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Cell-Free Synthesis of Polyoma Virus Capsid Proteins VP1 and VP2

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Polyadenylated RNA isolated from the cytoplasm of mouse 3T6 cells 28 h after infection with polyoma virus has been isolated and translated in vitro. Polyoma capsid proteins VP1 and VP2 have been identified in the cell-free product by polyacrylamide gel electrophoresis, specific immunoprecipitation, and tryptic peptide fingerprinting. Polyoma mRNA species have been isolated by preparative hybridization to purified viral DNA immobilized on cellulose nitrate filters and shown to code for both VP1 and VP2. These experiments establish conditions for the isolation of late polyoma mRNA and the cell-free synthesis of polyoma capsid proteins and indicate that the active mRNA species are at least partially virus coded.

Polyoma virus causes one of two responses when it infects cells grown in culture. In permissive hosts the virus replicates, producing progeny virions and killing the host cell. In certain semi- or nonpermissive cells viral DNA becomes stably integrated into the host genome and the cells become transformed, acquiring altered growth characteristics and an increased ability to cause tumors in animals. Polyoma virus induces a wide range of tumors when injected into animals of different species (26).

The genome of polyoma is composed of a single molecule of superhelical double-stranded DNA of molecular weight about 3.5×10^6, associated with an equal weight of histone derived from the host cell (6). This DNA-histone complex is contained in a particle with icosahedral symmetry made up of one major (VP1) and two minor (VP2 and VP3) capsid proteins (reviewed by Crawford, reference 2). The total coding capacity of the viral genome cannot exceed about 200,000 daltons of protein. Since the virus is apparently so simple and yet has such a profound effect on cellular metabolism both in culture and in whole animals, it is of great interest to establish what proteins are coded for by the viral DNA.

The molecular weights of the viral capsid proteins VP1, -2, and -3 are 45,000, 34,000, and 23,000, respectively (9). Tryptic peptide analysis suggests that although VP1 and VP2 are different proteins with non-overlapping sequences, VP2 and VP3 contain common peptide sequences (5, 7, 9). There is some evidence from biochemical (3) and genetic (16) experiments to suggest that the viral capsid protein is at least partially virus coded. If VP1 and VP2 were totally virus coded, about half of the viral genome would be required, leaving only 100,000 daltons of additional viral protein to perform the other viral functions observed during the early phase of replication and in transformed cells. In this paper we show that mRNA isolated from virus-infected cells directs the synthesis of the capsid proteins VP1 and VP2 and confirm that the active mRNA is predominantly virus coded.

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MATERIALS AND METHODS

Preparation of infected cell RNA. Plaque-purified polyoma virus of the A2 strain (8) was grown at low multiplicity in secondary mouse embryo cells. Mouse 3T6 cells (25) were grown in 80-ounce (ca. 2.3-liter) roller bottles and when almost confluent were infected at 50 to 100 PFU/cell in 10 ml of phosphate-buffered saline for 90 min at 37°C. A 200-ml amount of Dulbecco modified Eagle medium (E4) containing 5% fetal calf serum was then added. At 26 to 30 h after infection the medium was poured off, and the cells were removed by vigorous swirling with 25 ml of Versene (EDTA) buffer. The cells were pelleted and washed three times in Tris-buffered saline. The cell pellet was rapidly suspended in 5 to 10 volumes of NDS lysis buffer (20 mM Tris-hydrochloride, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% naphthalene 1,5-disulfonic acid [Eastman Kodak]) and swirled for 2 to 5 min, Nonidet P-40 was added to 1%, and the nuclear material was immediately sedimented by centrifugation at $1500 \times g$ for 5 min. The supernatant was poured off, and sodium dodecyl sulfate (SDS; Serva) was added to 0.5% and polyvinyl sulfate (Sigma) to 100 μg/ml. RNA was extracted by repeated shaking with phenol-chloroform-isoamyl

alcohol (50:50:1) and precipitated with alcohol. Polyadenylic acid [poly(A)] containing RNA was isolated by chromatography on polyurydilic acid-Sepharose (Pharmacia) as described by Lindberg and Persson (15). RNA from subconfluent uninfected 3T6 cells was prepared by the same method.

Preparation of [^{35}S]methionine-labeled polyoma virus and labeled cell extracts. Radioactive polyoma virus was grown and purified as described by Frearson and Crawford (6). Labeled cell extracts were made from cells infected as described above and grown in 25-mm plastic petri dishes (Nunc). At about 28 h postinfection the medium was removed and replaced with 0.5 ml of similar medium lacking unlabeled methionine and containing 30 μCi of [^{35}S]methionine (Radiochemical Centre, 150 to 500 Ci/mmol). After 1 h the cells were pelleted and lysed in electrophoresis sample buffer (50 mM Tris-hydrochloride, pH 6.8, 1% SDS, 10 mM dithiothreitol, 10% glycerol, 0.001% bromophenol blue).

Cell-free protein synthesis. Extracts were prepared from commercial wheat germ exactly as described by Roberts and Paterson (20). Cell-free incubations in 25 μl included 7.5 μl of wheat germ extract, 15 mM HEPES, pH 7.0, 85 mM KCl, 1.5 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 1 mM ATP, 0.2 mM GTP, 10 mM creatine phosphate, 50 μM spermine, 250 μM spermidine, and 1 mM dithiothreitol. Nineteen unlabeled amino acids, omitting methionine, were added to 200 μM, and [^{35}S]methionine was added at 500 μCi/ml. Approximately 40 μg of poly(A)-containing RNA per ml was added, and incubation was continued for 3 h at 22°C. The incorporation of [^{35}S]methionine into trichloroacetic acid-insoluble material was estimated by removing a 2-μl sample, treating it with 100 μl of 0.1 N KOH for 15 min at 37°C, and precipitating with 2 ml of trichloroacetic acid. Samples were collected on membrane filters (Millipore Corp.) and counted in a scintillation counter. Typically, 10 to 20 pmol of [^{35}S]methionine were incorporated per 1 μg of RNA. The remainder of the incubation mixture was processed for polyacrylamide gel electrophoresis, immunoprecipitation, or peptide mapping.

Polyacrylamide gels. Samples (10 μl) of the cell-free incubation were mixed with an equal volume of electrophoresis sample buffer and applied to 15% SDS-polyacrylamide slab gels. Electrophoresis, using the discontinuous pH, SDS-polyacrylamide gel system of Laemmli (13), staining, drying, and exposure of the autoradiographs have all been described previously (24).

Preparation of antibodies. (i) Anticapsid sera. Purified polyoma virions were prepared as described by Crawford (1), dialyzed against phosphate-buffered saline, and injected directly into rabbits.

(ii) Anti-VP2 sera. Purified polyoma virions were disrupted with electrophoresis sample buffer, and viral proteins were separated on polyacrylamide gels as described later for labeled virus. A thin strip was cut from the edge of the gel and stained. Regions of gel containing VP2 and VP3 were excised, crushed, and injected directly into rabbits to raise antibodies, as described by Lazarides and Weber (14).

Immunoprecipitation. All buffers, sera, and samples of the cell-free incubation mixtures to be used in immunoprecipitation reactions were clarified by centrifugation at 10,000 × *g* for 15 min. Typically, 10 μl of incubation mixture was mixed with an equal volume of appropriate antiserum and 5 to 10 volumes of phosphate-buffered saline containing 1 mg of bovine serum albumin per ml and 1% Triton X-100. After 60 min at 37°C, 10 volumes of sheep anti-rabbit immunoglobulin G (IgG) (Wellcome Reagents) was added, and incubation was continued 60 min at 37°C and overnight at 4°C. Under these conditions a flocculent immunoprecipitate developed. The precipitate was pelleted and washed at least three times in buffer (20 mM Tris, pH 7.5, 50 mM NaCl, 0.5% Nonidet P-40, 0.5% deoxycholate). The washed pellet was then resuspended in electrophoresis sample buffer, heated, and, if necessary, sonicated until completely redissolved. Precipitates prepared in this way could be applied to a 10- by 1-mm slot on the gel without overloading. Electrophoresis, processing of the gel, and autoradiography were all as described above.

Tryptic peptide fingerprinting. Samples of [^{35}S]methionine-labeled purified virus (~1 ml, containing approximately 10^7 cpm) were dialyzed for 2 h against 10 mM Tris-hydrochloride, pH 7.5, 100 mM NaCl, and 1 mM EDTA, 250 μl of electrophoresis sample buffer was added, and the mixture was heated at 80°C for 2 min. Large-scale cell-free reactions (500 μl) were treated in the same way, but heated to 100°C. Each sample (up to 1.5 ml) was applied to a single-slot SDS-polyacrylamide slab gel (14 cm by 2 mm) and subjected to electrophoresis as described above. The gel was wrapped in Saran Wrap and autoradiographed without drying, and the appropriate bands were excised and eluted with 5 ml of buffer (0.1% SDS, 2 mM phenylmethylsulfonyl fluoride, 50 μg of bovine serum albumin, 10 mM sodium bicarbonate) for 18 h at 37°C. A 500-μg amount of total wheat germ extract was added as carrier and precipitated by the addition of 100% trichloroacetic acid to 20%. The protein was reprecipitated three times from 1 N NaOH using 20% trichloroacetic acid and dried. It was then oxidized with performic acid and digested with trypsin as described previously (24).

After extensive washing, a sample of the digest containing 20,000 to 100,000 cpm was lyophilized and taken up in 2 μl of water containing a marker dye mixture (xylene cyanol FF, crystal violet, orange G) and applied directly to a cellulose thin-layer chromatogram sheet (Eastman Kodak) that was already wet with buffer. Electrophoresis was performed in a water-cooled Shandon thin-layer apparatus. At pH 2.1 electrophoresis was for 75 min at 600 V; at pH 3.5 and 6.5 it was for 2 h at 600 V. After being dried, the plates were developed by ascending chromatography using BAWP (butanol-acetic acid-water-pyridine, 15:3:12:10) or isoamyl alcohol-pyridine-water (7:7:6). The dried sheets were autoradiographed for 1 to 10 days. Two-dimensional fingerprints on paper were performed exactly as described previously (24).

Preparative hybridization. Polyoma DNA was extracted from cells using the method of Hirt (10), purified by CsCl density centrifugation, and recovered by alcohol precipitation. The DNA was di-

gested to completion with restriction endonuclease *Eco*RI and again precipitated. The DNA was taken up in 0.01× SSC, boiled for 10 min, and rapidly cooled to 0°C. At each of the stages described above approximately 0.5-μg samples were taken and subjected to electrophoresis on 1% agarose disc gels (as described by Sharp et al. [21]) to ensure that the expected products (form I DNA → linear double-stranded DNA → linear single-stranded DNA), each of which has a characteristic mobility, had been formed. The single-stranded DNA (25 μg) was diluted with 10 ml of 2× SSC and slowly passed through a 2.4-cm membrane filter (HAWP 02400; Millipore Corp.). The filter was washed with 25 ml of 2× SSC and dried in air for 4 h and under vacuum at 80°C for a further 4 h. A DNA-containing filter and a blank filter were placed in a siliconized pot and shaken in 1 ml of hybridization buffer (50% formamide [BDH Analar], 0.75 M NaCl, 0.5% SDS, 50 mM Tris-hydrochloride, pH 7.5) containing 25 μg of total infected-cell mRNA. After 16 h at 37°C, the hybridization buffer was removed and unbound RNA was collected by precipitation. The filters were shaken for a further 1 h in 10 ml of hybridization buffer and washed by filtering through 25 ml of 1× SSC containing 0.5% SDS from each side. RNA was eluted by shaking the filter in 1 ml of 90% formamide at 37°C for 1 h and precipitated with alcohol after addition of 25 μg of wheat germ tRNA. After repeated precipitation the RNA fractions were dried, dissolved in 25 μl of water, and translated in vitro.

RESULTS

In vitro translation of mRNA from polyoma-infected cells. Mouse 3T6 cells were infected at a multiplicity of 50 PFU/cell with seed virus grown up from plaque-purified stocks of the A2 strain of polyoma. RNA was isolated from the cells at 26 to 30 h after infection, at which time the synthesis of viral capsid protein is maximal. The RNA extraction buffer included the detergent naphthalene disulfonic acid (17) which we find helpful because it lyses cells, does not disrupt nuclei, and inhibits RNase. Poly(A)-containing RNA was isolated by chromatography through polyurydilic acid-Sepharose (15) and translated in vitro. Kamen and Shure (12) have previously shown that all species of cytoplasmic polyoma RNA contain a poly(A) tract. All of the work described here utilized a wheat germ system, similar to that of Roberts and Paterson (20) but supplemented with spermine and spermidine (T. Hunt and R. Jackson, personal communication), which we find to be essential for efficient synthesis of high-molecular-weight proteins.

The addition of poly(A)-containing mRNA isolated from uninfected 3T6 cells or cells lytically infected with polyoma causes a large stimulation of amino acid incorporation into trichloroacetic acid-insoluble material when added to the wheat germ cell-free system. The salt requirements for maximal amino acid incorporation (1.5 mM Mg^{2+}, 80 mM K^{+}; see Materials and Methods for further details) were similar to those found for a variety of other cellular and viral mRNA's. Each 25-μl incubation contained approximately 1 μg of mRNA, and amino acid incorporation was linear for 3 h at 22°C.

The proteins made in vitro were analyzed by SDS-polyacrylamide gel electrophoresis, in parallel with labeled extracts from infected and uninfected 3T6 cells and with purified labeled virions. Figure 1 shows that the pattern of proteins made in vitro in response to added mRNA is similar to that from whole cells. A prominent new protein, which migrates slightly slower

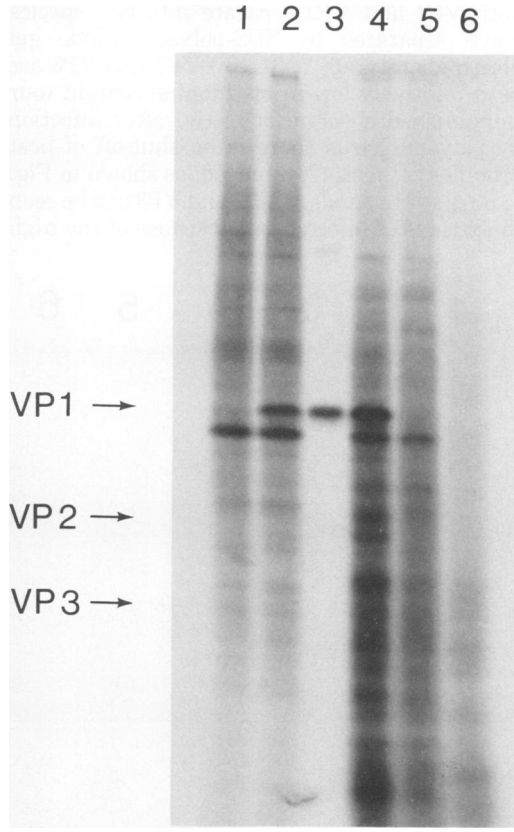

FIG. 1. *Comparison of the proteins made in uninfected and polyoma-infected 3T6 cells and in a wheat germ cell-free system primed with mRNA from the same cells. The autoradiograph of a 15% SDS-polyacrylamide gel shows the [^{35}S]methionine-labeled proteins from: (1) uninfected 3T6 cells; (2) infected 3T6 cells; (3) purified polyoma virus; proteins made in vitro in response to (4) infected 3T6 mRNA; (5) uninfected 3T6 mRNA; and (6) no RNA added. Autoradiography was for 2 days. Although the minor capsid proteins in track 3 are not visible here, they were detected after longer exposure. The positions of viral capsid proteins are indicated.*

than a major cellular protein, actin (molecular weight, 43,000), is present in the infected cell samples labeled both in vivo and in vitro. This infected-cell-specific protein has a molecular weight of 45,000 and comigrates with VP1 from purified polyoma virus. A similar major band comigrating with polyoma VP1 was synthesized in response to infected-cell mRNA in extracts from Krebs II mouse ascites cells, L cells, and rabbit reticulocytes, but this material has not been extensively characterized.

The synthesis of the minor capsid proteins is more difficult to study for several reasons: (i) the amount of viral proteins VP2 and VP3 in purified virions is considerably less than VP1, totalling about 10 to 20% by weight (9); (ii) often both VP2 and VP3 separate into two species when separated by SDS-polyacrylamide gel electrophoresis (5, 7, 9); (iii) VP2 and VP3 are both relatively low in methionine content (our unpublished observations); (iv) after infection by polyoma virus there is no shut-off of host protein synthesis (26). Indeed, as shown in Fig. 1, no discrete bands of VP2 and VP3 can be seen in extracts of infected cells because of the high background of host cell proteins. Similarly, no obvious band of the minor viral proteins was observed among the proteins made in vitro in response to total infected-cell, poly(A)-containing RNA.

The synthesis of several viral proteins shows a higher resistance to elevated concentrations of monovalent cation than does the synthesis of cellular proteins (G. Koch, H. Oppermann, P. Bilello, F. Koch, and D. Nuss, *in Modern Trends in Human Leukemia*, vol. 2, in press). When the proteins made in vitro in response to polyoma-infected cell mRNA at different K^+ concentrations were examined on polyacrylamide gels (Fig. 2), the synthesis of the band comigrating with polyoma VP1 was found to be resistant to high salt. At 65 mM K^+ approximately equal amounts of actin and the putative VP1 were made, but at 125 mM K^+, although total protein synthesis in vitro was inhibited, the synthesis of the VP1-like protein greatly exceeded that of actin. Under the high-salt conditions other proteins were also preferentially synthesized, and among these was a protein that comigrated with viral capsid protein VP2.

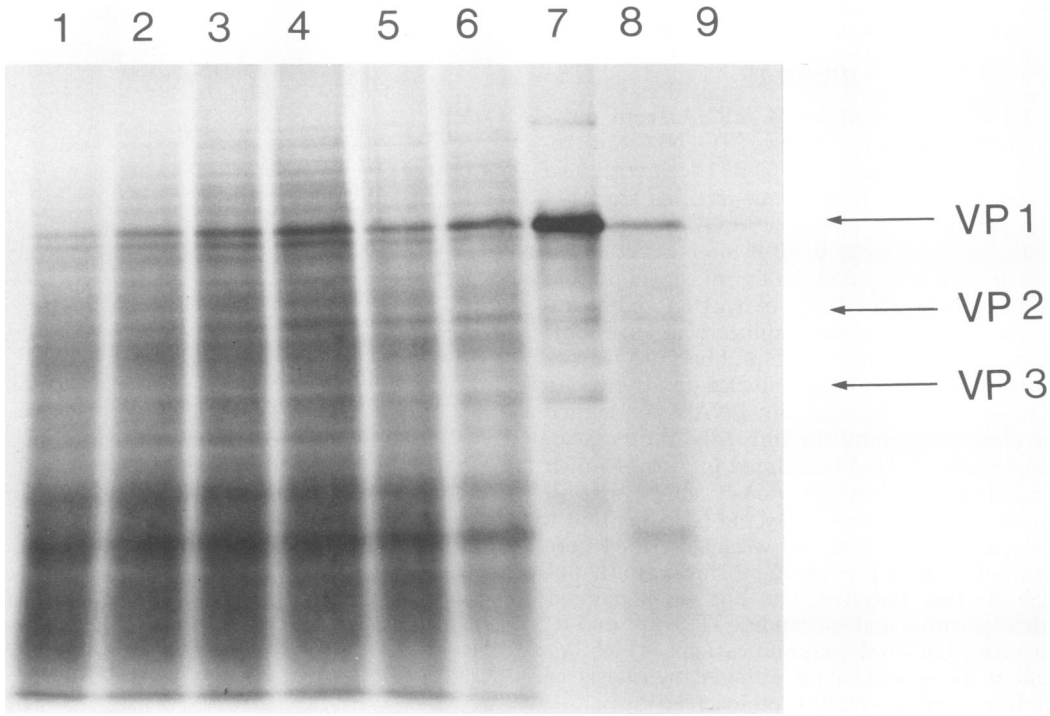

FIG. 2. *Polyacrylamide gel analysis of proteins made in vitro in response to infected-cell mRNA at different K^+ concentrations. Incubations included K^+ at the following concentrations (mM): (1) 35; (2) 50; (3) 65; (4) 80; (5) 95; (6) 110; (8) 125; (9) 140. (7) [^{35}S]methionine-labeled purified polyoma virus. The three capsid proteins are indicated. Autoradiography was for 4 days; this overexposed parts of the gel but was necessary to visualize minor bands.*

Because of this we considered it possible that in addition to a protein related to polyoma VP1, VP2 was also being synthesized in response to mRNA from infected cells. To further substantiate this possibility, we characterized the two proteins by more rigorous methods.

Immunoprecipitation of polyoma capsid proteins VP1 and VP2. Antiserum against purified polyoma virions was raised in rabbits and had a hemagglutination inhibition titer of 1/1,000,000. This antiserum was used to immunoprecipitate proteins made in vitro using a double-antibody sandwich technique. Figure 3 shows the proteins immunoprecipitated from incubations containing either uninfected or infected-cell mRNA. The protein made in vitro using infected-cell mRNA that comigrated with polyoma VP1 was specifically immunoprecipitated by the antivirion serum but not by control rabbit serum. VP1-like material was not detected in the product directed by uninfected-cell mRNA. No other proteins were reproducibly immunoprecipitated from the cell-free products using the antivirion serum, and the small amounts of actin that were occasionally present probably result from nonspecific aggregation.

Since the antivirion antisera did not react with anything related to the minor capsid proteins of polyoma, we prepared antiserum against purified polyoma VP2. Large quantities of polyoma virus were disrupted with SDS and reducing agent, and the viral proteins were separated by polyacrylamide gel electrophoresis. Polyoma VP2 was located by staining a strip cut from the edge of the gel, and a band of acrylamide containing the protein was excised, crushed, and injected into a rabbit to raise a specific antibody (14).

The proteins immunoprecipitated by VP2 antiserum from the products made in the wheat germ cell-free system in response to total infected-cell mRNA are shown in Fig. 3B. A band comigrating with VP2 is present as well as two other bands, one comigrating with VP3 and the other with VP1. This result indicates that cross-reacting material related to polyoma VP2 is made in vitro. It also shows that the antiserum is not monospecific and indicates that a protein similar to VP3 may be made in vitro in addition to VP1 and VP2.

The cross-reactivity of the anti VP2 serum with VP3-like material is not unexpected since it is known that the two proteins contain common polypeptide sequences (5, 7, 9). The cross-

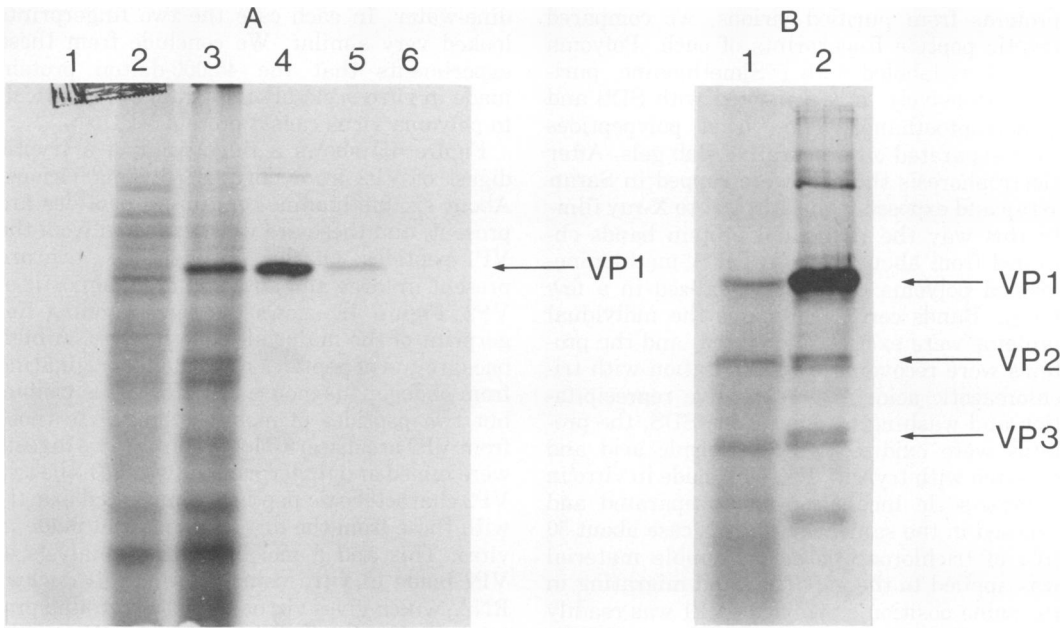

FIG. 3. *Immunoprecipitation of proteins made in vitro with polyoma antivirion serum and anti-VP2 serum. (A) Incubations contained 100 mM K^+. The autoradiograph of the polyacrylamide gel shows: (1) immunoprecipitate with rabbit antivirion serum of proteins made in response to uninfected 3T6 cell mRNA; proteins made in response to (2) uninfected 3T6 cell mRNA and (3) infected-cell mRNA; (4) [^{35}S]methionine-labeled polyoma virus; immunoprecipitates of proteins made in response to infected-cell mRNA with (5) rabbit antivirion serum and (6) control rabbit serum. (B) Incubations contained total infected-cell mRNA and included 80 mM K^+: (1) immunoprecipitate of proteins made in vitro with anti-VP2 serum; (2) [^{35}S]methionine-labeled polyoma virus. The capsid proteins are indicated. Autoradiography was for 10 days.*

reactivity with VP1 is more surprising. Perhaps the VP2 used to inoculate the rabbit contained contaminating VP1, since it is known that specific degradation products of VP1 comigrate with VP2 (9). To reduce this possibility, polyoma VP2 was prepared by an alternative, more gentle method. Purified virions were disrupted at room temperature with SDS in the absence of reducing agent. This procedure disrupts the virions, releasing the DNA, histones, and minor capsid proteins but leaving the viral shell, made up entirely of disulfide bridge-linked VP1, intact (27). The shells were removed by centrifugation, and the minor proteins were separated by polyacrylamide gel electrophoresis.

Since three additional preparations of anti-VP2 serum prepared using VP2 made by the more gentle procedure all immunoprecipitated the three bands comigrating with the three viral proteins, we find cross-contamination a rather unlikely explanation. It is possible that VP1 and VP2 share common antigenic determinants, but further work would be needed to establish this proposition firmly.

Tryptic peptide fingerprinting. To further establish the relationship between the putative viral proteins made in vitro and the separated proteins from purified virions, we compared tryptic peptide fingerprints of each. Polyoma virus was labeled with [^{35}S]methionine, purified extensively, and disrupted with SDS and β-mercaptoethanol. The virion polypeptides were separated on preparative slab gels. After electrophoresis the gels were rapped in Saran Wrap and exposed while still wet to X-ray film. In this way the individual protein bands obtained from about 10 μCi of [^{35}S]-methionine-labeled polyoma could be visualized in a few hours. Bands corresponding to the individual proteins were excised and eluted, and the proteins were recovered by precipitation with trichloroacetic acid. After extensive reprecipitation and washing to remove the SDS, the proteins were oxidized with performic acid and digested with trypsin. Proteins made in vitro in a large-scale incubation were separated and exposed in the same way. In this case about 50 μCi of trichloroacetic acid-insoluble material was applied to the gel. The band migrating in the same position as polyoma VP1 was readily located. No obvious band of VP2 was visible, but the region of the gel known from the autoradiograph to correspond to the molecular weight of VP2 was also cut out and processed as described above. The peptides from the various digests were separated in two dimensions by electrophoresis, followed by chromatography either on paper or on plastic-backed cellulose thin-layer sheets.

Figure 4A shows an autoradiograph of a fingerprint of the digest of VP1 from purified virus separated on cellulose by electrophoresis at pH 2.1 and ascending chromatography in butanol-acetic acid-water-pyridine. About .12 [^{35}S]-methionine-containing peptides are well separated under these conditions, and these give a characteristic and reproducible fingerprint. Figure 4B shows the corresponding fingerprint of the putative VP1 made in vitro. It shows a very similar pattern of major peptides with a very low background of minor peptides, which probably originate from cellular proteins with the same mobility as VP1. By way of contrast, Fig. 4C shows the corresponding fingerprint of actin made in vitro. Actin is a major protein in mouse cells and has a molecular weight very similar to that of VP1. The actin fingerprint is totally different from that of polyoma VP1. VP1 fingerprints of material made in vitro and from purified virus have been repeated on paper by using electrophoresis at pH 3.5, followed by descending chromatography with butanol-acetic acid-water, and on thin-layer cellulose using electrophoresis at pH 3.5 and chromatography with isoamyl alcohol-pyridine-water. In each case the two fingerprints looked very similar. We conclude from these experiments that the 45,000-dalton protein made in vitro is closely related, if not identical, to polyoma virus capsid protein VP1.

Figure 4D shows a fingerprint of a tryptic digest of VP2 from purified polyoma virions. About six methionine-containing peptides are present, and these are well separated from the VP1 peptides. Of the VP2 peptides, two are present in large amounts and are diagnostic of VP2. Figure 4E shows the corresponding fingerprint of the material made in vitro. A high background of peptides presumably originating from endogenous mouse cell proteins is visible, but two peptides of mobility similar to those from VP2 are discernible. When the two digests were mixed and fingerprinted (Fig. 4F), the two VP2 characteristic peptides comigrated exactly with those from the digest of material made in vitro. This and a more extensive analysis of VP2 made in vitro using infected-cell nuclear RNA, which gives virtually no background proteins (R. Kamen, T. Wheeler, and A. E. Smith, manuscript in preparation), strongly suggest that the protein made in vitro is very similar to polyoma VP2.

Purification of the mRNA coding for polyoma VP1 and VP2. The experiments described above show that proteins related to polyoma

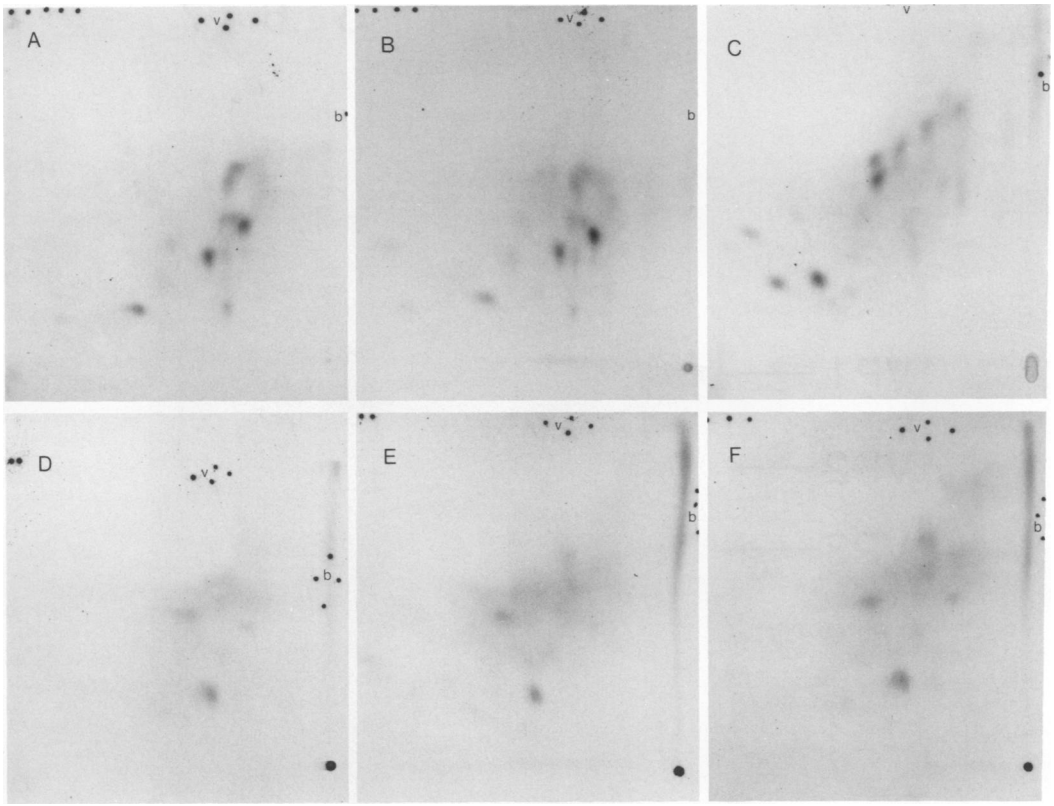

FIG. 4. *Comparison of tryptic peptide fingerprints of polyoma proteins made in vitro with those of purified viral proteins. The first dimension was right to left at pH 2.1 and chromatography was bottom to top, using BAWP. (A) VP1 from purified polyoma virus; (B) polyoma VP1 made in vitro; (C) mouse actin made in vitro; (D) VP2 from purified polyoma virus; (E) polyoma VP2 made in vitro; (F) mixture of equal cpm (15,000) of VP2 made in vitro and from purified virions. b and v indicate the positions of the blue (xylene cyanol FF) and violet (crystal violet) markers. Note that in sample 4D the solvent front has migrated rather less than in the other samples. Autoradiography was for 14 days.*

capsid proteins VP1 and VP2 can be made in vitro using as template mRNA isolated from lytically infected 3T6 mouse cells. We next investigated whether the mRNA's coding for the virion proteins were coded for by the viral genome by attempting to isolate the mRNA by hybridization to viral DNA. The technique used was a modification of that described by Prives et al. (19). Supercoiled polyoma (form 1) DNA was purified by equilibrium gradient centrifugation and cleaved with restriction endonuclease *Eco*RI to give linear molecules. The double-stranded DNA was denatured by boiling in low salt, and the single-stranded DNA was immobilized on cellulose nitrate filters. The polyoma DNA filters were incubated in a solution containing total cellular mRNA for 20 h at 37°C. The filters were then extensively washed, and the hybridized RNA was eluted, precipitated, and translated. In each case the material that did not hybridize was also recovered and translated. In addition, a filter containing no viral DNA was included in each hybridization incubation, and this acted as a negative control.

Figure 5 shows that the RNA purified by hybridization to polyoma DNA directs the synthesis of proteins that comigrate with polyoma VP1 and VP2, with molecular weights of 45,000 and 34,000, respectively. In addition, a smaller protein migrating between VP2 and VP3 and corresponding to a molecular weight of about 29,000 to 31,000 was reproducibly present, but we have not identified this. RNA recovered from the material remaining in solution directed the synthesis of many host cell proteins but very little VP1 mRNA activity remained, indicating that hybridization of the viral

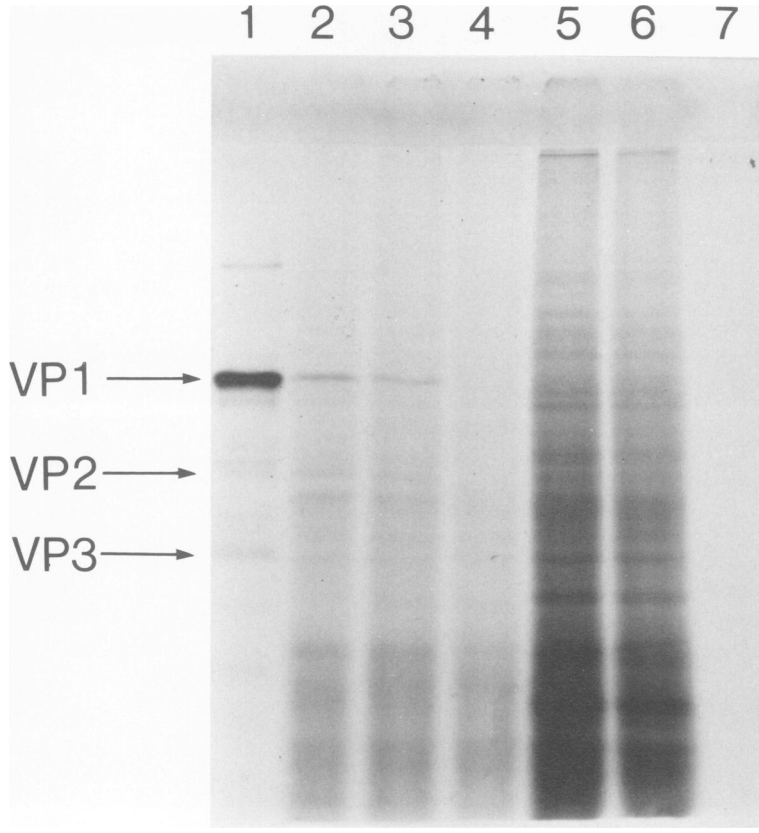

FIG. 5. *Proteins made in vitro in response to purified polyoma mRNA. A preparative hybridization experiment was performed exactly as described in the experimental procedures. The autoradiograph shows a polyacrylamide gel analysis of the proteins made in vitro at 80 mM K^+ in response to RNA from different fractions. (1) [^{35}S]-methionine-labeled polyoma virus; (2) 3 μl of RNA bound to a polyoma DNA-containing filter; (3) 1 μl of RNA as in slot 2; (4) 3 μl of RNA bound to a blank filter; (5) 3 μl of RNA not bound to either filter; (6) 1 μl of RNA as in slot 5; (7) no RNA added. Autoradiography was for 7 days.*

mRNA to the viral DNA was essentially complete. RNA bound under these conditions to blank filters gave no stimulation of amino acid incorporation. In other experiments (23), we have used *Hpa*II restriction endonuclease fragments of polyoma virus DNA, instead of complete linear molecules, and showed that the mRNA for VP1 will hybridize to fragment 1, which comes from the late region of viral DNA, but not to fragment 2, which is from the early region (11). These experiments argue that polyoma capsid proteins are at least partially virus coded since the mRNA coding for them hybridizes specifically to viral DNA.

DISCUSSION

In the work described here we have established that proteins closely related, if not identical, to polyoma virus capsid proteins VP1 and VP2 can be made in a wheat germ cell-free system using total poly(A)-containing RNA isolated from lytically infected mouse cells. As yet we have not confirmed the synthesis of polyoma VP3 by tryptic peptide mapping, but since this protein is probably derived from VP2 by proteolytic cleavage, the cell-free synthesis of VP2 can be considered as the synthesis of a VP3 precursor. A protein comigrating with polyoma VP1 was also made in extracts from Krebs II ascites cells, L cells, and rabbit reticulocytes in response to the same mRNA.

The synthesis of polyoma VP1 and VP2 in vitro shows a differential resistance to inhibition by high concentrations of K^+ in the incubation. We have found that several other viral mRNA's also have a higher K^+ optimum for maximal incorporation of amino acids into viral proteins than typical cellular mRNA's; these

include encephalomyocarditis virus, mengovirus and poliovirus RNAs, and simian virus 40 (SV40) mRNA (S. T. Bayley, L. Carrasco, and A. E. Smith, unpublished observations). Some adenovirus mRNA's also have a high potassium requirement in vitro (3). Other studies with intact cells have shown that the synthesis of several virus proteins, including SV40 capsid proteins, is resistant to hypertonic salt conditions in vivo (4; Koch et al., in press). It is not yet clear if the observed salt resistance has any physiological significance.

Our immunoprecipitation data indicate that the proteins made in vitro are closely related to authentic virion proteins. In addition to reacting with proteins comigrating with the minor proteins, the VP2 antiserum also reacts with VP1. Since, in spite of all the precautions taken, preparations of VP2 could contain specific degradation products of VP1, this finding is difficult to interpret, but it could mean that VP1 and VP2 share common antigenic sites. A protein exactly comigrating with polyoma VP3 is also immunoprecipitated by the anti-VP2 serum. However, we have not confirmed its identity by tryptic peptide mapping; neither do we know whether it is generated by cleavage of VP2 made in vitro, it represents incomplete translation of the VP2 cistron, or it is synthesized from a separate mRNA.

The conditions used to separate the peptides from polyoma capsid proteins gave well-resolved fingerprints and demonstrate that the methionine-containing tryptic peptides from VP1 and VP2 are unrelated. Fingerprints of VP2 and VP3, on the other hand, are virtually indistinguishable (data not shown). This is in agreement with earlier data showing that VP1 and the minor capsid proteins are different, but that VP2 and VP3 share common sequences (5, 7, 9). The fingerprint of polyoma VP1 made in vitro is very similar to that of virion VP1, but in the case of VP2 it is more difficult to distinguish all the viral peptides because of the high background. Nevertheless, the two major peptides of virion VP2 are clearly present in the digest of the cell-free product. RNA isolated from the nuclei of infected 3T6 cells also directs the synthesis of proteins in vitro (Kamen et al., manuscript in preparation). In this case, however, only a small number of different proteins are made, and material similar to polyoma VP2 is the major product. Peptide fingerprints of this material have very low backgrounds and show that the minor peptides are also present in the cell-free product. Taken together, these results show that the two proteins made in vitro are related to the corresponding viral proteins in molecular weight, immunological cross-reactivity, and tryptic peptide maps.

Since the small DNA tumor viruses have such a small genome and yet have such a profound effect on cellular metabolism both in lytic and transformed infections, there is considerable interest in establishing the nature of the proteins coded for by the viral DNA. Analysis of the peptides made in *E. coli* extracts using polyoma complementary RNA as template (3) suggests that the capsid proteins are at least partially virus coded, and this is supported by genetic evidence (16). We have also found that polyoma complementary RNA is translated in the wheat germ cell-free system to give complete VP1 and small amounts of VP2 (22, 23; W. F. Mangel, R. Hewick, M. D. Waterfield, S. T. Bayley, R. Harvey, T. Wheeler, and A. E. Smith, manuscript in preparation).

The purification of polyoma-specific RNA by preparative hybridization to purified viral DNA, and the subsequent translation of this RNA to give proteins that comigrate with viral capsid proteins, are consistent with the suggestion that the capsid proteins are virus coded. Potentially, the method of purification of the viral mRNA is very powerful and could be used to associate the viral proteins with RNAs purified using different DNA fragments, and thereby to map the proteins directly on the viral genome. We have used this technique to obtain preliminary maps of the capsid proteins on the *Hpa*II fragments of polyoma. We found that the capsid proteins were made from mRNA's that were purified using *Hpa*II fragments 1 and 3 but not from mRNA made with fragments 2 and 4 (23). This, as expected, positioned the capsid proteins in the late portion of the viral genome. To obtain more accurate mapping two problems have to be overcome: (i) the restriction enzyme fragments have to be of very high purity because the hybridization reaction is performed under conditions of DNA excess and, consequently, minor impurities become significant; and (ii) the viral mRNA tends to aggregate under the conditions described here and thus can be isolated by its association with another viral molecule rather than a direct interaction with the immobilized DNA. For this reason we have used sucrose gradient centrifugation under denaturing conditions to separate viral mRNA's and translated these to locate the capsid proteins unequivocally on the viral genome. These experiments will be described in a separate publication (23a).

Experiments similar to those described have been reported using the closely related virus SV40. In this case a protein identical to SV40

VP1 was synthesized in vitro in response (i) to total infected-cell mRNA and (ii) to mRNA purified by preparative hybridization (18, 19). A minor protein was also made in vitro using purified mRNA, and this comigrated with a minor protein present in SV40-infected cells. Surprisingly, the minor protein was not present in purified SV40 virions.

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