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New region of the simian virus 40 genome required for efficient viral transformation

(viable deletion mutants/soft agar assay/small t antigen)

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ABSTRACT Viable mutants of simian virus 40 with deletions in three regions of the virus genome (map coordinates 0.21–0.17, 0.59–0.54, and 0.67–0.74) have been tested for their ability to transform rat fibroblasts to anchorage independence. Only those mutants whose deletions occur between 0.59 and 0.55 in the proximal part of the early region are defective in transforming ability. The most severely defective of these transform with less than one-hundredth the efficiency of wild type. They retain their defect when tested in Chinese hamster lung cells and when infection is initiated with viral DNA instead of intact virions. Complementation for transformation can be observed between these transformation-defective deletions and a simian virus 40 temperature-sensitive *A* mutant.

Simian virus 40 (SV40) is a DNA tumor virus whose small genome makes it a simple system to analyze how a viral genome causes neoplastic transformation. Although SV40 has been extensively studied (1–3), only one viral function has been shown to be essential for successful transformation. Temperature-sensitive (ts) *A* mutants, whose lesions are located in the middle to distal part of the early region of the viral genome, have reduced transforming activity (4–7). This picture of SV40 transformation may be incomplete, however, because only mutants that have a defect in the viral reproductive cycle have been tested for transforming defects. Any viral functions required only for transformation may have been undetected.

Recently, unusual SV40 mutants were isolated; the mutants lack 12 to 200 base pairs in one of three distinct areas of the viral genome: (*a*) the proximal part of the late region (map coordinates 0.68–0.74); (*b*) the proximal part of the early region (map coordinates 0.59–0.54); or (*c*) the distal part of the early region (map coordinates 0.21–0.17) (8–10). These deletion mutants are viable, that is, they form plaques and produce nearly normal amounts of progeny virus. Each of the regions bearing the deletions is contained on the large SV40 DNA fragment (0.73–1.0) that is capable of transforming nonpermissive cells (11); these regions are also present and transcribed in SV40-transformed cell lines (12–14). Because one or more of these regions might play a role in transformation, we have tested the transforming activity of representatives of these deletion mutants.

To assess the function of the deleted regions we measured the transformation of normal nonpermissive rat cells (F111) to anchorage independence following their infection with wild-type (WT) or mutant genomes. Several of the mutants with deletions in the proximal part of the early region (0.59–0.55) were severely defective in their transforming ability; mutants whose deletions were in the proximal part of the late and distal part of the early regions were nearly as efficient as WT virus in transforming activity. Preliminary data indicate that the deletions in the proximal part of the early region affect a different function than the one altered in the ts *A* mutants.

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MATERIALS AND METHODS

Cells and Viruses. Fischer rat line F111 (15), kindly sent to us by A. Freeman, and passaged less than 25 times, was used for most transformation experiments. Chinese hamster lung cells, CHL (7), were obtained from R. Martin. The monkey kidney line BSC-1 was used routinely for growth and plaque assay of virus stocks.

Characteristics of SV40 WT 830 and deletion (dl) mutants in the 800 series (9) and in the 1200 series (10) have been published. The mutant dl 1410 is missing a nucleotide sequence from 0.585 to 0.54 on the SV40 DNA map, and also contains in its genome the dl 892 deletion. The ts *A*58 mutant (16) was a gift from P. Tegtmeyer.

Virus stocks were prepared using a multiplicity of infection (MOI) of 0.01–0.05 plaque-forming units per cell in Dulbecco's modified Eagle's medium (DME) plus 2% fetal calf serum. When maximum cytopathic effects were observed, the cells were harvested, frozen and thawed five times, clarified by low-speed centrifugation, and stored at −20°. Occasionally, concentrated purified stocks were prepared by banding the virions in CsCl.

Transformation with Virions. Cells, suspended at 1×10^6 per ml in Tris-buffered saline without Ca^{2+} or Mg^{2+} (17) and containing 1% filtered calf serum, were infected with various multiplicities of virus while being stirred in suspension at 37° for 1 hr. After disruption of any clumps, infected cells were diluted and plated in soft agar at $2–8 \times 10^4$ cells per 60-mm dish, following the procedure of Macpherson and Montagnier (18) using DME containing 5% dog serum and 5% fetal calf serum solidified with 0.3% agar. For CHL cells, the serum proportions were 4% dog serum and 6% fetal calf serum and the agar concentration was 0.34%. Colonies arising in agar (diameter >0.2 mm) were counted after 4- to 6-wk incubation at 38.5° or 8-wk at 32° for ts *A*58. The efficiency of transformation was expressed as the number of agar colonies per 100 cells plated.

Transformation was also assayed by a focus assay: 4×10^4 infected cells were plated per 60-mm dish in the same medium used for agar assay, but the agar was omitted. After 2 wk at 38.5°, the plates were rinsed with 0.9% NaCl, fixed in methanol, and stained with 10% filtered Giemsa. Densely stained foci were counted.

Transformation with DNA. DNA was extracted from infected cells by the Hirt procedure (19), ethanol precipitated, and purified in a CsCl/ethidium bromide gradient. Forms I and

Abbreviations: SV40, simian virus 40; MOI, multiplicity of infection; WT, wild type; ts, temperature-sensitive; dl, deletion; DME, Dulbecco's modified Eagle's medium.

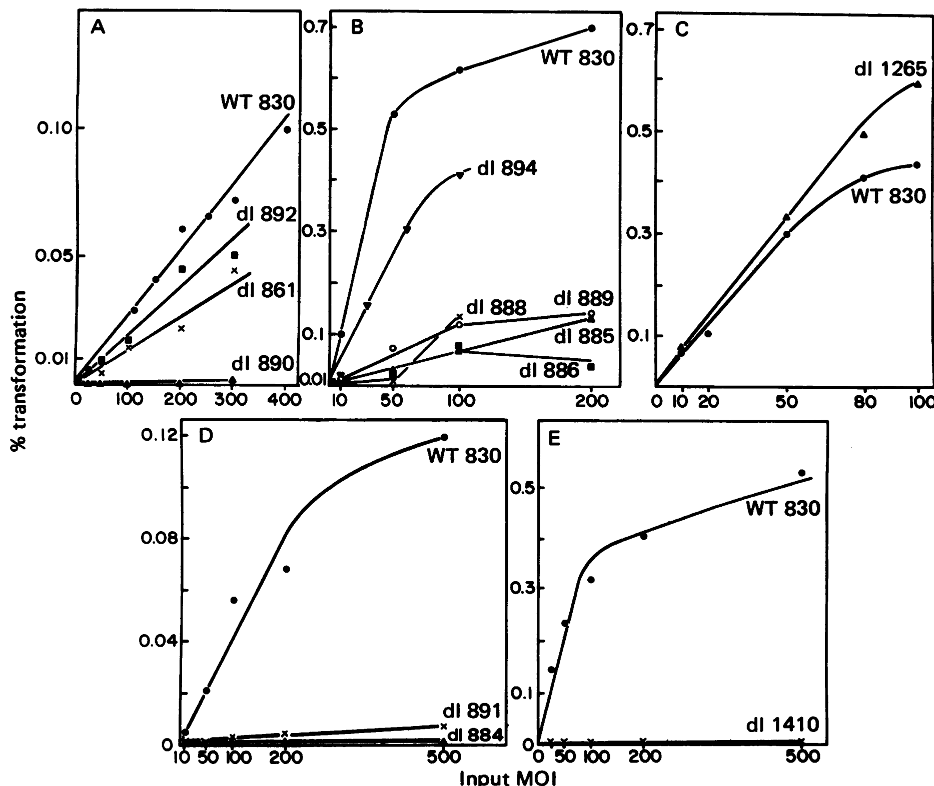

FIG. 1. Transformation of F111 by WT 830 and by deletion mutants from the proximal part of the late region (*A*, *B*), a mutant from the distal part of the early region (*C*), and mutants from the proximal part of the early region (*A*, *B*, *D*, *E*).

II were extracted with isopropanol and stored at −20° in 10 mM Tris·HCl/2 mM EDTA/10 mM NaCl, pH 8.0.

For transformation, a DNA–calcium precipitate was formed following the methods of Abrahams and van der Eb (20), using

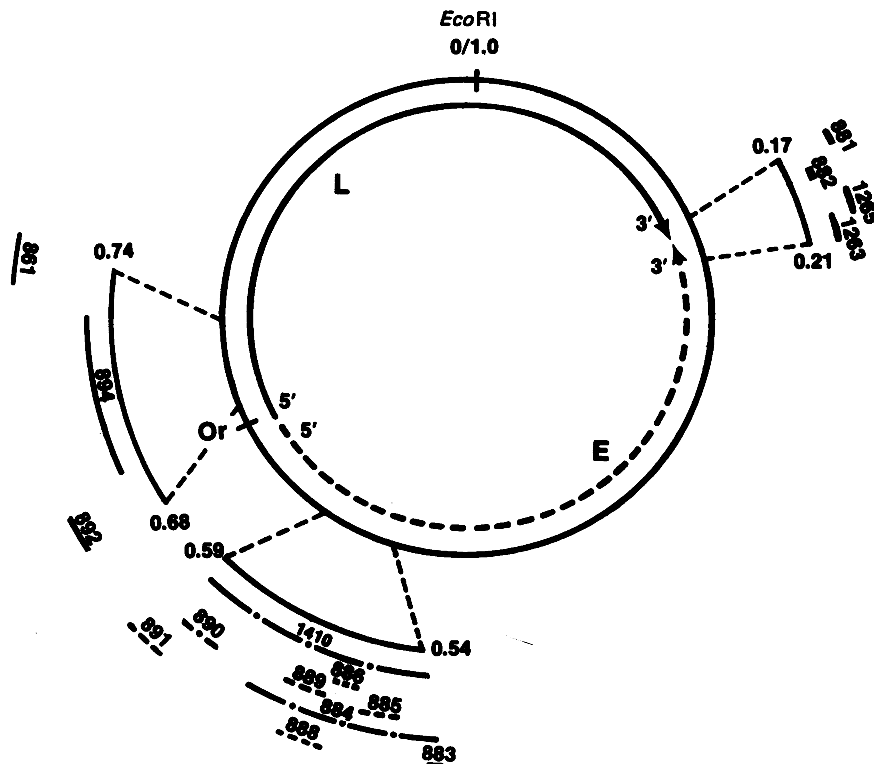

FIG. 2. Summary of the map location and approximate size of the viable deletion mutants (9), indicating those that transform F111 at a frequency within ±0.5 of WT (—), those that transform with frequencies 0.1–0.3 of WT (- - -), and those that transform with a frequency of less than 0.02 of WT (· — ·). E, Early region; L, late region; Or, origin of DNA replication; the *Eco*RI endonuclease cleavage site is shown at the top. The average values for dl transformation frequency as a proportion of WT, taken from dose reponse curves of two to nine experiments are: dl 881 (0.54); dl 882 (0.47); dl 1263 (1.6); dl 1265 (1.2); dl 883 (0.61); dl 884 (0.02); dl 885 (0.19); dl 886 (0.17); dl 888 (0.34); dl 889 (0.12); dl 890 (0.01); dl 891 (0.18); dl 1410 (0.004); dl 861 (0.71); dl 892 (0.58); dl 894 (0.66).

salmon sperm carrier DNA at 10–25 μg/ml. One-half milliliter of DNA precipitate was incubated for 20 min at 36.5° with a subconfluent monolayer of F111 (5×10^5 cells per 60-mm dish). The cells were re-fed with DME plus 2% fetal calf serum, incubated 1 hr, fed again with DME plus 10% fetal calf serum, and incubated overnight. The next day cells were trypsinized and assayed for colony-forming ability in DME plus 20% serum and in soft agar as described above. Due to variations in cell survival following Ca^{2+} treatment, the efficiency of transformation is expressed as the number of colonies formed in agar per 100 cells able to clone in liquid medium.

RESULTS

The Agar Assay. The ability to grow in soft agar has not previously been used to assay for SV40 transformants, although it is commonly used for other papovaviruses. This assay provides a direct measure of the number of independent transforming events; moreover, the ability to grow in agar correlates well with the ability to form tumors *in vivo* (21, 22). In our assay, SV40 can transform the rat fibroblast line F111 to grow in agar just as polyoma (18) and BK virus (23) transform baby hamster kidney (BHK) cells; the number of transformants increases linearly from zero to a plateau level as the input multiplicity of virus reaches several hundred per cell (see WT 830, Fig. 1). The plateau level varies from experiment to experiment, depending to some degree on the batch of fetal calf serum used. Transformation is significantly higher when 5% dog serum is included in the medium with 5% fetal calf serum.

When colonies arising in agar after SV40 infection are picked and grown through several passages in liquid culture, they retain their ability to clone in soft agar. Twenty-five out of 25 replated in soft agar with an average plating efficiency of 26% (range 4–71%), whereas the uninfected parent F111 has a plating efficiency in soft agar of <0.0003%. Viruses that form plaques whose morphology is indistinguishable from that of plaques produced by SV40 on BSC-1 cells can be rescued from these recloned transformants by cocultivation or polyethylene glycol-induced fusion with BSC-1 (data not shown).

Transformation by Deletion Mutants. Viable deletion mutants from the proximal part of the late region (0.68–0.74) (see Fig. 2) transform with a frequency similar to, but slightly lower than, WT. The mutant whose deletion maps closest to the origin (dl 892), and the mutant with the deletion located farthest from the origin (dl 861), have transformation frequencies within 50% of WT (Fig. 1*A*). The mutant with the largest deletion in the late region (dl 894, ref. 9) also transforms with good efficiency (Fig. 1*B*).

Mutants from the distal part of the early region (0.17–0.21) transform approximately as well as WT (see example, Fig. 1*C*), although one (dl 882), which occurs in the region where early and late messenger RNAs overlap (0.17), has in some experiments given transformation frequencies significantly below WT.

In contrast, most of the mutants whose deletions occur in the proximal part of the early region (0.59–0.54) are defective in transformation (see examples in Fig. 1). Three of the mutants with deletions in this region, dl 884 (−184 base pairs, Fig. 1*D*), dl 890 (−53 base pairs, Fig. 1*A*) and dl 1410 (−225 base pairs, Fig. 1*E*) are severely defective in transforming ability; five of the mutants with smaller deletions, dl 891 (Fig. 1*D*) and dl 885, dl 886, dl 888, and dl 889 (Fig. 1*B*) give transformation frequencies that are 10–30% of WT.

Even the most defective deletion mutants are not completely negative for transformation. With dl 884, dl 890, and dl 1410 the frequency is not below about 1% of the WT value. In the case of dl 884 and dl 890 the rare transformants are indistinguishable from WT transformants in that they reclone efficiently in soft agar and produce virions after fusion with BSC-1 (data not shown).

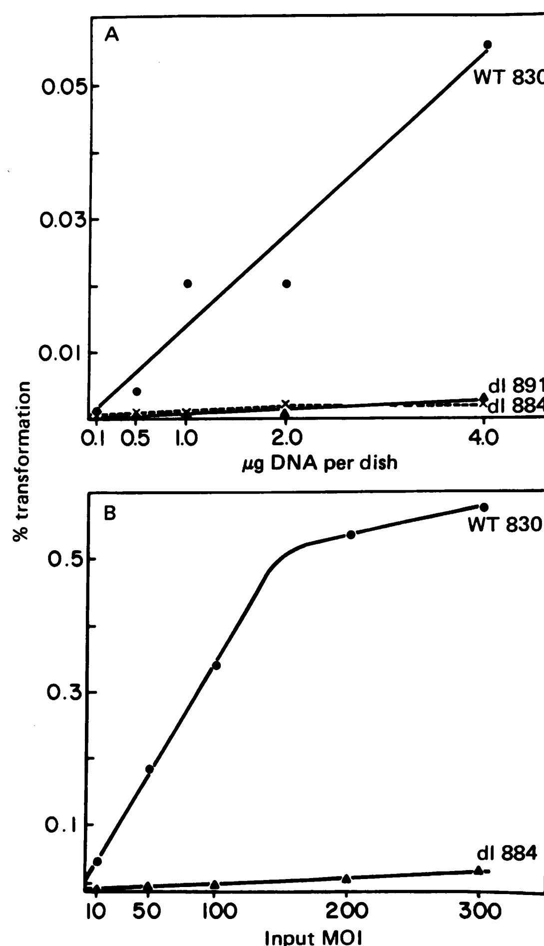

FIG. 3. (*A*) Transformation of F111 by DNA from WT 830 and two proximal early deletion mutants, dl 884 and dl 891. (*B*) Transformation of CHL cells by WT 830 and proximal early deletion mutant dl 884.

Data from many transformation experiments with the mutants carrying deletions in different parts of the genome are summarized in Fig. 2; the size and map location of the mutants tested are indicated by the position and length of the arcs and their relative transforming abilities, averaged over several experiments, are given in the legend.

The same low transformation frequencies with dl 884 and dl 890 were obtained after plaque purification of the virus stocks and with recloned F111 cells (data not shown). The transformation frequency with dl 884, or dl 891 DNA (0.1–4 μg per dish), was 5% or less of that found with WT DNA (Fig. 3*A*), and a similarly depressed value was found using intact virions on CHL cells with dl 884 and dl 890 (Fig. 3*B*; data for dl 890 not included). The somewhat higher transformation requency in CHL cells might result from the difference in permissiveness for SV40 growth between CHL and F111 (7).

Neither dl 884 nor dl 890 is defective for transformation as assayed by focus formation (Fig. 4*A*), whereas the defect is clearly demonstrated in the same experiment using the agar assay (Fig. 4*B*). Reconstruction experiments using WT 830-transformed clones selected in agar indicated that both assays are equally efficient in detecting transformed cells and that reseeding did not occur in the focus assay (data not shown).

Complementation between the Transformation-Defective

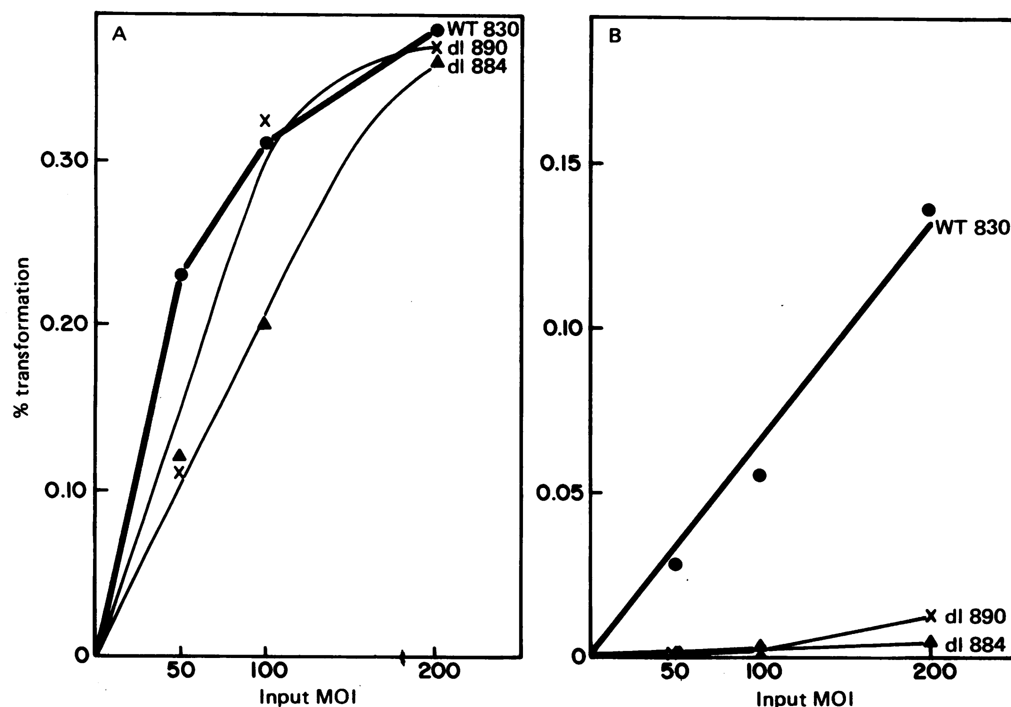

FIG. 4. Efficiency of transformation of F111 cells using focus formation (*A*) or colony formation in agar (*B*) to assay transformation.

and ts *A* Mutants. To determine if the transformation defect of the deletion mutants was in the *A* complementation group, F111 cells were infected with dl 884 or dl 890 with and without ts *A*58 at 32° and 38.5° (Table 1). There is a 6- to 15-fold enhancement of the transformation frequency in the mixed infections over the sum of the transformation frequencies of each mutant alone, indicating that these two transformation defects can complement one another. Unlike the analogous transformation-defective polyoma mutants (24), the frequency of transformation by WT virus is not depressed when cells are simultaneously infected with various MOIs of dl 884 (Fig. 5).

DISCUSSION

We have tested various mutants with deletions in three regions of the SV40 genome that are dispensable for viral multiplication (9) for their ability to transform nonpermissive cells to anchorage-independent growth. Mutant viruses with deletions at the end of or just beyond the early region transform nearly as well as WT virus. One of these (dl 1265) has about 10-fold reduced activity in the adenovirus-2 helper function (C. Cole and P. Berg, unpublished data) and another (dl 1263) produces a T-antigen that is smaller by 6 kilodaltons (10, 25). Mutants with deletions in the proximal part of the late region (0.68–0.74) are nearly normal in their transforming ability. Although the integrity of these two regions is not essential for transformation, additional studies with more mutants and correlations with nucleotide sequence data will be needed to fully document that conclusion.

By contrast, mutants with deletions in the proximal part of the early region (0.59–0.54), with the exception of the mutant with a small deletion at 0.54 (dl 883), are reduced in their transformation efficiency; three of these (dl 884, dl 890, and dl 1410) are only 1–2% as effective as the WT parent. Mutants dl 884 and dl 890 are also defective in transforming the semipermissive Chinese hamster cells, suggesting that the defect is not due to some trivial requirement specific to nonpermissive rat cells. Adsorption and/or uncoating problems cannot account for the deficiency because the mutant DNA is also defective and the mutant viruses complement ts *A*58 for transformation at high temperature.

Although mutants dl 884, dl 890, and dl 1410 are severely transformation defective, they are not completely inactive and do produce transformed clones, albeit at low frequency. These rare transformants could be derived from physiologically variant cells with lenient transformation requirements or from transforming virus in the deletion mutant stock. Transforming viruses could arise as evolutionary variants (3) or as a result of recombination with cryptic SV40 sequences present in the cell line in which the virus stocks were grown. Analysis of early viral proteins and rescued virus from the rare transformants may help to differentiate between these alternatives.

The transformation defect in dl 884 and dl 890 appears to be different from the SV40 early function defined by ts *A* mutants. These dl mutants (*a*) multiply efficiently in monkey

Table 1. Complementation in transformation of F111 rat cells by ts *A*58 and deletion mutants

MOI			Temperature,	% transformation			Enhancement
ts *A*58	dl 884	dl 890	°C	ts *A*58 alone	dl alone	ts *A*58 + dl	factor
10	90	—	38.5	0.01	<0.002	0.06	6
			32	0.20	<0.002	0.57	
10	—	90	38.5	0.003	<0.0008	0.026	8
			32	0.14	0.0006	0.16	
1	10	—	38.5	<0.0008	<0.0004	0.018	>15

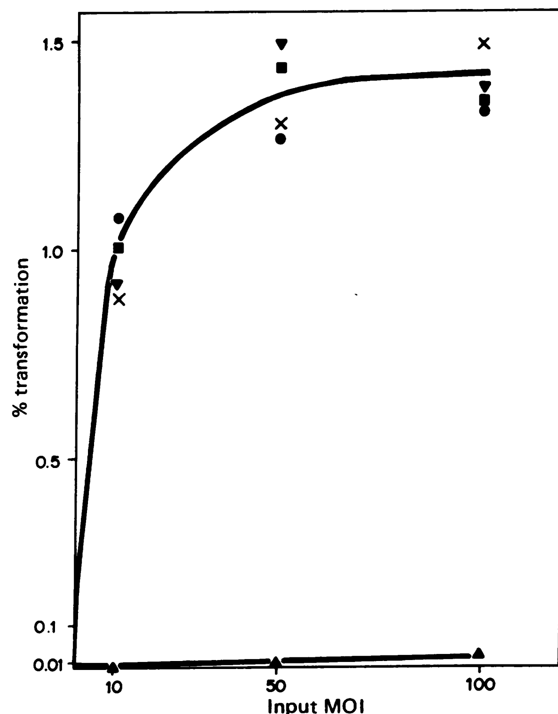

FIG. 5. Transformation-defective mutant dl 884 does not inhibit transformation by WT 830. F111 cells were infected with WT 830 at the MOI indicated on the abscissa and simultaneously with dl 884 at an MOI of 10 (▼), 50 (■) or 100 (×), and the number of colonies in agar was scored. Control infections were WT 830 alone (●) and dl 884 alone (▲).

cells (9), an ability that requires the *A* function; (*b*) produce T-antigen of apparently normal molecular weight (9, 10, 25); and (*c*) dl 884 and dl 890 are able to complement ts *A*58 (and other ts *A* mutants; M. Fluck and T. L. Benjamin, personal communication) for transformation in mixed infections. This latter result is inconsistent with the possibility that the 0.59–0.54 region is a *cis*-acting control region that regulates the level of T-antigen in the transformed cell.

The defective transformation by dl mutants observed with the agar assay is not seen when transformants are selected as foci grown from low density or as clones in 2% serum (9). These latter two traits are not consistently correlated with tumorigenicity in SV40-transformed rat lines (22). Clones selected in this manner may be quite different from agar-selected clones (26) and reflect a viral function which is not impaired in mutants dl 884 or dl 890.

The deletions responsible for defective transformation resemble the transformation-defective *hrt* mutants of the polyoma genome. The majority of the *hrt* mutants also have deletions, and these occur in the analogous position of the polyoma early region (27–29). Like the SV40 deletions, the *hrt* mutants can complement ts *A* mutants for transformation (24, 28, 29).

While these studies were in progress it was shown that SV40's early region codes for two proteins: a 15- to 20-kilodalton protein, small t, and the previously known 90- to 100-kilodalton protein, large T (25, 30). Mutants with deletions at map position 0.59–0.55 induce the formation of normal quantities of full-size large T-antigen but fail to produce small t after infection of monkey cells (25). Consequently there is good reason to believe that this region of the SV40 genome codes for part of the structure of small t but not for large T (25, 31). Because the mutants that fail to make small t are also defective for transformation, small t may be involved in the transformation of cells to anchorage independence and tumorigenicity.

The data so far do not distinguish (*a*) whether small t acts alone or in conjunction with other virus-coded products, or (*b*) whether small t initiates the transforming event or is responsible for the maintenance of the transformed phenotype.

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1. Eckhart, W. (1974) *Annu. Rev. Genet.* **8,** 301–317.
2. Fried, M. & Griffin, B. E. (1977) *Adv. Cancer Res.* **24,** 67–113.
3. Kelly, T. J. & Nathans, D. (1977) *Adv. Virus Res.* **21,** 85–173.
4. Kimura, G. & Dulbecco, R. (1973) *Virology* **52,** 529–534.
5. Tegtmeyer, P. (1972) *J. Virol.* **10,** 591–598.
6. Kimura, G. & Itagaki, A. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 673–677.
7. Martin, R. G. & Chou, J. Y. (1975) *J. Virol.* **15,** 599–612.
8. Mertz, J. E. & Berg, P. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 4879–4883.
9. Shenk, T. E., Carbon, J. & Berg, P. (1976) *J. Virol.* **18,** 664–671.
10. Cole, C. N., Landers, T., Goff, S. P., Manteuil-Brutlag, S. & Berg, P. (1977) *J. Virol.* **24,** 277–294.
11. Graham, F. L., Abrahams, P. J., Mulder, C., Heijneker, H. L., Warnaar, S. O., deVries, F. A. J., Fiers, W. & van der Eb, A. J. (1974) *Cold Spring Harbor Symp. Quant. Biol.* **39,** 637–650.
12. Botchan, M., Ozanne, B., Sugden, B., Sharp, P. A. & Sambrook, J. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 4183–4187.
13. Khoury, G., Martin, M. A., Lee, T. N. H. & Nathans, D. (1975) *Virology* **63,** 263–272.
14. Botchan, M., Topp, W. & Sambrook, J. (1976) *Cell* **9,** 269–287.
15. Freeman, A. E., Igel, H. J. & Price, P. J. (1975) *In Vitro* **11,** 107–116.
16. Tegtmeyer, P., Schwartz, M., Collins, J. K. & Rundell, K. (1975) *J. Virol.* **16,** 168–178.
17. diMayorca, G., Greenblatt, M., Trauthen, T., Soller, A. & Giordano, R. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 46–49.
18. Macpherson, I. & Montagnier, L. (1964) *Virology* **23,** 291–294.
19. Hirt, B. (1967) *J. Mol. Biol.* **26,** 365–369.
20. Abrahams, P. J. & van der Eb, A. J. (1975) *J. Virol.* **16,** 206–209.
21. Freedman, V. H. & Shin, S. (1974) *Cell* **3,** 355–359.
22. Shin, S., Freedman, V. H., Risser, R. & Pollack, R. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 4435–4439.
23. Major, E. O. & diMayorca, G. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 3210–3212.
24. Fluck, M. M., Staneloni, R. J. & Benjamin, T. L. (1977) *Virology* **77,** 610–624.
25. Crawford, L. V., Cole, C. N., Smith, A. E., Paucha, E., Tegtmeyer, P., Rundell, K. & Berg, P. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 117–121.
26. Seif, R. & Cuzin, F. (1977) *J. Virol.* **24,** 721–728.
27. Feunteun, J., Sompayrac, L., Fluck, M. & Benjamin, T. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 4169–4173.
28. Eckhart, W. (1977) *Virology* **77,** 589–597.
29. Staneloni, R. J., Fluck, M. M. & Benjamin, T. L. (1977) *Virology* **77,** 598–609.
30. Prives, C., Gilboa, E., Revel, M. & Winocour, E. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 457–461.
31. Berk, A. J. & Sharp, P. A. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 1274–1278.