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Protein interactions among the vaccinia virus late transcription factors

Stephanie Dellis[a], Kyle C. Strickland[a], William J. McCrary[b], Ashwin Patel[a], Eileen Stocum[a], Cynthia F. Wright[b,*]

[a]*Biology Department, College of Charleston, Charleston, SC 29401, USA*
[b]*Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, SC 29425, USA*

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Abstract

The viral proteins A1L, A2L, G8R, and H5R positively modulate vaccinia virus late gene expression. Host-encoded proteins hnRNP A2 and RBM3 may also interact with these viral factors to influence late gene expression. In these studies, a yeast two-hybrid screen and in vitro pulldown and crosslinking experiments were used to investigate protein–protein interactions among these factors. These studies confirmed a previous observation that G8R interacts with itself and A1L [McCraith, S., Holtzman, T., Moss, B., and Fields, S. (2000). Genome-wide analysis of vaccinia virus protein–protein interactions. Proc. Natl. Acad. Sci. U.S.A. 97 (9), 4879–4884]. However, self-interactions of A1L and H5R, and interactions between A2L and G8R, A2L and H5R, and H5R and G8R were also observed. In addition, the proteins hnRNP A2 and RBM3 both showed some interaction with A2L. Illustration of these interactions is a step toward understanding the architecture of the late gene transcription complex as it occurs in poxviruses.
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Keywords: Protein; Vaccinia virus; Transcription factor

Introduction

Gene expression in vaccinia virus is temporally regulated and three gene classes—early, intermediate, and late—have been identified based on their time of expression relative to viral DNA synthesis. Each gene class is transcribed by a virally encoded multi-subunit RNA polymerase, whose subunits have homology to eukaryotic RNA polymerase (Broyles and Moss, 1986; Patel and Pickup, 1989). However, the promoter regions and the accessory factors that cause transcription to initiate at DNA sequences specific for each gene class are different. By using an in vivo complementation assay, Keck et al. (1990) identified the A1L, A2L, and G8R viral genes, encoding proteins of 17-, 26-, and 30-kDa, respectively, as being necessary for late gene transcription. Subsequent biochemical studies using in vitro late transcription systems demonstrated a requirement for each of these proteins (Hubbs and Wright, 1996; Keck et al., 1993a,b; Passarelli et al., 1996; Wright et al., 1991). Interestingly, biochemical studies also elucidated the requirement for two additional proteins that were not identified by the in vivo assay. One of these was a viral protein, the product of the H5R gene (Kovacs and Moss, 1996). The other was identified as a factor present in uninfected cells, suggesting that this was a host cell factor. Subsequent work identified the proteins providing at least part of this cellular activity as heterogeneous ribonucleoproteins hnRNP A2 and RBM3, both of which can act independently to stimulate late transcription (Wright et al., 2001). Additional viral proteins, A18R, G2R, and J3R, function in the regulation of transcription elongation of the intermediate and late genes in vivo (Black and Condit, 1996; Lackner and Condit, 2000; Latner et al., 2000; Xiang et al., 1998).

Studies to date of vaccinia virus late transcription initiation have mainly focused on identification of the

* Corresponding author. Department of Pathology and Laboratory Medicine, Medical University of South Carolina, 165 Ashley Avenue, Suite 309, PO Box 250908, Charleston, SC 29425. Fax: +1 843 792 0368.
E-mail address: wrightcf@musc.edu (C.F. Wright).

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proteins participating in this process. The goal of this project was to investigate protein–protein interactions to begin to elucidate how these proteins function in a late transcription complex. A previous report (McCraith et al., 2000) employed a yeast two-hybrid system to conduct a genome-wide screen to investigate potential interactions between all vaccinia virus-encoded proteins. This screen was conducted in a high throughput fashion and, since optimization of interactions was not attempted, most likely only uncovered a fraction of interactions that may be occurring during a viral infection. Notably, none of the subunits that make up the viral RNA polymerase enzyme were seen to interact. However, a potential interaction between the G8R and A1L proteins and a self-interaction of the G8R protein was revealed. Here, we have employed a different version of the yeast two-hybrid screen and have identified several novel potential interactions among the viral late transcription factors and between these factors and the host-encoded factors. Furthermore, we have substantiated several of these interactions through in vitro pulldown and crosslinking experiments. Characterization of these interactions may assist in understanding the function of each of these proteins as they collaborate to activate late gene expression.

Results

Yeast two-hybrid analysis

The yeast two-hybrid system (Fields and Song, 1989) was used to screen for interactions among the late transcription factors. The open reading frames encoding the A1L, G8R, H5R, A2L, and hnRNP A2 proteins were cloned into the pGILDA vector (Clontech, Palo Alto, CA) to construct LexA (DNA binding domain) fusion bait proteins. These same open reading frames, and additionally that encoding RBM3, were cloned into the pJG4–5 vector (Origene, Rockville, MD) to create B42 fusion (activation domain) target proteins. For both of these vectors, growing the yeast in media containing galactose as a carbon source induces expression of the fusion protein. The bait plasmids were transformed into yeast along with the pSH18–34 reporter plasmid (Origene) in which the *lac*Z gene is under the control of an operator containing 8 LexA operator sequences. The bait yeast strains were tested to confirm that none activated the transcription of the reporter plasmid alone. Each bait strain was subsequently transformed separately with the target plasmids, and interactions were identified by streaking co-transformants onto plates containing galactose and X-GAL as an indicator. At least four independent colonies from each transformation were analyzed. Table 1 presents the characteristics of the yeast strains co-transformed with both bait and target plasmids. Results are not given with A2L as the target because that plasmid was unstable in bacteria and not likely to lead to meaningful results in the yeast screen. In several cases, the presence of particular bait and target combinations was apparently toxic to the yeast, resulting in colonies that grew poorly. Often, these plates contained a mixture of extremely small, interspersed blue and white colonies (designated SP for speckled in Table 1). This phenotype was especially prevalent in combinations with the hnRNP A2 and H5R proteins.

Table 1
Growth characteristics and β-galactosidase activity of yeast containing fusion proteins

Bait	Target	Colony color	Growth	β-Gal activity[a]	*P* value[b]
A2L	hnRNP A2	SP[c]	Poor	1.45 ± 0.781	0.0557
	RBM3	++	Good	3.93 ± 2.19	0.0276
	A1L	–	Good	0.870 ± 0.437	0.140
	G8R	++	Good	4.28 ± 0.842	0.00156
	H5R	+++	Poor	17.8 ± 1.36	6.80E−05
	vector	–	Good	0.582	
H5R	hnRNP A2	SP	Poor	0.215 ± 0.0317	
	RBM3	SP	Poor	0.564 ± 0.456	0.489
	A1L	–	Good	0.297 ± 0.0768	
	G8R	++	Good	2.028 ± 1.13	0.0221
	H5R	+++	Good	22.3 ± 21.4	0.0672
	None	–	Good	0.557	
G8R	hnRNP A2	–	Poor	0.146 ± 0.0861	
	RBM3	–	Good	0.102 ± 0.0560	
	A1L	–	Good	0.213 ± 0.0525	
	G8R	+++	Good	41.893 ± 7.844	0.000908
	H5R	SP	Poor	2.501 ± 0.9359	0.0328
	vector	–	Good	0.497 ± 0.268	
A1L	hnRNP A2	SP	Poor	0.225 ± 0.0627	
	RBM3	–	Poor	ND	
	A1L	+++	Good	13.3 ± 4.85	0.00200
	G8R	+++	Good	18.7 ± 9.86	0.00708
	H5R	–	Poor	4.09 ± 0.339	0.00138
	vector	–	Good	0.37	
hnRNP A2	hnRNP A2	+++	Good	48.9 ± 34.6	0.017
	RBM3	+++	Good	11.5 ± 10.5	0.038
	A1L	–	Good	ND	
	G8R	–	Good	ND	
	H5R	++	Good	2.46 ± 0.846	0.00976
	vector	–	Good	0.217 ± 0.066	

[a] β-Galactosidase units are expressed as μmol ONPG hydrolyzed per min per cell and include the standard deviation obtained from assaying four or five independent colonies from each co-transformation. Reactions with A2L as target were not done because of the instability of the plasmid.
[b] *P* values for those interactions above background are reported.
[c] The symbols used are as follows: +++, dark blue; ++, pale/medium blue; –, white; SP, speckled; ND, not done.

In addition to the plate assay, a liquid assay for β-galactosidase activity was also carried out to quantify the interactions in the independent clones from each co-transformation (Table 1). For each strain, the enzyme values obtained for the bait/target combinations were compared to the background value obtained for the bait in the absence of target. It can be seen from the table that the standard deviation in some of these results is rather broad. We believe that this reflects the variability between colonies: when the same colony was assayed in duplicate, the numbers were much more reproducible (data not shown). In general, the units of β-galactosidase activity calculated from the liquid

assay correlated well with the colony color. This assay demonstrated that the interactions that were significantly above background (P value < 0.05) included the self-interactions of A1L, G8R, and hnRNP A2, and the interactions of A1L with G8R, A2L with G8R, A2L with H5R, H5R with G8R, A2L with RBM3, and hnRNP A2 with RBM3. Two interactions are noteworthy in that they did not reach statistical significance in the β-galactosidase liquid assay, but they were confirmed in the experiments described below. The first was the interaction of A2L and hnRNP A2, which fell just short of significance. For this reason, this interaction is shown as a dashed line in Fig. 4. The second was the self-interaction of H5R, which was surprising since the colonies on the X-GAL plates were intensely blue. However, the assayed colonies distributed equally into high and low ranges for β-galactosidase activity, leading to a large standard deviation. For this reason, this interaction did not calculate as significant, yet all other indications are that this interaction is strong.

In vitro pulldown assays

The yeast two-hybrid screen identified several potential interactions between the late transcription factors, however, these results are subject to artifacts that may occur due to endogenous bridging molecules and/or because of variable in vivo expression patterns of the fusion proteins. Therefore, to confirm the interactions seen in the yeast two-hybrid system, a variety of in vitro pulldown experiments were performed. In these experiments, one protein was expressed as a fusion with a domain that would allow it to be immobilized on an affinity resin. Thus, the A2L, A1L, G8R, and H5R proteins were expressed as glutathione-*S*-transferase (GST) fusions and bound to glutathione agarose. The A2L protein was also expressed as a maltose binding protein (MBP) fusion and bound to an amylose resin. Experiments were performed to demonstrate that the GST•G8R, GST•A1L, GST•A2L, and MBP•A2L fusion proteins were all functional using in vitro transcription reactions (Fig. 1). The immobilized protein was then incubated with a ^{35}S-labeled target protein synthesized in a reticulocyte coupled transcription/translation system, the beads were washed, and the proteins eluted from the beads were visualized by SDS-PAGE followed by autoradiography. In some reactions, purified G8R target protein was incubated with an immobilized protein on a resin and the eluted protein was visualized by immunoblotting with anti-G8R antiserum. In all cases, the amount of protein retained by the beads was compared to that retained by control beads containing only the GST or MBP proteins. The results from these experiments are shown in Fig. 2: panel A shows interactions with immobilized A1L incubated with itself; panel B shows interactions with immobilized G8R incubated with itself, A1L and A2L; panel C shows interactions with immobilized H5R incubated with A1L, A2L, G8R and itself; panel D shows interactions with immobilized A2L incubated with hnRNP A2 and RBM3; and panel E shows interactions with immobilized A2L or A1L incubated with G8R. These experiments substantiated the interactions seen in the yeast two-hybrid screens between A1L and itself (panel A); G8R and itself (panel B); G8R and A1L (in both directions, panels B and E); G8R and A2L (in both directions, panels B and E); H5R and itself (panel C); H5R and A2L (panel C); H5R and G8R (panel C); and A2L and the host cell proteins hnRNP A2 and RBM3 (panel D).

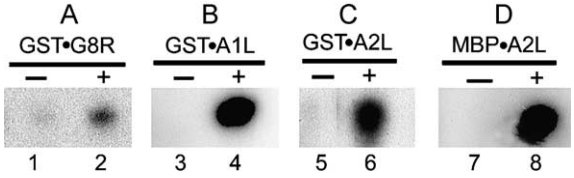

Fig. 1. Transcription reactions with fusion proteins. All reactions contained vaccinia virus RNA polymerase purified from infected HeLa cells and a partially purified extract containing host cell transcription factors. Each reaction also contained additional transcription factors, purified from either a baculovirus expression system or from bacteria, as indicated in the following descriptions. (A) Proteins A1L and A2L (baculovirus) with approximately 100 ng of GST•G8R (bacteria) added in lane 2. (B) MBP•A2L (280 ng, bacteria) with approximately 200 ng of GST•A1L protein (bacteria) added in lane 4. (C) G8R and A1L proteins (baculovirus) with approximately 100 ng GST•A2L protein (bacteria) added in lane 6. (D) A1L protein (baculovirus) with 280 ng MBP•A2L protein (bacteria) added in lane 8. For all reactions, the radiolabeled RNA transcript produced was subjected to electrophoresis on a 4% denaturing acrylamide gel and visualized by autoradiography.

Crosslinking experiments

The availability of a highly purified preparation of native (non-fusion) A1L protein allowed us to investigate the stoichiometry of its self-interaction. In these experiments, glutaraldehye, a homobifunctional amine crosslinker, was added to the purified A1L protein in increasing concentrations. The reactions were then subjected to SDS-PAGE and visualized by immunoblot analysis using an anti-A1L antibody. As a control, BSA was also exposed to glutaraldehyde at the same concentrations and the products were visualized by SDS-PAGE followed by silver staining. Previous studies have shown that BSA exists primarily as a monomer at low concentrations of glutaraldehyde (Friedman et al., 1993). Fig. 3 (panel A) shows the A1L preparation subjected to SDS-PAGE and either reduced (lane 1) or not reduced (lane 2). These reactions demonstrated that the protein migrates predominantly as a monomer under reducing conditions. However, two additional species slightly larger than 30 kDa appeared when the A1L protein was not reduced. Either of these forms could represent SDS-resistant dimers, and may reflect the fact that there are multiple subpopulations of A1L or different structures that can form when it interacts with itself. With the addition of 0.004% or 0.0008% glutaraldehyde, the predominant species observed was that of the faster-migrating dimer, perhaps reflecting compaction of the

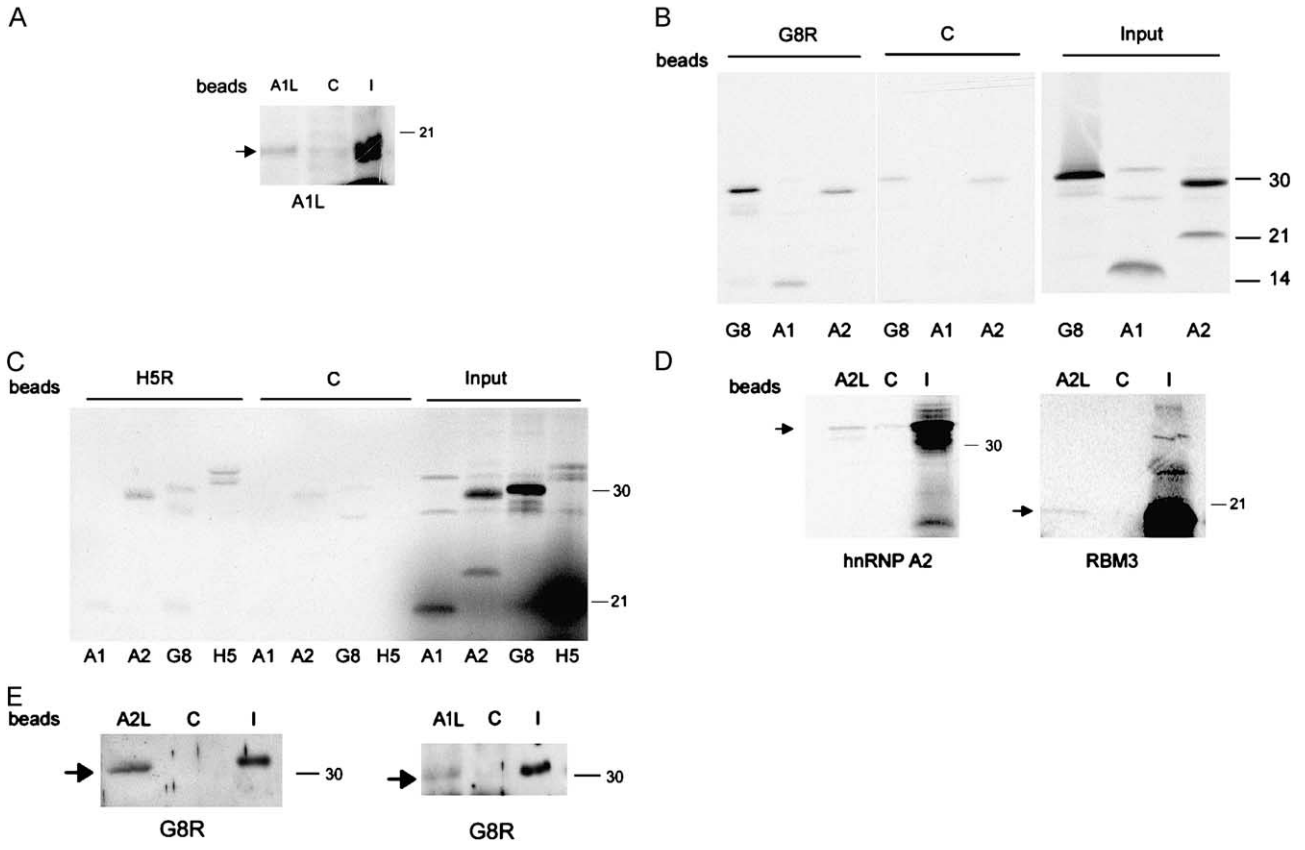

Fig. 2. Pulldown analyses. For all panels, the protein immobilized on beads is indicated above the lanes and the soluble protein is indicated below the lanes. The positions of selected molecular mass markers run in parallel are shown to the right of the gels. Results are representative of at least two independent reactions. (A) The GST • A1L protein (A1L) or GST alone (C) were bound to glutathione agarose and incubated with ^{35}S-labeled A1L in binding buffer containing 100 mM KCl. The beads were washed with the same buffer and eluted by boiling in SDS loading buffer. The input (I) lane represents 20% of the A1L protein added to the reactions. (B) The GST • G8R protein (G8R) or GST alone (C) were bound to glutathione agarose and incubated with ^{35}S-labeled G8R, A1L, or A2L in binding buffer containing 10 mM KCl. The beads were washed with the same buffer and eluted with SDS loading buffer. The input lanes represent the amount of labeled protein added to the reactions; however, the exposure time for these lanes was 2 h, while the exposure time for the remaining lanes was 60 h. (C) The GST • H5R protein (H5R) or GST alone (C) were incubated with ^{35}S-labeled A1L, A2L, G8R, or H5R in binding buffer containing 50 mM KCl. The beads were washed with the same buffer and eluted by boiling in SDS loading buffer. The input lanes represent the total amount of protein added to each reaction with the same exposure time as the reaction lanes. (D) The MBP • A2L (A2L) or MBP alone (C) were bound to an amylose resin and incubated with ^{35}S-labeled hnRNP A2 or RBM3 in binding buffer containing 100 mM KCl. The beads were washed in the same buffer and eluted with buffer containing 10 mM maltose. The input lanes represent 20% of the labeled protein added to the reaction. (E) Left panel: GST • A2L (A2L) or GST (C) were bound to glutathione agarose and incubated with purified G8R in binding buffer containing 50 mM KCl. The beads were washed with binding buffer containing 150 mM KCl and eluted with 20 mM reduced glutathione dissolved in Tris–HCl, pH 8. The input lane represents the total amount of G8R protein added to the reactions. Right panel: The GST • A1L (A1L) or GST (C) proteins were bound to glutathione agarose and incubated with purified G8R in binding buffer containing 100 mM KCl. The beads were washed with the same buffer and eluted by boiling in SDS loading buffer. The input lane represents the total amount of G8R protein added to the reactions. For both panels, the results were visualized by immunoblot analysis using anti-G8R antibody.

structure due to the crosslinking agent. At 0.02% glutaraldehyde, the protein was apparently crosslinked to the extent that it did not enter the gel. Less predominant species with the mobility of trimers and tetramers were also observed. In contrast, BSA was predominantly monomeric at 0.004% and 0.0008% glutaraldehyde concentrations (panel B, lanes 9 and 10). No bands were observed in the 120-kDa region that would be expected if BSA were dimerizing. Thus, our data indicate that a crosslinking agent preferentially stabilizes a dimeric form of A1L. This is consistent with our results from the yeast two-hybrid analysis and from the pulldown studies both of which suggested that A1L interacts with itself.

Discussion

The current study has expanded knowledge of the number of potential protein–protein interactions that may be occurring among the vaccinia virus late transcription factors. Fig. 4 diagrammatically illustrates the interactions that were observed. In summary, we confirm the self-interaction of the G8R protein and the A1L–G8R interaction first elucidated in the whole genome screen of McCraith et al. (2000). Additionally, however, self-interactions of A1L and H5R were also identified as well as interactions between A2L and G8R, A2L and H5R, and H5R and G8R. In general, it appears as though the whole genome

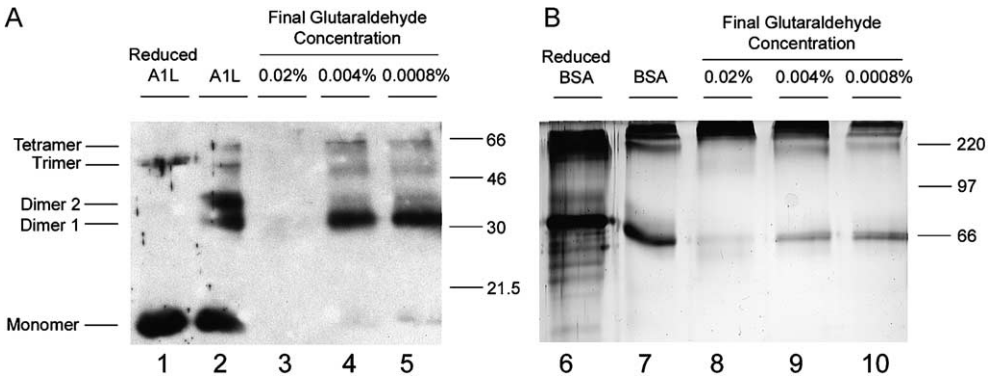

Fig. 3. Glutaraldehyde crosslinking of A1L. (A) Purified A1L was incubated in SDS loading buffer containing β-mercaptoethanol and boiled before SDS-PAGE (lane 1) or incubated in buffer lacking β-mercaptoethanol and not boiled before SDS-PAGE (lane 2). In lanes 3–5, the protein was dialyzed into PBS and incubated with the concentration of glutaraldehyde indicated above each lane, SDS loading buffer lacking β-mercaptoethanol was added, and the samples loaded onto the gel without boiling. After running, the gel was subjected to immunoblot analysis using anti-A1L polyclonal antibody and visualized using enhanced chemiluminescence. The positions of molecular mass markers run in parallel on the gel are shown on the right. (B) Purified BSA was treated in a similar manner only the results were visualized by silver staining the gel.

screen detected the stronger interactions that may potentially occur; the sensitivity of our screen uncovered many additional interactions.

The A1L protein has been identified as a late transcription factor both in vivo (Keck et al., 1990) and in vitro (Keck et al., 1993b; Wright and Coroneos, 1993). A temperature-sensitive mutation has been mapped to the open reading frame encoding this protein, demonstrating that it is essential to the viral life cycle (Carpenter and DeLange, 1992; Condit and Motyczka, 1981; Condit et al., 1983). Our screen identified three interactions of A1L with other factors. First, we confirmed the A1L-G8R interaction noted by McCraith et al. (2000) and substantiated it through in vitro pulldown experiments. The immobilized GST•A1L fusion protein interacted with soluble G8R; conversely, immobilized GST•G8R interacted with soluble A1L (Fig. 2, panels E and B). Second, we identified a strong interaction of A1L with itself, which was confirmed with pulldown experiments (Fig. 2, panel A). This interaction was further substantiated by glutaraldehyde crosslinking experiments, which demonstrated that A1L could form a dimer when crosslinked in solution (Fig. 3). Finally, we saw a potential interaction of A1L with H5R when A1L was used as the bait protein in the yeast two-hybrid screen. However, this interaction was not reciprocated in the H5R bait-A1L target configuration, nor was it convincing in pulldown experiments when the GST•H5R fusion was immobilized on beads and incubated with soluble A1L (Fig. 2, panel C) or when GST•A1L was immobilized on beads and incubated with soluble H5R (data not shown). Therefore, we think it likely that this interaction was a false-positive.

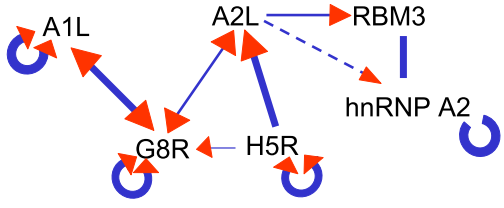

Fig. 4. Diagrammatic representation of the potential interactions of the late transcription factors. The strength of the interaction as determined by the yeast two-hybrid β-galactosidase activity is represented by the thickness of the line connecting the proteins. Self-interactions are represented by the looped semi-circle. The figure also shows the interactions corroborated by pulldown assays. A red arrowhead (when present) pointing to a protein shows that the soluble protein interacted with its partner immobilized on beads (soluble A2L interacted with bound H5R, for example). A red double-headed arrow shows that the two proteins interacted when the pulldown assay was performed in both directions (G8R and A1L, for example).

The G8R protein is another essential protein (Zhang et al., 1992) demonstrated to be a late transcription factor both in vivo (Keck et al., 1990) and in vitro (Wright et al., 1991). Our yeast two-hybrid screen confirmed the very strong interaction of G8R with itself and this was substantiated by in vitro pulldown experiments (Fig. 2, panel B). The yeast two-hybrid screen also identified a weaker interaction between G8R and H5R, which was also confirmed in vitro (Fig. 2, panel C). Interestingly, when G8R was used as the bait, we failed to see an interaction with A1L (Table 1). The reason for this is unknown since this same bait strain allowed the strong self-interaction of G8R to be observed. Thus, the G8R–A1L interaction as monitored by these fusion proteins was specific to the orientation of the viral proteins. However, the preponderance of data (including pulldown experiments performed in both directions) suggests that this result is a false-negative and that these two proteins do indeed interact.

The A2L and H5R proteins have also been shown to have late transcription stimulatory activities in vitro (Hubbs and Wright, 1996; Kovacs and Moss, 1996; Passarelli et al., 1996). Experiments conducted using the A2L protein as the bait in the yeast two-hybrid system revealed a strong interaction between this protein and H5R, as well as a moderate interaction with the G8R protein. Both of these interactions were also seen in the pulldown assays. Self-interaction of A2L was not detectable in the yeast two-

hybrid screen due to the fact that the target vector appeared to be unstable. Also, a convincing interaction between A2L and itself was not apparent by pulldown analysis (data not shown). However, others have reported a possible dimerization of the protein (Keck et al., 1993a) and we have noted anomalous sedimentation of the protein in glycerol gradients, suggesting multimerization or formation of an unusual structure (data not shown).

The H5R protein is an enigmatic protein that migrates anomalously on gels and is phosphorylated in vivo by the vaccinia virus B1 kinase (Beaud et al., 1995). In addition to stimulating late transcription in vitro, it interacts with the A18R and G2R proteins that modulate post-replicative transcription elongation (Black et al., 1998). Taken together, these experiments suggest that this protein is part of a late transcription complex. Our screen substantiated this by finding a strong interaction with the A2L protein and also revealed a strong self-association of this protein. A somewhat weaker interaction with the G8R protein as target was also seen in the yeast two-hybrid screen, which reciprocated the results seen with G8R as bait, and in pulldown experiments. The H5R protein may have additional roles, however, as it has been found that viruses with a temperature sensitive mutation in H5R are wild-type with respect to late transcription, but have defects in morphogenesis (DeMasi and Traktman, 2000).

Our previous studies have identified two host cell proteins, hnRNP A2 and RBM3, that are individually capable of stimulating late transcription in vitro (Wright et al., 2001). The current results show that hnRNP A2 strongly interacted both with itself and with RBM3. This confirmed a previous report that hnRNP A2 is self-interacting (Cartegni et al., 1996) and also demonstrated for the first time that hnRNP A2 and RBM3 could potentially interact in cells. Interestingly, we found an interaction of the viral A2L protein with these proteins both in the two-hybrid screen and in pulldown experiments. This interaction, albeit weak, is consistent with results from biochemical purification of cell extracts, in which we could not detect stimulation of late transcription by A2L until the host cell activity was purified from infected cells incubated with hydroxyurea or from uninfected cells (Hubbs and Wright, 1996). This suggested that A2L contaminated the host cell factor preparations, which lends further support to the idea that these proteins may interact during an infection.

In summary, the current screen has identified several previously unreported interactions that may potentially occur between these proteins as they interact to form a late transcription complex. All of these interactions are presumably direct, since it is unlikely that molecules bridging these interactions would be present both in yeast cells and in reticulocyte extracts. Also, the A2L–G8R, A1L–G8R, and A1L–A1L interactions were observed in vitro using purified proteins. It must be noted that the interactions we have observed have yet to be confirmed in infected cells. We have previously attempted to co-immunoprecipitate the G8R and A1L proteins from infected cell extracts and from lysates of coupled in vitro transcription/translation systems. These experiments were not successful (data not shown), although the yeast screens and in vitro experiments consistently show interactions between these proteins.

The association of transcription factors during initiation is likely to be quite complex; proteins may interact with themselves, with other proteins (including RNA polymerase) and/or with DNA. Current studies are focusing on which of these factors, or combination of factors, contacts DNA. These experiments may help to determine the order of the protein–protein interactions and ultimately the mechanism of transcriptional activation for the vaccinia virus late genes.

Materials and methods

Construction of recombinant plasmids

The vaccinia virus open reading frames (ORFs) encoding A1L and G8R had previously been cloned into the pET3a plasmid (Novagen; Madison, WI) by polymerase chain reaction (PCR) amplification using appropriate primer sets and vaccinia virus (WR strain) DNA as the template. The H5R ORF had similarly been cloned into the pRSETA vector (Invitrogen; Carlsbad, CA). To clone these genes into the plasmids for the yeast two-hybrid system, the ORFs were re-amplified using primers that would put appropriate restriction sites at the ends of the products. Table 2 shows the sequences of the primers that were used to amplify the A1L, G8R, and H5R ORFs using the pET3a or pRSETA plasmids as templates. PCR was performed using 0.4 mM forward and reverse primer, 0.1 mM dNTP's, 2 units of Taq

Table 2
Sequences of primer pairs used to create recombinant plasmids

ORF	Forward primer	Reverse primer
A1L[a]	5′: GGGGAATTCATGGCTAAGCGAGTAAG	5′: GGGCTCGAGTTACAATAAACTCCGTAGAG
G8R	5′: GGGGAATTCATGAGCATCCGTATAAAAAT	5′: GGGCTCGAGTTAATCTAAAAACGCCATAA
H5R	5′: GGGGAATTCATGGCGTGGTCAATTAC	5′: GGGCTCGAGTTACTTCTTACAAGTTTT
RBM3	5′: AAAGAATTCATGTCCTCTGAAGAAGGA	5′: AAACTCGAGTCAGTTGTCATAATTGTC
A2L	5′: GAGTCTTAACATGAATCTACG	5′: GCCATTTAATTACGGAACTAT

[a] For the A1L, G8R, H5R, and RBM3 genes, the forward primer of each set amplifies the 5′ end of the gene with an inserted *Eco*RI site; the reverse primer of each set amplifies the 3′ end of the gene with an inserted *Xho*I site.

Gold Polymerase (Applied Biosystems; Foster City, CA), 6 ng of plasmid template, and 1× Taq Gold Buffer. The PCR cycle was: 95 °C for 10 min; 35 cycles of 95 °C for 45 s, 42 °C for 60 s, 72 °C for 60 s; and a final cycle of 72 °C for 10 min. The amplified PCR product was then digested using *Eco*RI and *Xho*I, phenol extracted, ethanol precipitated, and resuspended in water. The bait (pGILDA; Clontech) and target (pJG4–5; Origene) plasmids for the yeast two-hybrid system were also digested with both *Eco*RI and *Xho*I. Each plasmid was then phenol extracted, ethanol precipitated, and treated with shrimp alkaline phosphatase (SAP, United States Biochemicals; Cleveland, OH) using 0.5 units of SAP per 400 ng of each plasmid. The SAP-treated plasmids were then phenol extracted, ethanol precipitated, resuspended in water, and ligated with the amplified PCR products from above using T4 DNA ligase (Invitrogen). Separate reactions were carried out for each of the late transcription factor genes.

The hnRNP A2 ORF was cloned by digesting the pET28a vector containing this gene (Wright et al., 2001) with *Eco*RI and *Sal*I and cloning the insert into *Eco*RI and *Xho*I digested pGILDA or pJG4–5. The RBM3 ORF was cloned into pJG4–5 by amplifying the ORF from a recombinant pET15b plasmid (Wright et al., 2001) using the primers shown in Table 2, digesting the PCR product with *Eco*RI and *Xho*I, and ligating into pJG4–5 digested with *Eco*RI and *Xho*I.

The A2L ORF was amplified from vaccinia virus DNA using the primers shown in Table 2. The pJG4–5 and pGILDA plasmids were digested with *Eco*RI; the digested plasmids and the A2L PCR product were filled in using the Klenow fragment of *Escherichia coli* DNA polymerase I. The vectors and insert were mixed and ligated in separate reactions. Restriction analysis confirmed the presence of insert in each vector; however, the pJG4–5 plasmid containing the A2L insert was unstable in bacteria and continually segregated colonies in which the insert had been lost.

All recombinant plasmids were verified by restriction digestion and sequence analysis.

Antibodies

Polyclonal antibodies to G8R and A1L were raised separately in rabbits by injecting a combination of purified native proteins and proteins eluted from SDS-PAGE gels following the schedule previously described (Wright et al., 1991). Antibodies were tested by verifying that they recognized their cognate purified proteins by immunoblot analysis, and that they recognized an appropriately sized protein in extracts from vaccinia virus-infected mammalian cells (but not in uninfected cell extracts). Both antibodies were also effective in immunoprecipitating their cognate proteins.

Yeast two-hybrid analysis

Saccharomyces cerevisiae yeast strain EGY48 (MATα *trp1 his3 ura3 leu2*::6 LexAop-*LEU2;* Origene) was simultaneously transformed with each recombinant bait plasmid and pSH18–34 (Origene), a *lac*Z reporter plasmid containing 8 copies of the Lex A operator sequence, and transformants were selected on (−)his (−)ura minimal agar plates containing 2% glucose as a carbon source. Tests for the autoactivation of bait plasmids were carried out on (−)his (−)ura minimal agar plates containing 2% galactose/1% raffinose and the indicator 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL). Each yeast strain was then transformed separately either with the empty pJG4–5 vector or a target plasmid containing the A1L, G8R, H5R, hnRNP A2 or RBM3 genes and colonies were selected on (−)his (−)ura (−)trp minimal agar plates containing glucose. As mentioned above, the pJG4–5 plasmid containing the A2L ORF was apparently unstable in bacteria and so was not used as a target in the yeast two-hybrid analysis. Co-transformants were then streaked on (−)his (−)ura (−)trp minimal agar plates containing galactose/raffinose and X-GAL and positive interactions were identified by the presence of blue colonies.

Liquid β-galactosidase assay

To quantify the protein–protein interactions, a β-galactosidase liquid culture assay was employed (Clontech, protocol #PT3024-1). Briefly, yeast strains containing bait, target, and reporter plasmids were grown to log phase in (−)his (−)ura (−)trp liquid media containing galactose/raffinose and the OD_{600} of the culture was recorded. Cells were centrifuged, washed in 1.5 ml Z-Buffer (62.5 mM Na_2HPO_4; 40 mM NaH_2PO_4; 10 mM KCl; 1.2 mM $MgSO_4$), and then resuspended in 0.3 ml Z-Buffer. A 0.1 ml aliquot of these cells was subjected to 3 freeze–thaw cycles using dry ice in methanol and a 37 °C water bath. Then 0.7 ml Z-buffer (containing 0.27% β-mercaptoethanol) and 160 μl *o*-nitrophenol-β-D-galactopyranoside (ONPG; 4 mg/ml in Z-buffer) were added to the cells. The samples were incubated at 30 °C until a yellow color developed and the time since addition of the ONPG was noted. At this point, 0.4 ml of 1 M Na_2CO_3 was added to stop the reaction. Cellular debris was pelleted, and the OD_{420} of the supernatant was measured. The β-galactosidase units (μmol ONPG hydrolyzed per min per cell) were then calculated according to the equation: $\beta\text{galunits} = 1000 * \frac{OD_{420}}{t * V * OD_{600}}$, where t is incubation time (in min) and V is the concentration factor. Statistical analysis of the β-galactosidase results was performed using Student's one-tailed t test and the P values for the interactions above background are reported in Table 1.

Fusion protein production

The A1L, H5R, A2L, and G8R ORFs were cloned into the plasmid pGEX-2TK (Amersham; Piscataway, NJ) and transformed into *E. coli* DH5α. The empty pGEX-2TK plasmid was similarly transformed. Bacterial cultures

containing the empty plasmid (to make glutathione-*S*-transferase (GST) protein alone) or the recombinant plasmids (to make the fusion proteins) were grown to mid-log phase in 400 ml Luria Broth containing ampicillin. Protein expression was then induced by adding 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cultures were harvested after 2.5 h, and the cells were then pelleted and lysed in PBS by sonication or by shaking in 20 ml B-PER reagent (Pierce, Rockford, IL) containing 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml aprotinin. After centrifugation (10 000 rpm for 20 min in a JA-17 rotor), 200 μl of a 50% slurry of glutathione–sepharose beads in PBS were added to the supernatant. The protein and beads were incubated on ice for 30 min with rocking, and then the beads were washed two times with 50 ml of cold PBS. After washing, the beads were suspended in PBS to make a 50% slurry. Aliquots of the beads were centrifuged, resuspended in SDS loading buffer (62 mM Tris–HCl, pH 6.8; 2% SDS; 10% glycerol; 0.0015% bromophenol blue; 5% β-mercaptoethanol), boiled, and the eluted protein subjected to SDS-PAGE and stained. The volume of beads was adjusted as necessary with empty beads so that approximately the same amount of fusion and GST proteins were added to pulldown reactions assayed in parallel. For use in transcription reactions, the proteins were eluted from the beads with 20 mM reduced glutathione dissolved in 0.05 M Tris–HCl, pH 7.5/100 mM NaCl and dialyzed against buffer A (50 mM Tris–HCl, pH 8; 100 mM NaCl; 0.01% IGEPAL (Sigma; St. Louis, MO); 2 mM dithiothreitol; 10% glycerol).

Due to difficulties with yield and stability of the GST•A2L protein, we examined whether an alternate expression system could be used. To that end, the A2L gene was amplified from vaccinia virus DNA using the primers shown in Table 2 and blunt-end cloned into the plasmid pUC18 digested with *Sma*I. The insert was sequenced and transferred as a *Bam*HI/*Eco*RI fragment into pRSETA. The insert was excised from pRSETA with a *Bam*HI/*Hin*dIII digestion and cloned into *Bam*HI/*Hin*dIII digested pMAL-c2X (New England Biolabs; Beverly, MA) to create a maltose binding protein (MBP)•A2L fusion vector. Growth, induction, and purification of the fusion protein over an amylose resin (and MBP alone from the empty vector) were conducted as described by the manufacturer. Both proteins were then further purified by chromatography on hydroxylapatite and re-bound to the amlyose resin as needed for the pulldown reactions. The soluble MBP•A2L protein was also used in transcription reactions after dialysis against buffer A. This strategy resulted in a higher yield of full-length fusion as compared to the protocol used to make the GST•A2L protein.

Transcription reactions

Late promoter-specific in vitro transcription reactions were conducted as previously described (Wright and Coroneos, 1993). The G8R, A1L, and A2L proteins were purified from the extracts of recombinant baculovirus-infected Sf9 cells as previously described (Hubbs and Wright, 1996; Wright and Coroneos, 1993).

Radiolabeled target proteins

Radiolabeled target proteins were synthesized by adding 2 μg of plasmid containing a T7 promoter driving expression of a particular gene (pET3a for A1L, A2L, or G8R; pRSETA for H5R; pET28a for hnRNP A2; or pET15b for RBM3) to 40 μl TNT or TNT Quick Reaction Mix (Promega; Madison, WI), containing 40 μCi ^{35}S-labeled methionine in a final volume of 50 μl. The reaction mixes were incubated at 30 °C for 90 min.

Pulldown assays

To test for interactions, the beads containing the GST fusions or GST control (or MBP fusion for A2L) were incubated with the radiolabeled target protein in separate reactions containing binding buffer (50 mM Tris pH 7.5; 5 mM $MgCl_2$; 0.1% IGEPAL; and from 10 to 100 mM KCl) in a total volume of 200 μl. Each tube was rotated end-over-end at 4 °C overnight. The beads were then washed with binding buffer from two to five times. Proteins were eluted from the beads by boiling the beads in SDS loading buffer at 95 °C for 3 min (for the GST proteins) or by adding buffer (20 mM Tris–HCl, pH 7.4; 200 mM NaCl; 1 mM EDTA) containing 10 mM maltose (for the MBP protein). The eluants were subjected to SDS-PAGE and examined by autoradiography. In some reactions, glycerol-gradient purified G8R protein (Wright and Coroneos, 1993) was added to the beads and the eluted protein was subjected to SDS-PAGE, blotted onto Immobilon-P (Millipore; Bedford, MA), and incubated with anti-G8R antibody. The bound antibody was detected using an anti-rabbit secondary antibody coupled to horseradish peroxidase (BioRad; Hercules, CA), followed by detection using an ECL kit from Amersham.

Glutaraldehyde crosslinking

The A1L protein (1 μg), purified as previously described (Wright and Coroneos, 1993) and dialyzed in PBS, was mixed in PBS with glutaraldehyde in 20 μl reactions to give final glutaraldehyde concentrations of 0.02%, 0.004%, and 0.0008%, respectively. The mixtures were incubated for 20 min at room temperature and subjected to SDS-PAGE. The protein was then visualized by immunoblot analysis as described above, but using anti-A1L antibody. As a negative control, 1 μg of bovine serum albumin (BSA) was treated in a similar manner except that the protein was visualized by silver staining.

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