Simian Virus 40 Gene A Regulates the Association Between a Highly Phosphorylated Protein and Chromatin and Ribosomes in Simian Virus 40-Transformed Cells

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Received for publication 23 November 1976

The species of proteins associated with chromatin and ribosomes of simian virus 40 (SV40)-transformed and untransformed monkey, mouse, and rat cells have been compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after phosphorylation of these proteins either *in vivo* or *in vitro*. *In vitro* phosphorylation was carried out by protein kinase associated with these organelles and [γ - 32 P]ATP as the phosphoryl donor. The reaction products contained both phosphoserine and phosphothreonine in approximately equal amounts. The electrophoretic analysis of the phosphorylated proteins revealed that the highly phosphorylated protein with a molecular weight of approximately 90,000 (90K protein) was associated with chromatin and ribosomes from transformed cells but not from untransformed cells. The 90K protein could be extracted from chromatin and ribosomes with 0.5 to 1.0 M NaCl or KCl. The 90K protein was still associated with the runoff ribosomes prepared by the puromycin reaction of the post-mitochondrial supernatant in the protein-synthesizing system. *In vitro* phosphorylation of chromatin and ribosomes from SV40 *tsA*-transformed mouse and rat cells indicated that the amounts of 90K protein associated with these organelles decreased greatly when the cells were cultivated at the restrictive temperature. A similar temperature-dependent decrease in the amount of 32 P-labeled 90K protein was observed in nonhistone chromosomal and ribosome-associated protein fractions prepared from SV40 *tsA*-transformed cells labeled with [3 H]leucine and [32 P]orthophosphate *in vivo*. *In vitro* phosphorylated 90K protein in nonhistone chromosomal and ribosome-associated proteins extracted with high salt was not immunoprecipitated with anti-SV40 T sera.

The function of chromatin and ribosomes, which are concerned with the expression of genetic information, is regulated by the proteins associated with these organelles (6, 24). These proteins, nonhistone chromosomal and ribosome-associated proteins, which can be extracted with 0.5 to 1 M NaCl or KCl, contain protein-modifying enzymes such as protein kinase (5, 11, 12, 16, 22, 38), acetyl transferase (29, 31), ADP-ribosyl transferase (27, 32, 37), and the substrate proteins for these enzymes. Phosphorylation of nonhistone chromosomal proteins seems to be concerned with the control of gene expression (33). Many species of ribosome-associated proteins are phosphorylated in eucaryotic cells as compared with those in procaryotic cells, suggesting that phosphorylation may play a role in the regulation of protein synthesis. It has been reported recently that two initiation factors from rabbit reticulocytes, IF-E2 and IF-E3, are phosphorylated (10, 17, 20, 21). Phosphorylation of the former results in a cessation of protein synthesis.

The modification of chromatin and ribosomes seems to occur in simian virus 40 (SV40)-transformed cells; the nonhistone proteins in the native chromatin of SV40-transformed human cells are immunologically different from those of their normal counterparts (45), and the ribosomes in SV40-transformed monkey cells are altered to translate a certain species of late adenovirus mRNA (26).

The expression of transformed phenotype in SV40-transformed cells is regulated by the function of SV40 gene A. The cells transformed by SV40 *tsA* mutants are unable to express the complete transformed phenotype at the restrictive temperature (3, 25, 28, 39). The pleiotropic alteration in the cellular systems by the function of a single gene product suggests that the gene product responsible for cell transformation interacts with cellular regulatory elements.

In the present paper, the species of proteins associated with chromatin and ribosomes are compared between SV40-transformed and un-

transformed cells after phosphorylation of these organelles either in vivo or in vitro. The results indicate that the protein of 90,000 daltons (90K protein), which is a good substrate for phosphorylation, is associated with chromatin and ribosomes of SV40-transformed cells, irrespective of the species of cell. The association is regulated by the function of the SV40 gene A.

MATERIALS AND METHODS

Cells. The following established cell lines were cultivated in Eagle medium with 10% calf or fetal calf serum at the temperature indicated in the text. The C3H2K-C4 cell line is a clonal line of C3H2K cells derived from C3H/He mouse kidney (44). A clonal derivative of the 3Y1-B cell line of Fisher rat embryo fibroblast, 3Y1-B, clone 1-6 (15), was kindly provided by G. Kimura. W-2K-11 and 900-2K-34 cells are the clonal lines of C3H2K-C4 cells transformed by the wild-type (WT) SV40 strain 777 and its temperature-sensitive early mutant, *tsA900* (42), respectively. W-3Y-23 and 900-3Y-11 cells are the clonal lines of 3Y1-B cells, clone 1-6, transformed by WT SV40 and *tsA900*, respectively. The mutant *tsA900* was classified into A complementation group, using *tsA58*, *tsB1* and *tsB11* as references, which were kindly provided by P. Tegtmeyer. Clones of C14 and T22 cells were lines of normal African green monkey kidney (AGMK) and SV40-transformed AGMK cells, respectively (36).

Preparation of chromatin and ribosomes. Cells were scraped with a rubber policeman and washed once with Earle solution and once with TKM buffer (10 mM Tris-hydrochloride [pH 7.4]–10 mM KCl–1.5 mM MgCl_2 –1 mM dithiothreitol [DTT]). The packed cells were suspended in 2 volumes of TKM and kept at 0°C for 5 min. The swollen cells were disrupted with about 20 strokes in a Dounce homogenizer, and 1/9 volume of 10 $\times$ medium K (1 $\times$ medium K contains 30 mM Tris-hydrochloride (pH 7.4)–125 mM KCl–5 mM $\text{Mg}(\text{OAc})_2$ and 1 mM DTT) was added immediately. The homogenate was centrifuged at 1,000 $\times g$ for 5 min, and the supernatant fluid was collected. The pellet was resuspended in 1 volume (equal to the volume of packed cells) of 1 $\times$ medium K and homogenized with about 10 strokes. After centrifugation, the two supernatant fluids were combined and the pellet was suspended in isotonic buffer (0.14 M NaCl–0.01 M Tris-hydrochloride [pH 7.4]–1.5 mM MgCl_2 –1 mM DTT) containing 0.5% Nonidet P-40 in 2 $\times 10^7$ to 4 $\times 10^7$ cells/ml (2). After standing at 0°C for 15 min, the nuclei were sedimented by centrifugation at 1,000 $\times g$ for 5 min, and the nuclei were washed with isotonic buffer. The nuclei were homogenized in about 10 volumes of 0.14 M NaCl and centrifuged at 9,000 $\times g$ for 10 min. This extraction was repeated once more. The pellet was similarly extracted twice with 0.02 M Tris-hydrochloride (pH 7.4) and once with 1 mM Tris-hydrochloride (pH 7.4). All of the solutions contained 5 mM sodium bisulfite and 1 mM DTT. The resulting pellet was used as chromatin.

The supernatant fluid of the cell homogenate was centrifuged at 10,000 $\times g$ for 5 min to remove mito-

chondria. The post-mitochondrial supernatant fluid was treated with 0.5% sodium deoxycholate and 1% Triton X-100 at 0°C for 30 min and centrifuged at 165,000 $\times g$ for 16 h through a cushion of 1.6 M sucrose in 25 mM Tris-hydrochloride (pH 7.5)–35 mM KCl–5 mM $\text{Mg}(\text{OAc})_2$ –1 mM DTT. The pellet was suspended in a small amount of buffer H [25 mM Tris-hydrochloride (pH 7.5)–35 mM KCl–5 mM $\text{Mg}(\text{OAc})_2$ –1 mM DTT] by homogenization in a Teflon homogenizer and clarified by centrifugation at 1,500 $\times g$ for 5 min. The resulting supernatant fluid was used as ribosomes. The ribosomes thus prepared consisted mainly of polysomes.

Preparation of nonhistone chromosomal proteins.

Nonhistone chromosomal proteins for acid hydrolysis and immunoprecipitation were prepared essentially by the method of Kish and Kleinsmith (16). All subsequent operations were carried out at 4°C. The chromatin was dispersed in 1 M NaCl–0.02 M Tris-hydrochloride (pH 7.5)–1 mM DTT (about 10⁸ cells/ml) by using a Polytron homogenizer (Brinkmann Instruments, Inc.) for 30 s at a setting of 5 and stirred for 1 h. The resulting solution was mixed with 1.5 volumes of 0.02 M Tris-hydrochloride (pH 7.5)–1 mM DTT and centrifuged at 81,000 $\times g$ for 90 min. To the supernatant fluid was added Bio-Rex 70 (Na^+), which had been equilibrated previously with 0.4 M NaCl–0.02 M Tris-hydrochloride (pH 7.5) in a ratio of 20 mg of Bio-Rex per mg of protein. After stirring for 5 min, the suspension was centrifuged at 6,000 $\times g$ for 10 min and the supernatant fluid was collected. The resin was washed by homogenizing it in 0.4 M NaCl–0.02 M Tris-hydrochloride (pH 7.5) and centrifuging at 6,000 $\times g$ for 10 min. The two supernatant fluids were combined and dialyzed overnight against 0.3 M NaCl–0.05 M Tris-hydrochloride (pH 7.5)–1 mM DTT–10% glycerol.

Nonhistone chromosomal proteins for polyacrylamide gel electrophoresis were prepared from the chromatin of ³²P-labeled cells by hydroxyapatite column chromatography after dissociation in 2 M NaCl–5 M urea–1 mM sodium phosphate (pH 6.8) to remove DNA and RNA by the method of MacGillivray et al. (23). After histones were eluted with the same equilibration buffer, nonhistone proteins were eluted with 2 M NaCl–5 M urea–50 mM sodium phosphate (pH 6.8) and subsequently with the same solution but containing 100 mM sodium phosphate.

Preparation of ribosome-associated proteins. Extraction of proteins associated with ribosomes was carried out with either acetic acid (8) or KCl. To the ribosome suspension was added 3 mM succinic acid, 0.1 volume of 1 M MgCl_2 , and 2 volumes of glacial acetic acid. The mixture was stirred at 0°C for 45 min and centrifuged at 10,000 $\times g$ for 10 min. The supernatant fluid was collected and the pellet was reextracted similarly in 0.5 original volume of 67% acetic acid containing 0.033 M MgCl_2 . After centrifugation, the two supernatant fluids were combined and dialyzed against 2% acetic acid overnight. The dialyzed extract containing both ribosome-associated proteins and their structural proteins was lyophilized to reduce the volume.

Extraction with KCl was carried out by stirring the ribosomes at 0°C for 1 h in 0.25 M sucrose containing 50 mM Tris-hydrochloride (pH 7.4)–10 mM

Mg(OAc)₂-0.5 M KCl-2 mM DTT. The mixture was centrifuged at $200,000 \times g$ for 4 h at 4°C through a cushion of 0.5 M sucrose in the same solution. The top layer was collected and dialyzed against the same solution but containing 0.3 M KCl.

Phosphorylation of chromatin- and ribosome-associated proteins in vitro. The basic reaction mixture contained 50 mM Tris-hydrochloride (pH 7.5), 10 mM MgCl₂, 2.5 mM DTT, 10 μ M [γ -³²P]ATP (50 to 500 Ci/mol), and 0.1 to 1.0 mg of protein per ml of chromatin or ribosomes in a total volume of 0.1 to 1.0 ml. The reaction was carried out at 30°C for 10 min. Under these conditions, the phosphorylation increased linearly with increasing concentrations of chromatin and ribosomes. When the extent of the phosphorylation was measured, the reaction was stopped by the addition of 10 volumes of 18% trichloroacetic acid containing 1% sodium pyrophosphate. After standing at 0°C for 30 min, the precipitate was collected and washed on a Whatman glass fiber filter (GF/C). The filters were dried and counted. When the phosphorylated proteins were to be subjected to gel electrophoresis, the reaction was terminated by the addition of 1.0% sodium dodecyl sulfate (SDS) containing 1% 2-mercaptoethanol. After complete dissociation of the chromatin or ribosomes, the sample was dialyzed against 0.1% SDS containing 8 M urea-1% 2-mercaptoethanol-0.1 M sodium phosphate (pH 7.0) at 37°C overnight. The sample was concentrated by evaporation. [γ -³²P]ATP was prepared by the procedure of Glynn and Chappel (7).

Electrophoresis. Gel electrophoresis was carried out in a 7.5% acrylamide gel (0.6 by 12 cm) containing 0.5 M urea at 80 V (15 mA/gel) for 6.5 h as previously described (9). The buffer used was 0.1 M sodium phosphate (pH 7.0) containing 0.1% SDS. After staining with amido black and scanning with an Ozum densitometer, the gels were cut into 1-mm slices. The slices were dissolved in 0.5 ml of 50% H₂O₂ at 80°C overnight and counted with 5 ml of Triton X-toluene scintillator.

Identification of phosphoserine and phosphothreonine was carried out by high-voltage paper electrophoresis. The nonhistone chromosomal and ribosome-associated proteins extracted from the phosphorylated chromatin and ribosomes were lyophilized and dissolved in 1 ml of 6 N HCl. The hydrolysis was carried out at 105°C for 6 h in vacuo. The hydrolysates were dried in a rotary evaporator and dissolved in 0.1 ml of 2.5% formic acid-7.8% acetic acid (pH 1.9) containing 30 μ g each of phosphoserine and phosphothreonine standards, which were kindly provided by Y. Nishizuka. Electrophoresis was carried out at 4°C on Whatman no. 3MM paper (59 by 6 cm) for 3 h at 3,000 V. After electrophoresis, the paper was dried and stained with Hanss and Ishewood reagent (60% perchloric acid-4% ammonium molybdate-1 N HCl). Amino acid spots were developed by irradiation with UV light. ³²P-labeled compounds were detected by autoradiography for 1 week.

Immunoprecipitation. Anti-T sera were obtained from Syrian hamsters bearing tumors induced by inoculation of SV40-induced tumor cells, clone SVH93 (18). The formalin-fixed and heat-killed bacterium *Staphylococcus aureus* (Cowan I strain,

kindly provided by Y. Arai) was used as an adsorbent for hamster immunoglobulin G as described by Kessler (14). In vitro phosphorylated nonhistone chromosomal and ribosome-associated proteins (100 μ l each) were allowed to react with either anti-T or control normal hamster sera (20 μ l) at 4°C for 1 h, followed by addition of the bacterial immunoadsorbent (10% [wt/vol], 200 μ l) to precipitate antiserum bound to T antigen. The precipitate was pelleted and washed four times with 50 mM Tris-hydrochloride (pH 7.4) containing 150 mM NaCl, 5 mM EDTA, 2 mM methionine, 0.05% Nonidet P-40, and 1 mg of bovine serum albumin per ml. Radiolabeled materials that were precipitated were solubilized in 1% SDS containing 0.1 M sodium phosphate (pH 7.0)-1% 2-mercaptoethanol at 37°C overnight and subjected to electrophoresis.

RESULTS

In vitro phosphorylation of chromatin- and ribosome-associated proteins. Chromatin- and ribosome-associated proteins can be phosphorylated in vitro by protein kinase associated with these organelles (5, 16, 22). To determine whether the pattern of phosphorylation of these proteins is affected by the transformation of cells with SV40, chromatin and ribosomes were prepared from SV40-transformed monkey (T22), mouse (W-2K-11), and rat (W-3Y-23) cells. The extent of in vitro phosphorylation was compared between these organelles and those prepared from parental untransformed (C14, C3H2K-C4, 3Y1-B) cells. Figure 1 shows that chromatin preparations from transformed cells were phosphorylated twice as much as those from untransformed cells. A greater difference was observed in the ribosomes. Ribosomes prepared from transformed cells were phosphorylated 2.5 to 8 times more than those from untransformed cells. The difference was especially marked in rat cells, owing to the very low phosphorylation in ribosomes of 3Y1-B cells. The extent of phosphorylation was not significantly altered by the cell growth phase. The maintenance of W-2K-11 cells in serum- and phosphate-free medium for 15 h, however, resulted in a 40% decrease in the phosphorylation of chromatin. A similar decrease has been reported in the phosphorylation of ribosomes from chicken embryo cells maintained in serum-free medium (22). To determine whether the phosphate incorporated into proteins associated with chromatin and ribosomes is covalently bound to amino acid residues, nonhistone chromosomal proteins and ribosomal proteins containing both associated and structural proteins extracted from the phosphorylated T22 cell chromatin and ribosomes were acid hydrolyzed and analyzed by high-voltage paper electrophoresis. A radioautograph (Fig. 2) indicated that hydrolysates of ribosomal proteins

FIG. 1. *In vitro* phosphorylation of protein associated with chromatin and ribosomes from SV40-transformed and untransformed cells. Chromatin and ribosomes were prepared from cells cultivated at 37°C and harvested at confluency. A zero-time value, which corresponds to 2 to 4% of the radioactivity incorporated, was subtracted from all measurements. The values are presented as averages of duplicate determinations.

contained radioactive material that migrated similarly to standard phosphoserine and phosphothreonine on electrophoresis. Approximately equal amounts of phosphoserine and phosphothreonine were formed. A large amount of inorganic phosphate was formed, probably by the hydrolysis of phosphorylated amino acid. Essentially the same results were obtained with the hydrolysates of nonhistone chromosomal proteins (data not shown).

The specific activities of protein kinases present in nonhistone chromosomal and ribosome-associated proteins were threefold higher in transformed cells than in untransformed cells when assayed with casein, an exogenous substrate. However, their specificities are not different in transformed and untransformed cells. This finding suggests that different species of proteins that are good substrates for phosphorylation are associated with the chromatin and ribosomes of SV40-transformed cells. The properties of protein kinase associated with these organelles will be described elsewhere.

Electrophoretic analysis of phosphorylated proteins associated with chromatin. *In vitro* phosphorylated chromatin from SV40-transformed and untransformed cells was dissociated in 1% SDS-8 M urea and subjected to electrophoresis on an SDS-polyacrylamide gel to determine the species of proteins that were highly phosphorylated (Fig. 3). The electrophoresis was carried out until histones migrated to the bottom of the gel. Under these conditions, a linear relationship was observed between protein mobility and molecular weight, in the range of molecular weights smaller than 70,000. The curve was skewed and no linearity was observed in the range of molecular weights larger than 70,000 (Fig. 3D). The highly phos-

FIG. 2. High-voltage electropherogram of acid hydrolysis products of ribosomal proteins phosphorylated *in vitro*. The acid hydrolysis products of ribosomal proteins extracted from *in vitro* phosphorylated T22 cell ribosomes with acetic acid were electrophoresed and autoradiographed as described in the text.

phorylated species with a molecular weight of approximately 90,000, which migrated between phosphorylase *a* (94,000) and bovine serum albumin (68,000), was observed only in chromatin prepared from transformed cells (Fig. 3A through C). Two highly phosphorylated species

FIG. 3. Electrophoresis of chromosomal proteins from SV40-transformed and untransformed cells phosphorylated *in vitro*. *In vitro* phosphorylated chromatin containing 300 to 400 μ g of protein was dissociated in 1% SDS containing 1% 2-mercaptoethanol and dialyzed against 8 M urea containing 1% SDS, 1% 2-mercaptoethanol, and 0.1 M sodium phosphate buffer (pH 7.0) at 37°C for 3 h. The sample was then dialyzed overnight against the same buffer, but containing 0.1% SDS. The dialyzed material was concentrated and subjected to electrophoresis, with bromophenol blue as a marker. Chromatin was prepared from the following cells: (A) T22, (B) W-3Y-

were observed in T22 cells (Fig. 3A). Most of the radioactivity was associated with proteins since Pronase digestion of dissociated chromatin proteins resulted in a rapid decrease in their molecular sizes, as will be shown in the next sections. The absence of the radioactive peak in the chromatin from untransformed cells (Fig. 3E through H) was not due to the presence of either inhibitor of protein phosphorylation or phosphatase, because the chromatin prepared from a mixture of transformed and untransformed cells showed the expected intermediate phosphorylation of the 90K protein, but did not abolish the radioactivity associated with this species (Fig. 3G).

The highly phosphorylated 90K protein associated with the chromatin from SV40-transformed monkey, mouse, and rat cells may have a similar function and is related to the transformed state of the cells since the radioactivity associated with this species of protein in SV40 *tsA*-transformed mouse (900-2K-34) and rat (900-3Y-11) cells greatly decreased when the cells were cultivated at the restrictive temperature (Fig. 4). In contrast, very little decrease in the radioactivity associated with the 90K protein was observed when the phosphorylation was carried out with chromatin prepared from SV40 WT-transformed rat cells, W-3Y-23 cultivated at the restrictive temperature of 40.5°C (Fig. 5). SV40 *tsA*-transformed cells grew to a high density at the permissive temperature, as do SV40 WT-transformed cells, but displayed limited growth, as do their parental untransformed cell lines at the restrictive temperature (Fig. 6). SV40 WT-transformed mouse (W-2K-11) and rat (W-3Y-23) cells grew to a high density, irrespective of the culture temperature (M. Miwa, K. Oda, K. Segawa, M. Tanaka, S. Irie, N. Yamaguchi, K. Kuchino, K. Shiroki, H. Shimojo, H. Sakura, T. Matsushima, and T. Sugimura, Arch. Biochem. Biophys., in press).

Electrophoretic analysis of phosphorylated proteins associated with ribosomes. *In vitro* phosphorylated ribosomes from SV40-transformed and untransformed cells were dissociated in SDS and urea, and the phosphorylated proteins were similarly analyzed by SDS-polyacrylamide gel electrophoresis. In SV40-trans-

23, (C) W-2K-11, (E) C14, (F) 3Y1-B, (G) W-2K-11 plus C3H2K-C4 (1:1), (H) C3H2K-C4. In (D), the relationship between protein mobility and molecular weight is shown by using the following marker proteins: $\circ$, β -galactosidase (130,000); $\bullet$, phosphorylase a (94,000); $\times$, bovine serum albumin (68,000); Δ , ovalbumin (45,000); $\blacktriangle$, chymotrypsinogen (25,000); $\square$, myoglobin (17,800). —, absorbance at 570 nm; $-\bullet-$, 32 P.

FIG. 4. Electrophoresis of chromosomal proteins from SV40 tsA-transformed cells phosphorylated *in vitro*. The electrophoresis was carried out as described in the legend to Fig. 3. Chromatin was prepared from the following cells cultivated at the indicated temperature at confluency: (A) 900-2K-34 (35°C), (B) 900-2K-34 (39.5°C), (C) 900-3Y-11 (35°C), (D) 900-3Y-11 (40.5°C). —, absorbance at 570 nm; —●—, ^{32}P .

FIG. 5. Electrophoresis of chromosomal proteins from SV40 WT-transformed cells phosphorylated *in vitro*. The electrophoresis was carried out as described in the legend to Fig. 3. Chromatin was prepared from W-3Y-23 cells cultivated either at (A) 35°C or (B) 40.5°C at confluency. —, absorbance at 570 nm; —●—, ^{32}P .

formed monkey cells, clone T22, a highly phosphorylated species of protein with a molecular weight of approximately 90,000 was also observed (Fig. 7A). Several other species were

FIG. 6. Growth curves of SV40-transformed and untransformed cells. The cells were seeded in 86-mm petri dishes at 5×10^5 to 7×10^5 cells/dish. At the times indicated, the number of cells was counted with a standard hemocytometer. (A) W-2K-11 (●) and C3H2K-C4 (○) cells; (B) W-3Y-23 (●) and 3Y1-B (○) cells; (C) 900-2K-34 cells cultivated at 35°C (●) or 39.5°C (○); (D) 900-3Y-11 cells cultivated at 35°C (●) or 40.5°C (○).

phosphorylated, but to a lesser extent. In contrast, no significant phosphorylation was observed in ribosomes from untransformed monkey cells, clone C14 (Fig. 7B). Many species of proteins distributed in fractions 20 to 50 consisted mainly of ribosomal structural proteins because almost all of these proteins were found in the 0.5 M KCl-washed ribosomes (Fig. 7D). As many as 72 ribosomal structural proteins, with molecular weights ranging from 53,700 to 10,000, have been identified in rat liver ribosomes (41). The highly phosphorylated 90K protein and other phosphorylated species were found in the 0.5 M KCl ribosomal wash (Fig. 7C). The phosphorylated species of protein with a molecular weight slightly smaller than 90,000 may have resulted by proteolytic cleavage during preparation of the ribosomes or extraction of the ribosome-associated proteins.

The highly phosphorylated 90K protein was also detected by *in vitro* phosphorylation of ribosomes prepared from SV40-transformed mouse cells, clone W-2K-11 (Fig. 8A). The other species of proteins were also phosphorylated,

FIG. 7. Electrophoresis of *in vitro* phosphorylated proteins associated with ribosomes from SV40-transformed and untransformed monkey cells. *In vitro* phosphorylated ribosomes containing 300 to 400 μ g of protein from T22 (A) and C14 (B) cells cultivated at 37°C were subjected to electrophoresis after dissociation and dialysis, as described in the legend to Fig. 3. Ribosome-associated proteins were extracted with 0.5 M KCl from *in vitro* phosphorylated T22 cell ribosomes and lyophilized after dialysis against 0.1% SDS. The extract (C) and washed ribosomes (D) were subjected to electrophoresis after processing, as indicated above. —, absorbance at 570 nm; —●—, 32 P.

but to a much lesser extent. Very little phosphorylation was observed in ribosomes of the parental untransformed cell line, clone C3H2K-C4 (Fig. 8B). The highly phosphorylated 90K protein was present in ribosomes of SV40 *tsA*-transformed mouse cells, clone 900-2K-34, when cultivated at the permissive temperature (35°C) (Fig. 8C). However, the radioactivity associated with the protein decreased greatly in ribosomes of 900-2K-34 cells cultivated at the restrictive temperature (39.5°C) (Fig. 8D). Cultivation of W-2K-11 cells at this restrictive temperature resulted in only a slight decrease in the radioactivity associated with the 90K protein (Fig. 8E), suggesting that either the association of this protein with the ribosomes or its phosphorylation is affected by the function of the SV40 A gene.

The highly phosphorylated 90K protein was

also associated with the ribosomes of SV40-transformed rat cells, clone W-3Y-23 (Fig. 9A). Besides this species, there existed about 10 species of phosphorylated proteins. No significant phosphorylation was observed in ribosomes of its parental cell line, 3Y1-B (Fig. 9B). Similar species of phosphorylated proteins were observed in SV40 *tsA*-transformed 3Y1-B cells, clone 900-3Y-11, cultivated at the permissive temperature (35°C) (Fig. 9C). When the cells were cultivated at the restrictive temperature (40.5°C), however, these phosphorylated proteins completely disappeared (Fig. 9D) and the pattern of phosphorylation became quite similar to that of 3Y1-B cells (Fig. 9B). In addition to the shift in the radioactivity profile, the optical density profile of ribosome-associated proteins in 900-3Y-11 cells was also shifted by the culture temperature. The optical density profiles of W-3Y-23 and 3Y1-B cells were quite similar to those of 900-3Y-11 cells cultivated at the permissive and restrictive temperatures, respectively (compare Fig. 9A with C and Fig. 9B with D). As noted above, the species of proteins with molecular weights larger than 60,000 (fraction around 18) are likely to consist of ribosome-associated proteins. The shift in the optical density profile in this region suggests that not only the association of the 90K protein but also the association of a set of proteins with the ribosomes is affected by the function of the SV40 A gene. The radioactivity associated with several species of proteins, including the 90K protein, decreased but to less than 50% when W-3Y-23 cells were cultivated at the restrictive temperature (40.5°C) (Fig. 9E), but did not completely disappear as observed in 900-3Y-11 cells. The association of the radioactivity with protein was shown by incubating the phosphorylated ribosomes with either Pronase or alkaline phosphatase. The radioactivity was greatly reduced without significant alteration in the optical density profile when the phosphorylated ribosomes were incubated with *Escherichia coli* alkaline phosphatase (Fig. 10B). An incomplete dephosphorylation by the alkaline phosphatase was also observed in 32 P-labeled ribosomes (12) and 32 P-labeled reverse transcriptase (19). Treatment with Pronase resulted in a complete degradation of proteins, and the radioactivity present in the high-molecular-weight region was shifted along with the oligopeptide to the low-molecular-weight end of the gel (Fig. 10C). No significant alteration in the radioactivity profile was observed after treatment of the phosphorylated ribosomes with pancreatic RNase (data not shown).

Characterization of highly phosphorylated protein associated with ribosomes. The highly

FIG. 8. Electrophoresis of *in vitro* phosphorylated proteins associated with ribosomes from SV40-transformed and untransformed mouse cells. Phosphorylation and electrophoresis were carried out as described in the legend to Fig. 3 and in the text. Ribosomes were prepared from the following cells cultivated at the indicated temperature at confluency: (A) W-2K-11 (37°C), (B) C3H2K-C4 (37°C), (C) 900-2K-34 (35°C), (D) 900-2K-34 (39.5°C), (E) W-2K-11 (39.5°C). —, absorbance at 570 nm; —●—, ^{32}P .

FIG. 9. Electrophoresis of *in vitro* phosphorylated proteins associated with ribosomes from SV40-transformed and untransformed rat cells. Ribosomes were prepared from the following cells cultivated at the indicated temperature at confluency: (A) W-3Y-23 (37°C), (B) 3Y1-B (37°C), (C) 900-3Y-11 (35°C), (D) 900-3Y-11 (40.5°C), (E) W-3Y-23 (40.5°C). —, absorbance at 570 nm; —●—, ^{32}P .

FIG. 10. Electrophoresis of *in vitro* phosphorylated ribosomal proteins after treatment with Pronase and alkaline phosphatase. Treatment of *in vitro* phosphorylated ribosomal proteins from W-2K-11 cells was carried out as follows. (A) No treatment. (B) Ribosomes (400 μ g of protein per ml) were treated with 1 mg of *E. coli* alkaline phosphatase (Worthington Biochemicals Corp.) per ml at 37°C for 30 min, as described by Kabat (12). After treatment, ribosomes were processed as described in the legend to Fig. 3. (C) Dissociated ribosomes, dialyzed against 8 M urea containing 0.1% SDS, 1% 2-mercaptoethanol, and 0.1 M sodium phosphate buffer (pH 7.0) were treated with 10 μ g of Pronase per ml at 37°C for 15 min and boiled at 100°C for 1 min just before electrophoresis. —, absorbance at 570 nm; —●—, 32 P.

phosphorylated 90K protein seems to be related to T antigen. Its molecular weight is very close to that estimated for T antigen, i.e., 86,000 by Ahmad-Zadeh et al. (1) and 94,000 by Carroll and Smith (4), and its association with chromatin and ribosomes in SV40 *tsA*-transformed cells depends on the culture temperature of the cells. The complement-fixing (CF) activity of T antigen in the ribosome preparations was therefore measured. Table 1 shows that the CF activity is present in ribosome preparations from transformed cells but not from untransformed cells. The number of CF units present in ribosome preparations from SV40 *tsA*-transformed mouse (900-2K-34) and rat (900-3Y-11) cells was decreased greatly when the cells were cultivated at the restrictive temperature. No significant CF activity was detected in mitochondrial and postribosomal fractions. To determine whether both the 90K protein and T antigen are associated with the ribosomes, the post-mitochondrial supernatant (S10) prepared from the cytoplasm of SV40-transformed rat cells was centrifuged on 10 to 30% (wt/wt) sucrose gradients with or without preincubation in the protein-synthesizing system containing puromycin (Fig. 11). The preincubation resulted in a disruption of polysomes and a concomitant increase in the amount of runoff ribosomes. Treatment of the post-mitochondrial supernatant with sodium deoxycholate and Triton X-100 did not alter the pattern of polysomes. The CF activity of T antigen in each fraction was measured. Most of the activity was found in the upper fractions of the gradient,

TABLE 1. CF activity of T antigen in ribosome preparations^a

Cells	Culture temp (°C)	CF units ^b
W-3Y-23	35	64 (3.36)
	40.5	45 (3.45)
900-3Y-11	35	64 (2.12)
	40.5	4 (2.16)
W-2K-11	37	128 (2.40)
	35	45 (2.50)
	39.5	2 (1.74)
C3H2K-C4	37	2 (1.96)

^a Ribosomes were prepared as described in the text.

^b CF titers are the average of duplicate determinations. Numbers in parentheses indicate milligrams of protein per milliliter.

whereas very little activity was detected in all classes of ribosomes. No phosphorylated 90K protein was detected in the upper fractions upon phosphorylation *in vitro*. The fractions indicated by the arrow in Fig. 11B were collected, and the monosomes were concentrated by sedimenting through a cushion of 1.6 M sucrose. The monosomes were phosphorylated *in vitro*, and the phosphorylated proteins were similarly analyzed by electrophoresis (Fig. 12). The result indicates that the phosphorylated 90K protein is associated with the monosomes and may be different from T antigen.

Immunoprecipitation of the phosphorylated 90K protein by anti-SV40 T antigen hamster sera was also carried out. The nonhistone chro-

FIG. 11. Sedimentation analysis of T antigen present in the cytoplasm of SV40-transformed rat cells. The post-mitochondrial supernatant prepared from W-3Y-23 cells cultivated at 37°C, as described in the text, was sedimented through 10 to 30% (wt/wt) sucrose gradient on 2 ml of 2 M sucrose in 25 mM HEPES buffer (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (pH 7.0) containing 25 mM KCl-5 mM Mg(OAc)₂-1 mM DTT at 23,000 rpm for 3 h at 4°C in a Spinco SW25.1 rotor with or without preincubation in the protein-synthesizing system. The preincubation of the post-mitochondrial supernatant supplemented with 5 μ M each of 20 amino acids, 2 mM ATP, 0.4 mM GTP, 4 mM phosphoenolpyruvate, 10 μ g of pyruvate kinase per ml, and 200 μ g of rat liver pH 5 enzyme per ml was carried out at 37°C for 5 min, and 0.8 mM puromycin was added. The mixture was further incubated at 37°C for 40 min. The CF activity of SV40 T antigen in each fraction was titrated with anti-T serum prepared from hamsters bearing virus-free, SV40-induced tumors. (A) Post-mitochondrial supernatant treated with 0.5% sodium deoxycholate and 1% Triton X-100 at 0°C for 30 min; (B) preincubated post-mitochondrial supernatant.

mosomal and ribosome-associated proteins extracted with 1 M NaCl and 0.5 M KCl, respectively, from the chromatin and ribosomes of W-3Y-23 cells were phosphorylated in vitro with endogenous protein kinase. These phosphorylated proteins were allowed to react with anti-T sera, and the antigen-antibody complexes were precipitated by the addition of bacterial immunoadsorbent. Radioactive materials solubilized from the precipitate by SDS were analyzed by SDS-gel electrophoresis. No significant amount of phosphorylated proteins was precipitated (Fig. 13A and B). T antigen in the extract of SV40-infected AGMK cells labeled with [³⁵S]methionine was specifically precipitated by anti-T sera (Fig. 13C, fraction 11), but not by normal hamster sera.

Phosphorylation of chromatin-associated

FIG. 12. Electrophoresis of in vitro phosphorylated proteins associated with runoff ribosomes from SV40-transformed rat cells. The runoff ribosomes in fractions indicated by arrows in Fig. 11B were collected by centrifugation through a cushion of 1.6 M sucrose after treatment with sodium deoxycholate and Triton X-100 as described. In vitro phosphorylation and electrophoresis were carried out as described. —, absorbance at 280 nm; —●—, ³²P.

proteins in vivo. SV40 *tsA*-transformed mouse cells, 900-2K-34, were cultivated at either the permissive (35°C) or restrictive (39.5°C) temperature. At near confluency, the cells were incubated with [³H]leucine and subsequently with [³²P]orthophosphate to provide ³H-labeled protein as markers in the electrophoretic analysis. The chromatin prepared from labeled cells was dissociated in 2 M NaCl-5 M urea, and the nonhistone protein fraction was separated from histones, DNA, and RNA by hydroxyapatite column chromatography (Fig. 14). The flow-through fraction consisted mainly of histones. The nonhistone proteins were eluted from the column with sodium phosphate at concentrations of 50 and then 100 mM. DNA was eluted with sodium phosphate at 500 mM. Elution patterns of these fractions were quite similar in the chromatin prepared from cells cultivated at either 35°C (Fig. 14A) or 39.5°C (Fig. 14B). The nonhistone protein fractions were subjected to electrophoresis after concentration by lyophilization (Fig. 15). The ³H and ³²P radioactivity profiles of nonhistone proteins eluted with 50 mM sodium phosphate from cells cultivated at either 35°C (Fig. 15A) or 39.5°C (Fig. 15B) were similar, except for that of the largest species of protein with a molecular weight of approximately 90,000 (fraction 10). Comigration of the ³H and ³²P radioactivities in this peak indicates that the protein is extensively phosphorylated in vivo. A great decrease in both radioactivities associated with the 90K protein

FIG. 13. Electrophoresis of *in vitro* phosphorylated nonhistone chromosomal and ribosome-associated proteins immunoprecipitated by anti-T sera. Phosphorylated nonhistone chromosomal (A) and ribosome-associated (B) proteins were electrophoresed before (●) or after (○) immunoprecipitation with anti-T sera. The extract (C) of SV40-infected AGMK cells, clone GC7 (42) (20 PFU/cell), labeled with 100 μ Ci of [35 S]methionine per ml per 86-mm dish (330 Ci/mmol) at 40 h postinfection was allowed to react with anti-T serum (●) or normal hamster serum (○). The extraction was carried out with 0.5% Nonidet P-40 in Tris-buffered saline at pH 8.0 (40).

FIG. 14. Hydroxyapatite column chromatography of chromosomal proteins from SV40 *tsA*-transformed mouse cells. 900-2K-34 cells were cultivated at either (A) 35°C or (B) 39.5°C. At near confluency, the medium was changed with prewarmed leucine-free medium and cells were labeled with 2 μ Ci of [3 H]leucine per ml (46 Ci/mmol) for 5 h. The medium was then replaced with prewarmed medium containing citrate (1 mM) instead of phosphate by the method of Yamashita et al. (43) and labeled with 30 μ Ci of [32 P]orthophosphate per ml for 2 h. Chromatin prepared from labeled cells was dissociated in 2 M NaCl-5 M urea and fractionated by hydroxyapatite column chromatography as described in the text.

in the cells cultivated at 39.5°C suggests that the synthesis and/or association of this protein is regulated by the function of the SV40 A gene. Very little of the phosphorylated 90K protein was recovered in the nonhistone protein fractions eluted with 100 mM sodium phosphate,

irrespective of the culture temperature of the cells (data not shown).

The ribosomal proteins from labeled 900-2K-34 cells were similarly analyzed by electrophoresis (Fig. 16). The proteins extracted with acetic acid contained both ribosome-associated

FIG. 15. Electrophoresis of nonhistone chromosomal proteins from SV40 tsA-transformed mouse cells phosphorylated *in vivo*. Nonhistone protein fractions eluted from the column with 50 mM sodium phosphate (Fig. 14) were concentrated by lyophilization and subjected to electrophoresis as described. Nonhistone proteins were prepared from cells cultivated at either (A) 35°C or (B) 39.5°C.

proteins and their structural proteins. The phosphorylated 90K protein was detected (fraction 10) in ribosomal proteins prepared from cells cultivated at either 35°C (Fig. 16A) or 39.5°C (Fig. 16B), although the 90K protein was not well resolved from other proteins of similar molecular size. The ^3H and ^{32}P radioactivities associated with the 90K protein were decreased at 39.5°C, but were not as marked as that observed in 50 mM nonhistone protein fractions. However, very little phosphorylation was observed in ribosomal proteins from untransformed parental cells, C3H2K-C4, similarly labeled *in vivo*, and no 90K protein was detected (Fig. 16C). A brief treatment of nonhistone and ribosomal proteins with Pronase resulted in a complete shift of the radioactivities to the low-molecular-weight end of the gel.

DISCUSSION

Chromatin- and ribosome-associated proteins can be phosphorylated *in vitro* by protein kinase associated with these organelles. Analysis of phosphorylated proteins by SDS-polyacryl-

FIG. 16. Electrophoresis of ribosomal proteins from SV40 tsA-transformed mouse cells phosphorylated *in vivo*. Ribosomal proteins were prepared from 900-2K-34 cells cultivated and labeled at either (A) 35°C or (B) 39.5°C as described in the legend to Fig. 14. (C) Ribosomal proteins prepared from untransformed parental cells, C3H2K-C4, cultivated and labeled at 37°C.

amide gel electrophoresis revealed that a protein of approximately 90,000 daltons, which is highly phosphorylated *in vitro*, is associated with the chromatin and ribosomes prepared from SV40-transformed cells, but not from untransformed cells. This finding is not caused by a difference in either the specificity of protein kinase or the amount of inhibitor of protein phosphorylation associated with these organelles. The specific activities of protein kinases in nonhistone chromosomal and ribosome-associated proteins extracted from these organelles with 0.5 to 1.0 M NaCl or KCl are two- to

threefold higher in transformed cells than in untransformed cells, but their specificities, e.g., K_m for substrate and metal requirements, are not different in transformed and untransformed cells (unpublished data). The mixing of these proteins extracted from transformed and untransformed cells did not result in an inhibition of protein phosphorylation. The 90K protein could not be detected by phosphorylation of proteins in the nuclear sap and postribosomal fractions from SV40-transformed cells with endogenous protein kinase.

The phosphorylated 90K protein could also be detected in nonhistone chromosomal and ribosome-associated proteins extracted from SV40-transformed cells but not from untransformed cells upon phosphorylation with endogenous protein kinase (Fig. 13). The results suggest that the absence of the phosphorylated 90K protein in the chromatin and ribosomes of untransformed cells is not due to the steric hindrance that makes the 90K protein and protein kinase inaccessible upon association with these organelles. In this context, it is interesting that a 90,000-dalton protein was found to be associated with the ribosomes of a plasmacytoma, a plasma cell tumor, and is highly phosphorylated by the protein kinase associated with ribosomes but not by soluble protein kinases I and II present in the cytoplasm (30). The 90K protein is still associated with the runoff ribosomes that are released by puromycin reaction and sedimented to the middle of the sucrose gradient. These results suggest that the 90K protein is specially bound to ribosomes in SV40-transformed cells. However, the possibility that the 90K protein is an aggregating protein, as has been demonstrated with T antigen (18, 28), forms complexes with the other proteins and/or RNA, and cosediments with chromatin and ribosomes without being associated with these organelles cannot be excluded completely. The decrease in the amount of 90K protein in SV40 *tsA*-transformed cells at the restrictive temperature, however, cannot be ascribed to loss of ability of 90K protein to aggregate at the restrictive temperature since the amount of 90K protein in SV40 WT-transformed cells was much less affected by the culture temperature.

The 90K protein associated with chromatin and ribosomes is also phosphorylated *in vivo*. The gel electrophoretic analysis of nonhistone proteins prepared from chromatin of SV40 *tsA*-transformed mouse cells (900-2K-34) labeled with [^3H]leucine and [^{32}P]orthophosphate *in vivo* showed that the peaks of the ^3H and ^{32}P radioactivities comigrating with a molecular weight of 90,000 greatly decreased concomitantly when the cells were cultivated at the

restrictive temperature (Fig. 15). This decrease was not as evident in ribosome-associated proteins; however, no phosphorylated 90K protein was detected in ribosome-associated proteins from untransformed parental cells (C3H2K-C4) similarly labeled *in vivo* (Fig. 16C). The results indicate that the function of the SV40 A gene is closely related to the synthesis and/or association of the 90K protein rather than to its phosphorylation.

It has been reported recently that the translation product of the 19S mRNA transcribed from the early region of SV40 DNA is immunoprecipitated by anti-T sera and has the same molecular weight as T antigen synthesized *in vivo* (cited in reference 4). The results strongly suggest that the product of the SV40 gene A is T antigen. The molecular weight of our phosphorylated protein is similar to that estimated for T antigen, i.e., 86,000 by Ahmad-Zadeh et al. (1) and 94,000 by Carroll and Smith (4). The temperature-dependent association of the 90K protein with chromatin and ribosomes in SV40 *tsA*-transformed cells suggested that the 90K protein might be T antigen. However, the following results suggest that the 90K protein is not T antigen. (i) *In vitro* phosphorylated 90K protein in nonhistone chromosomal and ribosome-associated proteins from SV40-transformed cells was not immunoprecipitated by anti-T sera under conditions in which T antigen in the extract of SV40-infected AGMK cells was specifically precipitated (Fig. 13). (ii) Sedimentation analysis of the post-mitochondrial supernatant from SV40-transformed cells on the sucrose gradient showed that most of the CF activity of T antigen is localized in the upper fractions of the gradient, whereas the 90K protein is bound to ribosomes (Fig. 11 and 12). (iii) The phosphorylated 90K protein was not detected in chromatin and ribosomes from SV40-infected AGMK cells upon phosphorylation *in vitro* (unpublished data).

The 90K protein was discriminated from the other phosphorylated proteins associated with these organelles by a preferential phosphorylation *in vitro*. The results suggest that the turnover of phosphorus groups associated with the 90K protein may occur more quickly than that of the other proteins and that the protein contains more serine and threonine residues than do the other proteins. A slow labeling and turnover of the protein phosphorus groups associated with ribosomes in rabbit reticulocytes (13) and Chinese hamster ovary cells (34) has been reported.

Whether the 90K protein associated with chromatin and ribosomes is the same protein is presently unknown. This possibility will be in-

vestigated by peptide map analysis of purified 90K protein from these organelles of SV40-transformed cells. The similarity of the 90K protein associated with these organelles of various species of SV40-transformed cells is also unknown at the present time. The 90K protein in the various species of cells, however, may have a similar function since its expression is regulated by the function of the SV40 A gene, which is closely related to the expression of transformed phenotypes in SV40 *tsA*-transformed cells (3, 25, 28, 39).

The results of the present paper suggest that different species of proteins are associated with chromatin and ribosomes in SV40-transformed cells as compared with those associated with these organelles in untransformed cells and are modified by the protein-modifying enzymes associated with these organelles. It is noteworthy that the activity of one of the protein-modifying enzymes, ADP-ribosyl transferase, which is also associated with the chromatin and ribosomes, is increased severalfold in SV40-transformed cells as compared with untransformed cells when measured with or without added DNA and histones (Miwa et al., in press). The involvement of tumor virus function in the modification of chromatin has also been suggested with polyoma virus. A host range mutant of polyoma virus, *hr-t*, which is defective in cell transformation, loses the ability to modulate the histone acetylation system (35). The phosphorylation of non-histone chromosomal proteins enhances the chromatin template activity (33) and may control the recognition of certain polynucleotide sequences in DNA. The phosphorylation of initiation factors associated with the ribosomes (10) may alter the species of mRNA to be translated. The phosphorylation of the 90K protein may have such a function.

ACKNOWLEDGMENTS

We thank H. Shimojo for support and Y. Kaziro, K. Iwasaki, and their colleagues for preparation of [γ - 32 P]ATP. We also thank G. Kimura for 3Y1-B cells, Y. Nishizuka for phosphoserine and phosphothreonine, and J. A. Robb for reading this manuscript.

This work was supported by grants from the Ministry of Education, Science and Culture of Japan.

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