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Interaction between the J3R Subunit of Vaccinia Virus Poly(A) Polymerase and the H4L Subunit of the Viral RNA Polymerase

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J3R, the 39-kDa subunit of vaccinia virus poly(A) polymerase, is a multifunctional protein that catalyzes (nucleoside-2′-*O*-)-methyltransferase activity, serves as a poly(A) polymerase stimulatory factor, and acts as a postreplicative positive transcription elongation factor. Prior results support an association between poly(A) polymerase and the virion RNA polymerase. A possible direct interaction between J3R and H4L subunit of virion RNA polymerase was evaluated. J3R was shown to specifically bind to H4L amino acids 235–256, C terminal to NPH I binding site on H4L. H4L binds to the C-terminal region of J3R between amino acids 169 and 333. The presence of a J3R binding site near to the NPH I binding region on H4L led us to evaluate a physical interaction between NPH I and J3R. The NPH I binding site was located on J3R between amino acids 169 and 249, and J3R was shown to bind to NPH I between amino acids 457 and 524. To evaluate a role for J3R in early gene mRNA synthesis, transcription termination, and/or release, a transcription-competent extract prepared from cells infected with mutant virus lacking J3R, *J3-7.* Analysis of transcription activity demonstrated that J3R is not required for early mRNA synthesis and is not an essential factor in early gene transcription termination or transcript release *in vitro.* J3R interaction with NPH I and H4L may serve as a docking site for J3R on the virion RNA polymerase, linking transcription to mRNA cap formation and poly(A) addition.

Key Words: vaccinia virus; poxvirus; RNA polymerase; poly(A) polymerase; capping enzyme.

INTRODUCTION

Vaccinia virus, the prototypical member of the orthopoxvirus family, is unique among DNA viruses in that it replicates entirely in the cytoplasm of the infected cell (for review, see Moss, 1996). As a consequence, vaccinia virus encodes most of the enzymes needed for viral gene expression and DNA replication. As a result, vaccinia virus has served as a useful model system for investigating the basic mechanisms of RNA and DNA metabolism.

Vaccinia virus gene expression is divided into three temporal classes, early, intermediate, and late. This temporal regulation of gene transcription is mediated by the timed synthesis of class-specific transcription initiation factors. Early genes are transcribed in the virus core immediately after infection; intermediate genes are transcribed in the cytoplasm subsequent to the onset of DNA replication and require prior early gene expression; late gene transcription follows the intermediate genes and requires the synthesis of three intermediate proteins (Moss, 1990). Host factors are also employed in both intermediate (Rosales *et al.,* 1994) and late (Gunasinghe *et al.,* 1998; Zhu *et al.,* 1998) mRNA synthesis. Initiation of early vaccinia virus transcription requires the early transcription factor VETF (Broyles *et al.,* 1988) and virion RNA polymerase possessing the RNA polymerase-associated protein RAP94, the product of H4L gene (Ahn and Moss, 1992; Deng and Shuman, 1994).

The structure of intermediate and late vaccinia virus mRNAs differs significantly from the structure of early viral mRNAs primarily as a result of differences in formation of mRNA 3′ ends (Mahr and Roberts, 1984). Early gene transcription is actively terminated in response to a conserved sequence element, UUUUUNU, in the nascent mRNA (Yuen and Moss, 1987). Early viral mRNAs are therefore discrete in size. In contrast, at intermediate and late times during infection, early termination signals are ignored, and most intermediate and late transcripts do not appear to be terminated at unique sites on the genome (Mahr and Roberts, 1984). However, analyses of the mechanism of 3′ end formation of the late mRNA encoding the major protein component of the A-type inclusions (in cowpox virus, the 160-kDa protein encoded by the *ati* gene) showed that these 3′ ends are generated

Abbreviations: GST, glutathione S-transferase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; NPH I, nucleoside triphosphate phosphohydrolase I; VTF, vaccinia termination factor; RAP94, RNA polymerase-associated protein 94 kDa; IBT, isatin-β-thiosemicarbazone; CTD, carboxy-terminal domain.

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by site-specific RNA cleavage (Antczak *et al.,* 1992; Howard *et al.,* 1999). *In vitro* only the virion RNA polymerase capable of recognizing early promoters is subject to signal-dependent termination, indicating that this form of RNA polymerase is uniquely termination competent (Condit *et al.,* 1996). Termination requires both the vaccinia termination factor (VTF; also serves as viral mRNA capping enzyme) (Shuman *et al.,* 1987) and nucleoside triphosphate phosphohydrolase I (NPH I), the product of D11L gene, as the ATPase employed in transcription termination (Christen *et al.,* 1998; Deng and Shuman, 1998). A recent report by Mohamed and Niles (2000) demonstrated that a physical interaction between H4L subunit of virion RNA polymerase and NPH I is required for transcript release *in vitro*. This interaction explains the observed restriction of transcription termination to early viral genes and defines H4L as a termination cofactor (Mohamed and Niles, 2000).

J3R, the small subunit of vaccinia virus poly(A) polymerase (Gershon *et al.,* 1991; Schnierle *et al.,* 1992), is a multifunctional polypeptide. In the virion, J3R is present in a fivefold molar excess over E1L, the catalytic subunit of poly(A) polymerase, and therefore exists both in monomeric form and in association with E1L (Gershon *et al.,* 1991). Although it lacks polyadenylation catalytic activity (Gershon *et al.,* 1991), J3R dramatically accelerates E1L catalytic extension of either mRNA 3′-end primers with short oligo(A) tails, or poly(A) primers (Gershon and Moss, 1993). J3R has a second, entirely unrelated function, catalyzing the conversion of the mRNA 5′ cap O [m^7G(5′)pppN] structure to its cap 1 counterpart [m^7G(5′)pppN_m] by methylation of the ribose moiety of the first transcribed nucleotide of the mRNA (Schnierle *et al.,* 1992). Both the monomeric and heterodimeric forms of J3R exhibit methyltransferase activity (Schnierle *et al.,* 1992). A recent report by Latner *et al.* (2000) demonstrates that J3R serves as a postreplicative positive transcription elongation factor during a normal virus infection. Compared to wild-type virus infections, J3R mutants synthesize reduced amounts of large late viral proteins and shorter-than-normal intermediate and late mRNAs (Xiang *et al.,* 2000). The elongation factor activity of J3R is independent of the poly(A) stimulatory activity (Xiang *et al.,* 2000).

Prior work by Zhang *et al.* (1994) showed that nascent RAP94-deficient virus core particles exhibit low or undetectable amounts of several viral enzymes, including viral RNA polymerase, capping enzyme, NPH I, poly(A) polymerase, topoisomerase, and RNA helicase. The presence of these unpackaged viral enzymes in the cytoplasm indicated that RAP94 (H4L) is required for targeting a complex of functionally related proteins involved in early mRNA synthesis. Several observations made over the past few years have forged strong molecular links between transcription by nuclear RNA polymerase II (Pol II) and pre-mRNA processing. The key findings support

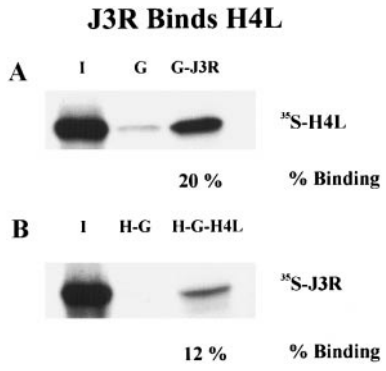

FIG. 1. J3R interacts with the H4L subunit of virion RNA polymerase. A coupled transcription/translation system was employed to synthesize ^{35}S-H4L or ^{35}S-J3R. GST-J3R and His-GST-H4L were purified from the S100 fraction of the induced *E. coli* cells by batchwise affinity to glutathione–Sepharose. One microliter of the translation mix was incubated with 25 μl of glutathione–Sepharose resin (50:50 slurry) coupled to 1 μg of GST-fusion protein of interest at 4°C overnight in binding buffer [25 mM Tris–HCl, pH 8.0, 1 mM EDTA, 10% glycerol (w/v), 50 mM NaCl]. The resin was washed and analyzed by SDS–PAGE followed by X-ray autoradiography. (A) The association of the H4L subunit of virion RNA polymerase, ^{35}S-H4L, with the small subunit of poly(A) polymerase, GST-J3R, is demonstrated. (B) The association of ^{35}S-J3R with His-GST-H4L is demonstrated. I, 50% of the input radioactivity; G, resin-bound GST; G-J3R, resin-bound GST-J3R; H-G, resin-bound His-GST; H-G-H4L, resin-bound His-GST-H4L. The percent binding (indicated below the autoradiograph) was quantified by scanning the autoradiogram with a PhosphorImager.

the view that the carboxy-terminal domain (CTD) of the large subunit of RNA polymerase II binds directly to protein factors essential for RNA processing (Neugebauer and Roth, 1997). The presence of a nuclear RNA pol II transcription/processing complex suggests the possibility of such a complex in vaccinia virus.

In this report, we demonstrate that J3R binds to both NPH I and the H4L subunit of the viral RNA polymerase. Furthermore, we show that J3R is not required for early mRNA synthesis, transcription termination or transcript release *in vitro.*

RESULTS

Interaction between J3R and H4L subunit of virion RNA polymerase

The GST-pulldown approach was employed to evaluate possible interactions between J3R, the small subunit of vaccinia virus poly(A) polymerase, and the H4L subunit of virion RNA polymerase. To this end, glutathione–Sepharose coupled with GST-J3R was mixed with ^{35}S-labeled, *in vitro*-synthesized H4L protein. As a negative control, glutathione–Sepharose resin coupled with GST was mixed with an equal amount of ^{35}S-H4L. Compared to a resin-linked GST, GST-J3R bound to H4L (Fig. 1A), indicating that there is a direct interaction between J3R and H4L subunit of virion RNA polymerase. To confirm this interaction, glutathione–Sepharose coupled with

His-GST-H4L was mixed with an ^{35}S-labeled, *in vitro*-synthesized J3R protein. Compared to a resin-linked His-GST, His-GST-H4L bound to J3R (Fig. 1B).

J3R binds to H4L amino acids 235–256

In an attempt to map the site of interaction of J3R on H4L, a battery of N- and C-terminal truncation mutations of H4L was constructed in pCITE-4a (Mohamed and Niles, 2000) (Fig. 2A). Each of these truncations was synthesized *in vitro* as ^{35}S-labeled protein. Each of the N- and C-terminal truncation mutations of H4L was mixed with GST-J3R coupled to glutathione–Sepharose. Compared to a GST negative control, all of the C-terminal deletion mutations of H4L, except the smallest N-terminal region of H4L (1–195), bound to GST-J3R (Fig. 2B). Furthermore, GST-J3R was able to pull down the N-terminal deletion mutation of H4L (235–795) mapping the site of interaction of J3R to H4L between amino acids 235 and 288. In confirmation, a mal fusion possessing the N-terminal third of H4L (representing amino acids 1–256) was able to pull down ^{35}S-J3R (Fig. 2C), demonstrating that the region between amino acids 235 and 256 is necessary for productive binding of J3R to the N-terminal region of H4L.

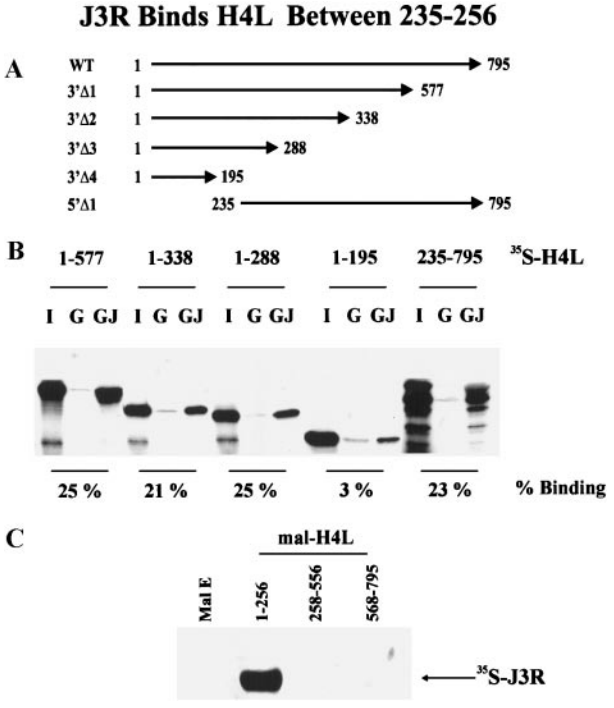

FIG. 2. J3R binds to H4L amino acids 235–256. (A) A series of N- and C-terminal truncation mutations of H4L was constructed and expressed *in vitro* in a coupled transcription/translation system. Numbers denote the H4L amino acids present in each protein fragment. (B) The association of GST-J3R with a set of ^{35}S-H4L truncation mutations is demonstrated. Resin-bound proteins were separated by gel electrophoresis, and H4L proteins were identified by autoradiography. The N-terminal truncation mutant of H4L (235–795) bound GST-J3R. I, 50% of the input radioactivity; G, resin-bound GST; GJ, resin-bound GST-J3R. The percent binding (indicated below the autoradiograph) was quantified by scanning the autoradiogram with a PhosphorImager. For ^{35}S-H4L$_{1\text{-}338}$, background value of 3.6% was subtracted. (C) The association of MalI-fusion of H4L fragments with ^{35}S-J3R is demonstrated.

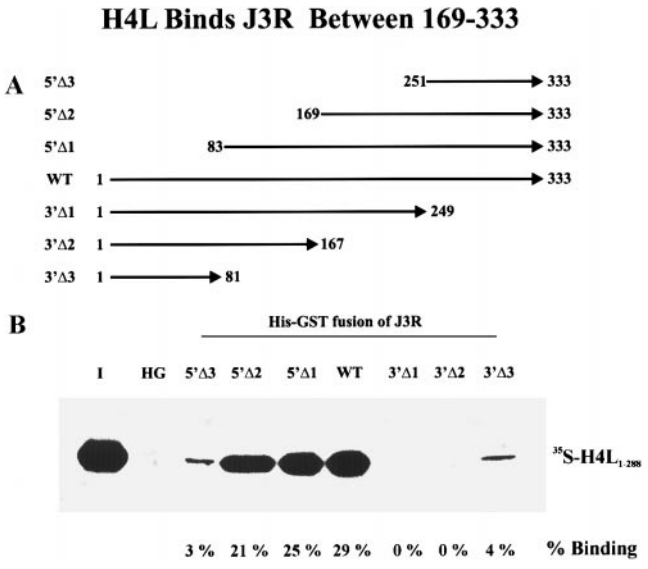

FIG. 3. H4L binds to J3R amino acids 169–333. (A) A series of N- and C-terminal truncation mutations of J3R was constructed and expressed *in vitro* in an *E. coli* expression system. Induction of GST fusion of wild type as well as truncation mutations of J3R was carried out at 20°C. His-GST fusions of full-length J3R or a set of truncation mutations were purified from the S100 fraction of the induced cells by batchwise affinity to glutathione–Sepharose. Numbers denote the J3R amino acids present in each protein fragment. (B) The association of His-GST fusions of full-length J3R or a set of truncation mutations with the ^{35}S-labeled N-terminal region of H4L (1–288) is demonstrated. I, 50% of the input radioactivity; HG, His-GST. The percent binding (indicated below the autoradiograph) was quantified by scanning the autoradiogram with a PhosphorImager.

H4L binds to J3R amino acids 169–333

To map the site on J3R that interacts with H4L, a battery of N- and C-terminal truncation mutations of J3R was constructed both in pET-42a and pCITE-4a vectors (Fig. 3A). Each truncation was either expressed *in vitro* in an *Escherichia coli* expression system or synthesized *in vitro* as ^{35}S-labeled protein. Glutathione–Sepharose resins, coupled with His-GST-fusion of either WT J3R or each truncation mutant, were mixed with ^{35}S-H4L$_{1\text{-}288}$ and binding was evaluated. Compared to a His-GST-negative control, only J3R truncations 5′Δ1 and 5′Δ2 (representing amino acids 83–333 and 169–333, respectively) were capable of pulling down ^{35}S-H4L$_{1\text{-}288}$ (Fig. 3B). These results demonstrate that the N-terminal region of H4L (1–288) interacts with the C-terminal region of J3R (169–333). The fact that J3R truncations 3′Δ1 and 5′Δ3 (representing amino acids 1–249 and 251–333, respectively) failed to bind ^{35}S-H4L$_{1\text{-}288}$ suggests that H4L binding site on J3R may reside in the region centered on amino acid 250.

J3R binds NPH I

A recent report by Mohamed and Niles (2000) demonstrated a physical interaction between H4L subunit of virion RNA polymerase and nucleoside triphosphate phosphohydrolase I (NPH I; a ssDNA-dependent ATPase). This interaction was mapped to the N-terminal 195 amino acids of H4L and the C-terminal end of NPH I (Mohamed and Niles, 2000). The finding that J3R binds to the N-terminal region of H4L between amino acids 235 and 256 (Figs. 2B and 2C), C-terminal to NPH I binding site, led us to test for a possible interaction between J3R and NPH I. To this end, glutathione–Sepharose coupled with GST-J3R was mixed with an ^{35}S-labelled, *in vitro*-synthesized NPH I protein. As a negative control, glutathione–Sepharose resin coupled with GST was mixed with an equal amount of ^{35}S-NPH I. Compared to a resin linked GST, GST-J3R bound to NPH I (Fig. 4A) demonstrating direct interaction between J3R and NPH I. In an attempt to confirm this observation, glutathione–Sepharose coupled with GST-NPH I was mixed with an ^{35}S-labelled, *in vitro*-synthesized J3R protein. Compared to a resin linked to GST, GST-NPH I bound to J3R (Fig. 4B).

NPH I binds to J3R amino acids 169–249

To map the site of NPH I interaction on J3R, we employed the battery of J3R truncation mutants (Fig. 3A) in a pull-down experiment. Each of the J3R truncations was synthesized *in vitro* as an ^{35}S-labeled protein. Glutathione–Sepharose coupled with GST-NPH I was mixed with each of the ^{35}S-labeled J3R truncations. Wild-type J3R was included as a positive control. As a negative control, glutathione–Sepharose resin coupled with GST was mixed with an equal amount of each of the ^{35}S-labeled proteins. Compared to a wild-type positive control, J3R

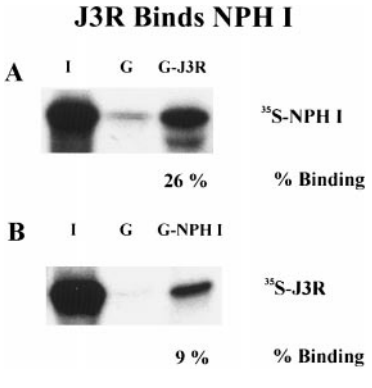

FIG. 4. J3R binds NPH I. A coupled transcription/translation system was employed to synthesize ^{35}S-NPH I or ^{35}S-J3R. Induction of GST-fusion of NPH I or J3R was carried out at 20°C. (A) The association of ^{35}S-NPH I, with the small subunit of poly(A) polymerase, GST-J3R, is demonstrated. (B) The association of ^{35}S-J3R with GST-NPH I is demonstrated. I, 50% of the input radioactivity; G, resin-bound GST; G-J3R, resin-bound GST-J3R; G-NPH I, resin-bound GST-NPH I. The percent binding (indicated below the autoradiograph) was quantified by scanning the autoradiogram with a PhosphorImager.

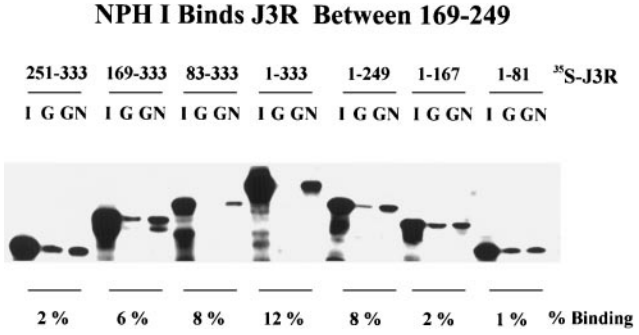

FIG. 5. NPH I binds to J3R amino acids 169–249. A coupled transcription/translation system was employed to synthesize full-length ^{35}S-J3R or a set of truncation mutations. The association of GST-NPH I with full-length J3R or a set of truncation mutations is demonstrated. I, 50% of the input radioactivity; G, resin-bound GST; GN, resin-bound GST-NPH I. The percent binding (indicated below the autoradiograph) was quantified by scanning the autoradiogram with a PhosphorImager.

truncations, 5′Δ1, 5′Δ2, and 3′Δ1 (representing amino acids, 83–333, 169–333, and 1–249), maintained a minimum of 50% of the WT binding efficiency to GST-NPH I (Fig. 5). These results map the site of interaction between NPH I and J3R to the C-terminal half of J3R between amino acids 169 and 249, apparently N-terminal to the H4L interaction region on J3R.

J3R binds to NPH I amino acids 457–524

Previous evaluation of NPH I carboxyl terminal deletion mutations indicated that the carboxyl terminal end of NPH I (603–631) interacts with H4L subunit of virion RNA polymerase (Mohamed and Niles, 2000). The ability of each NPH I C-terminal deletion mutation to interact with J3R was assessed directly. Each of these truncation mutants was expressed as GST fusion, coupled to glutathione–Sepharose and mixed with equal amounts of ^{35}S-J3R. GST fusion of wild-type NPH I was used as a positive control. Compared to a GST-negative control, NPH I C-terminal deletions, 3′Δ1, 3′Δ2, and 3′Δ3, representing amino acids 1–603, 1–563, and 1–524, respectively, bound to ^{35}S-J3R (Fig. 6, top). In confirmation of these results, GST-J3R bound to ^{35}S-labeled NPH I C-terminal deletions, 3′Δ1, 3′Δ2, and 3′Δ3 (representing amino acids 1–603, 1–563, and 1–524, respectively) (not shown). Moreover, a Ni–agarose resin coupled with His_6-tagged-NPH $I_{457-631}$ was capable of pulling down ^{35}S-J3R, compared to a Ni–Agarose-negative control (Fig. 6, bottom). These results demonstrate that J3R binds NPH I between amino acids 457 and 524, a site that is N-terminal to the H4L binding site on NPH I.

J3R is not required for early gene transcription termination *in vitro*

A recent report showed that an interaction between H4L subunit of virion RNA polymerase and NPH I, a

ssDNA-dependent ATPase, is required for early gene transcription termination and transcript release (Mohamed and Niles, 2000). Moreover, Latner *et al.* (2000) concluded that J3R is a postreplicative positive transcription elongation factor. Our observation that J3R interacts with both NPH I and H4L suggested a possible role for J3R in early gene mRNA synthesis and in transcription termination and transcript release. However, Xiang *et al.* (2000) observed apparently proper early gene transcription in J3R mutant virus-infected cells. In an attempt to further evaluate the role of J3R in early gene transcription, the following *in vitro* analyses were conducted. A transcription competent extract was prepared from cells infected with mutant virus lacking J3R (Latner *et al.,* 2000). The ability of the J3R mutant virus-infected cell extract to mediate early gene transcription termination and/or release was next assessed. To test the ability of the J3R mutant virus-infected cell extract to mediate early gene transcription termination, the pSB24-term plasmid template (Condit *et al.,* 1996) was used (Fig. 7A). The plasmid template was designed to contain a G-less cassette possessing a tandem early gene transcription termination signal downstream from a strong synthetic vaccinia virus early promoter (Condit *et al.,* 1996). Synthesis of a transcript that extends from the initiation site to the end of the G-less cassette would yield a product of ~540 bases in length. Signal-dependent termination would be expected to produce a family of RNA products ~450 bases in length. Transcription of pSB24-term was carried out in the presence or absence of exogenously added VTF and various levels of J3R, and the RNA products were analyzed by gel electrophoresis (Fig. 7B). Virus-infected cell extracts are deficient in VTF to various degrees (Condit *et al.,* 1996). Addition of VTF enhanced the level of termination to ~70%. However, addition of pure J3R did not alter total RNA synthesis and had no effect on the overall termination process. In addition, measurement of the early gene transcription elongation rate, *in vitro,* in the absence of J3R was indistinguishable from that done in the presence of J3R (not shown). These results demonstrate that J3R is not required for early mRNA synthesis or for transcription termination *in vitro.* Furthermore addition of J3R does not influence early

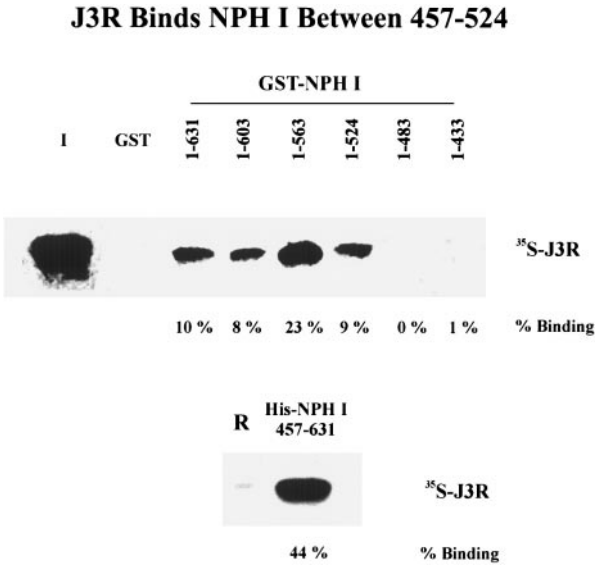

FIG. 6. J3R binds to NPH I amino acids 457–524. A coupled transcription/translation system was employed to synthesize ^{35}S-J3R. Top, the association of GST fusions of full-length NPH I or a set of C-terminal truncation mutations with ^{35}S-J3R is demonstrated. Bottom, the association of His_6 fusion of NPH $I_{457-631}$ with ^{35}S-J3R is demonstrated. Bottom panel: background value of 5% was subtracted. I, 50% of the input radioactivity; GST, resin-bound GST; R, Ni–agarose resin alone. The percent binding (indicated below the autoradiograph) was quantified by scanning the autoradiogram with a PhosphorImager.

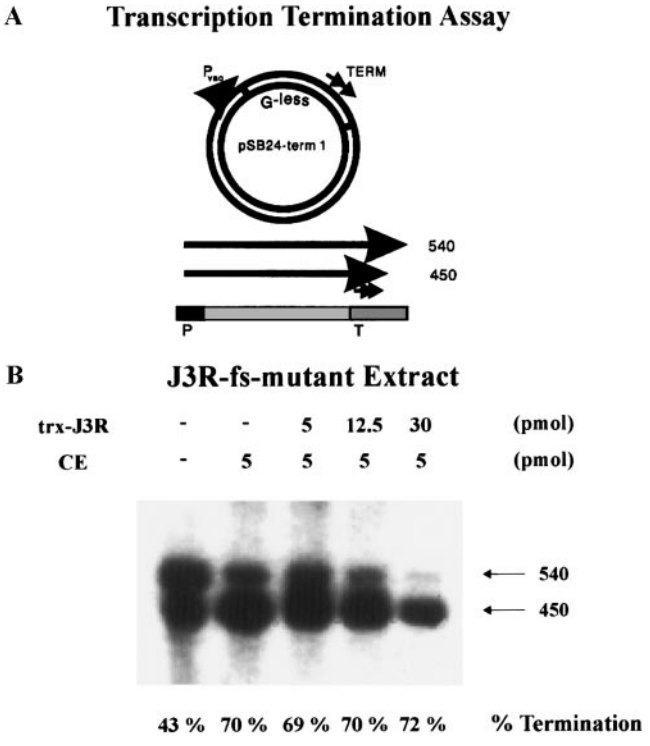

FIG. 7. J3R is not required for early gene transcription termination *in vitro.* (A) A map of the pSB24-term template is shown (Condit *et al.,* 1996). Synthesis of a transcript that extends from the initiation site to the end of the G-less cassette would yield a product of ~540 bases in length. Signal-dependent termination would be expected to produce a family of RNA products ~450 bases in length. P, early gene promoter; T, termination signal. (B) Transcription reactions were carried out in 20 μl total volume containing 6 μl of J3R-mutant extract (lacking J3R), 1 mM ATP, 0.1 mM UTP, 20 μM CTP, 4 μCi [α-^{32}P]CTP (800 Ci/mmole), 0.1 mM 3′OMeGTP, 0.2 μg supercoiled plasmid DNA, 20 mM Tris buffer, pH 8.0, 6 mM $MgCl_2$, 2 mM dithiolthreitol, and 8% glycerol. After proteinase K treatment, RNA was isolated by extraction with phenol/chloroform, precipitated with isopropanol, and resuspended in formamide dye. Samples were heated at 90°C, separated by electrophoresis in 5% acrylamide 8 M urea gels, and the RNA was visualized by autoradiography. Termination efficiency (indicated below the autoradiograph) was calculated as the molar ratio of terminated RNA to the sum of read through and terminated RNA, using a PhosphorImager. CE, capping enzyme.

gene transcription, indicating that it is not a regulator of these events.

The ability of the J3R mutant virus-infected cell extract to mediate early gene transcript release from bead bound ternary complexes, in the presence or absence of exogenously added J3R, was tested. The prototype G21(TER29)A78 transcription unit (Deng *et al.,* 1996) consists of a synthetic early promoter fused to a 20-nucleotide G-less cassette, which is flanked by a run of three G residues at positions +21 to +23. A 57-nucleotide A-less cassette was inserted downstream of the G-less cassette and flanked at its 3′ end by a run of four A residues at positions +78 to +81. A termination signal, TTTTTTTTT, was placed within the A-less cassette, spanning positions +29 to +37 (Fig. 8A). The use of bead-bound DNA template provided a convenient method to assay transcript release by magnetic separation of template-engaged ^{32}P-labeled RNA products (bead-bound) from released transcripts in the supernatant. The labeled RNAs that had extended to A78, in the J3R-mutant virus infected cell extracts lacking J3R (Latner *et al.,* 2000), were recovered in the template-bound fraction and then incubated with 1 mM dATP, VTF/CE, J3R, and E1L. The addition of VTF alone was capable of enhancing the level transcript release from the arrested ternary complex (Fig. 8B). However, the addition of J3R, either alone and in combination with E1L, failed to have any significant effect on the release process (Fig. 8B). These results indicate that J3R is not an essential cofactor for early gene transcript release *in vitro.*

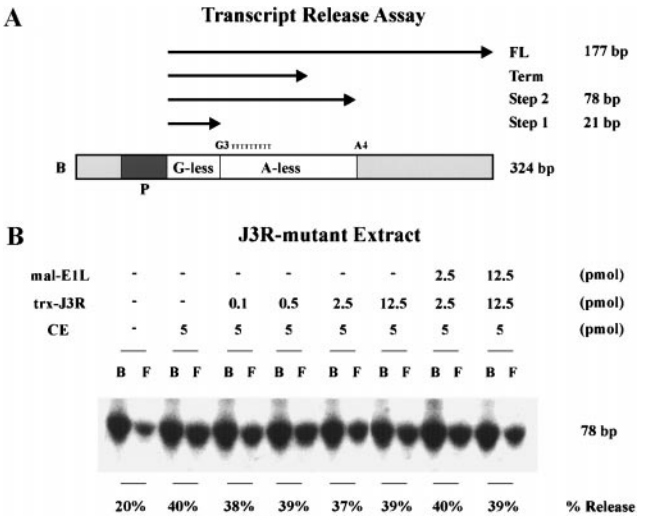

FIG. 8. J3R is not required for early gene transcript release from an arrested ternary complex *in vitro.* (A) A map of the bead bound G21(TER29) A78 DNA template is shown (Deng *et al.,* 1996). The DNA template is uniquely biotinylated at the 5′ end of the non template strand, which anchors the DNA to streptavidin-coated magnetic beads. The transcription unit consists of a synthetic early promoter fused to a 20-nucleotide G-less cassette, which is flanked by a run of three G residues at positions +21 to +23. A 57-nucleotide A-less cassette was inserted downstream of the G-less cassette and flanked at its 3′ end by four A residues at positions +78 to +81. A termination signal, TTTTTTTTT, was placed within the A-less cassette, spanning position +29 to +37. Arrows represent the products produced by the various reaction conditions. FL, full length; P, promoter; term, termination product. (B) Ternary complexes containing the G21 transcript, were synthesized in a J3R mutant virus-infected cell extract (lacking J3R), ATP, CTP, UTP, and 3′ OMeGTP. The ternary complexes were then isolated and the nascent transcript was extended through the A-less cassette, in the presence of UTP, GTP, CTP, and cordycepin triphosphate, to yield a bead-bound ternary complex containing the A78 transcript. Transcript release from the paused ternary complex was then assessed in the presence or absence of VTF, trx-J3R, or mal-E1L. The bead-bound A78 RNA (lane B; bound) was separated from released A78 RNA (lane F; free) by centrifugation. The transcription products were analyzed by electrophoresis through a 12% polyacrylamide gel containing 8 M urea. The labeled A78 transcript was visualized by autoradiography. The percent of RNA released (indicated below the autoradiograph) was quantified by scanning the autoradiogram with a PhosphorImager. CE, capping enzyme.

DISCUSSION

J3R, the small subunit of vaccinia virus poly(A) polymerase (Gershon *et al.,* 1991; Schnierle *et al.,* 1992), is a multifunctional protein. In the virion, J3R is present in a fivefold molar excess over E1L, the catalytic subunit of poly(A) polymerase, and therefore exists both free and associated with E1L (Gershon *et al.,* 1991). Although J3R lacks polyadenylation catalytic activity (Gershon *et al.,* 1991), it dramatically accelerates E1L catalytic extension of either mRNA 3′-end primers with short oligo(A) tails or poly(A) primers (Gershon and Moss, 1993). J3R has a second, entirely unrelated function, catalyzing the conversion of the mRNA 5′ cap O [m^7G(5′)pppN] structure to its cap 1 counterpart [m^7G(5′)pppN_m] by methylation of the ribose moiety of the first transcribed nucleotide of the mRNA (Schnierle *et al.,* 1992). Both the monomeric and heterodimeric forms of J3R exhibit methyltransferase activity (Schnierle *et al.,* 1992). In addition, a recent report by Latner *et al.* (2000) showed that J3R is a postreplicative positive transcription elongation factor during a normal virus infection. The elongation factor activity of J3R is independent of the poly(A) stimulatory activity (Xiang *et al.,* 2000).

Prior results support an interaction between poly(A) polymerase and the virion RNA polymerase. Work by Zhang *et al.* (1994) demonstrated that nascent RAP94-deficient virus core particles, lacking the H4L subunit, exhibit low or undetectable amounts of several viral enzymes including viral RNA polymerase, capping enzyme, NPH I, poly(A) polymerase, topoisomerase, and RNA helicase. The presence of these unpackaged viral enzymes in the cytoplasm indicated that RAP94 is required for targeting a complex of functionally related proteins required for early gene transcription and mRNA processing. Multiple proteins employed in mRNA synthesis and mRNA cap and poly(A) formation appear to bind either directly or indirectly to H4L subunit of virion RNA polymerase, suggesting the formation of a viral transcrip-

tion/mRNA processing complex. Results from our laboratory showed that E1L, the catalytic subunit of vaccinia poly(A) polymerase, does not bind H4L (unpublished data). Therefore, a possible direct interaction between J3R subunit of vaccinia virus poly(A) polymerase and H4L subunit of virion RNA polymerase was evaluated. J3R was shown to bind specifically to H4L, and, using a series of N- and C-terminal truncation mutations of H4L, the site of interaction of J3R was mapped to the N-terminal region of H4L between amino acids 235 and 256 and the site of interaction of H4L was mapped to the C-terminal region of J3R between amino acids 169 and 333. The J3R binding site on NPH I was mapped between amino acids 457 and 524, and the NPH I binding site on J3R was located between amino acids 169 and 249. The H4L interaction site on J3R appears to be C-terminal to the NPH I binding site on J3R.

X-ray crystal structure of J3R presents a compact single-domain protein with a typical α/β fold (Hodel *et al.,* 1996). The central portion of the polypeptide chain folds into a core structure in which a mixed seven-stranded twisted β-sheet structure is surrounded by parallel α helices. The protein envelope resembles a highly oblate sphere with one face bisected by a cleft, the cleft face (Hodel *et al.,* 1996). The face of J3R opposite to the cleft, the flat face, is formed from the loops N terminal to each of the seven β strands and by the three C-terminal helices appended to the core structure. E1L was reported to bind to the flat face of J3R, with a 3- to 5-residue "hot-spot," which is sensitive to amino acid substitutions, including Arg-55, His-56, Ile-58, Cys-272, and Tyr-271 (Shi *et al.,* 1997). In this report, we demonstrated that NPH I bound to the C-terminal region of J3R (169–249) that occupies a significant part of the flat face of J3R, yet away from E1L interaction site with J3R. In addition, none of the residues in the E1L interaction site is located within the mapped binding region for NPH I (169–249). Data indicate that H4L binds C terminal to NPH I on J3R. Therefore, the J3R N-terminal residues required for E1L interaction are not needed for H4L interaction. Moreover, E1L addition does not reduce the binding of either H4L or NPH I to J3R *in vitro* (not shown), demonstrating that the E1L binding site on J3R does not overlap that of NPH I and H4L.

Since the interaction between NPH I and H4L is required for early gene transcription termination and transcript release (Mohamed and Niles, 2000), the observed interaction of J3R with both NPH I and H4L suggested that J3R might play a role in early gene mRNA synthesis, transcription termination, transcript release, or regulation of these processes. To evaluate this possibility, RNA synthesis from an early promoter was measured in a transcription-competent extract prepared from cells infected with mutant virus lacking J3R (Latner *et al.,* 2000). Total RNA synthesis appeared normal in the absence of J3R. In addition, measurement of the early gene transcription elongation rate in the absence of J3R, *in vitro,* was indistinguishable from that done in the presence of J3R (not shown). Exogenously added VTF enhanced the level of termination to ~70%, in the absence of J3R. These results demonstrate that J3R is not required for early mRNA synthesis or for early gene transcription termination *in vitro.* Moreover, addition of pure J3R did not exhibit any effect on the overall termination process, demonstrating that J3R is not a modulator of early gene transcription termination *in vitro.* These results are consistent with the *in vivo* observation of Xiang *et al.* (2000), who showed that infection with J3R mutant viruses exhibited early gene expression that is indistinguishable from that in a wild-type virus infection. Analysis of transcript release activity of ternary complexes prepared from virus-infected cell extracts lacking J3R demonstrated that J3R is not required for this final step in transcription. These results demonstrate that J3R is not an essential factor in early gene mRNA synthesis, transcription termination and/or release *in vitro.* Alternatively, NPH I and H4L may serve as a docking site for J3R on the virion RNA polymerase, linking the transcription machinery to an enzyme employed in mRNA processing at both the 5′ and the 3′ ends.

The presence of nuclear transcription/RNA processing complex has been the subject of much research activity. Increasing evidence supports the presence of such complex in eukaryotic nuclei. Several observations made over the past few years have forged strong molecular links between transcription by RNA polymerase II (Pol II) and pre-mRNA processing. Biochemical studies have shown that RNA polymerase II, via the CTD, can physically interact with capping enzymes (Cho *et al.,* 1997; McCracken *et al.,* 1997a; Yue *et al.,* 1997), polyadenylation factors (McCracken *et al.,* 1997b), and splicing factors, including both snRNPs and SR-like proteins (Chabot *et al.,* 1995; Kim *et al.,* 1997; Mortillaro *et al.,* 1996; Yuryev *et al.,* 1996). In addition, *in vivo* studies using mammalian cells in culture have demonstrated that RNAs synthesized by RNA polymerase II with a shortened CTD undergo inefficient capping, splicing, and polyadenylation (McCracken *et al.,* 1997a,b) and that overexpression of phosphorylated CTD peptides inhibits splicing (Du and Warren, 1997).

Because of its large size and multisubunit composition (Baroudy and Moss, 1980; Nevins and Joklik, 1977; Spencer *et al.,* 1980), the vaccinia virus RNA polymerase closely resembles RNA polymerases of eukaryotic and prokaryotic organisms (Studier and Dunn, 1983). Prior studies demonstrate that the virion form of RNA polymerase bears structural and functional similarity to cellular RNA polymerase II (Moss *et al.,* 1991). In addition, the vaccinia mRNA capping enzyme, was shown to bind directly to its cognate viral RNA polymerase in solution (Broyles and Moss, 1987; Hagler and Shuman, 1992), which is consistent with the presence of a transcription/

mRNA processing complex. Moreover, a recent report by Katsafanas and Moss (1999) demonstrated that the vaccinia virus-encoded capping enzyme, early transcription factor, and nucleoside triphosphate phosphohydrolase I specifically co-eluted with a histidine-tagged RNA polymerase, consistent with their physical association. The fact that J3R, the small subunit of vaccinia poly(A) polymerase, is involved in both polyadenylation and capping processes, raises the possibility that its association with NPH I and the H4L subunit of the virion RNA polymerase serves as a docking site for J3R in its role in RNA processing. Further studies are underway to test the essential features of this model.

MATERIALS AND METHODS

Cells and viruses

The African green monkey kidney cell line BSC40 and conditions for cell culture have been previously described (Condit and Motyczka, 1981; Condit *et al.,* 1983). The J3R-fs mutant virus, *J3-7,* and the conditions for its growth, infection, and plaque titration have been previously described (Latner *et al.,* 2000). *J3-7* is phenotypically isatin-β-thiosemicarbazone-dependent (IBT^d) and contains a single nucleotide deletion in the J3R gene that causes a frameshift at codon 49 and truncation of the 333-amino-acid protein at position 58. IBT was prepared fresh before each use and applied at a final concentration of 45 μM as previously described (Pacha and Condit, 1985).

Transcription extracts

Extracts of virus-infected cells were prepared by lysolecithin treatment as described (Condit *et al.,* 1996). A549 cells were infected with *J3-7,* an IBT-dependent mutant, at a m.o.i. of 15, at 37°C in the presence of 45 μM IBT. After 24 h, cells were washed and treated with 250 μg/ml lysolecithin and extracts prepared.

Transcription termination assay

Termination of early gene transcription was monitored by using a plasmid template, pSBterm (Condit *et al.,* 1996), which possess tandem termination signals within the G-less cassette. Transcription reactions were carried out in 20 μl total volume containing 6 μl extract, 1 mM ATP, 0.1 mM UTP, 20 μM CTP, 4 μCi [α-^{32}P]CTP (800 Ci/mmole), 0.1 mM 3′OMeGTP, 0.2 μg supercoiled plasmid DNA, 20 mM Tris buffer, pH 8.0, 6 mM $MgCl_2$, 2 mM dithiolthreitol, and 8% glycerol for 30 min at 30°C. After proteinase K treatment, RNA was isolated by extraction with phenol/chloroform, precipitated with isopropanol, and resuspended in formamide dye. Samples were heated at 90°C, separated by electrophoresis in 5% acrylamide 8 M urea gels, and the RNA was visualized by autoradiography. Termination efficiency was calculated as the molar ratio of terminated RNA to the sum of read through and terminated RNA.

Transcript release assay

Construction of the G21(TER29)A78 plasmid containing a vaccinia early promoter was described by Deng *et al.* (1996). The prototype G21(TER29)A78 transcription unit consists of a synthetic early promoter fused to a 20-nucleotide G-less cassette, which is flanked by a run of three G residues at positions +21 to +23. A 57-nucleotide A-less cassette was inserted downstream of the G-less cassette and flanked at its 3′ end by four A residues at positions +78 to +81. A termination signal, TTTTTTTTT, was placed within the A-less cassette, spanning position +29 to +37. The biotinylated 324 bp DNA template was PCR amplified employing a 5′ biotin tag on the upstream primer and isolated by preparative agarose gel electrophoresis. The purified DNA fragment was then immobilized to streptavidin-coated magnetic beads (Dynabeads M280; Dynal) as described (Hagler *et al.,* 1994). The bead bound (B) template (typically, 100 fmol) was first incubated with 6 μl of J3R-fs virus-infected cell extract in the presence of 1 mM ATP, 4 μCi α^{32}P-CTP (800 Ci/mmol), 0.1 mM UTP, and 0.625 mM 3′OMeGTP to synthesize the G21 transcript. The ternary complex was then isolated and the nascent transcript was extended through the A-less cassette, in the presence of 1 mM UTP, 1 mM GTP, 4 μCi α^{32}P-CTP, and 1 mM cordycepin triphosphate (3′dATP), to yield a bead-bound ternary complex containing the A78 transcript. Elongation of the nascent chains beyond the arrest site at G21 depends on removal of the blocking 3′OMeGMP moiety by the hydrolytic activity intrinsic to the vaccinia RNA polymerase elongation complex (Hagler and Shuman, 1993). The ternary complexes were collected by centrifugation and resuspended, and transcript release from the paused ternary complex was then assessed (Deng *et al.,* 1996) in the presence or absence of VTF, trx-J3R, or mal-E1L. After incubation for 10 min at 30°C, the bound transcript was separated from the free by centrifugation and analyzed by gel electrophoresis. The percent of RNA released was quantified by scanning the autoradiogram with a PhosphorImager.

Plasmids

pGEX 4T1-D11L plasmids containing either full-length or truncated D11L (NPH I) coding sequence were described (Christen *et al.,* 1998). pET-30a-D11L$_{457\text{-}631}$ was constructed by excising the C-terminal coding region from pGEX 4T1-D11L by restriction digestion using *Eco*RV and *Sal*I restriction enzymes and then inserting the DNA fragment into pET-30a vector digested with the same enzymes. pET-42a-H4L and pCITE-4a-H4L plasmids containing full-length H4L were constructed by inserting a *Nco*I–*Sal*I DNA fragment derived from pET-14a-

H4L (obtained from Dr. Stewart Shuman), containing the coding sequence of H4L, into the *Nco*I–*Sal*I digested pET-42a and pCITE-4a, respectively. A series of H4L truncation mutations in pCITE-4a was constructed by restriction digestion of the original pCITE-4a-H4L construct with *Acc*I, *Bgl*II, *Hin*cII, *Spe*I, or *Msc*I–*Sna*BI restriction enzymes and religation of the digested construct. This gave rise to a series of H4L C- and N-terminal truncations representing amino acids, 1–195, 1–288, 1–338, 1–577, and 235–795. A series of 5′ and 3′ primers derived from different regions of J3R coding sequence was employed to amplify a battery of J3R truncation mutants (representing amino acids, 1–81, 1–167, 1–249, 83–333, 169–333, and 252–333) using a proofreading polymerase *Pfu.* The amplified blunt-end PCR fragments were ligated into pCR-Blunt II-TOPO plasmid vector (Shuman, 1994). The truncated J3R fragments were then excised using *Nco*I–*Sal*I restriction digestion and ligated into both pCITE-4a and pET-42a plasmids linearized with the same enzymes.

Resin preparation

Large-scale inductions of *E. coli* expressing a GST-fusion of wild-type NPH I or NPH I truncation mutations were carried out at 20°C as described previously (Higman *et al.,* 1992). Cells were collected by centrifugation and stored at −80°C until used. GST-NPH I fusions were then purified from an S100 fraction of the induced cells by batchwise affinity to glutathione–Sepharose. Large-scale inductions of His-GST fusion of wild-type J3R as well as truncation mutations, were carried out at 20°C using 0.5 mM IPTG. Cells were collected by centrifugation and stored at −80°C until used. His-GST fusions were then purified from an S100 fraction of the induced cells by batchwise affinity to Glutathione Sepharose. In case of NPH $I_{457-631}$, the His_6-tagged fusion was isolated by batchwise affinity to Ni–agarose. The protein-bound resins were tested by SDS–PAGE, and the volume used was adjusted, whenever required, by dilution with the respective resin to yield equivalent amounts of each protein. Resins were kept as 50% slurry at −20°C.

In vitro transcription/translation

Novagen Single Tube Protein system 3 (STP3) was used for the *in vitro* synthesis of ^{35}S-labeled proteins directly from DNA templates containing T7 RNA polymerase promoter. The DNA template (typically 0.5 μg) was transcribed in 10 μl at 30°C for 15 min followed by the addition of 40 μl translation mix and continued incubation for 60–90 min. Both pCITE-4a- and pET-30a-derived recombinant plasmids were used.

In vitro protein–protein interaction assay

The proteins were labeled *in vitro* with [^{35}S]methionine by the STP3 *in vitro* translation system, from Novagen. One microliter of the translation mix was incubated with 25 μl of glutathione–Sepharose or Ni-charged His-bind resins coupled to 1 μg of the protein of interest at 4°C overnight in binding buffer [25 mM Tris–HCl, pH 8.0, 1 mM EDTA, 10% glycerol (w/v), 50 mM NaCl]. After binding, the resin was washed four times, each with 500 μl of binding buffer. In case of Ni-charged His-bind resins, 50 mM imidazole was included in the buffer during the wash step. The washed resins were then boiled in 1× SDS-loading buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 0.72 M β-mercaptoethanol) for 5 min and analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). The gel was then soaked in 1 M salicyclic acid for 30 min and dried, and autoradiography was then performed at −80°C. The percent binding was quantified by scanning the autoradiogram with a PhosphorImager. Unless otherwise stated, the background values of between 1 and 3% were subtracted, yielding the resulting percentage of specific binding.

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