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Adding an Rb-Binding Site to an N-Terminally Truncated Simian Virus 40 T Antigen Restores Growth to High Cell Density, and the T Common Region in *trans* Provides Anchorage-Independent Growth and Rapid Growth in Low Serum Concentrations

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The simian virus 40 large T antigen is sufficient to confer on cells multiple transformed cell growth characteristics, including growth to a high cell density, rapid growth in medium containing low serum concentrations, and anchorage-independent growth. We showed previously that distinct regions of the protein were involved in conferring these properties and that removal of the first 127 amino acids of T antigen abrogated all three activities. At least three large-T-antigen transformation-related activities have been localized to that region: binding of the tumor suppressor gene product Rb and two independent activities contained within the common region shared by large T and small t antigens. The experiments described here were directed toward determining whether these were the only activities from the N terminus that were needed. To do so we reintroduced an Rb-binding region into the N-terminally truncated T antigen (T128-708) and examined the growth properties of cells immortalized by it in the presence and absence of small t antigen, which can provide the T-common-region transformation-related activities in *trans*. We show that an Rb-binding region consisting of amino acids 101 to 118, when introduced into a heterologous site in T128-708, is capable of physically binding Rb and that binding is sufficient for cells expressing the protein to acquire the ability to grow to a high saturation density. However, in low-serum medium, the growth rate of the cells and maximal cell density are reduced relative to those of wild-type-T-antigen-expressing cells, and the cells cannot divide without anchorage. This result suggests that although Rb binding is sufficient in the context of T128-708 to confer growth to a high density, one or more other N-terminally located T-antigen activities are needed for cells to acquire the additional growth properties. Small t antigen in *trans* supplied those activities. These results indicate that the T-common-region activities and Rb binding are the only activities from the T-antigen N terminus needed to restore full transforming activity to the N-terminally truncated T antigen.

The simian virus 40 (SV40) early gene region encodes three transformation-related proteins: the 94,000-molecular-weight (94K) multifunctional large T antigen, the 18K small t antigen (44), and a recently discovered 17K T antigen (48). These proteins, consisting of 708, 135, and 174 amino acids, respectively, are synthesized from alternatively spliced mRNAs and contain both shared and unique amino acid sequences (44, 48). All three proteins share the first 82 amino acids. The region containing amino acids 1 to 82, therefore, is called the T-antigen common region. The 17K T antigen shares amino acids 83 to 131 with large T antigen as well. The remaining amino acids in each protein are unique.

Each of the T antigens can participate in transforming cells in culture. Large T antigen is sufficient to fully transform actively growing primary cells in culture, conferring an indefinite life span (immortalization) and the capacities to grow to a high cell density, to grow well when serum concentrations are limited, to divide in the absence of anchorage, and to form tumors in immunocompromised or transgenic mice (reviewed in references 14, 29, and 44). Small t antigen, although it rarely independently confers transformed cell properties (36a), generally augments the transforming activity of large T antigen in quiescent cells (reviewed in reference 44) and when large T antigen is in limited supply (3, 47). The 17K T antigen confers on cells in culture the capacity to form small colonies when suspended in semisolid medium (48).

Among the T antigens, the transforming activities of the large T antigen have been investigated in greatest depth (for a review, see reference 29). Alterations in at least four regions of T antigen diminish one or more transforming activities. T antigens with small deletions or multiple amino acid substitutions within the T common region cannot form dense foci on continuous cell lines (23, 30, 31, 34, 36, 39, 49) or primary rat embryo fibroblasts (30). Genetic alteration of the region encompassing the Rb/p107/p130-binding site (amino acids 101 to 118) prevents dense focus formation (20, 37, 42) and anchorage-independent growth (5, 43). Specific changes in the region containing amino acids 127 to 250 diminish transformation in multiple assays (5, 20, 43). Alteration of T antigen's p53-binding capacity abrogates T antigen's ability to immortalize C57BL/6 mouse embryo fibroblasts (22, 42) and diminishes transformation of an established cell line (33).

Consistent with these findings, we showed previously that cells expressing a large T antigen missing amino acids 1 to 127 could not grow to a high density and that cells expressing T antigens missing either amino acids 1 to 127 or 127 to 250 also could not grow well in low-serum medium or when suspended in soft-agarose medium (43). This loss-of-function analysis suggested that the three transformed cell growth properties de-

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pend on one or more activities or specific T-antigen conformations contained within or dependent on the N-terminal 127 T-antigen amino acids and that both growth in low-serum medium and anchorage-independent growth require one or more activities or structural determinants dependent on amino acids 1 to 250.

A large number of activities reside in or are dependent on the N-terminal 250 amino acids of T antigen. These include binding the cellular proteins of the retinoblastoma (Rb) susceptibility family (Rb/p130/p107) (8, 11–13), a protein of unknown function (p185) (23), the heat shock 73 (Hsc73) protein (38), and the transcription factors TFIIB, Sp1, TBP, TEF-1, and the 140K subunit of RNA polymerase (2, 19), as well as the nuclear localization signal (21); binding to the viral origin of replication (1); activities needed for maximal stimulation of cell DNA synthesis (9, 10); and transcriptional transactivating activities (24–26, 36a, 49). Thus, it is not clear which one or several of these activities are needed to confer the altered growth properties.

In this study we investigated the role of the large-T-antigen Rb-binding region in acquisition of the three transformed cell growth properties, i.e., growth to a high density, efficient growth in low-serum medium, and anchorage-independent growth, by gain-of-function analysis. Cells expressing a T antigen consisting of amino acids 128 to 708 displayed none of these properties. When an Rb-binding region (T-antigen amino acids 101 to 118) was inserted between amino acids 650 and 651, cells expressing the T128-708Rb protein gained the ability to grow to a high cell density only. Addition of small t antigen to T1-708Rb-expressing cells resulted in efficient growth in low-serum medium and anchorage-independent growth.

MATERIALS AND METHODS

Plasmids and cell lines. The plasmids py2xmet128-70K, dl536, and pPVU0-RI650 have been described previously (4, 22, 43). They encode, respectively, a large T antigen consisting of amino acids 128 to 708 with the substitution of Met for Lys at position 128 (T128-708) only, small t only, or a large T antigen containing an *Eco*RI 8-mer linker between codons 650 and 651. The cell lines used are shown in Table 1.

Addition of an Rb-binding region to T128-708. First, an *Eco*RI 8-mer linker positioned between T-antigen codons 650 and 651 was transferred to the T-antigen-coding sequence in the plasmid py2xmet128-70K from the plasmid pPVU0-RI650 (22). Both py2xmet128-70K and pPVU0-RI650 were digested with *Pvu*II and *Bam*HI. The large py2xmet128-70K fragment, containing the vector backbone, the SV40 origin, the enhancer-promoter, and the T128met-708-coding sequence to nucleotide 3506, and the small pPVU0-RI650 fragment, containing the remainder of the early-region coding sequence and poly(A) signal (nucleotides 3507 to 2533), were gel purified as described previously (16) and ligated to produce the plasmid py2xmet128-70K-EcoRI650. Next, oligonucleotide pairs designed, when annealed, to encode T-antigen amino acids 101 to 118 or 105 to 114 with 5′AATT3′ single-stranded extensions were annealed and inserted at the *Eco*RI site of py2xmet128-70K-EcoRI650 to give the plasmids py2xmet128-70KRb101-118 and py2xmet128-70KRb105-114, respectively. In all cases the inserted sequences and maintenance of the reading frame were confirmed by DNA sequencing.

Immortalization of primary mouse embryo fibroblasts and generation of cell lines expressing small t antigen. Primary fibroblast cultures were generated from 14- to 16-day-old C57BL/6 embryos and were immortalized by transfecting them with py2xmet128-70KRb101-118 or py2xmet128-70KRb105-114 DNA exactly as described previously (22). Individual colonies of immortalized cells were expanded into cell lines. The cell lines were named (Table 1) according to the amino acids in the T antigen they expressed and a letter designation for the independent clone from which they were derived. The cell lines T128-708Rb+tA and T128-708Rb+tB through -E were generated by cotransfecting the T128-708Rb-A or T128-708Rb-B cell line, respectively, with the small-t-antigen expression plasmid dl536 (39) and the plasmid pSV2Neo (40). Colonies that arose following selection in medium containing 500 μg of G418 per ml were expanded into cell lines and examined for expression of small t antigen.

Steady-state levels of large T and small t antigens. The steady-state levels of T antigens were determined as described in detail previously (22). Basically, protein extracts were prepared from actively growing cultures exactly as described. Unless stated otherwise, extracts were precleared by incubating them with a monoclonal antibody directed against glycoprotein D of herpes simplex virus (18) as described previously (4). Large T antigens were immunoprecipitated by using a polyclonal rabbit anti-T antibody (a gift from S. S. Tevethia). Small t antigen was immunoprecipitated by using the monoclonal antibody PAb419, which recognizes a denaturation-resistant epitope within the T common region (17). Immunoprecipitated proteins were separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE). The proteins were transferred to polyvinylidene difluoride (PVDF) membranes and probed by using PAb901, which recognizes an epitope contained within T-antigen amino acids 690 to 698 (41a), or a cocktail of PAb419 and PAb901 as indicated in the figure legends. PAb419 was included to detect small t antigen. Reacting proteins were visualized by using the enhanced chemiluminescence system exactly as described previously (22).

Detection of Rb binding. For detection of Rb binding, protein extracts were incubated with either the mouse monoclonal anti-human Rb antibody PMG3-

TABLE 1. Cell lines used

Cell line[a]	T antigen(s)	Cells from which derived	Previous designation	Reference
S-1[b]	None	C57BL/6 MEF[c]	B6Scl1	43
S-7[b]	None	C57BL/6 MEF	B6Scl7	43
T1-708+tA	T, t	C57BL/6 MEF	B6/PVBETK1	43
T1-708+tB	T, t	C57BL/6 MEF	B6/pPVU0flacl1	22
T1-708-A	T	C57BL/6 MEF	B6/dl2005flbcl2	43
T1-708-B	T	C57BL/6 MEF	B6/dl2005flacl1	43
T128-708	T128-708[d]	C57BL/6 MEF	B6/y2xmet128-70Kflacl3	43
T128-708+tA	T128-708, t	T128-708	B6/y2xmet128-70Kflacl3/tcl3	43
T128-708+tB	T128-708, t	T128-708	B6/y2xmet128-70Kflacl3/tcl2	43
T128-708Rb105-114[e]	T128-708Rb105-114	C57BL/6 MEF		This study
T128-708Rb[f]-A	T128-708Rb	C57BL/6 MEF		This study
T128-708Rb-B	T128-708Rb	C57BL/6 MEF		This study
T128-708Rb+tA	T128-708Rb, t	T128-708Rb-A		This study
T128-708Rb+tB	T128-708Rb, t	T128-708Rb-B		This study
T128-708Rb+tC	T128-708Rb, t	T128-708Rb-B		This study
T128-708Rb+tD	T128-708Rb, t	T128-708Rb-B		This study
T128-708Rb+tE	T128-708Rb, t	T128-708Rb-B		This study

[a] Cell lines are named according to the amino acids in the T antigen expressed, the presence (+t) or absence of small t antigen, and the independent cell clone from which they were derived (A, B, C, etc.).
[b] S-1 and S-7 are independent spontaneously immortalized C57BL/6 mouse embryo fibroblast cell lines.
[c] MEF, mouse embryo fibroblasts.
[d] Authentic amino acids 129 to 708 are preceded by a methionine rather than a lysine.
[e] Rb-binding site (amino acids 105 to 114) inserted between T-antigen codons 650 and 651.
[f] Rb-binding site (amino acids 101 to 118) inserted between T-antigen codons 650 and 651.

245 (Pharmingen) or a rabbit polyclonal antibody raised against amino acids 914 to 928 of human Rb (Santa Cruz). When PMG3-245, which is of the immunoglobulin G1 (IgG1) subtype, was used, goat anti-mouse IgG1 was added to the immunoprecipitation reaction mixtures. After SDS-PAGE and transfer to membranes, blots were probed with anti-T-antigen monoclonal antibodies as indicated in the figure legends.

Assays for saturation density, growth in low-serum medium, and anchorage-independent growth. On the day preceding initiation of the assay, cultures of the individual cell lines were passaged 1:2 to bring them into a state of active growth. On the following day, the cells were removed from the flasks by using trypsin and were counted. For determining saturation density, 35-mm-diameter dishes were each seeded with 10^5 cells in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) supplemented with 100 μg of streptomycin per ml, 100 μg of kanamycin per ml, 100 U of penicillin per ml, 0.03% glutamine, 25 mM HEPES, and 0.15% $NaHCO_3$ (DMEM–10% FBS) and were incubated at 37°C. Daily, the cells in three dishes were removed by being treated with trypsin, and viable cells were counted by trypan blue exclusion. The medium in the remaining dishes was removed and replaced with fresh DMEM–10% FBS.

For determining growth in low-serum medium, cells were seeded at a density of 10^4 cells per 35-mm-diameter dish in DMEM supplemented as described above except with 2% FBS (DMEM–2% FBS) and incubated at 37°C. Every other day the cells in three dishes were removed by being treated with trypsin and were counted. On the alternate days, the medium in the remaining dishes was removed and replaced with fresh DMEM–2% FBS.

Assays for anchorage-independent growth were performed in 12-well cell culture plates (Limbro) essentially as described in detail previously (43). Specifically, 1 ml of DMEM–10% FBS containing 0.46% GTG-agarose (Sigma) was added to each well and allowed to harden at room temperature. Single-cell suspensions of each cell line were diluted to 1.5×10^5, 7.5×10^4, and 3.75×10^4 cells per ml, and then 0.5 ml of each dilution was added to a tube containing 4 ml of DMEM–10% FBS supplemented with 0.23% GTG-agarose (Sigma). One milliliter of the resulting suspension was added to each of six (experiment 1 in Table 2) or four (experiments 2 to 4 in Table 2) wells containing 0.46% GTG-agarose medium. The newly added agarose layer was allowed to harden, and the plates were incubated at 37°C in a 5% CO_2 atmosphere. Each week, 0.5 ml of fresh 0.23% GTG-agarose medium was added to each well. Colonies greater than 0.2 mm in diameter were counted at the times indicated in Results.

RESULTS

Rb binds to T128-70KRb101-118 but not to T128-70KRb105-114. At the outset, the extent of the Rb-binding region needed to function when transferred to a foreign context was not obvious. Specific amino acid substitutions or small deletions within the 105-114 sequence diminish Rb binding and abrogate T antigen's ability to transform cells in dense-focus assays (a property related to high-saturation-density growth) and to promote anchorage-independent growth (4, 6, 15, 27, 31, 32). In addition, a peptide containing only amino acids 102 to 115 competes effectively with T antigen for Rb binding (7). Moran (32) previously replaced the conserved region 2 (CR2) site in the adenovirus E1A protein with T-antigen amino acids 101 to 118 and showed that the resulting protein functionally inactivated Rb. In that instance, the SV40 Rb-binding region was positioned in the E1A protein at the site normally containing a homologous Rb-binding site. It was not clear, however, that Rb binding would occur if T-antigen amino acids 101 to 118 were inserted in a different context.

Although Moran (32) was able to convert the E1A CR2 region to an SV40 CR2-like region and retain functional Rb binding, the exchange of Rb-binding regions between transforming proteins has not uniformly produced the expected result. The human JC virus (JCV) and SV40 virus T antigens are closely related. Yet, JCV T antigen both binds Rb and transforms cells less efficiently than SV40 large T antigen. Travis et al. (41) converted the amino acids in the JCV T-antigen Rb-binding region to those found in the SV40 T antigen. The resulting T antigen, in contrast to expectations, bound Rb less efficiently than authentic JCV T antigen, and its transforming activity was unchanged by the conversion. Those results demonstrated that the context in which the Rb-binding sequence resides influences its activity. Since our objective was to introduce as few T-antigen amino acids as possible so as to ensure addition of a single T-antigen activity, two potential Rb-binding regions, amino acids 105 to 114 and 101 to 118, were introduced between amino acids 650 and 651 of large T antigen. That site for insertion was selected because we showed previously that the region containing the C-terminal 82 amino acids of T antigen was not needed to confer any of the classical transformed-cell growth behavior changes (42) and that heterologous sequences could be added between codons 650 and 651 without compromising either protein stability or immortalizing activity (16).

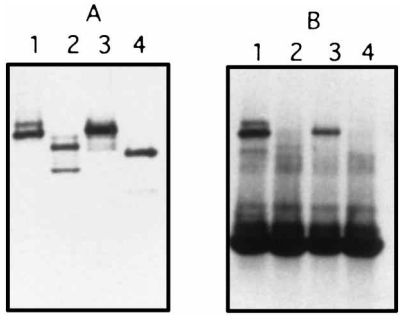

FIG. 1. Introduction of T-antigen amino acids 101 to 118 restores Rb-binding capacity to the N-terminally truncated T antigen T128-708. Equal amounts of protein (200 μg in panel A or 800 μg in panel B) from T1-708+tB cells expressing wild-type large T and small t antigens (lanes 1), cells expressing T128-708 (lanes 4), T128-708Rb105-114 expressing T128-708 with amino acids 105 to 114 inserted between amino acids 650 and 651 (lanes 2), or T128-708Rb101-118 expressing T128-708 with amino acids 101 to 118 inserted between amino acids 650 and 651 (lanes 3) were immunoprecipitated with a rabbit polyclonal anti-T antibody (A) or with anti-human Rb monoclonal antibody PMG3-245 (B) as described in Materials and Methods. Immunoprecipitated proteins were resolved by SDS–7.5% PAGE. The proteins were then transferred to PVDF membranes and probed with a cocktail of anti-T-antigen monoclonal antibodies PAb419 and PAb901. The secondary antibody was horseradish peroxidase-conjugated goat anti-mouse IgG. Detection was with the enhanced chemiluminescence system as described previously (22).

The abilities of T128-708, T128-708Rb105-114, T128-708Rb101-118, and wild-type large T antigen to bind mouse Rb were examined. Equal amounts of protein from cells immortalized by each T antigen were treated with a rabbit polyclonal anti-T antibody (Fig. 1A) or with the mouse monoclonal anti-human Rb antibody (Fig. 1B). Immunoprecipitated proteins were resolved by SDS-PAGE, transferred to membranes, and probed with anti-T-antigen monoclonal antibodies. The anti-T-antigen antibody recognized a protein with the appropriate migration characteristics in all lanes that received anti-T-antigen immunoprecipitates (Fig. 1A). However, in Fig. 1B, T antigen bands appear only in lanes that received anti-Rb immunoprecipitates from cells expressing wild-type T antigen (lane 1) or T128-708Rb101-118 (lane 3). Neither T128-708 (Fig. 1B, lane 4) nor T128-708Rb105-114 (lane 2) was immunoprecipitated by the anti-Rb antibody. Therefore, translocation of the Rb-binding region consisting of amino acids 101 to 118 to the C terminus of T128-708 restored the capacity to physically bind Rb. The shorter region (amino acids 105 to 114) was not sufficient to do so. Although not tested directly, it is reasonable to assume that binding to the Rb-related proteins p107 and p130 also would occur, since all three proteins bind to the CR2-like region containing the essential sequence LXCXE, and their binding has not been separated genetically (13, 15, 28). For simplicity, in naming mutant T antigens, the region consisting of amino acids 101 to 118 is referred to henceforth as Rb.

To determine whether binding to the translocated site altered the biological activities of T antigen, the steady-state

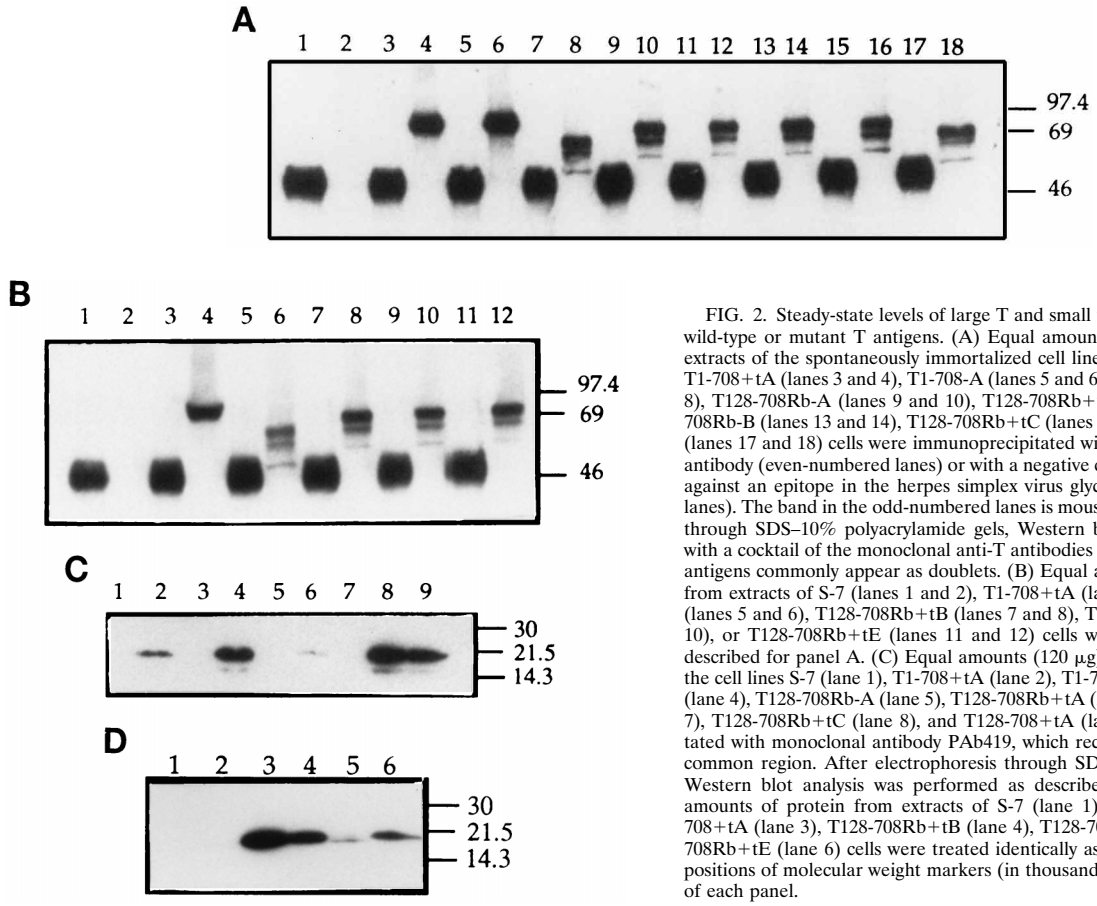

FIG. 2. Steady-state levels of large T and small t antigens in cells expressing wild-type or mutant T antigens. (A) Equal amounts of protein (120 μg) from extracts of the spontaneously immortalized cell line S7 (lanes 1 and 2) or from T1-708+tA (lanes 3 and 4), T1-708-A (lanes 5 and 6), T128-708+tB (lanes 7 and 8), T128-708Rb-A (lanes 9 and 10), T128-708Rb+tA (lanes 11 and 12), T128-708Rb-B (lanes 13 and 14), T128-708Rb+tC (lanes 15 and 16), or T128-708+tA (lanes 17 and 18) cells were immunoprecipitated with a rabbit polyclonal anti-T antibody (even-numbered lanes) or with a negative control monoclonal antibody against an epitope in the herpes simplex virus glycoprotein D (odd-numbered lanes). The band in the odd-numbered lanes is mouse IgG. After electrophoresis through SDS–10% polyacrylamide gels, Western blot analysis was performed with a cocktail of the monoclonal anti-T antibodies PAb901 and PAb419. The T antigens commonly appear as doublets. (B) Equal amounts (112 μg) of protein from extracts of S-7 (lanes 1 and 2), T1-708+tA (lanes 3 and 4), T128-708+tA (lanes 5 and 6), T128-708Rb+tB (lanes 7 and 8), T128-708Rb+tD (lanes 9 and 10), or T128-708Rb+tE (lanes 11 and 12) cells were processed identically as described for panel A. (C) Equal amounts (120 μg) of protein from extracts of the cell lines S-7 (lane 1), T1-708+tA (lane 2), T1-708-A (lane 3), T128-708+tB (lane 4), T128-708Rb-A (lane 5), T128-708Rb+tA (lane 6), T128-708Rb-B (lane 7), T128-708Rb+tC (lane 8), and T128-708+tA (lane 9) were immunoprecipitated with monoclonal antibody PAb419, which recognizes an epitope in the T common region. After electrophoresis through SDS-10% polyacrylamide gels, Western blot analysis was performed as described for panel A. (D) Equal amounts of protein from extracts of S-7 (lane 1), T1-708-A (lane 2), T128-708+tA (lane 3), T128-708Rb+tB (lane 4), T128-708Rb+tD (lane 5), or T128-708Rb+tE (lane 6) cells were treated identically as described for panel C. The positions of molecular weight markers (in thousands) are indicated on the right of each panel.

levels of the T antigen and Rb:T complexes were determined, and the growth properties of two clonally derived cell lines expressing T128-708Rb were investigated in three assays: growth to a high saturation density, growth in low-serum medium, and colony formation in soft-agarose medium. Since small t antigen can complement large T antigens missing the T common region (2, 31, 36a, 47), five clonally derived cell lines expressing both T128-708Rb and small t antigen were examined in parallel. The same cell lines were examined simultaneously in all three assays.

Steady-state expression levels of large T and small t antigens. The cell lines expanded from individual immortalized or G418-resistant colonies were examined for the steady-state levels of large T (Fig. 2A and B) and small t (Fig. 2C and D) antigens. A protein of the appropriate molecular mass for full-length large T antigen was immunoprecipitated from extracts of T1-708+tA and T1-708-A cells by the polyclonal rabbit anti-T antibody (Fig. 2A, lanes 4 and 6; Fig. 2B, lane 4). A protein (T128-708) migrating as expected for a 70K protein was immunoprecipitated from extracts of T128-708+tB (Fig. 2A, lane 8) and T128-708+tA (Fig. 2A, lane 18; Fig. 2B, lane 6). A protein migrating more slowly than the T128-708 band was immunoprecipitated from extracts of cell lines immortalized by T128-708Rb (Fig. 2A, lanes 10 and 14) and their small-t-antigen-expressing derivatives (Fig. 2A, lanes 12 and 16; Fig. 2B, lanes 8, 10, and 12). The decreased mobility of these T128-708Rb proteins is presumed to result from the reintroduction of 18 amino acids, some of which in their natural context are phosphorylated. It is not known whether the translocated amino acids are phosphorylated or, if so, whether they are phosphorylated in a manner identical to that in wild-type T antigen. Each of the T antigens accumulated to wild-type or near-wild-type levels. No T antigen was immunoprecipitated from extracts of S7 cells (Fig. 2A and B, lanes 2). No proteins were immunoprecipitated from extracts treated with a monoclonal antibody that recognizes an epitope on the herpes simplex virus glycoprotein D, which was used as a negative control antibody (Fig. 2A and B, odd-numbered lanes). The band seen in this case is mouse IgG.

A protein with the migration expected for small t antigen was immunoprecipitated by PAb419 from extracts of T1-708+tA (Fig. 2C, lane 2), T128-708+tB (Fig. 2C, lane 4), T128-708Rb+tA (Fig. 2C, lane 6), T128-708Rb+tC (Fig. 2C, lane 8), T128-708+tA (Fig. 2C, lane 9, and D, lane 3), T128-708Rb+tB (Fig. 2D, lane 4), T128-708Rb+tD (Fig. 2D, lane 5), and T128-708Rb+tE (Fig. 2D, lane 6) cells but not from extracts of S-7 (Fig. 2C and D, lanes 1), T1-708-A (Fig. 2C, lane 3, and D, lane 2), T128-708Rb-A (Fig. 2C, lane 5), or T128-708Rb-B (Fig. 2C, lane 7) cells. Separately (data not shown), we showed that the T antigen in all cell lines expected to express T128-708 or T128-708Rb protein could not be immunoprecipitated by PAb419, as these proteins are missing the region containing the PAb419 epitope, the majority of the small t coding sequence, and the small t donor splice site.

In addition, we examined the level of T:Rb complexes in each cell line. The results appear in Fig. 3. Equal amounts of protein from each cell line were treated with a rabbit polyclonal anti-Rb antibody. The resulting immunoprecipitates

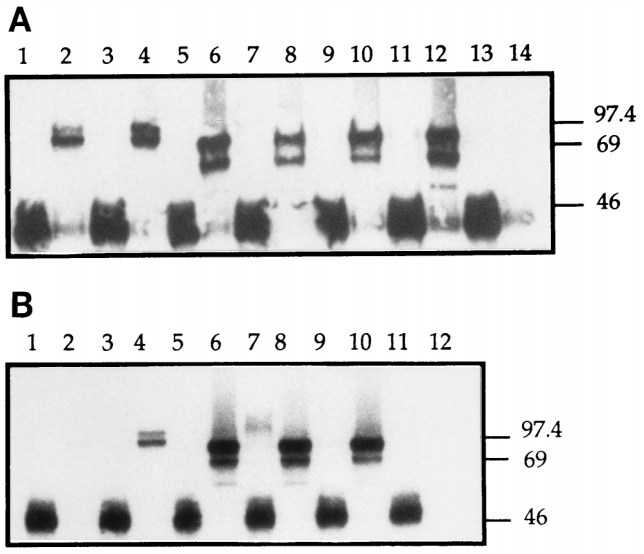

FIG. 3. Steady-state levels of T:Rb antigen complexes in cells expressing wild-type T antigens or T antigens with a translocated Rb-binding region. (A) Equal amounts (945 μg) of protein from extracts of T1-708+tA (lanes 1 and 2), T1-708-A (lanes 3 and 4), T128-708Rb-A (lanes 5 and 6), T128-708Rb+tA (lanes 7 and 8), T128-708Rb-B (lanes 9 and 10), T128-708Rb+tC (lanes 11 and 12), or T128-708+tB (lanes 13 and 14) cells were treated with a rabbit polyclonal anti-Rb antibody. The immunoprecipitates were resolved by SDS–10% PAGE and transferred to membranes, and the membranes were probed with the monoclonal antibody PAb901 and detected as described in the legend to Fig. 1. (B) Extracts (660 μg) of the cell lines S-7 (lanes 1 and 2), T1-708-A (lanes 5 and 6), T128-708Rb+tB (lanes 7 and 8), T128-708Rb+tD (lanes 9 and 10), T128-708Rb+tE (lanes 11 and 12), and T128-708+tB (lanes 13 and 14) were treated identically as described for panel A. The smaller amount of coprecipitated T1-708 (lane 4) was not typical, as it was not reproduced in other experiments. Migrations of molecular weight markers (in thousands) are indicated on the right of each panel.

were resolved by SDS-PAGE, transferred to PVDF membranes, and probed with monoclonal anti-T-antigen antibody. Levels of T antigen similar to those in immunoprecipitates of extracts containing wild-type T antigen were detected, indicating that equivalent amounts of T:Rb complexes were present in each cell line.

Effect of restoring Rb-binding capacity on growth to a high saturation density. Cells expressing T128-708 and small t or full-length T antigen or cells expressing T128-708Rb with and without small t antigen were examined for the ability to grow to a high cell density. The results appear in Fig. 4. Figure 4A compares the growth of cells expressing wild-type large T and small t antigens (T1-708+tA), large T antigen only (T1-708-A), or T128-708 and small t antigen (T128-708+tB) with that of cells expressing T128-708Rb (T128-708Rb-A and T128-708Rb-B). Both cell lines expressing a wild-type large T antigen grew to a maximal density of 3.75×10^6 and 4.66×10^6 cells per dish before the cell layers sloughed and could no longer be brought into single-cell suspension. Neither the growth rate nor the saturation density was enhanced by the presence of small t antigen. In contrast, the cell line expressing T128-708 and small t antigen grew more slowly and attained a maximal cell density of only 1.38×10^6 cells per dish. Cells expressing T128-708Rb (Fig. 4A) attained densities and grew at rates similar to those for cells expressing a wild-type T antigen. As shown in Fig. 4B, the presence of small t antigen in cells expressing T128-708Rb (T128-708Rb+tA to -E) did not consistently further enhance either the rate of growth or the maximal cell density attained. Restoration of Rb binding, therefore, was sufficient to restore the wild-type growth rate and the capacity to grow to a high saturation density in medium containing 10% FBS.

Rb-binding and transforming activities in the T common region are required to restore rapid growth in low-serum medium and for anchorage-independent growth. Figure 5 shows the growth behaviors of the same cell lines in medium containing 2% FBS. Figure 5A compares the growth of cells expressing wild-type large T and small t antigens, wild-type large T antigen only, or T128-708 and small t antigen with that of cells expressing T128-708Rb. Cell lines expressing wild-type T antigen, with or without small t antigen present, grew rapidly and attained a high cell density (1.16×10^6 and 4.47×10^6 cells per dish, respectively). In contrast, cells expressing T128-708 and small t antigen grew more slowly and attained a lower maximal cell density (2.57×10^5 cells per dish). The growth rate of cells expressing T128-708Rb (T128-708Rb-A and T128-708Rb-B) paralleled that of cells expressing T128-708 and small t antigen, although both cell lines expressing T128-708Rb grew to higher density than the cells expressing T128-708 and small t antigen. In contrast (Fig. 5B), the growth rate of cells expressing T128-708Rb and small t antigen (T128-708Rb+tA to -E) generally paralleled that of cells expressing wild-type T antigen. In addition, with the exception of T128-708Rb+tA, the cell lines reached a high density characteristic of cells expressing a wild-type T antigen.

The ability of cells to form colonies in semisolid medium depends on at least two activities of T antigen, i.e., Rb binding and an activity contained within the T common region. We showed previously that removal of amino acids 1 to 127 from T antigen prevented cells expressing the mutant T antigen from dividing in soft-agarose medium. To determine whether the only activities from the deleted region that were needed were the Rb-binding and T-common-region transformation activities, we examined the ability of cells expressing T128-708Rb alone or T128-708Rb and small t antigen to form colonies in soft-agarose medium. The results appear in Table 2.

As is typical for this type of assay, the percentage of cells that generated colonies varied from assay to assay. For this reason, multiple assays were performed to establish the patterns of growth behavior for the individual cell lines. Cells expressing wild-type T antigen, whether or not small t antigen was present, divided to form colonies. Thus, under the conditions used, colony formation in semisolid medium by cells expressing a full-length large T antigen is small-t-antigen independent. In contrast, as we showed previously (43), cells expressing T128-708 alone or T128-708 and small t antigen remained as single cells or rarely developed into colonies. In no case did the number of colonies significantly exceed that generated by a spontaneously immortalized cell line (S7). Cells expressing T128-708Rb formed low numbers of colonies relative to cells expressing full-length T antigen in the same experiment. These results indicated that neither Rb binding nor small t antigen alone was sufficient to fully restore the mutant T antigen's ability to promote anchorage-independent growth. However, cells expressing T128-708Rb and small t antigen divided efficiently in the semisolid medium to produce colonies. The results were essentially the same whether colonies were counted after 15 to 17 days of incubation (Table 2, experiments 1 to 3) or after 9 weeks of incubation (Table 2, experiment 4). As we showed previously (43), an occasional cell line (e.g., T128-708Rb+tA) deviates from the general pattern that emerges for the properties of clonally derived cell lines.

We conclude that, as was the case for growth in low-serum medium, restoration of Rb binding is necessary but not sufficient to confer the ability to divide in the absence of anchorage.

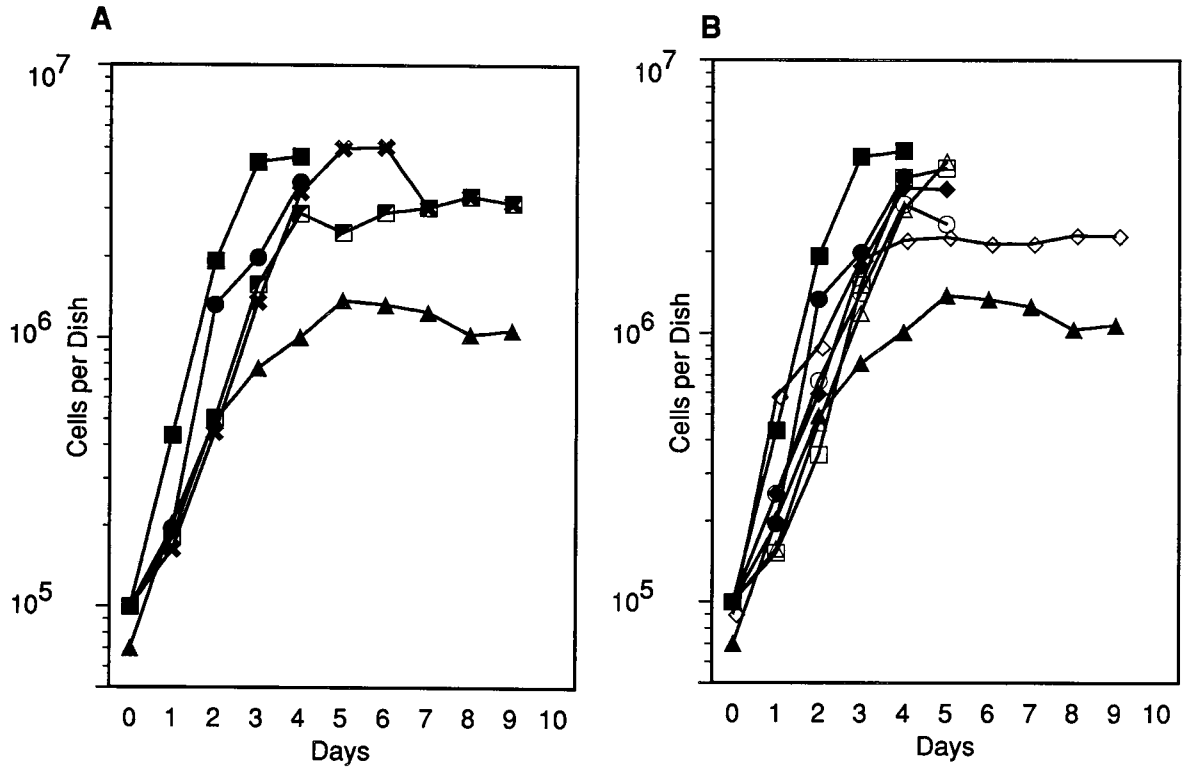

FIG. 4. Growth characteristics of cells with a translocated Rb-binding region in medium containing 10% FBS. Each point is the average of the cell numbers in three cultures. (A) Cells expressing wild-type large T and small t antigens (T1-708+tA) (circles), large T antigen alone (T1-708-A) (closed squares), T128-708 and small t antigen (T128-708+tB) (triangles), or T128-708Rb (T128-708Rb-A [half-closed squares] and T128-708Rb-B [crosses]). (B) Cells expressing T128-708Rb and small t antigen (T128-708Rb+tB) (closed diamonds), T128-708Rb+tE (open squares), T128-708Rb+tC (open circles), T128-708Rb+tD (open triangles), or T128-708Rb+tA (open diamonds). Growth curves for cells expressing wild-type large T and small t antigens (T1-708+tA) (closed circles), large T antigen alone (T1-708A) (closed squares), or T128-708 and small t antigen (T128-708+tB) (closed triangles) are reproduced from panel A for comparison.

Small t antigen complements T128-708Rb for anchorage-independent growth.

DISCUSSION

We showed previously that Rb binding and the transforming activities within the T common region were not needed for either immortalization or enhanced tumorigenicity in nude mice (43). However, removal of the first 127 amino acids of T antigen, containing both the Rb-binding region and the T common region, resulted in a T antigen that could not confer the altered growth behaviors monitored as rapid growth in reduced-serum medium, anchorage-independent cell division, and growth to a high cell density. To determine whether these behaviors depended on only Rb binding, the T common region, or additional N-terminal T-antigen activities, we introduced an Rb-binding region into a T antigen missing the N-terminal 127 amino acids (T128-708) and examined the growth behavior of cells expressing the protein in the presence or absence of small t antigen.

The results presented here show that T-antigen amino acids 101 to 118 constitute an Rb-binding site when translocated to the C terminus of T antigen. Insertion of T-antigen amino acids 105 to 114 between residues 650 and 651 in the T128-708 T antigen did not result in Rb binding. It is possible that amino acids 101 to 104 and 115 to 118 are not essential to Rb binding at the natural site in T antigen but are needed in order to form a functional Rb-binding site at a foreign location. Similarly, the possibility that the shorter Rb-binding region binds Rb more weakly than does the longer region so that the complexes do not remain associated during extraction cannot be ruled out.

The Rb-related proteins p107 and p130, in addition to Rb, bind to the E1A CR2-like portion of T antigen (reviewed in reference 28), and all depend on the same sequence, LXCXE, for binding (13, 15). It is likely that p107, p130, or both must be inactivated or modified in order for T antigen to transform cells. T antigen confers growth advantages on Rb-null mouse cells (6, 46); however, alteration of the LXCXE sequence abrogates the transformation. Thus, Rb binding alone is not sufficient to confer the altered growth behavior. Although we did not directly measure this, we presume that the Rb-binding region used here will bind Rb, p107, and p130, as it contains the CR2-like domain.

Previous loss-of-function analysis showed that mutations within or including the Rb-binding region diminish T antigen's ability to transform in dense-focus assays, grow to a high saturation density, grow with wild-type kinetics in low-serum medium, and form colonies in semisolid medium (5, 20, 43). It was not clear from those investigations whether Rb binding was the only activity from the N terminus needed to confer these properties. To make that determination, we generated clonally derived cell lines expressing T128-708 and small t antigen, T128-708Rb, or T128-708Rb and small t antigen and compared their growth to that of cells expressing either wild-type T antigen alone or wild-type large T and small t antigens. Restoration of Rb/p107/p130-binding capacity was sufficient to restore T128-708's ability to confer on cells growth to high density. Small t antigen did not enhance that ability. These results indicate that

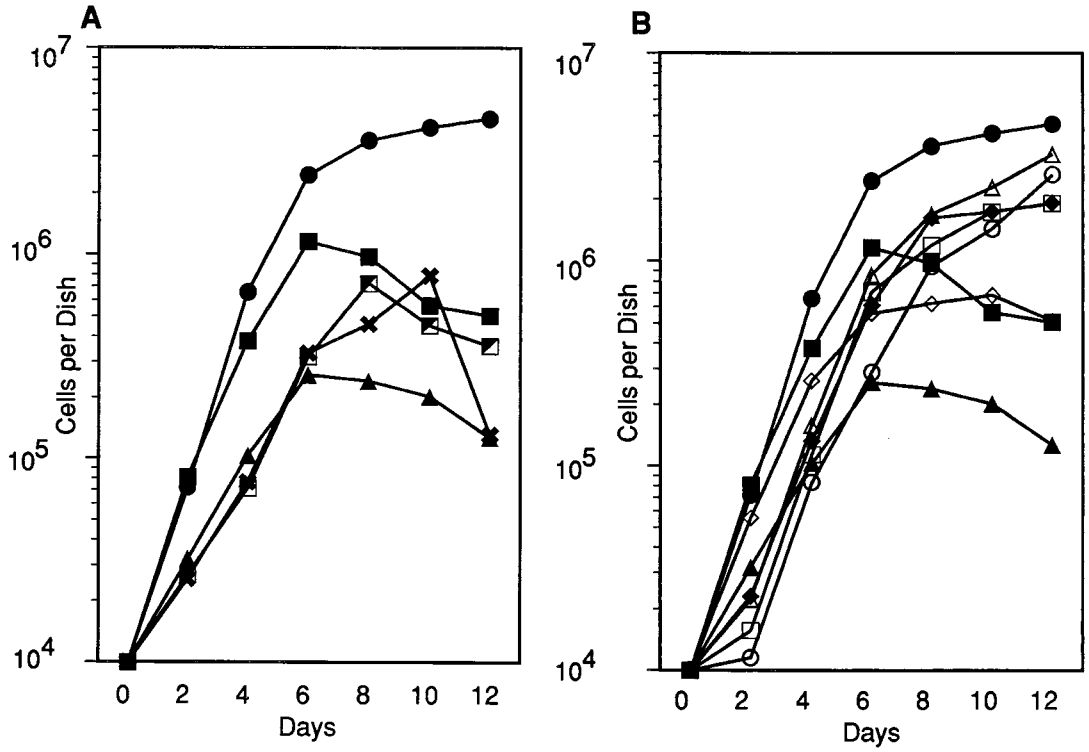

FIG. 5. Growth characteristics of cells with a translocated Rb-binding region in medium containing 2% FBS. Each point is the average of the cell numbers in three cultures. (A) Cells expressing wild-type large T and small t antigens (T1-708+tA) (closed squares), large T antigen alone (T1-708A) (circles), T128-708 and small t antigen (T128-708+tB) (triangles), or T128-708Rb (T128-708Rb-A [half-filled squares] and T128-708Rb-B [crosses]). (B) Cells expressing T128-708Rb and small t antigen (T128-708Rb+tD) (open triangles), T128-708Rb+tE (open squares), T128-708Rb+tB (closed diamonds), T128-708Rb+tA (open diamonds), or T128-708Rb+tC (open circles). Growth curves for cells expressing wild-type large T and small t antigens (T1-708+tA) (closed circles), large T antigen alone (T1-708A) (closed squares), or T128-708 and small t antigen (T128-708+tB) (closed triangles) are reproduced from panel A for comparison.

no additional activities contained within the first 127 amino acids are essential for cells to grow to a high density. The results are consistent with current understanding of events during contact inhibition of growth. In response to the negative growth signal sent by contact inhibition, the cyclin-dependent kinase inhibitor $p27^{KIPI}$ prevents the assembly of cyclin E-CDK2 complexes (reviewed in reference 16a). The result is accumulation of the active, hypophosphorylated Rb and halting of the cell cycle. Introduction of the T-antigen Rb-binding region, which associates exclusively with hypophosphorylated Rb (27), would be expected to overcome contact inhibition of cell division.

TABLE 2. Influence of Rb-binding capacity and small t antigen on colony formation in soft-agarose medium

Cell line	T antigen(s)	% Cells forming colonies in expt[a]:			
		1	2	3	4
S-1	None	0.002	ND[b]	ND	ND
S-7	None	0.01	0.039	0.008	<0.0003
T1-708+tA	T, t	5.0	5.9	ND	1.88
T1-708+tB	T, t	1.24	ND	ND	ND
T1-708-B	T	10.8	ND	ND	ND
T1-708-A	T	14.4	3.9	2.35	ND
T128-708	T128-708	<0.0008	ND	ND	ND
T128-708+tA	T128-708, t	<0.003	ND	ND	ND
T128-708+tB	T128-708, t	<0.0007	<0.042	<0.0003	ND
T128-708Rb-A	T128-708Rb	0.8	ND	ND	<0.003
T128-708Rb-B	T128-708Rb	0.16	0.31	0.045	0.01
T128-708Rb+tA	T128-708Rb, t	<0.0006	<0.0003	<0.0003	ND
T128-708Rb+tB	T128-708Rb, t	14	3.19	1.32	4.72
T128-708Rb+tC	T128-708Rb, t	ND	0.412	2.66	2.0
T128-708Rb+tD	T128-708Rb, t	ND	ND	0.40	3.27
T128-708Rb+tE	T128-708Rb, t	ND	ND	1.37	0.56

[a] In experiments 1 to 3 colonies were counted after 15 to 17 days; in experiment 4 colonies were counted after 9 weeks.
[b] ND, not determined.

Neither anchorage-independent growth nor rapid growth in low-serum medium, in contrast, was restored by addition of the Rb-binding region to T128-708. Neither was restored by addition of the T-common-region transforming activities in *trans*. However, the combination of the Rb-binding site and small t antigen fully restored these capabilities. The involvement of both Rb-binding capacity and an activity in the T common region in anchorage-independent growth was shown previously by loss-of-function analyses (31). We show here that only these regions from the N-terminal 127 amino acids are needed. We cannot rule out the possibility, however, that in addition to the transforming activities of the T common region, the ability of small t antigen to transactivate (24–26) or repress (45) heterologous promoters distinct from those modulated by large T antigen affects its ability to complement T128-708Rb. Since a T antigen containing only the first 82 amino acids cannot complement a large T antigen containing amino acids 83 to 708 (21), it is not clear as yet whether the complementing activity in small t antigen depends on the same function as that represented by amino acids 1 to 82 of large T antigen or, alternatively, whether small t antigen contains an activity which is analogous in function but not identical biochemically to the N terminus of T antigen.

None of the N-terminally truncated mutant constructs used in this study is capable of producing the 17K T antigen. The extent to which the 17K T antigen contributes to transformed cell growth when wild-type T antigen is present is not known. The first 131 amino acids of the 17K T antigen (48) are identical to those of large T antigen and would be expected to have both Rb-binding capacity and the T-common-region transformation activities. The 17K T antigen probably is not essential for growth to high density in cell culture under the conditions employed in this study. Cells expressing T128-708Rb and those expressing wild-type T antigens grew to similar densities. Therefore, the level of T:Rb complexes immunoprecipitated from extracts of cells expressing T128-708Rb was sufficient in the absence of the 17K T antigen. It remains likely, nonetheless, that the 17K T antigen could provide a selective growth advantage under different in vitro culture conditions or in vivo.

Both rapid growth in low-serum medium and anchorage-independent growth depend on one or more activities in the T common region as well as on Rb binding. These activities should be contained within the 17K T antigen, and the 17K T antigen alone is sufficient to confer anchorage-independent growth (48). In several cases, cells expressing T128-708Rb and small t antigen generated fewer colonies in soft-agarose medium than did their counterparts expressing wild-type T antigen. While we did not measure this precisely, we observed that in those cases the colonies grew more slowly. A comparison of the levels of small t antigen in the various cell lines reveals that the two cell lines that generated the fewest colonies (T128-708Rb+tA and T128-708Rb+tD) accumulated very low levels of small t antigen. Differences in the levels of small t antigen in clonally derived cell lines probably reflect transcriptional activity at the chromosomal site of integration of the small-t-antigen-expressing plasmid. Since the presence of small t antigen provides no growth advantage in high-serum medium, which was the condition under which the clonal cell lines were derived, selection for cell lines expressing high levels of the protein would not be expected. Since the levels of mutant large T antigen were similar to (Fig. 2) and levels of T:Rb complexes were equivalent to (Fig. 3) those in cells expressing wild-type T antigen, we conclude that the level of the complementing transforming activity in the T common region may be a limiting factor, at least in some clonally derived lines. Therefore, it is reasonable to expect that the 17K T antigen, if it was made, could assist in anchorage-independent growth.

It should be pointed out that all of the mutant T antigens used in this investigation accumulate in the cell cytoplasm. Deletion of the first 127 amino acids and conversion of amino acid 128 from Lys to Met inactivates the nuclear localization signal. Clearly, accumulation of T antigen in the nucleus is not needed for accumulation of wild-type levels of T:Rb complexes (Fig. 3) or for growth to a high cell density (Fig. 4). Presumably, T antigen and Rb form complexes at their site of synthesis in the cell cytoplasm. It is unlikely that T128met-70K, if targeted to the nucleus, would confer anchorage-independent growth in the absence of small t antigen. Montano et al. (31) showed previously that a T antigen containing amino acids 83 to 708, which accumulates in the nucleus, could not confer on cells anchorage-independent growth unless the cells also expressed the complementing small t antigen.

The nature of the transforming activity in the common region that complements T128-708Rb for anchorage-independent growth remains to be determined. Two transformation-related activities within the common region have been identified. Amino acid substitutions at residues 19 and 28 in both large T and small t antigens prevent dense focus formation (35, 36a). Recently, Porras et al. (36a) defined an additional transforming activity in the T common region. They showed that the hexapeptide sequence HPDKGG (amino acids 42 to 47), which is conserved among papovavirus T antigens, as well as the activity marked by the substitutions at positions 19 and 28, is required in small-t-antigen-dependent dense-focus transformation assays. They showed further that a wild-type small t antigen could complement both a large T antigen with amino acid substitutions at positions 19 and 28 and a large T antigen with amino acid substitutions in the hexapeptide sequence in a small-t-dependent focus formation assay, whereas a small t antigen with the corresponding substitutions could not. The T-common-region activity marked by mutations in the hexapeptide sequence is needed for T-antigen-mediated transactivation of the cyclin A promoter (36a). This activity may in part account for small t antigen's ability to complement T128-708Rb in conferring anchorage-independent growth, since in normal cells, transcription of the cyclin A gene is anchorage dependent (38a). It will be of considerable interest to determine whether either or both of the T-common-region activities provided in *trans* participate in anchorage-independent division and wild-type growth in low-serum medium.

It is likely that one or more T-antigen activities in addition to Rb binding and the transforming function(s) in the T common region are needed for anchorage-independent growth and efficient growth in low-serum medium. We showed previously (43) that a T antigen missing amino acids 127 to 250 (T1-708dl127-250) could not confer those transformed cell properties. Similarly, Kalderon and Smith (20) found that mutations within that region decreased focus formation on Rat-1 cells. Two conclusions were possible: either deleting amino acids 127 to 250 altered the conformation of the Rb-binding region or the T common region, or an additional required activity was disturbed by the deletion. It seems unlikely that disruption of the Rb-binding region or the T-common-region transforming activity by deleting amino acids 127 to 250 could account for the growth behavior, for two reasons. First, the T1-708dl127-250 protein contains an Rb-binding site, and cells expressing the protein grew to a high saturation density (43), a growth property that requires Rb binding. Second, cells immortalized by the T1-708dl127-250-expressing construct synthesize small t antigen abundantly (43). Small t antigen would provide the T-common-region activities in *trans*. Since the cells expressing T1-708dl127-250 produce wild-type amounts of large T and small t antigens, it also is unlikely that the quantity of mutant T antigen is limiting (43). Thus, the results presented here in combination with our previous characterization of cells expressing T1-708dl127-250 lend support the hypothesis that the region containing amino acids 127 to 250 defines an additional transformation-related region of T antigen. Additional mutational and biochemical analysis will be needed to define the limits of the activity in order to probe its function.

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