Metallopanstatulin Gene Product Produced in a Baculovirus Expression System Is a Nuclear Phosphoprotein That Binds to DNA

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Abstract

The protein product of the human Metallopanstatulin (MPS-1) gene was produced in the insect cell line Spodoptera frugiperda (SF9) using the baculovirus expression vector system Autographa californica nuclear polyhedrosis virus (AcNPV). When a cloned MPS-1 complementary DNA sequence was inserted into the AcNPV viral genome downstream from the promoter of the polyhedrin gene, a polypeptide with an apparent molecular weight of approximately 10,000 was observed in extracts of infected SF9 cells. This protein was not detected in SF9 infected cells with AcNPV-MPS-1-Del, a vector in which the MPS-1 gene was deleted. The MPS-1 protein was produced at high levels in this host-vector system (~ 12% of total labeled soluble protein).

Characterization of the MPS-1 protein extracted from SF9 infected cells showed that it: binds zinc ions specifically; is phosphorylated; accumulates in the nucleus; is tightly bound to the nucleus; and binds to calf thymus DNA-cellulose. The MPS-1 protein constitutes one of the major proteins in the nuclear fraction of SF9 cells and can be purified from this source to near homogeneity by a two-step procedure composed of high-performance liquid chromatography and gel electrophoresis. Antibodies were raised against selected peptide sequences of the MPS-1 protein and used to detect MPS-1 in insect cells and in various cultured mammalian cell types. Western analysis demonstrated that the baculovirus-produced protein had electrophoretic and immunological properties equivalent to those of MPS-1 spontaneously expressed in various human mammalian cell lines. Finally, recombinant MPS-1 generated a specific protein-DNA complex with a duplex oligomer containing a cyclic AMP-responsive element, as assessed by gel mobility shift assays. These results support the hypothesis that the MPS-1 protein may act, at least in part, by interacting with genes possessing the cyclic AMP-responsive element sequence.

Introduction

We have recently identified by cDNA cloning a gene, denoted Metallopanstatulin-1, that is activated in cultured human MDA-468 cells by fetal calf serum, transforming growth factor βs, and cAMP analogues (1). The MPS-1 gene was found to have a nucleotide sequence that predicts a M, 10,000 protein with homology to transcriptional regulatory DNA-binding proteins (1). MPS-1 protein contains one zinc finger domain of the C4 type, similar to those present in proteins involved in recognition of damage DNA, the adenovirus E1A gene family, and the steroid/thyroid hormone receptor superfamily (1). Thus, MPS-1 presumably belongs to the C4 class of DNA-binding proteins that function as transcriptional enhancers (reviewed in Refs. 2–6).

Further insights into the role of the MPS-1 gene in control of cell growth could be achieved by studying the structure and function of this protein. However, isolation of sufficient amounts of MPS-1 protein for detailed biochemical and structural studies is not feasible because of the low abundance of the protein in the mammalian tissues studied. To circumvent this problem, we decided to explore the applicability and capacity of the baculovirus vector expression system (7) for the expression of the MPS-1 protein.

The baculovirus vector system, AcNPV, is a potentially useful vector for expressing high levels of biologically active recombinant proteins (7). This virus propagates in cultured insect cells and has powerful, temporally regulated promoters, and the coding sequence of the gene of interest can be inserted by in vivo recombination (7). Proteins expressed in insect cells are usually correctly folded and contain appropriate posttranslational modifications (7).

In this study, we have introduced the cDNA corresponding to the open reading frame of the MPS-1 gene into an AcNPV expression vector and have propagated the recombinant virus in insect cells. We found that MPS-1 protein is expressed at high levels in this host-vector system (at least 100-fold higher than that observed in mammalian cells). The MPS-1 protein produced in this system binds zinc ions specifically, is phosphorylated, is tightly bound to the nucleus, and binds to single- and double-stranded calf thymus DNA-cellulose. In addition, we have generated antibodies against selected peptide sequences of the MPS-1 protein to detect MPS-1 in various preparations. Analysis of the highly purified baculovirus-produced MPS-1 protein confirms that it has authentic mammalian characteristics with respect to

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2 The abbreviations used are: cDNA, complementary DNA; MPS, metallopanstatulin; AcNPV, Autographa californica nuclear polyhedrosis virus; CRE, cyclic AMP-responsive element; bp, base pairs; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; BEVS, baculovirus expression vector system; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; UV, ultraviolet; XP, xeroderma pigmentosum; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMEM, Dulbecco's modified Eagle's medium; EBSS, Earle's balanced salt solution.
**DNA Translation**

**MPS-1 cDNA**

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**Fig. 1.** Nucleotide sequence of the 329-bp fragment of plasmid ST1H2-pcDNA-II containing the exon coding for the 84 amino acids of human MPS-1 and the 5' and 3' flanking regions (GenBank accession no. L19739). The deduced amino acid sequence is shown in one-letter code. The translational initiation site ATG starts at nucleotide position 21 and the TAA termination signal begins at nucleotide 273. The underlined amino acid residues in regions 2-17 and 67-77 correspond to two synthetic peptides designated A and C, respectively, that were used for antibody production. The numbers in each line refer to the nucleotide (upper) and amino acid (lower) positions. The methionine at position 21 constitutes the NH2-terminus.

**Fig. 2.** Cloning of the human MPS-1 (ST1H2) sequence into the baculovirus transfer vector pJVEI to construct the recombinant expression vector pJVEI-ST1H2/P17.

mobility and immunological properties. Finally, gel mobility shift assays showed that MPS-1 protein present in insect nuclear extracts interacts specifically with duplex DNA containing the CRE consensus sequence (8). Taken together, these results suggest that one of the functions of the MPS-1 protein in mammalian cells may be in the regulation of genes possessing the CRE consensus sequence.

**Results**

**Construction of AcNPV-MPS-1/P17.** Fig. 1 shows the nucleotide sequence of the 329-bp fragment of plasmid pcDNA-II/ST1H2, containing the coding region for the human MPS-1 gene and the 5' and 3' flanking regions (GenBank accession no. L19739). The 329-bp MPS-1 cDNA was inserted during library construction into the Bst XI cloning site of pcDNA-II (Ref. 1; Fig. 2).

The strategy used to construct the protein MPS-1 baculovirus expression vector is outlined in Fig. 2. A baculovirus transfer vector, designated pJVEI/MPS-1/P17, was constructed from pJVEI and a 0.4-kb restriction fragment containing the MPS-1 gene. The nucleotide sequences surrounding the site of recombination and the initiation codons of the wild-type polyhedrin gene and pJVEI/MPS-1 are shown in Table 1. From the analysis of these sequences, it may be inferred that the pJVEI/MPS-1/P17 vector codes for two distinct but related proteins: (a) a peptide containing the wild-type form of the MPS-1 protein; and (b) a fusion peptide which contains the MPS-1 sequence coupled to 17 additional amino acid residues at the NH2-terminus, due to an in-frame start codon (ATG) present in the pcDNA-II 5'-linker used to make the cDNA library (Table 1; Ref. 1). This fusion peptide was referred to as the MPS-1/P17 pep-
Table 1 Cloning of the MPS-1 (ST1H2) sequence into pIVETL to construct the recombinant expression vector pIVETL-MPS-1/P17 containing a fusion peptide

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<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>21</td>
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<tr>
<td></td>
<td>... GAT AAAAAAA 3'</td>
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<tr>
<td>pcDNA II linker</td>
<td>CTTTAGACGACACTGCCGCGCCTTTA 3'</td>
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To transfer the MPS-1 gene into the AcNPV baculovirus genome, the pIVETL/MPS-1/P17 was cotransfected with wild-type AcNPV DNA into SF9 cells, and the recombinant AcNPV-MPS-1/P17 was detected initially by visual discrimination of plaque properties (blue color and occlusion negative). Subsequently, the presence of AcNPV-MPS-1/P17 in selected clones was verified by PCR as described in “Materials and Methods.” Finally, screening of lysates prepared from SF9 cells infected with cloned AcNPV-MPS-1/P17 confirmed that RNA from infected cells contained sequences that hybridized to a human MPS-1 cDNA probe, whereas cells infected with wild-type AcNPV or AcNPV-Del did not (data not shown).

Gel Electrophoresis and Western Analysis. Western analysis was performed to determine if the AcNPV-MPS-1/P17 generated full-length, intact native human MPS-1 protein with electrophoretic mobility and immunological properties similar to those of naturally occurring mammalian MPS-1. As illustrated in Fig. 3A, Lanes 1 and 2, the affinity-purified anti-peptide A antibody recognized two proteins of M, 10,000 and 13,000 only in extracts from SF9 cells infected with AcNPV-MPS-1/P17 vector containing the MPS-1 cDNA sequence but not in extracts from cells infected with the AcNPV-Del vector without the MPS-1 insert. Immunoreactive proteins were not detected in lysates from wild-type virus infections (data not shown). The recognition of the M, 10,000 and 13,000 proteins in infected cells by the anti-peptide A antibody (Fig. 3C, Lane 1) was neutralized in a concentration-dependent manner by incubation of the antibody with increasing concentrations of the peptide A antigen used to raise the antibody (Fig. 3C, Lanes 2–4). These results clearly showed that the anti-peptide A antibody is highly specific for detection of MPS-1 protein species. The unique species of low molecular weight (Fig. 3A, Lane 2) had an apparent molecular mass which coincides with the value determined previously by computer calculations for the human MPS-1 protein (1). Moreover, this band comigrated with an immunoreactive species from the human mammary carcinoma cell line MDA-468 (Fig. 3B, Lanes 5 and 6), which was used to generate the library from which the MPS-1 gene was isolated (1). Likewise, the protein of M, 10,000 in insect cells comigrated with an immunoreactive species detected by the same anti-peptide A antibody in SK-MEL-28 melanoma, LAN-5 neuroblastoma, and tongue carcinoma SCC-15 cells (data not shown). In distinction, the immunoreactive protein of M, 13,000 found in insect cells was not detected in cell lysates from MDA-468 (Fig. 3B, Lane 5) or other mammalian cell lines studied here (data not shown). Similar results were obtained when the anti-peptide C antibody, raised against the COOH-terminus of the MPS-1 protein, was used in the immunoblot procedures (data not shown).

Studies on MPS-1 Protein Localization in Insect Cells. To evaluate the subcellular localization of the MPS-1 protein, insect cells were infected with AcNPV-MPS-1/P17, AcNPV wild-type, or AcNPV-Del, a virus in which the MPS-1 gene is deleted. After labeling with [35S]cysteine, total cell extracts, culture media, and cytoplasmic and nuclear extracts were separated and analyzed by SDS-PAGE and autoradiography. Analysis of whole cell extracts showed two labeled proteins of M, 10,000 and 13,000 corresponding to the natural form MPS-1 and the MPS-1/P17 fusion protein, respectively, in cells infected with the recombinant virus containing the MPS-1 sequence (Fig. 4, Lane 4). These proteins, which represent ∼37% of the total labeled cellular protein, were not detected in extracts from cells infected with AcNPV wild-type or AcNPV-Del (Fig. 4, Lanes 2 and 3, respectively). Both proteins have identical mobilities on SDS-PAGE as those detected by immunoblot analysis (Fig. 3A).

The results, shown in Fig. 4, reveal several important characteristics of both the natural form MPS-1 protein and the fused MPS-1 protein having 17 extra amino acids: (a) as indicated by the heavy double bands (Fig. 4, arrowheads) in Lane 17, most of the MPS-1 protein species are localized in the nucleus of the cell (∼32% of labeled nuclear proteins). Clearly, the natural form and the fused protein are either transported inside the nucleus, are bound to nuclear components, or both; (b) as indicated by the lighter but still substantial double bands (Fig. 4, Lane 8, arrowheads), a considerable portion of the natural and fused MPS-1 proteins are secreted by cells into the extracellular fluid (≈41% of labeled secreted proteins); and (c) the absence of heavy bands indicating either natural or fused MPS-1 in the cytosol (Fig. 4, Lane 13) suggests that any transport mechanisms that cause the proteins to be taken into the nucleus or secreted by the cell are relatively efficient. Thus, as predicted by computer analyses (1) and subsequently confirmed in the laboratory (Fig. 4, Lane 17), the MPS-1 is a protein capable of reaching the nucleus. It is also secreted by the cells into the extracellular fluid (Fig. 4, Lane 8). These results were further verified by Western analysis of nuclei.
Fig. 3. A, Western immunoblot analysis of BEVS-expressed MPS-1 proteins. Whole-cell extracts were prepared from cells infected with AcNPV-Del (Lane 1) or AcNPV-MPS-1/P17 (Lane 2). The extracts (70 μg protein/lane) were electrophoresed and processed by Western blot protocols as described under “Materials and Methods.” Both lanes were exposed to anti-peptide A antibodies (1:5000 dilution). Arrows 1 and 2 indicate MPS-1 proteins of ~ M, 13,000 and 10,000, respectively. Molecular weight standards used to determine the M, of MPS-1 proteins were ovalbumin (M, 43,000), carbonic anhydrase (M, 29,000), β-lactoglobulin (M, 18,400), lysozyme (M, 14,300), bovine trypsin inhibitor (M, 6,200), and insulin chains A and B (M, 2,300–3,000). B, Western immunoblot analysis of native MPS-1 protein expressed in human mammary MDA-468 cells. Lanes 3, 4, and 5 contain equal amounts of protein (70 μg/lane) from a whole-cell extract prepared from MDA-468 cells. Lane 3, no antibodies added; Lane 4, preimmune sera (1:2,500); Lane 5, anti-peptide A antibodies (1:2,500). Lane 6, whole-cell extracts (70 μg protein) from cells infected with AcNPV-MPS-1/P17 and exposed to anti-peptide A antibodies (1:2,500). Arrow, MPS-1 protein of ~ M, 10,000 in insect cells (Lane 6) which comigrates with MPS-1 protein of identical molecular weight in MDA-468 cells (Lane 5). C, Western immunoblot analysis of specific neutralization of anti-peptide A antibodies by peptide A. Antibody to peptide A (4.0 μg/tube) was incubated with peptide A at the indicated molar ratios of antibody:antigen for 30 min at room temperature. The mixtures were then added to the blots containing equal amounts of protein extracts from Sf9 cells infected with AcNPV-MPS-1/P17. The Western blots were subsequently processed as described under “Materials and Methods.” Arrows 1 and 2 indicate MPS-1 proteins of ~ M, 13,000 and 10,000, respectively.

Fig. 4. Subcellular localization of radiolabeled MPS-1 proteins in Sf9 insect cells infected with a viral vector containing the MPS-1 gene. Insect cells were infected with recombinant viruses prior to radioactive labeling with [14C]lysine. The labeled proteins were analyzed by electrophoresis in a 15% polyacrylamide gel under reducing conditions; 5 × 10^5 cpm (A) or 1 × 10^5 cpm (B) were added per lane. Proteins were visualized by autoradiography (96-h exposure). For details, see “Materials and Methods.” A, total cellular protein (Lanes 1–4) and secreted protein (Lanes 5–8). Analysis of total cell extracts show that two protein bands of M, 10,000 and 13,000 were only found in cells infected with AcNPV-MPS-1/P17 (Lane 4). As indicated by the two MPS-1 bands in Lane 8, a substantial quantity of MPS-1 proteins of M, 13,000 (upper band) and M, 10,000 (lower band) are secreted by the cells. B, protein isolated from the cytosol (Lanes 10–13) and from the nucleus (Lanes 14–17). As indicated by the absence of MPS-1 bands in Lane 13 (cytosol) and the heavy MPS-1 bands in Lane 17 (nucleus), the major portion of MPS-1 proteins inside the cell are associated with the nucleus. Lanes 1, 5, 10, and 14 show control uninfected Sf9 cells. Lanes 2, 6, 11, and 15 show control cells infected with AcNPV wild-type (WT). Lanes 3, 7, 12, and 16 show control cells infected with AcNPV-Del. Lanes 4, 8, 13, and 17 show cells infected with AcNPV-MPS-1/P17. The arrowheads to the left of Lanes 4, 8, and 17 indicate the position of two proteins that were exclusively found in the cells infected with AcNPV-MPS-1/P17. The arrow on the right of Lanes 9 and 18 indicate the position of a 12,5-kilodalton size marker in parallel lanes. 14C-labeled molecular mass markers were loaded in lanes 9 and 18; the markers were ovalbumin (M, 48,000), lactoglobulin A (M, 18,400), cytochrome c (M, 12,500), and insulin (M, 5,800).
Detection of MPS-1 Protein Species in Nuclei of Sf9 Cells by Immunofluorescence. The nuclear localization of the MPS-1 protein species was confirmed by indirect immunofluorescent staining of insect cells infected with AcNPV-MPS-1/P17. As shown in Fig. 5A, intense immunofluorescent staining is observed exclusively over the large nuclei of cells infected with AcNPV-MPS-1/P17. When cells infected with AcNPV-Del were incubated with the anti-MPS-1 antibodies, no specific binding was observed (Fig. 5B). In addition, when cells infected with AcNPV-MPS-1/P17 were incubated with control nonspecific IgG antibodies, only background binding was observed (data not shown). The immunofluorescence results were further verified by Western analysis of nuclei from Sf9 cells infected with AcNPV-MPS-1/P17 (Fig. 5C, Lane 1). We conclude that the MPS-1 protein species reside in the nucleus of Sf9 cells.

Phosphorylation of MPS-1 Proteins in AcNPV-MPS-1/P17-infected Insect Cells. Our previous results showed that the amino acid sequence of MPS-1 contains a number of potential phosphorylation sites (1). Therefore, we investigated whether MPS-1 was phosphorylated in infected insect cells by incubating AcNPV-MPS-1/P17-infected cells with $^{32}$P in a phosphate-free medium for 6 h at 48 h after infection. Total cell extracts from the infected cells were then analyzed by SDS-PAGE. As shown in Fig. 6A, phosphoprotein bands of $\approx M_r$ 10,000 and 13,000 were detected in extracts from cells infected with AcNPV-MPS-1/P17 but not in uninfected cells. Moreover, these bands were not observed in extracts from cells infected with AcNPV-Del (data not shown). These observations suggest that the MPS-1 proteins produced in insect cells infected with AcNPV-MPS-1/P17 are phosphorylated.

To confirm that the MPS-1 protein species are phosphoproteins, the protein extracts were incubated with calf intestinal alkaline phosphatase in the presence or absence of the phosphatase inhibitors sodium pyrophosphate, sodium fluoride, and sodium vanadate (9). The results shown in Fig. 6B, Lane 2, show that the two bands of MPS-1 proteins disappeared when the protein extract was treated with alkaline phosphatase. The inclusion of phosphatase inhibitors during the incubation with phosphatase prevented the disappearance of the bands (Fig. 6B, Lane 3), indicating that the decrease in phosphate-containing MPS-1 proteins (Fig. 6B, Lane 2) was specific. These results demonstrate that the MPS-1 protein species are specifically phosphorylated in insect cells.

Partial Purification of Baculovirus-expressed MPS-1 Protein from Nuclear Extracts. To determine the optimal conditions for isolation of the MPS-1 protein from nuclei of AcNPV-MPS-1-infected cells, the time course of expression of recombinant protein was monitored in nuclear extracts by immunoblotting with anti-peptide A antibodies. The re-
Characterization of Recombinant Metallopanstimulin

Results showed that these antibodies detected maximal expression of nuclear MPS-1 protein species at 48- to 72-h postinfection (data not shown).

A variety of extraction conditions (salt concentration, nonionic detergents, and pH) led us to the conclusion that the MPS-1 protein species are tightly bound to nuclear components (data not shown). We found that most of the MPS-1 nuclear protein species could be efficiently recovered in a soluble form by extracting with 50% (v/v) acetonitrile in a 0.3 M ammonium carbonate buffer at pH 9. The BEVS yielded 4.5 mg of acetonitrile extracted MPS-1 nuclear protein from 1.4 × 10^10 baculovirus-infected cells.

The MPS-1 protein was further purified from the acetonitrile nuclear extracts by reverse phase HPLC (Fig. 7). The lyophilized fractions (void volume) containing MPS-1 protein species from the gel filtration step were reconstituted in 0.1% TFA in water and chromatographed on a μBondapak C18 column using a linear gradient of acetonitrile containing 0.1% TFA (Fig. 7A). Radioactive assay of ^15S[MP]-protein species demonstrated that the major peak of labeled material corresponded to a small protein peak eluting in fraction 14 (Fig. 7A). Analysis of the C18 column fractions by Western blot with anti-peptide A antibodies showed two bands of Mr. 10,000 and 13,000 under reducing conditions in fractions 14 to 18 (Fig. 7B). The largest amount of immunoreactive MPS-1 protein of Mr. 10,000 eluted in fraction 14, with decreasing concentrations of this protein in fractions 15 and 16 (Fig. 7B). Most of the immunoreactive protein of Mr. 13,000 eluted in fraction 17 (Fig. 7B). Silver stain demonstrated the presence of one major protein band of Mr. 10,000 in fraction 14 (Fig. 7A, Inset, Lane 2) which comigrated with the MPS-1 band detected by Western analysis in the same fraction (Fig. 7B). The protein recovered from fraction 14 was about 95% pure as judged by silver staining after SDS-PAGE (Fig. 7A, Inset, Lane 2).

**DNA Binding Activity of the MPS-1 Protein.** To determine whether the solubilized MPS-1 protein binds to DNA, it was chromatographed on a DNA-cellulose column containing double- and single-stranded calf thymus DNA. The acetonitrile-extracted MPS-1 protein was desalted and subsequently loaded onto DNA cellulose. The column was washed, and the bound proteins were eluted with 1 M NaCl. The fractions were concentrated by trichloroacetic acid precipitation, washed with acetone, and then analyzed by immunoblotting using the anti-peptide A antibodies. The Mr. 10,000 MPS-1 protein was detected in the fraction eluted with 1 M NaCl (data not shown). We conclude that the solubilized MPS-1 protein is capable of binding to single- or double-stranded DNA.

**Ability of Baculovirus-expressed MPS-1 Protein Species to Bind a Specific DNA Sequence.** Sequence-specific DNA binding of baculovirus-expressed MPS-1 protein species to the minimal CRE was examined in gel mobility shift assays. Nuclear extracts from Sf9 cells infected with either AcNPV-MPS-1/P17 or AcNPV-Del were prepared by the method of Dignam et al. (10). A double-stranded oligonucleotide consisting of two complementary synthetic oligonucleotide strands, 5′-GATTGGCTGACGTCAGAGAGCT-3′ and 3′-CTAACGGACTGCAGTCCTCAG-5′, containing the CRE consensus sequence was tested. When nuclear extracts of baculovirus-expressed MPS-1 protein species were incubated with duplex oligonucleotides containing the CRE and analyzed by nondenaturing PAGE, one major protein:DNA complex was observed (Fig. 8A, Lane 1). Addition of cold competing CRE oligonucleotide (20-fold molar excess) eliminated the protein:DNA complex formation (Fig. 8A, Lane 2). Further evidence suggesting that the complex is the result of MPS-1 proteins:CRE interactions came from mobility shift assays in the presence of antibodies against MPS-1 proteins. When nuclear extracts of MPS-1 proteins were incubated with anti-peptide A antibodies (final concentration, 0.5 μg), a significant reduction (≈60%) in the oligonucleotide binding activity of the MPS-1 proteins present in the nuclear extracts was observed (Fig. 8A, Lane 3). A similar nuclear extract prepared from Sf9 cells infected with AcNPV-Del yielded no protein:DNA complex that comigrated with the complex obtained in AcNPV-MPS-1/P17-infected cells (Fig. 8A, Lane 4), demonstrating that the protein:DNA complex observed was dependent on the AcNPV-MPS-1/P17 expressing virus. Acetonitrile-extracted or HPLC-purified MPS-1 nuclear proteins species generated a specific protein:DNA complex of identical mobility and intensity as that shown in Fig. 8A, Lane 1 (data not shown).

DNA:protein complexes were not observed when consensus sequences of DNA binding sites for SP-1, NF1/CTF, NFκB, CRE, OCT-1, and AP1 were used (Fig. 8B, Lanes 1-12). To establish the specificity of MPS-1 binding to CRE, competition assays with target sequences other than CRE were performed. While addition of cold competing CRE oligonucleotide (20-fold molar excess) eliminated the MPS-1:CRE complex formation (Fig. 8C, Lane 2), addition of a series of cold competing oligonucleotides (50-fold molar excess) did not eliminate the MPS-1:CRE complex formation (Fig. 8C, Lanes 3-9), suggesting that the MPS-1:CRE complex has substantial specificity. In summary, MPS-1 binds to CRE but not to an AP1 site. Considering the canonical CRE (5′-TGACGTCA-3′) and AP1 (5′-TGGAGTCA-3′) sequences.
3') sites vary by only one nucleotide, the results presented here strongly indicate specificity of MPS-1 for CRE.

Zinc Binding Studies with the MPS-1 Protein. To demonstrate that the MPS-1 protein binds zinc, gel-purified MPS-1 apoprotein was reconstituted with radioactive $^{65}$Zn in the absence and presence of 2 mM ZnCl$_2$. Table 2 shows that recombinant MPS-1 protein binds $^{65}$Zn, as demonstrated by the 33 to 40% increase in $^{65}$Zn cpm in the

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**Fig. 7.** Partial purification of human baculovirus-generated MPS proteins by HPLC. A, chromatography. Acetonitrile nuclear extracts (1.6 mg protein) from the previous purification step (gel filtration) were reconstituted in 2 ml of 0.1% TFA. Gel-purified [35S]MPS-1 protein species ($1 \times 10^7$ cpm) were added to the reconstituted sample as a marker to determine the position of elution of these proteins. Then, the sample was chromatographed on a µBondapak reverse-phase C$_{18}$ column. The sample was eluted with a linear gradient of 5 to 50% acetonitrile containing 0.1% TFA at a flow rate of 0.8 ml/min; approximately 3-ml fractions were collected. Aliquots of 100 μl were removed from each fraction for radioactive measurements. Then, the fractions were lyophilized and reconstituted; aliquots were subjected to SDS-PAGE. Inset, SDS-PAGE of fraction 14 on a 15% acrylamide gel under reducing conditions. After electrophoresis, the gel was fixed and stained with silver. Lane 1, molecular weight standards ovalbumin (M, 43,000), carbonic anhydrase (M, 29,000), β-lactoglobulin (M, 18,400), lysozyme (M, 14,300), bovine trypsin inhibitor (M, 20,000), and insulin chains A and B (M, 2,300-3,400). Lane 2, 1 μg of protein was analyzed; arrow, the position of MPS-1 protein of M, 10,000. B, Western blot analysis of eluted fractions. Reconstituted material (25 ul) from each fraction were analyzed by Western blot analysis. This analysis was done using anti-peptide A antibody as described under "Materials and Methods." Arrow, the position of MPS-1 protein of M, 13,000. The numbers correspond to the column fraction numbers shown in (A).
Table 2  Binding of zinc to MPS-1 (65Zn, cpm)

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Percent Increase

33.3  39.9  33.7

presence of MPS-1 protein. As shown in Table 2, an excess of nonradioactive Zn2+ abolished the binding of 65Zn to MPS-1, demonstrating specific binding of zinc to MPS-1 protein. Calculations based on the specific activity of 65Zn, the molecular mass and concentration of MPS-1 (Table 2) indicates that MPS-1 binds one zinc atom per molecule of MPS-1. These results are consistent with the hypothesis that Zn2+ coordinates to the four cysteine residues present in the zinc finger sequence (1).

**Subcellular Localization of MPS-1 Protein in Cultured Mammalian Cells.** For some proteins expressed in S9 cells, the mere overexpression can theoretically force a fraction of the protein into the nucleus. To determine whether the MPS-1 protein resides in the nucleus of cells that spontaneously express the MPS-1 protein, we performed the following induction experiments.

First, we examined the immunocytochemical localization of MPS-1 protein in SK-MEL-28 cells using antibodies that recognize MPS-1 (Fig. 9). Most uninduced cells were negative (Fig. 9, A and E), although a few cells did show some weak cytoplasmic staining (Fig. 9C), probably because not all cells were completely quiescent under low-serum conditions. In striking contrast, treatment of cells with dibutyryl cAMP resulted in a time-dependent appearance and redistribution of the MPS-1 protein with a specific increase in nuclear MPS-1 (Fig. 9, D and F). After a 6-h dibutyryl cAMP treatment, a specific increase in the fluorescence of the cytoplasm adjacent to the nucleus, in portions of the nuclear envelope, and over the nucleus were observed (Fig. 9D). After 24 h, the nucleus was strongly stained (Fig. 9F). The staining pattern covered the surface of the nuclei in a punctuated manner, and the intensity was somewhat heterogeneous among the nuclei (Fig. 9F). These results indicate that after activation with dibutyryl cAMP, an agent which has been previously shown to induce mRNA for MPS-1 (1), the MPS-1 protein is preferentially associated with the nucleus. These results are in agreement with earlier

sample shown in Lane 1 with excess unlabeled CRE DNA (20-fold molar excess). Lanes 3 to 9 repeat the condition in Lane 1 with the addition of excess unlabeled API (Lane 3), AP2 (Lane 4), NFkB (Lane 5), GRE (Lane 6), OCT-1 (Lane 7), and NF1/CTF (Lane 8) and SPI (Lane 9; 50-fold molar excess each). Cells were infected for 72 h.
results on nuclear localization of the MPS-1 protein obtained with MDA-468 cells stimulated with serum (1).

Second, we have examined the effect of UVC radiation (254 nm) on MPS-1 gene expression in culture XP skin cells for the following reasons: (a) cells from patients with this condition are hypersensitive to UV radiation because of a defect in DNA repair systems (11); (b) UV radiation has been shown to induce complex responses in human skin
cells, including increased expression of numerous repair genes and oncogenes (12); and (c) the homology of MPS-1 to various proteins involved in DNA repair systems that respond to UV light (1) suggest that the MPS-1 protein may be involved in DNA repair. We found that the MPS-1 protein is induced by brief exposure to UVC light in XP cells (Fig. 10). Sixteen h after exposure to UVC light, a prominent increase in fluorescence in the cytoplasm contiguous to the nucleus, in portions of the nuclear envelope, and over the nucleus were observed (Fig. 10B). In contrast, in control cells, diffuse cytoplasmic staining with some weak nuclear staining was noted (Fig. 10A). It is worth noting that greater than 95% of the cells were viable after 16 h of exposure to UVC light, as determined by trypan blue dye exclusion test in three separate experiments (control, >99% viability, n = 3; UVC, >95% viability, n = 3), indicating that the effect of UVC light on MPS-1 is not the result of cell death after such exposure. These results indicate that MPS-1 protein is preferentially associated with the nucleus after exposure to UVC light.

In summary, the results with SK-MEL and XP cells showed that after chemical or physical stimulation, the MPS-1 protein is particularly associated with the nucleus in two widely different cell types which naturally express the MPS-1 protein (Figs. 9G and 10C). Thus, these results demonstrate that nuclear accumulation of MPS-1 protein is not restricted to the condition observed with infected Sf9 cells which overproduce MPS-1 protein.

Discussion

The present studies are consistent with previous computer-generated predictions on the MPS-1 gene product concerning molecular weight, zinc binding, nuclear localization, and DNA binding activity in MDA-468 mammalian cells (1). In this study, we have shown that the human MPS-1 zinc finger protein can be produced in relatively large amounts in cultured insect Sf9 cells using the BEVs. We found that the yields of Sf9 cell-generated MPS-1 protein were at least 100-fold higher than those observed in cultured mammalian cells. Furthermore, we have found conditions for the efficient recovery of soluble protein from nuclear extracts that encompass extraction with acetonitrile in high salt, as has been done for other DNA-binding proteins (13). Most likely, the MPS-1 protein isolated in this fashion is in its native form, since the acetonitrile has been used in many occasions for the purification of native proteins (13–15). Moreover, Western analysis indicated that MPS-1 protein was expressed in the BEVs with the appropriate molecular mass of 10 kilodaltons, coinciding with the mobility of native MPS-1 protein obtained from a variety of mammalian sources. Thus, the results suggest that recombinant MPS-1 produced in the BEVs has the functional and structural characteristics of authentic mammalian MPS-1.

We showed that when MPS-1 is overexpressed by Sf9 insect cells, a significant fraction of this protein (approaching 30% of total extracellular labeled proteins) was detected and 3 contain equal amounts of protein (70 μg/lane) from a whole-cell extract prepared from XP cells. Lane 1, no antibodies added; Lane 2, pre-immune sera (1:2,500); Lane 3, anti-peptide A antibodies (1:2,500). The arrow indicates the MPS-1 protein of M, 10,000. Molecular weight standards used to determine the molecular weight of MPS-1 were as described in Fig. 3.
in culture fluids (Fig. 4, Lane B). Whether this reflects an
authentic ability of MPS-1 to be secreted by the insect cells
or some inherent experimental conditions in the insect cell
system is not known. In any case, the fact that significant
amounts of MPS-1 protein were released from Sf9 cells in
serum-free media made it possible, in a two-step ultrafiltration-
concentration purification scheme, to enrich the pro-
tein by at least 100-fold.

The MPS-1 gene expressed in the baculovirus construct
appears as a doublet of M, 10,000–13,000 on SDS-PAGE.
This is a somewhat expected finding since the construct
possesses two in-frame initiation sites (Table 1), suggesting
that two size classes should be observed in this construct:
(a) the fusion protein containing 17 additional amino acids,
MPS-1/17, of M, = 13,000; and (b) the native authentic
MPS-1 protein of M, = 10,000. In support of this contention
are the findings with anti-peptide antibodies which showed
that antibodies directed against the NH2- and the COOH-
terminus of MPS-1 both react with the low molecular
weight protein which comigrates with authentic mamma-
lian MPS-1. These findings indicate that the native form of
M, 10,000 does not contain any appreciable NH2-terminal
or COOH-terminal modification; thus, it is not a degrada-
tion product of the larger fusion form. It would be highly
unlikely that the MPS-1/17 fusion protein is cleaved in
such a manner that precisely results in a protein that loses
the 17 additional amino acids. Furthermore, the results
indicate that the size heterogeneity of the two products is
too large to be due to phosphorylation.

The significance of the phosphorylation of the MPS-1 pro-
tein observed in insect cells infected with the recombinant
MPS-1 virus is not known. It is worth noting that various
eukaryotic regulatory proteins are phosphorylated in the BEVS
as well as in their natural condition (6, 7), suggesting that
MPS-1 may be a phosphoprotein in its native state in vivo. In
agreement with this contention are our unpublished results
which indicate that the MPS-1 protein is also phosphorylated
in human MDA-468 cells in vivo. Phosphorylation has been
demonstrated to modulate the function of numerous DNA
binding proteins (16, 17). For example, phosphorylation of the
nuclear protein CRE-B results in changes in the affinity of
this protein for CRE on DNA (16). Thus, our findings, taken
in conjunction with previous results, raise the possibility of
the significance of phosphorylation with regard to the regulation
of MPS-1 activity.

Although the mechanism of action of MPS-1 is not
known, the presence of a zinc-binding finger domain, the
nuclear localization under certain experimental conditions,
and the close relationships to nuclear zinc finger DNA
binding proteins suggest that this mechanism may involve
binding to DNA (2–6). Comparable suggestions, subse-
quently supported by experimental findings, have been
made for other zinc finger proteins such as those encoded
by several growth-related genes to which MPS-1 shows a
considerable degree of homology (1, 18). Analysis of the
MPS-1 protein produced with the BEVS and additional
results with mammalian cells are consistent with this conten-
tion: (a) MPS-1 is localized preferentially in the nuclear
fraction of insect cells infected with the MPS-1-AcNPV virus
(Fig. 4, Lane 17); (b) indirect immunofluorescence staining
observations using antibodies against selected peptide se-
quencies of the MPS-1 protein demonstrated that this protein
accumulates in the nucleus of MPS-1-AcNPV-infected cells
(Fig. 5A); (c) nuclear accumulation of MPS-1 was not re-
stricted to MPS-1-AcNPV-infected insect cells which may
have an anomalous nuclear distribution of MPS-1 protein
resulting from overproduction of this protein. Additional
immunocytochemical studies with mammalian cells clearly
demonstrated that the MPS-1 protein is located in the cy-
toplasm contiguous to the nucleus, in the nuclear envelope,
and in the nucleus of various cell types as a function of time
after stimulation with various agents such as serum (1),
dibutyryl cAMP (Fig. 9, B, D, and F), and UVC light (Fig.
10B). It would appear, therefore, that the nuclear expres-
sion of MPS-1 protein may play a role in the response of
mammalian cells to a variety of environmental factors, in-
cluding agents that damage DNA such as UVC light. In this
consideration, our findings are consistent with other studies
that showed that UV irradiation of cells induces an increase
of proteins that bind DNA, such as various nuclear onco-
proteins and DNA repair proteins (12); (d) we showed that
the recombinant MPS-1 protein binds to single- and double-
stranded calf thymus DNA-cellulose; and (e) we found that
the baculovirus-expressed nuclear MPS-1 protein was ca-
pable of high affinity, sequence-specific interaction with
a DNA oligomer containing a consensus CRE sequence
(8, 16, 19–22) as assessed by gel mobility shift assays
(Fig. 8). The nuclear localization and DNA-binding prop-
erties of the MPS-1 product support the contention that this
gen product might be involved in the regulation of trans-
scription of genes possessing the CRE sequence. However,
other mechanisms of action are also possible and may
include interaction with signal transduction pathways in the
cytoplasm and nuclear envelope, as has been suggested for
several other zinc-finger proteins to which MPS-1 is related
by cellular location and sequence homology (1, 23, 24).

In conclusion, the BEVS is an excellent expression system
capable of providing large amounts of recombinant MPS-1
for detailed mechanistic studies. The availability of suffi-
cient quantities of highly purified recombinant MPS-1 pro-
tein should provide the opportunity to characterize further
the molecular properties and mechanism(s) of action of
MPS-1. Several of those studies are currently under way in
this laboratory.

Materials and Methods

Materials. 65Zn as ZnCl2 (2.56 mCi/mg) was purchased from
DuPont NEN (Boston, MA). PO4,H2102Pi inorganic
phosphate (285 Ci/mg; 709 mCi/ml) was purchased from ICN
Radiochemicals (Irvine, CA). 35S]-cysteine (11 mCi/
ml; 1,200 Ci/mmol) was purchased from DuPont NEN. The
restriction endonucleases were purchased from New
England Biolabs (Beverly, MA). Calf intestinal alkaline
phosphatase was obtained from Promega (Madison, WI). T4
DNA ligase was from Invitrogen (San Diego, CA). DNA
primers were synthesized using an Applied Biosystems (Fos-
ter City, CA) ABI 391 PCR Mate apparatus; purity of pre-
parations was determined by electrophoresis. Peptides for
antibody production were synthesized on an Applied Bio-
systems peptide synthesizer (Model 430A) using terbu-
tyloxy carbonyl chemistry (25); purity of the preparations
was evaluated by HPLC. Sources of other materials were as
described elsewhere (1, 26, 27).

Cells and Cell Culture. The insect ovarian cell line Spod-
optera frugiperda (Sf9) was obtained from Invitrogen and
was propagated in monolayer or suspension cultures in
complete Grace medium (GIBCO Laboratories, Gaithers-
burg, MD) at 27°C as described (28). For MPS-1 protein
production, the suspension cultures were maintained in EX-CELL 400 media (JRH Biosciences, Lenexa, KS).

The human breast carcinoma MDA-468 cells and other human mammalian cell lines spontaneously expressing the MPS-1 gene (1) were used for comparative studies with the MPS-1 protein produced by the baculovirus-infected SF9 cells. All mammalian cell lines were obtained from the American Type Culture Collection (Rockville, MD) and were cultured previously as described (26). The complementation group of the XP17BE cell line (American Type Culture Collection No. CRL 1360) is not known.

**BEVS.** The MaxBac BEVS was used to produce the MPS-1 protein in accordance with the procedures described in the MaxBac BEVS manual, version 1.3 (Invitrogen). This system consisted of the baculovirus polyhedrin expression vector, pBlueBac pVETL, and the wild-type AcNPV. Extracellular virions and viral genomic DNA were prepared as described previously (28). AcNPV stocks were grown, and titers were determined in SF9 cells using the standard procedures of Summers and Smith (28).

**Construction of pVETL-MPS-i/P17.** All DNA manipulations and transfers were accomplished using established methods (29). The plasmid pcDNA-II/St1H2 contains a 329-bp fragment of the human MPS-1 cDNA which was inserted during library construction into the BstXI cloning site of pcDNA-II (Fig. 1; Ref. 1).

The steps used to transfer a 0.4-kb segment of human-derived cDNA out of the pcDNA-II/St1H2 plasmid and into the pBlueBac/pVETL vector are shown in Fig. 2. In one series of reactions, the *Escherichia coli* cells were cultured and lysed, and the pcDNA-II/St1H2 plasmid was purified by CsCl centrifugation. The purified plasmid was digested with *SpeI* and *XbaI*, which cleaved the plasmid at cleavage sites supplied by the polylinkers of the pcDNA-II cloning vector (Fig. 2; Table 1; Ref. 1).

The DNA fragments that resulted from the *SpeI* and *XbaI* digestion were size-separated using gel electrophoresis, and a double-stranded 0.4-kb fragment was eluted from the gel. The 0.4-kb fragment contained the complete MPS-1 coding region as well as 20 base pairs of 5′ untranslated sequence and 57 base pairs of 3′ untranslated sequence (Fig. 1; Table 1; Ref. 1).

In a separate set of reactions, the baculovirus polyhedrin expression vector pVETL was digested with *NheI*, which generates overhangs that are compatible with both *XbaI* and *SpeI* overhangs (Fig. 2). The resulting linearized plasmid was mixed with the 0.4-kb fragment from the pcDNA-II/St1H2 plasmid, and the DNA fragments were ligated using T4 DNA ligase (Fig. 2). This generated a variety of recombinant plasmids that were transfected into and grown in clonal colonies of *E. coli* INV a F′ cells (Invitrogen). Forty sets of clonal cells were lysed, and their plasmid DNA was purified and analyzed so that a clonal line having a vector with the desired structure could be selected.

The proper orientation of the ST1H2 insert in the pVETL vector was confirmed by PCR using combinations of both one polyhedrin primer (7, 28) and two ST1H2 primers (1). The sequence of the polyhedrin primer was 5′-GAT ATC ATG AAT ATT AAA ATG AT-3′. The sequences of one of the ST1H2 primers was 5′-AAG GAA CAT CCT TCT GTA AGC-3′, whereas the sequence of the other ST1H2 primer was 5′-ATG CCT CTC GCA AAG GAT CTC-3′.

The ST1H2 primer sequences were selected based on a computerized analysis, using the "OligoPrimer" software (described by W. Rychlik in 1990 and sold by National Biosciences, Hamel, MN). That computerized analysis indicated that optimal hybridization of these primers to the target sequence will occur. PCR analysis was done using a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT) as described (1). Proper orientation of the insert in clones selected for further work was also confirmed by restriction enzyme mapping (1). Nine of forty clones had the ST1H2 sequence in the proper orientation. After these analyses, one plasmid, denoted pVETL-MPS-i/P17, was selected for further use. pTLV-MPS-i/P17 was propagated in the *E. coli* INV a F′ strain and purified as described (29).

The reference to "P17" in the plasmid name indicates that this plasmid may generate two different forms of MPS-1: (a) the endogenous form; and (b) a larger variant that contains an additional 17 amino acids at the NH2-terminus, due to a start codon spuriously located in one of the oligonucleotide linkers used for the library construction (1), which is in the same reading frame as the natural MPS-1 start codon (Table 1).

**Generation and Purification of AcNPV-MPS-1.** To transfer the MPS-1 gene into the baculovirus genome, the pVETL-MPS-i/P17 plasmid was cotransfected with wild-type AcNPV viral DNA into SF9 cells. One ml of SF9 cells (2 × 10⁶ cells/ml) were cotransfected with 1 µg of wild-type AcNPV baculovirus genomic DNA and 2 µg of pVETL-MPS-i/P17 plasmid DNA by the calcium phosphate transfection method (28). Two days after transfection, extracellular recombinant virions were collected and allowed to form plaques on SF9 cells as described previously (28). Polyhedrin-negative blue plaques were picked and used to infect SF9 cells in 24-well culture plates. Cells were lysed and screened for MPS-1 sequences by PCR using appropriate primers. Recombinant virus was further purified by performing two more rounds of plaque purification. Stocks of recombinant virus were grown on SF9 cells to a titer of 10⁷ to 10⁸ plaque-forming units/ml for use in all subsequent experiments.

**Metabolic Labeling and Cell Fractionation.** SF9 cells were seeded at a density of 6 × 10⁶ cells per 16-mm well in complete Grace medium and infected at a multiplicity of ~10 viral particles per cell with either wild-type AcNPV, AcNPV-Del (a virus in which the MPS-1 gene is deleted), or AcNPV-MPS-1 as described (28). At 48 h postinfection, the cells were labeled for 5 h with [15N]L-cysteine (50 µCi/ml) in cysteine-free EX-CELL 400 medium. After labeling, cell-free culture supernatants were analyzed to determine whether labeled proteins were being secreted by the cells into the extracellular fluid (28, 30). Furthermore, the cells were lysed and processed to isolate either cytoplasmic or nuclear extracts using previously described methods (30). The isolated extracts were processed by SDS-PAGE to separate the protein mixtures (31). The gels were autoradiographed as described (27).

**Preparation of Anti-MPS-1 Antibodies Using Synthetic Peptides with Partial MPS-1 Sequences and Affinity Purification of Anti-MPS-1 Antibodies.** Peptide antisera against the amino terminus or carboxy terminus of the MPS-1 protein have been prepared as follows. Two different amino acid sequences were used (Fig. 1). One sequence, PLAK-DLLHSPEEEK, corresponding to MPS-1 amino acid residues 2–17, was designated as the A peptide. It was derived from the NH2-terminal region of the protein located between the NH2-terminus and the zinc finger domain, as shown in Fig. 1. The second synthetic peptide had the sequence TGGKARLTEG, which corresponds to MPS-1 amino acid sequence 67–77, located between the zinc
finger and the COOH-terminus as indicated in Fig. 1. It was designated as peptide C. The A (NH₂-terminal) and C (COOH-terminal) peptides were selected so that they would not contain any portion of the zinc finger domain of the MPS-1 protein (Fig. 1), because the zinc finger domain is a highly conserved structure (2–5) and antisera against it might cross-react with other zinc finger proteins. The A or C peptides, to which a cysteine moiety was added at the NH₂-terminus, were cross-linked to a protein which stimulates the immune system, keyhole limpet hemocyanin, by the use of sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Pierce, Rockford, IL).

To produce anti-peptide antibodies, New Zealand rabbits were immunized by perilymph node injection with 300 μg each of the A or C peptide-keyhole limpet hemocyanin complex and Freund’s complete adjuvant. The rabbits were given a booster injection (150 μg) after 3 weeks and bled at 4 weeks. The sera obtained from the injected rabbits were screened for anti-peptide antibodies by enzyme-linked immunosorbent assay on peptide A- or ovalbumin covalently bound to peptide C-coated polyvinylchloride 96-well plates (32). Sera from the rabbits were positive for anti-peptide antibodies. Subsequently, booster injections were given as needed. The sera was obtained by centrifugation, and the IgG antibodies were purified by affinity chromatography on Affi-Gel-Protein A agarose essentially following the recommendations of the manufacturer (Bio-Rad, Richmond, CA). Specificity of the IgG antibodies was determined by peptide-anti-peptide antibody neutralization studies using Western blot analysis of MPS-1 proteins.

**Western Analysis of Insect and Mammalian Cell Lysates.** Sf9 cells were plated at 9 × 10⁶ cells/100-mm culture dish and were infected for 72 h with 9 × 10⁶ plaque-forming units of either wild-type AcNPV, AcNPV-Del, or AcNPV-MPS-1 as described (28). Cell monolayers were rinsed with 10 ml of ice-cold PBS and then lysed by the addition of 2 ml of 0.625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 1 mM PMSF. Aliquots were removed for protein determinations, then dithiothreitol was added to 0.1 M, and the samples were heated to 100°C for 3 min. Cellular protein (70 μg) was separated on a discontinuous 15% polyacrylamide gel, and the proteins were transferred to a 0.45 μm cellulose membrane (Schleicher & Schuell, Keene, NH) in a Bio-Rad electrobolot apparatus as described previously (33).

After transfer, membranes were blocked with 5% nonfat dry milk at 4°C for 16 h. After rinsing in TNC buffer (50 mM Tris, pH 7.5, 200 mM NaCl, and 0.05% Tween 20; 5 ml/strip), primary antibody was added at a final concentration of 1.6 μg/ml or 3.2 μg/ml in 2.5 ml of TNC buffer for 1 h at 24°C. The strips were rinsed with 2 × 5 ml of TNC buffer for 15 min each. For detection of the primary antibody-antigen reaction, the StrAviGen Super Sensitive System (BioGenex, San Ramon, CA) with biotinylated goat anti-rabbit antibody and streptavidin-alkaline phosphatase was used. After rinsing with TNC buffer, enzymatic activity of alkaline phosphatase was detected by using 5-Bromo-4-Chloro-3-indolyolphosphate p-toluene and nitroblue tetrazolium chloride as chromogenic substrates (GIBCO-BRL, Gaithersburg, MD).

To compare migration properties of BEVS-expressed MPS-1 and mammalian-expressed human MPS-1, extracts were obtained from MDA-468 cells and various other cultured cell lines, and the extracts were processed to detect the MPS-1 proteins by Western analysis as described above.

**Immunocytochemistry.** Sf9 cells were infected with AcNPV-Del or AcNPV-MPS-1 and grown in spinner cultures at 2 × 10⁶ cells/ml in complete Grace medium as described above. At 72 h postinfection, the cells were centrifuged, rinsed with PBS, and fixed in 50% ethanol-2% polyethylene glycol 6000 (10 min at 24°C). After fixation, the cells were centrifuged onto microscope slides in a Cytospin-3 centrifuge (Shandon, Pittsburg, PA). Then, the cells were treated with 95% ethanol (15 min at 24°C) and permeabilized with acetone (5 min at −20°C). Subsequently, the cells were rinsed three times in PBS and treated with 2% normal goat serum for 20 min to block nonspecific cellular binding sites (31). Then, the cells were incubated with antiserum (1:250 dilution of either preimmune sera, control IgGs, or affinity-purified anti-peptide antibodies) for 1 h at 24°C. After rinsing three times in PBS, the cells were incubated with rhodamine-labeled goat anti-rabbit IgGs (Accurate, Westbury, NY; final concentration, 10 μg/ml) for 30 min at 24°C. After rinsing three times in PBS, the cells were embedded in standard mounting media, covered with slips, and photographed with a fluorescence microscope (Nikon Co.).

**Isolation of MPS-1 Protein from Sf9 Culture Fluids.** Sf9 cells (2 × 10⁶ cells/ml) were infected with AcNPV-MPS-1 and grown in serum-free EX-CELL-400 media in spinner cultures as described above. Seventy-two h postinfection, the cells were removed by centrifugation, and the culture media (7 liters) were collected. The media were centrifuged at 100,000 × g for 1 h and filtered through a M₈, 30,000 cut-off Diaflow hollow fiber cartridge using a DC-2 apparatus (Amicon, Danvers, MA). The ultrafiltrate-containing materials of M₈ < 30,000 were concentrated to 60 ml with a M₈, 3,000 cut-off hollow fiber in the same apparatus. After dialysis in 0.1% acetic acid or 10 mM sodium phosphate buffer, pH 7.4, the material containing MPS-1 protein was lyophilized and stored at 4°C.

**Preparation of Sf9 Nuclear Extract.** A nuclear extract was obtained from virus-infected Sf9 cells by hypotonic Dounce homogenization, low-speed centrifugation, and acetonitrile extraction essentially as described by Ollo and Maniatis (13). Briefly, the cells contained in 7 liters of media were harvested by low-speed centrifugation, the supernatant was removed with 5 volumes of hypotonic buffer (10 mM HEPES pH 7.8, 3 mM MgCl₂, 10 mM ZnCl₂, 50 mM KCl, and 0.5 mM PMSF), and the cells were homogenized by Dounce homogenization. The homogenate was centrifuged, the soluble cytoplasmic fraction was separated, and the nuclear pellet was washed once with the same hypotonic buffer. The nuclear pellet was extracted overnight at 4°C with 50% acetonitrile (v/v)-0.3 M ammonium carbonate (pH 9.0) and subsequently centrifuged for 4 h at 30,000 × g to eliminate insoluble proteins. The resulting crude nuclear extract was chromatographed on a P-4 column (2.5 × 40 cm; exclusion M₈ > 4,000; Bio-Rad) equilibrated with 10 mM Tris buffer, pH 8.0. The void volume containing the nuclear extract (80 mg of protein) was collected and lyophilized.

**Purification of Baculovirus-expressed MPS-1 Protein by Reverse-Phase HPLC.** Partial purification of MPS-1 was achieved by the application of HPLC (14). Chromatography was performed using a Waters HPLC system consisting of two Model 45A pumps, a Model 680 microprocessor solvent controller-programmer, and a U6K injector system. The effluent was monitored at 280 nm using a Waters Model 441 analytical wavelength detector. The output of
this detector was coupled to an LKB Model 2210 dual pen recorder.

The pooled lyophilized fractions from the Bio-Gel P-4 step containing 1.6 mg of protein were reconstituted in 500 μl of 0.1% TFA in water, centrifuged at 3500 × g, and applied through the sample injector onto a Waters uBondapak-C18 (3.9 x 30 cm) fitted with a Waters guard column. The solvents used were HPLC grade and were degassed by sonication prior to use. The column was equilibrated in 5% acetonitrile and 0.1% TFA. The column was eluted at 22°C with a linear acetonitrile gradient (5 to 50%) containing 0.1% TFA at a flow rate of 0.8 ml/min. The column effluent was collected in =3-ml fractions. Aliquots from these fractions were lyophilized for subsequent determination of protein content, radioactivity, Western blot analysis, and specific DNA-binding activity.

**DNA-Binding Assay.** The binding assay was performed essentially as described by Ollo and Maniatis (13) with minor modifications. Briefly, a nuclear extract from cells infected with AcNPV-MPS-1 was prepared using the acetonitrile procedure as described above. The nuclear protein fraction (230 μg) was dissolved in binding buffer (20 mM HEPES (pH 8.4), 0.1 M NaCl, 3 mM MgCl2, 10 μM ZnCl2, and 0.2 mM PMSF) and chromatographed on a column (2-ml) containing a mixture of single- and double-strand calf thymus DNA-cellulose (Pharmacia, Piscatway, N J) which was equilibrated with binding buffer. The flow-through was applied again to the same column and then collected. The column was washed with 4 volumes of binding buffer. Subsequently, the proteins bound to the column were eluted with 20 mM HEPES buffer (pH 8.4), containing 1 M NaCl, 3 mM MgCl2, 10 μM ZnCl2, and 0.2 mM PMSF. Aliquots from the flow-through, the washed, and the eluted fractions were precipitated with 20% trichloroacetic acid and washed with acetone. They were then electrophoresed in a 15% acrylamide gel and used for immunoblot analysis to identify MPS-1 proteins.

**Gel Mobility Shift Analysis.** Nuclear extracts from S9 cells infected with either AcNPV-Del or AcNPV-MPS-1 were obtained by the method of Dignam et al. (10). Partially purified MPS-1 nuclear protein obtained by acetonitrile extraction and HPLC as indicated above were also tested. The gel shift mobility assay (34) was performed using the Gelshift kit from Stratagene (La Jolla, CA), essentially following the manufacturer’s specifications. Briefly, consensus sequences of DNA binding sites for SP-1, AP1, AP2, AP3, NFI/CTF, NFκB, GRE, OCT-1, and CREB were used. Double-stranded oligomers were labeled to high specific activity by T4 polynucleotide kinase and [gamma-32P]ATP (6000 Ci/mmol; DuPont). Unincorporated label was removed by phenol-chloroform extraction followed by acetone-ethanol precipitation. Binding reactions were carried out with 5 μl of nuclear protein extracts (25 μg) in 17 μl of incubation buffer (25 mM Tris-HCl (pH 7.5), 100 mM KCl, 12.5 mM MgCl2, 10 μM ZnSO4, 1.0 mM dithiothreitol, 15% glycerol, 0.1% Nonidet P-40, and 4.5 μg/ml poly[d(dC)].DNA probe (1 μl; =0.5 ng; 50,000–100,000 dpm) was added, and the samples were incubated at 22°C for 30 min. The entire incubate was subjected to nondenaturing electrophoresis on a 5% polyacrylamide gel using Tris-glycine-EDTA buffer at 15 mA/gel for 2 h. Dried gels were used to exposed Kodak XR-5 film at −80°C.

**Phosphorylation and Dephosphorylation Analysis of MPS-1 Protein Species.** To investigate the possibility that the MPS-1 protein is phosphorylated in S9 cells, the cells were infected in 60-mm dishes with AcNPV-MPS-1 as described above. Forty-eight h after infection, the cells were labeled with 100 μCi of [32P]orthophosphate in 3 ml of phosphate-free DMEM for 6 h. Then, the cells were solubilized in 62.5 μM Tris (pH 6.5), 2% SDS, 10% glycerol, and 1 mM PMSF. The cell lysates were analyzed by SDS-PAGE and autoradiography.

The removal of phosphate from 32P-labeled proteins was performed by incubation with 6 to 20 units of calf intestinal alkaline phosphatase (Promega; Ref. 9) in phosphatase buffer (50 mM Tris-Cl (pH 9.0), 1 mM MgCl2, 0.1 mM ZnCl2, and 1 mM spermidine) for 40 min at 37°C. When indicated, phosphatase inhibitors (100 mM NaF, 10 mM Na2P2O7, and 1 mM NaVO3) were added to the incubation mixtures.

**Zinc Binding Analysis.** Zinc-binding studies were performed essentially as described by Thiesen and Bach (35).

**Irradiation of Cells with UV light.** Irradiation of XP cells was performed on monolayer cultures with lids removed using a Stratalinker (Stratagene, La Jolla, CA) with a fluorescent light source of 254 nm, essentially as described previously (36).

**Other Procedures.** 32P-labeled DNA probes having the M13 region sequence were used for Northern blot analysis and were prepared by PCR as described previously (1). Other methods used in this investigation were performed as described in Refs. 26, 27, and 31.

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**References**


