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Identification of a Domain of the Herpes Simplex Virus *trans*-Activator Vmw65 Required for Protein-DNA Complex Formation through the Use of Protein A Fusion Proteins

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In order to identify structural domains of the herpes simplex virus *trans*-activator Vmw65 required for protein-DNA complex formation, subfragments of Vmw65 were expressed in *Escherichia coli* as fusion polypeptides with protein A of *Staphylococcus aureus*, and the purified hybrids were used in a band shift assay. The results indicate that a region near the amino terminus of Vmw65 between amino acids 141 and 185 is necessary for complex formation.

Vmw65, a phosphoprotein with an apparent molecular weight of 65,000, is a major structural component of herpes simplex virus which, during an infection, *trans* activates the expression of the viral immediate-early genes (2, 4, 5). Genetic and biochemical investigations have determined that responsiveness of immediate-early genes to Vmw65 *trans* induction depends on the presence of *cis*-acting TAATGA RAT (R = purine) sequence elements, which are present at least once in the promoter-regulatory domains of all immediate-early genes (3, 8, 17, 18, 22). While the function of Vmw65 is correlated with its ability to associate with these *cis*-acting target sites, Vmw65 does not have any intrinsic DNA-binding properties (12). Instead, Vmw65 interacts with host factors to form a complex which specifically binds to TAATGARAT elements (9, 11, 13, 15, 19). One of the host factors involved in this protein-DNA complex assembly has been identified as the ubiquitous octamer-binding protein oct-1 (or TRF) (9, 16). Thus, the activity of Vmw65 depends on protein-protein interactions as well as sequence-specific DNA recognition.

We have previously constructed a series of linker insertion, deletion, and nonsense mutations in a plasmid-borne copy of the Vmw65 gene, and by using a transient transfection assay, we identified regions of the polypeptide which are important for its activity in vivo (23). This work, as well as work by others, has shown that the highly acidic domain present at the COOH terminus of Vmw65 is essential for its *trans*-activation properties (1, 21). It was also demonstrated that an amino-terminal domain of Vmw65 could act as a *trans*-dominant inhibitor of the wild-type (WT) protein, which suggests that a region close to the amino terminus is capable of competing with the (WT) protein for some type of protein-protein interaction (21, 23).

In this report, we sought to identify the region of Vmw65 which is required for protein-DNA complex assembly. The procedure involved expressing domains of Vmw65 in *Escherichia coli* as soluble fusion proteins with protein A of *Staphylococcus aureus* by using the inducible expression vector pRIT2 (14). Fusion proteins were then purified on immunoglobulin G (IgG) Sepharose columns and tested for their ability to assemble into a protein-DNA complex by a mobility shift assay.

The fusion vectors which were constructed for this study are shown in Fig. 1. The sources of Vmw65-coding sequences were plasmid pMC1 or mutant derivatives of pMC1 described previously (5, 23). The protein A-Vmw65 fusion junction for each hybrid comprised the *Sal*I site in the multiple cloning site of pRIT2 and the *Sal*I site at amino acid codon 4 of Vmw65. This allows in-frame fusions in each case. All of the fusion plasmids, with the exception of pRIT65, were constructed by cloning the *Sal*I fragment contained within the coding portion of Vmw65 from pMC1 or pMC1 mutants into the *Sal*I site of pMC1. pRITsal contains amino acid codons 5 through 411 of Vmw65; pRIT178 is identical to pRITsal but contains a *Bam*HI dodecameric linker inserted after amino acid codon 178; pRITΔ 25-141 contains a deletion encompassing amino acid codons 25 through 141; pRITΔ 173-241 is missing amino acids 173 through 241. For these constructs, the translational termination signals are provided by the vector. The plasmids described above are also missing the C-terminal 79 amino acids of Vmw65, which include the acidic domain. pRITam186 contains an amber termination codon after amino acid codon 185. pRIT65 contains the entire coding sequence of Vmw65 except for the first four amino acids. It was generated by reconstructing pRITsal through cloning the 1,417-nucleotide-long *Kpn*I fragment from pMC1 into the unique *Kpn*I site in pRITsal.

To examine the expression of the fusion proteins on a small scale, *E. coli* N4830-1 (which carries the thermosensitive *c*I857 repressor) transformants harboring the various plasmids were incubated at 30°C until the cultures reached an optical density at 600 nm of 0.5. Samples (1 ml) were induced by shifting the temperature to 42°C for 30 min, and samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Fusion proteins were detected by Western blotting (immunoblotting) with horseradish peroxidase-conjugated goat anti-rabbit IgG, which will bind to the protein A moiety of all the fusion proteins (Fig. 2). Figure 2, lanes a and b, corresponded, respectively, to uninduced and induced cultures harboring pRIT2, which, as demonstrated, synthesizes unfused protein A only after induction. The various fusion vectors direct the synthesis of substantial amounts of protein A fusion proteins of the expected sizes (Fig. 2, lanes c through g). pRITΔ 173-241 produced somewhat lower amounts of fusion protein than the others, and some degradation was evident as well (Fig. 2, lane e).

For large-scale purification of fusion proteins, cultures

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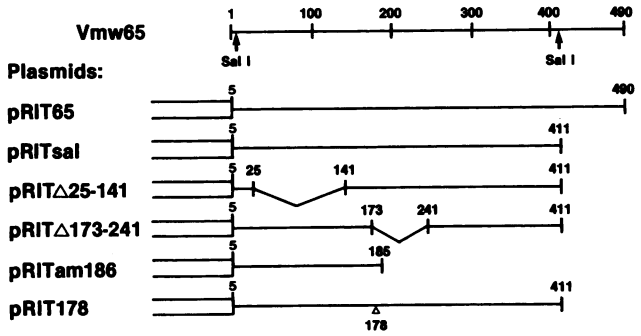

FIG. 1. Structure of protein A fusion proteins. The top line represents the coding region of Vmw65 starting with the ATG at position 1 according to the published sequence (5). Fusion plasmids were constructed as described in the text. The numbers shown for each fusion correspond to the amino acid codons of Vmw65. The open box represents protein A.

carrying the various plasmids were grown to an optical density at 600 nm of 0.5 in 500 ml of Luria broth at 30°C. Cultures were induced by the addition of an equal volume of medium prewarmed to 60°C, and incubation was continued at 42°C for 30 min. All subsequent purification steps were carried out at 4°C. Cells were harvested at 2,000 × *g* for 10 min, and the pellets were washed with 50 mM Tris hydrochloride (pH 7.6)–100 mM NaCl–1 mM EDTA and recovered by centrifugation, as described above. Cell pellets were

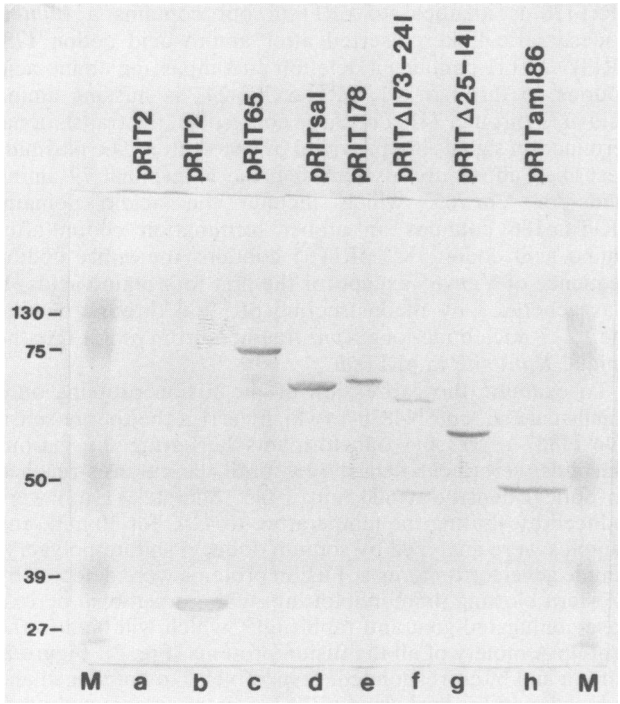

FIG. 2. Expression of protein A-Vmw65 fusion proteins in *E. coli*. Cultures harboring plasmids indicated above each lane were induced at 42°C, and samples were run on an 8% polyacrylamide gel. Following electrophoresis, proteins were transferred to nitrocellulose and fusion proteins were detected with rabbit anti-mouse IgG conjugated with horseradish peroxidase. Lane a is an uninduced control. The sizes of prestained molecular size markers (lane m; Bio-Rad Laboratories) are given in kilodaltons.

frozen at −70°C, and after the cells were thawed on ice, they were lysed in 50 ml of lysis buffer (13% sucrose, 250 mM NaCl, 10 mM EDTA, 50 mM Tris hydrochloride [pH 7.6], 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 100 U of Trasylol per ml, 0.25 mg of lysozyme per ml). After the cells were incubated for 30 min on ice, cellular debris was removed by centrifugation at 100,000 × *g* for 1.5 h by a Beckman VTi50.2 rotor. The supernatant was applied directly onto a column (0.7 by 10 cm) of IgG Sepharose 6 Fast Flow (Pharmacia) pre-equilibrated with TST (50 mM Tris hydrochloride (pH 7.8), 150 mM NaCl, 0.05% Tween 20). The column was washed extensively with TST to remove unbound material, and fusion proteins were eluted with 0.5 M acetic acid (pH 3.6). The eluted material was immediately desalted on Sephadex PD10 columns (Pharmacia) with 20 mM Tris hydrochloride (pH 7.8)–100 mM NaCl–1 mM dithiothreitol–1 mM phenylmethylsulfonyl fluoride–100 U of Trasylol per ml–10% glycerol and used directly in band shift assays. In general, approximately 3 to 5 mg of purified fusion protein was obtained from 1 liter of induced culture.

Mobility shift assays were carried out with HeLa cell nuclear extracts as described previously (6, 20). Reaction mixtures contained, in a final volume of 20 μl, 0.5 ng of ^{32}P-end-labeled oligonucleotide probe, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9), 60 mM KCl, 2 mM EDTA, 0.1% bovine serum albumin, 4% (vol/vol) glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 2 μg of poly(dI-dC) · (poly dI-dC), 4 μg of nuclear extract, and 0.5 μg of purified fusion protein. Complete binding reaction mixtures were preincubated at room temperature for 5 min prior to the addition of radioactive probe. After the reaction mixtures were incubated at 30°C for 20 min, reaction mixtures were loaded on a 3.5% polyacrylamide gel (30:1 acrylamide:*N*,*N*′-bisacrylamide) and electrophoresed with 0.5× TBE (1× TBE is 0.09 M Tris-borate–2 mM EDTA).

Oligonucleotide probes used for mobility shift assays were synthesized on a 380A DNA synthesizer (Applied Biosystems) and purified by gel electrophoresis. The following duplex oligonucleotides were used:

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WT, 5′- GATCCCGTGCATGCTAATGATATTCTTT
        GGCACGTACGATTACTATAAGAAAGATC
GA⁻, 5′-GATCCCGTGCATGCTAATTACCGTCTTT
        GGCACGTACGATTAATGGCAGAAAGATC
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The WT oligonucleotide contains sequences corresponding to positions −170 to −143 of the promoter-proximal TAAT GARAT motif of the hepes simplex virus type 1 IE110 gene (9). The GA$^-$ oligonucleotide contains mutations in the GARAT portion of the motif (underlined) which, while allowing TRF complex formation, prevent the association of Vmw65 with the complex, as demonstrated by others (9, 16). The corresponding complementary oligonucleotides were annealed, labeled at the 3′ ends with [α-^{32}P]dATP with Klenow polymerase, and purified by gel electrophoresis.

The results of the gel retardation assays with purified fusion proteins and the WT oligonucleotide probe are shown in Fig. 3A. In the presence of nuclear extract only, a major protein-DNA complex which represents the binding of oct-1, as previously demonstrated (9, 15, 16; Fig. 3A, lane a), is observed. This complex is referred to as TRF, as described by O'Hare and Goding (15). The addition of partially purified Vmw65, obtained from insect cells infected with a recombinant baculovirus expressing full-length Vmw65 (J. P. Capone and G. Werstuck, Mol. Cell. Biochem.,in press),

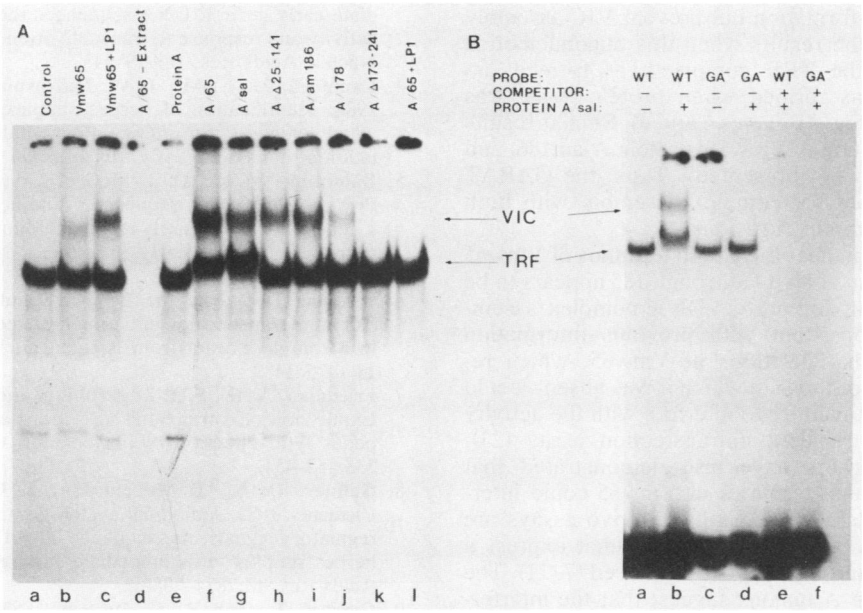

FIG. 3. Protein-DNA complex formation with purified protein A-Vmw65 fusion proteins. (A) HeLa cell nuclear extract was incubated with 3'-end-labeled WT oligonucleotide with various additions as indicated at the top of each lane. Vmw65, expressed in insect cells, was present at 0.5 μg (lanes b and c). Protein A or the protein A-Vmw65 fusion proteins were present at 0.5 μg (lanes d through l). Lane a, Incubation carried out with extract only; lane d, protein A-Vmw65 but no nuclear extract. TRF, TAATGARAT recognition factor. (B) Complete binding reactions were carried out with labeled WT probe or labeled GA^- probe as indicated. The competitor used in lanes e and f was the unlabeled WT oligonucleotide which was present at a 50-fold molar excess with respect to the labeled probe.

resulted in the formation of a new complex which is referred to as VIC, for Vmw65-induced complex (9; Fig. 3A, lane b). That Vmw65 is part of this complex is shown in lane c, for which LP1, a monoclonal antibody to Vmw65 (12), was included in the binding reaction. This results in a noticeable shift in VIC to a slower mobility, as demonstrated by others (19). Figure 3A, lanes e through l, shows the results obtained when 0.5 μg of protein A or the various fusion proteins was included in the reactions. While protein A itself had no effect (Fig. 3A, lane e), protein A-Vmw65 generated a novel complex which had a slightly slower mobility than VIC and whose appearance was dependent on the presence of nuclear extract (Fig. 3A, lanes d and f). This is consistent with the larger predicted molecular weight of the fusion protein (84,000) compared with that of WT Vmw65 (65,000). This complex was shown to be specific, since it could be completely excluded from binding by unlabeled probe (Fig. 3B, lane e). Including LP1 in the reaction resulted in disruption of VIC, presumably because of complexing of the antibody to the protein A tail (Fig. 3A, lane l). The same effect was observed when nonspecific IgG was included in the binding reaction (not shown). Protein A-sal and protein A-am186 were also able to assemble into specific complexes with specific activities similar to that of the full-length fusion (Fig. 3A, lanes g and i). Thus, the information necessary for complex formation resides within the amino-terminal 185 amino acids of Vmw65. In addition, the data demonstrate that the acidic COOH-terminal domain, which was shown previously to be essential for the transactivation function of Vmw65, is not critical for protein-DNA complex formation. Interestingly, while these fusion proteins differ in size by up to 35,000 daltons, the hybrid protein-DNA complexes formed had similar mobilities.

In order to further define the domain responsible for this interaction, fusion proteins derived from pRIT178, pRITΔ 25-141, and pRITΔ173-241 were tested. The fusion protein in which amino acids 25 through 141 were deleted was still able to form a complex, whereas removal of amino acids 173 through 241 resulted in the loss of complex formation ability (Fig. 3A, lanes h and k, respectively). This defines amino acids 141 through 185 as being critical for assembly. That this region is important is further demonstrated in Fig. 3A, lane j, for which protein A-178 was used. This mutant fusion protein had a specific activity at least 20-fold lower than the equivalent amount of protein A-Vmw65. Previously, we demonstrated that this mutation in Vmw65 inactivates its transactivating ability in vivo; thus, in this case, defective transactivation may be correlated with inefficient complex formation (23). Ace et al. recently reached a similar conclusion with derivatives of Vmw65 synthesized in vitro from the cloned gene (1). They showed that mutants of Vmw65 which contained linker insertion mutations at amino acid 173 or 178 were unable to assemble into a complex. Another interesting observation, which was made consistently, was that protein A-Vmw65 and protein A-sal caused a slight reduction in the mobility of TRF (Fig. 3A, lanes f and g, and B, lane b), whereas all of the other fusion protein derivatives tested had no such effect on TRF. This effect was highly reproducible but was observed only when large amounts of the corresponding fusion proteins (more than 0.2 μg) were used in the binding reaction (data not presented). The significance of this finding is not clear at present, but the facts suggest that an additional protein-DNA complex which requires amino acids other than or in addition to 141 through 185 of Vmw65 may form under appropriate conditions. We are further investigating this with additional fusion protein mutants.

In order to further demonstrate the specificity of the protein A fusions in complex formation, oligonucleotides containing mutations in the GARAT sequence were used in the band shift assays. As shown by others, these mutations

have no effect on TRF formation but prevent VIC assembly (9). Figure 3B shows the results when this oligonucleotide was used. Although the TRF complex was formed, no additional complex was formed when protein A-sal was added to the reaction (Fig. 3B, lanes c and d). Similar results were obtained with protein A-Vmw65, protein A-am186, and protein A–25-141 (data not presented). Thus, the GARAT sequences are important for complex assembly with both WT Vmw65 and the protein A fusions.

In conclusion, a region near the amino terminus of Vmw65 that encompasses amino acids 141 through 185 appears to be necessary and sufficient for protein-DNA complex assembly. This result is consistent with previous information which demonstrated that deletions of Vmw65, which retained this region but not those in which it was absent, could act in a *trans*-dominant manner to interfere with the activity of WT protein in a transient cotransfection assay (23). McKnight and co-workers have also demonstrated that sequences near the amino terminus of Vmw65 could interfere with the WT protein when similar in vivo assays are used, as well as when permanent cell lines that express a mutant of Vmw65 lacking the acidic tail are used (7, 21). The results with the protein A fusions suggest that the interference observed in vivo may be directly related to competition for complex assembly.

After this work was completed, Greaves and O'Hare reported on the expression of COOH-terminal truncations of Vmw65 in COS cells and examination of these mutants for complex assembly (10). In agreement with our data, they show that the acidic domain is dispensable for complex assembly but further suggest that amino acids 317 through 403 are important. This was based on the finding that deletion mutants which extended upstream from amino acid 403 were unable to form a complex in vitro. However, it was also found that these extended deletions resulted in substantially reduced levels of synthesis of the truncated proteins relative to those which contained downstream amino acids. It is possible that these mutants are unstable in vivo or in an improper conformation and that this may underlie their inability to assemble into a complex. In light of our results, it is possible that fusion of a large soluble protein, in this case protein A, to subfragments of Vmw65 stabilizes the binding domain and maintains it in a proper conformation for complex assembly to proceed.

The ability to synthesize large amounts of soluble protein A-Vmw65 fusion polypeptides and the facile purification afforded by the protein A affinity tail will be extremely valuable in further defining the critical biochemical parameters involved in protein-DNA complex assembly of this important eucaryotic *trans* activator.

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