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Characterization of the Interactions among Vaccinia Virus Transcription Factors G2R, A18R, and H5R

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Prior genetic analysis suggests that there may exist an interaction between the products of the vaccinia virus genes A18R, a putative negative transcription elongation factor, and G2R, a putative positive transcription elongation factor. In addition, affinity purification of polyhistidine-tagged G2R protein overexpressed in vaccinia virus-infected cells, reported here, results in copurification of the vaccinia H5R protein, previously characterized as a late viral transcription factor. We have therefore used several methods to screen further for interactions among the G2R, A18R, and H5R proteins. Methods include copurification or co-immunoprecipitation of proteins overexpressed during vaccinia virus infection, activation of the gal 4 promoter by gal 4 fusions in the yeast two-hybrid system, and co-immunoprecipitation of proteins synthesized *in vitro* in a rabbit reticulocyte lysate. The results reveal interactions which include all possible pairwise combinations of the three proteins G2R, A18R, and H5R; however, not all possible permutations of the interactions are observed and the interactions are not observed in all environments tested. The results suggest that the vaccinia virus proteins G2R, A18R, and H5R interact as part of a higher order transcription complex.

INTRODUCTION

Control of gene expression in prokaryotes, eukaryotes, and many viral systems is accomplished primarily by the interaction of regulatory proteins with RNA polymerase. Trans-acting factors that modulate initiation, elongation, and termination of nascent RNA molecules by both bacterial and eukaryotic RNA polymerases are frequently associated with the core polymerase in a large complex of proteins (Burgess *et al.,* 1969; McCracken *et al.,* 1997; Uptain *et al.,* 1997; Zawel and Reinberg, 1993). For example, several trans-acting factors intimately associated with the yeast RNA polymerase II have been identified using a combination of genetic and biochemical studies. The genetic experiments uncovered cold-sensitive extragenic suppressors of cells containing a truncated form of the carboxy-terminal domain (CTD) of the RNA polymerase II (Koleske and Young, 1995). Purification of the products of the suppressing genes, known as SRB (suppressors of RNA polymerase B), yielded a large RNA polymerase II-containing complex which was stable throughout the initial purification steps. Many of the proteins in the complex were subsequently identified as previously characterized GTFs (general transcription factors) which are necessary for controlled transcription initiation and elongation. Recent evidence shows that the mammalian core RNA polymerase, several GTFs, and auxiliary kinases copurify on an affinity column containing bound TFIIS, an RNA polymerase II elongation factor (McCracken *et al.,* 1997). Together these experiments suggest that the eukaryotic RNA polymerase II functions *in vivo* as a high-order complex in association with numerous initiation and elongation factors.

Gene transcription during a vaccinia virus infection is regulated predominately by the action of stage-specific trans-activating factors (Moss, 1996). Viral early gene expression takes place in the infecting virion particle, and early transcripts are extruded into the host cytoplasm. The early genes encode protein factors responsible for trans-activating intermediate gene expression, the proteins encoded by intermediate genes trans-activate late viral genes, and late genes encode the early transcription factors which are packaged into virions along with RNA polymerase for the subsequent round of infection. Intermediate and late transcription are both coupled to viral DNA replication, and thus these gene classes can be referred to collectively as "postreplicative." Highly purified *in vitro* systems are available for study of early gene transcription initiation, elongation, and termination (Baldick *et al.,* 1994; Luo *et al.,* 1991; Hagler and Shuman, 1992; Deng and Shuman, 1996; Li and Broyles, 1995). Relatively pure *in vitro* systems exist for study of intermediate and late gene transcription initiation (Kovacs and Moss, 1996; Passarelli *et al.,* 1996;

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Hubbs and Wright, 1996; Rosales *et al.,* 1994), but the precise molecular function of the transacting factors required for expression of postreplicative genes is not fully understood at the biochemical level.

Biochemical analysis of vaccinia early gene transcription suggests that, as in the eukaryotic host, virus transcription may be carried out by high-order RNA polymerase complexes that are specific for each class of gene expression. For example, purification of RNA polymerase from vaccinia virions results in copurification of the mRNA capping enzyme, early gene transcription termination activity, viral early transcription initiation factor activity, and RAP94, a additional protein required for initiation of early gene transcription (Ahn and Moss, 1992; Broyles and Moss, 1987; Deng and Shuman, 1996). Analogous RNA polymerase complexes have not yet been elucidated for transcription of the intermediate and late classes of viral genes.

In vivo characterization of vaccinia viruses containing mutations in the A18R and G2R genes suggests that the proteins encoded by A18R and G2R are necessary for the regulation of postreplicative viral transcription elongation and/or termination and that they have complementary activities (Black and Condit, 1996; Condit *et al.,* 1996b; Xiang *et al.,* submitted for publication). A18R mutant viruses exhibit a phenotype termed "promiscuous transcription," defined as transcription of regions of the genome that are normally transcriptionally silent late during infection (Bayliss and Condit, 1993). Recent experiments suggest that promiscuous transcription results from readthrough synthesis of longer-than-normal intermediate RNAs from upstream genes (Xiang *et al.,* submitted for publication). Thus the A18R protein may normally serve as a negative regulator of transcription elongation or possibly as a termination factor. Interestingly, treatment of wt infections with the antipoxviral drug IBT produces the same phenotype as A18R mutant virus infections (Pacha and Condit, 1985; Bayliss and Condit, 1993). Thus IBT seems to effect intermediate and late transcription elongation by promoting readthrough transcription or by inhibiting termination. Conversely, the G2R mutant viruses synthesize abnormally short intermediate and late viral RNAs which are trucated specifically at their 3′ ends (Black and Condit, 1996). Treatment with IBT rescues growth of G2R mutant viruses (Meis and Condit, 1991), possibly by promoting elongation of the truncated postreplicative mRNAs. Based on these observations we hypothesize that the G2R protein functions as a positive transcription elongation factor at postreplicative times of a vaccinia infection.

Additional genetic data support the hypothesis that the G2R and A18R proteins may interact either directly or indirectly in a common pathway of regulation of postreplicative transcription. First, mutants in the G2R gene serve as extragenic suppressors of mutations in the A18R gene, suggesting at least a functional interaction between the two proteins (Condit *et al.,* 1996b). Second, a mutation that confers resistance to IBT maps to the second largest subunit of the RNA polymerase (Condit *et al.,* 1991). The action of IBT on a wt infection and the IBT phenotype of the G2R and RNA polymerase mutant viruses suggests a functional interaction among G2R, A18R, and RNA polymerase proteins.

The goal of this work was to explore the interactions among several vaccinia proteins, including A18R and G2R, that have been implicated in the regulation of postreplicative viral transcription. We used several methods to screen for protein–protein interactions both *in vivo* and *in vitro* including copurification of proteins overexpressed in vaccinia virus, the yeast two-hybrid system (Fields and Song, 1989), and co-immunoprecipitation of specific viral proteins. The results reveal interactions among A18R, G2R, and an additional late viral transcription factor, H5R, and suggest that these proteins may exist in a complex involved in the regulation of viral postreplicative transcription elongation. Characterization of these interactions may assist us in understanding the role of the proteins in the regulation of transcription elongation at postreplicative times of a viral infection.

RESULTS

Overexpression of hisG2R; monoclonal antibody production

In order to obtain antigen for anti-G2R monoclonal antibody production, we overexpressed an amino-terminal polyhistidine-tagged G2R protein from a T7 promoter in the vaccinia-T7 system. Purification of hisG2R, a 32-kDa protein, resulted in copurification of a second protein of 44 kDa from the Ni^{2+} affinity column (Fig. 1A). The 44-kDa protein was not seen in preparations of hisA18R purified from infected cells by the same protocol (Fig. 1A), suggesting that the 44-kDa protein specifically interacts with the hisG2R protein *in vivo.* The 44-kDa protein was transferred from SDS–PAGE gels to PVDF membrane following instructions from Ariad Pharmaceuticals, Inc. and sent to Ariad for microsequencing. The N-terminus of the protein was found to be blocked, but an N-terminal sequence from two internal cyanogen bromide fragments was obtained. The sequences obtained, AEIRAHLKNS and VQVEAGKVNH, both match perfectly with internal regions of the predicted translation product of the H5R gene of vaccinia virus. The identity of the 44-kDa protein was confirmed by subsequent Western blot analysis (Fig. 1B), which showed that the 44-kDa protein reacts specifically with an antiserum raised against the H5R protein. The H5R protein is a known transactivator of late viral gene expression (Kovacs and Moss, 1996). These data stimulated further experiments to define protein–protein interactions among the G2R, H5R, and A18R proteins.

We generated a monoclonal antibody using the

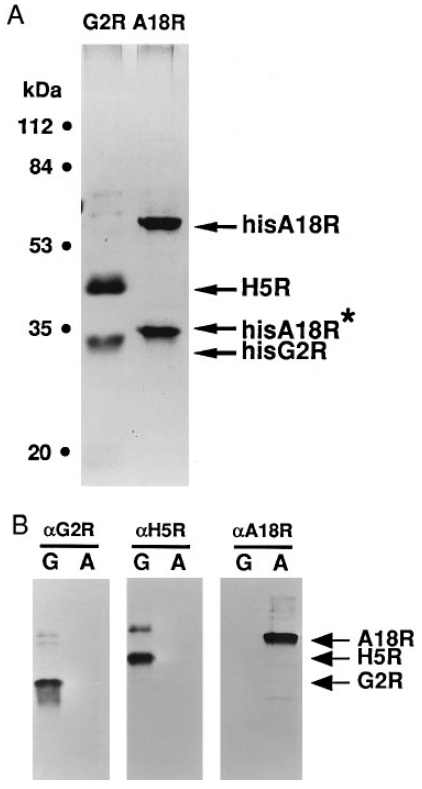

FIG. 1 Electrophoretic and Western blot analysis of the hisG2R protein preparation. Monolayers of BSC40 cells were infected with both vTF7.3 and vvhisG2 or vvhisA18 (control) viruses at an m.o.i. of 10. Cytoplasmic extracts were prepared and the his-tagged proteins were purified by Ni^{2+} affinity chromatography. The fractions were collected from the column and the peak fractions were pooled and dialyzed. (A) An aliquot of the hisG2R or hisA18R was analyzed by SDS–PAGE followed by silver staining. The known proteins in each fraction are identified on the right side of the figure. HisA18R* is an N-terminal fragment of hisA18R resulting from premature termination by T7 RNA polymerase (Bayliss and Condit, 1995). (B) A gel equivalent to (A) was transferred to nitrocellulose and probed with anti-G2R (left), anti-H5R (middle), and anti-A18R (right) antibodies. The same filter was used for each Western, stripped between exposure to different primary antibodies. Lanes labeled G are loaded with hisG2R protein; lanes labeled A are loaded with hisA18R protein. The known proteins in each fraction are identified on the right side of the figure.

hisG2R protein as antigen and screened the resulting hybridomas by Western analysis against both the hisG2R preparation and wt-infected cell extracts. In a Western analysis, the resulting monoclonal antibody recognizes a 26-kDa protein in wt-infected cell extracts but not in extracts of cells infected with the G2R mutant virus, G2A, which contains a frameshifting deletion that truncates the C-terminal two-thirds of the protein (Meis and Condit, 1991) (Fig. 2). Western blots also show that the monoclonal antibody also recognizes hisG2R overexpressed during a vvhisG2R infection. The antibody also immunoprecipitates radiolabeled hisG2R synthesized *in vitro* in a rabbit reticulocyte lysate (data not shown). However, we were unable to immunoprecipitate the *in vivo* synthesized native 26-kDa G2R protein from wt infections or the 32-kDa hisG2R overexpressed from vvhisG2R infections. Also, the monoclonal antibody specific for G2R does not immunoprecipitate G2R or hisG2R from denatured infected cell extracts. There are two possible explanations for the failure of the monoclonal antibody to immunoprecipitate the G2R protein from infected cells. First, the epitope recognized by the G2R monoclonal antibody may be obscured *in vivo,* perhaps masked by other proteins, but is available for binding *in vitro.* Second, the antigen–antibody interaction may be of relatively low affinity and therefore disrupted by the relatively stringent conditions used for immunoprecipitation of proteins synthesized *in vivo* but stable to the less stringent conditions used for immunoprecipitation of proteins synthesized *in vitro.*

Because we were interested in exploring the interactions among the proteins G2R, A18R, and H5R, we analyzed infected cell extracts by Western analysis using antisera specific for each protein to determine the steady-state levels of each protein. Initially, the G2R monoclonal antibody was used in a Western blot analysis of cell extracts infected with wt, G2A, vvhisG2R, and vvhisA18R. Figure 2 shows that the native G2R protein is visible in the wt extracts made at 6, 8, 10, 12, 16, and 24 hpi, but not in the G2A extract. The hisG2R protein, slowed to an apparent molecular weight of 32 kDa due to the amino-terminal polyhistidine tag, is visible in the vvhisG2R lane. The blot was stripped and probed with a monoclonal antibody specific for the A18R protein and then stripped and probed with a polyclonal serum specific for the H5R protein. The G2R, A18R, and H5R proteins are all found in wt-infected cells at late times of infection. Western blot analysis of purified proteins (Fig. 1B) indicates that the anti-G2R, -H5R, and -A18R antibod-

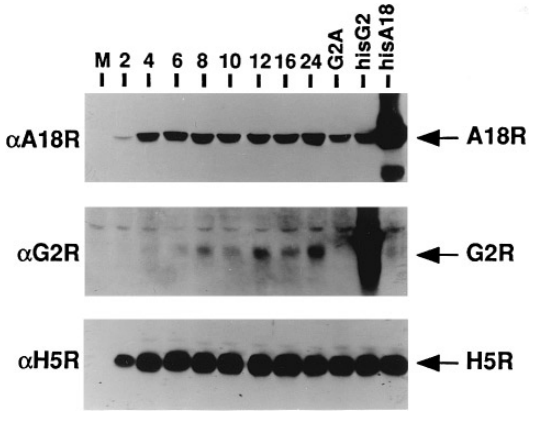

FIG. 2. Western analysis of G2R, A18R, and H5R proteins in virus-infected cells. Monolayers of BSC40 cells were infected with wt, G2A, vvhisG2, or vvhisA18 viruses. Cell extracts were prepared from wt infected cells at the times indicated above the lanes or from G2A-, vvhisG2 + vvTF7.3-, or vvhisA18 + vvTF7.3-infected cells at 9 hpi. The proteins were separated on SDS–PAGE and transferred to nitrocellulose. The blots were probed with the G2R or A18R monoclonal antibodies or an H5R polyclonal sera, as indicated at left. Only relevant portions of the blots are shown.

TABLE 1

G2R H5R Interaction in the Yeast Two Hybrid System

DNA binding domain hybrid	Activation domain hybrid	Colony color[a]	β-Gal activity[b]
GBN	GADN	−	nd
GBNG2R	GADN	−	1.3
GBNG2R	GADNH5R	+	96.4
GBNH5	GADNG2R	−	0.0
GBN	GADNH5R	−	1.1
GBNH5	GADN	−	nd
p53	T antigen	++	1441.2

Note. nd, not done.

[a] (−) white, (+) light blue, (++) deep blue.

[b] β-Gal activity expressed in nmol/mg protein/min.

ies yield similar signals when reacted with equivalent amounts of protein, and therefore the relative intracellular concentrations of G2R, H5R, and A18R can be approximated by visual inspection of the blots in Fig. 2. Thus the H5R protein is present in amounts equal to or slightly greater than the A18R protein, while the G2R protein is present in much lower amounts than either A18R or H5R. We have used the antibodies specific for all three proteins in subsequent experiments to further assay for protein–protein interactions.

Protein–protein interactions in the two-hybrid system

The yeast two-hybrid system (Fields and Song, 1989) was utilized to screen for interactions among the G2R, H5R, and A18R proteins. Each of the viral genes was cloned into both the DNA binding domain (GBN) and the activation domain (GADN) vectors. The positive control for the system was cotransformation of T antigen subcloned in the activation domain construct and p53 subcloned into the binding domain construct. Negative controls include cotransformation of each construct with the cognate vector lacking insert. A positive interaction in both the filter assay and the liquid assay was seen when the H5R gene was subcloned into the GADN vector and cotransformed with the G2R gene subcloned into the GBN vector (Table 1). However, when the H5R gene was subcloned into the GBN vector and cotransformed with the G2R gene subcloned in the GADN vector, no β-gal activity was observed in either assay. Thus, the G2/H5 interaction was specific to the orientation of the viral proteins in the fusion constructs. Additionally, the A18R gene was subcloned into both the GBN and GADN vectors and cotransformed in all combinations with both the G2R and H5R constructs. None of these cotransformations resulted in β-gal activity by filter or liquid assay (data not shown).

The data gathered from the two-hybrid screen is consistent with the interaction between H5R and G2R observed when G2R is overexpressed by a recombinant vaccinia virus. Nevertheless, we chose to explore the hypothesized protein–protein interactions further by alternative approaches.

In vivo co-immunoprecipitations

We have used co-immunoprecipitation experiments to test for interactions among the G2R, A18R, and H5R proteins *in vivo* (Fig. 3). Cells were infected with wt, G2A, vvhisG2R, vvhisA18R, or a mixture of vvhisG2R and vvhisA18R viruses. The infected cells were harvested at 3 or 9 h postinfection and lysates were immunoprecipitated with antisera specific for the G2R, A18R, or H5R proteins. The immune complexes were separated on SDS–PAGE and transferred to nitrocellulose for Western analysis. The Western blots were probed with various antibodies to determine co-immunoprecipitating proteins. The Western analyses were controlled by the use of total cytoplasmic extracts from wt- and G2A-infected cells electrophoresed alongside the immunoprecipitated proteins. Background immunopositive proteins of approximately 25 and 50–60 kDa seen in the mock lanes in the Western analyses are immunoglobulin chains from the initial immunoprecipitation that react with secondary antibody in the Western blot. Nonspecific co-immunoprecipitating proteins were controlled for by the use of a monoclonal antibody specific for a yeast protein in the initial immunopreicpitation followed by Western analysis with antisera specific for the candidate proteins G2R, A18R, and H5R.

Initially, infected cell extracts were immunoprecipitated with the A18R monoclonal antibody (Fig. 3, top two panels). The 56-kDa native A18R and the 57-kDa hisA18R proteins can be precipitated with the A18R antibody from infected cell extracts but not mock-infected cell extracts, as confirmed by both radioimmunoprecipitation and by Western analysis of immunoprecipitated proteins with the A18R monoclonal antibody (data not shown). The proteins immunoprecipitated by the A18R monoclonal antibody were analyzed by Western blots using either the G2R monoclonal antibody (Fig. 3, top left) or the H5R polyclonal serum (Fig. 3, top right). Western analysis with the G2R antibody reveals a 32-kDa band corresponding to the hisG2R protein in infections with vvhisG2R (lane 6). This same 32-kDa hisG2R band is present in abundance in immunoprecipitates from cells infected with both vvhisG2R and vvhisA18R (lane 8). It is noteworthy that more hisG2R is present in immunoprecipitates from mixed infections with both vvhisG2R and vvhisA18R than is present in immunoprecipitates from cells infected with vvhisG2R alone. This observation indicates that the quantity of G2R protein co-immunoprecipitated is proportional to the amount of A18R present in extracts. A very small amount of native G2R protein, which runs at 26 kDa (lane 10), was reproducibly observed in immunoprecipitates from cells in which A18R was overexpressed (lanes

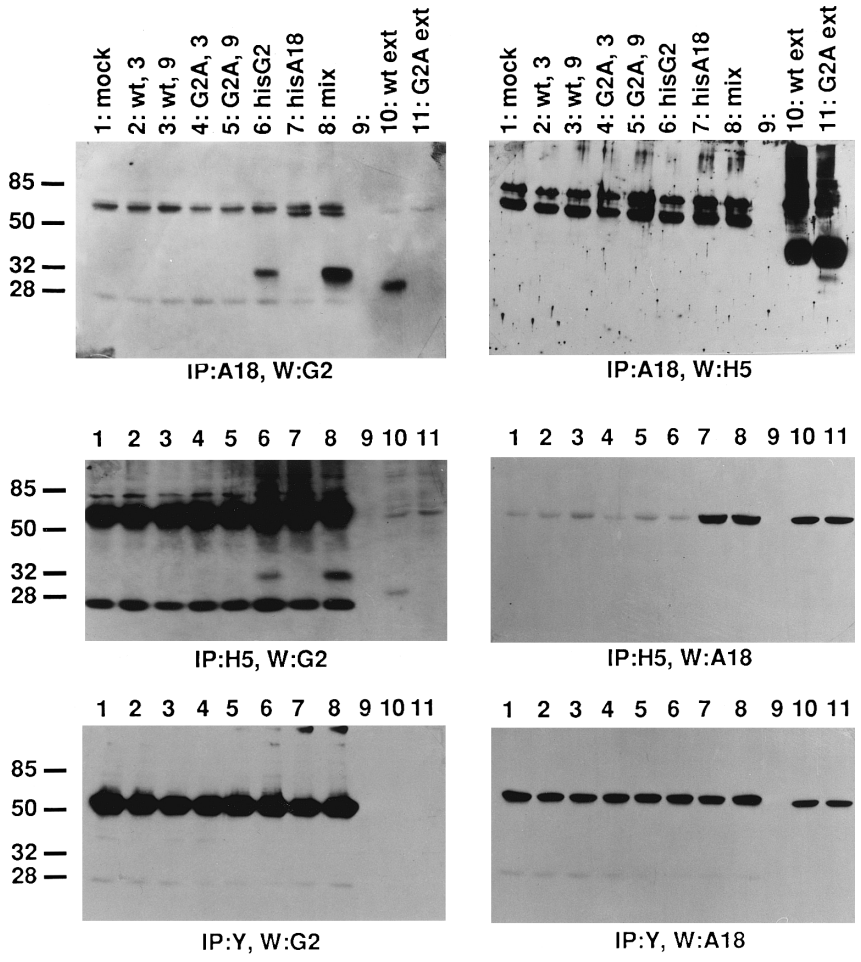

FIG. 3. Co-immunoprecipitation analysis of viral proteins synthesized *in vivo.* Monolayers of BSC40 cells were infected with wt, G2A, vvhisG2, or vvhisA18 or coinfected with vvhisG2 plus vvhisA18. Cells infected with recombinant viruses expressing polyhistidine-tagged proteins were also co-infected with vvTF7.3. The cells were harvested into RIPA buffer at 3 or 9 h postinfection and treated with benzonase. The insoluble material was pelleted by centrifugation and the cleared lysate was immunoprecipitated with A18R monoclonal antibody, H5R polyclonal sera, or a monoclonal antibody specific for a yeast protein. The immunoprecipitated proteins were separated on SDS–PAGE and transferred to nitrocellulose for Western analysis. The antibodies used for the immunoprecipitation (IP) or Western (W) analysis are indicated at the bottom of each panel. For each panel: lane 1, mock-infected extract; lane 2, wt infection, 3 hpi; lane 3, wt infection, 9 hpi; lane 4, G2A infection, 3 hpi; lane 5, G2A infection, 9 hpi; lane 6, vvhisG2 infection, 9 hpi; lane 7, vvhisA18 infection, 9 hpi; lane 8, vvhisG2 + vvhisA18 infection, 9 hpi; lane 9, blank; lane 10, wt total cytoplasmic extract, not immunoprecipitated; lane 11, G2A total cytoplasmic extract, not immunoprecipitated. Molecular weight markers are shown on the left side of the panels.

7 and 8). The proteins immunoprecipitated by the A18R monoclonal antibody did not include the H5R protein (Fig. 3, top right) although the control cell extracts demonstrated that H5R was recognized in a Western analysis (lanes 10 and 11). In summary, these data show that immunoprecipitation of the A18R protein from infected cell extracts results in co-immunoprecipitation of G2R, and that H5R does not co-immunoprecipitate with A18R.

Incubation of infected cell extracts with H5R polyclonal serum immunoprecipitates the 44-kDa H5R protein in wt, G2A, vvhisG2R, vvhisA18R, and mixed infections but not mock-infected cell extracts as determined by both radioimmunoprecipitation and by Western analysis of immunoprecipitated proteins (data not shown). Western analysis of proteins immunoprecipitated with the H5R polyclonal serum done with either the G2R (Fig. 3, middle left) or A18R (Fig. 3, middle right) monoclonal antibody shows that hisG2R and hisA18R are included in the immunoprecipitated proteins, respectively. Native G2R protein is undetectable in the Western analysis of proteins immunoprecipitated by the H5R antibody, although the G2R protein is present in the control extracts (lane 10). Interestingly, overexpression of both hisA18R and hisG2R results in increased co-immunoprecipitation of hisG2R relative to overexpression of hisG2R alone (compare lanes 8 and 6, middle left panel). This suggests that at least a portion of the hisG2R protein exists as part of a higher order complex which also contains A18R and that overexpression of hisA18R increases the amount of this complex and thus the amount of immunoprecipitated

hisG2R. Unfortunately, hisA18R (lanes 7 and 8, middle right panel) coelectrophoreses with the background of rabbit Ig heavy chain; however, the 57-kDa signal in these vvhisA18R immunoprecipitates is reproducibly significantly greater than the background. It is unclear whether native A18R is included in the proteins immunoprecipitated by the H5R antisera, although it is present in the control extracts (lanes 10 and 11). In summary, these data show that immunoprecipitation of H5R results in co-immunoprecipitation of both hisG2R and hisA18R.

A monoclonal antibody specific for a yeast protein was used as a control to test for nonspecific co-immunoprecipitation of G2R and A18R (Fig. 3, bottom panels). Western analysis of the proteins immunoprecipitated with the yeast monoclonal antibody reveal neither G2R nor hisG2R, A18R, or hisA18R (lanes 1–8), though G2R and A18R are recognized in the control extracts (lanes 10 and 11). We conclude based on this control that the co-immunoprecipitating proteins observed in the previous experiments are not nonspecifically precipitated by the monoclonal antibodies.

As noted above, we are unable to immunoprecipitate the G2R protein from either wt or vvhisG2R recombinant virus-infected cell extracts. Therefore, we did not undertake the task of Western analyses of G2R immunoprecipitates with the A18R or H5R antisera.

In vitro co-immunoprecipitations

While the *in vivo* immunoprecipitations suggest that interactions involving G2R, A18R, and H5R occur *in vivo,* we wanted to further analyze the protein–protein interactions in the absence of other viral proteins. To accomplish this, we synthesized hisG2R, hisA18R, or H5R individually, in pairs, or in triplicate *in vitro* and immunoprecipitated with the individual antibodies. We found that while the G2R, A18R, and H5R antibodies each immunoprecipitated their cognate protein *in vitro,* in no case did we observe co-immunoprecipitating proteins (data not shown).

GST-G2 bead binding

We tested the hypothesis that G2R interacts with H5R and A18R *in vivo* in a GST-G2 cobinding experiment (Fig. 4). A549 cells were infected with a vaccinia virus expressing the T7 RNA polymerase (vTF7.3) and transfected with pTM1GST-G2, a plasmid DNA encoding a GST-G2 fusion protein driven by a T7 promoter. The vTF7.3 infection provides wt vaccinia proteins in addition to supplying T7 RNA polymerase for expression of the GST-tagged proteins. A control experiment was done by infecting A549 cells with vTF7.3 and transfecting pTM1GST-Sendai P, a plasmid DNA containing a fusion of GST with the Sendai virus P protein driven by a T7 promoter. The GST-G2 or GST-P proteins were overexpressed in the infected cells and

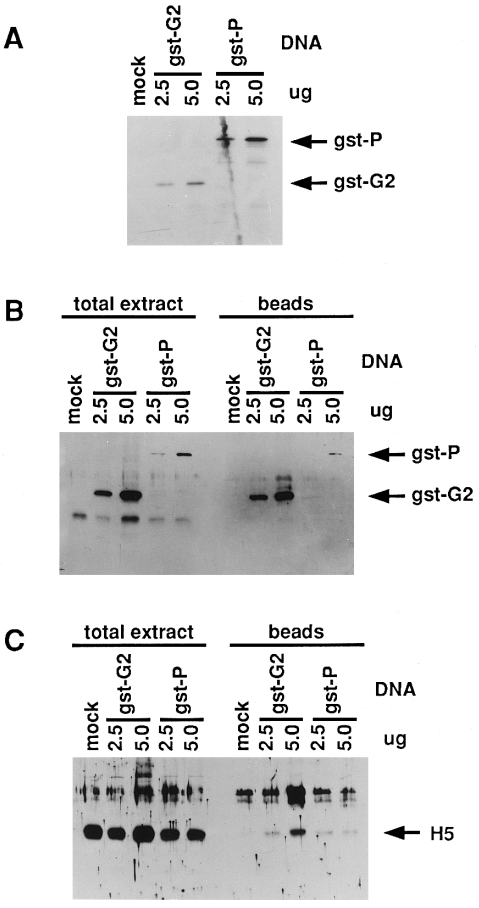

FIG. 4. Cobinding of H5R with GST-G2. Monolayers of A549 cells were infected with vTF7.3 at 37°C and transfected with pTM1GST-G2 (gst-G2) or pTM1GST-Sendai P (gst-P) DNA. The infected/transfected cells were labeled with Trans[^{35}S]methionine for 16–18 h. Following the labeling period, cytoplasmic extracts were made using lysolecithin permeabilization. (A) The extracts were incubated with glutathione–Sepharose 4B and the bead-bound proteins were solubilized, analyzed on SDS–PAGE, dried, and exposed to film. (B and C) The bead-bound proteins were analyzed on SDS–PAGE, transferred to nitrocellulose, and probed in a Western analysis with a mix of monoclonal antibodies specific for G2 and Sendai P (B) or polyclonal sera specific for H5R (C). For (B) and (C), the Western analyses were done using both total extract and bead-bound protein as indicated. For (A–C), both mock transfections and transfections at two concentrations of plasmid DNA were done as indicated in the figure. The relative mobilities of the proteins of interest are shown at the right of each panel.

purified from lysolecithin extracts of the infected/transfected cells using the glutathione–Sepharose beads as described under Materials and Methods. Figure 4A shows the total radiolabeled proteins which cobind GST-G2 or GST-Sendai P from infected/transfected cell extracts. GST-G2 migrates at approximately 60 kDa and GST-P migrates at approximately 100 kD. No co-binding proteins were detected using this assay. Figure 4B shows a Western analysis of total infected/transfected cell extract and glutathione–Sepharose bead-bound protein analyzed with both the G2R monoclonal antibody and the Sendai P monoclonal antibody

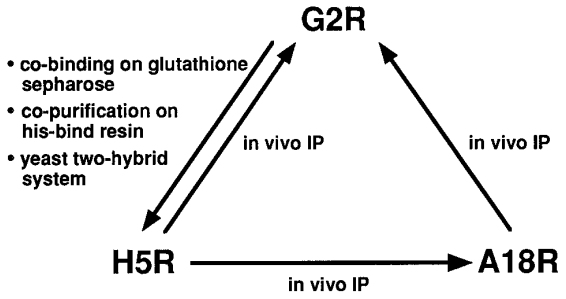

FIG. 5. Diagrammatic representation of the protein interaction data. The three viral proteins of interest (G2R, A18R, and H5R) are shown at the corners of the triangle. Interactions between the proteins were assayed by using one protein as "bait" and testing for the capture of the other proteins. In the diagram, the "bait" is found at the tail of the arrow and the "captured" protein is found at the head of the arrow. The assay(s) utilized is found immediately adjacent to the relevant arrow.

in the same reaction. Western analysis fails to show the native G2R protein at approximately 26 kDa in an infected but mock-transfected extract, probably because lesser amounts of cell extract are analyzed in these experiments than in the control Western blots in Fig. 3. GST-G2 is immunoreactive with the G2R antibody and the Sendai P monoclonal antibody detects GST-P. The Western blots in Fig. 4B were stripped and reprobed with the H5R polyclonal sera (Fig. 4C). The vaccinia protein H5R cobinds GST-G2 protein on glutathione–Sepharose beads as detected by Western analysis. There is some H5R cobinding GST-P but an increased quantity reproducibly cobinds GST-G2. The blot was stripped of the secondary horseradish peroxidase antibody and reprobed with the monoclonal antibody specific for the A18R protein. A18R cobinds neither GST-P nor GST-G2 (data not shown). In summary, these experiments provide further evidence for an interaction between the vaccinia proteins H5R and G2R expressed *in vivo.*

DISCUSSION

We have obtained biochemical evidence suggesting that the vaccinia virus proteins G2R, A18R, and H5R interact as part of a higher order transcription complex. In general, the experimental approach is to use one protein as a "bait," namely, conjugated to the yeast gal4 DNA binding domain, a histidine or GST tag, or specifically immobilized on protein A with an antibody, then present the bait to a selection of other proteins, and determine what if any proteins are captured. We have tested for interactions in several different environments, including synthesis in vaccinia virus-infected cells, in yeast, or *in vitro.* Figure 5 provides a diagrammatic summary of the interactions we have observed. The results (Fig. 5) reveal interactions which include all possible pairwise combinations of the three proteins, G2R, A18R, or H5R; however, not all possible permutations of the interactions are observed and the interactions are not observed in all environments tested. For example, we have found evidence for an interaction between A18R and H5R; however, the interactions are seen only via immunoprecipitation from virus-infected cells and not in yeast or *in vitro,* and the interactions are unidirectional, that is, A18R coprecipitates in an anti-H5R antibody-mediated immunoprecipitation, while H5R does not coprecipitate in an anti-A18R antibody-mediated immunoprecipitation.

The conditions under which we detect protein–protein interaction may provide information about the nature of the interaction, specifically whether it represents a direct physical contact between two proteins or an association mediated by another macromolecule. Two experiments strongly suggest that the interaction between the G2R and H5R proteins represents a direct interaction. First, purification of an amino-terminal histidine-tagged G2R protein synthesized *in vivo* using the vaccinia-T7 overexpression system results in copurification of H5R in the absence of any other detectable proteins or nucleic acids. Second, the GBNG2R–GADNH5R interaction observed in the yeast two-hybrid system most likely represents a direct interaction, since no other vaccinia proteins are present and the interaction is unlikely to be mediated by another yeast protein. By contrast, all of the other interactions, namely, A18R with H5R and G2R with A18R, are observed only via immunoprecipitation from vaccinia virus-infected cells and not in yeast or *in vitro.* While such negative results must be interpreted with extreme caution, it is nevertheless tempting to speculate that these latter interactions may be mediated by additional macromolecules (protein, DNA, or RNA) found only in vaccinia virus-infected cells. A logical and provacative candidate for a mediating macromolecule would be the viral RNA polymerase.

Based on prior genetic experiments (see Introduction) and on the results reported here, we envision postreplicative vaccinia transcription elongation to be regulated by a dynamic complex of proteins which includes at least H5R, G2R, and A18R and which may interact directly with the viral RNA polymerase. The interaction between G2R, a putative positive transcription elongation factor, and A18R, a putative negative transcription elongation factor, was predicted based on genetic experiments revealing both complementary transcription elongation phenotypes and genetic suppression (Black and Condit, 1996; Condit *et al.,* 1996b). The discovery of an interaction between these proteins and H5R, a viral late transcription factor (Kovacs and Moss, 1996), lends support to the notion that A18R and G2R may function in a higher order complex containing additional transcription factors. Hopefully, further genetic and biochemical characterization of the vaccinia virus transcription elongation factors will pro-

vide additional insights into transcription elongation and its relation to both initiation and termination.

MATERIALS AND METHODS

Cells, viruses, bacterial hosts, and antibodies

A549, BSC40, and Rat2 cells, vaccinia virus wild-type strain WR, the G2R deletion mutant G2A, the recombinant vaccinia virus vTF7.3 (Fuerst *et al.,* 1986), which expresses the bacteriophage T7 RNA polymerase, and the conditions for their growth, infection, and plaque titration have been described previously (Condit and Motyczka, 1981; Condit *et al.,* 1983, 1996a; Meis and Condit, 1991; Bayliss and Condit, 1993). IBT was prepared as described previously (Pacha and Condit, 1985) and used at a final concentration of 45 μM.

The vaccinia recombinant vvhisG2R was constructed by transfecting wild-type virus-infected cells with the plasmid pTM1-G2R (see below) and selecting for thymidine kinase-negative recombinants on Rat2 cells (Ausubel *et al.,* 1987). The resulting recombinant virus contains a amino-terminal polyhistidine–G2R fusion, driven by a T7 RNA polymerase promoter, located in the vaccinia virus thymidine kinase gene. The structure of the recombinant was confirmed by PCR analysis using appropriate primers specific for the G2R ORF.

Monoclonal antibodies were prepared by the University of Florida Interdisciplinary Center for Biotechnology Research (ICBR) hybridoma laboratory using histidine tagged A18R and G2R (see below) proteins purified by nickel column affinity chromatogrpahy as antigens. The polyclonal serum specific for the H5R protein was kindly provided by Dr. Paula Traktman (Cornell University). The yeast monoclonal antibody was kindly provided by Dr. Maurice Swanson (University of Florida).

Plasmid constructs

pET16bG2R contains the vaccinia virus G2R coding sequence inserted in frame downstream from an amino-terminal polyhistidine tag in the vector pET16b (Novagen). The G2R coding sequence was subcloned from the plasmid pgG2rap (Condit *et al.,* 1996b). pgG2rap was digested with *Nde*I and *Bam*HI. The *Nde*I site at the 5′ end includes the authentic translation initiation codon of G2R. The *Nde*I, *Bam*HI fragment was subcloned into cleaved pET16b generating pET16bG2R.

The plasmid construct pTM1his-G2 contains the polyhistidine–G2R fusion driven by a T7 RNA polymerase promoter flanked by the 5′ and 3′ terminal segments of the vaccinia virus thymidine kinase gene. pTM1his-G2 was constructed by cloning the *Nco*I, *Bam*HI fragment from pET16bG2R, which contains the polyhistidine–G2 fusion, into pTMI cleaved with *Nco*I and *Bam*HI (Ausubel *et al.,* 1987).

pGEMH5 was constructed to contain the vaccinia virus H5R coding sequence inserted in the multiple cloning site of pGEM3Zf-. The H5R coding sequence was initially PCR amplified from vaccinia virus WR DNA using primers that inserted a *Eco*RI site at the 5′ end and a *Bam*HI site at the 3′ end. The *Eco*RI, *Bam*HI-cleaved PCR product was cloned into *Eco*RI, *Bam*HI-cleaved pGEM3Zf- generating pGEMH5. The cloned H5R gene was sequenced to confirm the fidelity of the PCR reaction.

pGADN-H5 and pGBN-H5 were constructed by PCR amplification of the H5R ORF from vaccinia virus WR DNA using primers that inserted an *Nde*I site at the 5′ end and a *Bam*HI site at the 3′ end of the H5R gene. To create pGADN-H5, the PCR product was cleaved with *Nde*I and *Bam*HI and cloned into the *Nde*I, *Bam*HI-cleaved pGADN vector. The cloned H5R gene was sequenced to confirm the fidelity of the PCR reaction. pGBN-H5 was constructed by subcloning the H5 ORF-containing *Nde*I, *Bam*HI fragment from pGADN-H5 into *Nde*I, *Bam*HI-cleaved pGBN. The pGADN and pGBN vectors (a gift from P. Traktman, Cornell University) are modifications of the pGAD24 and pGBT9 plasmids (Clonetech), respectively, in which the *Eco*RI site in each vector was removed and replaced with an *Nde*I site. pGADN-G2 and pGBN-G2 were constructed by subcloning the G2R ORF from pgG2Rap, generated by cleaving the plasmid DNA with *Nde*I and *Bam*HI, into the vectors pGADN and pGBN cleaved with *Nde*I and *Bam*HI.

pTM1GST-G2 contains the vaccinia virus G2R open reading frame inserted in frame downstream from the GST cassette. The pTM1GST vector contains the GST gene inserted into pTM1 multiple cloning site (Chandrika *et al.,* 1995; Stein *et al.,* 1989) (Merchilinsky, unpublished data). The G2R open reading frame was subcloned into pTM1GST from pgG2rap by PCR amplification of the G2R open reading frame which generated a *Bam*HI site at the 5′ end of the open reading frame and a *Pst*I site at the 3′ end. The *Bam*HI, *Pst*I-cleaved PCR product was ligated into the *Bam*HI, *Pst*I-digested pTM1GST vector generating pTM1GST-G2. The insert was sequenced to verify the fidelity of the PCR reaction.

Overexpression and purification of hisG2R protein

The amino-terminal polyhistidine–G2R fusion protein (hisG2R) was purified from BSC40 cells coinfected with recombinant viruses vTF7.3 and vvhisG2R as described previously (Simpson and Condit, 1995; Bayliss and Condit, 1995). Cytoplasmic extracts of the virus-infected cells were prepared and applied to the His-Bind column (Novagen). The hisG2R protein was eluted from the column with increasing concentrations of imidazole. Peak fractions were identified by Bradford protein assay (Bio-Rad), pooled, and dialyzed against 20 mM Tris–HCl, pH 8.0, 40 mM KCl, and 40%

glycerol over a period of 24 h. The protein was stored at −20°C.

Two-hybrid screen

Transformations of *Saccharomyces cerevisae,* Y190 strain (Clonetech), were done with combinations of the GBN and GADN constructs using the LiOAc/PEG method as described (Rose *et al.,* 1990). The β-galactosidase filter assays were done as previously described in the Matchmaker Library protocol (Clonetech). The β-galactosidase liquid assays were done as previously described (Rose *et al.,* 1990).

In vivo immunoprecipitation and Western blot analysis

Confluent monolayers of BSC40 cells in 35-mm dishes were infected with vaccinia virus at an m.o.i. of 30 or mock-infected. Cells were harvested in RIPA lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 μg/ml aprotinin (Sigma)). The lysates were frozen and thawed, treated with 100U/ml benzonase (Sigma) for 30 min at 37°C, and then clarified by centrifugation for 10 min at 15,000 rpm in a microfuge at 4°C. Clarified lysates were incubated with antiserum for 2 h at 4°C with rotation. Following monoclonal antibody incubation, a secondary antibody specific for mouse Ig was added to the lysates at 4°C with rotation. Protein A–Sepharose was added for 30 min at 4°C. The immune complexes were harvested by centrifugation and washed three times with RIPA lysis buffer. Samples were resuspended in SDS loading buffer, boiled, and analyzed by SDS–PAGE using 10% separating gels. The proteins were transferred to nitrocellulose in 25 mM Tris–HCl, 192 mM glycine, 20% methanol at 4°C overnight. Nitrocellulose filters were incubated with primary antibody, and the bound antibody was detected using polyclonal anti-mouse or anti-rabbit horseradish peroxidase-conjugated antibody (1:5000; Amersham) and enhanced chemiluminescence. Western blotting reagents (Amersham) were used as described by the manufacturer.

Coupled *in vitro* transcription/translation

The G2R, A18R, and H5R proteins were made *in vitro* using the TnT Quick coupled reticulocyte lysate system (Promega) as described. Briefly, pET16bG2R (expresses hisG2R), pTM1GST-G2 (expresses GST-G2), pET16bA18R (expresses hisA18R) (Simpson and Condit, 1995; Bayliss and Condit, 1995), and pGEMH5 (expresses native H5), each driven by a T7 promoter, were used to program the *in vitro* transcription/translation reaction. Supercoiled plasmid DNA was added at 0.5-1.0 μg per 12.5-μl reaction and the reaction was incubated with 10 μl TnT Quick Lysate (Promega), 5 μCi Redivue Pro-Mix [^{35}S] (Amersham) and incubated for 2 h at 30°C. The resulting proteins were immunoprecipitated in NP-40 buffer (0.25% NP-40, 150 mM NaCl, 50 mM Tris–HCl (pH 7.5)) and analyzed by SDS–PAGE using a 10% separating gel, fluorographed, dried, and autoradiographed.

Affinity purification of GST-G2 and interacting proteins

GST-G2 or GST-P (kindly provided by Dr. Sue Moyer, University of Florida), GST fusion proteins driven by a T7 promoter, was synthesized by transfection into vTF7.3-infected A549 cells. Confluent monolayers of A549 cells were infected with vTF7.3 at an m.o.i. of 10. The cells were incubated at 37°C for 4 h in DME and 10% Hyclone. The cells were washed in DME and transfected with 0, 2.5, or 5 μg of pTM1GST-G2 or pTM1GST-P in DME. The cells were labeled with 100 μCi of Tran[^{35}S]label (ICN Biochemical, Irvine, CA) for 16–18 h in DME and 2% Hyclone. Lysolethicin extracts of the infected/transfected cells were made as for *in vitro* transcription as described (Condit *et al.,* 1996a). For both the *in vitro* and *in vivo* synthesized GST-G2, purification on glutathione–Sepharose 4B was done using the same technique. Specifically, an aliquot of glutathione–Sepharose beads was washed in transcription wash solution [25 mM Hepes (pH 7.4), 50 mM potassium acetate] and then incubated in transcription wash solution containing 0.1% NP-40, 0.5% dry milk, and 1% BSA on ice for 15 min. The beads were washed again in transcription wash solution containing 0.1% NP-40. An aliquot of beads in 50 μl of transcription wash solution containing 0.1% NP-40 was added to either *in vitro* synthesized protein in 350 μl transcription wash solution containing 0.1% NP-40 or 100 μl cell extract in 250 μl transcription wash solution containing 0.1% NP-40. The proteins were allowed to bind beads for 15 min on ice. The bead-bound protein was washed three times in transcription wash solution containing 0.1% NP-40, and the bound protein was solubilized in SDS loading buffer. The solubilized protein was separated on 10% SDS–PAGE, fluorographed, and autoradiographed or transferred to nitrocellulose and probed in a Western analysis.

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