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Expression of simian virus 40 early genes in transformed rat cells is correlated with maintenance of the transformed phenotype*

(tumor antigen/immunoprecipitation/temperature-sensitive A mutants/regulation of gene expression)

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ABSTRACT Early viral polypeptides synthesized in simian virus 40 rat transformants were identified by immunoprecipitation using anti-T (tumor) antigen immune serum. Four polypeptide classes could be identified, which were not detectable in extracts of nontransformed cells and were not precipitated from transformed cell extracts by nonimmune serum. Their apparent M_r were 92,000, 63,000, 56,000, and 19,000. A similar pattern was observed in extracts from lytically infected cells, but the relative rate of radioactive labeling of the M_r 63,000 and 56,000 species was in this case significantly lower than in transformed cells. In *tsA30* transformants of type A, which maintain the transformed phenotype at high temperature, only minor quantitative variations of this pattern were observed when the cultures were shifted from 33° to 40.5°. In contrast, the rate of labeling of the four virus-specific polypeptides was decreased by 90% or more at high temperature in the temperature-sensitive N transformants. In all cases, a coordinated variation of the radioactivity associated with the different polypeptide classes was observed. These results suggest that the synthesis or processing, or both, of the viral early proteins may be controlled by different mechanisms in various types of simian virus 40 transformants and, furthermore, that it may be under the positive control of a virus-coded protein in transformed cells of type N.

Transformation of rat fibroblasts with either polyoma virus or simian virus 40 (SV40) may lead to two distinct types of transformed derivatives, designated as A and N, respectively (1, 2). They can be distinguished operationally by using cells transformed with the early temperature-sensitive (*ts*) mutants *tsa* of polyoma (3) and *tsA30* of SV40 (4). Polyoma *tsa* and SV40 *tsA30* transformants of the A type were selected by colony formation in agar medium. They appeared as phenotypically transformed at both low and high temperatures, whereas the corresponding type N transformants, derived from foci overgrowing attached monolayers of normal cells, reverted at high temperature to the normal phenotype for various characters that define the transformed state: saturation density of growth, cloning ability on plastic, ability to grow in agar medium, growth dependence on serum factors, generation time, and rate of 2-deoxyglucose transport. Transformants of both classes carry *ts* mutated viral genomes and the A state does not appear to result from the independent selection, in agar medium, of cellular mutations epistatic to the virus transforming function(s). Determination of the A *vs.* N transformed states occurs during the first 4 days after virus infection and depends on both the physiological state of the infected cells and the multiplicity of infection (unpublished results).

The viral function identified by the *tsa*(*A*) mutations therefore appears to be required not only for the establishment of the A transformed state, as originally demonstrated by Fried (3), but also for the maintenance of the transformed phenotype in N cell lines. The N and A states appear as two distinct mechanisms regulating the expression of the transformed phenotype. They could not be distinguished on the basis of the expression of a particular set of transformation characters: *ts*-A and *ts*-N transformants exhibit quite similar phenotypes at the permissive temperature, as do wild type (wt) polyoma or SV40 transformants isolated under conditions leading to either the A or the N transformed states (wt-A and wt-N lines).

Independently from the mutations belonging to the A complementation group, the transforming activity of SV40 virus recently has been shown to be severely depressed in viable deletion mutants mapping in the 0.54–0.59 region of the virus genome (5–7). These mutations correspond to the *hrt* mutations of polyoma previously isolated by Benjamin and his coworkers (8): genetic localization (9, see Fig. 1), effect on transformation, and ability to complement with mutations of group A are similar for both viruses (6, 10, 11). No *ts* mutant has so far been described in the 0.54–0.59 region. Only the establishment of the transformed state can therefore be proven to require the corresponding gene function. On the basis of the available data, one may nevertheless consider as a working hypothesis that this product may be the essential transforming protein of polyoma and SV40 viruses (6–8, 11).

Early viral polypeptides can be identified by immunoprecipitation using antibodies against the virus-specific T antigens. Best characterized (12–20) are the 90,000- to 100,000-dalton and the 17,000- to 19,000-dalton T-antigen polypeptides, which correspond to two distinct early mRNA species with different spliced regions (21, 22). The region of the genome corresponding to the *hrt*/*dl*54-59 mutations codes for the COOH-terminal part of the small T polypeptide, whereas the largest T polypeptide is mostly derived from sequences on the 3′ side of the deleted region, sequences where all the mutations in the A complementation group are located (Fig. 1).

Several other bands can be reproducibly observed after sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis of polyoma and SV40 T antigens (14, 20, 24; this report). Their apparent M_r range from 22,000 to 63,000. No published data allow us to conclude that these "middle T" species are virus-coded. However, this hypothesis is suggested by their discrete and reproducible size distribution, their antigenic specificity, and their presence in amounts quantitatively correlated with those of the large and small T products (15, 20; this report).

The hypothesis that the small T polypeptide, affected by the *hrt* type mutations, could be responsible for at least part of the virus transforming properties apparently contradicts the fact that the *tsa*(*A*) mutations, which do not affect this polypeptide, may lead to a fully temperature-dependent transformed phenotype (1, 2). One therefore has to assume either that both the large and small T polypeptides are necessary for the maintenance of transformation or that the activity of a protein affected by the *tsa*(*A*) mutation is necessary for the synthesis of the small T polypeptide.

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Abbreviations: SV40, simian virus 40; *ts*, temperature-sensitive; wt, wild type; $NaDodSO_4$, sodium dodecyl sulfate.

* Part of a thesis submitted by P.G. and M.R. to the University of Nice (France).

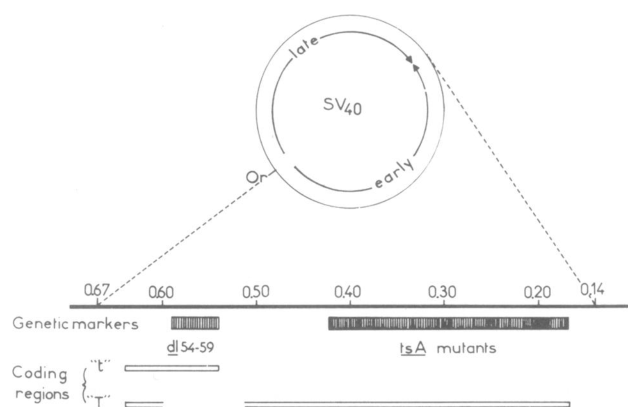

FIG. 1. Genetic map (23) of early region of SV40 genome, indicating origin of autonomous replication (Or), fractional lengths respective to *Eco*RI cleavage site and regions corresponding to primary sequences of the small and large T polypeptides ("t" and "T").

Data presented in this report show a clear temperature-dependence of the synthesis of all the immunoprecipitable T antigenic polypeptides in *tsA-N* but not in *tsA*-A SV40 transformants. They suggest that the A and N states may correspond to distinct modes of regulation of the synthesis of early proteins from integrated viral genomes.

MATERIAL AND METHODS

Viruses and Cells. wt and *tsA30* mutant (4) of SV40 were grown in CV1 cells infected at low multiplicity of infection (<0.01 plaque-forming unit per cell). For T antigen studies, the Vero African green monkey cell line (Flow Laboratories) was used as a permissive host. wt- and *tsA30*-transformants were isolated from the Fisher rat FR 3T3 cell line (1) and grown as previously described (2) in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% calf serum.

Radioactive Labeling of Proteins. Cultures were prepared by seeding 2×10^4 cells in 6-cm petri plates (Nunclon) and grown at 33° for 72 hr. After the plates were washed three times with methionine-free Dulbecco's modified Eagle's medium, 0.5 ml of the same medium containing 100 μCi of [^{35}S]methionine (700–1000 Ci/mmol, Amersham) per ml was added to the cultures. Two hours later they were quickly washed three times at 4° with Tris-buffered saline, and 0.5 ml of extraction buffer [0.01 M Tris·HCl, pH 8.0/0.1 M NaCl/0.5% Nonidet P40 (B.R.L. Inc.)/2 mM phenylmethylsulfonyl fluoride (Sigma)] was added to each plate. After 10 min at 4°, the cell lysates were centrifuged (15,000 × g, 6 min) and the supernatants were subjected to immunoprecipitation.

Large T Antigen Immunoprecipitation. Immunoprecipitation was performed by using a modification of the method described by Schwyzer (25). Extract (200 μl) was incubated for 15 min at 20° with 4 μl of either nonimmune or anti-SV40 T hamster serum (kindly given to us by M. Kress, Institut de Recherches Scientifiques sur le Cancer, Villejuif, France). Then, 20 μl of settled staphylococcal protein A-Sepharose CL-4B (Pharmacia) in 40 μl of extraction buffer was added. After 1 hr at 4° with constant stirring, the Sepharose pellet was washed repeatedly with 0.1 M Tris·HCl, pH 8.6/0.5 M LiCl/0.5% Nonidet P40, and once with 50 mM Tris·HCl, pH 6.8. Immune complexes were then eluted in 25 μl of 50 mM Tris·HCl, pH 6.8/0.2 M dithiothreitol/4% $NaDodSO_4$/10% glycerol.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. This was performed according to Laemmli (26), with 1.5-mm-thick slab gels containing 12.5% acrylamide and 0.33% bisacrylamide. Samples were heated at 80° for 5 min and bromphenol blue was added to a final concentration of 0.001%. After 3 hr of electrophoresis under a constant power of 6 W, the gels were stained with Coomassie brilliant blue R 250, destained, and prepared for fluorography on Kodak RP Royal-X-Omat emulsions without preflashing, as described by Bonner and Laskey (27). The gels were calibrated by using the following M_r standards: β-galactosidase (130,000), phosphorylase a (94,000), bovine serum albumin (68,000), gamma globulin heavy chain (53,000), ovalbumin (43,000), and β-lactoglobulin (18,500). Fluorograms were scanned by using a Vernon densitometer.

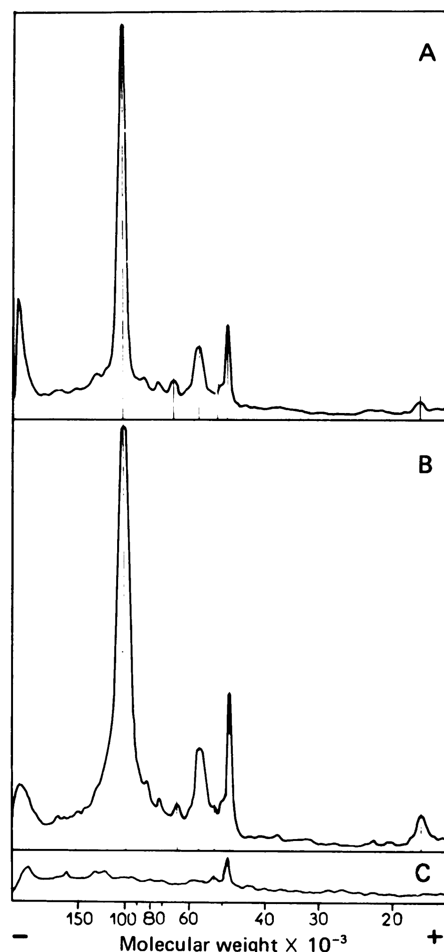

FIG. 2. $NaDodSO_4$ gel electrophoresis profiles of T antigenic immunoprecipitated proteins from SV40-transformed FR 3T3 rat cells. Cultures of the wt-N1 and wt-A1 transformed lines were prepared by seeding 2×10^4 cells in 6-cm Petri plates (Nunclon) at 33°. The cells were labeled with [^{35}S]methionine. Extracts were precipitated by addition of excess anti-T hamster serum and staphylococcal protein A coupled to Sepharose. After electrophoresis, the radioactive bands were revealed by fluorography (see Fig. 3) and the fluorograms were scanned. (*A*) wt-N1; (*B*) wt-A1; (*C*) immunoprecipitation of the wt-N1 cell extract with nonimmune hamster serum.

RESULTS

Immunoprecipitation of T Antigen from wt SV40 Transformants. FR 3T3 rat fibroblasts transformed by wt SV40 (wt-A and wt-N cell lines) were labeled with [^{35}S]methionine during exponential growth at 33°. The $NaDodSO_4$ gel electrophoresis profiles after immunoprecipitation of T antigen revealed six major polypeptide species (Figs. 2 and 3) with apparent M_r of 19,000, 48,000, 52,000, 56,000, 63,000, and 92,000. Neither the total amounts of immunoprecipitable radioactive material nor the relative amounts of the different polypeptides were found to vary in a significant way when the concentration of anti-T antiserum used for immunoprecipitation was increased (data not shown).

This profile was qualitatively similar to that observed after immunoprecipitation of T antigen from extracts of lytically

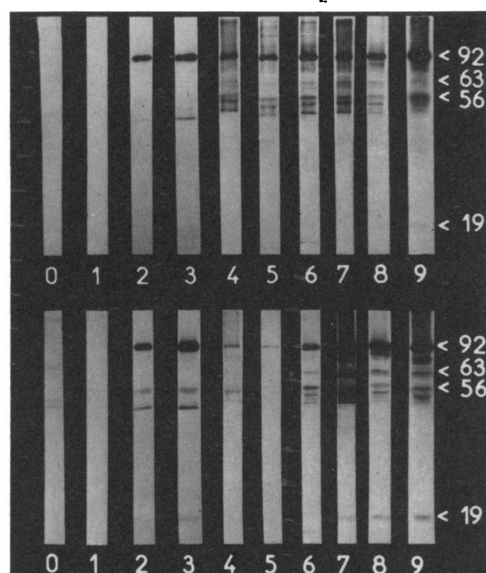

FIG. 3. ^{35}S-Labeled T antigenic proteins in FR 3T3 cells and their various SV40-transformed derivatives. Same experiment as in Figs. 2 and 4, after labeling for 2 hr with [^{35}S]methionine at 33° (*Upper*) or at 40.5° 72 hr after shift up (*Lower*). $M_r \times 10^{-3}$ shown at right. Lanes: 0, FR 3T3 + immune serum; 1, wt-N1 + nonimmune serum; 2, wt-N1 + immune serum; 3, wt-A1 (as in 2); 4, *tsA30*-N1 (as in 2); 5, *tsA30*-N2 (as in 2); 6, *tsA30*-An3 (as in 2); 7, *tsA30*-A1 (as in 2); 8, *tsA30*-A2 (as in 2); 9, *tsA30*-A3 (as in 2).

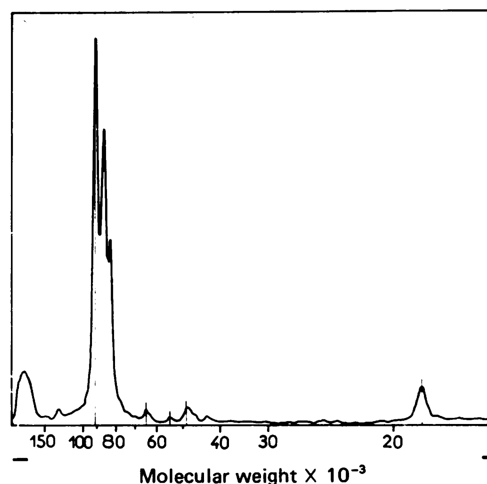

FIG. 4. NaDodSO$_4$ gel electrophoresis of T antigenic immunoprecipitated proteins from lytically infected permissive cells. Vero cells were infected at a multiplicity of 50 plaque-forming units per cell and incubated at 33°; 48 hr after infection, they were labeled with [^{35}S]methionine and extracts were prepared and processed as indicated in the legend of Fig. 2. Immunoprecipitation with nonimmune hamster serum produced electrophoretic profiles undistinguishable from Fig. 2*C* (see also Fig. 3).

infected Vero cells (Fig. 4). However, the M_r 48,000–63,000 polypeptides were consistently present in lesser amounts in the latter. These "middle T" species exhibit apparent M_rs identical to those observed by electrophoresis of either immunoprecipitated (14, 20) or extensively purified (24) polyoma T antigen. However, because two minor bands were detected at positions corresponding to the 48,000- and 52,000-dalton polypeptides in control experiments using nonimmune hamster serum (Figs. 2 and 3), only the 92,000 (large T), 63,000, and 56,000 (middle T), and 19,000 (small t) dalton species may be considered as virus-specific at this stage. No detectable polypeptide with electrophoretic mobilities corresponding to any of these species could be detected after immunoprecipitation of labeled extracts from nontransformed FR 3T3 cells (Fig. 3).

No significant differences were found in the electrophoretic profiles of T antigenic polypeptides from two A transformants and two N transformants, all independently isolated (data not shown). In all wt transformants, similar profiles were observed at 33 and 40.5° (Fig. 3).

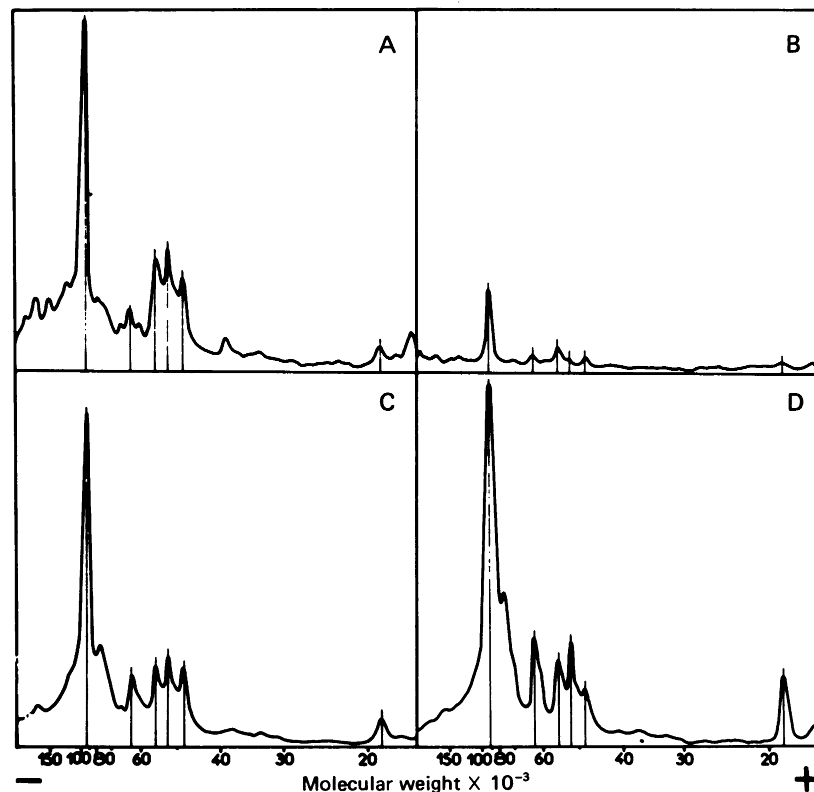

FIG. 5. Densitometer tracing of the fluorograms corresponding to the *tsA30*-N2 and *tsA30*-A1 lines in Fig. 3. (*A*) *tsA30*-N2, 33°; (*B*) *tsA30*-N2, 40.5°; (*C*) *tsA30*-A1, 33°; (*D*) *tsA30*-A1, 40.5°.

Table 1. Relative amounts of [^{35}S]methionine-labeled polypeptides in T antigen from *tsA30*-A and *tsA30*-N transformants grown at 33° and 40.5°

Cell line	Temp., °C	T polypeptides amounts immunoprecipitated cpm/10^5 input cpm					Ratio, 40.5°/33°			
		92,000	63,000	56,000	19,000	Total	92,000	63,000	56,000	19,000
tsA30-A1	33	39	7	12	5	63				
	40.5	66	11	11	8	96	1.7	1.6	0.9	1.6
tsA30-A2	33	8	<0.1	<0.1	1	9				
	40.5	12	<0.1	<0.1	1	13	1.5	—	—	1.9
tsA30-A3	33	33	3	5	3	44				
	40.5	32	3	3	3	41	1.0	1.1	0.5	1.1
tsA30-An3	33	24	2	5	1	32				
	40.5	16	3	3	2	24	0.7	1.2	0.7	1.5
tsA30-N1	33	5	<0.1	<0.1	1	6				
	40.5	≤0.1	<0.1	<0.1	≤0.1	<1	≤0.02	—	—	≤0.1
tsA30-N2	33	35	5	9	5	54				
	40.5	3	0.5	0.7	≤0.5	4	0.1	0.1	0.1	≤0.1

Cells were grown and labeled with [^{35}S]methionine. After fluorographic exposure, the bands of the gels corresponding to the indicated apparent M_rs were cut out, and their radioactivity was measured by scintillation counting. The results are shown as the ratio to the total input radioactivity in the sample subjected to immunoprecipitation. Total incorporated radioactivity varied between 1 and 4 × 10^7 cpm per 2 × 10^5 cells, and no significant difference was observed between one cell line and another or, for one given line, between low and high temperatures.

T Antigen Synthesized in *tsA30* Transformants at 33°. As shown in Fig. 3, qualitatively similar patterns were observed after immunoprecipitation and subsequent gel electrophoresis of T antigen from six different *tsA30* transformants, four of type A (temperature-independent) and two of type N (temperature-dependent for the maintenance of the transformed phenotype). Densitometer tracings are shown in Fig. 5 for one representative line of the A and N classes.

After fluorographic exposure, the bands corresponding to the 92,000, 63,000, 56,000, and 19,000 dalton polypeptides were cut from the gels and their radioactivity was measured by scintillation counting. The total immunoprecipitable radioactivity in these polypeptides, expressed as a fraction of the radioactivity in the aliquots subjected to analysis, varied between 6 × 10^{-4} and 6 × 10^{-5} from one cell line to the other (Table 1). The same range of variation was observed among transformants of either N or A phenotype.

Synthesis of T Antigen in *tsA30* A and N Transformants at High Temperature. Cultures of the same six *tsA30* transformants were seeded at 33° and shifted to 40.5° 24 hr later. They were labeled with [^{35}S]methionine 72 hr after the shift up. Growth rates of *tsA30* A and N transformants are similar at 33° and 40.5° (2). In all cases, cultures at low and high temperatures were therefore, at the time of labeling, at the same cell density, $\frac{1}{10}$th to $\frac{1}{5}$th of the saturation density of the phenotypically normal cell cultures.

Fluorograms obtained under these conditions are shown in Fig. 3, and the densitometer scanning of two representative lines are in Fig. 5. The relative amounts of radioactivity associated with the 92,000, 63,000, 56,000, and 19,000 dalton species are listed in Table 1.

Transformants of types A and N clearly exhibited two distinct patterns of T antigen synthesis, with only minor variations from one cell line to another. The profiles obtained at 33° and 40.5° were similar in all the A lines: the relative amounts of the four virus-specific polypeptides were either not affected, increased 1.5- to 2-fold, or slightly decreased after shift up. In contrast, the amount of radioactivity found associated with these polypeptides was decreased at 40.5 by at least 90% in the two independent N lines.

In all cases, a strong correlation was observed between the amounts of radioactivity in individual polypeptides. Measurements on the six *tsA30* transformants and on various wt-A and wt-N transformants are plotted in Fig. 6. The rank correlation coefficients computed by the nonparametric tests of Spearman and Kendall (28) indicate significance ($P > 0.99$). This observation excludes the possibility that the middle T polypeptides may represent adventitious contaminants or result from random proteolysis in cell extracts. It indicates that the amount of the small T polypeptide, which is not structurally

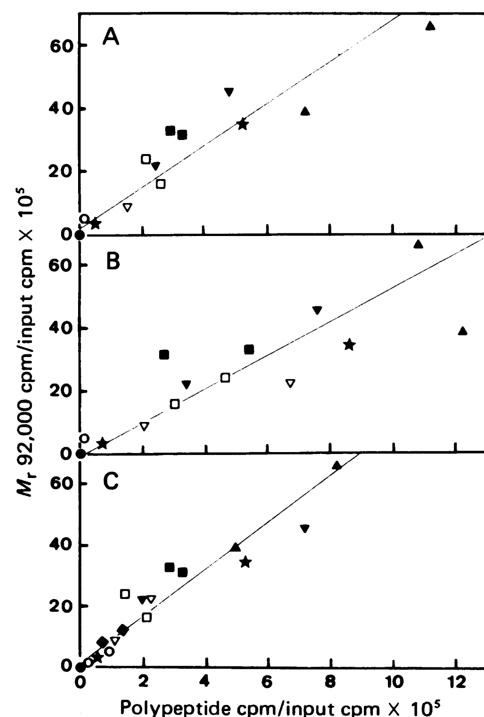

FIG. 6. Correlation between the amount of labeled 92,000 dalton and 63,000 (*A*), 56,000 (*B*), and 19,000 (*C*) dalton polypeptides in various transformants at either 33° or 40.5°. Numerical values are those from Table 1 for *tsA30* transformants and from similar experiments performed with wt transformants. The least square lines are shown for each regression. ●, FR 3T3; ▼, WT-A1; ▽, WT-N1; ▲, *tsA30*-A1; ♦, *tsA30*-A2; ■, *tsA30*-A3; □, *tsA30*-An3; ○, *tsA30*-N1; ★, *tsA30*-N2.

modified by the *tsA* mutation, is regulated in *tsA30*-N cells by a mechanism depending on the activity of a viral *ts* protein.

DISCUSSION

A common pattern of T antigenic polypeptides was observed in all SV40-transformed FR 3T3 cells that were expressing the transformed phenotype—namely, wt and *tsA*-A transformants at either 33° or 40.5° and *tsA*-N transformants at 33°.

These electrophoretic distributions were qualitatively similar to those found in lytically infected permissive cells (Figs. 2–4), in agreement with recent reports on various SV40 rat transformants (17, 18, 29). However, we observed a reproducible quantitative difference between the relative amounts of immunoprecipitable polypeptide species in lytic and transformed cell extracts: the middle T 56,000 and 63,000 dalton species amounted to 5–8% of the total T antigen-associated radioactivity in lytic cell extracts, compared with 20–30% in transformed cells. It appears unlikely that these components may result from random proteolysis or any other chemical modification in extracts. These processes are known to generate products in the 80,000- to 90,000-dalton and 20,000- to 40,000-dalton ranges (18, 30), which are not detectable in the above fluorograms. These middle T polypeptides represent, in addition, a constant fraction of the total immunoprecipitable radioactivity in transformed cells and their apparent M_rs closely correspond to those of recently described components of polyoma T antigen (14, 20). They may therefore represent virus-coded polypeptides, which might be produced either by translation of independent spliced mRNAs or by post-translational processing of a larger viral polypeptide (large T). Alternatively, they may be cellular gene products in a specific and stoichiometric association with viral proteins.

A pleiotropic and coordinated variation in the relative amounts of antigenic polypeptides was observed in all the lines we studied. A strong decrease in these amounts was correlated, in *tsA*-N transformants grown at 40.5°, with their phenotypic reversion to the normal growth type (1, 2). In contrast, *tsA*-A cells, which are phenotypically transformed at 40.5°, also maintain their pattern of T antigen synthesis at this temperature. These results are compatible with the observation by Brockman (31) of a higher T antigen steady-state level at high temperature in one temperature-independent mouse cell line transformed by the *tsA30* mutant.

Distinct mechanisms thus appear to control the amount of T antigen synthesized in N and A transformants. In N cells, a positive regulation of the expression of the integrated early viral genes by a *tsA* mutated gene product appears as a likely possibility. Such a regulation would not operate in A transformed cells. It would be different from the negative regulation of its own synthesis exerted by the *A* gene product in lytically infected cells (12, 32).

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