Triton X-100–300 mM sucrose in phosphate-buffered saline. Proteins were visualized by using the rabbit anti-*sis* serum mentioned above, followed by treatment with rhodamine-conjugated goat anti-rabbit immunoglobulin G. Over 35 positive cells from each transfection were examined for protein subcellular localization.

RESULTS

Sequences required for nuclear localization of the v-*sis* gene product. We have previously described mutations which encode proteins with alterations in the signal peptide of the v-*sis* gene product (20). Two of these mutations are nontransforming as a result of the protein products not being translocated across the membrane of the ER. The proteins

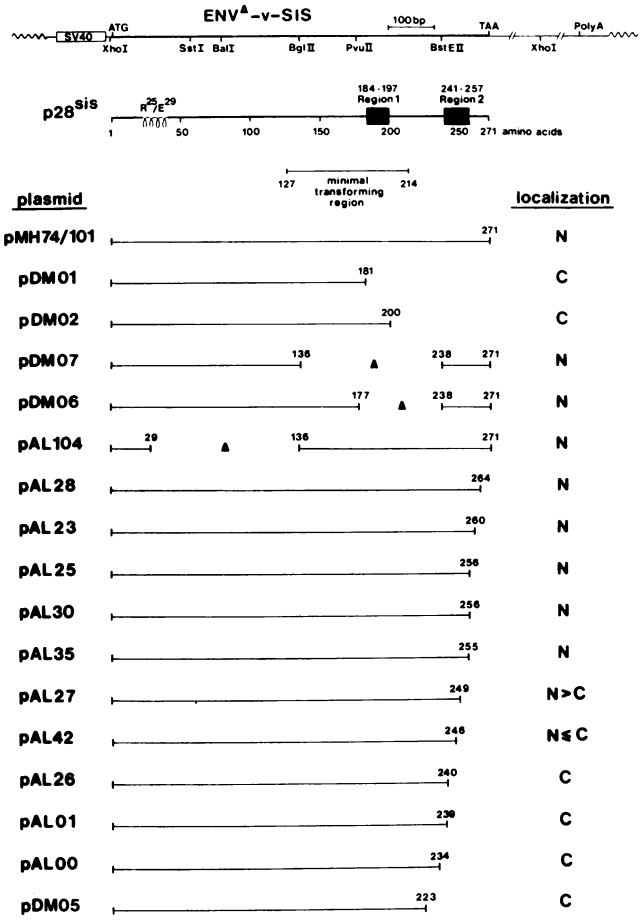

FIG. 1. Deletion mutations of v-*sis* identify a C-terminal basic region as an NTS. A series of deletion mutations in the v-*sis* gene was constructed as described in Materials and Methods. These mutant genes were put under the control of the SV40 late promoter and transiently expressed in COS-1 cells by using the SV40-derived expression vector pJC119 (55). The wild-type v-*sis* gene and expression vector are represented schematically at the top of the figure. Initiation and termination codons are noted, as well as unique restriction enzyme sites, the SV40 late promoter, and the polyadenylation signal. Also represented is the full-length v-*sis* protein $p28^{sis}$. The helix represents the signal peptide sequence, with two point mutations indicated. The black boxes indicate short stretches of basic amino acids. The minimal transforming region, as previously determined by N- and C-terminal deletions (22, 49), is indicated. Also represented are the protein products of all of the deletion mutations. Amino acid endpoints are indicated and represent the last v-*sis*-encoded amino acids. Subcellular localization was determined by indirect immunofluorescence. N represents predominantly nuclear accumulation, while C indicates cytoplasmic localization. N ≤ C represents a range of localization from equal nuclear and cytoplasmic localization to cytoplasmic localization alone. N > C represents nuclear accumulation with significant amount of additional cytoplasmic protein. bp, Base pairs.

encoded by these two mutations are not glycosylated, do not dimerize, and are no longer proteolytically processed as compared with the wild-type v-*sis* protein. These mutation-encoded proteins contain charged amino acid residues in place of hydrophobic residues within the signal peptide. The protein encoded by pMH74 contains a glutamic acid residue in place of a valine residue at amino acid position 29, while pMH101 encodes a protein which contains an arginine residue in place of a leucine residue at position 25 (Fig. 1). Indirect immunofluorescence of COS-1 cells expressing the wild-type v-*sis* protein showed perinuclear Golgi complex and reticular staining with an antibody directed against the v-*sis* gene product (Fig. 2A). When proteins encoded by pMH74 and pMH101 were examined by immunofluorescence, these proteins were found to localize to the nucleus and specifically seemed to accumulate in nucleoli (Fig. 2B).

A consensus NTS has not been defined. However, basic amino acids and proline residues appear to be common in many of the described NTSs (18, 30, 31, 46). With this in mind, it was possible to identify two regions of the v-*sis* gene product as candidates for an NTS. In Fig. 1, these are identified as regions 1 (residues 184 to 197: RKIEIVRK KPIFKK) and 2 (residues 241 to 257: RTVRVRRPPKGKH RKCK). To investigate the possible presence of an NTS in the v-*sis* gene product, we constructed a number of deletion mutations encoding truncated proteins. These mutations contain sequences encoding nonfunctional signal peptides obtained from either pMH74 or pMH101 (Table 1). The proteins encoded by these deletion mutations were assayed for subcellular localization by indirect immunofluorescence in COS-1 cells (Fig. 1 and 2). Several large deletions were first constructed to determine whether either region 1 or 2 was responsible for nuclear localization of the v-*sis* gene product. When sequences encoding residues 182 to 271 were deleted (pDM01), removing both regions 1 and 2, the resulting protein exhibited cytoplasmic staining which was characteristically punctate (Fig. 2I). When sequences encoding residues 201 to 271 were deleted (pDM02), removing region 2 but leaving region 1 intact, the resulting protein was still cytoplasmic in localization (Fig. 2H). These results suggested that region 1 is responsible for nuclear localization of the v-*sis* protein. These results also suggested that an amino acid sequence responsible for nuclear transport likely is contained between residue 200 and the C terminus of the protein.

To confirm that region 1 was not responsible for nuclear transport and to show the involvement of region 2, we constructed two mutations resulting in the deletion of internal portions of the v-*sis* protein. The protein encoded by pDM07 had residues 137 to 237 deleted, while the protein specified by pDM06 had residues 178 to 237 deleted (Fig. 1). Both of these internal deletions removed region 1 but left region 2 intact. When the resulting proteins were examined for subcellular localization by immunofluorescence, both accumulated in the nucleus (Fig. 2C). These results indicate that nuclear localization of the v-*sis* gene product is not due to region 1 and that region 2 is likely responsible for nuclear transport.

To further define the amino acid residues necessary for nuclear localization, additional deletion mutations were constructed by using BAL 31 exonuclease. These data are summarized in Fig. 1. Mutant proteins with C-terminal deletions up to residue 255 were nuclear in localization, while mutant proteins with C-terminal deletions up to and beyond residue 240 exhibited cytoplasmic staining by immunofluorescence. It should be noted that the staining observed for proteins showing nuclear accumulation varied from a diffuse pattern to one showing accumulation in what appeared to be nucleoli. This nucleolar designation was based on comparison of phase-contrast and immunofluorescence images (data not shown). The significance of this nucleolar accumulation is not known; however, it is presently being investigated in greater detail.

Although most mutant proteins displayed either predomi-

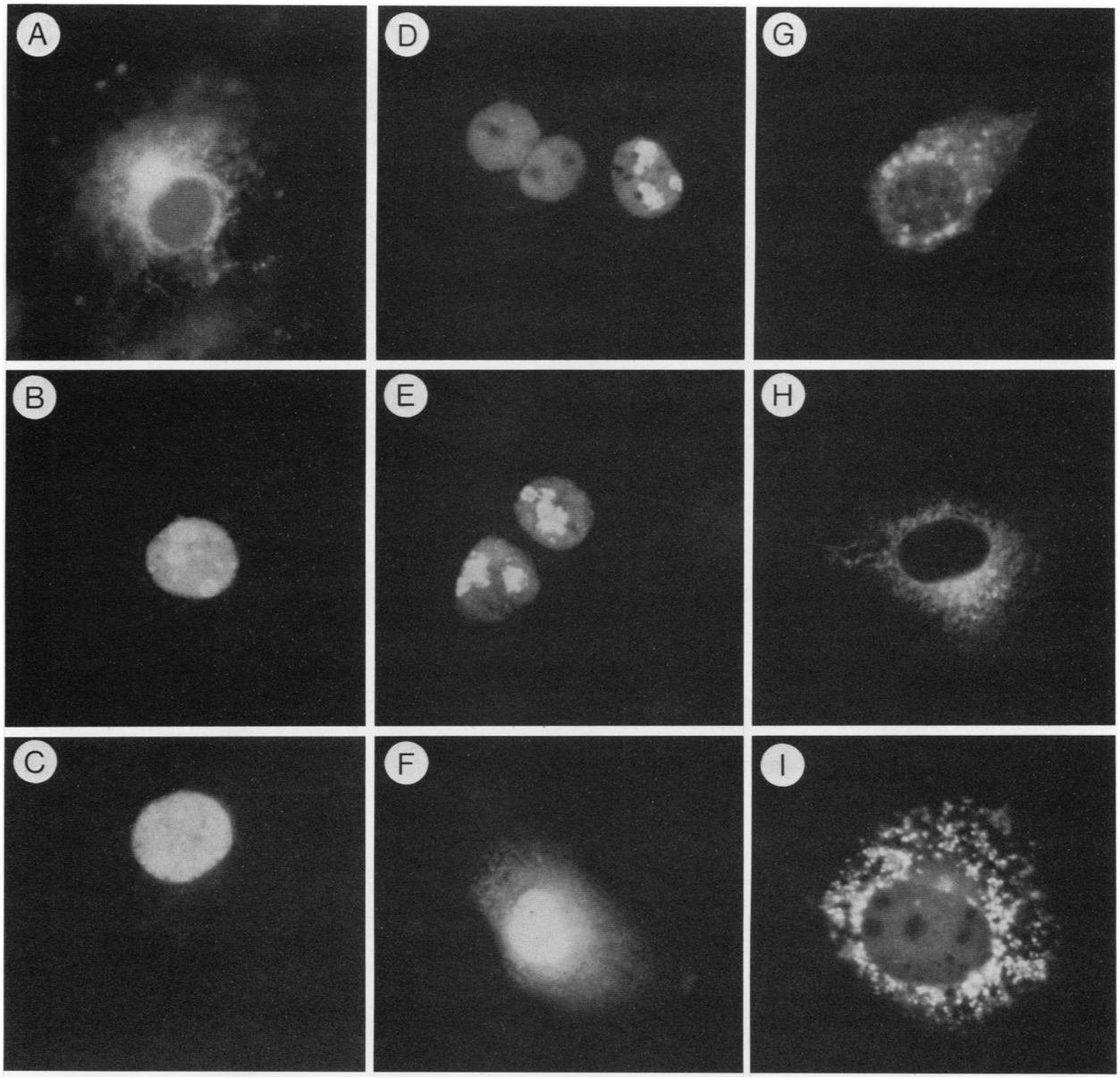

FIG. 2. Indirect immunofluorescence of mutant v-*sis* gene products. Deletion mutations of the v-*sis* gene were transiently expressed in COS-1 cells. Protein was detected by indirect immunofluorescence as described in Materials and Methods. Shown here are immunofluorescence micrographs of COS-1 cells transfected with the following (panel): A, pMH29, encoding the v-*sis* wild-type protein; B, pMH74; C, pDM07; D, pAL28; E, pAL30; F, pAL27; G, pAL01; H, pDM02; I, pDM01.

nantly nuclear or cytoplasmic localization, two showed mixed staining patterns. The protein encoded by pAL42 has a C-terminal endpoint at residue 246 and exhibited mixed nuclear and cytoplasmic staining. This staining ranged from cells showing equal nuclear and cytoplasmic staining to ones exhibiting only cytoplasmic staining (data not shown). The protein encoded by pAL27 has a C-terminal endpoint at residue 249 and exhibited nuclear staining with significant amounts of additional cytoplasmic staining (Fig. 2F). Presumably, these two deletions partially disrupt amino acid sequences responsible for nuclear transport, thus preventing complete nuclear accumulation of these proteins. The C-terminal deletion analysis results indicate that the C-terminal limit of the putative v-*sis* NTS lies between residues 240 and 256.

Cell fractionation confirms nuclear localization of the v-*sis* gene product. Cell fractionation was performed to confirm the subcellular localization observed by indirect immunofluorescence. COS-1 cells were transfected with plasmids encoding proteins which showed nuclear, cytoplasmic, or mixed localization by immunofluorescence. Two days post-transfection, cells were labeled with [^{35}S]methionine and [^{35}S]cysteine, lysed, and separated into nuclear and cytoplasmic fractions. Each fraction was then immunoprecipitated and analyzed by SDS-PAGE. The wild-type v-*sis* protein encoded by pMH29 fractionated with the cytoplasmic fraction which contained membranes of the ER, the Golgi apparatus, and other vesicles (Fig. 3, lane A2). This pro-*sis* protein, which has undergone N-linked glycosylation and signal sequence cleavage, is about 32 kilodaltons (kDa)

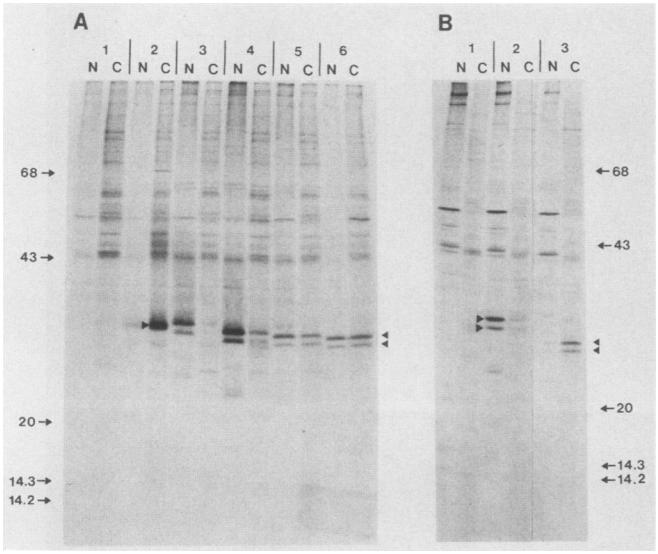

FIG. 3. Subcellular fractionation of nuclear and cytoplasmic forms of the v-*sis* gene product. COS-1 cells were transfected with plasmids encoding v-*sis* mutant proteins. At 48 h posttransfection, cells were labeled with [^{35}S]cysteine and [^{35}S]methionine, lysed, and fractionated into nuclear and cytoplasmic fractions. The fractions were immunoprecipitated with anti-*sis* serum and subsequently analyzed by SDS-PAGE. (A) Lanes: 1, mock transfection with vector pJC119; 2, pMH29, encoding the v-*sis* wild-type protein; 3, pMH74; 4, pAL35; 5, pAL27; 6, pAL42. (B) Lanes: 1, pJC119; 2, pMH74; 3, pAL26. A and B represent two separate fractionation experiments. N and C indicate nuclear and cytoplasmic fractions, respectively. Molecular mass markers (in kilodaltons) are indicated. The arrowheads indicate specifically immunoprecipitated v-*sis*-related protein.

in molecular mass. The proteins encoded by pMH74 and pAL35 were concentrated in the nuclear fraction as expected from the nuclear-nucleolar localization observed by immunofluorescence (Fig. 3, lanes A3, A4, and B2). Note the 33- and 31-kDa protein doublet shown in the nuclear fractions of Fig. 3, lanes A3 and B2. The 33-kDa protein represents the full-length v-*sis* gene product, including an uncleaved signal sequence, while the 31-kDa protein results from internal initiation at the second methionine codon of v-*sis*. This internally initiated protein was also observed with in vitro transcribed-translated v-*sis* (data not shown). Similar doublets of lower molecular mass were also observed for truncated v-*sis* proteins (Fig. 3, lanes A4, A5, A6, and B3). A protein synthesized from internal initiation at the second methionine was not observed for the wild-type protein (Fig. 3, lane A2). This is because the second methionine codon occurs before the signal sequence, and thus, such a protein would not be observed because of subsequent signal sequence cleavage. The protein encoded by pAL26, which is cytoplasmic by immunofluorescence, fractionated with the cytoplasm as expected (Fig. 3, lane B3). Proteins encoded by pAL27 and pAL42 showed fractionation profiles in which the proteins seemed to be equally distributed between the nuclear and cytoplasmic fractions (Fig. 3, lanes A5 and A6). This is consistent with the additional cytoplasmic staining observed for these proteins by immunofluorescence. However, we did not attempt to directly quantitate the amount of protein localized to different subcellular compartments by immunofluorescence. Thus, the cell fractionation data shown in Fig. 3 only support the results obtained by immunofluorescence in that both techniques show a mixed localization pattern for the proteins encoded by pAL27 and pAL42. The fractionation data also suggest that the nuclear v-*sis* protein is associated tightly with some component of the nuclear fraction, since repeated washing did not remove the nucleus-associated v-*sis* protein.

Cytoplasmic mutations of the v-*sis* gene product can be targeted to the nucleus by insertion of region 2 sequences. To further test the importance of region 2 in nuclear transport, sequences encoding residues 237 to 256 were fused to two mutations whose encoded proteins were cytoplasmic in localization. Three fusions were constructed by inserting these sequences at the *Bal*I and *Bgl*II sites of pAL26 and at the *Bgl*II site of pDM01. The proteins encoded by these fusions were assayed for subcellular localization in COS-1 cells by indirect immunofluorescence, and the results are summarized in Fig. 4. Whereas the proteins encoded by pAL26 and pDM01 showed cytoplasmic localization (Fig. 5A and 2I), all of the fusion proteins were found to be located in the nuclei and specifically seemed to accumulate in the nucleoli (Fig. 5B, C, and D). Fusion proteins with multiple copies of this amino acid sequence were also targeted to the nuclei (data not shown). The fusion protein encoded by pDM57 lacks region 1 and was localized to the nuclei. This supports the idea that region 2 alone is sufficient for nuclear transport and that region 1 has no role in nuclear localization of the v-*sis* protein. These results indicate that sequences encoding amino acid residues 237 to 256 of the v-*sis* gene product are capable of directing cytoplasmic forms of the v-*sis* protein to the nucleus.

Nuclear localization of PK by region 2 sequences. To further

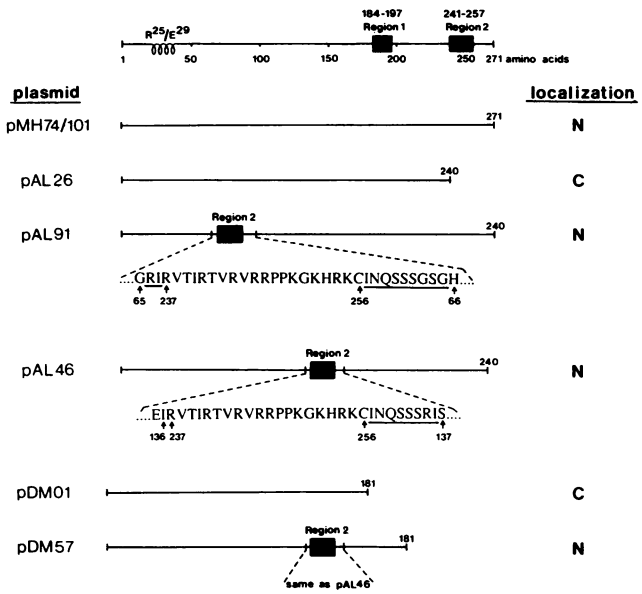

FIG. 4. Relocalization of cytoplasmic forms of the v-*sis* gene product by insertion of region 2 sequences. Cytoplasmic v-*sis* mutant proteins encoded by pAL26 and pDM01 were redirected to the nucleus by insertion of DNA sequences encoding region 2 amino acids 237 to 256. These DNA sequences were inserted into the *Bal*I and *Bgl*II sites of pAL26 and into the *Bgl*II site of pDM01 to give pAL91, pAL46, and pDM57, respectively. A schematic representation of the resulting fusion proteins is shown here. The amino acid sequence encoded by each insert is given. The underlined amino acids represent non-v-*sis*-encoded residues contributed by linkers. The subcellular localization of each fusion protein was determined by indirect immunofluorescence as described in Materials and Methods. N, Nuclear localization; C, cytoplasmic localization.

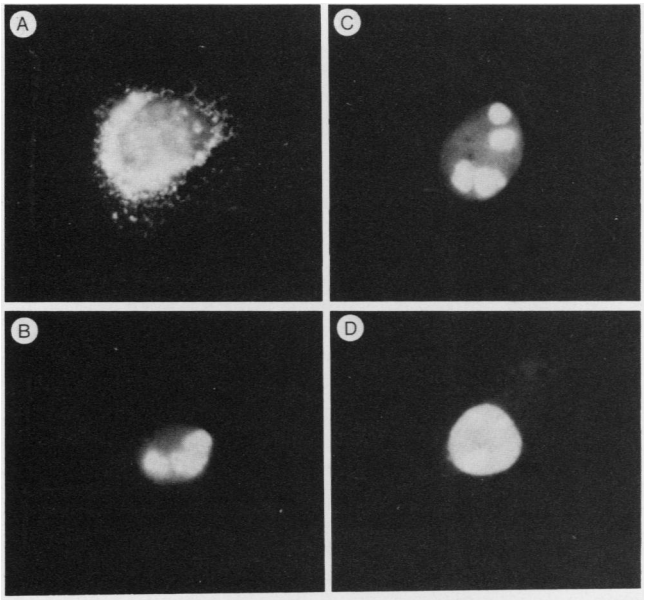

FIG. 5. Indirect immunofluorescence of relocalized v-*sis* proteins. COS-1 cells were transfected with the following (panel): A, pAL26; B, pAL91; C, pAL46; D, pDM57. pAL26 encodes a cytoplasmic v-*sis* mutant protein, while pAL91, pAL46, and pDM57 encode the fusion proteins depicted in Fig. 4. Protein was detected by indirect immunofluorescence as described in Materials and Methods.

test the putative v-*sis* NTS, we examined its ability to target a cytoplasmic protein to the nucleus. The chicken muscle PK gene has been cloned and sequenced and is known to encode a protein with a cytoplasmic location (36). Kalderon et al. have used the PK gene to produce fusion proteins containing the SV40 large-T antigen NTS and have shown that these fusion proteins are efficiently transported to the nuclei of Vero cells (31). The clone XR30PK encodes an SV40 NTS-PK fusion protein containing amino acid residues 126 to 135 of the large-T antigen (31). When this protein was expressed in COS-1 cells, strong nuclear localization was observed by immunofluorescence using an antiserum directed against chicken muscle PK (Fig. 6A). When sequences encoding the large-T antigen NTS were deleted (yielding pAL79), the protein was cytoplasmic in localization (Fig. 6B). To construct a v-*sis* NTS-PK fusion, sequences encoding amino acid residues 237 to 256 of the v-*sis* protein were ligated to sequences encoding cytoplasmic PK, starting at amino acid residue 17. A fusion protein which contained a single copy of the putative v-*sis* protein NTS showed primarily cytoplasmic staining, with a small pecentage of cells exhibiting an equal amount of nuclear and cytoplasmic staining (data not shown). Another fusion protein, encoded by pAL101, contained two copies of the sequence and exhibited primarily equal nuclear and cytoplasmic staining, with some cells (approximately 10%) showing mostly nuclear staining with accumulation in nucleoli (Fig. 6C and D). These results indicate that sequences encoding the putative NTS of the v-*sis* protein can partially target PK to the nuclei of COS-1 cells when present in multiple copies. Two copies of this NTS are necessary to direct PK to the nucleus, presumably because one copy is not efficiently exposed or folded properly for interaction with putative transport machinery.

Determination of the half-lives of nuclear and cytoplasmic forms of the v-*sis* protein. To determine the relative rates of turnover of nuclear and cytoplasmic forms of the v-*sis* protein, the half-lives of representative proteins were measured by pulse-chase analysis. COS-1 cells transfected with pAL35 (encoding a nuclear form of the v-*sis* protein) and pAL26 (encoding a cytoplasmic form of the v-*sis* protein) were labeled for 30 min, followed by chase periods with unlabeled medium for 0, 1, and 2 h. Cells were then lysed, the cell lysates were immunoprecipitated with anti-*sis* serum, and the recovered proteins were analyzed by SDS-PAGE. The cytoplasmic and nuclear proteins were found to have similar half-lives of approximately 35 min. (Fig. 7). This result demonstrates that preferential degradation or cleavage of the protein lacking the putative NTS is not responsible for its inability to migrate to the nucleus.

Kinetics of transport to the nucleus. To examine the rate of import of the nuclear v-*sis* protein, we took advantage of the ability of the *Drosophila hsp70* promoter to be rapidly induced under heat shock conditions. The full-length v-*sis* gene contained in pMH74 was put under control of the *hsp70* promoter on a plasmid containing an SV40 origin (yielding pAL68) to allow replication in COS-1 cells. This plasmid was then introduced into COS-1 cells and examined for inducible expression of the v-*sis* protein. Cells were heat shocked at 45°C for 10 min and allowed to recover at 37°C for various lengths of time before immunofluorescence was performed. Whereas mock-transfected cells showed no specific staining with anti-*sis* serum, cells transfected with pAL68 showed v-*sis* protein in the nuclei as soon as 30 min after induction (Table 2). This result indicates that nuclear transport of the v-*sis* protein was rapid and that some type of transport mechanism was in effect. Although some expressing cells showed predominantly nuclear accumulation of the v-*sis*

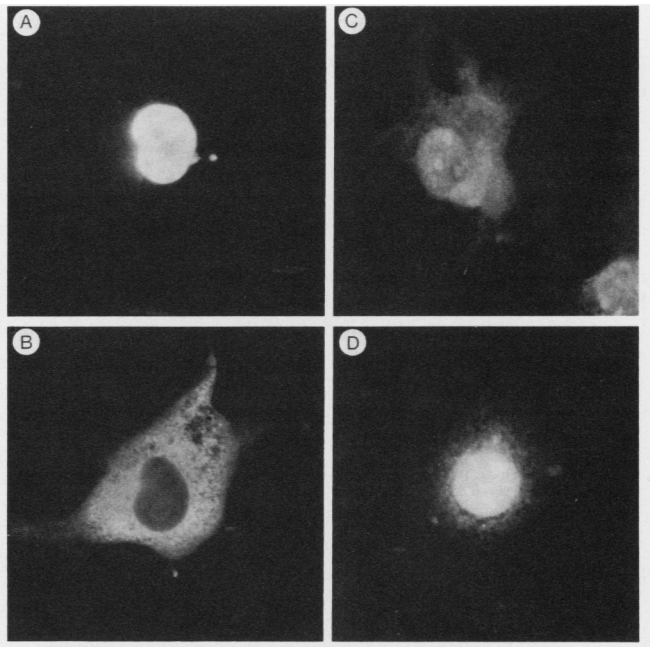

FIG. 6. Indirect immunofluorescence of relocalized PK. COS-1 cells were transfected with the following (panel): A, XR30PK; B, pAL79; C and D, pAL101. XR30PK encodes an SV40 NTS-PK fusion protein, pAL79 encodes PK lacking any NTS, and pAL101 encodes a region 2-PK fusion protein containing two copies of the v-*sis* NTS. Immunofluorescence was performed as described in Materials and Methods.

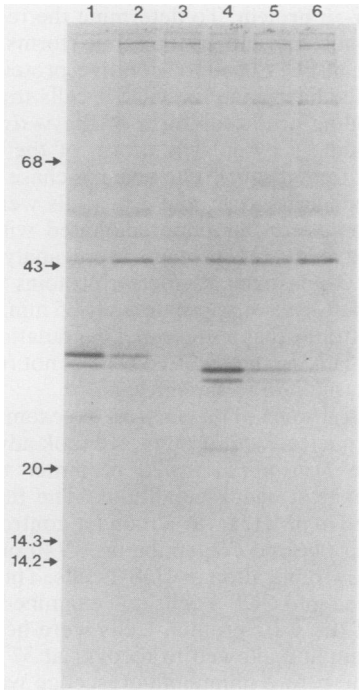

FIG. 7. Pulse-chase analysis of nuclear and cytoplasmic forms of mutant v-*sis* gene products. COS-1 cells were transfected with pAL35 (lanes 1, 2, and 3), which encodes a nucleus-localized v-*sis* protein, or pAL26 (lanes 4, 5, and 6), which encodes a cytoplasm-localized v-*sis* protein. The cells were pulse-labeled with [^{35}S] methionine and [^{35}S]cysteine for 30 min (lanes 1 and 4), followed by a chase with unlabeled medium for 1 h (lanes 2 and 5) or 2 h (lanes 3 and 6). Cell lysates were immunoprecipitated with anti-*sis* serum and analyzed by SDS-PAGE. The v-*sis* proteins are located between the 43- and 20-kDa molecular mass markers.

protein shortly after heat shock, a substantial portion exhibited additional cytoplasmic staining similar to that seen with pAL27 (Fig. 2F). This suggests that nuclear transport of the v-*sis* protein was not complete after 30 min. Longer periods of recovery, up to 5 h (data not shown), did not allow complete transport of the v-*sis* protein into the nuclei in 100% of the cells. It is possible that heat shock disrupts the ability of the v-*sis* protein to accumulate completely in the nucleus because some portion of the protein is improperly folded or partially degraded. Heat shock has been shown to induce expression of ubiquitin, a protein involved in protein degradation (3, 43a). Thus, heat shock conditions may result in partial degradation of the v-*sis* protein or some other component of the nuclear transport system.

DISCUSSION

The data presented in this paper identified an NTS in the v-*sis* gene product. This NTS became apparent after transport across the membrane of the ER was blocked by mutation of the signal peptide. Indirect immunofluorescence and cell fractionation demonstrated that the NTS was contained in the C-terminal portion of the protein. Furthermore, fusion proteins containing this C-terminal region demonstrated that amino acid residues 237 to 256 of the v-*sis* protein can function as an NTS. The C-terminal boundary of the transport signal can be delimited to between residues 249 and 256, based on cell fractionation and deletion analysis. The N-terminal limit of the transport signal was not determined directly; however, fusion proteins indicated that an N-terminal limit at residue 237 can still provide a functional NTS. Thus, on the basis of these data, an NTS is contained within residues 237 to 255 (RVTIRTVRVRRPPKGKHRK).

The amino acid sequence of the v-*sis* protein responsible for nuclear transport contains residues which are common to many NTSs. The 19-amino-acid-long sequence contains 6 arginine residues, 3 lysine residues, and 2 proline residues. Although a consensus sequence for nuclear transport has not been determined, many of the known NTSs contain a significant number of these three residues. For example, the SV40 large-T antigen NTS consists of PKKKRKV (31), and the two NTSs of polyomavirus large-T antigen consist of VSRKRPA and PKKARED (46). The 74 N-terminal amino acids of *S. cerevisiae GAL4* function as an NTS in *S. cerevisiae* and contain a preponderance of lysine, arginine, and proline residues (35, 51), as do the 21 N-terminal amino acids of the *S. cerevisiae* ribosomal protein L3 (38, 50). Thus, the v-*sis* protein NTS fits the general pattern of known nuclear targeting signals.

Proteins are thought to enter the nucleus via nuclear pores. Microinjected gold particles coated with a nuclear protein (nucleoplasmin) have been observed to enter the nuclei of *Xenopus* oocytes through nuclear pores (14). Also, in vitro nuclear transport has been blocked by a lectin which interacts specifically with the nuclear pore complex (15). Globular proteins smaller than 70 kDa are believed to be able to diffuse passively through these pores, as predicted from a functional pore radius of 4.5 nm and microinjection studies of different-size proteins (4, 43). The v-*sis* mutant proteins described in this paper are less than 35 kDa. Thus, it is possible that these mutant proteins enter the nucleus through passive diffusion and accumulate by nonselective binding to DNA as a result of the overall positive charge of the protein. However, it should be noted that both the nuclear and cytoplasmic forms of the v-*sis* proteins have an overall positive charge. For instance, the cytoplasmic v-*sis* protein encoded by pAL26 has a calculated pI of 8.3, compared with a calculated pI of 8.6 for the nuclear v-*sis* protein encoded by pAL35. Thus, it seems unlikely that the nuclear localization reported here is due solely to the net positive charge of the proteins. The partial nuclear localization of PK due to the v-*sis* NTS also strongly argues against this possibility.

The result from the heat shock-induced synthesis of nuclear v-*sis* protein indicates that most of the protein does not enter the nucleus by passive diffusion. That the protein was detected in the nucleus 30 min after induction demonstrates that nuclear transport occurs quickly. This suggests that some type of transport system is involved, since diffusion alone cannot account for this rapid nuclear accumulation

TABLE 2. Import of nuclear v-*sis* protein

Time (min) of:		Subcellular localization[a]	
Heat shock (45°C)	Recovery (37°C)	N	N ≥ C
0	0	0	0
10	30	5	95
10	60	12	88
10	90	16	84
10	120	21	79

[a] Subcellular localization is given as a percentage of positively staining cells. N represents cells exhibiting only nuclear localization as determined by indirect immunofluorescence, while N ≥ C represents cells showing nuclear localization with additional cytoplasmic localization. An average of 30 positively staining cells was examined for each time point.

(10, 41, 42). A general receptor-mediated nuclear transport system has been postulated by others, and this is consistent with the heat shock experiment discussed here.

The v-*sis* NTS may not only be responsible for nuclear transport but may also function to target the protein to nucleoli. This hypothesis is based on the fact that certain nuclear forms of the v-*sis* protein accumulate specifically in nucleoli. However, a sequence comparison of the putative v-*sis* NTS with other nucleolar proteins showed no obvious homologies. The nucleolar accumulation observed is particularly striking in the nucleus targeted v-*sis* proteins and the PK fusion which was partially targeted to the nucleus (Fig. 5 and 6). As stated in Results, nuclear staining varied from a strong nucleolar pattern to a diffuse pattern in which nucleoli were excluded (Fig. 2D). The structure of nucleoli is known to be cell cycle dependent (53); thus, it is possible that this heterogeneous staining is the result of some cell cycle-dependent phenomenon. The various staining patterns may also be due to differences in the levels of protein expression in different cells.

It is possible that the v-*sis* protein sequence responsible for nuclear transport is a cryptic NTS and thus may have no biological function. Recently, random amino acid sequences were examined for their ability to function as a signal sequence in *S. cerevisiae* (29). One-fifth of these sequences were found to function as a secretion signal. Thus, the specificity of signal sequence recognition in *S. cerevisiae* must be low. A similar situation may exist for nuclear transport signals, although this has not been tested directly.

The region of amino acids shown to contain the v-*sis* NTS is completely conserved between the v-*sis* protein and the full-length human c-*sis* protein predicted from its nucleotide sequence (7, 9, 27, 28). It is interesting to speculate, therefore, that the NTS described in this paper functions in both PDGF and the wild-type v-*sis* protein and that these proteins have important functions in the nucleus. This region is not required, however, for transformation by the v-*sis* gene product (22) (Fig. 1, minimal transforming region). It should be noted that the reported C-terminal amino acid sequence of the B chain of PDGF (27) is truncated compared with that predicted from its nucleotide sequence (7, 27, 28). This may be due to a C-terminal processing event (47) or incomplete amino acid sequence data from the C terminus of the B chain of PDGF. If C-terminal truncation does occur, then the region containing the NTS would be absent from mature PDGF.

PDGF has a number of effects on cells. PDGF is known to initiate a round of DNA replication when allowed to interact with cells bearing PDGF receptors. PDGF has also been shown to induce or modulate the expression of a wide variety of genes. Some of these include c-*fos*, c-*myc*, β_2 interferon, collagenase, and the JE and KC genes described by Cochran et al. (1, 5, 13, 32, 33, 39, 59). The mechanism(s) by which these nuclear events are mediated by PDGF is not understood. It should also be noted that ^{125}I-labeled PDGF has been reported to accumulate in the nuclei of cells bearing the appropriate receptor (45). In addition, proteins related to the v-*sis* gene product have been identified in the nuclei of SSV-transformed normal rat kidney cells (58). The above observations suggest that forms of the v-*sis* protein which specifically localize to the nucleus play a role in mediating physiologically significant nuclear events.

How might a normally secreted growth factor get to the nucleus? One model suggests that PDGF or the wild-type v-*sis* protein enters target cells by receptor-mediated endocytosis of a receptor-ligand complex, followed by release from endosomes or secondary lysosomes. These proteins could then be transported to the nucleus if they contained functional NTSs. In the event that C-terminal processing occurs during maturation of PDGF, it may also be possible that nuclear localization occurs for the C-terminal fragment, containing the NTS, which is released during processing in the ER-Golgi apparatus.

A second model can also be proposed, in which the monomeric nuclear v-*sis* protein described in this paper has a normal function in the nucleus. Similar proteins are likely produced in cells which normally secrete PDGF or the v-*sis* protein. These proteins could result if the signal recognition particle, involved in targeting proteins to the ER, fails to quantitatively recognize the signal peptide at the N terminus of nascent v-*sis* polypeptides. A similar situation would result if internal initiation occurred 3′ of sequences encoding the signal peptide. Under these conditions, the proteins produced should be transported to the nucleus as are the mutant v-*sis* proteins reported in this work. It should be noted that nuclear fluorescence was detected in a small percentage of COS-1 cells that transiently express large amounts of the wild-type v-*sis* protein (data not shown). It is possible that the internal initiation or nonquantitative signal recognition particle interaction discussed above is responsible for this observation. Thus, it seems possible that similar proteins can be synthesized in a physiologically relevant system.

The identification of an NTS in the v-*sis* protein suggests that PDGF or PDGF-related molecules may have a role in the nuclei of certain cells. The role that these growth factors may play in the nucleus remains to be investigated.

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