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In vitro assay for protein–protein interaction: Carboxyl-terminal 40 residues of simian virus 40 structural protein VP3 contain a determinant for interaction with VP1

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ABSTRACT **Intermolecular interactions between polypeptide chains play essential roles in the functioning of proteins. We describe here an *in vitro* assay system for identifying and characterizing such interactions. Such interactions are difficult to study *in vivo*. We have translated synthetic, nonmethyl-capped RNAs in a cell-free protein-synthesizing system. The translation products were allowed to interact posttranslationally to form protein–protein complexes. The chemical nature of the protein interaction(s) was determined by coimmunoprecipitation of associating proteins, sedimentation through sucrose gradients, followed by $NaDodSO_4$/polyacrylamide gel electrophoresis or by nonreducing $NaDodSO_4$/polyacrylamide gel electrophoresis. The system has been utilized to show the self-assembly of monomeric VP1, the major structural protein of simian virus 40, into disulfide-linked pentamers and to show the noncovalent interaction of another structural protein, VP3, with VP1 at low monomer concentrations. Additionally, we show that the carboxyl-terminal 40 amino acids of VP3 are essential and sufficient for its interaction with VP1 *in vitro*. The *in vitro* assay system described here provides a method for identifying the domains involved in, and the molecular nature of, protein–protein interactions, which play an important role in such biological phenomena as replication, transcription, translation, transport, ligand binding, and assembly.**

Within the cell, intramolecular and intermolecular interactions of polypeptide chains are essential for a large array of biological events ranging from enzymatic activity to viral assembly. How protein chains contribute to noncovalent and/or covalent interactions among polypeptide subunits of a protein molecule is difficult to study *in vivo*, where a functional assay system for a multistep pathway is often lacking. It has been established that the amino acid sequence of a protein determines its three-dimensional structure (1), and there is evidence that a polypeptide chain folds into a native form via distinct pathways and intermediates (2, 3). A role of the amino acid sequence in the folding pathway or subunit association has been determined for the tail-spike protein of *Salmonella* phage P22 by using temperature-sensitive mutants (4). Thus, in principle, recent advances in protein engineering via coding sequence modification make protein folding and subunit association studies possible *in vivo* as well as *in vitro*. However, mutated proteins are often susceptible to protein degradation *in vivo*, and this makes an assessment of the role of amino acid alteration in protein folding and in protein–protein interaction difficult. Thus, it is important to develop an *in vitro* assay system in which one can observe protein–protein interaction in a controlled way, examine the chemical environment for the interaction, and examine the contribution of the amino acid context of a polypeptide chain to the interaction. Accordingly, we have tested a eukaryotic *in vitro* translation system for the study of protein–protein interactions. Specifically, we have synthesized RNAs for the structural proteins of simian virus 40 (SV40), and translated them in a reticulocyte lysate. The translation products, which are the monomeric viral proteins, were then allowed to interact. In such reactions, where the concentration of protein is low, we were able to observe covalent interactions among VP1 chains and noncovalent interactions between VP1 and VP3. Using such a protein interaction assay system, we have been able to localize a protein domain on VP3 that contains a determinant for VP1 affinity.

Due to the relative simplicity of its gene structure, SV40 has been used as a model system in a number of studies of virus assembly, structure, and function (5). The three viral structural proteins, VP1, VP2, and VP3, are synthesized in the cytoplasm during infection and are transported into the nucleus, where assembly into a virion with an SV40 minichromosome takes place. In addition to their functions as viral structural proteins, VP1, VP2, and VP3 appear to play a role in transcriptional activity (6, 7), in nucleosome unfolding (8), and in nucleosome spacing (9). During virion assembly, VP3 replaces the host histone H1 in the SV40 minichromosome and is encapsidated with VP1 capsomeres (10, 11). Thus, it appears that there are several functions encoded in the sequences of these proteins. Furthermore, the viral structural proteins are transported to the nucleus in a precise molar proportion that reflects their ratio in the virion particle, irrespective of the amount synthesized in the cytoplasm (12). Thus, the interactions of coat proteins with each other appear to take place in the cytoplasm. The *in vitro* protein interaction assay system described here provides a method for identifying the domains involved in, and the molecular nature of, protein–protein interactions. A system providing such information should have many useful applications.

MATERIALS AND METHODS

Plasmids. Plasmids Sp6VP1, Sp6VP3, and Sp6VP3ΔC35 are derived from the transcription plasmid pSp64 vector (13) and contain coding sequences for SV40 VP1 (SV40 DNA nucleotides 1467–2666), VP3 (SV40 nucleotides 833–1716), and VP3ΔC35 (SV40 nucleotides 1078–1509), respectively. The construction of Sp6VP1 will be described elsewhere (unpublished data). For the construction of Sp6VP3, the *Hha* I–*Pvu* II fragment of SV40 DNA (SV40 nucleotides 833–1716) was inserted into pSp64 via its *Pst* I and *Sma* I polylinker sites. Sp6VP3ΔC35 was constructed by exchanging the *Avr* II–*Eco*RI fragment of Sp6VP3 (SV40 nucleotides 1078–1716) with that of pSVP23ΔC35 (SV40 nucleotides 1078–1509) (15). Thus, VP3ΔC35, a truncated SV40 VP3, lacks its carboxyl-terminal 35 amino acids. Prior to *in vitro* transcriptions,

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Abbreviation: SV40, simian virus 40.

Sp6VP1 was linearized with *Sst* I, and both Sp6VP3 and Sp6VP3ΔC35 were linearized with *Eco*RI.

***In Vitro* Transcription and Translation.** Linearized Sp6VP1, Sp6VP3, and Sp6VP3ΔC35 DNAs (1 μg) were transcribed as described by Melton *et al.* (13) except for the reduction of GTP to 0.1 mM and for the additional presence of 0.5 mM nonmethylated cap analogs [G(5')ppp(5')G; Pharmacia]. One microgram of each of the synthetic capped RNAs was translated in rabbit reticulocyte lysates as recommended by the manufacturer (New England Nuclear) in the presence of 50 μCi (1 Ci = 37 GBq) of [^{35}S]methionine (3000 Ci/mmol) or 150 μCi of [^{3}H]leucine (147 Ci/mmol), 150 mM potassium acetate, 1 mM magnesium acetate, and 7 units of RNasin. In some experiments, reticulocyte lysates from Promega Biotec (Madison, WI) were used according to the manufacturer's specifications. Translation was carried out for 1 hr at 30°C.

Purification of β-Galactosidase and Truncated VP3 Fusion Proteins. Plasmids B290E1 and B290F6 are derivatives of PUR290 (16) that respectively contain SV40 nucleotides 1046 to 1493 and 1493 to 1708 (ligated through a *Hin*dIII site of PUR290) and lead to the expression of β-galactosidase fusion proteins E1 and F6, which respectively contain an internal 150 amino acids of VP3 (aa44 to 194) and the carboxyl-terminal 40 residues of VP3 (aa194 to 234). The fusion proteins were isolated from *Escherichia coli* BMH71-18 harboring the plasmid as described by Fowler and Zabin (17), except that fusion proteins were induced with 250 μM of isopropyl β-D-thiogalactopyranoside (Boehringer Mannheim) for 3 hr. Fusion proteins were precipitated by the slow addition of an equal volume of a saturated solution of ammonium sulfate. The protein pellet was resuspended to a final concentration of 20 mg/ml [as determined by the Lowry protein assay (18)] in 1:1 (vol/vol) DEAE buffer and saturated ammonium sulfate solution, and the mixture was stored at 4°C. Prior to use, the fusion protein was diluted to 0.25 mg/ml with doubly distilled water.

Protein–Protein Interaction Assays. Labeled *in vitro* translated products were allowed to interact for 2 hr at room temperature in the same translation reaction mixture or in reaction mixtures containing reducing agent (10 mM dithiothreitol) (see figure legends). Under these reaction conditions, proteolysis is minimal. (Exogenously added radiolabeled proteins were not degraded over 8 hr of incubation in the protein–protein interaction assay system.) Interactions of proteins *in vitro* were determined by coimmunoprecipitation of two proteins (for example, coimmunoprecipitation of VP1: VP3 with anti-VP3), by sedimentation through sucrose gradients followed by immunoprecipitation, $NaDodSO_4$/PAGE analyses (19), and fluorography (20) or by nonreducing $NaDodSO_4$/PAGE (see figure legends). When the interaction of labeled translation products with *E. coli* β-galactosidase–VP3 fusion proteins were to be tested, an appropriate amount (see figure legends) of fusion proteins were added to the VP1 translation products prior to the additional protein–protein interaction incubation period. The interacting VP1 was coimmunoprecipitated with anti-β-galactosidase antibody. Anti-VP antibodies used in these studies have been described (21).

RESULTS

Monomeric VP1 Molecules Undergo Covalent Interaction to Form VP1 Pentamers *in Vitro*. We synthesized nonmethyl-capped Sp6VP1 RNA and translated the synthetic RNA in a cell-free protein-synthesizing system. The resulting translation products were assayed for intermolecular interactions by sucrose gradient sedimentation, followed by immunoprecipitations and $NaDodSO_4$/PAGE. Immediately after translation, *in vitro*-synthesized VP1 was found as 4S to 6S species (Fig. 1*A*), which were found to be monomeric VP1s of 45 kDa by nonreducing $NaDodSO_4$/PAGE (Fig. 2*A*, lane 1). Detailed studies on the nature and kinetics of VP1 pentamer formation will be reported elsewhere. Briefly, the concentration of monomeric VP1 was in the range of 0.5 to 2 μM in the translation mix. When these monomeric VP1 molecules were allowed to interact for 2 hr at 24°C, VP1 sedimented broadly in the gradient ranging from 4S to 12S species (Fig. 1*B*). When the incubated VP1 translation products were analyzed for the formation of covalent disulfide linkage by nonreducing $NaDodSO_4$/PAGE, a band corresponding to a pentameric VP1 with an apparent molecular mass of 220 kDa was observed (Fig. 2*A*, lane 2). Larger amounts of VP1 pentamers relative to VP1 monomers were formed with increased incubation periods: the oligomer formation was dependent on the concentration of VP1 monomers and on the incubation time (unpublished data). The covalent mutual interactions of VP1 could be disrupted by the reducing agent dithiothreitol (Fig. 2*A*, lane 3). The denatured and alkylated VP1 was incapable of the oligomer formation (unpublished data). Thus, monomeric VP1 molecules self-assemble into disulfide-linked VP1 pentamers *in vitro*.

VP3 Interacts with VP1 Noncovalently *in Vitro*. It has been shown that VP1 of polyoma virus, a virus closely related to SV40, contains sufficient structural information for association into pentamers and subsequently into empty viral capsomeres under conditions favoring crystallization *in vitro* (24, 25). As indicated above, we have shown that in SV40, the covalently-linked pentamer formation via disulfide bridge(s) occurs even at low monomer concentration. Thus, in both viruses, the coding sequence of the major coat protein, VP1, possesses determinants for pentamer formation even at low protein concentration.

During virus assembly, the structural proteins VP1, VP2, and VP3 and an SV40 minichromosome interact to form a virion particle. Thus, at some stage after synthesis, VP1 and

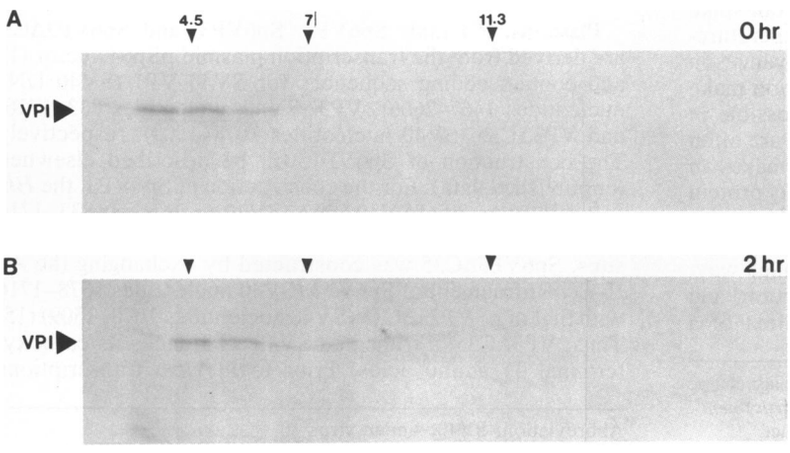

FIG. 1. Monomeric VP1s interact to form VP1 oligomers *in vitro*. *In vitro* translated, ^{35}S-labeled VP1 (a typical translation product from 1 μg of synthetic VP1 RNA) was layered onto 5–20% sucrose gradients immediately after translation (*A*) or subsequent to a 2-hr incubation (*B*) and was allowed to sediment for 23 hr at 35,000 rpm in a Beckman SW 41 rotor at 4°C. Fractions (0.75 ml) were collected and immunoprecipitated with polyclonal anti-VP1 antisera (10 μl) as described (14). Immunoprecipitates were washed, eluted, and subjected to $NaDodSO_4$/PAGE and fluorography. Sedimentation markers were bovine serum albumin (4.5 S), IgG (7 S), and catalase B (11.3 S). The position for VP1 is indicated at the left of each gradient.

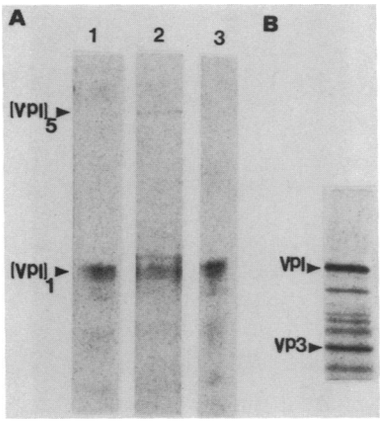

FIG. 2. VP1 self-assembles into disulfide-linked oligomers and interacts with VP3 *in vitro*. (*A*) One-fifth (10 μl) of the translation reaction containing [^{3}H]leucine-labeled VP1 was subjected to nonreducing (no dithiothreitol) NaDodSO$_4$/PAGE either immediately after translation (lane 1), subsequent to a 2-hr incubation at 24°C (lane 2), or subsequent to a 2-hr incubation in the presence of 10 mM dithiothreitol (lane 3). Prior to loading onto 8% gels, an equal volume of double-strength Laemmli buffer without dithiothreitol was added to each sample, and the samples were incubated for an additional 15 min at 30°C. Immediately after translation, only VP1 monomers [$(VP1)_1$] are present (lane 1). After a 2-hr incubation, VP1 pentamers [$(VP1)_5$] have emerged (lane 2). The pentamers are disrupted in the presence of dithiothreitol (lane 3). (*B*) [^{35}S]Methionine-labeled cotranslation products of Sp6VP1 and Sp6VP3 RNAs were subjected to immunoprecipitation with affinity-purified anti-VP3 antibody. Affinity-purified anti-VP3 was a gift of B. Fung (Tufts University School of Medicine, Medford, MA) and was prepared by the procedure described by Olmsted (22) using NaDodSO$_4$/PAGE-purified VP3. The smaller molecular mass non-VP3 bands are early termination products (23) of VP1 and make up 10% to 30% of the total translation products depending on the quality of lysate used (see also Fig. 4). The molar ratio of VP1:VP3, as determined by densitometry, is 1:2.

VP2 or VP1 and VP3 are expected to interact *in vivo*. Unlike VP1, which contains seven cysteines, neither VP2 nor VP3 contain any cysteine residues; and the interactions of VP1 with VP2 or with VP3 are expected to be purely noncovalent. To test whether an interaction between VP1 and VP3 may be detectable in our *in vitro* protein interaction assay system, synthetic capped Sp6VP1 and Sp6VP3 transcripts were cotranslated *in vitro*, and the translation products were then incubated for 2 hr and assayed for complex formation in sucrose sedimentation gradients. Upon cotranslation with VP1, the sedimentation profile of VP3 shifted drastically with a peak in the region of 10S to 12S species, while VP1 sedimented broadly in the gradient (4S to 15S species) (Fig. 3*B*) with a sedimentation profile similar to that observed in Fig. 1*B*. Under identical translation and incubation reactions, *in vitro* synthesized VP3 by itself remained as 4S to 5S species (Fig. 3*A*); after incubation, the VP3 sedimentation profile was unchanged from that obtained immediately after translation (data not shown). A sedimentation profile similar to that observed after cotranslation was observed when synthetic VP1 RNA and VP3 RNA were separately translated and then translation products were mixed during the incubation period (data not presented). After cotranslation, when proteins were reacted with affinity-purified anti-VP3 antibody, VP1 coprecipitated with VP3 (Fig. 2*B*), confirming that the sedimentation shift of VP3 involves interaction with VP1. Thus, VP3 interacts with VP1 *in vitro*. The interaction of VP3 with VP1 is noncovalent because the VP3 monomer migrated separately from the VP1 pentamer in nonreducing and denaturing NaDodSO$_4$/PAGE (not shown).

The sedimentation profiles for VP1 and VP3 in Fig. 3*B* suggest that VP3 interacts more with VP1 oligomers than with VP1 monomers. Densitometric measurements for both VP1 and VP3 bands in the 12S region indicated the relative VP1/VP3 molar intensity ratio to be about 1 to 2. This ratio is clearly less than that observed in virions or in cell nuclei (12, 26). This observation agrees with the immunoprecipitation study with affinity-purified anti-VP3 (Fig. 2*B*). In the presence of reduced and alkylated VP1, however, VP3 did not form higher sedimenting complexes (preliminary results). Thus, it appears that VP3 is interacting with disulfide-linked oligomeric VP1 and not monomeric VP1.

The Carboxyl-Terminal 40 Amino Acids of VP3 Are Essential and Sufficient for Its Interaction with VP1. We used the *in vitro* protein interaction assay system to investigate the contribution of the carboxyl-terminal region of VP3 to its affinity for VP1. The plasmid Sp6VP3ΔC35 encoding a truncated VP3 (VP3ΔC35) lacking its carboxyl-terminal 35 amino acids was constructed. Synthetic capped Sp6VP3ΔC35 transcripts were synthesized and translated *in vitro*. Analogous to wild-type VP3, VP3ΔC35 remained a 4S to 5S species after translation and after a subsequent 2-hr incubation (Fig. 4*A*). In contrast to wild-type VP3, however, VP3ΔC35 did not show a shift in its sedimentation profile upon cotranslation with VP1 (Fig. 4*B*). While cotranslated VP1 oligomerized as expected, VP3ΔC35 remained a 4S to 5S species. The lack of interaction between VP3ΔC35 and VP1 is further shown in Fig. 4*C*, in which the top seven fractions

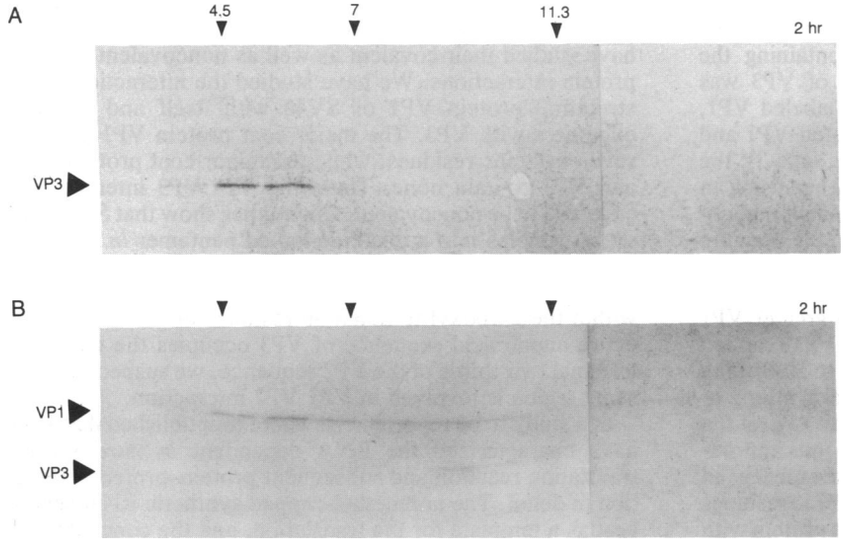

FIG. 3. VP3 interacts with VP1 *in vitro*. (*A*) *In vitro* translated ^{35}S-labeled VP3 was incubated for 2 hr at room temperature prior to sucrose gradient sedimentation. Proteins in fractions were immunoprecipitated with 15 μl of anti-VP3 antisera as described for Fig. 1*A*. (*B*) *In vitro* ^{35}S-labeled VP1 and VP3 (cotranslation products of Sp6-VP1 and Sp6-VP3 RNAs) were allowed to interact for 2 hr at room temperature prior to sucrose gradient sedimentation. Proteins were immunoprecipitated with a mixture of 10 μl of anti-VP1 and 15 μl of anti-VP3 antisera. Positions for VP1 and VP3 polypeptide chains are marked as are sedimentation markers of 4.5, 7, and 11.3 S as in Fig. 1.

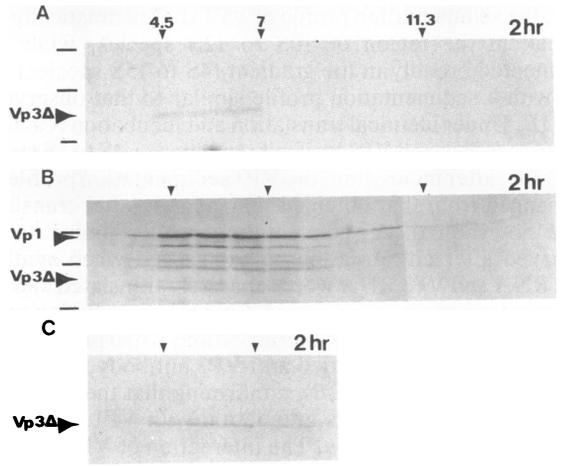

FIG. 4. A truncated VP3ΔC35 does not interact with VP1. (*A*) *In vitro* translated, labeled VP3ΔC35 was allowed to interact for 2 hr prior to sucrose gradient sedimentation and was immunoprecipitated with anti-VP3 antisera. (*B*) *In vitro* synthesized, labeled VP1 and VP3ΔC35 were allowed to interact for 2 hr prior to sucrose gradient sedimentation and were immunoreacted with anti-VP1 and anti-VP3 antibodies. The VP3ΔC35 band is seen as a faint band just above the smallest truncated VP1 band. The non-VP3ΔC35 lower molecular mass bands (truncated VP1 bands) are early termination products of VP1 translation and do not immunoreact with anti-VP3 (see below). (*C*) The top seven fractions from a sucrose gradient similar to that shown in *B* were immunoreacted with affinity-purified anti-VP3, and the immunoprecipitates were subjected to $NaDodSO_4$/PAGE. Positions for VP1, VP3, and VP3ΔC35 (VP3Δ) are marked as are sedimentation markers of 4.5, 7, and 11.3 S as in Fig. 1. Horizontal bars in *A* and *B* indicate from top to bottom the position of molecular mass markers ovalbumin (46 kDa), carbonic anhydrase (30 kDa), and lysozyme (14.3 kDa).

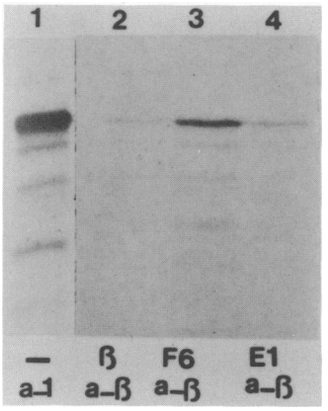

FIG. 5. Interaction of VP1 with β-galactosidase-VP3 fusion proteins *in vitro*. One-tenth (5 μl) of the translation reaction containing labeled VP1 was allowed to interact with 0.5 μg (2 μl) of β-galactosidase (β), F6 (F6), or E1 (E1) (lanes 2, 3, and 4, respectively) for 2 hr at 30°C in a total of 7 μl. The reaction mixtures were then treated with 15 μl of anti-β-galactosidase antisera (a-β), and the volumes were increased to 200 μl. Final concentrations in the immunoreaction mixtures were 0.15 M NaCl, 0.5% Triton X-100, 0.5% deoxycholate, and 10 mM EDTA. The immunoreaction was carried out for 2 hr at 4°C. Thirty microliters of a 50% solution of *Staphylococcus aureus* cells were then added, and incubation was continued for another hour. Bacterial cells were centrifuged and washed five times with 400 μl of 0.15 M NaCl/0.5% Triton X-100, and the immunoprecipitate was eluted as described (12) and subjected to $NaDodSO_4$/PAGE and fluorography. Labeled VP1 immunoprecipitated with anti-VP1 antiserum (a-1) is shown in lane 1. For quantitations, the intensities of the coprecipitated VP1 bands were compared by densitometry. An equivalent amount of VP1 was immunoreacted with excess anti-VP1 antibodies (lane 1), and the precipitated VP1 was designated as the total labeled VP1. Of the labeled VP1, 35% was coimmunoprecipitated with F6 in an F6 excess reaction by the anti-β-galactosidase antiserum (lane 3), while only 7% to 8% of the labeled VP1 coprecipitated in the presence of β-galactosidase (lane 2) or E1 (lane 4).

from a gradient similar to the one in Fig. 4*B* were immunoprecipitated with an affinity-purified anti-VP3 antibody. The antibody precipitated truncated VP3ΔC35 but not the VP1 present in those fractions. Thus, the carboxyl-terminal 35 amino acids of VP3 are essential for VP1:VP3 interaction *in vitro*.

The lack of interaction of the truncated VP3ΔC35 with VP1 could imply that the carboxyl-terminal residues contain a determinant for the VP1:VP3 interaction. Alternatively, the carboxyl-terminal deletion could result in a gross structural alteration that in turn affected the VP1:VP3 interaction. To distinguish between these two alternatives, we tested *in vitro* for the interaction of VP1 with *E. coli* β-galactosidase-VP3 fusion proteins, denoted F6 and E1, in which coding sequences for segments of VP3 occupy the carboxyl-terminal portion of the fusion proteins. When F6 containing the carboxyl-terminal 40 residues (aa194 to 234) of VP3 was allowed to interact with *in vitro* synthesized, labeled VP1, anti-β-galactosidase antibody immunoprecipitated VP1 and truncated VP1 molecules as well as F6 (Fig. 5, lane 3); the VP1 band pattern was similar to that observed when using an anti-VP1 reaction (Fig. 5, lane 1). Although a small amount of VP1 (7% to 8% of labeled VP1) was found in the reaction with unfused β-galactosidase or with E1 fusion proteins, which contain an internal 150 residues (aa44 to 194) of VP3 (Fig. 5, lane 2 or 4, respectively), 5- to 6-fold greater VP1 (35% to 45% labeled VP1) was found in the F6-VP1 reaction than in the above two reactions. Since only 40% to 50% of the *in vitro* synthesized VP1 was in an oligomeric form after 2 hr of incubation (unpublished data), then the 35% to 45% of the labeled VP1 that interacted with the F6 corresponds approximately to the entire oligomeric VP1 available (see the legend to Fig. 5). Thus, while the addition of the internal 150 residues of VP3 did not affect the interaction of the fusion protein with VP1, the addition of the carboxyl-terminal 40 residues of VP3 enhanced that interaction by 5-fold. We thus conclude that the carboxyl-terminal 40 residues of VP3 are sufficient for its interaction with VP1 *in vitro* and, hence, contain a determinant for the VP1:VP3 interaction.

DISCUSSION

We have taken advantage of a eukaryotic *in vitro* translation system to establish an *in vitro* protein–protein interaction assay system. We have used synthetic RNAs and cell-free protein-synthesizing reactions to produce monomeric polypeptide chains, have allowed them to interact *in vitro*, and have studied their covalent as well as noncovalent protein–protein interactions. We have studied the interaction of the structural protein VP1 of SV40 with itself and of these oligomers with VP3. The major coat protein VP1 contains seven cysteine residues, while the minor coat proteins VP2 and VP3 contain none. Thus, the VP1:VP3 interaction is expected to be noncovalent. Our studies show that SV40 VP1 self-assembles into a disulfide-linked pentamer *in vitro* and that a noncovalent interaction between VP1 and VP3 occurs *in vitro*. A determinant for the interaction with VP1 residues within the carboxyl-terminal 40 residues of VP3. Since the entire amino acid sequence of VP3 occupies the carboxyl-terminal two-thirds of the VP2 sequence, we suspect that the same region is involved in VP1:VP2 interaction.

In a study to be reported elsewhere (unpublished data), we have characterized the RNA dependent *in vitro* protein translation reaction and subsequent protein–protein interaction in detail. The nonmethyl-capped synthetic RNA served best as a template for the translation, and the concentration

of monomeric polypeptide chains in a typical translation reaction is low, in the range of 0.5 to 2 μM. This relatively low-yield translation reaction or low monomer concentration made the interaction study feasible. Unlike *in vivo* situations, the protein degradation is minimal in the *in vitro* system; thus, the protein–protein interaction is readily detected at low concentrations of polypeptide chains.

In the studies presented here, protein–protein interactions were allowed to take place in the medium used for cell-free protein synthesis, which includes a moderate amount of thiol reagent (0.04–0.66 mM dithiothreitol, as specified by the manufacturers). Thus, the VP1 self-assembly into disulfide-linked pentamers appears to be quite tolerant of the presence of moderate amounts of reducing agent. However, this finding may not be a surprising one, since the virion assembly takes place in an environment with a relatively high concentration of intracellular glutathione (27). Our studies presented here and elsewhere (unpublished data) support the idea that the observed VP1 pentamer formation *in vitro* is nonrandom with regard to disulfide bond formation. Whether the disulfide bridge(s) reflects true covalent linkage that occurs *in vivo* or reflects nonnative disulfide linkage(s) must be determined by further study.

The same reaction conditions also supported a noncovalent VP1:VP3 interaction. We have shown that the carboxyl-terminal 40 residues of VP3 contain the determinant for this interaction. The presence of a cluster of positively charged residues in the carboxyl-terminal domain suggests that the VP1:VP3 interaction might involve electrostatic interactions. However, the precise chemical nature of the interaction, the location of the interaction site on VP1, and the precise amino acid residues of VP1 and VP3 carrying the instructions for the VP1:VP3 interaction await further studies.

The same carboxyl-terminal residues of VP3 that cause association with VP1 have also been shown to carry a nuclear localization signal (15, 28). Our preliminary results indicate that the F6 fusion protein carrying these residues promotes the nuclear localization of the otherwise cytoplasmic β-galactosidase and its binding to DNA (unpublished results). Thus, these residues contain a nuclear transport signal, the VP1 interaction determinant, and perhaps a DNA binding signal. Of the 40 amino acid residues at the carboxyl-terminal end of SV40 VP3, only the first 13 residues share homology with polyoma virus VP3. The homology includes the putative karyophilic signal of VP3. The remaining 27 residues, including clusters of positively charged amino acids, are totally absent from the polyoma VP3. It will be surprising if all three activities found in the carboxyl-terminal SV40 VP3 residues are confined to those 13 residues that share homology with the polyoma VP3. Alternatively, the way VP1:VP3 interacts in polyoma may be quite different from that in SV40 with respect to the amino acid sequence and the location of the interactive domains. Only further experiments will clarify the nature of the interactions.

Although the structure of many proteins will eventually be elucidated to atomic resolution by x-ray analysis, the nature of the folding determinants remains an unsolved problem. Many interesting examples of enzyme catalysis involve larger enzymes that catalyze complex multisubstrate reactions. Furthermore, many enzymes are oligomers with interesting subunit interactions that are responsible for the multisubstrate reactions. Thus, critical protein–protein contacts are likely to play an important role in the activation function of many biological events, including replication, transcription, translation, transport, ligand binding, and assembly. How overall interactions in forming the assembled structure take place *in vivo* depends on the primary amino acid sequence of each polypeptide chain and on many intricate controls exerted by cellular organelles. Recent advancements in genetic engineering have allowed the expression and production of subunit proteins in bacteria. Thus, in principle, the study of protein–protein interaction should be feasible *in vitro* with pure subunit polypeptide chains. However, protein overproduction in microbial cells often leads to a situation where active protein is obtainable only by denaturation and renaturation processes. Our approach appears to overcome some of these difficulties, since the protein products are monomeric and native polypeptide chains. By this approach, the identification of determinants for many intra- and intermolecular interactions should be possible. Furthermore, the chemical composition of the protein–protein interaction reaction mixture (i.e., ionic strength, pH, or presence or absence of reducing agent) can be altered by the addition of appropriate reagents after translation. Thus, the method enables one to determine and identify interactive domains and to assess the nature of the interactions. We note that it should be possible to establish an analogous bacterial protein–protein interaction assay system by using prokaryotic transcription and translation systems.

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