THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 279, No. 50, Issue of December 10, pp. 52210–52217, 2004
Printed in U.S.A.

Vaccinia Virus Intermediate Stage Transcription Is Complemented by Ras-GTPase-activating Protein SH3 Domain-binding Protein (G3BP) and Cytoplasmic Activation/Proliferation-associated Protein (p137) Individually or as a Heterodimer*

Received for publication, September 24, 2004
Published, JBC Papers in Press, October 6, 2004, DOI 10.1074/jbc.M411033200

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Transcription of the DNA genome of vaccinia virus occurs in the cytoplasm and is temporally programmed by early, intermediate, and late stage-specific transcription factors in conjunction with a viral multisubunit RNA polymerase. The RNA polymerase, capping enzyme, and three factors (VITF-1, VITF-2, and VITF-3) are sufficient for *in vitro* transcription of a DNA template containing an intermediate stage promoter. Vaccinia virus intermediate transcription factor (VITF)-1 and -3 are virus-encoded, whereas VITF-2 was partially purified from extracts of uninfected HeLa cells. Using purified and recombinant viral proteins, we showed that the HeLa cell factor was required for transcription of linear or nicked circular templates but not of super coiled DNA. HeLa cell polypeptides of ~110 and 66 kDa co-purified with VITF-2 activity through multiple chromatographic steps. The polypeptides were separated by SDS-polyacrylamide gel electrophoresis and identified by mass spectrometry as Ras-GTPase-activating protein SH3 domain-binding protein (G3BP) and p137, recently named cytoplasmic activation/proliferation-associated protein-1. The co-purification of the two polypeptides with transcription-complementing activity was confirmed with specific antibodies, and their association with each other was demonstrated by affinity chromatography of tagged recombinant forms. Furthermore, recombinant G3BP and p137 expressed individually or together in mammalian or bacterial cells complemented the activity of the viral RNA polymerase and transcription factors. The involvement of cellular proteins in transcription of intermediate stage genes may regulate the transition between early and late phases of vaccinia virus replication.

Poxviruses contain double-stranded DNA genomes of about 200 kbp, replicate in the cytoplasm of vertebrate or insect cells, and encode enzymes and factors for DNA replication and transcription (1). Most of the information regarding poxvirus gene expression comes from studies of vaccinia virus (VAC),[1] which is the vaccine used to prevent smallpox caused by variola virus, a closely related orthopoxvirus. The VAC transcriptional program is divided into three sequentially regulated stages: early, intermediate, and late. The products of more than 20 VAC genes, including a multisubunit DNA-dependent RNA polymerase (RPO), stage-specific transcription factors, termination factors, 5′-capping and methylating enzymes, and a poly(A) polymerase are involved in the biosynthesis of viral mRNA (2). Transcription of early stage genes is mediated by proteins that are carried into the cell within the core of infectious virions. In contrast, *de novo* DNA, RNA, and protein synthesis are required for transcription of viral intermediate and late stage genes. The viral RPO, containing an associated polypeptide called RAP94, together with the viral early transcription factor heterodimer are sufficient to initiate transcription from early stage promoters *in vitro* (3–6). The viral intermediate transcription factors (VITFs) are products of early stage genes, allowing the second stage of transcription to commence when viral DNA replication occurs. The viral proteins needed for *in vitro* transcription at an intermediate promoter include the viral RPO, capping enzyme, VITF-1, and VITF-3 (7–9). VITF-1 corresponds to the RPO 30 subunit of the viral RNA polymerase, and VITF-3 is a heterodimer of 34- and 45-kDa polypeptides. An additional factor, VITF-2, was partially purified from extracts of uninfected HeLa cells (10). Some promoters originally thought to be late stage but subsequently defined as intermediate stage have eukaryotic transcription factor YinYang1 binding sites (2, 11). However, there are no data linking VITF-2 and YinYang1. Three intermediate stage proteins required for transcription of late stage genes by the viral RPO were identified through *in vitro* and *in vivo* studies (12–18). In addition, an early viral protein (19) and heterogeneous nuclear ribonucleoproteins A2/B1 and RBM3 stimulate late stage transcription *in vitro* (20).

The objectives of the present study were to confirm the requirement for a cellular intermediate stage transcription factor, purify the protein, and identify the gene encoding it. We enhanced the specificity of the *in vitro* transcription reaction, making it entirely dependent on a factor from extracts of uninfected HeLa cells. This assay was then used to monitor the presence of the transcription factor in column chromatography fractions. Polypeptides of 110- and 66-kDa co-purified as a heterodimer with VITF-2 activity and were identified as Ras-GTPase-activating protein SH3 domain-binding protein (G3BP) (21) and p137 (22) recently named cytoplasmic activation/proliferation-associated protein-1 (23). Recombinant forms of the two polypeptides were expressed in a heterolo-

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[1] The abbreviations used are: VAC, vaccinia virus; RPO, RNA polymerase; VITF, viral intermediate transcription factor; G3BP, Ras-GTPase-activating protein SH3 domain-binding protein; Ni-NTA, nickel-nitrilotriacetic acid; PMSF, phenylmethylsulfonyl fluoride; ORF, open reading frame; HA, hemagglutinin; mAb, monoclonal antibody; HPLC, high pressure liquid chromatography.

gous system and shown to have VITF-2 activity in transcription assays.

EXPERIMENTAL PROCEDURES

Transcription Assay—The VAC RPO, with 10 histidine residues attached to the C terminus of the RPO22 subunit, was isolated with associated capping enzyme and VITF-1 activities by chromatography on nickel-nitrilotriacetic acid (Ni-NTA)-agarose (Qiagen) as described (24). Recombinant VITF-3 was produced in bacteria and purified with Talon (Clontech) metal affinity resin (8). The template used for *in vitro* transcription consisted of a pUC-13-based plasmid containing the G8R intermediate promoter followed by an ~300-bp sequence lacking guanosine residues in the non-template strand (G-less cassette), which was linearized by HindIII digestion. VAC RPO, recombinant VITF-3, and cellular extract in 20 μl of 40 mM Tris-HCl, pH 8.0, 50 mM NaCl, 15% glycerol, 2 mM dithiothreitol, 0.2 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) were held on ice for 10 min. Then 0.1 mg of linear DNA template was added; after 10 more min on ice, 20 μl of solution containing 5 mM Tris-HCl, pH 8.0, 0.35 mM EDTA, 5 mM $MgCl_2$, 5 mM ATP, 0.5 mM CTP, 0.05 mM UTP, 10 μCi of [α-^{32}P]UTP, and 4.8% (v/v) polyvinyl alcohol was added, and the reaction was allowed to proceed for 30 min at 30 °C. The RNA was digested with 500 units of RNase T1 for 15 min at 37 °C and then 0.18 ml of stop solution (0.3% SDS, 0.15 M NaCl), 0.1 ml of phenol, and 0.1 ml of chloroform/isoamyl alcohol (24:1) were added. After mixing vigorously for 15 s, the material was centrifuged; the aqueous phase was recovered, and 10 μg of rRNA, 20 μl of 3 M NaOAc, and 0.5 ml of ethyl alcohol were added. The mixture was stored at −20 °C overnight and centrifuged; the pellet was dried in a Speedvac (Savant) and suspended in 30 μl of 0.025% xylene cyanol, 0.025% bromphenol blue, 10 mM EDTA, and 98% formamide. The samples were applied to a 4% polyacrylamide/urea gel and electrophoresis was conducted at 300 V for 1.5 h. The gel was washed in water and dried onto DE81 chromatography paper (Whatman), placed into a cassette, and exposed to x-ray film for 12 h.

Preparation of Cell Extract and Purification of VITF-2—HeLa S3 cells (3.6×10^{10}) were collected by centrifugation, washed twice in phosphate-buffered saline, and washed once in 5 volumes of 10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 1.5 mM $MgCl_2$, and 2 mM β-mercaptoethanol. The cells were resuspended in 2 volumes of above buffer, Dounce homogenized, and centrifuged. The pellet was resuspended in 0.5 volume of 20 mM Tris-HCl, pH 8.0, 20% (v/v) glycerol, 0.6 M NaCl, 1.5 mM $MgCl_2$, 2 mM β-mercaptoethanol, and 0.5 mM PMSF, Dounce homogenized, and left on ice for 30 min with gentle vortexing every 5 min. The lysate was centrifuged at 27,000 × *g* for 30 min, and the supernatant (75 ml) was collected.

Approximately 945 mg of extract protein (12.6 mg/ml × 75 ml) was mixed with 20 ml of Ni-NTA agarose at 4 °C overnight. The slurry was poured into a column and washed with 10 volumes of buffer A (20 mM Tris-HCl, pH 8.0, 15% glycerol, 0.5 M NaCl, 1.5 mM $MgCl_2$, 2 mM β-mercaptoethanol, 0.01% Triton X-100, and 0.5 mM PMSF). The beads were washed successively with 10-column volumes of buffer A with 0.1 M NaCl and 20 mM imidazole and 10-column volumes of buffer A with 0.1 M NaCl, 80 mM imidazole. The peak activity fractions eluting with 20 mM imidazole were pooled (90 ml, 5.22 mg/ml of protein) and dialyzed against buffer B (20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 10% glycerol, 0.1 mM EDTA, 0.01% Triton X-100, and 1 mM dithiothreitol). All subsequent column chromatography steps were carried out with 10-column volumes of linear NaCl gradients (0.05–0.5 M NaCl) in buffer B with dialysis against buffer B between each step. The protein was divided into two aliquots and applied to two 15-ml phosphocellulose columns (P11, Whatman); maximal transcriptional activity was eluted between 0.24 and 0.32 M NaCl. Pooled peak fractions (56 ml, 0.69 mg/ml) were applied to a 7.9-ml HQ/M column (Poros) and maximal transcriptional activity was eluted between 0.39 and 0.46 M NaCl. Pooled peak fractions (20 ml, 0.4 mg/ml) were applied to a 1.7-ml heparin (HE/M, Poros) column. Maximal transcriptional activity (5 ml, 0.69 mg/ml) was eluted between 0.35 and 0.48 M NaCl. A 0.3-ml sample was layered onto an 11-ml, 15–35% glycerol gradient in buffer B, which was centrifuged in a SW41 rotor in a Beckman L8–80 M ultracentrifuge for 26 h at 41,000 rev/min. Fractions were collected from the bottom, and peak activity in fractions 12–15 was pooled. An additional 0.8 ml of the pooled heparin fractions was applied to a 0.8-ml column of poly(A)-Sepharose (Amersham Biosciences) and peak transcriptional activity was eluted between 0.32 and 0.39 M NaCl.

Construction of VAC G3BP and p137 Expression Vectors and Purification of Recombinant Proteins—PCR primers were used to copy the G3BP open reading frame (ORF) in the plasmid pDual GC+G3BP (Stratagene) and append a NdeI site to the N terminus and a 10-histidine tag to the C terminus followed by a BamHI site. In addition a mutation in pDual GC+G3BP was corrected by changing guanosine at position 470 of the ORF to adenosine. The NdeI/BamHI fragment was inserted into the corresponding site of VAC transfer vector pVOTE.2 (25) forming pVOTE-G3BP10H. The p137 open reading frame was obtained from the hsGPI137 plasmid kindly provided by J. P. Luzio (University of Cambridge). PCR primers were used to add an NdeI site at the N terminus of the ORF and an HA tag followed by a BamHI site at the C terminus. The NdeI/BamHI fragment was then inserted into pVOTE.2 to form pVOTEp137HA. In addition, two corrections were made to the p137 open reading frame: Asp at position 210 was changed to Ala and Asp at 317 to Tyr forming pVOTEp137HAay. The latter plasmid was modified by substituting a 10-histidine tag for the HA tag and the new plasmid called pVOTEp137HisAY. The transfer vectors were used to construct recombinant VAC vG3BP10H, vp13710H, and vp137HA, which express G3BP10His, p13710His, and p137HA, respectively (25, 26).

Suspended HeLa S3 cells (3×10^9) were infected with three plaque-forming units of vG3BP10H or vp13710H in the presence of 0.5 mM isopropyl 1-thio-β-D-galactopyranoside for 20 h. The cells were harvested by low speed centrifugation, washed twice with phosphate-buffered saline, suspended in buffer C (20 mM Tris-HCl, pH 8.0, 10 mM NaCl, 1 mM β-mercaptoethanol, 0.5 mM PMSF, and 2.5 mM $MgCl_2$), placed on ice for 10 min, and Dounce homogenized. An equal volume of buffer C plus 0.83 M NaCl, 30% glycerol, 0.02% Triton X-100, and 2 mM imidazole was mixed with the lysate for 1 h, after which the lysate was centrifuged in a swinging bucket rotor at 21,000 × *g* for 30 min. The soluble material was mixed overnight with 4.5 ml of Ni-NTA-agarose, which had been pre-equilibrated with the latter buffer. The column was sequentially washed with 10 volumes of buffer D (20 mM Tris-HCl, pH 8.0, 0.42 M NaCl, 15% glycerol, 1 mM β-mercaptoethanol, 0.01% Triton

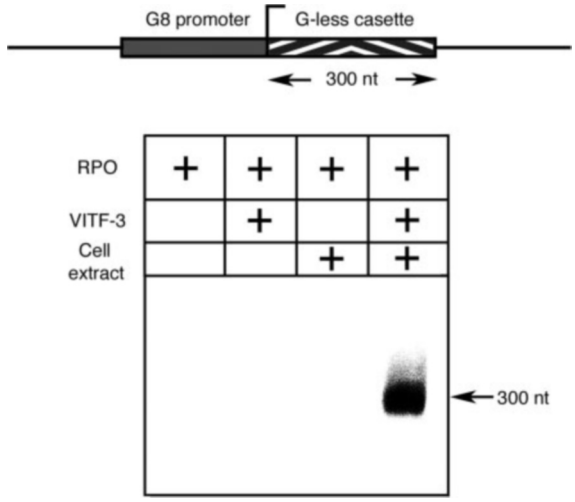

FIG. 1. **Reconstitution of intermediate transcription activity.** The *upper diagram* represents part of a plasmid containing the promoter sequence of the VAC intermediate gene G8R adjacent to a G-less cassette of ~300 bp. The plasmid was linearized by digestion with HindIII restriction enzyme and used as the template for the transcription assay. The reactions were carried out with purified viral RPO complex containing capping enzyme and VITF-1, recombinant VITF-3 made in *E. coli*, and the cell factor was eluted from Ni-NTA-agarose and ribonucleoside triphosphates including [α-^{32}P]UTP. The RNA products were digested with RNase T1 and analyzed by PAGE. An autoradiogram is shown with the expected transcript size of 300 nt indicated by an *arrow*.

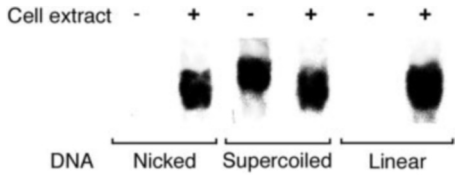

FIG. 2. **Effect of DNA topology on intermediate transcription.** Transcription reactions were carried out as described in the legend to Fig. 1 with a template that was either nicked, supercoiled, or linearized with (+) or without (−) an extract from uninfected HeLa cells.

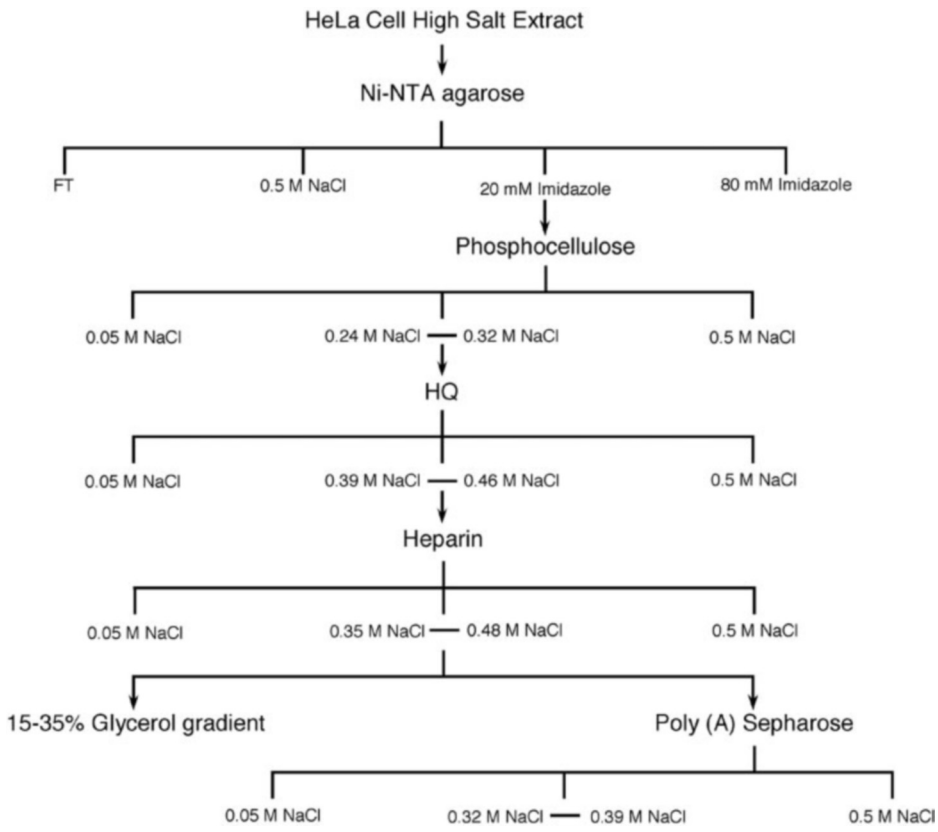

FIG. 3. **Purification scheme.** VITF-2 was purified from a high salt extract of the nuclear fraction of 3.6×10^{10} HeLa cells. Activity eluted from Ni-NTA-agarose with 20 mM imidazole and VITF-2 was further purified by column chromatography. The salt gradient and the concentration at which VITF-2 was eluted are indicated. *FT*, flow through.

X-100, 40 mM imidazole, and 0.5 mM PMSF), 10 volumes of buffer D plus 1 M NaCl, and buffer D plus 0.15 M NaCl and 0.2 M imidazole. The proteins eluted in the last wash were pooled and dialyzed against buffer E (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 10% glycerol, 1 mM dithiothreitol, 0.01% Triton X-100, 0.5 mM PMSF, and 0.1 mM EDTA), and 0.5 ml of the proteins was layered on an 11-ml 15–35% (v/v) glycerol gradient in buffer E. The gradient was centrifuged in a SW41 rotor in a Beckman L8–80M ultracentrifuge for 26 h at 41,000 rpm. Additionally, suspension HeLa S3 cells (3×10^9) were infected with a mixture of vG3BP10H and vp137HA each at a multiplicity of 1.5 plaque-forming units per cell, and the proteins were purified as above except the 1 M NaCl wash and the glycerol gradient purification step were omitted.

Construction of Recombinant Escherichia coli G3BP and p37 Expression Vectors—The plasmids pVOTE-G3BP10H and pVOTEp137HisAY were digested with restriction enzymes NdeI and BamHI, and the fragments containing the G3BP and p137 ORFs were ligated to pET-11a (Novagen), which had been digested with the same enzymes, to form pET11a-G3BP10H and pET11a-p13710H. *E. coli* Bl21 Star (DE3) (Invitrogen) cells were transformed with either pET11a-G3BP10H or pET11a-p13710H, grown at 37 °C to an optical density (A_{600}) of 0.5, and induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside. After 3 h, the cells were collected by centrifugation at $3{,}440 \times g$ for 10 min and resuspended in B-PER reagent (Pierce). NaCl, β-mercaptoethanol, and PMSF were added to final concentrations of 200, 1, and 0.5 mM, respectively. After rotating for 10 min at room temperature, the mixture was centrifuged at $27{,}000 \times g$ for 15 min. The soluble fraction was collected, and glycerol and imidazole were added to 10% (v/v) and 20 mM, respectively. The proteins were mixed overnight at 4 °C with Ni-NTA-agarose that had been equilibrated with sample buffer. The mixture was poured into a column, and the beads were washed with buffer F (20 mM Tris-HCl, pH 8.0, 15% glycerol (v/v), 0.5 M NaCl, 1 mM β-mercaptoethanol, 0.01% Triton X-100, 20 mM imidazole, and 0.5 mM PMSF). Recombinant proteins were eluted with buffer F containing 0.15 M NaCl and 0.2 M imidazole. Proteins were dialyzed against buffer F without imidazole and containing 0.15 M NaCl to make antibodies in rabbits or 40 mM Tris-HCl, pH 8.0, 15% glycerol, 50 mM NaCl, 2 mM dithiothreitol, 0.02 mM EDTA, and 0.5 mM PMSF for transcription reactions.

Expression of Proteins in Transfected Mammalian Cells—HeLa cells were grown in four T162 cm^2 flasks (Costar). Each flask with $\sim 2 \times 10^7$ cells was infected with 10^8 plaque-forming units of recombinant VAC vTF7.3 and incubated at 37 °C for 2 h. The unadsorbed virus was then removed, and two flasks were each transfected with 40 μg of vector DNA pVOTE.2 or a combination of 20 μg of pVOTE-G3BP10H and 20 μg pVOTEp137HAay with 0.2 ml of Lipofectamine 2000 (Invitrogen) in 20 ml of Opti-MEM I. After 20 h, cell monolayers were washed in phosphate-buffered saline, and 10 ml of fresh phosphate-buffered saline was applied to each flask. Cells were harvested, and the pairs were pooled and resuspended in buffer C to a final volume of 2 ml and Dounce homogenized. The lysates were placed into tubes with an equal volume of buffer C plus 0.83 M NaCl, 30% glycerol, 0.02% Triton X-100, and 1 mM imidazole. The lysate was centrifuged at $20{,}200 \times g$ for 14 min, and the supernatant was mixed with an equal volume of buffer G (20 mM Tris-HCl, pH 8.0, 15% glycerol, 1 mM β-mercaptoethanol, 0.01% Triton X-100, 0.5 mM imidazole, 0.5 mM PMSF, 2.5 mM $MgCl_2$), and 0.7 ml of Ni-NTA-agarose, which had been equilibrated with the same buffer, and incubated over night with rotational mixing. The mixture was poured into a column and washed with 15-column volumes of buffer G plus 0.42 M NaCl, followed by sequential elution in buffer H (20 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 15% glycerol, 1 mM β-mercaptoethanol, 0.01% Triton X-100, 60 mM imidazole, and 0.5 mM PMSF) and buffer H with 0.2 M imidazole.

SDS-PAGE—Proteins were resolved on a 4–20%, 10–20% gradient, or 6% polyacrylamide SDS gel (Invitrogen) and detected using the Silver Stain Plus kit (Bio-Rad Laboratories), SimplyBlue Safe Stain reagent (Invitrogen), or Coomassie Blue R-250. For immunoblotting, proteins were transferred to a nitrocellulose membrane (Micron Separations) and probed with the appropriate antibodies. The following primary antibodies were used: anti-G3BP mouse monoclonal antibody (mAb) (Pharmingen), rabbit polyclonal anti-G3BP, rabbit polyclonal anti p137, rat anti-HA-peroxidase high affinity mAb (3F10, Roche Applied Science), and anti-His6-peroxidase mouse mAb (Roche Applied Science). The secondary antibodies were either donkey anti-rabbit-peroxidase IgG or donkey anti-mouse-peroxidase IgG (Amersham Biosciences). The membranes were developed using the SuperSignal West Pico chemiluminescent substrate (Pierce) and autoradiographs prepared.

Mass Spectrometric Analysis and Protein Sequencing—Approximately 5 μg of protein was resolved in a 6% SDS-polyacrylamide gel and stained with Coomassie Blue R-250. Stained bands were excised and subjected to in-gel tryptic digestion. The samples were analyzed with a ThermoFinnigan ProteomeX HPLC/electrospray ionization mass spectrometer system consisting of a surveyor Capillary HPLC interfaced to an LCQ ion trap electrospray ionization mass spectrometer. An additional 5 μg of protein was prepared as above and subjected to in-gel tryptic digestion (27) and analyzed by automated Edman degradation on a 477A sequencer (Applied Biosystems, Foster City, CA).

RESULTS

In Vitro Transcription Assay—The requirement for a cell factor, called VITF-2, in transcription assays has varied in part because of the use of incompletely purified proteins. To purify VITF-2 it was essential to develop an assay that exhibits a high degree of dependence on the cellular protein. We previously constructed a recombinant VAC in which a histidine tag had been appended to the C terminus of the RPO22 subunit of the viral RPO, allowing us to purify the enzyme with its tightly bound capping enzyme by Ni-NTA chromatography (24). In those studies, we discovered that VITF-2 bound to Ni-NTA-agarose and was eluted with a low concentration of imidazole (unpublished). VITF-1 and VITF-3 with polyhistidine tags were expressed in bacteria and purified using metal affinity chromatography (8). VITF-1 proved not to be required in our *in vitro* intermediate transcription assay, presumably because of its presence with capping enzyme in the polymerase fraction. Synthesis of an appropriate size RNA from a linearized plasmid template containing the VAC G8R intermediate promoter followed by a 300 bp G-less cassette is shown in Fig. 1. Transcription was dependent on the cellular factor, which had been partially purified on Ni-NTA-agarose, as well as the RPO-capping enzyme complex and VITF-3. The requirement for the cellular factor depended on the topological state of the DNA. We found that the viral components were sufficient for transcription of a super coiled plasmid but that the cell factor was needed for transcription of a nicked circular or linear plasmid (Fig. 2).

Purification of VITF-2—The purification scheme starting with a high salt extract of a nuclear fraction of HeLa cells is shown in Fig. 3. The extract was passed through a Ni-NTA-agarose column, which apparently removes inhibitory factors. The unbound material was collected, and the column was washed with high salt followed by elution with 20 and 80 mM imidazole. The polypeptide composition of fractions was determined by SDS-PAGE (Fig. 4*A*). Fractions were assayed for their ability to complement viral intermediate transcription *in vitro*. No activity was detected in the unbound fractions, whereas considerable activity was eluted in 20 mM imidazole (Fig. 4*B*). Only low activity was found in the 80 mM imidazole fractions (data not shown). Fractions comprising the middle of the activity peak were negative in the transcription assay, suggesting that excess VITF-2 or another component of the fraction was inhibitory. This interpretation was confirmed by detecting activity with lower amounts of protein. Activity increased, reached a maximum, and then decreased over a 10-fold range of protein (Fig. 4*C*).

Fractions with the highest activity from the Ni-NTA-agarose column were pooled, and VITF-2 was purified successively by chromatography on phosphocellulose, HQ anion exchanger, and heparin (Fig. 3). In each step, the fractions with the highest activity were pooled. Some material from the heparin column was analyzed by glycerol gradient sedimentation (Fig. 5, *A* and *B*), and some was analyzed by chromatography on poly(A)-Sepharose (Fig. 5, *C* and *D*). Silver staining of SDS-polyacrylamide gels revealed bands of ~110 and 66 kDa in the active fractions obtained by each purification procedure. The co-purification of these two polypeptides can be seen by Coomassie Blue staining of 1 μg of protein from each step of the purification (Fig. 6*A*).

Identification of Cellular Genes Encoding the 110- and 66-kDa Polypeptides—The glycerol gradient-purified 110- and 66-kDa polypeptides were excised from the polyacrylamide gel and digested with trypsin. The resulting digests were analyzed by HPLC and mass spectrometry. Two tryptic peptides from the 66-kDa protein corresponded to the Ras-GTPase-activating protein SH3-domain-binding protein (G3BP) (21) with a high degree of certainty. A chymotryptic peptide derived from the 110-kDa polypeptide matched protein p137 (22). The latter result was confirmed by sequencing HPLC-purified peptides by the Edman degradation method.

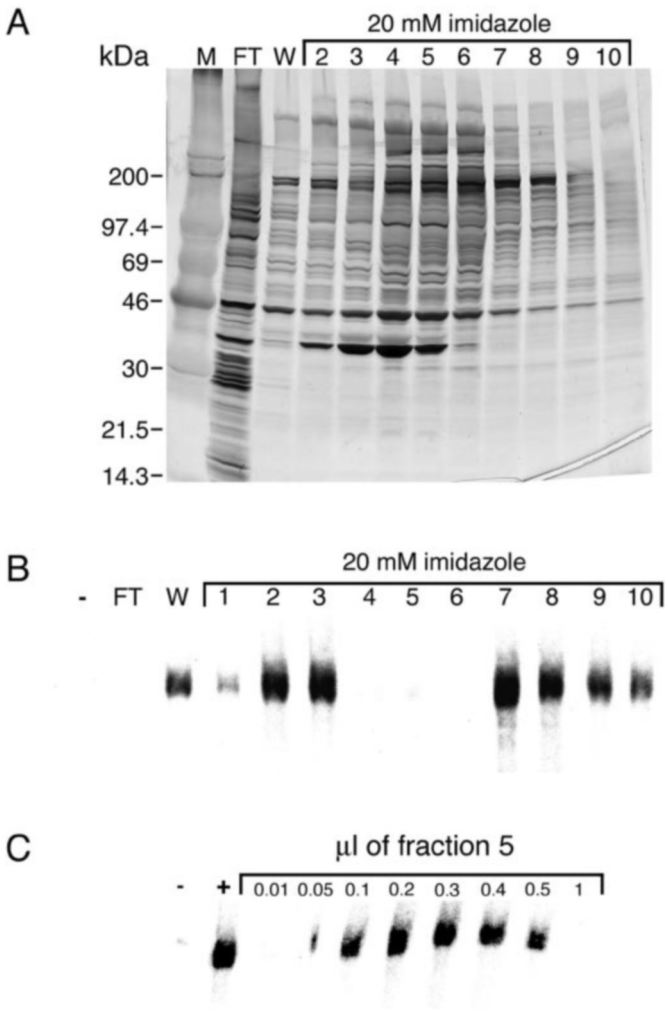

FIG. 4. **Partial purification of VITF-2 on Ni-NTA-agarose.** *A*, high salt nuclear extract of HeLa cells was incubated with Ni-NTA-agarose, and the flow through (*FT*), wash (*W*), and fractions eluted with 20 mM imidazole were collected and analyzed by SDS-PAGE and silver staining. The masses in kDa of marker proteins are shown on the *left*. *B*, fractions obtained in *A* were analyzed in the intermediate transcription assay. (−) indicates omission of uninfected cell extract. *C*, various amounts of protein from *fraction 5* were added to the transcription assay, and resultant RNAs were analyzed. Positive control (+) is a duplicate of *fraction 3* from *B*.

To confirm the identities of the 66-kDa and 110-kDa polypeptides, we expressed recombinant forms. A plasmid with the G3BP ORF from Stratagene was corrected by changing the glutamic acid at position 470 to glycine. A plasmid containing the p137 ORF was obtained from Dr. J. Paul Luzio (University of Cambridge). Based on his personal communication and our sequencing data we found a nucleotide insertion after position 1726. In addition, a BLAST search revealed a human cDNA (GenBank™ accession number BC001731) with a sequence corresponding to the p137 ORF except for two amino acid differences at position 210 and 317. Because the differences were also present in a mouse homolog, we altered the sequence of p137 at these positions. Polyhistidine tags were appended to the C termini of the G3BP and p137 ORFs, and they were inserted into a bacterial

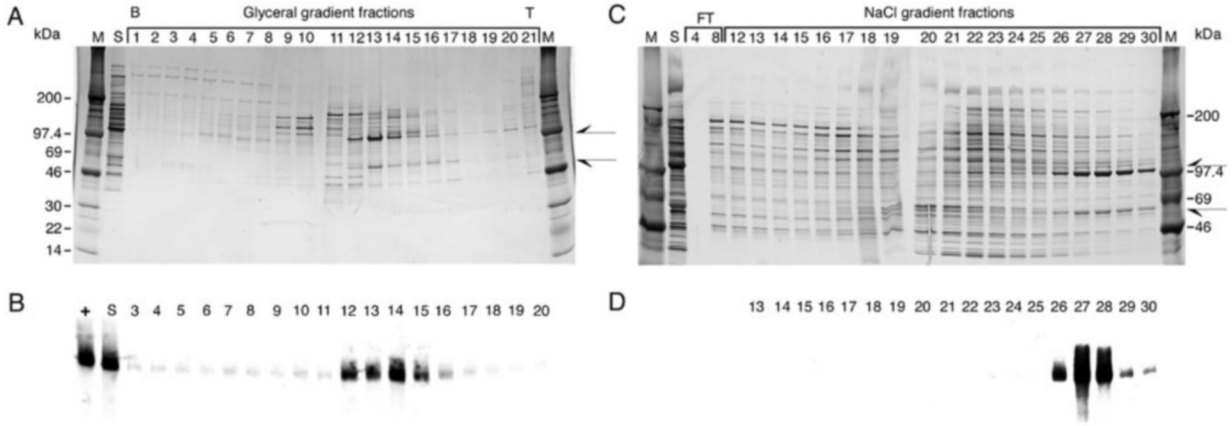

FIG. 5. **Purification of VITF-2 by glycerol gradient sedimentation and poly(A)-Sepharose chromatography.** *A*, fractions with peak transcriptional activity from the heparin column were pooled and analyzed by glycerol gradient centrifugation. Starting material (*S*) and fractions from the gradient were subjected to SDS-PAGE (4–20% gel) and silver stained. *B*, bottom; *T*, top. Masses in kDa of marker proteins (*M*) are shown on the *left*. *Arrows* point to 110- and 66-kDa bands. *B*, protein fractions from *A* were analyzed for VITF-2 activity. (+) indicates positive VITF-2 control. *C*, fractions with peak transcriptional activity from the heparin column were pooled and applied to a poly(A)-Sepharose column. Flow through (*FT*) was collected, and the column was washed with 50 mM NaCl and was eluted with a linear 50–500 mM NaCl gradient. The indicated fractions were subjected to SDS-PAGE and silver stained. Masses in kDa of marker proteins (*M*) are on the *right*. *Arrows* point to 110- and 66-kDa bands. *D*, protein fractions 13–30 from *C* were assayed for VITF-2 activity.

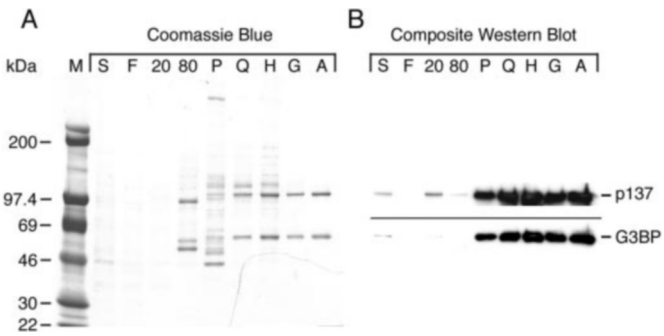

FIG. 6. **SDS-PAGE and Western blotting of proteins from successive steps of the purification.** *A*, the same quantity of protein (1 μg) of starting material (*S*), flow-through material (*F*), 20 mM imidazole (*20*), 80 mM imidazole (*80*), phosphocellulose (*P*), anion exchanger (*Q*), heparin (*H*), glycerol gradient (*G*), and poly(A)-Sepharose (*A*) were analyzed by SDS-PAGE and stained with Coomassie Blue. Masses in kDa of marker proteins (*M*) are shown on the *left*. *B*, the proteins were also analyzed by SDS-PAGE and Western blotting with rabbit polyclonal antibody to p137 and mouse mAb to G3BP. A composite of the Western blots is shown.

expression vector. The expressed proteins were purified and injected into rabbits for the production of antibodies. These antibodies, as well as a purchased mAb to G3BP, specifically reacted with the polypeptides in the purified VITF-2 fractions (Fig. 6*B*).

Recombinant VITF-2 Polypeptides Form an Active Complex—The data obtained so far indicated that G3BP and p137 co-purified with VITF-2 activity. Several approaches were used to determine whether recombinant polypeptides could replace VITF-2 in transcription assays. First we used a system in which HeLa cells were infected with recombinant VAC vTF7.3, which expresses bacteriophage T7 RNA polymerase, and co-transfected with plasmids encoding polyhistidine-tagged G3BP (pVOTE-G3BP10H) and HA-tagged p137 (pVOTEp137HAay) regulated by a T7 promoter or a control pVOTE vector plasmid (28). Cell extracts were incubated with Ni-NTA-agarose, and proteins were eluted with 60 and 200 mM imidazole. Knowing that endogenous G3BP and p137 elute at 20 mM imidizole, we anticipated that the polyhistidine-tagged G3BP would elute at a higher concentration of imidazole. Although p137 was tagged with HA instead of polyhistidine, we considered that it too would elute at higher imidazole if it were complexed to histidine-tagged G3BP. Proteins eluting at 60 and 200 mM imidazole were analyzed by SDS-PAGE. Bands corresponding to both p137HA and G3BP10H were eluted at 200 mM imidazole, and these bands were not present in the corresponding fractions from the control transfected with the pVOTE vector (Fig. 7*A*). Western blot analysis confirmed p137HA and G3BP10H as the two protein visualized by silver staining (data not shown). Importantly, the proteins eluting with 200 mM imidazole contained VITF-2 activity in the transcription assay, whereas the corresponding fractions from the control did not (Fig. 7*B*). To further establish that G3BP10H and p137HA form a heterodimer, we analyzed the two proteins expressed alone and together. Alone, G3BP10H was eluted with 180 mM imidazole, and most of p137HA eluted with 60 mM imidazole (data not shown). When both recombinant proteins were expressed together, there was a shift in the p137HA elution profile with most eluting in the 180 mM imidazole fractions together with G3BP10H as in Fig. 7.

Recombinant G3BP and p137 Expressed by Bacteria Are Active Transcription Factors—The mammalian transfection system allowed us to express G3BP and p137 together. However, it was difficult to obtain them completely free of each other because of the endogenous proteins and their binding affinity for each other. To circumvent this problem, we expressed G3BP10H and p13710H separately in *E. coli*. Each recombinant protein was purified by Ni-NTA-agarose chromatography. As a control the vector without insert was mock-expressed, and extracts were processed identically. Fractions with the highest amount of protein from each preparation were dialyzed and analyzed for VITF-2 activity. The control extract had none, whereas both recombinant G3BP and p137 exhibited activity (Fig. 8). This was surprising because we had anticipated that only one of the two polypeptides or the combination would be active.

Further Comparisons of Transcriptional Activities of G3BP and p137 Alone and Together—Having established that recombinant G3BP and p137 complement VAC intermediate transcription, we wanted to determine their relative activities as individual proteins and as a heterodimer. Because the transfection system is difficult to scale up, we made recombinant VACs vG3BP10H, vp13710H, and vp137HA that encode T7 RNA polymerase and express histidine-tagged G3BP, histidine-tagged p137, or HA-tagged p137, respectively, from induc-

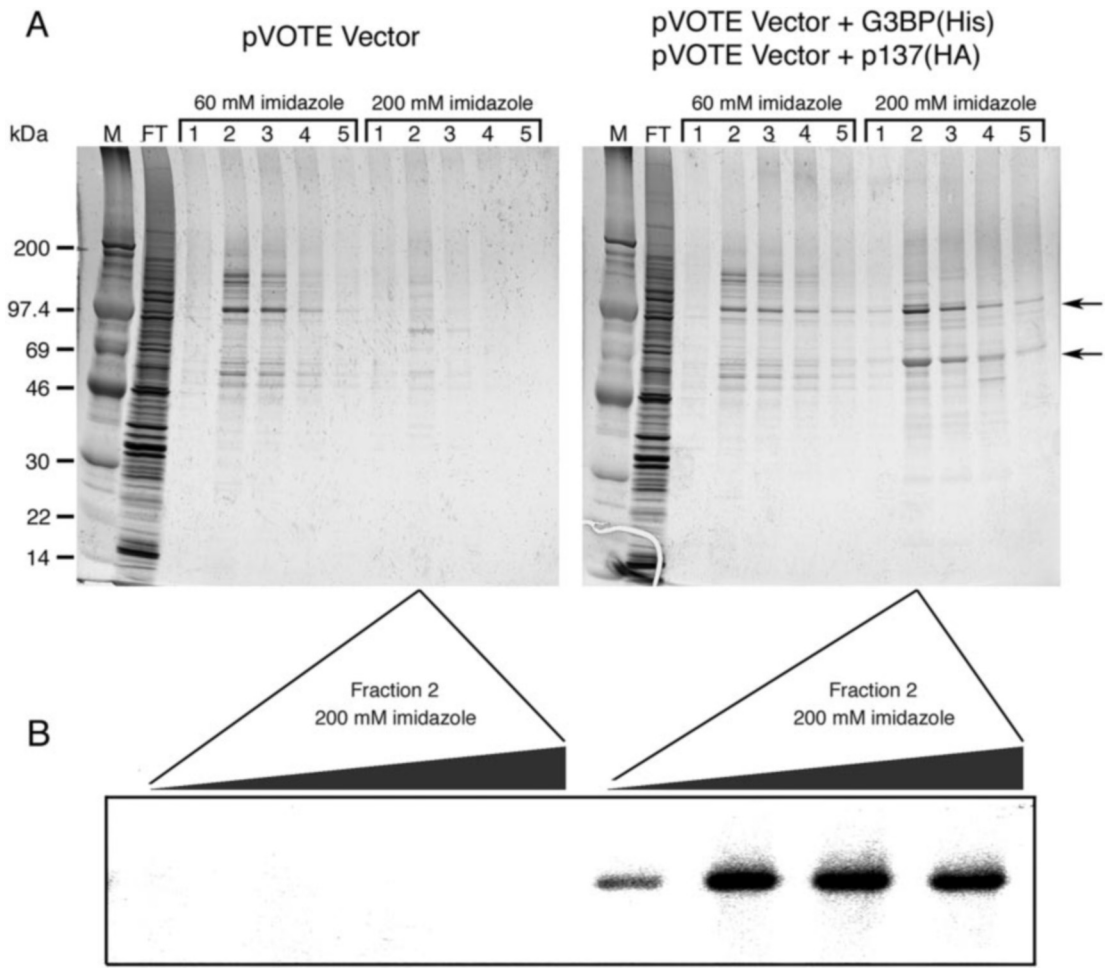

FIG. 7. **Recombinant G3BP and p137 expressed in HeLa cells form a complex with VITF-2 activity.** *A*, HeLa cells were infected with vTF7.3 and transfected with the vector plasmid pVOTE.2 or with pVOTE-G3BP10H and pVOTEp137HAay. After 20 h, a total cell extract was prepared and incubated with Ni-NTA-agarose. The beads were packed in a column and eluted with 60 and 200 mM imidazole. Proteins were visualized after SDS-PAGE by silver staining. *Arrows* on *right* indicate migration of p137 and G3BP. Masses in kDa of marker proteins (*M*) are shown on the *left*. *FT*, flow through. *B*, increasing amounts of protein from *fraction 2* of the 200 mM imidazole elution from the two Ni-NTA columns were tested for VITF-2 activity.

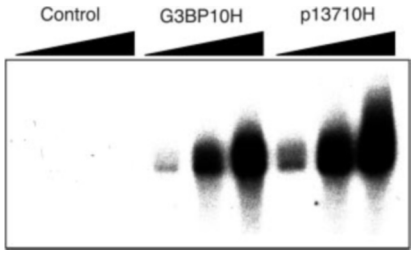

FIG. 8. **Bacterially expressed G3BP10H and p13710H have VITF-2 activity.** Bacterial cells were separately transformed with pET11a, pET11a-G3BP10H, and pET11a-p13710H. After induction of expression, cells were lysed, and extract from each was mixed with Ni-NTA-agarose. Proteins were eluted with 200 mM imidazole, and corresponding fractions were dialyzed and tested for VITF-2 activity. Increasing amounts of the imidazole fraction are indicated.

ible T7 promoters. HeLa cells were infected with vG3BP10H or vp13710H or with a 1:1 ratio of vG3BP10H and vp137HA. Extracts were prepared and proteins were purified by Ni-NTA chromatography. Because of the overexpression of the recombinant proteins compared with the endogenous levels in HeLa cells, the G3BP10H and p13710H were greater than 90% monomeric and appeared to sediment as monomers by glycerol gradient centrifugation (data not shown). When recombinant proteins were expressed together, the two polypeptides co-purified. Equal amounts of G3BP or p137 made in HeLa cells or *E. coli* or the heterodimer made in HeLa cells were added to transcription assays. Within a 2-fold range, the proteins had similar activity (Fig. 9).

DISCUSSION

The purification of VITF-2 was facilitated by improvements to the transcription assay. The use of affinity-purified histidine-tagged RPO and recombinant VITF-3 made in *E. coli* eliminated contaminating cellular proteins with VITF-2 activity. In addition, substitution of a linear DNA template for a super coiled plasmid eliminated VITF-2-independent transcriptional activity. Under the new conditions, transcription of a G-less cassette with an intermediate stage promoter was entirely dependent on a factor from uninfected HeLa cells. Although we used a high salt wash nuclear fraction as starting material for the purification, activity was also present in the cytoplasmic fraction, and the experimental conditions were not designed to determine the true intracellular distribution of VITF-2. We found that two polypeptides of 110 and 66 kDa co-purified with VITF-2 activity. Mass spectroscopy, chemical protein sequencing, and Western blotting identified the polypeptides as G3BP (21) and p137 (22). Moreover, recombinant forms of the proteins made in eukaryotic or prokaryotic cells had transcription factor activity. Evidence was obtained that the two polypeptides formed a heterodimer, explaining their co-purification. We expected, however, that the het-

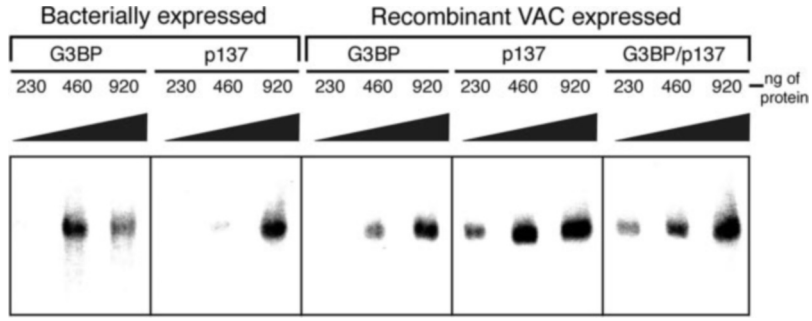

FIG. 9. **VITF-2 activity of G3BP and p137 expressed from bacteria and HeLa cells.** Proteins from bacteria were prepared as in Fig. 8. HeLa cells were infected separately with vG3BP10H, vp13710H, or with a one to one ratio of both. Cell extracts were mixed with Ni-NTA, and proteins were eluted with 200 mM imidazole and dialyzed. The amounts of protein used in VITF-2-dependent transcription assays are indicated.

erodimer or one of its constituent polypeptides would exhibit activity in the transcription assay. The finding that each polypeptide exhibited activity was unexpected and suggests they have a common feature.

The sequence corresponding to p137 was originally associated with a glycerol phosphate inositol-anchored membrane protein (22, 29). A number of errors in the published sequence of this protein were corrected in making the recombinant expression vectors (see "Experimental Procedures"). Two of the corrections, Asp at position 210 mutated to Ala and Asp at 317 mutated to Tyr, were necessary for the association of recombinant p137 with G3BP,[2] demonstrating the importance of these changes. Our studies did not suggest that the protein encoded by the gene is membrane-associated. A recent report (23) concludes that the original identification of p137 as a GPI-anchored membrane protein was an error; they found that the product of this ORF is a cytoplasmic 116-kDa protein that is up-regulated when resting T or B cells or hematopoietic progenitors are activated and offered the descriptive name cytoplasmic activation/proliferation-associated protein-1. Based on consensus sequences obtained from the National Center for Biotechnology information, Grill *et al.* (23) suggested that the protein contains an additional 53 amino acids at its N terminus, which were not present in our cDNA. Although we have not experimentally verified the additional amino acids, the protein from HeLa cells migrated slightly slower than the recombinant forms.

Considerable information is available regarding G3BP, which was first identified by its co-immunoprecipitation with Ras-GTPase-activating protein (21). This interaction was only detected in growing cells and occurs via the SH3 binding domain. The N terminus of G3BP contains a nuclear transport factor 2 domain, and the C terminus has an RNA recognition motif. G3BP has a phosphorylation-dependent selective RNase activity (30) and assembles stress granules that are believed to play an important role in RNA metabolism (31). G3BP has also been identified as part of a protein complex associated with the 3′-end of tau mRNA in neuronal cells (32) and with a ubiquitin-specific protease (33). In addition to RNA binding and RNase activity, G3BP was identified as human DNA/RNA helicase VIII (34). Given the amount of work done with G3BP, we are surprised that the association with p137 was not reported previously.

The finding that G3BP and p137 can complement viral factors for intermediate transcription raises many interesting questions. The first concerns the mechanism of action. The RNA binding and putative helicase activity of G3BP could be involved. The little information regarding p137 provides no clues regarding its role in transcription. We noticed, however, that the C termini of both p137 and G3BP are rich in Arg and Gly residues including some RGG RNA binding motifs. Possibly both proteins act by binding to the nascent RNA and facilitating elongation. In this context, viral intermediate (35) and late (36–39) mRNAs have a variable length 5′-poly(A) leader presumably produced by a slippage mechanism. We hypothesize that the cellular proteins facilitate the transition between reiterative adenylation and RNA synthesis. A second question is whether G3BP and p137 are unique in their ability to complement intermediate stage transcription. In this context, heterogeneous nuclear ribonucleoproteins A2/B1 and RBM3 have been reported to stimulate late stage transcription *in vitro* (20). It will be interesting to test the latter proteins for ability to stimulate intermediate transcription and G3BP and p137 to stimulate late transcription. Another question concerns the role in virus replication of G3BP and p137 or possibly other proteins with similar activities. Why should a virus that encodes a multisubunit RNA polymerase and numerous transcription factors need an additional cellular protein? Moreover, why does VAC need an intermediate stage of transcription between the early stage, which provides enzymes and factors for DNA replication, and the late stage, which provides the proteins for assembly of virus particles? Because both G3BP and p137 seem to be most highly expressed in growing cells, we suggest that the intermediate stage of transcription and the requirement for a cell factor serves as a checkpoint, preventing premature or abortive entry into the late phase of virus replication in a resting cell. In this model, the cellular transcription factors would become available only after a resting cell is activated and capable of manufacturing a large number of virus particles.

Acknowledgments—We thank H. Jaffe for peptide analysis and J. P. Luzio for the hsGPI137 plasmid.

[2] G. C. Katsafanas and B. Moss, unpublished data.

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