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TUMOR ANTIGEN ACTIVITY IN VIRAL DNA-PROTEIN COMPLEXES FROM CELLS PRODUCTIVELY INFECTED WITH PAPOVAVIRUSES[1]

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Summary. — A polypeptide with a molecular weight in the range from 70,000 to 90,000 daltons, previously identified in DNA-protein complex isolated from polyoma virus- and SV40-infected cells, was isolated after deoxyribonuclease treatment of the complex followed by extensive dialysis and hydroxylapatite chromatography. The nonstructural protein was specifically retained by columns of protein A-Sepharose CL-4B onto which antibodies directed against SV40 T-antigen had been bound. After absorption with this protein, antiserum against the SV40 T-antigen was unable to react with SV40-transformed cells in an immunofluorescence test. Finally the same antiserum reacted positively with the partially purified protein in the radioimmunoassay. These data are interpreted as indicating that, in productively infected cells, some of the nascent papovavirus DNA is complexed with T-antigen and viral structural polypeptides.

Key words: polyomavirus; DNA-protein complex; T-antigen, protein A; hydroxylapatite chromatography; radioimmunoassay; immunofluorescence

Introduction

During productive infections by polyoma virus and SV40 (genus *Polyomavirus*), the viral genome replicates and accumulates as part of a DNA-protein complex (Green *et al.*, 1971; Green, 1972; Frost and Bourgaux, 1973; Goldstein *et al.*, 1973; Shmookler *et al.*, 1974). Such complexes have been reported to contain viral structural polypeptides (McMillen and Consigli, 1974; Qureshi and Bourgaux, 1976) and even host-cell histone (McMillen and Consigli, 1974). Recently, we have detected, in both polyoma and SV40 DNA-protein complexes, the presence of a protein which, in sodium dodecyl sulphate-(SDS-) polyacrylamide gels, migrates as a single polypeptide with a molecular

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weight in the range from 70,000 to 90,000 daltons (Qureshi and Bourgaux, 1976, 1977).

Using antiserum from animals bearing SV40-induced tumors, a tumor (T) antigen could be detected in virus-infected or transformed cells (Black *et al.*, 1963; Rapp *et al.*, 1964). This antigen is now known to be a product of SV40 early gene in the lytically infected cells (Tegtmeyer, 1974; Rundell *et al.*, 1977; Paucha *et al.*, 1978*b*). It is also reported to be involved in the regulation of viral genome (Chou *et al.*, 1974; Alwine *et al.*, 1977; Tegtmeyer *et al.*, 1977) and probably be responsible for both initiation and maintenance of the transformed state (Brugge and Butel, 1975; Martin and Chou, 1975; Osborn and Weber, 1975; Tegtmeyer, 1975). Moreover, the T-antigen protein binds specifically to the viral DNA at or near the origin of DNA replication (Tegtmeyer, 1974; Reed *et al.*, 1975; Carroll and Smith, 1976; Jessel *et al.*, 1976).

To study this protein so vitally important in the replication of the virus, many investigators have attempted to isolate and purify it by biochemical and serological techniques. Using immunoprecipitation methods, several proteins ranging in size from 15,000 to 100,000 daltons have been claimed to have viral T-antigen activity (Tegtmeyer *et al.*, 1975, 1977; Ahmed-Zadeh *et al.*, 1976; Prives and Beck, 1977; Prives *et al.*, 1977; Robb, 1977; Paucha *et al.*, 1978*a*).

I am presenting evidence that the large nonstructural polypeptide detected in the DNA-protein complex of the two papovaviruses (Qureshi and Bourgaux, 1976, 1977) actually displays the serological reactivity of the respective tumor antigens.

Materials and Methods

Virus and cell cultures. The procedures used for propagation and purification of the TSP-1 small-plaque variant (Stanners, 1963) of polyoma virus (Py) have been described (Bourgaux and Bourgaux-Ramoisy, 1971; Bourgaux *et al.*, 1971). The large-plaque variant of SV40, obtained from Dr. P. Tegtmeyer (Stonybrook Medical Center, New York), was propagated in CV-1 cells (Flow Laboratories, Rockville, Maryland). Confluent monolayers were exposed to SV40 at an input multiplicity of 0.1 PFU/cell for 90 min at 37 °C, and covered with Dulbecco's Minimum Essential Medium (DMEM) containing 10 % calf serum. After one week of incubation at 37 °C, cultures were frozen and thawed 3 times, centrifuged at 800 × g for 20 min at 4 °C and the supernantant was used as virus stock.

Isolation and purification of DNA-protein complex. The Py complex was labeled, extracted and purified as before (Qureshi and Bourgaux, 1976). Thirty-two hr post-infection with SV40, CV-1 cells were pulse-labeled with ^{3}H-thymidine (5 μCi/ml, 53.6 Ci/mole) for another 8 hr period. The DNA-protein complex was extracted with Triton X-100 and purified by sucrose gradient centrifugation and ion-exchange chromatography as described for the Py complex (Qureshi and Bourgaux, 1976).

Partial purification of large molecular weight, nonstructural polypeptide. After ion-exchange chromatography, the protein moiety of the purified complex was labeled in vitro with ^{125}I as described (Qureshi and Bourgaux, 1976). The iodinated complex was then treated with calf thymus deoxyribonuclease (DNase, 20 μg/ml) for 2 hr at 37 °C in 0.015 M $MgCl_2$, 0.01 M phosphate buffer, pH 7.2. After DNase treatment, the reaction mixture was dialyzed overnight against 0.01 M phosphate buffer, pH 7.2, in a standard dialysis bag. The remaining protein was concentrated under vacuum using a collodion membrane (Sartorius) and applied onto a 0.5 × 8 cm hydroxylapatite column (Bio-Rad), equilibrated with 0.01 M phosphate buffer, pH 7.2. After

washing with 50 ml of 0.01 M phosphate buffer, pH 7.2, a linear concentration gradient of 0.1 to 0.4 M of the same buffer was applied to the column. Portions from the fractions (3 ml each) collected were counted in a scintillation spectrometer. The fractions containing radioactivive material corresponding to peaks were pooled, dialyzed, concentrated and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Qureshi and Bourgaux, 1976).

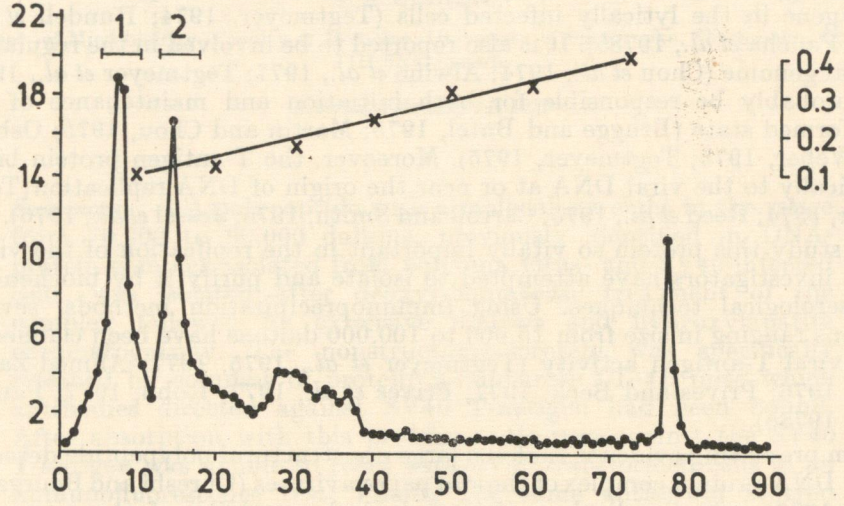

Fig. 1.

Hydroxylapatite chromatography of protein from the Py DNA-protein complex
Purified complex was labeled with ^{125}I using chloramine T, treated with DNase and dialyzed extensively as described in Materials and Methods. It was loaded on a hydroxylapatite column that was first washed with 0.01 M phosphate buffer. A linear concentration gradient of 0.1 to 0.4 M phosphate buffer was then applied (×, right ordinate).
Abscissa: fraction number; left ordinate: ^{125}I count/min × 10^{-3} (●)
Bars 1 and 2: the respective fractions were pooled.

Serological identification of partially purified nonstructural polypeptide. Antibodies against SV40 and Py T-antigens were prepared by injecting hamsters with wild type SV40- or Py-transformed hamster cells developed in this laboratory. The tumor-bearing animals were bled six weeks later. Immunoglobulin (IgG) was precipitated from the sera by $(NH_4)_2SO_4$ at 40% saturation. The resulting precipitate was redissolved and dialyzed overnight at 4 °C against the same buffer. Rabbit anti-hamster IgG and fluorescein isothiocyanate- (FITC-) conjugated hamster anti-SV40 T antibodies were obtained commercially (Flow). Anti-Py T antibodies were FITC-conjugated by methods of Liu (1964).

Immunoabsorption chromatography. Protein A-Sepharose CL-4B (PAS) was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden, swollen and washed with 0.01 M phosphate buffer, pH 7.2. To 75 µl of packed PAS, 100 µl of hamster anti-SV40 or Py-T serum was added and incubated in a shaker for 10 min at room temperature. The PAS was separated from unadsorbed serum by low speed centrifugation, washed with 0.01 M phosphate buffer, pH 7.2 and 1 ml of the QAE-Sephadex column concentrate was mixed with the pellet. After incubation in a shaker for 1 hr at room temperature, the mixture was poured into a small column made out of a Pasteur pipette which contained 100 µl of packed Sepharose 4B as a bed support for antigen-antibody adsorbed PAS. The column was washed first with 50 bed volumes of 0.15 M NaCl in 0.1 M Tris-HCl pH 8.0 followed by another 50 volumes of 0.5 M LiCl in Tris-HCl pH 8.0. Usually, negligible

amounts of radioactivity washed out of the column by this time. The antigen was eluted from the column by 2.5 % SDS and 5 % 2-β mercaptoethanol in 0.1 M Tris-HCl pH 8.0 (elution buffer). The eluate was concentrated by adding 0.1 volume of a 2 M KCl solution and the precipitate was washed 3 times with cold acetone before dissolving in 100 µl of sample buffer (2% SDS and 2 % 2-β-mercaptoethanol in 0.1 M Tris-HCl pH 8.0).

Fluorescent-antibody blocking test. FITC-conjugated anti-SV40-T or anti-Py-T serum (50 µl) was mixed with an equal volume of partially purified nonstructural polypeptide or other necessary control samples and incubated overnight at 4 °C. To remove any precipitate that may have formed during this incubation, the reaction mixture was centrifuged at maximum speed for 5 min in a Beckman Microfuge maintained at room temperature. The supernatant was used in a direct immunofluorescence test with Py- or SV-40-transformed hamster cells, known to exhibit a strongly positive fluorescence with the same batch of conjugated serum (unpublished data).

Radioimmunoassay (RIA). The partially purified nonstructural polypeptides from SV40 and Py complexes were also used as antigens in RIA, performed according to the method of Trent *et al.* (1976) and statistically analyzed as follows. Based on the fact that in a Poisson distribution of data, the mean of a population of values is approximately equal to the variances, the RIA index is then:

$$\text{RIA} = \frac{\bar{x}_t - \bar{x}_c}{\bar{x}_c}$$

where $\bar{x}_c$ is the mean of the test serum or sample's value, and $\bar{x}_c$ is the mean of the control serum or sample's values. This index then corresponds to "titre" in conventional serological procedures: it indicates how positive or negative a test serum or sample is with respect to the negative control. In general, sera or samples with an RIA index > 50 are positive for antibody or antigen depending upon the appropriate sample.

Results

Purification of the nonstructural proteins

Triton extracts of Py-infected mouse embryo cells were purified by neutral sucrose gradients and ion-exchange chromatography on QAE-Sephadex columns (Qureshi and Bourgaux, 1976). The peak material from such a column was then dialyzed, iodinated in vitro with ^{125}I (Qureshi and Bourgaux, 1976), digested with calf thymus DNase, and charged onto a hydroxylapatite column. Approximately one third of the total ^{125}I counts loaded on the column were washed out with 0.01 M phosphate buffer (Fig. 1). Another third of the total counts were eluted as a prominent peak at approximately 0.1 M phosphate and the rest of the ^{125}I radioactivity was eluted at various molarities of phosphate. Each elution peak was then concentrated in collodion membrane under vacuum and subjected to SDS-PAGE. The electropherogram of the column wash revealed polypeptides of various molecular sizes (Fig. 2-I). Majority of radioactivity was found at a place where proteins of lower molecular size would have migrated. The major peak eluted at 0.1 M phosphate was mixed with purified ^{131}I-labeled Py virus, denatured and electrophoresed under identical conditions. All of the ^{131}I-labeled virus structural proteins were detected at their respective places reported previously (Qureshi and Bourgaux, 1976). More than 90 % of the ^{125}I label migrated as a single discrete peak which did not comigrate with any of the viral structural proteins (Fig. 2-II). However, a slight smearing of radioactivity indicated the presence of a very small amount of contaminating protein(s) of lower molecular weight(s). The material eluted between 0.2 to 0.3 M phosphate did

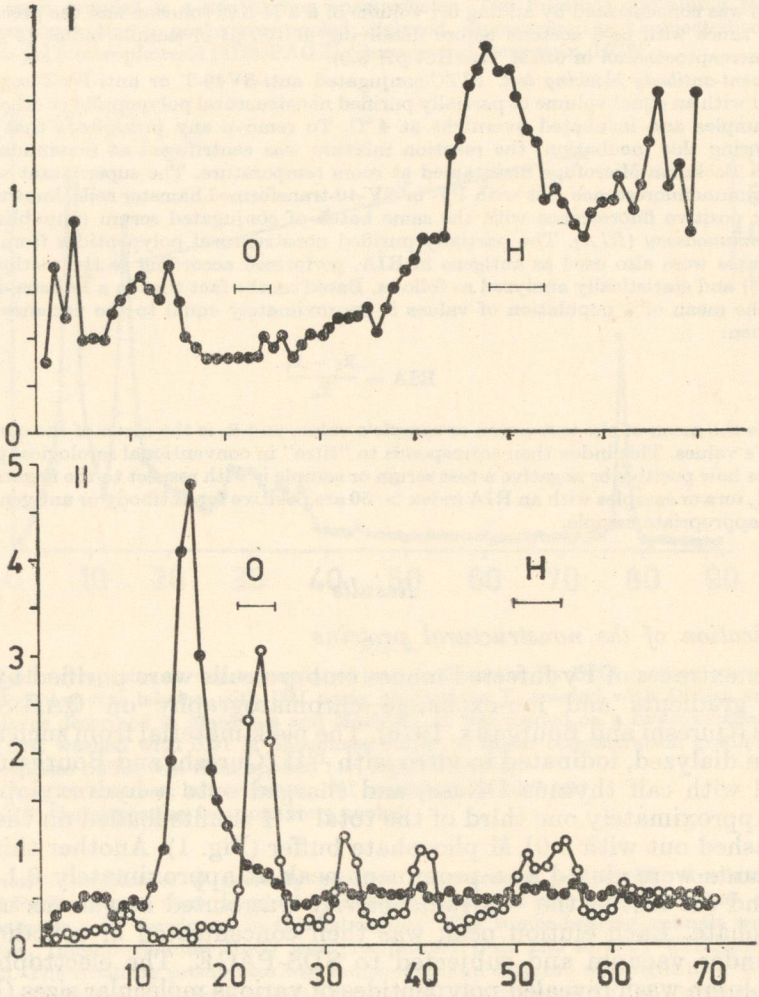

Fig. 2.

SDS-PAGE of ^{125}I-labeled protein from Py nucleoprotein complex, after hydroxylapatite chromatography

Fractions from the hydroxylapatite column were pooled (pool 1 and 2, see Fig. 1) and subjected to electrophoresis through cylindrical 10% SDS-polyacrylamide gels (Qureshi and Bourgaux, 1976). The figure shows the radioactivity of the various slices cut out from the gels, as well as the positions of the Ramazol Blue-stained (Griffith, 1972) markers ovalbumin (O) and histone (H) in the gel. I and II: analysis of pools 1 and 2, respectively

Abscissa: migration (mm); ordinate: ^{125}I (●) or ^{131}I (○) count/min × 10^{-3}

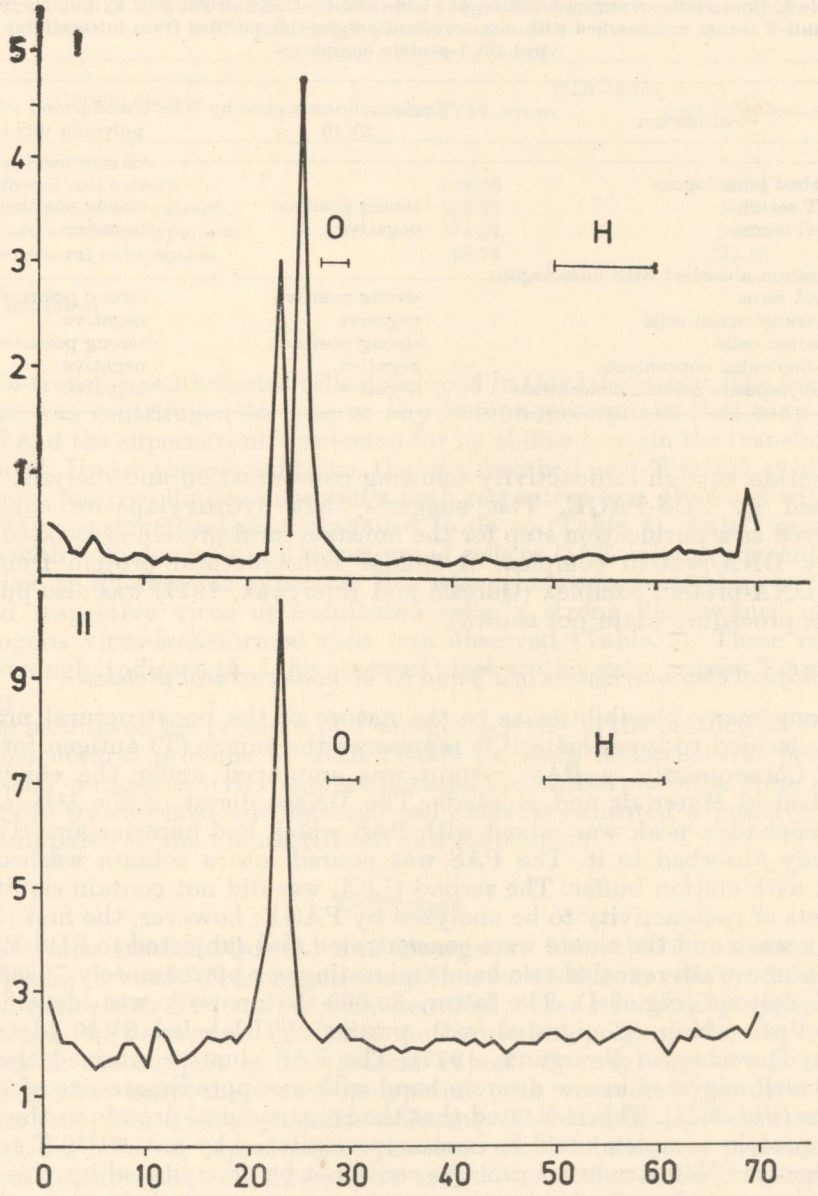

Fig. 3.

SDS-PAGE of two main fractions from the antibody — PAS column chromatography of SV40 nucleoprotein complex

I: Column wash (NaCl), II: column eluate

Abscissa: migration (mm); ordinate: ^{125}I count/min $\times 10^{-2}$

Table 1. Immunoffuorescence blocking of SV40- and Py-transformed cells by homologous anti-T serum preabsorbed with nonstructural polypeptide purified from intracellular viral DNA-protein complexes

Antiserum	Immunofluorescence by cells transformed with SV40	polyoma virus
Unabsorbed homologous		
Anti-T serum	strong positive	strong positive
Control serum	negative	negative
Anti-T serum absorbed with homologous		
purified virus	strong positive	strong positive
virus-transformed cells	negative	negative
uninfected cells	strong positive	strong positive
QAE-Sephadex concentrate	negative	negative
hydroxylapatite column concentrate	negative	negative

not contain enough radioactivity folowing concentration and dialysis, to be analyzed by SDS-PAGE. This suggests that hydroxylapatite could be employed as a purification step for the nonstructural protein associated with the Py DNA-protein complex. A similar nonstructural protein from the SV40 DNA-protein complex (Qureshi and Bourgaux, 1977) was also purified by this procedure (data not shown).

Serological characterization of Py and SV40 nonstructural proteins

Among many possibilities as to the nature of the nonstructural protein, it was decided to investigate if it represents the tumor (T) antigen for each virus. Consequently, a PAS column was employed under the conditions described in Materials and Methods. The DNase digest of the ^{125}I-labeled QAE-Sephadex peak was mixed with PAS which had hamster anti-SV40-T antibody absorbed to it. The PAS was poured into a column washed and eluted with elution buffer. The second (LiCl) was did not contain significant amounts of radioactivity to be analyzed by PAGE; however, the first (NaCl) column wash and the eluate were concentrated and subjected to SDS-PAGE. The column wash revealed two bands migrating at approximately 70,000 and 50,000 daltons (Fig. 3-I). The latter, 50,000 dalton peak was identified as SV40 Vp-1 which co-migrated with purified ^{131}I-labeled SV40 (data not shown; Qureshi and Bourgaux, 1977). The PAS eluate contained the protein which migrated in one discrete band with an approximate size of 70,000 daltons (Fig. 3-II). This indicated that the nonstructural protein in the SV40 nucleoprotein complex could be immunoprecipitated by anti-SV40-T antibody, whereas SV40 structural proteins could not be precipitated by this antibody (data not shown). Analogous experiments were carried out on the Py nucleoprotein complex with similar results.

In other experiments, homologous FITC-conjugated hamster Py or SV40 anti-T sera were absorbed with the hydroxylapatite column-purified SV40 nonstructural protein to specifically block the fluorescent staining of the Py-

Table 2. RIA of SV40 and Py nonstructural polypeptide purified from intracellular viral DNA-protein complexes

Protein sample	RIA Index	
	Anti-SV40 serum	Anti-Py T-serum
Uninfected cell extract	–	N.D.
SV40-infected cell extract	109.96	N.D.
SV40-transformed cell extract	105.22	N.D.
SV40 nonstructural polypeptide	274.04	39.25
Py nonstructural polypeptide	28.78	223.35

N.D. = not done.

or SV40-transformed hamster cells developed in this laboratory. The reaction mixture was centrifuged to remove any immunoprecipitate that may have formed and the supernatant was tested for its ability to stain the transformed cell nuclei. Under these conditions, the nonabsorbed anti-T serum exhibited a strongly positive fluorescence while both respective sera absorbed with Py and SV40 nonstructural proteins failed to do so (Table 1). Anti-T sera absorbed with homologous virus-transformed cells or QAE-column concentrates also failed to fluoresce as expected. But when these sera were absorbed with purified respective virus or uninfected cells, a strong fluorescence of the homologous virus-transformed cells was observed (Table 1). These results again strongly indicate that the nonstructural protein may possess T-antigen activity.

I also monitored the presence of T-antigen activity of the purified SV40 and Py nonstructural proteins by RIA (Table 2). Both nonstructural proteins exhitited T-antigen activity against homologous antiserum only. Here again both SV40-transformed and infected cell extracts exhitited a positive RIA index compared to the uninfected cell extract control.

Discussion

In previous studies (Qureshi and Bourgaux, 1976, 1977), at least four polypeptides in viral DNA-protein complexes isolated from cells productively infected with either SV40 or Py were identified: three structural polypeptides also present in virus, and one large nonstructural polypeptide, the origin of which is the subject of the present report. It may appear somewhat surprising that only the latter polypetide was recovered in detectable amounts from the hydroxylapatite columns. The largest of the structural polypeptides however represents at the most 45,000 daltons of protein, and it is thus possible that all three structural polypeptides leaked through, or stuck to, the tubing during the extensive dialysis preceding chromatography. Whatever the reason, fortunately the procedure adopted happened to yield preparations of nonstructural polypeptide of interesting homogeneity.

This nonstructural polypeptide was specifically absorbed by *Staphylococcus aureus* protein A column only in presence of homologous anti-T antibody and

immunofluorescence was also specifically blocked when anti-T sera were absorbed with it prior to testing. This serological behaviour was further demonstrated by the highly sensitive RIA where both SV40 and Py virus nonstructural polypeptides specifically bound the homologous antibody only, indicating an absence of common antigenic sites. Both nonstructural polypeptides failed to exhibit a positive RIA index with antiserum against respective virus structural proteins (Qureshi, unpublished data). Thus all three serological tests: immunoabsorption, blocking of immunofluorescence and RIA concur in designating this nonstructural polypeptide as possessing T-antigen activity.

Serologically reactive proteins can be detected in SV40- and Py-infected or transformed cells with sera from animals bearing tumours induced by the respective viruses. Several proteins exhibiting T-antigen activity have been identified for SV40 and Py in polyacrylamide gel electropherograms of immunoprecipitated (Tegtmeyer *et al.*, 1975, 1977; Ahmed-Zadeh *et al.*, 1976; Ito *et al.*, 1977*b*; Prives and Beck, 1977; Prives *et al.*, 1977; Robb, 1977; Turler and Salomon, 1977; Paucha *et al.*, 1978*a*; Smith *et al.*, 1978). The major species has an apparent molecular weight of 100,000 daltons in polyacrylamide gels, the small species approximately 15,000 to 22,000 daltons and an intermediate species in the range of 60,000 daltons (Tegtmeyer *et al.*, 1975, 1977; Ito *et al.*, 1977*a*, *b*; Prives and Beck, 1977; Prives *et al.*, 1977; Turler and Salomon, 1977; Hutchinson *et al.*, 1978; Paucha *et al.*, 1978*a*, *b*; Schaffhausen *et al.*, 1978; Smith *et al.*, 1978). The viral nonstructural polypeptides we have isolated (76,000 daltons for SV40 and 86,000 daltons for Py — Qureshi and Bourgaux, 1977), seem to be the major T-antigen protein. This conclusion is based on that (*i*) various sizes of major species of T-antigen were actually dependent on the degree of proteolysis during the extraction and handling of samples (Ahmed-Zadeh *et al.*, 1976; Carroll and Smith, 1976; Robb, 1977; Tegtmeyer *et al.*, 1977; Smith *et al.*, 1978); (*ii*) depending on conditions of extraction (i. e. buffer composition and pH) and method of analysis, the major species has been reported to vary from 100,000 to 81,000 daltons (Schaffhausen *et al.*, 1978; Smith *et al.*, 1978); and (*iii*) the intermediate species of T-antigen is associated at least in part with the membrane fraction of infected and transformed cells (Chang *et al.*, 1977; Ito *et al.*, 1977*a*; Hutchinson *et al.*, 1978; Qureshi, unpublished data). Using different conditions for extraction, Smith *et al.* (1978) also detected in their Fig. 1, polypeptides of approximately 75,000 to 78,000, 81,000 and 86,000 daltons as the major T-antigen species in SV40-infected cells. Since our detergent extraction procedure and purification steps were somewhat different from those of others, the discrepancy in the apparent molecular size of the T-antigen at least in the case of Py, does not seem impossible.

Presence of T antigen in the DNA protein complexes may offer two possible interpretations. The first is that this complex may be a viral precursor and will be packaged into a mature virion. If this were the case, then one should find T antigen in the mature virus. However, T antigen was not detected in purified SV40 by PAGE (Qureshi and Bourgaux, 1976, 1977). This may suggest that the DNA protein complexes containing T antigen do not take part in

virus morphogenesis. The second possibility is that presence of T antigen in the complexes may represent an intermediary step in the initiation of viral DNA synthesis. It is known that tsA mutants are temperature sensitive for initiation but not the completion of DNA chains (Tegtmeyer, 1972; Chou *et al.*, 1974). The functional role of T antigen was studied in SV40 tsA_{58}-infected cells grown at permissive temperature and then shifted up at nonpermissive temperature before extraction of the nucleoprotein complexes (Mann and Hunter, 1979). These results with that of Tegtmeyer *et al.* (1975) indicate that presence of T antigen in the DNA protein complexes is functional. Therefore, association of T antigen with the DNA protein complexes is not an artifactual coincidence.

As I did not detect any small t antigen in the nucleoprotein complexes and since it does not bind to viral DNA (Prives and Beck, 1977) it may have the function of initiation of viral DNA synthesis (Mann and Hunter ,1979). This is consistent with the reports that SV40 mutants lacking t antigen protein multiply slightly slower than the wild-type virus (Shenk *et al.*, 1976; Bouck *et al.*, 1978; Sleigh *et al.*, 1978).

T antigen is thought to be a viral-coded protein that regulates the expression of the papovavirus genome by binding to it (Tegtmeyer, 1974; Reed *et al.*, 1975; Carroll and Smith, 1976; Jessel *et al.*, 1976). Our observations confirm that of Mann and Hunter (1979) that T antigen actually binds to papovavirus DNA in vivo. It would be interesting to study the exact stoichiometry and location of T antigen binding to viral DNA.

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