Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 7448–7452, October 1990
Biochemistry

Simian virus 40 small tumor antigen and an amino-terminal domain of large tumor antigen share a common transforming function

(transformation helper effect)

X. MONTANO*†, R. MILLIKAN*, J. M. MILHAVEN*‡, D. A. NEWSOME*, J. W. LUDLOW*, A. K. ARTHUR§, E. FANNING§, I. BIKEL*, AND D. M. LIVINGSTON*

*The Dana–Farber Cancer Institute and Departments of Medicine and Pathology, Harvard Medical School, Boston, MA 02115; and §Institut für Biochemie der Ludwig-Maximilians-Universität München, Karlstrasse 23, D-8000 Munich 2, Federal Republic of Germany

Communicated by George J. Todaro, June 8, 1990

ABSTRACT **The 82-residue amino-terminal sequences of simian virus 40 large tumor antigen (TAg) and small tumor antigen (tAg) are identical. Genetic analysis of TAg lacking amino acids 1–82 revealed that it was transformation-defective, as revealed by the agar growth assay, except when introduced in the presence of tAg. Since the latter, alone, lacks overt transforming activity, it would appear that the function of the sequence common to TAg and tAg is necessary, but not sufficient, for TAg transforming activity and that tAg can provide that function or its equivalent in trans. Thus, tAg may, in part, be viewed as a "portable" copy of a TAg functional domain.**

The simian virus 40 transforming region encodes two products: the large tumor antigen (TAg) and the small tumor antigen (tAg) (1, 2). TAg can immortalize certain cell types (2–5). It can also induce G_1 phase-arrested cells to synthesize DNA and divide (6, 7), and some fibroblasts and epithelial cell lines that synthesize TAg in large amounts can overgrow a monolayer of untransformed cells (2), grow in semisolid medium (2), and are tumorigenic when injected into a suitable host (2, 8, 9).

tAg, alone, cannot transform established lines or immortalize primary cells, but it can, directly or indirectly, enhance the transforming activity of limiting quantities of TAg (10–14). Thus, it can cooperate with TAg in the transformation of some cell types, a property termed the "tAg-helper effect" (13). In addition, a report (15) suggests that tAg can increase the repertoire of cells in which TAg can exert its transforming effects in transgenic mice (15). The biochemical basis for these biological effects is unknown. Indeed, although tAg does have discrete transcription activation activity (16) and forms complexes with protein phosphatase 2A (17, 18), the role of these two properties in the aforementioned biological activities is not known.

Recent evidence (19–23) indicates that the amino-terminal ≈140 residues of TAg can exert full transforming effects in certain cell types, albeit with reduced frequency by comparison with intact TAg. TAg and tAg share the identical 82-residue amino-terminal sequence due to the partially overlapping nature of their coding units. Little is known of the role of this segment in the function of either protein or whether it operates in the TAg transforming process. An obvious question, then, is whether all or part of it is active in the expression of TAg transforming function and, if so, whether tAg can provide the function of this segment to cells. Here we report evidence consistent with the suggestion that in TAg the sequence common to TAg and tAg contains a domain needed for TAg transforming activity and that tAg can deliver the function of that unit in trans.

MATERIALS AND METHODS

Construction of pZIP-T_{83-708}. Standard methods of molecular cloning were used throughout (32). The construction of $pSVT_{83-708}$, originally called pTx, has been described (24). It is a 4.7-kilobase pUC9-based plasmid containing the DNA sequence encoding amino acids 83–708 of TAg fused, through a unique *Bam*HI site, to the sequence encoding the 12 amino-terminal amino acids of LacZ. The synthetic, double-stranded oligonucleotide

Sal I *Bam*HI *Hin*fI *Pfl*MI
5′-TCGACGGATCCATGATTCCAACCT-3′
3′-GCCTAGGTACTAAGGTT-5′

encoding amino acids 83–85 of TAg (Ile-Pro-Thr), as well as an upstream, ribosome-binding sequence, were inserted between the *Pfl*MI site in the TAg coding sequence and the *Sal* I site of the pUC9 polylinker to give rise to a sequence encoding amino acids 83–708 of TAg [T-(83–708)]. Translation of T-(83–708) would be expected to start at the inserted methionine codon and would then proceed from residue 83 to the normal carboxyl terminus of the protein. The T-(83–708) (with methionine at position 82) sequence, flanked at both the 5′ and 3′ ends by *Bam*HI sites, was excised from $pSV\text{-}T_{83-708}$ by *Bam*HI digestion and cloned into the *Bam*HI site of the pZipNeoSV(X)I vector (25) to yield $pZIP\text{-}T_{83-708}$.

Production of a T-(83–708) Recombinant Retrovirus and Generation of BALB/c 3T3 Cl.A31 Mouse Cell Lines Constitutively Expressing Simian Virus 40 T-(83–708). Transfection of the ψ-2 packaging cell line (26), infection of subconfluent A31 cell cultures with ψ-2 culture supernatant fluids, and selection of G418-resistant clones were performed as described for the small tAg-expressing recombinant retrovirus pZIPSV40t (13). Some T-(83–708)-producing A31 clones were isolated by directly transfecting $pZIP\text{-}T_{83-708}$ onto A31 and selecting G418-resistant clones. All cells were grown at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) calf serum (Colorado Serum, Denver) in a 10% CO_2/90% air humidified atmosphere. G418-resistant lines were grown in G418 at 100 μg/ml. ψ2 $ZIP\text{-}t_{5-10}$ and ψ-2 ZIP are wild-type tAg and backbone ZIP control-retroviral-producer cell lines (13) used to generate tAg and backbone retrovirus (ZIP) control retroviral stocks. Retroviral titers

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Abbreviations: TAg, large tumor antigen; tAg, small tumor antigen; wt, wild type; pRB, retinoblastoma gene product.

†Present address: Squibb Institute for Medical Research, Department of Molecular Biology, Princeton, NJ 08540.
‡Present address: Transkaryotic Therapies, Inc., 195 Albany Street, Cambridge, MA 02139.

Table 1. Colony formation in soft agar of clonal lines of A31 cells constitutively expressing T-(83–708) superinfected with ZIP backbone or ZIP-t retroviruses

Cell line	Virus	Virus input, ml	% microcolonies*
		Experiment 1	
A31	—	—	<1
	ZIP	0.6	<1
	ZIP-t_{5-10}	0.6	<1
A31-TEXS6	—	—	40–50
	ZIP	0.6	40–50
	ZIP-t_{5-10}	0.6	60–75
A31-TWT4	—	—	30
	ZIP	0.6	30
	ZIP-t_{5-10}	0.6	50
A31-7inf	—	—	<1
	ZIP	0.6	<1
	ZIP-t_{5-10}	0.6	5
		Experiment 2	
A31-7inf	ZIP	10	<1
	ZIP-t_{5-10}	10	40–50
	ZIP-t_{common}	10	<1

*Plate-to-plate range of microcolony formation values (rounded off to the nearest 5%) is shown. Where it is not, the variation from plate to plate was ≤5%.

were measured by assaying for G418 colony formation in NIH 3T3 cells after infection with serially diluted stocks (25).

Anchorage-Independent Growth. Where indicated, A31 cells were seeded, fed, and observed in 1.8% (wt/vol) soft agar as described (11–13). Colony formation was observed and quantitated as described (11–13). Colonies that were scored positive contained at least 8–16 cells. Growth in 0.8% methylcellulose and in soft agar was measured in the same manner.

***In Situ* Immunostaining and Immunoprecipitation.** These procedures were performed as described (13).

RESULTS

Biochemical Properties of T-(83–708). The second exon of TAg, encoding the unique TAg amino acid sequence residues 83–708, was linked to an amino-terminal methionine codon as an upstream translational control sequence. The resulting construct was then cloned into the pZIP backbone retroviral vector (25) to yield pZIP-T_{83-708}. BALB/c 3T3 Cl.A31 cells were infected with a recombinant retrovirus encoding this truncated TAg product or transfected directly with pZIP-T_{83-708}. G418-resistant clonal derivatives of A31 cells constitutively expressing T-(83–708) are listed in Table 1, and the biological properties of these cell lines are discussed below.

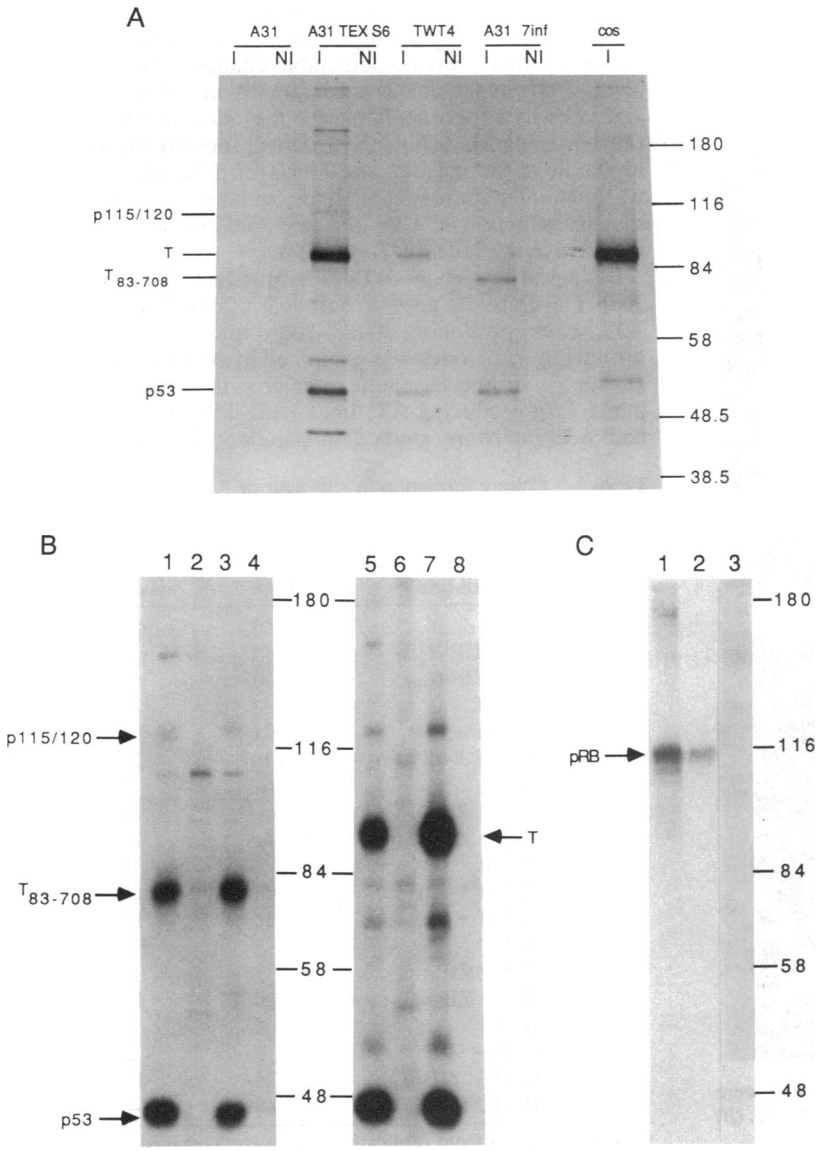

FIG. 1. Immunoprecipitation of [^{35}S]methionine-labeled extracts of various cultures. (*A*) Subconfluent cultures were labeled, washed, and extracted, and the extracts were immunoprecipitated with the anti-TAg monoclonal antibody, PAb423 (lanes I) or with nonimmune hybridoma culture fluid (lanes NI). Immunoprecipitates were electrophoresed through a 10% polyacrylamide gel containing SDS, an autoradiograph of which is shown. Identical amounts of trichloroacetic acid-precipitable radioactivity were used for the immunoprecipitation of A31, A31-TEXS6, A31-TWT4, and A31-7inf. An extract of [^{35}S]methionine-labeled COS-1 cells, immunoprecipitated and electrophoresed in parallel, is also shown. Note the greater electrophoretic mobility of simian cell p53 compared with its mouse cell counterpart. (*B*) Immunoprecipitates of various ^{35}S-labeled extracts. Lanes: 1–4, A31-7inf; 5–8, A31-TEXS6. Extracts were immunoprecipitated with PAb421 (anti-p53; lanes 1 and 5), RB-PMG3-245 (anti-RB; lanes 2 and 6), PAb423 (anti-TAg; lanes 3 and 7), and nonimmune myeloma cell culture fluid (lanes 4 and 8). An autoradiograph of a 7.5% polyacrylamide gel containing SDS is shown. (*C*) Immunoprecipitation of a mixture of equivalent amounts of protein from [^{35}S]-methionine-labeled CV-1P cells and A31-7inf cells. The extracts were incubated for 30 min at 4°C before immunoprecipitation with RB-PMG3-245 (lane 1) or PAb423 (lane 2). Lane 3 contains an immunoprecipitate, generated with PAb423, of an unmixed sample of [^{35}S]methionine-labeled CV-1P extract.

The generation and biological properties of the cell line A31-TEXS6 have been described (13).

As shown in Fig. 1, stably infected cells produced an 80-kDa anti-TAg-reactive protein. Like its wild-type counterpart, T-(83–708) reacted with both PAb416 (data not shown) and -423 (Fig. 1*A*), monoclonal antibodies directed at epitopes present, respectively, at each end of the sequence of T-(83–708) (27). By contrast, T-(83–708) failed to react with PAb419, an antibody that binds to an epitope in the common sequence of TAg and tAg (amino acids 1–82). Among the various wild-type (wt) TAg- and T-(83–708)-expressing A31 cell lines that were generated, two lines, termed A31-TWT4 and A31-7inf, were repeatedly found to contain comparable quantities of the wt and truncated early region products, respectively. This is apparent from the similar intensities of the TAg bands present in these two lines, as detected in an antibody-excess immunoprecipitation experiment in which identical quantities of total protein from each extract were tested. In addition, like wt TAg, T-(83–708) also bound tightly to p53 in A31 cells (Fig. 1*A*), and both proteins bound p115/120, the murine equivalent of human p107/120, a protein suspected, from the results of genetic analyses, of contributing to the transforming functions of TAg and adenovirus protein E1A (refs. 28 and 29 and Fig. 1*B*). In these cell lines, the levels of the retinoblastoma gene product (pRB) bound by wt TAg were sufficiently low and difficult to detect that it was not possible to reproducibly demonstrate TAg–pRB complexes. However, TAg-(83–708) from A31-7inf, readily bound monkey pRB when added to crude extracts of CV-1P cells (Fig. 1*C*). In addition, mutants containing, in the aggregate, deletions of much of the common sequence also bound pRB effectively in primate cell extracts (ref. 28; E. Marsilio and D.M.L., unpublished data). Finally, as shown in Fig. 2, *in situ* immunostaining of A31-TWT4 and A31-7inf showed that, like intact TAg, T-(83–708) accumulated in the nucleus.

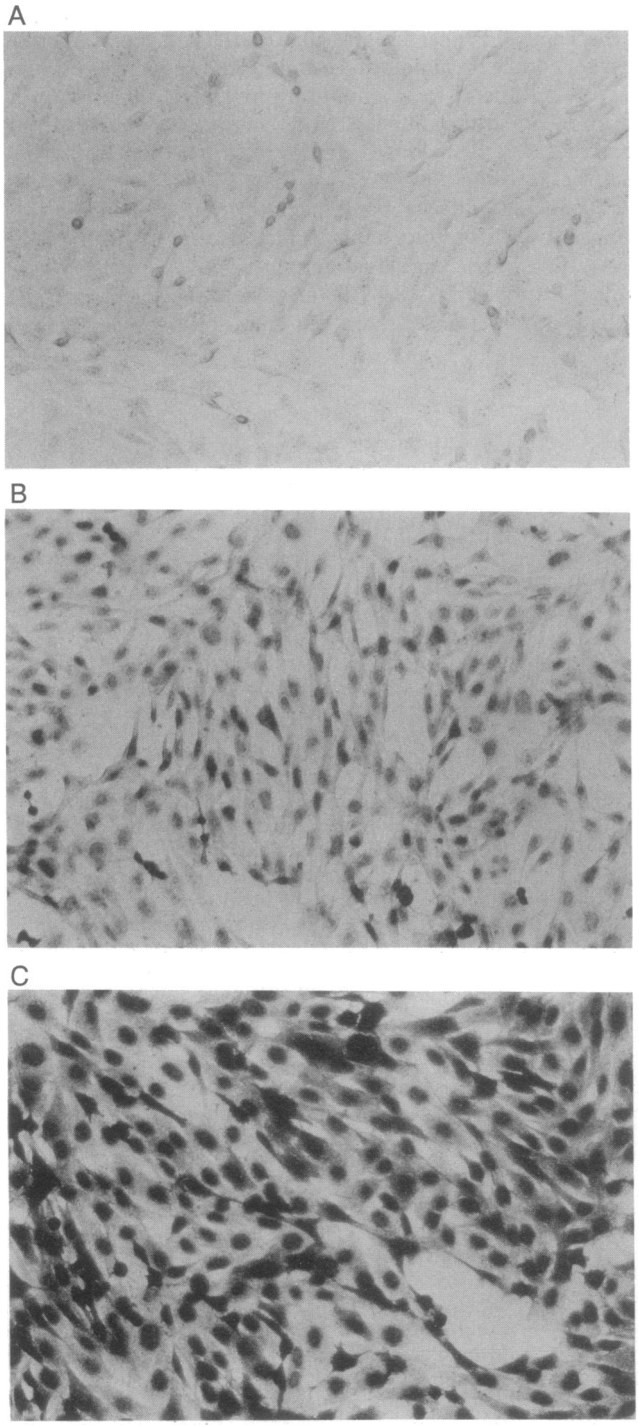

FIG. 2. Microscopic appearance of mouse cell clones that were fixed and immunostained with PAb423. (*A*) BALB/c 3T3 Cl.A31. (*B*) A31-7inf. (*C*) A31-TEXS6. (×110.)

When the stabilities of TAg and of T-(83–708) were compared in pulse–chase experiments performed on A31-TWT4 and A31-7inf, stability of the latter (1–2 hr) was clearly reduced relative to stability of wt (>6 hr; data not shown). However, there was no detectable change in the half-life of T-(83–708) when tAg was subsequently introduced into A31-7inf cells by superinfection with the relevant tAg-encoding retrovirus (data not shown). Moreover, despite the difference in stability, the steady-state levels of TAg and T-(83–708) were similar in these two cell lines, and there was no evidence that introduction of tAg into A31-7inf led to a significant increase in the level of T-(83–708).

Biological Properties of Cells Synthesizing TAg and T-(83–708). The ability to grow in soft agar of clonal derivatives of A31, each producing T-(83–708), and of two wt-TAg-producing A31 clones was examined in multiple experiments (Table 1). In one case, the agar growth properties of two intact-TAg-producing A31 lines (A31-TWT4 and -TEX S6) and A31-7inf were studied in parallel. Both of the intact

Table 2. Colony formation in soft agar of A31 clonal derviatives infected with ZIP backbone or ZIP-t retroviruses

Cell line	% microcolonies* Exp. 1	Exp. 2
A31	<1	<1
A31 + ZIP	<1	<1
A31 + ZIP-t_{5-10}	<1	<1
A31-1inf	1	4
A31-1inf + ZIP	<1	3
A31-1inf + ZIP-t_{5-10}	16	27
A31-9	—	2
A31-9 + ZIP	—	1
A31-9 + ZIP-t_{5-10}	—	18
A31-7inf	3	1
A31-7inf + ZIP	<1	<1
A31-7inf + ZIP-t_{5-10}	15	15
A31-2	2	7
A31-2 + ZIP	3	4
A31-2 + ZIP-t_{5-10}	10	15
A31-13	4	2
A31-13 + ZIP	5	2
A31-13 + ZIP-t_{5-10}	25	13
A31-TEXS6	18	—

*Values obtained from replicate plates were averaged.

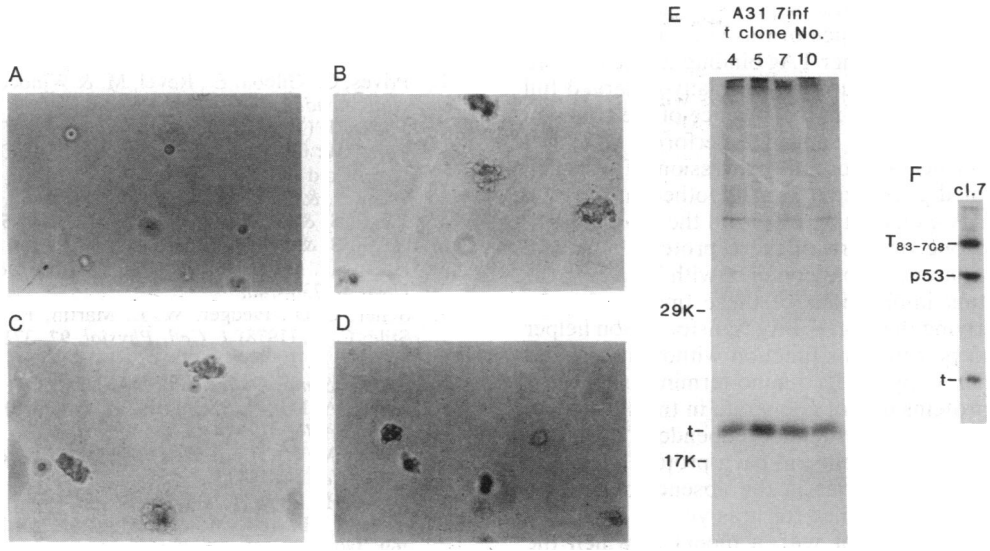

FIG. 3. (*A–D*) Microscopic appearance of various mouse cell clones in soft agar. (*A*) B2-8.2, an A31 clone constitutively expressing wt tAg. (*B*) A31-7inf wt tAg Cl.5, an A31-7inf clone superinfected with ZIP-t retrovirus and constitutively expressing wt tAg. (*C*) A31-7inf wt tAg Cl.7 (same as *B*, but Cl.7). (*D*) A31-TWT4. (*A–D*, ×140.) (*E*) Autoradiogram of 15% polyacrylamide gel containing SDS of PAb419 immunoprecipitates of [^{35}S]methionine-labeled extracts of A31-7inf cells superinfected with ZIP-t retrovirus. After superinfection, A31-7inf cells were seeded in methylcellulose. Thereafter, cell colonies containing >8–16 cells were picked, grown to mass cultures, labeled, and immunoprecipitated as described in Fig. 1. PAb419 recognizes epitopes in the common TAg/tAg sequence exclusively. (*F*) Autoradiogram of 12.5% polyacrylamide gel containing SDS of an immunoprecipitate of [^{35}S]methionine-labeled extract of A31-7inf wt tAg Cl.7. A mixture of PAb419, -423, and -416 was used. The positions of tAg, T-(83–708), and p53 are indicated.

TAg-producing lines grew relatively well in agar by comparison with A31-7inf. In a separate experiment, none of the T-(83–708)-containing lines grew as effectively as A31-TEXS6 (Table 2). However, when tAg was tested to determine whether it could restore the function lost by the deletion of the common sequence from TAg, each T-(83–708)-containing clone grew much more efficiently in agar after infection with a tAg-encoding retrovirus (Tables 1 and 2 and Fig. 3).

That tAg-retroviral infection actually did lead to agar-growing colonies that synthesize tAg is apparent from the results shown in Fig. 3 *E* and *F*. After tAg-retroviral infection of A31-7inf, several colonies growing in methylcellulose were picked, grown to mass culture, and subjected to immunoprecipitation with antibodies to TAg and tAg. As can be seen from the autoradiogram shown in Fig. 3*E*, the four colonies shown synthesized tAg, unlike the parental line (data not shown). The relative abundance of tAg in these clones was similar. When a mixture of monoclonal antibodies that recognize T-(83–708) or tAg was used, it was possible to demonstrate T-(83–708) and tAg in the same immunoprecipitate (Fig. 3*F*). Moreover, when two of the A31 clones that produced both T-(83–708) and tAg were reseeded in agar, each grew efficiently by comparison with A31-TWT4 (Fig. 3 *B* and *C*), demonstrating the stability of the agar growth property. By contrast as expected, a stable tAg-producing A31 clone, B2-8.2, which lacks a TAg gene product, failed to form colonies in parallel (Fig. 3*A*).

Finally, we asked whether the common sequence of tAg can substitute in trans for the same sequence of TAg without the addition of any tAg unique sequences. Specifically, parallel cultures of A31-7inf were again infected with tAg-sequence-encoding retroviruses. Two viruses were used, one encoding wt tAg and the other encoding only the common sequence (t_{common}). It has been shown (16) that the product of the common sequence could accumulate to wt tAg levels in unselected murine cells (16). When A31-7inf cultures infected, in parallel, by these viruses were studied, only those infected by the wt tAg virus grew in agar (Table 1, experiment 2). In a separate experiment, we found that both viruses led to the synthesis of comparable amounts of the predicted tAg products (data not shown), much as had been shown (16) when these two proteins were introduced by plasmid transfection. Therefore, tAg-unique sequences are required for the expression of the helper effect in the presence of T-(83–708), as had been documented for intact TAg (12).

DISCUSSION

These data indicate that the amino-terminal 82 residues of TAg constitute a domain needed for the full expression of its transforming activity, as measured by the criterion of anchorage-independent growth. A deletion mutant lacking this segment encodes a nuclear protein, which, while less stable than wt TAg as defined in formal pulse–chase experiments, bound p53 and p115/120, two known TAg-binding elements. Moreover, its pRB-binding activity also appeared to be grossly intact. Thus, this mutant TAg species is not likely to be completely denatured or inert *in vivo*, a conclusion strongly supported by the finding that, in the presence of intact tAg, it could perform an established aspect of its transforming function. Indeed, that the half-life of T-(83–708) did not change in the presence of tAg and that it was possible to identify a clone bearing the same steady-state concentration of T-(83–708) as a suitable wt TAg-containing control strongly imply that the reduced half-life is not the reason that T-(83–708) cannot promote agar growth like its intact progenitor.

How does the common sequence operate in TAg? The function of this sequence is not clear from any of the data reported herein. On the other hand, it seems reasonable to argue that it does not play a major quantitative role in the binding of T to three different proteins, each of which contributes to the expression of TAg transforming activity. Specifically, earlier data indicated that much of the common sequence is not required for pRB and p107 binding, and our present results reinforce this observation with regard to the latter. The data also show that the common sequence is nonessential for p53 binding. Thus, complex formation with

two, and possibly three, tumor suppressor gene products seems to proceed in the absence of this sequence.

What is not known is whether TAg binding to one or more of these proteins fails to induce the normally observed full perturbation of their function in the absence of the common segment. If so, perhaps this sequence performs an as yet undefined function needed for full expression of a simian virus 40-transformed phenotype. This hypothetical function could be defined as a qualitative effect on the growth regulatory activity of one or more of these proteins over and above the formation of a stable complex with TAg.

There are certain implications of these findings for the mechanism underlying the tAg → TAg transformation helper effect. (*i*) tAg can perform this function without its partner containing another copy of its amino-terminal sequence. Hence, the two proteins do not cooperate in transformation by forming a transient heterodimer dependent upon the existence of a common sequence in each protein. Conceivably, such a heterodimer forms in the absence of this sequence, but there is no evidence for it as yet.

(*ii*) The data are consistent with a model in which the common sequences in tAg and TAg serve a similar biochemical function. On the other hand, perhaps intact tAg and the common sequence of TAg provide a wholly different biochemical function(s), but the final biological effects are the same. There is presently no evidence as to which of these possibilities is correct.

What is also clear is that unique tAg sequences are needed for the biological effect. Whether they are necessary for the biological effect of the common segment is not clear. Conceivably, they are not and the biological requirement for them leads to a defect in a critical support function for tAg, such as proper stability and/or intracellular trafficking.

The nature of the function of the common region is unclear. Among the various possibilities is a transcription modulation function, given the known transactivation property of tAg (16). Although possible, the other known biochemical function of tAg (i.e., the ability to bind protein phosphatase 2A) cannot be immediately invoked, because there is, as yet, no evidence that TAg interacts with this enzyme. Perhaps the function of the common region contributes to an activity unique to each protein. In that case both of these functions must, in the end, be essential to the appearance of anchorage-independent growth.

Finally, if tAg is, in part, a portable copy of a discrete TAg domain, then one wonders whether the same kind of functional duplication exists for the three polyomavirus tumor antigens and other such partially duplicated viral transforming products. In this regard, it can be assumed that the function attributed to tAg and to TAg must contribute to the survival and, possibly, the replication of the virus in its natural host (30, 31). Conceivably, the existence of tAg was selected for, in part, because of its ability to perform the function of the common region more efficiently than TAg soon after natural infection of cells in the appropriate organ(s), when the intracellular concentration of TAg is likely to be relatively low.

We thank Ann Desai for her expert assistance in preparing the manuscript and James Figge for helpful discussions. This work was supported by Grant CA24715 from the National Institutes of Health.

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