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A Poxvirus Protein with a RING Finger Motif Binds Zinc and Localizes in Virus Factories

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Shope fibroma virus (SFV) is a *Leporipoxvirus* closely related to the highly virulent myxoma virus. The DNA sequence of the *Bam*HI N fragment of the SFV DNA genome was determined, and the single complete open reading frame (N1R) was characterized. The protein encoded by the N1R gene was found to contain a C_3HC_4 RING finger motif at the C terminus. This C_3HC_4 motif is the hallmark of a growing family of proteins, many of which are involved in regulation of gene expression, DNA repair, or DNA recombination. Complete homologs of the SFV N1R gene were also detected in variola virus, myxoma virus, and vaccinia virus strain IHD-W. In contrast, the gene is completely absent from vaccinia virus strain Copenhagen, and in vaccinia virus strain WR, the open reading frame is truncated prior to the zinc binding domain because of an 11-bp deletion, thus producing a frameshift and premature stop codon. Recombinant N1R protein from SFV was expressed in *Escherichia coli* and shown to bind zinc in a specific manner. Using fluorescence microscopy to visualize a peptide epitope tag (derived from ICP27 of herpes simplex virus) fused to the N terminus of the poxvirus proteins, we observed that the N1R protein of SFV and its homologs in myxoma virus and vaccinia virus IHD-W were localized primarily to the virus factories in the cytoplasm of infected cells and, to a lesser degree, the host cell nucleus. The truncated protein of vaccinia virus strain WR failed to localize in this manner but instead was observed throughout the cytoplasm.

The archetypal poxvirus genome is a single linear double-stranded DNA molecule in excess of 150 kb capable of encoding over 200 proteins (23). Poxviruses are distinguished from other eukaryotic DNA viruses by their complexity and their site of viral macromolecular synthesis, which occurs exclusively within the cytoplasm of infected cells. Upon infection, a virus factory or virosome is established in the cytoplasm, often close to the nuclear envelope, and viral genes are transcribed in a carefully controlled cascade by a fully competent transcription complex encoded by the virus. The *Poxviridae*, as a family, are ubiquitous, infecting mammals, birds, reptiles, and invertebrates (13). These viruses compose a disparate group produced by evolutionary selection pressures acting within a variety of individual complex host-parasite relationships.

Certain poxviruses are more restricted in host range or tissue specificity than others, and investigations into several virus-host systems are necessary to assess the contribution of particular genes to virulence. A significant proportion of poxvirus genes are not required for growth in a tissue culture environment (reviewed in references 6 and 33), and many of these genes tend to be localized in the terminal regions of the genome whereas the essential poxvirus genes are clustered within the central portion of the genome (32). Recently, a number of nonessential genes have been shown to contribute to virulence within an animal host (6, 29), some of which encode proteins that bind important host cytokines such as tumor necrosis factor, interleukin-1, or gamma interferon, and a protein that mimics the effect of epidermal growth factor (reviewed in reference 6).

Examination of proteins that contribute to the virulence of poxviruses can help elucidate the specificity determinants of host-virus interactions and may not only provide information about the host inflammatory and acquired immune functions that are most important in combating viral infections but also give insights into how patterns of gene expression by the host and virus interact in specific target cells. In this paper, we describe a novel Shope fibroma virus (SFV) gene product with a C_3HC_4 zinc binding RING finger motif and provide preliminary studies on members of this protein family from SFV, myxoma virus, and vaccinia virus. Since many of the other eukaryotic proteins with this motif are involved in the regulation of gene expression, we have analyzed the expression and localization of this protein to deduce whether it has the potential to be a direct regulator of virus and/or host gene expression.

MATERIALS AND METHODS

Cells and viruses. SFV (strain Kasza), myxoma virus (strain Lausanne), and vaccinia virus (strains WR and IHD-W) were obtained from the American Type Culture Collection and propagated in BGMK cells (provided by S. Dales) with Dulbecco's minimal Eagle's medium supplemented with 10% newborn bovine serum.

Cloning and DNA sequencing. The cloning and restriction mapping of the SFV genome have been described previously (10, 34). The SFV *Bam*HI N fragment was subcloned from pKBN (34) into the *Bam*HI site of pUC13 (both orientations), and sets of unidirectional nested deletions for DNA sequencing were produced by the exonuclease III-VII method, by using *Pst*I and *Xba*I to orient the deletions (15a). *Taq* cycle sequencing was performed on double-stranded DNA templates with an ABI 373A DNA sequencer to produce complete sequence

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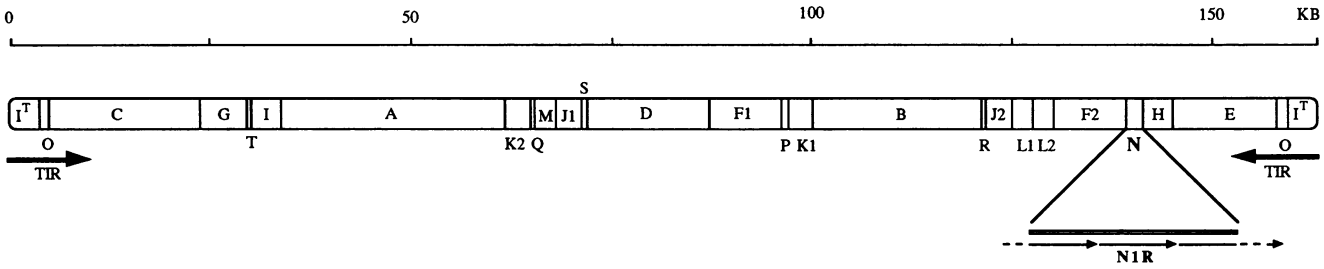

FIG. 1. SFV *Bam*HI genome map indicating the position of the N1R gene. The positions of the terminal inverted repeat (TIR) sequences are indicated by boldface arrows. Note that the orientation of the *Bam*HI map has been reversed from that in the original report (10) to coincide with that of vaccinia virus.

information from both strands. Myxoma virus and vaccinia virus homologs were sequenced with a series of oligonucleotide primers. The raw DNA sequence data were managed with the programs of Staden (9), and the final sequence was analyzed by programs in the Genetics Computer Group package (3, 11). Data base searches were performed with BLAST (2) and NW_Align of the SEQSEE package (developed by D. Wishart and R. Boyko, University of Alberta).

Epitope tagging of proteins. Complete homologs of the SFV N1R open reading frame (ORF) were cloned from SFV, myxoma virus, and vaccinia virus strains IHD-W and WR from PCR products into the vector pT7-Blue (Novagen). The PCR primers incorporated a *Nco*I site at the first methionine codon of the ORF and a *Bam*HI site immediately 3′ of the termination codon. Two complementary oligonucleotides containing the 12 N-terminal amino acids of the herpes simplex virus type 1 ICP27 gene (22a) were synthesized to have overhangs complementary to those of *Sal*I and *Nco*I restriction sites. Insertion of the annealed oligonucleotides 5′ to the poxvirus ORF (see Fig. 6) resulted in a single contiguous ORF which encoded a fusion protein. The epitope for the mouse monoclonal antibody H1119 (Goodwin Institute for Cancer Research, Plantation, Fla.) has been mapped to this region of ICP27 (11a).

For expression in poxvirus-infected cells, a *Sal*I-*Bam*HI fragment containing the region encoding the epitope tag fused to the appropriate viral zinc finger ORF was cloned into pMJ601 (see Fig. 6). In plasmid pMSN1 (for SFV N1R), this novel gene was driven by a strong synthetic poxvirus late promoter (8). Confluent BGMK cells in six-well dishes were infected with SFV or vaccinia virus strain WR (multiplicity of infection = 10) and after 2 h were transfected with 10 μg of plasmid, with Lipofectin (Gibco BRL). A protein of the expected molecular weight could be readily detected in 12-h lysates by probing Western immunoblots for the epitope tag with H1119 (data not shown). Other poxvirus homologs of the SFV N1R ORF were fused to the epitope tag directly in the expression plasmid by replacement of the *Nco*I-*Bam*HI SFV N1R cassette. Such constructs were confirmed by DNA sequencing. Plasmids pMIN1 and pMWN1 contain the RING finger homologs of vaccinia virus strains IHD-W and WR, respectively.

For localization of the tagged proteins, transfections and infections were performed by the basic procedure outlined above, except that cells were grown on coverslips in 12-well dishes. The cells were fixed, permeabilized, and processed for immunofluorescence as described by Quinlan et al. (23a). The cells were stained with a 1:200 dilution of H1119, followed by a 1:200 dilution of rhodamine (tetramethyl-rhodamine isothiocyanate)-conjugated anti-mouse immunoglobulin G (Jackson Immunoresearch Laboratories). As a control, the localization of *Escherichia coli* β-galactosidase, which is also produced from the pMJ601-based vectors, was monitored with a second mouse monoclonal antibody (Promega Corp.). A Zeiss Axioskop 20 fluorescence microscope equipped with a Plan Neofluor 63× objective lens was used to visualize the epitope-tagged proteins in infected-transfected cells.

Assay of zinc binding by SFV N1R protein. The complete SFV and myxoma virus ORFs were expressed in *E. coli* with an inducible T7 expression system (30). The *Nco*I-*Bam*HI fragments containing these ORFs were cloned into *Nco*I-*Bam*HI-digested pET19b (Novagen Corp.). Proteins of the expected molecular weight were expressed in *E. coli* BL21(DE3)lysS following IPTG (isopropyl-β-D-thiogalactopyranoside) induction and were recovered in insoluble inclusion bodies (15). Inclusion body protein preparations were subjected to discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (19), electroblotted to nitrocellulose, and probed with $^{65}ZnCl_2$ as previously described (25).

Nucleotide sequence accession number. The DNA sequence has been submitted to GenBank with the accession number L26342.

RESULTS

Analysis of the SFV *Bam*HI N fragment DNA sequence. The *Bam*HI N fragment is located approximately 22 kb from the right end of the SFV genome (Fig. 1). This fragment was of interest because sequencing of the *Bam*HI H fragment indicated that it was predicted to contain the N-terminal half of an ORF related to the B5R complement-binding protein in vaccinia virus (17, 22). The partial ORF designated SFV N2R (Fig. 2) is indeed related to the N terminus of B5R of vaccinia virus strains WR and Copenhagen. At the other end of the SFV *Bam*HI N fragment is the C terminus of a second incomplete ORF which originates within the SFV *Bam*HI F2 fragment. This is highly homologous to the vaccinia virus B1R protein kinase (5, 24).

The primary focus of this paper concerns the single complete ORF N1R present in the middle of the SFV *Bam*HI N fragment (Fig. 2). Initial sequence analysis of this ORF suggested that this is a nonessential gene for poxviruses in general since no homolog is present in the complete DNA sequence of vaccinia virus strain Copenhagen (14). Additionally, a predicted zinc finger motif was detected toward the C terminus of the N1R protein, encoded by nucleotides beginning at 1178 (Fig. 2). This motif belongs to a class that has been recently designated the RING finger motif (20) and consists of the C_3HC_4 signature (3). Data base searches indicated several poxvirus homologs of the SFV N1R gene (discussed below),

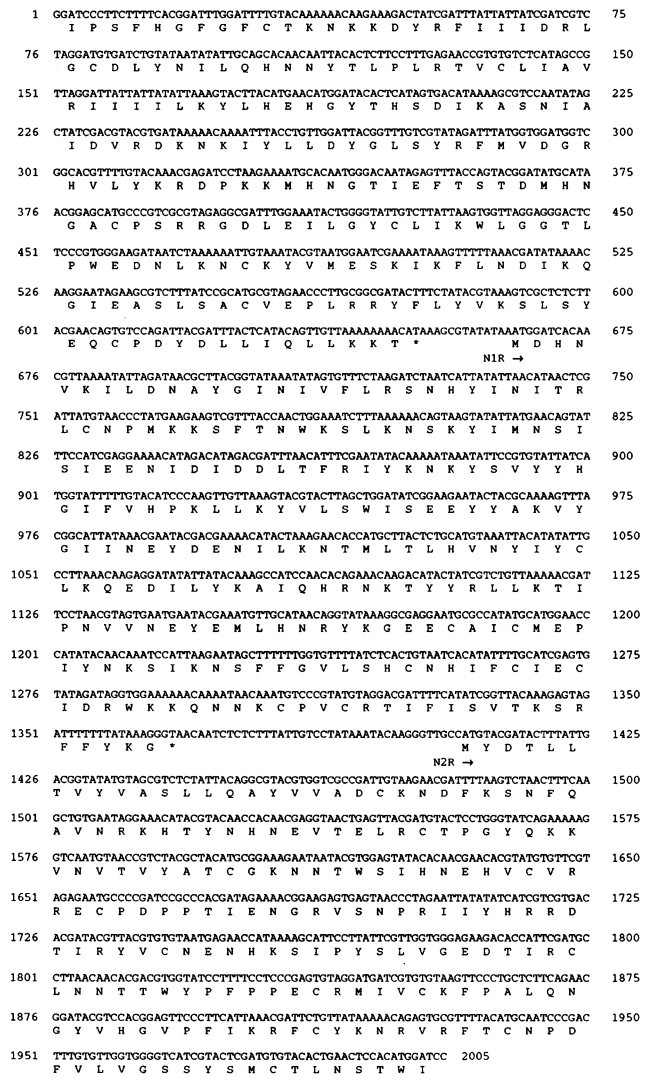

FIG. 2. DNA sequence of the SFV *Bam*HI N fragment. The complete translation (single-letter amino acid code) of the N1R ORF is shown together with the incomplete upstream and downstream (N2R) ORFs. Translation termination codons are denoted by asterisks.

but, other than what is presumed to be a structural relatedness by virtue of the RING finger motif, no other significant similarities to known nonviral proteins were detected.

Poxvirus ORFs related to SFV N1R. Two fowlpox ORFs which map in the near-terminal region of the genome (31) have homology to the N-terminal portion of N1R, but neither has a RING finger motif. It is unknown whether a complete version of the ORF is present elsewhere in the fowlpox genome. Complete homologs of the SFV N1R gene are present in the genomes of at least two strains of variola major virus, the agent of smallpox, Bangladesh-1975 (21) and India-1967 (28). In addition, sequencing data from a myxoma virus clone derived by PCR indicate that this leporipoxvirus also possesses a full-length homolog with 91% amino acid identity to the SFV N1R gene (data not shown). No counterpart exists in vaccinia virus strain Copenhagen (14), although a truncated version of the ORF is present in vaccinia virus strain WR, one

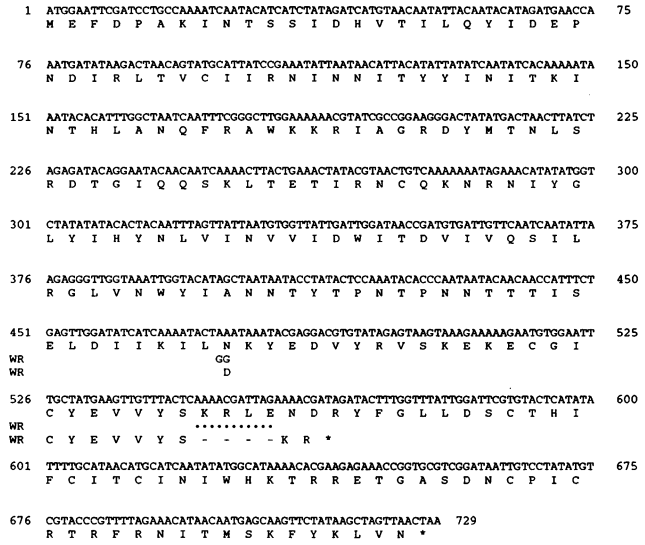

FIG. 3. DNA sequence and translation of the vaccinia virus strain IHD-W counterpart of the SFV *Bam*HI N1R gene. Differences between vaccinia virus strains IHD-W and WR are indicated below the IHD-W sequence. Nucleotides deleted from the WR sequence are shown as dots. Translation termination codons are denoted by asterisks.

which was previously designated the 21.7K gene (18). We analyzed the three possible translation frames 3′ of the vaccinia virus WR 21.7K gene and determined that a single frameshift close to the point of truncation could restore the full ORF, including the RING finger motif at the C terminus of the protein. To determine whether the published vaccinia virus strain WR DNA sequence was representative of those of other WR isolates and vaccinia virus strains, we designed primers to allow for PCR amplification of the complete gene from vaccinia virus. A *Nco*I site, encompassing the initiating ATG, was incorporated into the 5′ primer, and a *Bam*HI site was present in the 3′ primer which was placed after the termination codon of the untranslated RING finger motif found in the WR strain sequence. Vaccinia virus strains WR and IHD-W, from American Type Culture Collection stocks, were used to prepare genomic DNA templates, and three PCR products from independent PCRs were cloned and sequenced. Consensus sequences for each VV strain were derived to correct errors due to the PCR procedure. The data clearly show (Fig. 3) that a complete homolog of the SFV N1R gene is present in vaccinia virus strain IHD-W and that the American Type Culture Collection WR isolate had the DNA sequence identical to that previously published for this region (18). Two differences exist between the sequences of the vaccinia virus strains tested; (i) 2 adjacent nucleotides are altered (Fig. 3,

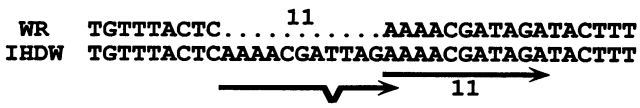

FIG. 4. DNA sequences from vaccinia virus strains IHD-W and WR showing the presence of a deletion of 11 nucleotides within the WR homolog of the SFV N1R ORF. The deletion composes one unit of an almost perfect direct repeat (arrows) of 11 nucleotides in the IHD-W sequence.

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IHD-W   MEFDPAKINT SSIDH-VTIL QYIDEPNDIR LTVCIIRNIN NITYYINITK
                    || | ||       |         |       |||||
SFV                 MDHNVKIL D-----NAYG INIVFLRS-- --NHYINITR

IHD-W   INTHLANQFR AWKKRIAGRD YMTNLSRDTG IQQSKLTETI RNCQKNRNIY
              |   ||          |   |     |    ||  |
SFV     LCNPMKKSFT NWKSLKNSKY IMNSISIEEN IDIDDLTFRI YKNKYSVYYH

IHD-W   GLYIH----- ---------- YNLVINVVID WITDVIVQSI LRGLVNWYI-
        |   |                |  |                |   ||
SFV     GIFVHPKLLK YVLSWISEEY YAKVYGIINE YDENILKNTM LTLHVNYIYC

IHD-W   ---ANNTYTP NTPNNTTTIS ELDII-KILN KYEDVYRVSK EKECGICYEV
              |       | |     |  |    |  ||       |   || || |
SFV     LKQEDILYKA IQHRNKTYYR LLKTIPNVVN EYEMLHNRYK GEECAICMEP
                                                    ↑  ↑

IHD-W   VYSKRLENDR YFGLLDSCTH IFCITCINIW HKTRRETGAS DNCPICRTRF
         | |   |    || |  | | |||| ||  |  |           || ||| |
SFV     IYNKSIKNS- FFGVLSHCNH IFCIECIDRW KK------QN NKCPVCRTIF
                     ↑ ↑   ↑  ↑                     ↑  ↑

IHD-W   RNITMSKFYK LVN
           | | |
SFV     ISVTKSRFFY KG
```

FIG. 5. Alignment of the SFV N1R protein and the vaccinia virus strain IHD-W homolog. Vertical lines between the sequences indicate identical amino acids, and arrows show members of the C_3HC_4 motif.

nucleotides 474 and 475), resulting in a single amino acid substitution, and (ii) 11 nucleotides have been deleted from the WR genome, which results in a frameshift and truncation of the ORF. An inspection of the sequence around the deletion site reveals the presence of an almost perfect 11-bp direct repeat in the IHD-W strain and the loss of one of these repeats from the WR strain (Fig. 4). The loss of one of a pair of direct repeats has been observed before (1), and such sequences appear to be inherently unstable in poxviruses.

Analysis of the SFV N1R protein sequence. Alignment of the SFV N1R protein sequence and its homolog in vaccinia virus strain IHD-W indicates 28% identity between these proteins (Fig. 5) whereas SFV-myxoma virus and variola virus–IHD-W alignments (not shown) have 91 and 96% identity, respectively. However, despite the relatively low conservation between the leporipoxvirus and orthopoxvirus proteins the cysteine and histidine residues of the RING finger motif are absolutely conserved (arrows in Fig. 5), as they are in the growing list of proteins bearing this motif (20). The C_3HC_4 has been observed to date in more than 30 proteins, many of which are thought to be involved in the regulation of gene expression, DNA recombination, or DNA repair. It is unknown how this RING motif is involved in these processes. Preliminary evidence suggests that the RING1 C_3HC_4 domain by itself can bind DNA (20), but there is no evidence that the equivalent domains from a family of herpesvirus proteins bind DNA (12). Structural studies on one herpesvirus C_3HC_4 domain indicate that the domain is unlikely to be able to bind directly to normal A- or B-form DNA (5b). It is quite feasible that the C_3HC_4 motif functions by interacting with other proteins whose function is to bind DNA.

Localization of the SFV N1R protein. To determine the distribution of the SFV N1R protein within infected cells, a plasmid expression vector was constructed in which an epitope tag derived from ICP27 of herpes simplex virus type 1 was fused to the N terminus of the protein (Fig. 6). The protein could then be recognized by a monoclonal antibody (H1119) specific for the ICP27 epitope and visualized by indirect immunofluorescence. The tagged protein was expressed following transfection of the vector into poxvirus-infected cells. Since H1119 also cross-reacts with a cellular protein localized to the plasma membrane, this results in a low but useful background fluorescence that outlines or produces spots on the surfaces of all cells, depending on the focus plane. Although cells were infected at a sufficiently high multiplicity of infection to infect more than 95% of the monolayer, the relatively inefficient transfection procedure resulted in only a few cells in any one field expressing the transfected tagged protein, while the remainder serve as negative controls.

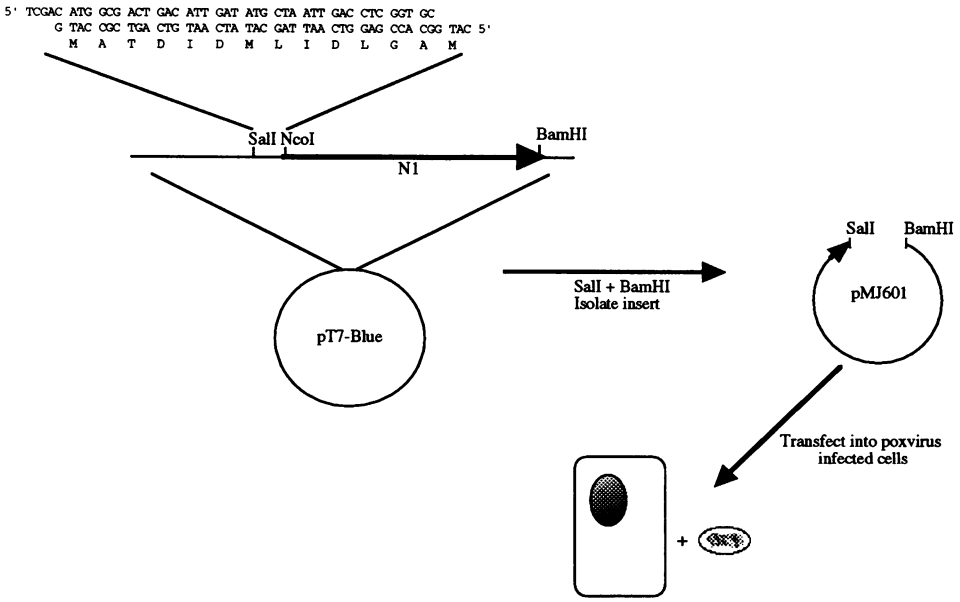

FIG. 6. Construction of expression vectors for epitope tagging of poxvirus RING finger proteins. The first 12 amino acids encoded by the *SalI-NcoI* fragment are identical to those at the N terminus of the herpes simplex virus type 1 ICP27 protein (23a). Amino acid 14 is thus the first residue which corresponds to the poxvirus ORF.

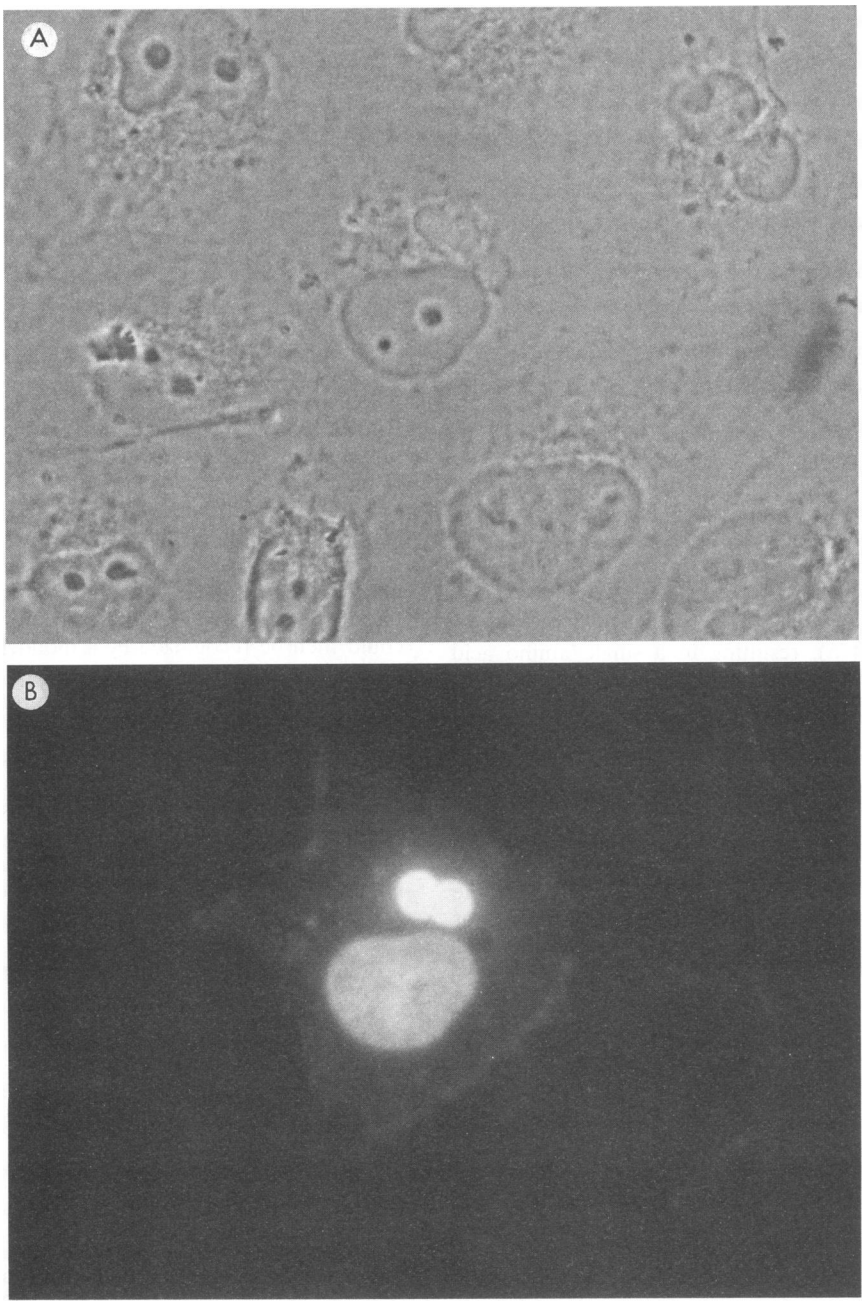

FIG. 7. Expression of epitope-tagged poxvirus RING proteins in infected cells. Panel A-B, C-D, and E-F pairs show identical fields with phase-contrast and immunofluorescence photographs, respectively. In panels C and E, the immunofluorescent signal was not blocked out for the phase-contrast image. Panels: (A and B) BGMK cells infected with SFV and transfected with pMSN1 to express epitope-tagged SFV N1R; (C and D and E and F) BGMK cells infected with vaccinia virus strain WR and transfected with pMIN1 to express the epitope-tagged intact IHD-W homolog (C and D) or transfected with pMWN1 to express the epitope-tagged truncated WR RING finger homolog (E and F).

When SFV-infected cells were transfected with the epitope-tagged SFV N1R gene, the majority of the fluorescence was observed to localize in the cytoplasmic viral factories by 12 h (Fig. 7A and B), but a significant amount of labeling of the cell nucleus was also observed. Similar results were found with the identically tagged myxoma virus homolog of the SFV N1R protein (not shown). In contrast, a control protein, *E. coli* β-galactosidase which was expressed from the same plasmid as the SFV N1R protein, did not localize to any particular cellular organelle and remained distributed throughout the infected cells (not shown).

To study the effect of the zinc finger domain on localization of this protein, we compared the distribution of the epitope-tagged vaccinia virus IHD-W protein with that of the naturally truncated WR homolog. In this case, the cells were infected with vaccinia virus strain WR and then transfected with either IHD-W or WR tagged constructs. To a large extent, the localization of the IHD-W protein was similar to that observed

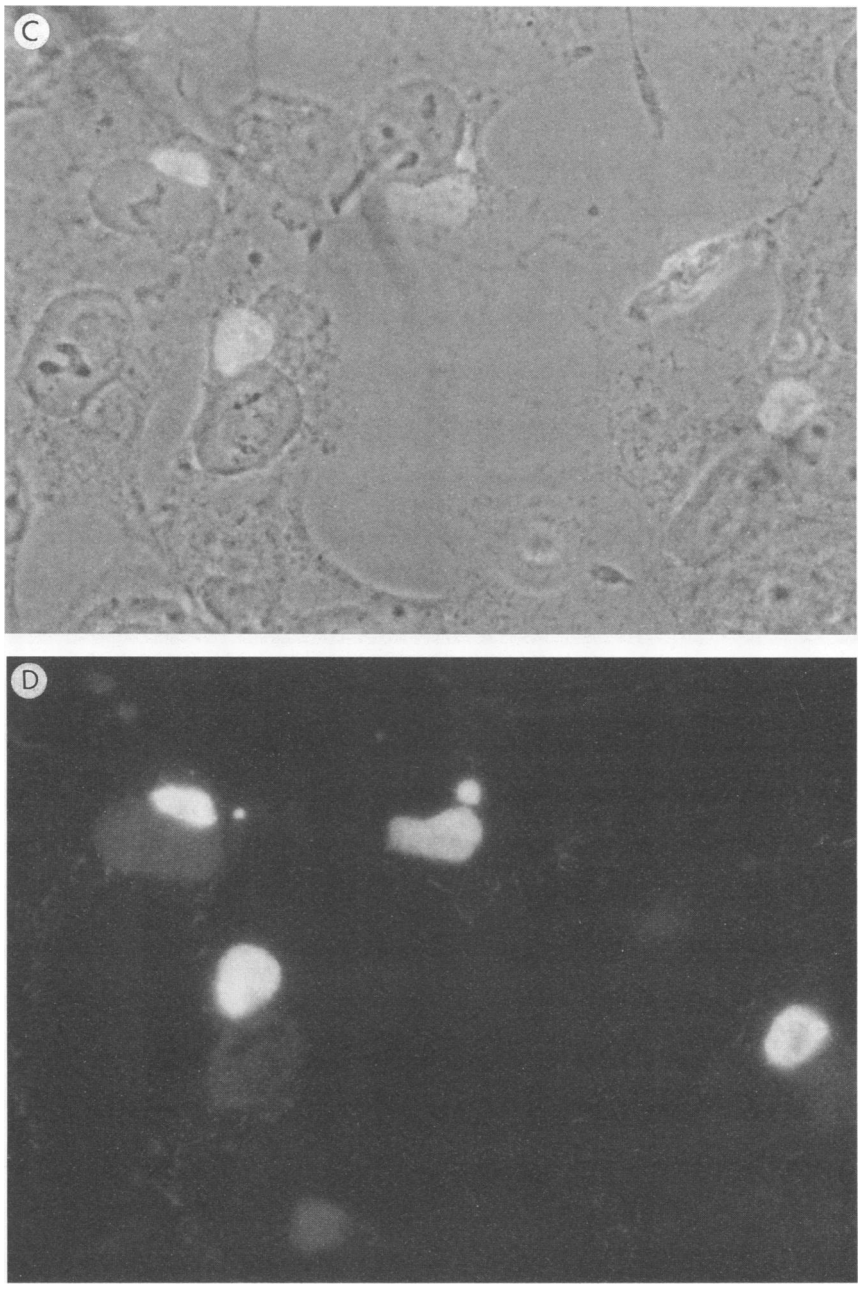

FIG. 7—*Continued.*

for the myxoma virus and SFV N1R proteins, primarily in virus factories and to a lesser degree in the nuclei of transfected cells (Fig. 7C and D). However, there were somewhat fewer cells in which the nuclear localization could be observed. In stark contrast, the truncated WR homolog of this protein, which possesses only the first two cysteine residues of the zinc finger, failed to localize to the virus factories, but was observed throughout the cytoplasm (Fig. 7E and F). Western blotting of cell lysates detected similar amounts of the WR and IHD-W proteins, suggesting that the truncation in WR does not alter the overall steady-state levels of the two vaccinia virus proteins (not shown). These results indicate that the C-terminal region which contains the RING finger motif is important for the localization pattern of this protein.

Assay of zinc binding by the SFV N1R protein. To determine whether the predicted RING finger motif functioned to bind zinc, the SFV N1R protein and its homolog from myxoma virus were expressed in *E. coli* from a T7 vector (30). Proteins of the expected molecular mass (28 kDa) were produced as insoluble inclusion bodies (Fig. 8A, lanes 4 and 5). *E. coli* cells expressing the myxoma virus Serp1 gene product from the same vector and cells containing the control vector were similarly prepared (Fig. 8A, lanes 6 and 3, respectively). These preparations (15) were used to assay zinc binding by a blotting technique (5a, 25). Following SDS-PAGE and electrophoretic transfer to nitrocellulose, we assayed binding under conditions that had previously been used to demonstrate zinc binding by TFIIIA-like zinc-binding proteins (25) as well as under reducing conditions

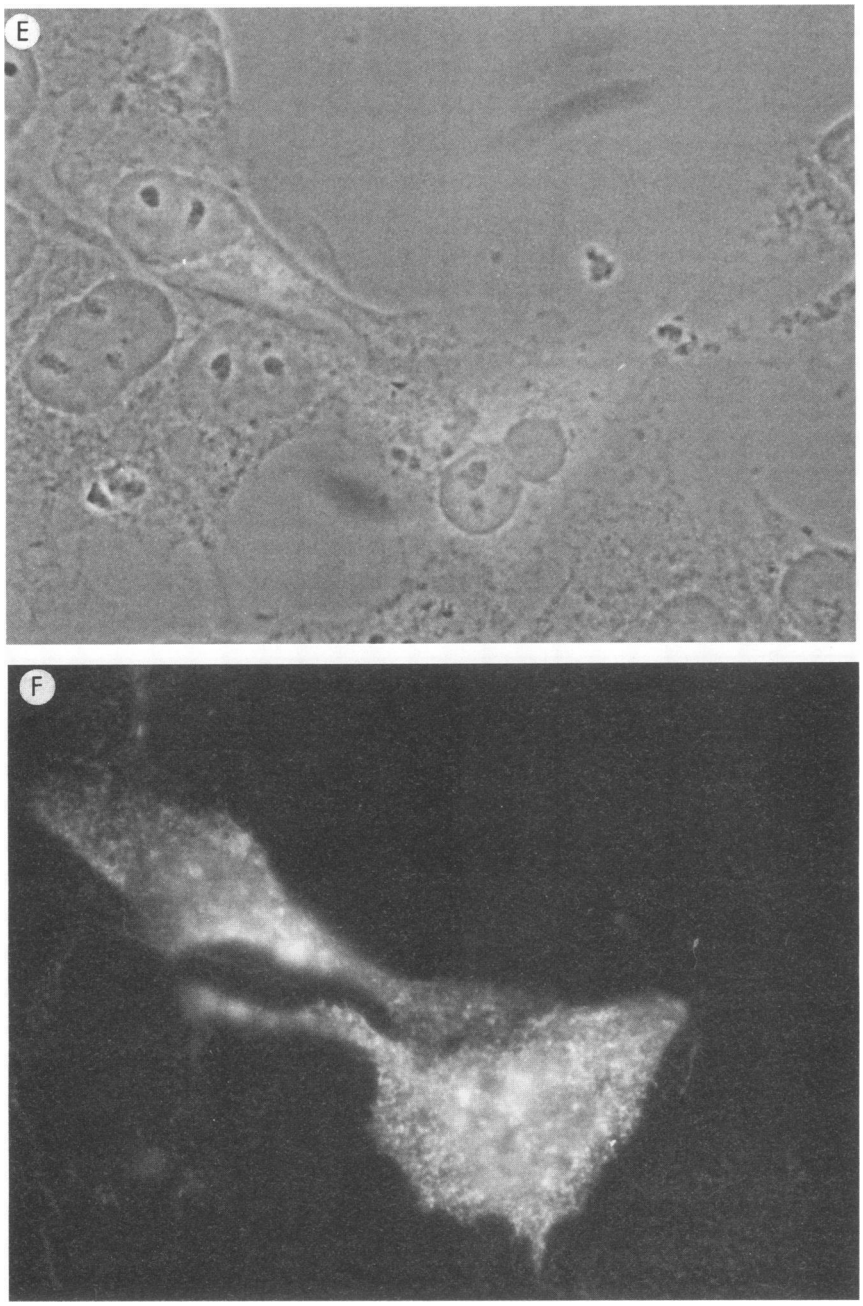

FIG. 7—*Continued.*

that have been used to demonstrate zinc binding by proteins whose metal ion coordination depends wholly or predominantly on cysteine residues (5a).

When the nitrocellulose filter was incubated with $^{65}ZnCl_2$ after being subjected to mild reducing conditions (Fig. 8B), we found that the induced protein from the SFV N1R ORF (lane 5) and that from the myxoma virus homolog (lane 4) both bound $^{65}Zn^{2+}$ (arrow). The level of binding was comparable to that of control zinc-binding proteins: carbonic anhydrase (c), superoxide dismutase (f), and alcohol dehydrogenase (d). Minor higher-molecular-weight zinc-binding proteins were detected in the lysates from strains expressing N1R proteins (Fig. 8B, lanes 4 and 5). The lack of similar proteins in the control bacterial extracts (Fig. 8B, lanes 3 and 6) suggests that these are related to the N1R proteins and are not endogenous bacterial zinc-binding proteins. Like alcohol dehydrogenase, the N1R proteins only bind detectable levels of zinc under reducing conditions (data not shown). This result may reflect the nature of the C_3HC_4 motif or the inclusion body source of protein. No significant binding was detected (Fig. 8B) by bacterial proteins induced in a strain carrying a control vector (lane 3), by an independent control protein (Serp1 of myxoma virus) expressed from the same vector (lane 6), or by the control proteins ovalbumin and glyceraldehyde-3-phosphate (lane 1, a and b, respectively). The large number of proteins present on the filter that do not bind detectable amounts of

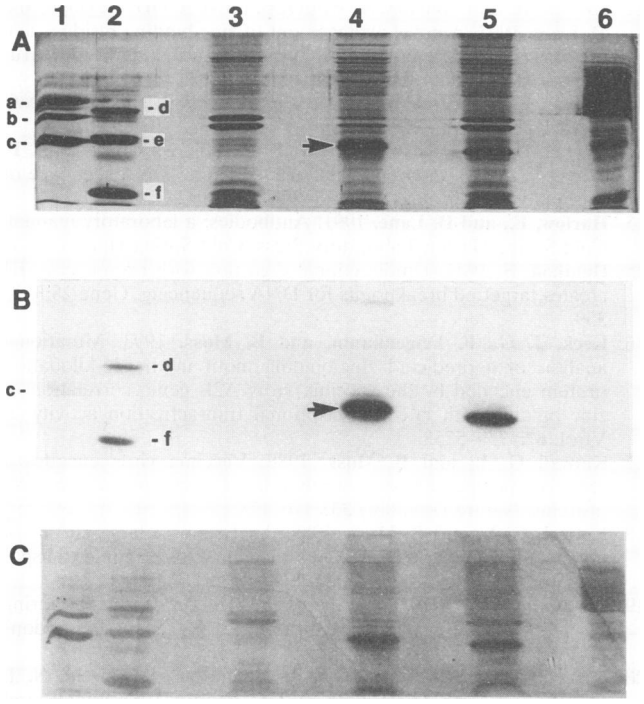

FIG. 8. Zinc binding by the SFV and myxoma virus RING finger proteins expressed in *E. coli*. Cells harboring T7-based expression plasmids were induced by IPTG, and the proteins were displayed on a Coomassie blue-stained SDS-polyacrylamide gel (A). The control proteins are marked as follows: lane 1 includes ovalbumin (a), 43 kDa; glucose 6-phosphate dehydrogenase (b), 36 kDa; and carbonic anhydrase (c), 29 kDa; lane 2 includes alcohol dehydrogenase (d), 40 kDa; carboxypeptidase A (e), 35 kDa; and superoxide dismutase (f), 17 kDa. Inclusion body proteins from *E. coli* expressing the SFV N1R gene product (lane 5), the myxoma virus N1R homolog (lane 4), and the myxoma virus Serp1 gene product (lane 6) are shown. An arrow indicates the position of the recombinant N1R viral protein (28 kDa). Lane 3 contains a sample isolated from cells (which contained a control vector) treated similarly. (B) Autoradiogram of a zinc blot from a polyacrylamide gel identical to that shown in panel A. The proteins were transferred to a nitrocellulose filter which was probed with $^{65}Zn^{2+}$ under mild reducing conditions, washed, and exposed to film overnight. (C) Amido black-stained proteins on the nitrocellulose blot after probing with $^{65}Zn^{2+}$, to illustrate transfer efficiency of the blotting procedure.

zinc was evident when the filter was stained with amido black following autoradiography (Fig. 8C).

DISCUSSION

This paper describes the analysis of the SFV N1R ORF which maps within the *Bam*HI N fragment and is located approximately 22 kb from the right terminus of the linear viral genome. The N1R ORF is distinguished by the presence of a C-terminal C_3HC_4 motif characteristic of a large family of RING finger zinc-binding proteins (20). Analysis of the complete sequences of vaccinia and variola viruses indicates that this particular motif, when present, is found only once per genome although a different zinc binding motif ($CX_2CX_{13}CX_2C$) has been described for the vaccinia virus A2L late transcriptional activator (16). Here, we have confirmed by ligand blotting that both the SFV N1R protein and the homologous protein from myxoma virus bind zinc, suggesting that the RING motif in these proteins is a functional zinc binding element.

The analysis of published poxvirus sequences together with the cloning and sequencing of PCR products from vaccinia virus strains reported here has established that a complete ORF homologous to the SFV N1R gene is present in myxoma virus, variola virus, and vaccinia virus strain IHD-W. In contrast, the ORF is completely absent from vaccinia virus strain Copenhagen and is truncated by a deletion-frameshift 5′ to the zinc finger motif in vaccinia virus strain WR, indicating that the intact protein is nonessential for poxvirus replication. The near-terminal location of the SFV N1R gene and the fact that the ORF is nonessential for virus growth in tissue culture are suggestive of a role for this protein in virus virulence or for propagation in an animal host. Indeed, it has recently been found that deletion of the ectromelia virus homolog of this ORF (27) greatly reduces the virulence of the knockout virus in mice, the natural host (26). However, the presence of this ORF in vaccinia virus strain IHD-W indicates that this RING motif protein cannot be solely responsible for the differences in virulence characteristics between variola virus and vaccinia virus. More likely, the pathogenic phenotype of any particular poxvirus in its natural host will be modulated by a great many viral gene products, and the N1R family described in this report will be but one of many contributors to the specificity of virus-host interactions.

Given that many of the proteins with the C_3HC_4 motif are believed to be involved in regulation of gene expression, DNA repair, or DNA recombination, it was of considerable importance to determine the location of the SFV N1R protein within the infected cell. The results presented in Fig. 7 clearly show a preferential localization of the protein in virus factories, and to a lesser extent the infected cell nucleus. Similarly, the full-length vaccinia virus IHD-W protein localized to these viral factories, whereas the truncated protein of vaccinia virus WR was observed throughout the infected cell. Thus, the C terminus of the full-length viral proteins which contain an intact zinc finger domain plays a direct role in the localization of N1R to virus factories. The smaller amounts of the SFV N1R protein and vaccinia virus IHD-W homolog observed in the nuclei of infected cells were a consistent observation, but it is still unknown whether any of these proteins play a role in modulating host gene expression during infection. Although it is reasonable to predict that SFV N1R protein possesses a DNA or protein binding activity, it is not yet known whether these activities are general or specific in nature, and therefore, a number of scenarios regarding the function of this protein remain open. One intriguing possibility is that while the protein may have a primary role in the virus factory, a second function of regulating host gene expression could be performed by the nuclear fraction of the protein.

Transcription of the SFV N1R gene has not yet been characterized. However, there is an early termination sequence for transcription (35) in the middle of the ORF (Fig. 2, nucleotides 906 to 912), suggesting that it is unlikely to be efficiently transcribed as an early gene. At the start of the SFV N1R ORF, there is the consensus TAAAT motif required for late transcription (7), and additionally, the sequence 5′ of this region is similar to that required for intermediate transcription (4). Interestingly, two of three intermediate gene products (A1L and A2L) that are required for activation of late gene transcription have also been shown to possess specific affinity for zinc (16) although they do not possess the C_3HC_4 motif found in SFV N1R. However, in contrast to these transactivators of late gene expression, the vaccinia virus homolog of the SFV N1R protein is nonessential and is therefore unlikely to

have a similar function for regulation of transcription cascades common to all poxviruses.

In these preliminary studies, we have clearly shown that the SFV N1R protein localizes within the virus replication factories and that the protein specifically binds zinc, presumably utilizing the C_3HC_4 RING finger motif. Using the homologous proteins from vaccinia virus strains IHD-W and WR, we have also demonstrated that the C-terminal domain, which contains the C_3HC_4 motif, is required for this localization. However, the presence of the tagged SFV, myxoma virus, and vaccinia virus IHD-W proteins in the nucleus of infected cells is less well understood, and further experimentation must be undertaken to determine whether this localization is a functional characteristic of this protein family. Similarly, the possibility of DNA, RNA, or protein binding activities associated with this zinc finger motif protein and its role in virus virulence must await further studies.

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