JOURNAL OF VIROLOGY, Mar. 1975, p. 636-644

Vol. 15, No. 3
Printed in U.S.A.

Simian Virus 40 Gene A Function and Maintenance of Transformation

MARY OSBORN*[1] AND KLAUS WEBER[1]

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

Received for publication 30 September 1974

Transformants have been isolated after infection of rat embryo cells at 33 C with either wild-type simian virus 40 or with the temperature-sensitive gene *A* mutants, ts*A*7 and ts*A*28. Examination of properties usually associated with transformation such as growth in 1% serum, growth rate, saturation density, and morphology show that these properties are temperature dependent in the ts*A* transformants characterized, but are not temperature dependent in the wild-type transformants that have been examined. In the most thoroughly characterized ts*A* transformants the expression of T antigen also appears to be temperature dependent. These data suggest that an active *A* function is required for the maintenance of transformation in these cells. In the lytic cycle, the *A* function is involved in the initiation of DNA synthesis. Thus transformation by simian virus 40 may be the direct consequence of the introduction of the simian virus 40 replicon and the presence of its DNA initiator function, which causes the cell to express a transformed phenotype.

Certain viruses have the property that, although they contain only a very small amount of DNA, they are capable of transforming various types of cells in culture, thus causing profound and lasting changes in the growth properties of such cells. In trying to understand the virus host relationships that may lead to such changes, one approach is to attempt to characterize the viral contribution to the transforming process with temperature-sensitive mutants of the virus. If transformants can be isolated that are temperature sensitive for certain properties usually associated with transformation, it is probable that the product of the temperature-sensitive viral gene is actively required for the maintenance of transformation. Such an approach has been useful in identifying those genes of the oncogenic RNA viruses which are associated with transformation (14, 24).

That the small DNA viruses, such as simian virus 40 (SV40), might control transformation through the continuous production of a gene product is suggested by the observation that all cells transformed by SV40 thus far examined express early SV40 RNA (9, 19). It seemed promising, therefore, to construct transformants with temperature-sensitive mutants of the only early complementation group thus far identified in the lytic cycle of SV40, the *A* gene (3, 23). Since the *A* function is involved in the initiation of DNA synthesis, *A* mutants do not replicate viral DNA or make viral capsids at the nonpermissive temperature (2, 21).

We have used wild-type SV40, and two ts*A* mutants, to isolate transformants of rat embryo cells. The transformants have been characterized at both permissive and nonpermissive temperatures with respect to growth rate, saturation density, morphology, and T antigen. Our results suggest that these parameters are temperature sensitive in the ts*A* transformants but not in the wild-type transformants. Thus it seems that at least in these cells the *A* function may be involved, either directly or indirectly, in the maintenance of the transformed state. Another account of the work will appear elsewhere (M. Osborn and K. Weber, Cold Spring Harbor Symp. Quant. Biol., in press).

MATERIALS AND METHODS

Virus. Stocks of the SV40 *A* gene mutants ts*A*7 and ts*A*28 (23) and of the SV40 wild-type strain were very kindly provided by Peter Tegtmeyer (Case Western Reserve University). Virus stocks were prepared at 33 C by infecting confluent monolayers of BSC-1 or CV-1 cells (at a multiplicity of infection of ~1 PFU per cell). When cells showed cytopathic effect they were scraped off the plates, disrupted by two freeze-thaw cycles, and stored at −80 C.

Isolation of transformants. The procedures for obtaining cells from embryos of CDF albino inbred

[1] Present address: Max-Planck Institut für Biophysikalische Chemie, Göttingen, West Germany.

rats (Charles River), as well as for the handling of the cells prior to infection with virus, and the procedures used for transformation are those described in detail by R. Risser, D. Rifkin, and R. Pollack (18a). Usually cells from 15- or 16-day-old rat embryos were used. After several in vitro passages, almost confluent monolayers of rat embryo cells were infected at low multiplicity (~ 1 or less) in parallel with either wild-type SV40 or with tsA7 or tsA28. Infection proceeded for 3 h at 33 C with shaking. After overnight incubation at 33 C the cells were trypsinized and diluted to approximately 4,000 cells per 6-cm plate. After 2 to 3 weeks at 33 C there was a 4- to 10-fold difference in the number of colonies on the virus-infected plate when compared to the mock-infected control plate. Colonies of two types were detected on both wild-type and tsA-infected plates. The first type stained densely with Harris hematoxylin, and on examination under the microscope cells in these colonies had a typically transformed morphology, i.e., they were piled up and disoriented. On subculture, cells from such colonies grew well and were all T antigen positive at 33 C. The second type of colony stained less well, and the cells had a flat morphology. On subculture, cells from the second type frequently failed to grow, and furthermore, as shown by Risser et al. (Cold Spring Harbor Symp. Quant. Biol., in press), such cells lack T antigen. Mock-infected cells appeared to have only the second type of colony. Several clones of the dense type were picked from wild-type, infected, and from tsA7- and tsA28-infected cells with steel cloning cylinders and were subcultured. Growth medium was Dulbecco modified Eagle medium (Gibco H-21) containing 10% fetal calf serum (Rehatuin) and 150 U of penicillin and 150 μg of streptomycin per ml. The growth curves and size distributions were measured on a Coulter counter (model ZB). Medium was changed every 4 days.

Staining of cells. Cells were fixed in phosphate-buffered saline (PBS) containing 3.5% formaldehyde for 30 min, washed with water, and then stained with Harris hemotoxylin (30 min to overnight). They were then washed with water followed by 1% ammonium hydroxide and a final water wash.

Morphology. Cells were fixed in PBS containing 3.5% formaldehyde for 30 min, washed with PBS, mounted in Elvanol, and examined by phase contrast with a microscope (Zeiss PM II).

Indirect immunofluoresence. Cells on 12-mm glass cover slips were fixed and stained as indicated below. Incubation with all antibodies was for 1 h at 37 C, and the cover slips were washed well with PBS after each antibody. After a final rinse, the cover slips were mounted in Elvanol and examined under dark field UV illumination with a microscope (Zeiss PM II). Photographs were taken on Plus-X film.

T antigen. T antigen was assayed by indirect immunofluorescence essentially as described by Robb (17). Hamster anti-T antibody (Flow Laboratories) was diluted 1:5 in PBS. Fluorescein conjugated goat anti-hamster globulins (Antibodies Inc.) were used at a 1:10 dilution in PBS. The cover slips were counterstained with Evans Blue (1:100 in PBS) for 30 s.

Actin. The expression of actin-containing fibers in cells was assayed by immunofluoresence essentially as described by Lazarides and Weber (12). Preparation of the rabbit anti-actin antibody has been described (12). This antibody was used at a 1:20 dilution in PBS. Fluorescein conjugated goat anti-rabbit globulins (Miles Laboratories) were used at a 1:10 dilution in PBS.

RESULTS

Elegant studies (R. Pollack, R. Risser, S. Conlon, and D. Rifkin, Proc. Nat. Acad. Sci. U.S.A., in press; 18a) have established the conditions necessary for transformation of rat embryo cells by wild-type SV40, and have shown that the transformants obtained can be characterized in a manner similar to those obtained from established cell lines (18). In addition, a test of wild-type SV40-transformed rat embryo cells showed that they would grow at 41 C, and we thought this property might be important since the tsA mutants were originally isolated at a nonpermissive temperature of 41 C and at least some of the mutants are known to be leaky below this temperature (23).

Transformants were isolated after parallel infections of rat embryo cells with either wild-type SV40, or with tsA28 or tsA7. Several clones of the dense type (see above) were picked from each plate, and stocks of the transformants were maintained by serial passage at 33 C. The transformants were then examined for differences in their growth properties, morphology, and expression of T antigen at 33 and 41 C as described below. Though the results are described in terms of a single tsA28 transformant, temperature-dependent effects have been seen with other tsA28 transformants and with some tsA7 transformants, whereas they have not been observed with wild-type transformants. At least some of the transformants have a chromosome number close to that of the normal rat diploid complement (Pollack et al., Proc. Nat. Acad. Sci. U.S.A., in press).

Growth rate and saturation density. Cells were seeded in 10% fetal calf serum at approximately 10^5 cells per 6-cm dish and allowed to attach overnight at 33 C. Half of the plates were shifted to 41 C the next morning, and the remainder were held at 33 C. Growth curves for a wild-type and for a tsA28 transformant are shown in Fig. 1. The growth rates of the wild-type and the tsA transformant were equal at 33 C. The wild-type transformant grew slightly faster at 41 than at 33 C. In contrast, the tsA28 transformant appears to grow slower

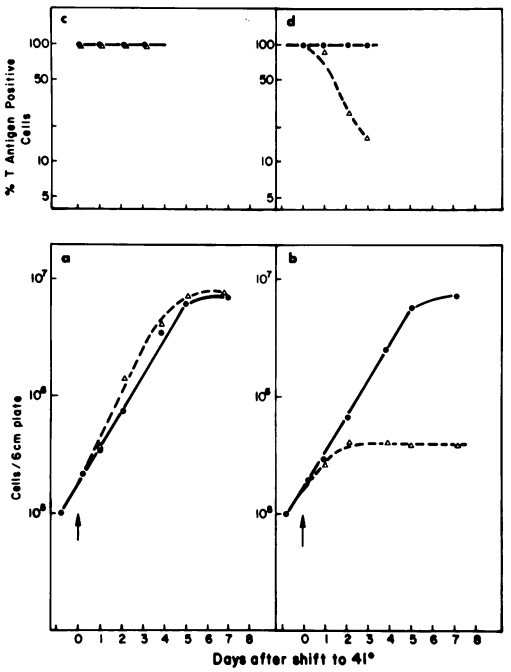

FIG. 1. *(a and b) Growth curves of (a) wild-type transformant WT4 and (b) tsA transformant tsA28.3 were measured at 33 C (●) or at 41 C (△). Cells were seeded at 10^5 per 6-cm dish and allowed to attach at 33 C. Half the plates were moved to 41 C at zero time. Cell number is expressed as the total number per 6 cm-dish and was measured on the Coulter counter. (c and d) The percentages of cells that were T antigen positive in (c) WT4 and (d) tsA28.3 cultures were measured at 33 C (●) or at 41 C (△). Cells were seeded on glass cover slips as described under (a and b). T antigen was assayed by indirect immunofluorescence as described in the text (see also Fig. 5).*

at 41 than at 33 C and appears to limit at a 10- to 12-fold lower density at 41 than at 33 C (see Fig. 1). Raising the serum concentration to 30% did not significantly change the growth properties of the tsA28 transformant at 41 C.

The same experiment was repeated with 1% fetal calf serum rather than 10% (data not shown). The wild-type transformant grew well at both temperatures, as did the tsA28 transformant at 33 C. Again, however, growth of the tsA28 transformant was very much reduced at 41 C.

The lower saturation density apparent for the tsA transformant at 41 C might be caused either by changed growth properties due to a shift to the nonpermissive temperature or to the cells only completing a single round of division after shift (Fig. 1). To distinguish between these possibilities, the saturation densities of the wild-type and of the tsA transformant were examined as a function of the initial cell inoculum. The characteristic saturation densities of the wild-type and of the tsA transformant at 41 C were independent of the initial plating density in the range 5×10^5 to 3×10^4 cells per 6-cm plate (Fig. 2). Below a starting inoculum of 3×10^4 cells per plate, neither the wild type nor the mutant grow to cover the dish. However, the individual colonies of wild-type cells stain much more heavily than the individual colonies of tsA28 transformant cells after equal times at 41 C. The existence of individual colonies for the tsA transformant at 41 C argues that the cells, when shifted to 41 C, go through several cell divisions.

Morphology by phase microscopy. Wild-type transformants appeared similar at the two temperatures and similar to the tsA transformant at 33 C. At 41 C the morphology of cells of the tsA transformant changed, and most of the cells became very large and flat. The pictures shown in Fig. 3 were taken 6 days after the shift to 41 C.

Size. The increase in size of these cells could also be assayed with the Coulter counter. The size distributions of the wild-type transformants at 33 and 41 C and of the tsA transformant were very similar. The size distributions were normal and peaked at an arbitrary setting of 30, with only 2% of the cells having a size greater than 85. In contrast, the size distribution of the tsA transformant 4 days after the shift to 41 C was abnormal; no real peak was seen, and 30% of the population had a size greater than 85.

Staining with antibody to actin. A difference in morphology can also be demonstrated by indirect immunofluorescence with actin antibody (12). The wild-type transformant at both temperatures and the tsA transformant at 33 C show few thick actin-containing fibers (Fig. 4). In contrast, the tsA transformant at 41 C shows very long and very thick fibers (>1 μm in diameter). Similar cables, so-called "stress fibers," can be seen also in the phase microscopy pictures of the tsA transformant at 41 C (Fig. 3b).

T antigen. T antigen was examined by indirect immunofluorescence microscopy in both the wild-type and the tsA28 transformant as a function of time after the shift to 41 C (Fig. 1, top frame; Fig. 5). For the wild-type transformant, greater than 98% of the cells of a given clone had T antigen at both temperatures at all times tested (Fig. 1c and 5b). For the tsA transformant shown in Fig. 1, expression of T antigen appeared to be temperature sensitive. Thus, at 33 C greater than 98% of the cells were T antigen positive. However, at 41 C the per-

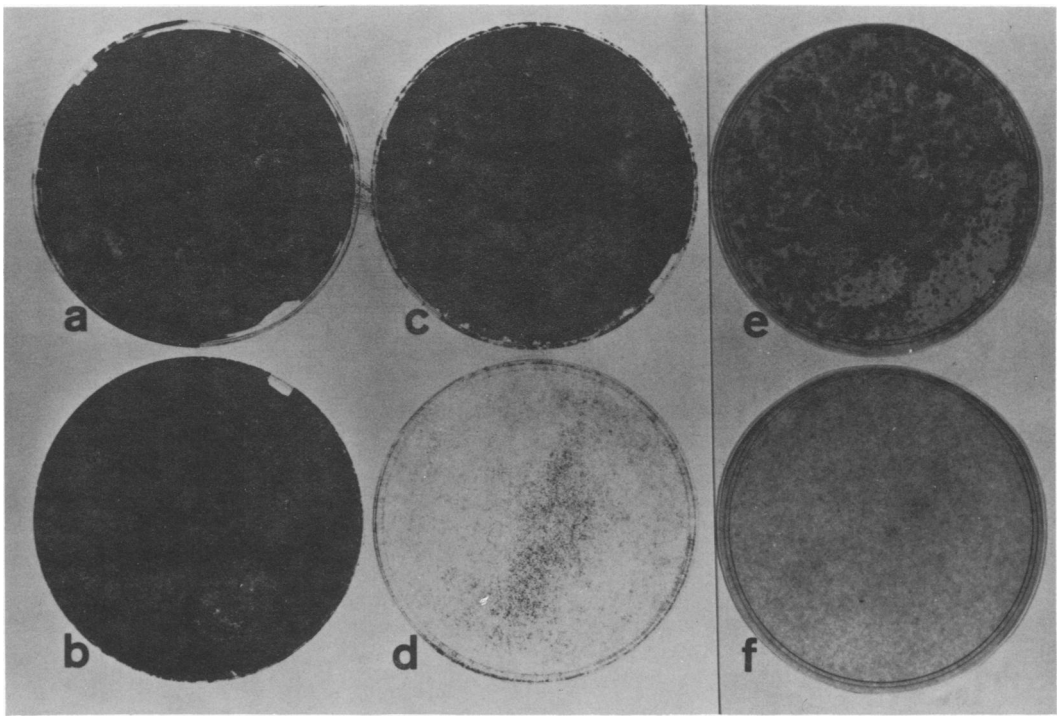

FIG. 2. *Saturation densities of WT4 and tsA28.3 cells as a function of starting inoculum and temperature. (a) WT4, 33 C, 10^5; (b) WT4, 41 C, 10^5; (c) tsA28.3, 33 C, 10^5; (d) tsA28.3, 41 C, 10^5; (e) tsA28.3, 33 C, 3×10^4; (f) tsA28.3, 41 C, 3×10^4. Cells were seeded at the indicated densities as described in the legend to Fig. 1 and stained with Harris hematoxylin after 10 days.*

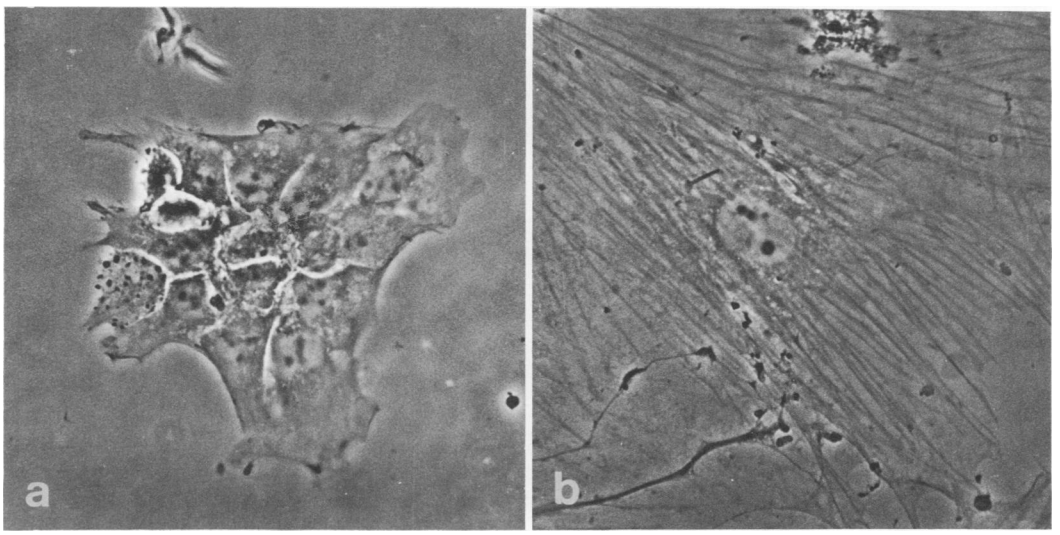

FIG. 3. *Morphology of (a) WT4 at 41 C and (b) tsA28.3 at 41 C. Cells were seeded as in the legend to Fig. 1, and the morphology was examined 6 days after the shift to 41 C. For (a) the cells were diluted 4 days after the shift so colony morphology could be clearly seen. Morphology of WT4 and tsA28.3 at 33 C is similar to that shown for WT4 at 41 C in (a).*

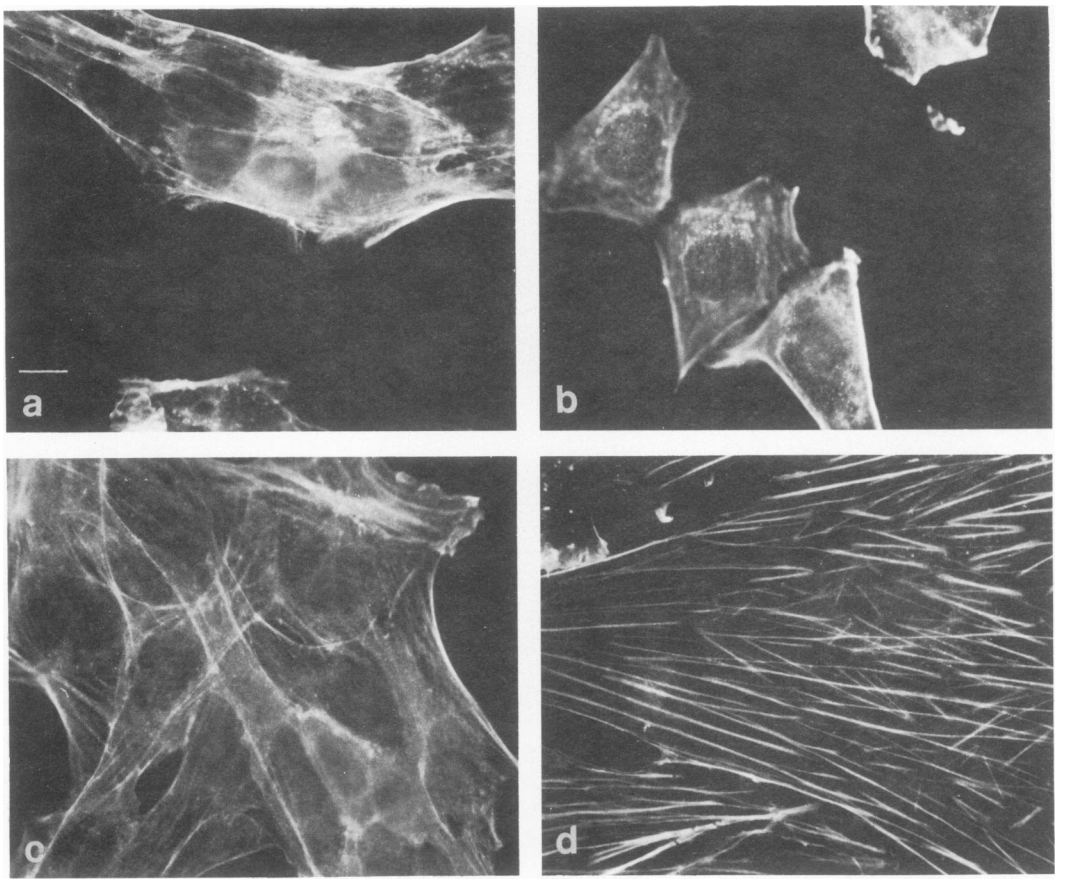

FIG. 4. *Indirect immunofluorescence using actin antibody (12) of (a) WT4, 33 C; (b) WT4, 41 C; (c) tsA28.3, 33 C; (d) tsA28.3, 41 C. Cells were seeded as in the legend to Fig. 1 and processed for immunofluorescence 6 days after the shift to 41 C. For a, b and c, cells were diluted after 4 days so staining pattern could be clearly seen. All four plates were taken at the same magnification, and the bar represents 10 μm. Cells of the tsA transformant at 41 C have very thick cables (d).*

centage of cells having T antigen appeared to decrease with the length of time the cells were kept at the elevated temperature. Complement fixation assays of T antigen at 41 and 33 C 4 days after the shift to 41 C also showed that the amount of T antigen was considerably reduced in the ts*A* transformant at 41 C.

Both the extent of the morphological changes and the fraction of cells showing loss of T antigen expression depend on the length of time the cells are held at 41 C. Several days at 41 C were required to demonstrate the changes shown in Fig. 4 and 5 for the transformant ts*A*28.3. In addition, 15% of the cells of the ts*A* transformant still have T antigen 3 days after the shift to 41 C (Fig. 1). Therefore caution is necessary in interpreting the data and particularly in deciding whether the changes described are fully reversible.

The following preliminary experiments suggest that cells of the ts*A* transformant remain viable after several days at 41 C. Cells were plated at approximately 10^5 cells per plate and shifted to 33 C after 4 days at 41 C. After further incubation at 33 C, dense areas of growth were seen. The same experiment was repeated, but the cells were trypsinized and replated after the 41 C incubation at dilutions of 10^{-1}, 10^{-2}, and 10^{-3} at 33 C. After 10 days, cells replated at 33 C formed visible colonies.

DISCUSSION

Our results suggest that wild-type transformants and ts*A* transformants of rat embryo cells behave very similarly, if not identically, at 33 C, a temperature which is permissive for both wild-type SV40 and for SV40 ts*A* mutants

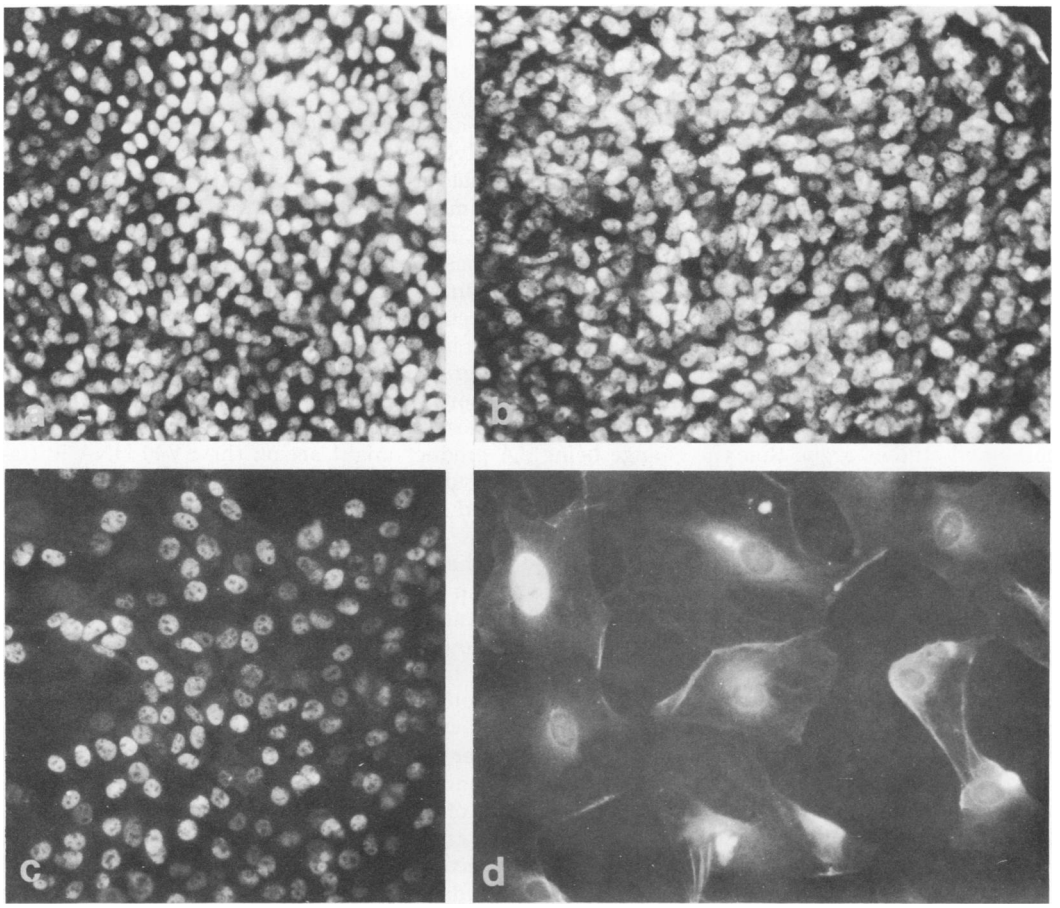

FIG. 5. *T antigen staining of (a) WT4 at 33 C, (b) tsA28.3 at 33 C, (c) WT4 at 41 C, and (d) tsA28.3 at 41 C. The cells were seeded as in the legend to Fig. 1 and processed for immunofluorescence 3 days (d) or 5 days (a–c) after the shift to 41 C. All four plates were taken at the same magnification. Almost all cells of the tsA transformant at 41 C are negative for T antigen (d; see also Fig. 1c and 1d).*

in the lytic cycle. Their properties at 33 C are very similar to those reported previously for a series of transformed rat clones isolated after infection of rat embryo cells with wild-type SV40 at 37 C (Pollack et al., Proc. Nat. Acad. Sci., U.S.A., in press; 18a). By criteria shown to be valid for the rat embryo system (Pollack et al., Proc. Nat. Acad. Sci. U.S.A., in press; 18a) and which are commonly used to judge transformation in vitro (i.e., low serum requirement, faster growth rate, higher saturation density, changed morphology, and expression of SV40 T antigen) both WT4 and tsA28.3 cells are transformed at 33 C. However, the wild-type transformant and the tsA transformants behave very differently at 41 C, a temperature which is nonpermissive for the tsA mutants in the lytic cycle (23). Thus the wild-type transformants remain transformed at 41 C, whereas the tsA transformants show reduced saturation density, changed morphology, and loss of expression of T antigen.

It will be interesting in the future to determine whether the tsA transformants at 41 C resemble rat embryo cells prior to transformation. Such a characterization was not done in the present study because of the difficulty of defining the properties of a population of embryonic cells. Also, since the transformation of wild-type SV40 and tsA mutants was done in parallel on the same cells, it seemed sufficient to use as a criteria for transformation the behavior of the wild-type cells, particularly since the behavior of these cells has been well documented by others (Pollack et al., Proc. Nat. Acad. Sci. U.S.A., in press; 18a). How-

ever, the apparent temperature sensitivity of properties usually associated with transformation is found not only in the tsA transformants of rat embryo cells, but also as shown in the accompanying manuscripts (1, 15, 22) for tsA transformants of several established cell lines. In these cases, the properties of the tsA transformants at the nonpermissive temperature appear to resemble those of the cell lines prior to transformation.

It is important that in all cases (1, 15, 22; this study) the original isolation of transformants was at the permissive temperature and the differences in properties of wild-type and tsA transformants only became apparent after a shift to the nonpermissive temperature. These combined studies argue that the change being observed is due to a viral gene and not a cellular one. They suggest that a mutation in the *A* gene interferes indirectly with the maintenance of transformation, or alternatively that a functional gene *A* product may be directly required for the maintenance of the transformed state.

Such a conclusion was unexpected in view of the results of Kimura and Dulbecco (10) with SV40 and of other earlier results (5–7) with polyoma, where transformants isolated at the permissive temperature after infection of certain cell lines with mutants of the tsA type appeared not to be temperature dependent for the maintenance of transformation. Additional work is needed to clarify whether the discrepancy between the earlier results and the results reported here and in the accompanying papers is due to the different viral mutants used, to the different cell lines used, or to the tests used to assay transformation. For example, it may be that, as previously shown with a temperature-sensitive mutant of avian sarcoma virus (8), the host cell can strongly influence the temperature at which temperature-dependent behavior of transformation is observed.

The data suggest that transformation requires more than the presence of an integrated site of viral replication in the transformed cell. The origin of replication of SV40 is 0.67 map units from the Eco RI site, whereas the *A* mutants mapped to date lie 0.32 to 0.42 map units from the same site (11), so the site of the origin of viral replication is clearly separate from the site at which the *A* mutants map. Therefore, it seems very likely that it is the product of the *A* gene that is actively required for the maintenance of transformation. In this context it should be recalled that all SV40 transformants characterized so far by RNA hybridization have been found to express the early SV40 RNA (9, 19) and that recent mapping of tsA mutants has shown that these mutations occur exclusively within the early SV40 RNA (11).

What is the function of the SV40 *A* gene? In the lytic cycle, the *A* function is required for the initiation of viral DNA synthesis (2, 21). If one assumes that in transformed cells the *A* function is also involved in some way in DNA replication, then it may be that the *A* function perturbs or alters the normal DNA replication process of the host cell. Taking into account that in transformed cells the SV40 genome is integrated into host cell DNA (20), there are two obvious ways in which a virus-specific influence on host DNA synthesis may occur. (i) The gene *A* product might act on the SV40 DNA initiation site and DNA synthesis might be propagated beyond the virus genome into host cell DNA. This interpretation would be analogous to that given by Nishimura et al. (16) for a strain of *E. coli* carrying a temperature-sensitive mutation in DNA initiation. DNA synthesis could be rendered temperature insensitive in this mutant by an autonomously replicating episome only if the episome was integrated (i.e., only when the bacterial chromosome had come under control of the episomal replicon). (ii) The *A* function might act on one or more host DNA loci of similar sequence to the viral initiation site, thus producing new initiator sites for DNA replication in the host genome. The latter model would be supported by the report that the induction of host DNA synthesis in permissive cells seems to depend on expression of the *A* function (4).

The *A* gene product might also act to perturb the normal cell cycle by either acting directly on host cell factors normally associated with cell division, or indirectly as a consequence of the introduction of new sites of DNA replication into the host cell genome. Thus, a transformed cell might require a functional gene *A* product to pass through a particular point on the cell cycle, e.g., from G_1 (or G_0) into S, or through mitosis. In this connection, it will be interesting to see if the cells of the tsA transformants that become very large after the shift to 41 C are blocked at a particular point in the cell cycle.

Are the *A* gene product and T antigen related? The ubiquitous presence of T antigen in the nuclei of cells transformed by SV40 indicates that T antigen is an expression of transformation of SV40. It is interesting, therefore, that at least some of our rat embryo tsA transformants lose T antigen as a function of time held at 41 C. Parallel to this loss of T antigen

the cells show strongly reduced growth properties, a decreased saturation density, a drastically flattened out morphology, and an increased expression of actin-containing fibers. Thus, in the rat embryo tsA transformants we describe, expression of T antigen appears to be correlated with the expression of the transformed state. A similar correlation of expression of T antigen with growth properties associated with transformation has been reported in other systems (17, 18). In addition, loss of T antigen in the tsA transformants at 41 C seems to be correlated with a gain in actin fibers, suggesting that in these cells these properties may be related. K. Weber, E. Lazarides, R. E. Goldman, A. Vogel, and R. Pollack (Cold Spring Harbor Symp. Quant. Biol., in press) have suggested that the decrease of actin-containing fibers may be correlated with transformation, again suggesting by this criteria for transformation that the tsA transformants are temperature-sensitive for the maintenance of transformation. Other evidence suggesting a possible relationship between A function and T antigen is our report that T antigen from cells infected at 41 C with certain tsA mutants has an aberrant sedimentation value (Osborn and Weber, Cold Spring Harbor Symp. Quant. Biol., in press).

Although the data are consistent with the interpretation that T antigen is the direct product of the A gene of SV40, several points should be discussed. (i) tsA transformants of other cell types described in the accompanying manuscripts do not appear to lose T antigen upon shift-up experiments (1, 15, 22). It may be that the loss of immunological properties requires a higher temperature than the loss of T function or loss of A function, or that the temperature required for inactivation depends on the host cell. Alternatively, the sample of tsA transformants we have characterized so far may be too small to detect clones which stay T positive at 41 C. (ii) Robb has reported (17) that certain SV40 D mutants show a similar loss of T antigen when transformants are shifted to 41 C, and since these mutants are now known to map in the late region (11) it has to be explained how such mutants can regulate the expression of an early event. (iii) All the A mutants mapped thus far lie in the Hin H and Hin I fragments (11), and this part of the SV40 DNA is generally assumed to induce TSTA antigen rather than T antigen (11, 13). This might be explained if the whole early region is translated into a precursor polypeptide chain which is secondarily cleaved to give rise to T, TSTA, and U antigens (P. Tegtmeyer, Cold Spring Harbor Symp. Quant. Biol., in press; Osborn and Weber, Cold Spring Harbor Symp. Quant. Biol., in press). (iv) An alternative explanation of the above data, which is also consistent with the currently known properties of T antigen, is that T antigen is not virus coded but is a conservative host protein modified or induced by early events after SV40 infection. In this case, we would expect the gene A product to be involved in some way in the modification or induction of T antigen. It is important, therefore, to isolate both T antigen and the gene A product and see if they are related biochemically.

Although the data suggest that the A function is required for the maintenance of SV40 transformation, they do not show that the gene A product is sufficient for maintenance of transformation or that the expression of the gene A product is not regulated by another viral gene function. In addition, by analogy with the RNA viruses, it may be that SV40 mutants affecting transformation, but not the lytic cycle, remain to be isolated. However, the finding that the A function most probably affects maintenance of transformation should serve to focus attention on the proteins coded by the early part of the SV40 genome. Hopefully, the understanding of the function of such proteins, and of their interactions with cellular components, may allow an explanation of transformation by SV40.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant CA 13106 from the National Cancer Institute.

We thank Peter Tegtmeyer for giving us the tsA mutants and Rex Risser and Bob Pollack for sharing their expertise on the rat embryo system with us. Enjoyable discussions with Peter Tegtmeyer, Janet Butel, and Bob Martin at the Tumour Virus Symposium at Cold Spring Harbor in June 1974, where these results and the results in the accompanying papers were first presented, are also acknowledged.

LITERATURE CITED

1. Brugge, J. S., and J. S. Butel. 1975. Role of simian virus 40 gene A function in the maintenance of transformation. J. Virol. **15**:619–635.
2. Chou, J. Y., J. Avila, and R. G. Martin. 1974. Viral DNA synthesis in temperature-sensitive mutants of simian virus 40. J. Virol. **14**:116–124.
3. Chou, J. Y., and R. G. Martin. 1974. Complementation analysis of simian virus 40 mutants. J. Virol. **13**:1101–1109.
4. Chou, J. Y., and R. G. Martin. 1975. DNA infectivity and the induction of host DNA synthesis with temperature-sensitive mutants of simian virus 40. J. Virol. **15**:145–151.
5. DiMayorca, G., J. Callender, G. Marin, and R. Giordano. 1969. Temperature sensitive mutants of polyoma virus. Virology **38**:126–133.
6. Eckhart, W. 1969. Complementation and transformation

by temperature sensitive mutants of polyoma virus. Virology **38**:120–125.
7. Fried, M. 1965. Cell transforming ability of a temperature sensitive mutant of polyoma virus. Proc. Nat. Acad. Sci. U.S.A. **53**:486–491.
8. Graf, T., and R. R. Friis. 1973. Differential expression of transformation in rat and chicken cells infected with an avian sarcoma virus *ts* mutant. Virology **56**:369–374.
9. Khoury, G., J. C. Byrne, K. K. Takemoto, and M. A. Martin. 1973. Patterns of simian virus 40 deoxyribonucleic acid transcription. II. In transformed cells. J. Virol. **11**:54–60.
10. Kimura, G., and R. Dulbecco. 1973. A temperature sensitive mutant of simian virus 40 affecting transforming ability. Virology **52**:529–534.
11. Lai, C-J., and D. Nathans. 1974. Mapping temperature sensitive mutants of simian virus 40: rescue of mutants by fragments of viral DNA. Virology **60**:466–475.
12. Lazarides, E., and K. Weber. 1974. Actin antibody: the specific visualization of actin filaments in non-muscle cells. Proc. Nat. Acad. Sci. U.S.A. **71**:2268–2272.
13. Lebowitz, P., T. J. Kelly, Jr., D. Nathans, T. N. H. Lee, and A. Lewis. 1974. A colinear map relating the SV40 DNA segments of six adenovirus SV40 hybrids to the DNA fragments produced by restriction endonuclease cleavage of SV40 DNA. Proc. Nat. Acad. Sci. U.S.A. **71**:441–448.
14. Martin, G. S. 1970. Rous sarcoma virus: a function required for the maintenance of the transformed state. Nature (London) **227**:1021–1023.
15. Martin, R. G., and J. Y. Chou. 1975. Simian virus 40 functions required for the establishment and maintenance of malignant transformation. J. Virol. **15**:599–612.
16. Nishimura, Y., L. Caro, C. M. Berg, and Y. Hirota. 1971. Chromosome replication in *E. coli*. IV. Control of chromosome replication and cell division by an integrated episome. J. Mol. Biol. **55**:441–456.
17. Robb, J. A. 1973. Simian virus 40 host-cell interactions. I. Temperature-sensitive regulation of SV40 T antigen in 3T3 mouse cells transformed by the *ts*101* temperature-sensitive early mutant of SV40. J. Virol. **12**:1187–1190.
18. Risser, R., and R. Pollack. 1974. A nonselective analysis of SV40 transformation of mouse 3T3 cells. Virology **59**:477–489.
18a. Risser, R., D. Rifkin, and R. Pollack. 1974. The stable classes of transformed cells induced by SV40 infection of established 3T3 cells and primary rat embryonic cells. Cold Spring Harbor Symp. Quant. Biol. **39**:317–324.
19. Sambrook, J., P. A. Sharp, and W. Keller. 1972. Transcription of simian virus 40. I. Separation of the strands of SV40 DNA and hybridization of the separated strands to RNA extracted from lytically infected and transformed cells. J. Mol. Biol. **70**:57–71.
20. Sambrook, J., H. Westphal, P. R. Srinivasan, and R. Dulbecco. 1968. The integrated state of viral DNA in SV40 transformed cells. Proc. Nat. Acad. Sci. U.S.A. **60**:1288–1295.
21. Tegtmeyer, P. 1972. Simian virus 40 deoxyribonucleic acid synthesis: the viral replicon. J. Virol. **10**:591–598.
22. Tegtmeyer, P. 1975. Function of simian virus 40 gene *A* in transforming infection. J. Virol. **15**:613–618.
23. Tegtmeyer, P., and H. L. Ozer. 1971. Temperature-sensitive mutants of simian virus 40: infection of permissive cells. J. Virol. **8**:516–524.
24. Wyke, J. A., and M. Linial. 1973. Temperature sensitive avian sarcoma viruses: a physiological comparison of twenty mutants. Virology **53**:152–161.