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Cell-Dependent Efficiency of Reiterated Nuclear Signals in a Mutant Simian Virus 40 Oncoprotein Targeted to the Nucleus

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We investigated the requisites for, and functional consequences of, the relocation to the nucleus of a transforming nonkaryophilic mutant of the simian virus 40 large T antigen (a natural deletion mutant lacking an internal large-T-antigen domain that includes the signal for nuclear transport). Synthetic oligonucleotides were used to obtain gene variants with one or more copies of the signal-specifying sequence inserted near the gene 3′ end, in a region dispensable for the main large-T-antigen functions. The analysis of stable transfectant populations showed that mouse NIH 3T3 cells, rat embryo fibroblasts, and simian CS cells (a subclone of CV1 cells) differed considerably in their ability to localize some variant molecules into the nucleus. CS cells were always the most efficient, and NIH 3T3 cells were the least efficient. The nuclear localization improved either with reiteration of the signal or with a left-flank modification of the signal amino acid context. Three signals appeared to be necessary and sufficient, even in NIH 3T3 cells, to obtain a nuclear accumulation comparable to that of wild-type simian virus 40 large T antigen; other signal-cell combinations caused a large variability in subcellular localization among cells of the same population, as if the nuclear uptake of some molecules depended on individual cell states. The effect of the modified location on the competence of the protein to alter cell growth was examined by comparing the activity of variants containing either the normal signal or a signal with a mutation (corresponding to large-T-antigen amino acid 128) that prevented nuclear transport. It was found that the nuclear variant was slightly more active than the cytoplasmic variants in rat embryo fibroblasts and NIH 3T3 cells and was notably less active in CS cells.

The nuclear localization of a number of eucaryotic proteins is known to depend on the presence in their polypeptide chains of determinants that vary in sequence and number; these are generally referred to as nuclear signals (3, 8, 9, 15, 18, 20, 24, 25, 30, 31, 39, 45, 46, 52–54). The simian virus 40 (SV40) large T antigen, a predominantly nuclear protein that performs essential regulatory tasks in viral growth and that is able to act as an oncoprotein in many animal cells (4, 48), carries one of the best-defined signals, a short stretch of mostly basic amino acids (Pro-Lys-Lys-Lys-Arg-Lys-Val), occupying positions 126 to 132 in the polypeptide chain (20). The insertion of such a codon sequence into the coding sequences of some cytoplasmic proteins has been shown to suffice for the nuclear targeting of these proteins (20, 41). Nuclear transport has been induced in monkey cells that were microinjected with carrier proteins conjugated to multiple copies of a synthetic peptide homologous to the signal described above (23). A similar transport of peptide conjugates microinjected into *Xenopus* oocytes suggests that the SV40 large-T-antigen signal can be recognized by rather distant species (17). Recent evidence also indicates that while a minimal nuclear signal is sufficient for nuclear localization, its activity is crucially dependent on the protein context within which it is present (41). We previously characterized a mutant of the SV40 large T antigen which lacks the normal large-T-antigen karyophily and accumulates in the cytoplasm (because of a natural deletion in the gene, which was originally found as a viral insertion in the DNA of transformed cells) (12). This nonkaryophilic large T antigen (NKLT) lacks amino acids 110 through 152 of the SV40 large T antigen, cannot bind to SV40 origin DNA (11, 12), is phosphorylated in vivo at only two sites (11), and predominantly forms dimers and cytoplasmic complexes with the cellular protein p53 (29). From a functional point of view, NKLT behaves as a cytoplasmic oncoprotein that is able either to immortalize primary cells or to transform some established cells (but not to perform both functions in the same primary cells), and cooperates better with H-*ras* or polyomavirus medium T (PyMT) than with nuclear oncoproteins in transformation (13, 28). In the normal large T antigen, the NKLT deleted region has in its center the nuclear signal, four phosphorylation sites of the NH_2-terminal site cluster (42) at one end, and at the opposite end a domain whose integrity is required for binding to SV40 origin DNA (34, 47, 52).

The SV40 large T antigen carries a number of tightly packed activities (4, 48) whose structural and functional connections are only partially understood; the presence of a normal nuclear signal, in particular, presumably affects the functioning of the rest of the large-T-antigen molecule, while in turn the signal is influenced by the surrounding molecular domains. Because NKLT is a cytoplasmic oncoprotein with a limited but distinct competence of its own and is simultaneously the defective derivative of a more potent nuclear oncoprotein, retargeting this molecule to the nucleus presented a special opportunity to answer some questions on the requirements and consequences of nuclear localization.

The specific points we investigated in the present study were as follows: (i) to what extent the functioning of the large-T-antigen nuclear signal is cell independent, (ii) whether a pattern of manipulations improving deficient transport (such as signal reiteration or changes in the signal flanking regions) could be recognized, and (iii) whether the NKLT intracellular localization per se contributed in some degree to its transforming defectiveness. The SV40 large-T-antigen signal was inserted in a region near the NKLT COOH end that is known to be dispensable for both the lytic and the transforming competence of SV40 (35, 37). The

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subcellular location analyzed here represents in all cases the steady-state situation in stable populations of transfectants. The information obtained thus differs in a complementary fashion from that provided by studies based on the microinjection of synthetic peptides.

It was found that the efficiency of nuclear accumulation for certain protein variants markedly differed among three types of cells examined: under varied signal conditions, simian CS cells always exhibited the highest efficiency, and mouse NIH 3T3 cells exhibited the lowest efficiency, while rat embryo fibroblasts (REFs) behaved in an intermediate fashion. When we raised to three the number of tandem signals, it promoted the full nuclear localization of the protein in all cells. Quite variable subcellular distributions were found in cells of the same population when signal-cell combinations did not result in full nuclear localization in all cells. Nuclear localization was always improved by either reiterating the signal or interposing a group of nonspecific amino acids between the signal and the left-flanking region. The ability of the nucleus-relocated protein to stimulate cell growth was found to be just minimally increased in the two rodent cell types and markedly decreased in the simian cells.

MATERIALS AND METHODS

Plasmid structure and mutagenesis. Numbers of SV40 nucleotides (nt) always refer to those of the wild-type (wt) genome (48). Mutagenesis was carried out by inserting the synthetic sequences indicated below at a *Bcl*I site (corresponding to SV40 nt 2770) of pACTSV2. This is a previously described construct (11) containing an entire SV40 genome carrying the NKLT deletion (SV40 nt 4362 to 4490) cloned in pAT153; this deletion is the only difference between the wt large T antigen and the NKLT gene. We used two pairs of synthetic 24-nt oligomers, each pair of which was constituted by complementary sequences except for 4 unpaired bases (GATC) at the 5′ end. One pair carried the genetic information for the SV40 large-T-antigen nuclear signal, and the other carried a similar sequence that was altered by the substitution of a threonine for the lysine codon in position 128 of the large T antigen. This kind of substitution is known to impede nuclear transport (20, 22). The ligation between recipient DNA and insert DNA was carried out with various molar proportions of the two (1:2 and 1:6). Plasmids carrying the desired inserts were screened by codigestion with *Eco*RI and *Sty*I endonucleases; this allowed the electrophoretic identification of a 291-base-pair fragment (from SV40 nt 2812 to pAT153 *Eco*RI site) whose migration varied proportionally to the number of synthetic DNA inserts. Mutants containing one to three inserts were picked for further use. Because all links occurred via GATC-protruding ends, inverted inserts either following or preceding inserts in the correct orientation could be spotted by the formation of a unique *Bgl*II site in the first case and of a new *Bam*HI site in the second. Wholly correct orientations were finally detected because a cut by *Mbo*II within the insert generated DNA fragments whose sizes depended on the insert orientation. The indications derived from restriction analyses were subsequently confirmed by chemical nt sequence determinations (26). RLT1, RLT2, and RLT3 were the names given to NKLT gene products containing one, two, or three tandem wt signals, respectively (identical names preceded by a p refer to the corresponding plasmids). Some other mutants with a similar structure were also constructed and used in this study. YLT3 contained three mutated tandem signals (whereby Thr replaced Lys-128), and RLTi1 and RLT1i each contained a single wt signal that was preceded or followed, respectively, by a sequence of 8 nonspecific amino acids (Leu-Pro-Phe-Ser-Ser-Phe-Leu-Asp, which were encoded by the DNA sequence complementary to that of the nuclear signal). Recombinants between the PyMT gene and some of the mutant genes described above were obtained by replacing the SV40 late region (SV40 nt 771 to 2533) in pACTSV2, pRLT3, and pYLT3 with a DNA segment from PyMT1 (55) comprised of nt 4631 through 2964 of the polyomavirus genome. The resulting constructs were designated pCTSV2MT, pRLT3MT, and pYLT3MT, respectively.

Hydrophilicity profiles. The procedure used to analyze protein hydrophilicity described by Hopp and Woods (19) was applied to the predicted amino acid sequence of the indicated protein domains by using the Microgenie program (Beckman Instruments, Inc., Fullerton, Calif.), and the results were plotted on a personal computer (XT2; International Business Machines).

Cell cultivation and transfection. All cells were cultured in Dulbecco medium containing 10% calf serum, 10% tryptose phosphate broth, and nonessential amino acids. Early-passage REFs were from a stock purchased from Flow Laboratories, Irvine, United Kingdom, that was stored frozen. CS cells were a subclone that we isolated from the established simian line CV1 (it yields a larger number of transfectants than parental cells and may also be easier to transform). Mouse NIH 3T3 and BALB/c 3T3 cells, rat F2408 cells, and simian BSC1 cells are standard established lines that were originally donated by C. Basilico. DNA transfections were carried out in REFs by the calcium method as described previously (13, 33) and in other cells by the polybrene method (5). About 4×10^5 cells in 6-cm-diameter dishes received 1 μg of DNA from pIBW3, a plasmid carrying *neo* (6, 13), and 5 μg of the indicated plasmid DNA. Transfectants could thus be selected by subculturing cells in medium containing 0.5 mg of G418 (Sigma Chemical Co., St. Louis, Mo.) per ml. After 9 to 12 days the surviving cells, which had meanwhile formed numerous small colonies, were pooled for further cultivation on cover slips or in semisolid medium, as indicated below. REFs for growth control assays underwent a different treatment, which is also indicated below.

Transformation and immortalization assays. Transformation and immortalization assays have been described previously in more detail (13). Briefly, transformation in CS and NIH 3T3 cells was determined by suspending G418-resistant cells with 0.33% agar (Difco Laboratories, Detroit, Mich.) in normal medium at a density of 2×10^4 cells per 6-cm-diameter dish; NIH 3T3 cell colonies growing in agar were counted after 4 weeks, and CS cell colonies were counted after 6 weeks. Transformation of nonestablished REFs was determined by an assay (not involving drug selection) of foci formation in dense monolayers; at 2 days posttransfection the cells from each 6-cm-diameter dish were replated into three similar dishes, where they reached confluence and eventually formed dense foci. Staining with Giemsa and counting were performed 2 weeks later. Immortalization of nonestablished REFs was tested by colony formation after sparse seeding in liquid medium (13). Two days posttransfection the cultures were exposed to G-418, and after another 4 days, cells were replated in 10-cm-diameter dishes; colonies were stained with Giemsa and counted 2 weeks later.

Indirect immunofluorescence. Indirect immunofluorescence was carried out as described previously (12) with anti-SV40 large-T-antigen monoclonal antibody Pab416 (On-

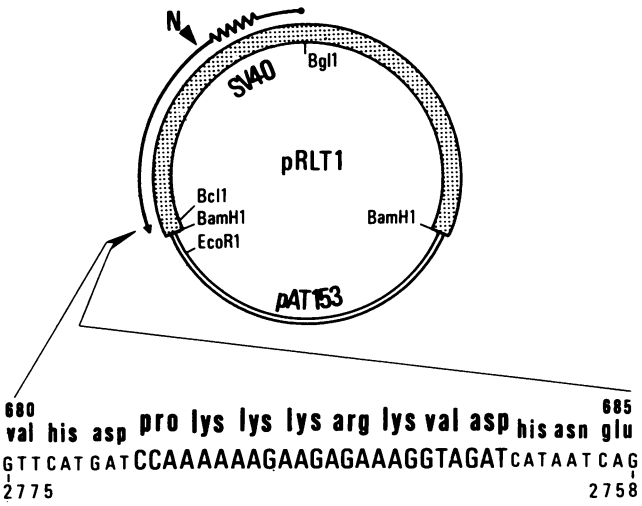

FIG. 1. Structure of the prototype gene variant used in this study. The circle represents an SV40 genome cloned in pAT153, with N indicating the NKLT deletion within the large T-antigen mRNA map, which is shown by the outer line. Such a plasmid (pACTSV2 [11]) was modified by synthetic oligonucleotide insertion at the indicated position. The inserted nucleotides (encoding the wt large-T-antigen nuclear signal plus a linking Asp codon) are shown by large letters, while small letters indicate the preexisting normal sequence. Nucleotide and amino acid numbers are those of reference 48. The illustrated structure is that of the simpler variant that was used (pRLT1), which contained one copy of the insert; the other variants used had a similar structure but contained either two tandem copies (pRLT2) or three tandem copies (pRLT3) of the same insert; pYLT3 contained three tandem copies of the same insert, with a Thr replacing the second Lys (equivalent to residue 128 of wt large T antigen). Further variants and experimental details are described in the text.

cogene Science, Mineola, N.Y.), which does not react with small t antigen, followed by treatment with fluorescein isothiocyanate-conjugated second antibody.

RESULTS

The alteration made in the NKLT gene, as illustrated in Fig. 1, inserted a sequence of codons after the position corresponding to amino acid 682 of the wt SV40 large T antigen. The added sequence encodes the minimum nuclear signal encompassing codons from Pro to Val (20), in addition to an extra Asp codon linking in-frame the insert 3′ end to the rest of the gene. The structure of the simpler mutant used, pRLT1, contained a single insert (Fig. 1), whereas in similar mutants, pRLT2 and pRLT3, the Pro to Asp sequence was repeated two or three times, respectively. The rest of the mutants used here shared the same basic structure described above. pYLT3 was identical to pRLT3 except for a Thr replacing the second Lys in the signal (corresponding to large T antigen position 128); this substitution prevents nuclear transport (7, 20, 22). pRLTil and pRLT1i had a nonspecific 8-residue sequence (Leu-Pro-Phe-Ser-Ser-Phe-Leu-Asp) interposed between their single signal sequence and either the upstream (pRLTi1)- or the downstream (pRLT1i)-flanking regions. These two mutants were used to explore the effect of a context change on either side of the signal.

Three signals are sufficient for full nuclear localization in simian and rodent cells. The anti-T-antigen immunofluorescence displayed by the original protein NKLT in NIH 3T3 cells (Fig. 2A) and that displayed by variant RLT3 in cells of three different species, NIH 3T3 cells (Fig. 2B), REFs (Fig. 2C), and CS cells (Fig. 2D), is shown. It appears that RLT3 was efficiently located in the nucleus of all cells, with immunofluorescence patterns very similar to those commonly observed with the wt SV40 large T antigen (12). The RLT3 nuclear localization was very uniform among these cells, although they represented the uncloned survivors of G418 selection. The experiment thus showed that three tandemly reiterated nuclear signals could efficiently offset the possible disadvantage posed by this unnatural protein context in all three cell types. As expected, a similar trio of signals carrying the Lys to Thr mutation indicated above left the protein (YLT3; Fig. 2E) in the cytoplasm.

Cell-dependent response to the nuclear signal number and context. The nuclear transport of NKLT variants that carried two signals (RLT2), one signal (RLT1), or one signal with nonspecific residues added on its left (RLTi1) or right (RLT1i) side differed considerably from that of RLT3 and actually differed for each variant. The distribution of these proteins between the nucleus and the cytoplasm appeared to depend strongly not only on the signal number and context but also on the cell type. Moreover, such suboptimal cell-signal combinations did not result in uniform pictures of subcellular distribution but disclosed large individual variability within the same cell population. These results are shown in Fig. 3 and 4. To express quantitatively cell-dependent transport variations, we scored the percentage of cells displaying stronger nuclear than cytoplasmic immunofluorescence; this was done in G418-selected populations of transfectants, in which immunopositive cells constituted 80 to 100% of the total. The histogram in Fig. 3 shows that two tandem signals contained in variant RLT2 were sufficient to relocate the protein predominantly in the nucleus of nearly 90% of CS cells, but only in 38% of REFs and in less than 5% of NIH 3T3 cells. The single signal of RLT1 rendered only 47% of CS cells and a negligible fraction of NIH 3T3 cells predominantly nucleus positive. Nuclear accumulation in REFs had an intermediate efficiency. Distinct cellular differences also appeared in response to context variations; when 8 nonspecific residues were interposed to the left of one signal in RLTi1 (see above for details), nuclear uptake became quite efficient in CS cells (as good as that of RLT2), but remained rather inefficient in NIH 3T3 cells. The behavior of REFs did not substantially change. The same residue interposition at the other end of the signal (in RLT1i) had negligible quantitative effects in all cases.

Figure 4 illustrates in more detail the response of the cells described above to signal conditions producing less than maximum transport. Since the pictures of CS cells (Fig. 4A), REFs (Fig. 4B), and NIH 3T3 cells (Fig. 4C) display the expression of the same variants in the same order, it is again possible to observe, despite the small cell number, that the efficiency of protein nuclearization generally decreases from CS cells to REFs to NIH 3T3 cells. Because similar variations were also observed in experiments with other closely related cell lines (mouse BALB/c 3T3, rat F2408, and simian BSC1), a likely possibility is that at least part of these differences depend on cell species rather than on cell lines. A more specific consequence of suboptimal signal-cell combinations, however, was to make evident the coexistence, in the same population, of cells with widely different distributions of the same protein (Fig. 4A3, B1, B2, B3, and C1), together with some morphological peculiarities (Fig. 4A4). As far as the first aspect is concerned, it should be added that in clones that were isolated from populations showing loca-

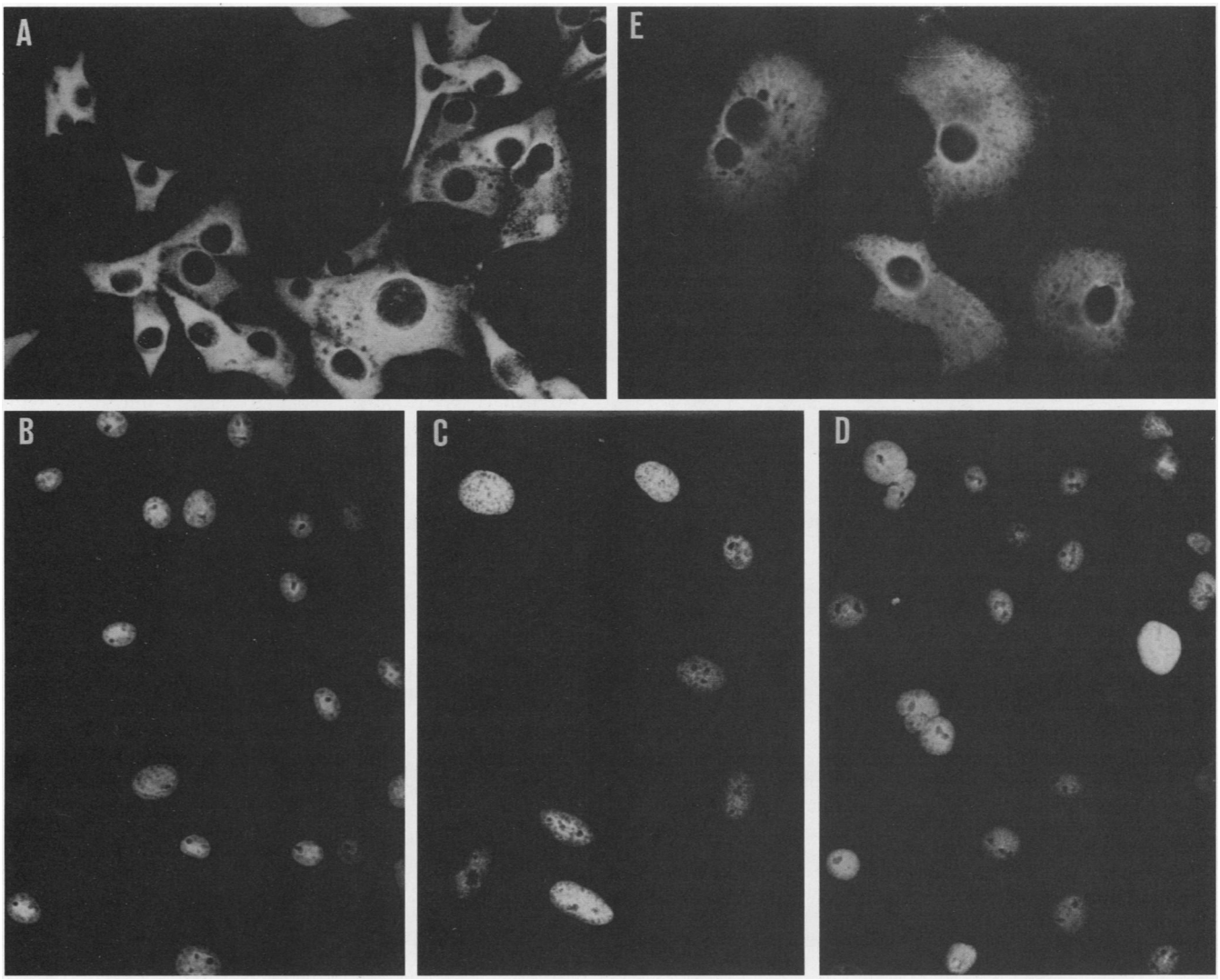

FIG. 2. Three nuclear signals promote complete nuclear transport in different cell types. Cells were cotransfected with the indicated plasmids and a Neo gene (see text), selected with G418, and assayed for anti-T-antigen immunofluorescence. (A) NIH 3T3 cells transfected with pACTSV2; NIH 3T3 cells (B), early-passage REFs (C), and CS simian cells (D) transfected with pRLT3; (E) CS simian cells transfected with pYLT3.

tion heterogeneity (CS cells with RLT1, REFs with RLT2, and NIH 3T3 cells with RLTi1), the heterogeneity was found to be only somewhat reduced but definitely not abolished (data not shown). Apparently, the barrier to be crossed during nuclear transport allows a degree of structural tolerance (or is influenced by unknown oscillations of cell metabolism), with critical consequences when signal conditions are less than optimal. Figure 4 also shows some strikingly ringed nuclear images (especially with variant RLT1i; Fig. 4A4, C3, and C4) that often occurred with suboptimal signals, but not in their total absence. These images suggest that an imperfectly signaled molecule can accumulate, because of binding or an equivalent mechanism, against a barrier whose crossing constitutes a separate step.

The conclusions drawn from the results presented thus far are as follows. (i) Three nuclear signals are both necessary and sufficient to compensate for the disadvantage of the unnatural amino acid context in all three types of cells examined; (ii) with less than three signals, marked differences in nuclear localization become manifest among some cell lines, and similar differences appear in response to context modifications; and (iii) within each cell line, a subset of individual cell variations emerges when signal conditions are insufficient to determine full nuclear localization in all cells.

Structural features of the signal-carrying domains. The hypothesis that protein folding strongly affects the efficiency of nuclear signals has been recently corroborated by experimental evidence (41, 44). Conceivably, nuclear transport determinants share some basic structural features with antigenic determinants, since the efficiency of both is likely to depend on the propensity to protrude from the molecular surface into the aqueous medium. The method of Hopp and Woods (19) of predicting antigenic determinants made it possible to show that myoglobin epitopes coincide with peaks of hydrophilicity in the primary structure; a similar method was recently used to characterize the nuclear signal of yeast histone 2B (30). To obtain information on the

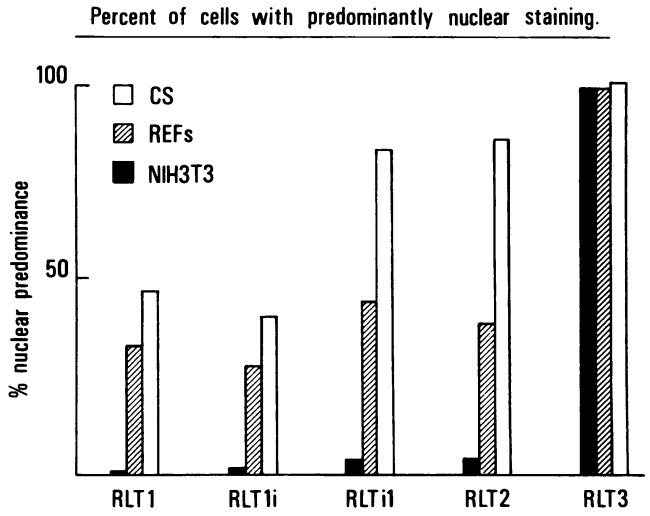

FIG. 3. Comparative transport efficiency shown by different signal-cell combinations. NIH 3T3 mouse cells, early-passage REFs, and CS simian cells expressing the indicated variants were obtained by transfection, as indicated in the legend to Fig. 2, and analyzed by anti-T-antigen immunofluorescence. Cells with stronger staining in the nucleus than in the cytoplasm were scored by microscopy as the percentage of the total number of stained cells, which represented 80 to 100% of the total number of cells; about 500 cells were counted for each sample.

secondary structure of signal-carrying domains, we applied the analysis of Hopp and Woods (19) to the predicted amino acid sequence of these domains in our mutants and in wt SV40 large T antigen and obtained the results shown in Fig. 5. The profile from the wt large T antigen shows that in its natural position, the nuclear signal sequence constitutes a high point of hydrophilicity, followed by a hydrophobic stretch. This feature is conserved with secondary variations in the other profiles, all coming from mutants that were transported (with different efficiencies) to the nucleus. These curves also resemble the curve displayed by the nuclear signal in yeast histone 2B (30), suggesting that this general shape does not occur fortuitously. Moreover, although no specific rule can be offered as a result of such a small number of examples, it seems worth noting that the least karyophilic variants (RLT1 and RLT1i) also had the smallest areas protruding from minimum hydrophilicity valleys. An obvious peak alteration occurs when the critical Lys-128 in the signal is replaced by the mildly hydrophobic Thr, and this could compound the effect of the altered signal recognition (data not shown). It seems safe to conclude that a prominent hydrophilic peak likely represents an inherent feature of a well-functioning signal and that prior analyses of this parameter may indicate the most effective way to use the signal in genetic manipulations.

Cell growth response to intracellular localization variants. An interesting part of this study was to establish whether the change of intracellular location affected the activity of NKLT as an oncoprotein. Using standard assays of immortalization and transformation, we compared the competence of NKLT nuclear and cytoplasmic variants to alter the proliferation of cells already examined for transport properties. A preliminary problem was posed by the fact that simian cells of the established lines we tested (CS cells, their parental CV1 cells, and BSC1 cells) seemed refractory to the induction of anchorage-independent growth, and neither NKLT nor PyMT or H-*ras*, which are each capable of transforming many established cells (2, 13, 14, 21, 51), could induce any colonies in semisolid medium. We found, however, that NKLT, if coexpressed with PyMT, was able to induce a low but significant number of CS colonies in soft agar; thus, this made a transformation assay possible. Early-passage REFs were used to test both immortalization and cooperative transformation with PyMT (13, 50), and NIH 3T3 cells were used to test the transformation of established mouse cells. The oncoprotein variants whose activities were compared were NKLT, RLT3, and YLT3; their intracellular localization was depicted in Fig. 2. Typical results obtained in a set of four assays described above are illustrated in Table 1. The activity of the nucleus-relocated protein RLT3 relative to that of cytoplasmic mutant YLT3 appeared to be consistently but minimally increased in the two assays with REFs and in that with NIH 3T3 cells (Table 1). The higher increase (only about fourfold) was found in the NIH 3T3 cell assay, which, more so than the others, tends to amplify activity differences (because of an enrichment in cells with a shorter division time, which occurred in polyclonal cell populations selected with G418 before suspension in agar). It was thus concluded that, although minor activity differences may be present among these variants in rodent cells (and whether or not the pathways that were interfered with were the same), the growth-releasing effects of the nuclear protein do not fall into a more powerful category than those of the cytoplasmic proteins. More surprising was the finding that in CS cells the activity exerted by the cytoplasmic proteins NKLT and YLT3 decreased almost to nil with the nuclear protein RLT3 (Table 1). This prompted us to check in similar experiments whether agar colony formation was repressed in CS cells that carried the oncoprotein both in the nucleus and in the cytoplasm; the result, however, was that the ability to form these colonies was conserved (data not shown). Since the same plasmid (pRLT3MT) preparation was perfectly active in REFs and its CS transfectants grew normally in monolayers, we concluded from the results presented above that the RLT3 mechanism that stimulates rodent cell growth is either missing in CS cells or prevailed upon by antagonistic regulators. It seems worth noting that even SV40 wt large T antigen, which by itself can easily transform many rodent cell types, including primary cells, to anchorage independence, has a characteristic but limited effect on simian cell transformation. CS cells transfected with the wt large-T-antigen gene and suspended in agar as in the assay described above formed a large number of early microcolonies (2,000 to 3,000 per dish), which then stopped growing without ever forming normal agar colonies (at least 0.15 mm in diameter); cotransfection of the PyMT gene also did not visibly alter this pattern (unpublished data).

DISCUSSION

The nuclear localization of large proteins appears to depend on a mechanism that basically involves the interaction between a discriminating barrier at or near the nuclear pore and an admission signal contained in the protein that is destined for the nucleus (10, 27, 32, 40). The functional efficiency of the SV40 large-T-antigen signal has recently been shown to be influenced by variables such as the signal number and the amino acid context. Raising the signal number in pyruvate kinase improved its transport, offsetting context disadvantages and even some mutations in the signal itself (41). Transport kinetics, which were determined with carrier proteins conjugated to various amounts of signal peptides, showed that an increase from two to six in the

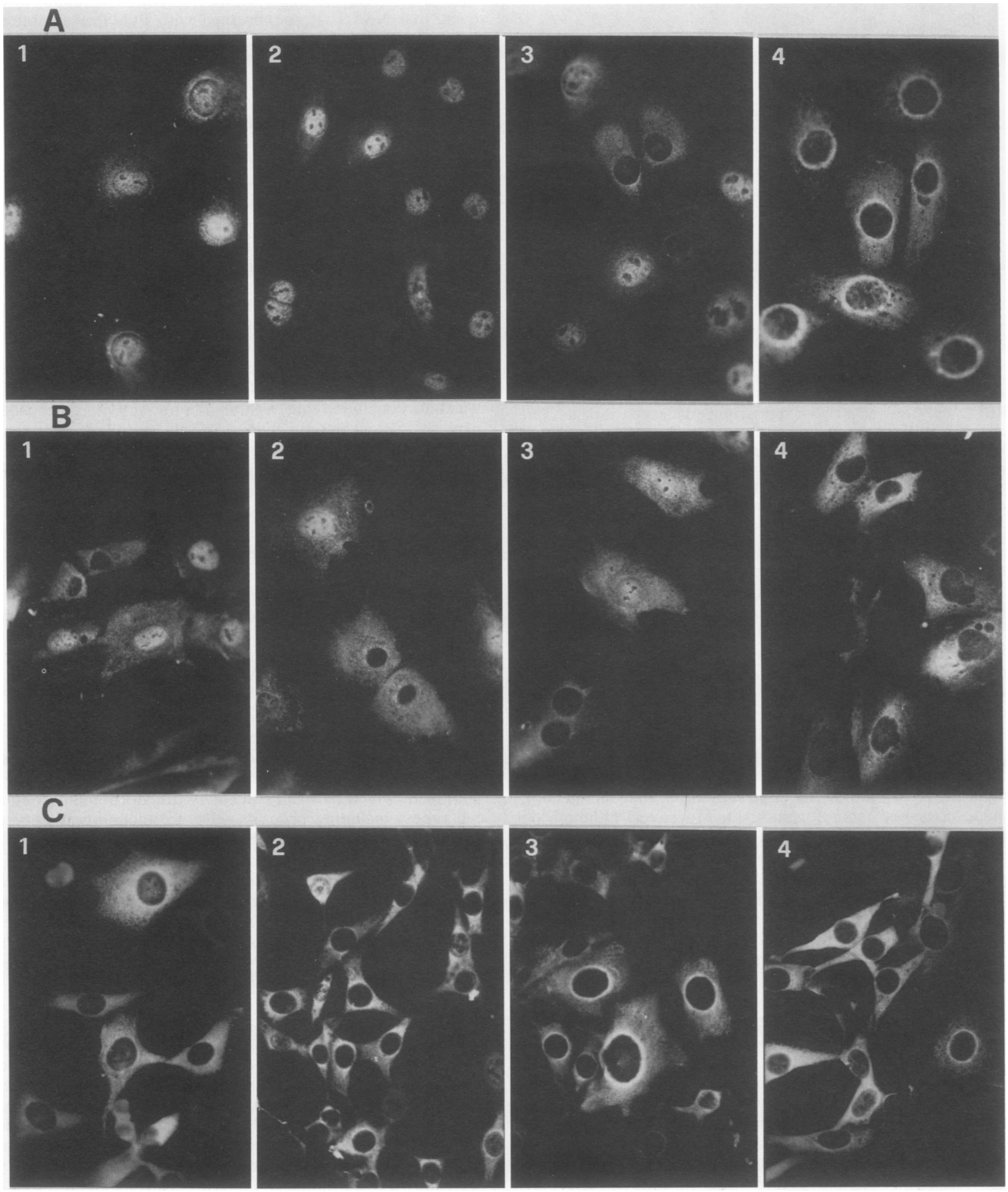

FIG. 4. Effect of suboptimal signal conditions on cells of the same populations. CS cells (A), early-passage REFs (B), and NIH 3T3 cells (C) were transfected with the following plasmids: pRLT2 (panels 1), pRLTi1 (panels 2), pRLT1 (panels 3), and pRLT1i (panels 4). The transfectants were selected and stained by anti-T-antigen immunofluorescence, as indicated in the legend to Fig. 2.

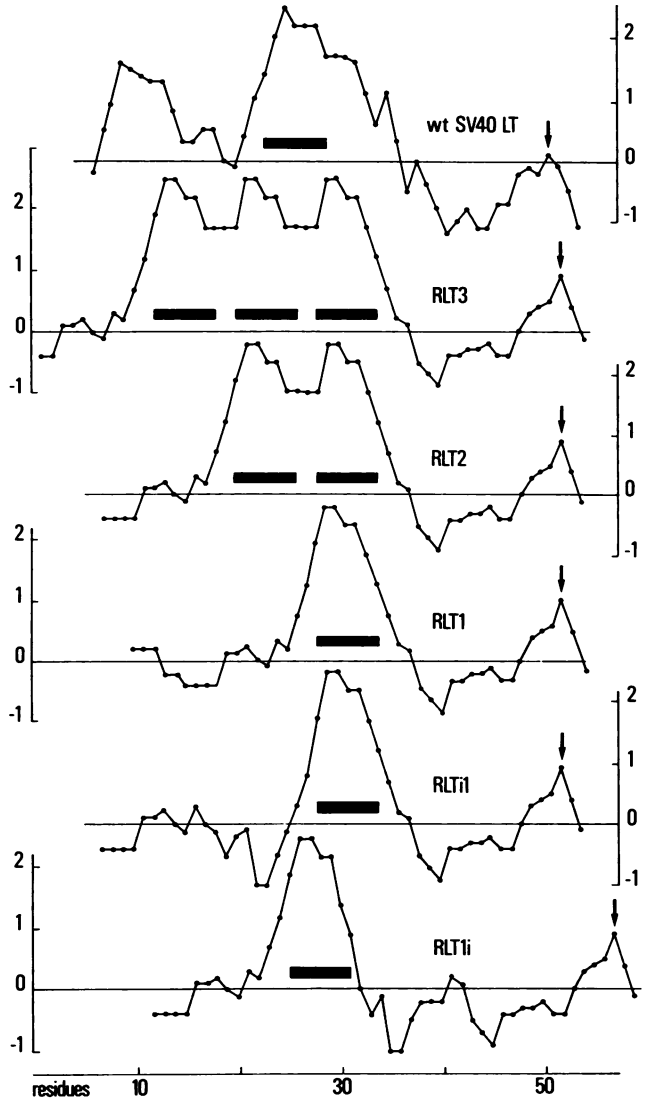

FIG. 5. Hydrophilicity profiles of signal-carrying domains. The method of Hopp and Woods (19) to predict antigenic determinants was applied to the domains containing the nuclear signals (black boxes) of the indicated proteins. The *x*-axis values indicate relative amino acid distances (in the NH_2- to COOH-end direction), while the true residue numbers in the protein sequences can be established with reference to the arrow; this corresponds to Arg-154 for the wt large T antigen (top sequence) and to Pro-699 (number of equivalent wt positions) for the remaining sequences. The hydrophilicity scale, on the *y*-axis, has lower and upper limits of −3.4 and +3.0, respectively.

number of signals per molecule increased the transport rate about eightfold (23). In this study we examined the requisites for nuclear targeting of a mutant nonkaryophilic large T antigen of SV40 in different cell types; the use of stable transfectants (selected with a selectable marker) allowed us to study the behavior of large cell populations. Our results confirm, on the one hand, the transport improvement brought about in general by signal reiteration, but show, on the other hand, that the need for reiteration and the negative effect of some amino acid contexts are not identical in all animal cells. Under some conditions of signal number or context, over 80% of simian CS cells could efficiently accumulate the protein in the nucleus, whereas only 5% of mouse NIH 3T3 cells could do so. The accumulation improvement observed after the signal number was raised from 2 to 3 was also much stronger in NIH 3T3 cells than in REFs or CS cells. The fact that nuclear uptake in simian CS cells appeared to be generally more efficient than in the other cells examined might reflect a special adaptation of this signal, since the efficient large-T-antigen transport (43) must be essential for regulation of the lytic cycle. However, sizable differences were also evident between two other cell types and within each population, suggesting that unequal cell efficiency in using a commonly recognized signal may not be exceptional. The neat nuclear or cytoplasmic localization of RLT3 and YLT3, together with the fact that the location heterogeneity of variants with suboptimal signals was mainly represented by individual cell variations (rather than by ubiquitous intracellular distribution), indicated that no significant role was played by possible protein degradation and diffusion.

The observed variations among cell lines and those among cells of the same population should be considered in conceiving possible models of nuclear transport. The concomitant aspects of signal specificity and response variability are somewhat easier to interpret in molecular terms if distinct structures are postulated for binding and translocation at the nuclear pores. Indeed, recent results show that functional steps of binding and translocation can be distinguished in nuclear transport (32, 40). Specific binding implies mutual recognition by two determinants (one acting as the signal in the transported protein and the other acting as the receptor near the translocating structure) and a certain degree of stability in their interaction. Genetic variations in the receptor determinant might vary the interaction stability, with results being equivalent to those produced either by some mutations in the nuclear signal or by the signal location in suboptimal protein contexts. In any of these cases, reiterated signals might increase the average number of molecules held in place long enough to start the next step, i.e., translocation. The variable subcellular distribution found in cells of the same population is harder to rationalize because it persists after cell cloning; it can be more plausibly envisioned, however, as occurring at the translocation rather than at the binding step. If the actual protein passage through the nuclear pores involves a resistance (to be overcome with energy expenditure [27, 32, 40]), it is possible, for instance, that the levels of usable energy are not identical at all times in all cells, varying the promptness of channel opening and producing variable transport of molecules that are less stably bound. Perhaps the most useful consideration is that it may not be correct to assume that normal cell physiology gives constant and automatic nuclear entry to any cell protein bearing one of the different nuclear signals. The behavior of suboptimal mutants shown here might then reflect not simply imperfect functioning but the potential existence of a control mechanism. The ring images shown in Fig. 4 are most easily explained if recognition and binding occur independently of the actual protein passage into the nucleus; this last step can thus become a barrier that is possibly sensitive to variations in cell metabolism. Finally, it should be noted that the present analysis of protein variants depicts their steady-state subcellular distribution, which in turn is presumably the net result of several variables (such as concentration of the protein to be transported, amount and type of receptors, rate of translocation through pores, lifetime as a nuclear protein, or consequences of mitosis), which could vary independently in different cell types.

We also investigated how the change of intracellular

TABLE 1. Relative activities of NKLT cytoplasmic and nuclear variants

Gene variant[a]	Complementation of PyMT in REF foci induction (no. of foci/dish)[b]	REF immortalization (no. of colonies/dish)[c]	Agar colony induction in NIH 3T3 cells (no. of colonies/dish)[d]	Complementation of PyMT to induce agar colonies in CS cells (no. of colonies/dish)[d]
NKLT	31	105	124	84
YLT3	25	130	96	102
RLT3	81	368	407	3
− DNA or complementing DNA[e]	0	9	5	0

[a] The indicated gene variants were contained either in plasmids carrying the variant genes alone (NKLT in pACTSV2, YLT3 in pYLT3, and RLT3 in pRLT3), which were used for the immortalization and agar colony induction assays, or in recombinants carrying also the PyMT gene of polyomavirus (NKLT in pAPyMTCT2, YLT3 in pYLT3MT, and RLT3 in pRLT3MT), which were used for the complementation assays. DNAs were transfected into the indicated cells, as described in the text.

[b] Dense foci in confluent monolayers derived from 3×10^5 cells seeded in 6-cm-diameter dishes after transfection with the indicated DNAs (see text).

[c] Each 9-cm-diameter dish received 10^3 cells that were selected with G-418.

[d] Each 6-cm-diameter dish received a suspension in soft agar of 2×10^4 cells that were selected with G-418 after transfection.

[e] Controls in the immortalization and agar colony induction assays were constituted by cells that were transfected with Neo DNA only. In the complementation assays, each assay included two controls: one of cells transfected with the PyMT gene alone carried in plasmid PyMT1 (55) and the other of cells transfected with the variant gene alone (there was no activity in either case).

location affected the competence of NKLT to alter cell growth. In the two types of rodent cells examined, the activity of the oncoprotein after relocation to the nucleus persisted at least at the previous (or somewhat higher) level, whereas in simian CS cells the activity was markedly decreased. Conceivably, the wt SV40 large T antigen can alter cell growth by using more than one pathway and/or mode of interfering with pathways; in addition, its stimulatory functions might not be so clearly differentiated as are the classical functions of immortalization and transformation in polyomavirus T antigens (2, 21, 38, 50, 51). The nuclear variant RLT3 and the cytoplasmic variants NKLT and YLT3 might thus act on pathways that are possibly different but that have roughly equivalent effects, at least in rodent cells. The competence of both variants apparently lacks a further stimulus that is dependent on the NKLT deleted domain (not on the protein location per se). Another possible interpretation is that the NKLT and YLT3 activities might depend on only a few molecules that have somehow diffused into the nucleus, while the mass of observable oncoprotein would be superfluous in this respect. This possibility is hard to exclude, because diffusion below 1 or 2% of the total is nearly impossible to detect, even when relying on the distinctive pattern of NKLT phosphorylation (11). It is not a very likely possibility, however, because different studies indicate that low oncoprotein levels prevent transformation by SV40 (1, 36, 49), and furthermore, the reduced activity of the nuclear NKLT variant in CS cells suggests that the presence of NKLT in the cytoplasm is not functionally irrelevant. This behavior of simian CS cells bears some apparent analogy with the restrained growth behavior shown by COS cells, which constitutively express the nuclear wt large T antigen of SV40 (16). It must be remembered, however, that the wt large T antigen might potentially act on simian cell growth also by direct interaction with cell DNA sequences that are homologous to SV40 origin DNA, an ability that has been lost by NKLT and its variants (11).

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