Virology **303,** 232–239 (2002)
doi:10.1006/viro.2002.1721

Mapping Interaction Sites of the A20R Protein Component of the Vaccinia Virus DNA Replication Complex

Koji Ishii[1] and Bernard Moss[2]

Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892-0445

Received June 13, 2002; returned to author for revision July 9, 2002; accepted July 18, 2002

The vaccinia virus A20R protein is required for DNA replication, is associated with the processive form of the viral DNA polymerase, and directly interacts with the viral proteins encoded by the D4R, D5R, and H5R open reading frames as determined by a genome-wide yeast two-hybrid analysis. The purpose of the present study was to further analyze the latter protein–protein interactions. Association of an epitope-tagged A20R protein with an epitope-tagged D4R or H5R protein, expressed in vaccinia virus-infected cells, was demonstrated by binding the complex to one mAb followed by Western blotting with another. Interaction between the A20R and D5R proteins, which was weakest in the yeast two-hybrid analysis, could not be demonstrated by this method. A panel of N- and C-terminal truncated forms of the A20R protein was tested for interaction with the D4R, H5R, and D5R proteins using the yeast two-hybrid system. These studies revealed that nonoverlapping regions of A20R comprising amino acids 1 to 25, 26 to 76, and 201 to 251 were required for binding of D4R, H5R, and D5R, respectively. By contrast, no interaction of A20R with D4R could be detected after deletion of only 25 codons from either end of the latter open reading frame. A fusion protein containing either full-length A20R or only the N-terminal 25 amino acids of A20R was sufficient to capture the D4R protein, whereas the fusion protein containing A20R amino acids 26 to 426 was not, confirming the results of the yeast two-hybrid analysis. The distinct protein binding domains of the A20R protein may contribute to the assembly or stability of the multiprotein DNA replication complex.

INTRODUCTION

Poxviruses, of which vaccinia virus is the prototype, are unique among DNA viruses in that they reproduce exclusively in the cytoplasm of host cells and consequently encode most of the enzymes and factors for transcription and genome replication (Moss, 2001). Many, but probably not all, of the proteins required for viral RNA and DNA synthesis have been identified. Several complementation groups of vaccinia virus temperature-sensitive (ts) mutants that express viral early proteins but are impaired in DNA synthesis have been isolated (Condit and Motyczka, 1981; McFadden and Dales, 1980). One group was mapped to the E9L open reading frame (ORF), which encodes the DNA polymerase with a mass of 110 kDa, an associated 3′ exonuclease activity, and sequence similarities with other eukaryotic and viral DNA polymerases (Challberg and Englund, 1979; Earl *et al.,* 1986; Jones and Moss, 1984; McDonald and Traktman, 1994; Sridhar and Condit, 1983; Traktman *et al.,* 1984). Another DNA replication-negative complementation group was mapped to the D4R ORF, which encodes a functional uracil DNA glycosylase (Ellison *et al.,* 1996; Millns *et al.,* 1994; Stuart *et al.,* 1993; Upton *et al.,* 1993). A third complementation group was mapped to the D5R ORF, which encodes a nucleic acid independent nucleoside triphosphatase (Evans *et al.,* 1995; Evans and Traktman, 1987, 1992). A fourth complementation group was mapped to the B1R ORF, which encodes a serine-threonine protein kinase that is packaged in virions (Banham and Smith, 1992; Lin *et al.,* 1992; Rempel *et al.,* 1990; Rempel and Traktman, 1992; Traktman *et al.,* 1989).

A role for a fifth viral protein in DNA replication was suggested by the results of a vaccinia virus genome-wide yeast two-hybrid screen (McCraith *et al.,* 2000). The A20R protein interacted with two proteins known to be involved in DNA replication, D4R and D5R, and with the H5R protein, which is a substrate of the B1R protein kinase (Beaud *et al.,* 1995) and has one or more roles in transcription (Black *et al.,* 1998; Kovacs and Moss, 1996) and virus assembly (DeMasi and Traktman, 2000). More direct evidence for the role of the A20R protein was obtained by Traktman and co-workers (Klemperer *et al.,* 2001), who identified this protein as a previously described component of the processive form of DNA polymerase (McDonald *et al.,* 1997). Subsequently, directed mutagenesis of the A20R ORF was used to isolate conditional lethal ts mutants that are blocked in viral DNA replication (Ishii and Moss, 2001; Punjabi *et al.,* 2001). Taken together these data suggest that the A20R protein

[1] Present address: Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan.

[2] To whom correspondence and reprint requests should be addressed at Laboratory of Viral Diseases, 4 Center Drive, MSC 045, National Institutes of Health, Bethesda, MD 20892-0445. Fax: (301) 480-1147. E-mail: bmoss@nih.gov.

0042-6822/02

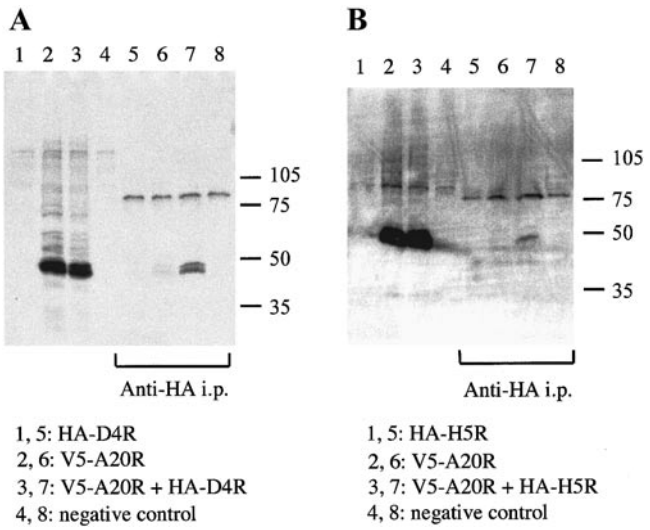

FIG. 1. Detection of protein–protein interactions by co-immunoprecipitation. Monolayers of BS-C-1 cells were infected with vTF7-3 and transfected or cotransfected with pCRV5A20R (V5-A20R), pCRHAD4R (HA-D4R), or pCRHAH5R (HA-H5R). After 24 h, the cells were harvested and cleared lysates were analyzed directly or incubated with anti-HA mAb followed by Protein A–Sepharose. The bound proteins were separated by SDS–PAGE and transferred to nitrocellulose for Western analysis with V5 mAb. Keys to the lanes are shown below the autoradiographs. The positions and masses in kDa of marker proteins are indicated on the right.

could be a key element of a multiprotein DNA replication complex that includes the DNA polymerase, the D4R and D5R proteins, and possibly the H5R protein. For A20R to assemble such a complex, however, the sites for interaction with the other proteins must be nonoverlapping. The purpose of the present work was to confirm and further dissect the interactions between the A20R and the D4R, D5R, and H5R proteins.

RESULTS

Interactions of the A20R protein in vaccinia virus-infected cells

We used an independent method to confirm the interactions of the A20R protein with the D4R, D5R, and H5R proteins, which had been determined with the yeast two-hybrid system. BS-C-1 cells were infected with vTF7-3, a recombinant vaccinia virus that expresses the bacteriophage T7 RNA polymerase (Fuerst *et al.,* 1986) and then transfected with one or two plasmids containing a T7 promoter regulating the V5 epitope-tagged A20R ORF or influenza virus hemagglutinin (HA) epitope-tagged D4R, D5R, or H5R ORFs. When extracts of cells that had expressed V5-A20R alone or coexpressed V5-A20R and HA-D4R were analyzed directly by Western blotting with V5 monoclonal antibody (mAb), a doublet band corresponding to the 49-kDa V5-A20R protein was visualized (Fig. 1A, Lanes 2, 3). When lysates of cells that had expressed HA-D4R and V5-A20R proteins were first incubated with HA mAb and the bound proteins examined by Western blotting with the V5 mAb, the V5-A20R protein was easily detected (Fig. 1A, Lane 7). In contrast, the protein was visible only as a faint background band in lysates of cells that had expressed V5-A20R alone (Fig. 1A, Lane 6). Thus, the complex of V5-A20R and HA-D4R was captured by the HA mAb. An additional protein with an estimated mass of greater than 75 kDa was detected by Western blotting in all lanes including controls, indicating that it is unrelated to either A20R or D4R expression. The V5-A20R-HA-D4R complex could also be captured with V5 mAb, although the band intensity was weaker on Western blotting with the HA mAb than with the V5 mAb (data not shown).

We used the same procedure to analyze the interaction of A20R with the H5R and D5R proteins. When V5-A20R and HA-H5R were coexpressed, the complex captured by the HA MAb contained the V5-A20R protein as detected by Western blotting with V5 mAb (Fig. 1B, Lane 7). Similarly, the complex captured by the V5 mAb contained the HA-H5R protein as detected by Western blotting with the HA mAb, although the band was fainter (data not shown). The signal detected by Western blotting was weaker for the A20R–H5R interaction than for the A20R–D4R interaction. Moreover, no interaction could be detected between epitope-tagged A20R and D5R proteins by this procedure (data not shown). The relative strengths of the interactions determined by binding of the complex to mAbs corresponded with the yeast two-hybrid analysis in which the sizes of the diploid yeast colonies in selective medium were A20R-D4R > A20R-H5R > A20R-D5R (McCraith *et al.,* 2000).

Analysis of the A20R-binding domains using the yeast two-hybrid system

To identify regions of the A20R protein required for its interaction with D4R, H5R, and D5R proteins, we fused C-terminal and N-terminal truncated A20R ORFs with the GAL4-binding domain. The ability of a panel of these constructs to interact with D4R fused to the activation domain was tested in the yeast two-hybrid system. Initially, the results were scored as positive or negative based on colony growth under selection conditions and a filter lift assay for β-galactosidase. Using the C-terminal truncation set (Fig. 2), we observed an interaction even when only the N-terminal 1 to 25 amino acids remained. Complementing this, removal of 25 or 100 amino acids from the N-terminus of A20R prevented the interaction with the D4R protein. To quantify the interactions, the yeast two-hybrid experiments were repeated using a liquid β-galactosidase assay. In Fig. 2, the data are presented as the percentage of β-galactosidase produced with the full-length A20R protein. With the fusion protein containing 1 to 50 N-terminal amino acids of A20R, the activity was even higher than with full-length,

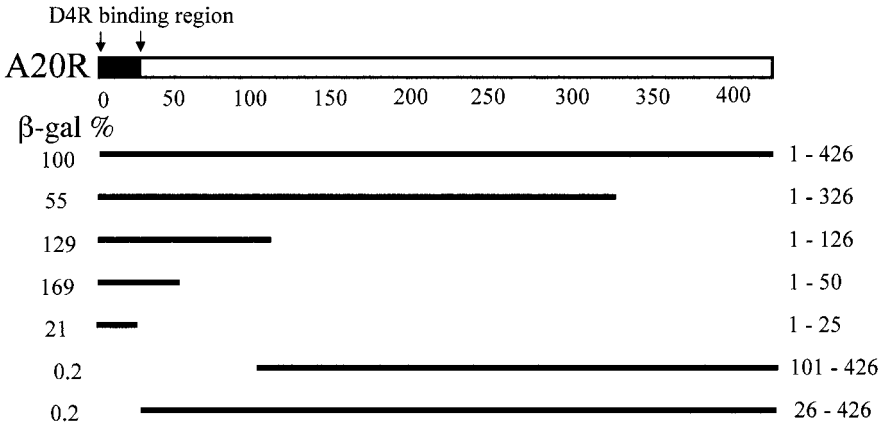

FIG. 2. Mapping the D4R-binding site of the A20R protein by yeast two-hybrid analysis. The full-length A20R ORF is shown at the top with amino acid numbers below. The filled box indicates the deduced D4R minimal binding region. Representations of the complete A20R ORF and truncated versions expressed in the yeast two-hybrid system are shown as bars. Segments of the A20R ORF are defined by the amino acid numbers indicated on the right. The expression of *lac*Z, resulting from the activation of a GAL4-dependent *lac*Z gene, is shown on the left. The value obtained using the full-length A20R ORF was defined as 100%.

whereas lower but still reproducible activity was obtained with only 1 to 25 N-terminal amino acids (Fig. 2). These data indicated that the minimal binding region for the D4R protein is within the N-terminal 25 amino acids of A20R, although for optimal binding it may extend somewhat further.

Similar studies were used to analyze the interactions of fusion proteins containing A20R truncations with the H5R protein. Again, the results were first scored as positive or negative by colony formation under selection conditions and a filter lift assay for β-galactosidase. We found interactions of all C-terminal truncations that retained 76 or more N-terminal amino acids but not when only 50 N-terminal amino acids remained. The panel of N-terminal truncations of A20R was also tested. Interaction with H5R still occurred when 25 amino acids were removed but not when 100 amino acids were removed. These results were confirmed using the liquid β-galactosidase assay (Fig. 3). The fusion protein containing only 76 N-terminal amino acids retained about half of the full-length activity, whereas background activity was obtained with only 50 N-terminal amino acids (Fig. 3). In addition, the fusion protein containing amino acids 26 to 426 retained activity (Fig. 3). Taken together these results suggested that the minimal binding region for the H5R protein is between the N-terminal 26 to 76 amino acids of A20R.

The different results obtained with D4R and H5R proteins provided an internal control. Since the N-terminal 1 to 50 amino acids of A20R mediated binding to D4R, the failure of the same construct to bind to H5R could not be due to poor expression or instability. Likewise, the dele-

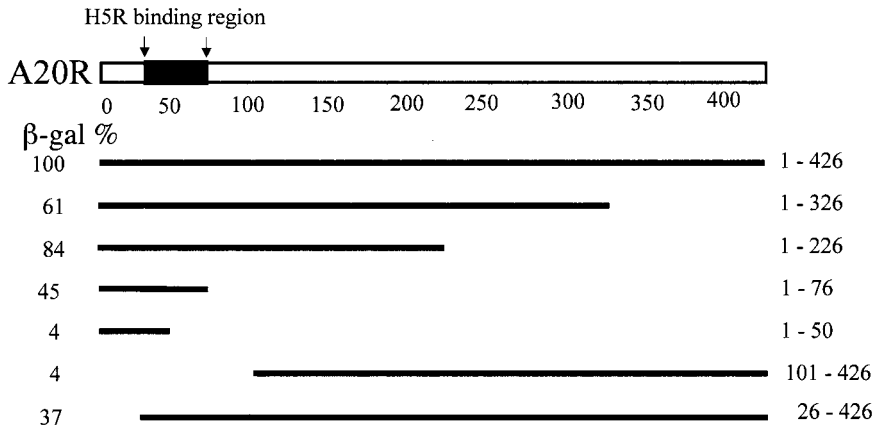

FIG. 3. Mapping the H5R-binding site of the A20R protein by yeast two-hybrid analysis. The full-length A20R ORF is shown at the top with amino acid numbers below. The filled box indicates the deduced H5R minimal binding region. Representations of the complete A20R ORF and truncated versions expressed in the yeast two-hybrid system are shown as bars. Segments of the A20R ORF are defined by the amino acid numbers indicated on the right. The expression of *lac*Z, resulting from the activation of a GAL4-dependent *lac*Z gene, is shown on the left. The value obtained using the full-length A20R ORF was defined as 100%.

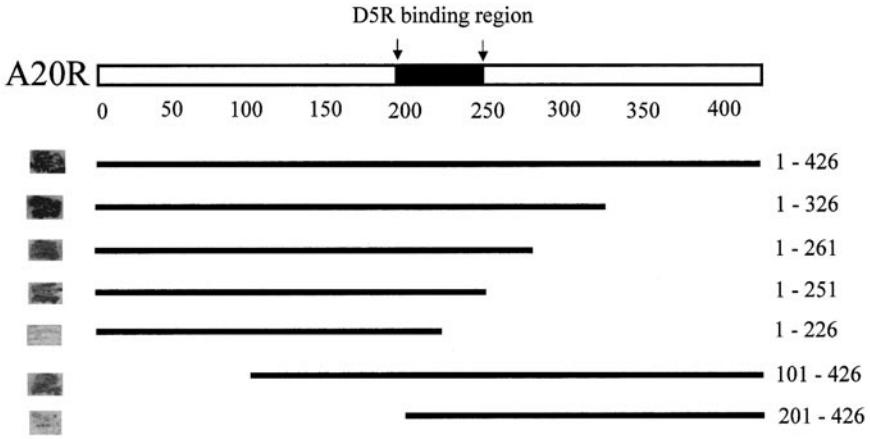

FIG. 4. Mapping the D5R binding site of the A20R protein by yeast two-hybrid analysis. The full-length A20R ORF is shown at the top with amino acid numbers below. The filled box indicates the deduced D5R minimal binding region. Representations of the complete A20R ORF and truncated versions expressed in the yeast two-hybrid system are shown as bars. Segments of the A20R ORF are defined by the amino acid numbers indicated on the right. Positive interactions are indicated by growth of yeast on His^- plates as shown on the left.

tion of the N-terminal 25 amino acids of A20R did not prevent binding to H5R and therefore failure of this construct to bind D4R could not be attributed to trivial reasons.

The same panel of A20R truncations was used to analyze binding to the D5R protein (Fig. 4). The fusion protein containing the N-terminal 1 to 251 amino acids of A20R retained binding activity, whereas one containing 1 to 226 amino acids did not. Activity was retained after deletion of the N-terminal 100 amino acids and was weaker but detectable after deletion of 200 amino acids. The liquid β-galactosidase assay was not sensitive enough to quantify the interaction of the A20R and D5R proteins and therefore photographs of colonies that formed in histidine-deficient medium are shown in Fig. 4. These results suggested that the minimal interaction region for D5R occurs between amino acids 200 and 250 of A20R.

We also tried to map the site on the D4R protein that is necessary for binding A20R. Four D4R truncations were made in which either 25 or 100 amino acids were deleted from the N- or C-terminal end. However, none of the D4R truncated proteins exhibited a detectable interaction with A20R.

Analysis of the A20R-binding domains using maltose-binding protein (MBP) fusions

We prepared fusions of the MBP with full-length and truncated forms of the A20R protein to confirm results obtained in the yeast two-hybrid analysis. Radioactively labeled D4R, D5R, and H5R proteins were synthesized in rabbit reticulocyte lysates. In each case, the predominant radioactive band detected by SDS–PAGE and autoradiography was of the expected size and expressed only with the appropriate template (data not shown). The MBP fusion proteins were immobilized on amylose resin and incubated with the *in vitro* synthesized D4R, D5R, or H5R protein. The D4R protein bound to full-length MBP-A20R protein and to the fusion containing only the N-terminal 25 amino acids of A20R. Although only a small percentage of the D4R protein applied to the resin was recovered after the extensive washing steps, the binding was specific as none bound either to the MPB fusion produced with amino acids 26 to 426 of A20R or to MPB alone (Fig. 5). Curiously, radioactively labeled bands that were minor components of the total translation mixture were concentrated by absorption to the amylose resin. However, these bands were present in similar amounts regardless of whether MPB or MPB-A20R proteins were

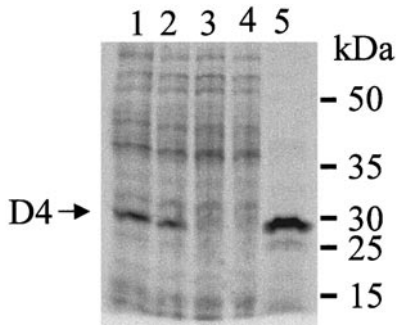

FIG. 5. Pull-down experiment demonstrating the interaction of full-length and truncated A20R fusion proteins with the D4R protein. Segments of the A20R ORF representing amino acids 1 to 426 (full length), 1 to 25, and 26 to 426 were expressed as MBP fusions and bound to amylose resin. As a control, MBP was expressed without A20R sequences and also bound to resin. An *in vitro* transcription-translation system was used to synthesize radioactively labeled D4R protein. A 20-μl sample was diluted with 200 μl of binding buffer, incubated with immobilized MPB fusion protein, and extensively washed. The bound proteins were eluted in 40 μl SDS buffer and 20 μl was analyzed by SDS–PAGE. Lane 1, MPB-A20R (1 to 426); Lane 2, MPB-A20R (1 to 25); Lane 3, MBP-A20R (26 to 426); Lane 4; MBP. Lane 5 contained 0.5 μl of the total *in vitro* translation reaction.

immobilized. Thus, we concluded that the first 25 amino acids of the A20R protein are both necessary and sufficient for binding the D4R protein. We failed, however, to show the interaction between A20R and D5R, A20R and H5R with this pull-down system, presumably because the interactions were not stable enough to withstand the resin washing steps.

DISCUSSION

The interactions of the vaccinia virus A20R protein with the D4R, D5R, and H5R proteins were discovered during a genome-wide yeast two-hybrid analysis (McCraith *et al.,* 2000). Because the D4R and D5R proteins are involved in viral DNA synthesis, these data suggested that the A20R protein is associated with the DNA replication complex. More direct evidence for this idea came from the finding that the A20R protein is associated with the processive form of viral DNA polymerase isolated from infected cells (Klemperer *et al.,* 2001). The latter authors (Klemperer *et al.,* 2001) suggested that the failure of the yeast two-hybrid screen to detect an interaction of the A20R protein with DNA polymerase could have been due to the presence of a yeast transcriptional terminator within the polymerase ORF. As synthesis of the DNA polymerase in the yeast system was not investigated and no interactions with the DNA polymerase were detected, the suggested explanation or another in which the DNA polymerase was not expressed well in yeast seems likely. However, the possibility remains that association of the A20R protein with DNA polymerase is mediated through binding to some intermediate protein such as D4R, H5R, or D5R.

In the first part of the present study, we confirmed the interaction of D4R and H5R proteins with the A20R protein by co-immunoprecipitation from lysates of vaccinia virus infected cells. Our failure to detect co-immunoprecipitation of the A20R protein with the D5R protein is likely due to the weakness of the interaction, since this was also the weakest of the A20R interaction in the yeast two-hybrid system. We cannot exclude the possibility, however, that the epitope tag on the D5R protein diminished the interaction.

The major emphasis of the present study was mapping the protein–protein interaction domains of the A20R ORF. This was achieved by employing a panel of A20R N- and C-terminal truncations in the yeast two-hybrid system. These studies revealed that the N-terminal 25 amino acids of A20R are necessary and sufficient for interaction with the D4R protein, although binding might be more efficient with additional amino acids. The ability of the 25 amino acid N-terminal domain fused with MPB to mediate binding to the D4R protein was demonstrated by a pull-down experiment, confirming the interaction. Additional yeast two-hybrid analyses suggested that the minimal A20R-binding domain is between amino acids 26 and 76 for the H5R protein and between amino acids 201 and 251 for the D5R protein, although these could not be evaluated by pull down. Attempts to use a similar yeast two-hybrid strategy to map the site on D4R that interacts with A20R, however, was unsuccessful as deletion of only 25 amino acids from either end abrogated binding.

Recently, A20R ts mutants were constructed by substituting alanine residues for clusters of charged amino acids (Ishii and Moss, 2001; Punjabi *et al.,* 2001). Such clusters could be involved in inter- as well as intraprotein interactions. There are three charged clusters within the determined binding region of A20R for D5R. Of these, mutants with alanine substitutions of two clusters could not be isolated, suggesting lethality and the mutant with substitution of the third cluster had the most stringent temperature sensitivity and grew poorly even at the permissive temperature. There was no charged cluster within the determined D4R-binding region and substitutions of the one within the H5R-binding region did not alter the plaque phenotype or temperature sensitivity. A multiple alignment analysis showed considerable amino acid identity between the D4R binding region of A20R orthologs of all chordopoxviruses. However, at this time we do not know whether the amino acid identities represent contact sites between A20R and D4R or conserved structural features. Nor do we know whether the A20R orthologs can bind vaccinia virus D4R or exhibit species or genus specificity.

In summary, we have confirmed and extended previous studies demonstrating the interaction of the A20R protein with at least three proteins, namely D4R, D5R, and H5R. Because the determined binding domains for the D4R, D5R, and H5R proteins do not overlap, the A20R protein may play an important role in forming or stabilizing the DNA replication complex.

MATERIALS AND METHODS

Plasmid constructions

pVOTED4R, pVOTED5R, and pVOTEH5R were constructed by PCR amplification of the D4R, D5R, and H5R ORF from vaccinia virus WR DNA using the primers:

D4R-fN: AACCATGGATTCAGTGACTGTATCACA
D4R-r: AAGGATTCTTAATAAATAAACCCTTGAG
D5R-fN: AACCATGGATGCGGCTATTAGAGGTAAT
D5R-r: AAGGATTCTTACGGAGATGAAATATCCT
H5R-fN: AACCATGGCGTGGTCAATTACAAATAAA
H5R-r: AAGGATTCTTACTTCTTACAAGTTTTAA

The PCR products were cleaved with *Nco*I and *Bam*HI and cloned into the *Nco*I, *Bam*HI-cleaved pVOTE1 vector (Ward *et al.,* 1995).

pCRV5A20R, pCRHAD4R, pCRHAD5R, and pCRHAH5R were constructed by PCR amplification of the A20R, D4R, D5R, and H5R ORFs from vaccinia virus strain Western

Reserve (WR) DNA using the primers D4R-r, D5R-r, and H5R-r shown above and the additional primers:

A20R-fV5: AAATGGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACGACTTCTAGCGCTGATTTAACTAACTTAAAA
A20R-r: AAGGATCCTCACTCGAATAATCTTTTTT
D4R-fHA: AAATGTATCCATATGATGTTCCAGACTATGCTAATTCAGTGACTGTATCACACGCGCCATAT
D5R-fHA: AAATGTATCCATATGATGTTCCAGACTATGCTGATGCGGCTA TTAGAGGTAATGATGTTATC
H5R-fHA: AAATGTATCCATATGATGTTCCAGACTATGCTGCGTGGTCAATTACAAATAAAGCGGATACT

The PCR products were ligated to pCR3.1 vector by using the eukaryotic TA Cloning Kit (Invitrogen/Life Technologies).

pGADD4R, pGADD5R, and pGADH5R were constructed by PCR amplification of the D4R, D5R, and H5R ORF from vaccinia virus WR DNA using the primers D4R-r, D5R-r, and H5R-r shown above and the additional primers:

D4R-fE: AAGAATTCATGAATTCAGTGACTGTATC
D5R-fE: AAGAATTCATGGATGCGGCTATTAGAGG
H5R-fE: AAGAATTCATGGCGTGGTCAATTACAAA

The PCR products were cleaved with *Eco*RI and *Bam*HI and cloned into the *Eco*RI, *Bam*HI-cleaved pGAD vector (Clontech).

pACTD5R and pACTH5R were constructed by PCR amplification of the D5R and H5R ORF from vaccinia virus WR DNA using primers shown in the methods describing construction of pVOTE derivatives. To create pACTD5R and pACTH5R, the PCR products were cleaved with *Nco*I and *Bam*HI and cloned into the *Nco*I, *Bam*HI-cleaved pACT vector (Clontech).

pASA20R and related plasmids containing truncations of the A20R ORF were constructed by PCR amplification of the A20R ORF from vaccinia virus WR DNA using the primers:

A20R-f: AAGAATTCATGACTTCTAGCGCTGATTT
A20R-r: AAGGATCCTCACTCGAATAATCTTTTTT
A20R-f25: AAGAATTCGCGGCTATAGAAAAGTATAA
A20R-f50: AAGAATTCGCTAATGTCGAGACGTCAATA
A20R-f100: AAGAATTCGGAAACTCTTTTCAAATACC
A20R-f150: AAGAATTCATATTTAAACTAGCTAGTGAGTAC
A20R-f200: AAGAATTCAAGTTGGGAGAACTTAGGCGGCAA
A20R-r326: AAGGATCCTCACTCATTAATATCTAATTTAT
A20R-r276: AAGGATCCTCAATTAATATCTAATTTATTAGTC
A20R-r251: AAGGATCCTCAGTCTACATCTTTTACTAATATC
A20R-r226: AAGGATCCTCAACGATCTACCTTGATGGACTC
A20R-r126: AAGGATCCTCACAGAAAGTCTATTCCGTTAT
A20R-r75: AAGGATCCTCAGTAATAGCCCGGATCAATAT
A20R-r50: AAGGATCCTCATACCTTTTGCACGCCTATT
A20R-r25: AAGGATCCTCAAGAATCTGAAAATTTCAAA

For yeast two-hybrid analysis, PCR fragments of A20R ORFs with N-terminal truncations were amplified by using one of A20R-fs and A20R-r, and those with C-terminal truncations were amplified by using one of A20R-rs and A20R-f. The PCR products were cleaved with *Eco*RI and *Bam*HI and cloned into the *Eco*RI, *Bam*HI-cleaved pAS vector (Clontech).

To make MBP fusions, PCR fragments of the vaccinia virus entire A20R coding sequence, coding sequence of N-terminal 25 amino acids of A20R, and coding sequence of C-terminal 400 amino acids of A20R were amplified by using sets of primers A20R-f and A20R-r, A20R-f and A20R-r25, and A20-f25 and A20-r, respectively. These fragments were digested with *Eco*RI and *Bam*HI and inserted in-frame downstream of MBP sequences in the vector pMALc2 (New England Biolabs), yielding pMALc2A20R, pMALc2A20R-1/25, and pMALc2A20R-26/426, respectively.

In all cases, cloned genes were sequenced to confirm the fidelity of the PCR reactions.

Yeast strains and transformation

Yeast strains Y187 and HF7C were used for transformation of Gal4-BD fusion proteins and Gal4-binding domain fusion proteins. The yeast strains were maintained at 30°C on Yeast extract, Peptone, Dextrose (YPD) plates. Transformation was performed as described in the Clontech Matchmaker System manual. Briefly, a stationary-phase yeast culture was diluted into 30 ml YPD medium to an optical density at 600 nm (OD_{600}) of between 0.2 and 0.3 and grown for 3 h at 30°C. To prepare competent yeast, cells were collected by centrifugation at 1000 *g* for 5 min at room temperature, washed once with H_2O, and suspended in 1.5 ml of 1× TE-LiAc (10× TE is 0.1 M Tris–HCl plus 10 mM EDTA, pH 7.5; 10× LiAc is 1 M LiAc, pH 7.5). For small-scale cotransformation, 0.5 μg of each DNA and 100 μg of herring sperm carrier DNA were mixed with 100 μl of competent yeast cells. After adding 600 μl of a polyethylene glycol (PEG)-LiAc solution (1× LiAc, 1× TE, 40% PEG 4000), the mix was incubated at 30°C for 0.5 h. Cells were then added to 70 μl dimethyl sulfoxide and incubated at 42°C for 15 min. The cells were then collected by centrifugation and suspended in 1× TE for selection on Leu Trp SD (Minimal Synthetic Dropout) plates.

Filter lift assay for β-galactosidase activity in yeast two-hybrid system

The yeast strain Y187 was used for this assay. Four to six days after transformation, the yeast colonies were

lifted onto filter paper (VWR, West Chester, PA), and the cells were lysed by freezing at 80°C for 20 min and thawing at room temperature. The filter disks were placed onto filter paper soaked in 2 ml of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 0.3%-mercaptoethanol) containing 0.33 mg 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal) per milliliter and incubated at 37°C for 1 to 3 h.

Liquid β-galactosidase assay in yeast two-hybrid system

Individual yeast colonies were transferred into 3 ml of Leu Trp SD medium and grown to stationary phase. They were then diluted 1:10 in medium and grown to A_{600} of between 0.4 and 0.6. The cells were collected by centrifugation at 1500 *g* for 5 min at 4°C and washed once with Z-buffer. The cell pellets were suspended in 250 μl of Z-buffer, and the cells were lysed following addition of 200 μl of ice-cold acid-washed glass beads by vortexing three times for 1 min each. The cell lysates were clarified by centrifugation at 3000 *g* for 5 min at 4°C. Total protein concentrations were determined by the method of Bradford (1976). For β-galactosidase assays, 200 μl of 4-mg/ml *o*-nitrophenyl-D-galactopyranoside (ONPG) was added to 150 μl of cell extract diluted in 800 μl of Z-buffer, and the mixture was incubated at 30°C until a pale yellow color developed. The reaction was stopped by adding 500 μl of 1 M Na_2CO_3, and the A_{420} was determined. Z-buffer alone incubated with ONPG was used as a control for measuring OD_{420}.

HIS3 assay

The yeast strain HF7C was used and the presence of an interaction between two proteins was indicated by the activation of the reporter genes HIS3, which allow for growth on medium lacking histidine. Colonies grown on SD/-Leu/-Trp plates were streaked on SD/-Leu/-trp/-His plates and growth on the plates was checked as described in the Matchmaker Library protocol (Clontech).

Overexpression and purification of MBP-fused A20R proteins

Bacterial synthesis of recombinant A20R was carried out with the T7 RNA polymerase expression system (Rosenberg *et al.,* 1987). *Escherichia coli* strain BL-21(DE3) pLysS was transformed with pMALc2A20R and transformants were grown in LB broth plus 100 μg of ampicillin per milliliter. Expression of recombinant proteins was induced with 0.4 mM isopropyl thiogalactoside for 2 to 3 h. A 10-ml culture of induced bacteria was centrifuged and the pellet was suspended in 0.01 M Tris–HCl, pH 7.5 containing 0.15 M NaCl, 1% sodium deoxycholate, 1% NP-40, 0.1% SDS (RIPA buffer). Bacteria were disrupted by four freeze-thaw cycles and the suspension was then forced through a 20-gauge needle to shear the bacterial DNA. Further steps were carried out at 4°C. The insoluble residue was removed by centrifugation at 100,000 *g* for 45 min, and the supernatant was mixed with 0.5 ml of amylose resin (New England BioLabs) for 2 h at 4°C. The suspension was centrifuged and the supernatant was discarded. The resin was washed five times with RIPA and used as the A20R-immobilized resin.

Coupled *in vitro* transcription and translation

The D4R, D5R, and H5R proteins were synthesized *in vitro* using the TnT Quick-coupled reticulocyte lysate system (Promega). Briefly, pVOTED4R, pVOTED5R, and pVOTEH5R, regulated 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 100 μl reaction and the mixture was incubated with 50 μl TnT Quick Lysate (Promega), 5 mCi Redivue Pro-Mix ^{35}S (Amersham), and incubated for 2 h at 30°C. Aliquots (20 μl) of the reaction mixture were diluted to 200 μl with RIPA buffer, mixed with 20 μl of MPB-A20R-immobilized resin, and incubated overnight at 4°C. The suspension was centrifuged and the supernatant was discarded. The resin was washed five times with RIPA, boiled in SDS loading buffer, and analyzed by SDS–PAGE using 4–20% separating gels. Gels were dried and autoradiographed.

Immune complexes and Western blot analysis

Confluent monolayers of BS-C-1 cells in 35-mm dishes were infected with vTF7-3 at a multiplicity of 10 and incubated at 37°C for 2 h. Infected cells were transfected with 0.5 μg of pCRV5A20R and 0.5 μg of either pCRHAD4R or pCRHAD5R or pCRHAH5R in Lipofectamine (Invitrogen/Life Technologies) and incubated at 37°C for 22 h. Cells were harvested in lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1 mg/ml aprotinin; Aldrich). The lysates were frozen and thawed and then clarified by centrifugation for 10 min at 15,000 rpm at 4°C. Clarified lysates were incubated with anti-HA (Covance) or anti-V5 (Invitrogen) mAb for 2 h at 4°C with rotation. Protein A–Sepharose was added for 2 h at 4°C. The immune complexes were harvested by centrifugation and washed three times with lysis buffer. Samples were suspended in SDS loading buffer, boiled, and analyzed by SDS–PAGE using 4–20% gradient separating gels. The proteins were transferred to nitrocellulose in 25 mM Tris–HCl, 192 mM glycine, 20% methanol for 1 h at 4°C. Nitrocellulose filters were incubated with primary mAb, and the bound mAb was detected using polyclonal anti-mouse horseradish peroxidase-conjugated antibody (1:5000; Amersham). Supersignal West Pico Chemiluminescence Substrates (Pierce) were used as described by the manufacturer.

ACKNOWLEDGMENT

Koji Ishii was supported in part by a Japan Science and Technology Overseas Research Fellowship.

REFERENCES

Banham, A., and Smith, G. L. (1992). Vaccinia virus gene B1R encodes a 34-kDa serine/threonine protein kinase that localizes in cytoplasmic factories and is packaged into virions. *Virology* **191,** 803–812.

Beaud, G., Beaud, R., and Leader, D. P. (1995). Vaccinia virus gene H5R encodes a protein that is phosphorylated by the multisubstrate vaccinia virus B1R protein kinase. *J. Virol.* **69,** 1819–1826.

Black, E. P., Moussatche, N., and Condit, R. C. (1998). Characterization of the interactions among vaccinia virus transcription factors G2R, A18R, and H5R. *Virology* **245,** 313–322.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Biochemistry* **72,** 248–254.

Challberg, M. D., and Englund, P. T. (1979). Purification and properties of the deoxyribonucleic acid polymerase induced by vaccinia virus. *J. Biol. Chem.* **254,** 7812–7819.

Condit, R. C., and Motyczka, A. (1981). Isolation and preliminary characterization of temperature-sensitive mutants of vaccinia virus. *Virology* **113,** 224–241.

DeMasi, J., and Traktman, P. (2000). Clustered charge-to-alanine mutagenesis of the vaccinia virus H5 gene: Isolation of a dominant, temperature-sensitive mutant with a profound defect in morphogenesis. *J. Virol.* **74,** 2393–2405.

Earl, P. L., Jones, E. V., and Moss, B. (1986). Homology between DNA polymerases of poxviruses, herpesviruses, and adenoviruses: Nucleotide sequence of the vaccinia virus DNA polymerase gene. *Proc. Natl. Acad. Sci. USA* **83,** 3659–3663.

Ellison, K. S., Peng, W., and McFadden, G. (1996). Mutations in active-site residue of the uracil-DNA glycosylase encoded by vaccinia virus are incompatible with virus viability. *J. Virol.* **70,** 7965–7973.

Evans, E., Klemperer, N., Ghosh, R., and Traktman, P. (1995). The vaccinia virus D5 protein, which is required for DNA replication, is a nucleic acid-independent nucleotide triphosphatase. *J. Virol.* **69,** 5353–5361.

Evans, E., and Traktman, P. (1987). Molecular genetic analysis of a vaccinia virus gene with an essential role in DNA replication. *J. Virol.* **61,** 3152–3162.

Evans, E., and Traktman, P. (1992). Characterization of vaccinia virus DNA replication mutants with lesions in the D5 gene. *Chromosoma* **102,** S72–S82.

Fuerst, T. R., Niles, E. G., Studier, F. W., and Moss, B. (1986). Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA* **83,** 8122–8126.

Ishii, K., and Moss, B. (2001). Role of vaccinia virus A20R protein in DNA replication: Construction and characterization of temperature-sensitive mutants. *J. Virol.* **75,** 1656–1663.

Jones, E. V., and Moss, B. (1984). Mapping of the vaccinia virus DNA polymerase gene by marker rescue and cell-free translation of selected mRNA. *J. Virol.* **49,** 72–77.

Klemperer, N., McDonald, W., Boyle, K., Unger, B., and Traktman, P. (2001). The A20R protein is a stoichiometric component of the processive form of vaccinia virus DNA polymerase. *J. Virol.* **75,** 12298–12307.

Kovacs, G. R., and Moss, B. (1996). The vaccinia virus H5R gene encodes late gene transcription factor 4: Purification, cloning and overexpression. *J. Virol.* **70,** 6796–6802.

Lin, S., Chen, W., and Broyles, S. S. (1992). The vaccinia virus B1R gene product is a serine/threonine protein kinase. *J. Virol.* **66,** 2717–2723.

McCraith, S., Holtzman, T., Moss, B., and Fields, S. (2000). Genome-wide analysis of vaccinia virus protein-protein interactions. *Proc. Natl. Acad. Sci. USA* **97,** 4879–4884.

McDonald, W. F., Klemperer, N., and Traktman, P. (1997). Characterization of a processive form of the vaccinia virus DNA polymerase. *Virology* **234,** 168–175.

McDonald, W. F., and Traktman, P. (1994). Vaccinia virus DNA polymerase. *J. Biol. Chem.* **269,** 1–8.

McFadden, G., and Dales, S. (1980). Biogenesis of poxviruses: Preliminary characterization of conditional lethal mutants of vaccinia virus defective in DNA synthesis. *Virology* **103,** 68–79.

Millns, A. K., Carpenter, M. S., and DeLange, A. M. (1994). The vaccinia virus-encoded uracil DNA glycosylase has an essential role in viral DNA replication. *Virology* **198,** 504–513.

Moss, B. (2001). Poxviridae: The viruses and their replication. *In* "Fields Virology" (D. M. Knipe, and P. M. Howley, Eds.), Vol. 2, 4th ed., pp. 2849–2883. 2 vols. Lippincott, Williams, & Wilkins, Philadelphia.

Punjabi, A., Boyle, K., DeMasi, J., Grubisha, O., Unger, B., Khanna, M., and Traktman, P. (2001). Clustered charge-to-alanine mutagenesis of the vaccinia virus A20 gene: Temperature-sensitive mutants have a DNA-minus phenotype and are defective in the production of processive DNA polymerase activity. *J. Virol.* **75,** 12308–12318.

Rempel, R. E., Anderson, M. K., Evans, E., and Traktman, P. (1990). Temperature-sensitive vaccinia virus mutants identify a gene with an essential role in viral replication. *J. Virol.* **64,** 574–583.

Rempel, R. E., and Traktman, P. (1992). Vaccinia virus-B1 kinase—Phenotypic analysis of temperature-sensitive mutants and enzymatic characterization of recombinant proteins. *J. Virol.* **66,** 4413–4426.

Rosenberg, A. H., Lade, B. N., Chui, D. O., Lin, S. W., Dunn, J. J., and Studier, F. W. (1987). Vectors for selective expression of cloned DNAs by T7 RNA polymerase. *Gene* **56,** 125–135.

Sridhar, P., and Condit, R. C. (1983). Selection for temperature-sensitive mutations in specific vaccinia virus genes: Isolation and characterization of a virus mutant which encodes a phosphonoacetic acid-resistant, temperature-sensitive DNA polymerase. *Virology* **128,** 444–457.

Stuart, D. T., Upton, C., Higman, M. A., Niles, E. G., and McFadden, G. (1993). A poxvirus-encoded uracil DNA glycosylase is essential for virus viability. *J. Virol.* **67,** 2503–2512.

Traktman, P., Anderson, M. K., and Rempel, R. E. (1989). Vaccinia virus encodes an essential gene with strong homology to protein kinases. *J. Biol. Chem.* **264,** 21458–21461.

Traktman, P., Sridhar, R. C., and Roberts, B. E. (1984). Transcriptional mapping of the DNA polymerase gene of vaccinia virus. *J. Virol.* **49,** 125–131.

Upton, C., Stuart, D. T., and McFadden, G. (1993). Identification of a poxvirus gene encoding a uracil DNA glycosylase. *Proc. Natl. Acad. Sci. USA* **90,** 4518–4522.

Ward, G. A., Stover, C. K., Moss, B., and Fuerst, T. R. (1995). Stringent chemical and thermal regulation of recombinant gene expression by vaccinia virus vectors in mammalian cells. *Proc. Natl. Acad. Sci. USA* **92,** 6773–6777.