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Separation of Requirements for Protein-DNA Complex Assembly from Those for Functional Activity in the Herpes Simplex Virus Regulatory Protein Vmw65

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A transient expression system was developed which results in efficient synthesis of the regulatory protein Vmw65 of herpes simplex virus type 1 in eucaryotic cells. The gene for Vmw65 was linked to the cytomegalovirus immediate-early (IE) promoter-enhancer region in a plasmid containing the simian virus 40 origin of replication. When transfected into COS cells, Vmw65 was expressed from this vector in 25 to 50% of the cells, with total levels of the protein approaching 20% of those observed in infected cells. Vmw65 expressed in this system is functional for specific DNA-binding complex formation with the host cell octamer-binding protein TRF and for transactivation of IE gene expression. We therefore produced a series of carboxy-terminal truncated forms of Vmw65 to examine the structural requirements of the protein for these activities. Deletion of the acidic carboxy-terminal 56 amino acids had no effect on DNA-binding complex formation but completely abolished the ability to transactivate. Amino acids between residues 434 and 453, a region which exhibits a high negative charge, were critical for IE transactivation. In contrast, the requirements for complex formation are located entirely within the N-terminal 403 amino acids, and our results indicate a requirement for this activity for residues between 316 and 403. Together with our previous work, the results presented here indicate that recruitment of TRF into a specific DNA-binding complex on IE consensus signals is required but not sufficient for functional IE transactivation by Vmw65.

Productive infection with herpes simplex virus (HSV) results in a complex cascade of positive and negative regulation of expression of virus gene products (1, 11, 23–25). Regulation of transcriptional initiation provides the main level of control of the coordinate induction of HSV gene expression, and results from classical genetic approaches and cotransfection analyses have identified regulatory roles for a number of the virus immediate-early (IE) polypeptides in the induction of delayed-early and late groups of virus genes (15–17, 42, 46, 48, 53–56).

However, in addition to induction of delayed-early and late genes, IE genes are themselves positively regulated by a structural component of the virion, as first demonstrated by analyses of virus infection of cell lines containing selected hybrid target genes controlled by IE promoters (33, 47). Sequence data from upstream regions of IE genes revealed IE gene-specific TAATGARAT consensus motifs (R = purine) present in the 5′ region of each of the five HSV IE genes, and these consensus regions were predicted to be involved in the induction of IE transcription (34, 39, 59). This prediction was confirmed by several studies demonstrating a requirement for the TAATGARAT consensus sequence in the stimulation of IE expression (7, 28, 43, 49, 58). The identity of the structural component mediating IE induction was revealed by Campbell et al. (8) as the major late phosphoprotein Vmw65 (UL48 in the nomenclature of McGeoch et al. [36]), which is assembled into the tegument region of the virion (22). Therefore, unlike the more complex situation in the induction of delayed-early and late expression, HSV IE expression is a system in which specific DNA sequence requirements have been identified and a single virus protein has been shown to be sufficient for coordinate induction of the group.

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However, despite the demonstration of specific *cis*-acting target sequences, Vmw65 does not exhibit intrinsic DNA-binding properties (35, 37, 50). Therefore, we previously examined the possibility that a cellular protein(s) binds the TAATGARAT motif and mediates responsiveness to Vmw65. From analysis of DNA-binding complexes using wild-type and mutant oligonucleotide probes which encompassed TAATGARAT motifs and parallel examination of the abilities of these probes to confer Vmw65 responsiveness on a nonresponsive promoter, we identified a specific host cell-binding factor, TRF (TAATGARAT recognition factor), which we proposed mediates Vmw65 transactivation (40). Subsequently, we demonstrated that Vmw65 is assembled into a DNA-binding complex with TRF, dependent on sequences flanking the core TRF-binding site and that formation of this complex correlates with Vmw65 functional transactivation (40a). Kristie and Roizman (27) have also identified a host cell factor, αH1, which specifically binds to the IE TAATGARAT region and may be assembled into a complex with Vmw65 (37). Comparison of their contact point analysis results (29) with our findings on the effects of various point mutations on TRF binding indicates that αH1 and TRF are identical. Preston et al. (50) also demonstrated a host cell TAATGARAT-binding factor (HC3) present in uninfected cells and a Vmw65-containing binding complex (IEC) in extracts of infected cells or in uninfected extracts supplemented with Vmw65. However, they concluded that the HC3 factor present in uninfected cells may not be recruited into the IEC complex but rather that Vmw65 induced the binding of a factor which did not bind DNA independently. Subsequent findings by these workers, however, have indicated that the cellular factor observed in the absence of Vmw65 is a precursor of the IEC complex, in agreement with our results. On the basis of a homology indicated by Pruijn et al. (51), we further provided evidence

(40) that TRF was similar or identical to the ubiquitous octamer-binding protein, a transcription factor which interacts with regulatory sequences in a number of cellular genes (2–4, 9, 18, 21) and has also been implicated in initiation of DNA replication from the origin of replication in adenovirus (44, 52). Our results (40a) indicated that for the induction of HSV IE expression, Vmw65 promotes selective interaction of the octamer-binding factor with the subset of appropriately flanked octamer-binding sites present in each of the IE genes.

In this work, we establish a system to analyze the structural requirements of Vmw65 for functional transactivation of IE gene expression and formation of the DNA-binding complex containing TRF. By overexpressing Vmw65 from the constitutively strong cytomegalovirus (CMV) IE promoter in a plasmid containing the simian virus 40 (SV40) origin of replication, we demonstrate Vmw65-TRF complex formation in extracts of transiently transfected cells. The Vmw65 expressed in the system also functions in the transactivation of IE expression. Furthermore, using vectors containing deletion mutants of Vmw65, we show that the requirements for complex formation are separable from those for functional activity. Thus, the highly acidic COOH-terminal region of the protein is dispensable for the formation of the TRF-DNA-binding complex while being stringently required for IE transactivation. On the basis of these results, a model for the mechanism of action of Vmw65 is presented and discussed with reference to findings from other systems which indicate that a number of transcriptional regulatory proteins comprise multiple domains, one of which may be an acidic domain required for activation of transcription.

MATERIALS AND METHODS

Plasmid construction. Coding sequences of the Vmw65 gene (originating from the *Bam*HI F fragment [map units 0.647 to 0.697] of HSV type 1 [HSV-1]), from the *Eco*RV site in the untranslated mRNA leader sequence to the *Pst*I site downstream of the polyadenylation signal, were inserted into the *Hin*dIII site of the vector pCMV-IL2 (12) to give the vector pRG3 (see Fig. 1). The Vmw65 sequences were supplied from the *Hin*dIII fragment of pCA21, kindly donated by Collette Ap Rhyss. The N-terminal portion of gene UL47 (20, 36) downstream of Vmw65 was removed by cleavage of pRG3 with *Bam*HI and *Asu*II, mung bean nuclease treatment, and blunt-end ligation to give the parent vector pRG4. Carboxyl-terminal deletion mutants were generated by digestion of pRG4 with the appropriate restriction enzyme, blunting of overhanging ends by incubation with either mung bean nuclease or T4 DNA polymerase, and ligation to the blunt end of the triple-frame stop linker

5′ TAGCTAGCTAG 3′
3′ ATCGATCGATCCTAG 5′.

The fragments generated, which thus contained a *Bam*HI overhang, were then digested with either *Apa*I or *Hin*dIII, and appropriate fragments were isolated by phenol extraction from low-gelling-temperature agarose gels. These fragments were then inserted between either the *Apa*I and *Bam*HI sites or the *Hin*dIII and *Bam*HI sites of pRG3, replacing full-length coding sequences and the N-terminal region of UL47. The mutant vectors were therefore identical to the parent pRG4, with the exception of the carboxy-terminal truncations.

Plasmid pCMV19, used as a control in transfections, contains the polylinker from pUC19 inserted between the *Hin*dIII site and the pBR322-derived *Eco*RI site of pRG4 and thus contains the SV40 origin of replication and the CMV promoter but lacks Vmw65-coding sequences. Plasmid pAB5, containing promoter-regulatory sequences from HSV-1 IE110 fused to coding sequences for chloramphenicol acetyltransferase (CAT), was constructed as previously described (40).

Analysis of expression vector products. The protein products of the vectors expressing full-length and truncated Vmw65 proteins were analyzed both by indirect immunofluorescence of transfected cells and by Western blotting (immunoblotting) of transfected-cell proteins with monoclonal antibody LP1 (38), kindly provided by T. Minson.

Transfected COS cell monolayers were fixed at 42 h after transfection by immersion in 50% acetone-50% methanol. Fixed monolayers were incubated with diluted LP1 ascites fluid (1/5,000) for 1 h, followed by incubation for 1 h with a goat anti-mouse immunoglobulin-fluorescein isothiocyanate conjugate. Staining was visualized by epifluorescence microscopy.

For Western blot analysis, 4×10^5 COS cells transfected with 1 μg of the expression vector were harvested at 42 h after transfection and prepared for electrophoresis by being boiled for 5 min in sample buffer (30). An equal number of COS cells infected with 5 PFU of HSV-1 (MP) per cell were harvested at 16 h postinfection and prepared for electrophoresis in the same way. Each sample (20 μl) was then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (30), and the separated proteins were blotted onto nitrocellulose filters as described by Burnette (6). The filters were blocked for 1 h in 3% bovine serum albumin fraction V in phosphate-buffered saline, incubated for 3 h at 20°C in diluted LP1 (1:5,000), washed, and incubated for a further 3 h at 20°C in a diluted goat anti-mouse immunoglobulin-horseradish peroxidase conjugate. After further washing, the filters were developed with diaminobenzidine and hydrogen peroxide as the substrate with nickel enhancement. Washing steps were performed at 20°C in several changes of 1% Nonidet P-40 in phosphate-buffered saline over a period of 15 min. Newborn calf serum (10%) in phosphate-buffered saline was used as a diluent in all of the antibody incubations. LP1 is a suitable antibody for analysis of expression of the constructs used in the present work since it reacts against an epitope of Vmw65 that is close to the N terminus (data not shown) and gives positive immunofluorescence against all of the carboxy-terminal truncated products.

Extract preparation and gel retardation analysis. Whole-cell extracts of transfected COS cells were made essentially as described by Wu (60). Cells in 140-mm-diameter dishes ($\sim 10^7$ cells per dish) were transfected with 20 μg of the expression plasmids and harvested approximately 42 h later. The cells were pelleted and washed by suspension in phosphate-buffered saline, pelleted, frozen for 10 min on dry ice, and then lysed by suspension in ice-cold 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9)–0.4 M NaCl–0.1 mM ethylene glycol-bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)–0.5 mM dithiothreitol–5% glycerol–0.5 mM phenylmethylsulfonyl fluoride (0.2 ml/10^7 transfected cells). The lysate was spun at 11,000 × *g* for 30 min at 4°C, and the supernatant was retained and stored at −70°C.

Nuclear extracts of uninfected HeLa cells and HeLa cells infected with 5 PFU of HSV-1 (MP) per cell were prepared as previously described (40). Extracts were stored at −70°C until use.

Gel retardation analysis was performed as previously described (40), except that 1 μg of sheared salmon sperm DNA was used in all of the assays in place of poly(dI-dC) and that when an antibody was included, it was added after the initial extract-probe-binding step. All of the oligonucleotide probes and competitor DNAs were generated as previously described (40).

Cells, transfection procedures, and transient expression assays. HeLa cells and COS cells were routinely cultured in Dulbecco modified Eagle minimal essential medium containing 10% newborn calf serum. Transfections were performed in COS cells by the method of Chen and Okayama (10) by using *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES)-buffered saline. COS cells were seeded overnight at 4 × 10^5 cells per 3-cm well or 10^7 cells per 15-cm-diameter dish, and new medium was added 2 h before transfection. The BES-buffered saline transfection cocktail was added to the medium, and the cells were incubated overnight. The medium was then replaced with fresh medium, and after a further 24 h, the transfected cells were harvested. Transient expression assays were otherwise performed as described by O'Hare and Hayward (41). In transient assays, the total amounts of DNA transfected were equalized by addition of pUC19 carrier DNA, as indicated in the figure legends. A quantitative estimate of chloramphenicol acetylation was obtained by excision of the substrate and products from the thin-layer chromatography plate, followed by liquid scintillation counting in an LKB 216 counter.

RESULTS

Efficient expression of Vmw65 in transfected cells. To produce efficient expression of Vmw65 and analyze the requirements for complex formation and transactivation, we constructed a plasmid (pRG4) which contains the coding region for Vmw65 (driven by the constitutively strong CMV IE promoter-regulatory region) and the SV40 origin of replication (Fig. 1). Introduction of plasmids containing the SV40 origin into COS cells, which contain an integrated SV40 genome expressing T antigen, results in high-efficiency replication of the plasmid DNA (19) and has been used for efficient expression of a number of eucaryotic genes.

Approximately 1 μg of pRG4 or the parent vector was transfected into semiconfluent COS cells in 30-mm-diameter dishes (4 × 10^5 cells per dish). Cells were harvested 48 h later and lysed, and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Estimations of the amounts of Vmw65 expressed were made by transfer of the separated proteins to nitrocellulose and detection with the monoclonal antibody LP1, which is specific for Vmw65, and horseradish peroxidase-coupled anti-mouse antibody. Typical results from such an analysis are shown in Fig. 2a. Vmw65 was easily detected in cells transfected with pRG4 (Fig. 2a, lane pRG4, solid arrowhead), while no reaction was observed in the control transfection (lane Con.). As an estimate of the relative efficiency of expression, extracts of cells infected with HSV-1 at a multiplicity of 5 PFU per cell were analyzed in parallel. Comparison of the transfected cells (Fig. 2a, lane pRG4) with an equivalent sample of HSV-infected cells (lane Inf.) indicated that expression in the transfected cells was extremely efficient, resulting in synthesis of Vmw65 at levels 10 to 20% of those observed in infected cells. Consistent with the relatively efficient expression of Vmw65 as determined by Western blot analyses, examination of transfected cells by immunofluorescence demonstrated that approximately

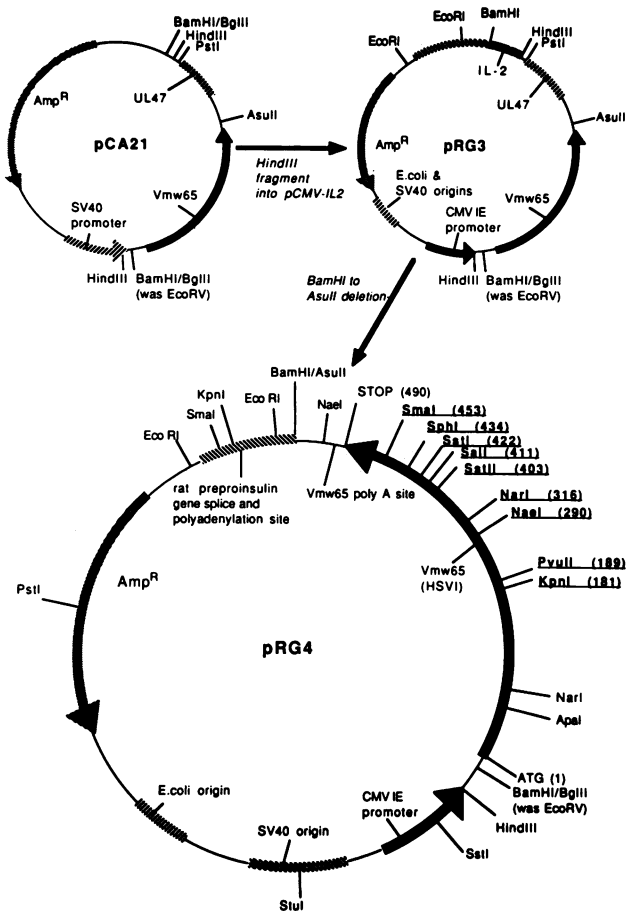

FIG. 1. Construction of Vmw65 expression vector pRG4. Plasmid pCA21, which contains the gene for Vmw65 under control of the SV40 early enhancer-promoter region, was cleaved with *Hin*dIII, and the appropriate fragment was inserted into the *Hin*dIII site of pCMV-IL2 to give plasmid pRG3. The N-terminal position of the gene for UL47 was removed from pRG3 by deletion of the *Bam*-to-*Asu*II fragment and blunt-end ligation to give pRG4, as described in Materials and Methods. IL-2, Interleukin 2; E. coli, *Escherichia coli*.

25% of the cells expressed Vmw65 (Fig. 2b). Over the course of this work, efficient expression in ≥50% of transfected cells was achieved. The pattern of fluorescence observed in transfected cells (Fig. 2b) was similar to that for infected cells (38; data not shown) in that Vmw65 was detected in both the cytoplasm and nucleus and exhibited no obvious compartmentalization.

Functional activity in transactivation and complex formation. To analyze the functional activity of the CMV-Vmw65 expression vector, dose-response experiments were performed in which increasing amounts of pRG4 were cotransfected with a CAT target gene under the control of the IE110K promoter-regulatory region. We have previously shown that this recombinant target gene exhibits an efficient IE-type response to Vmw65 through the TAATGARAT consensus sequence located between −165 and −130 (40).

Typical results from analysis of cotransfection of pRG4 with pAB5 are shown in Fig. 2c. CAT expression from the IE CAT recombinant was induced by pRG4. Optimal CAT activity was observed with relatively low input doses of pRG4 (10 to 50 ng; Fig. 2c, lanes 1 and 2). Cotransfection

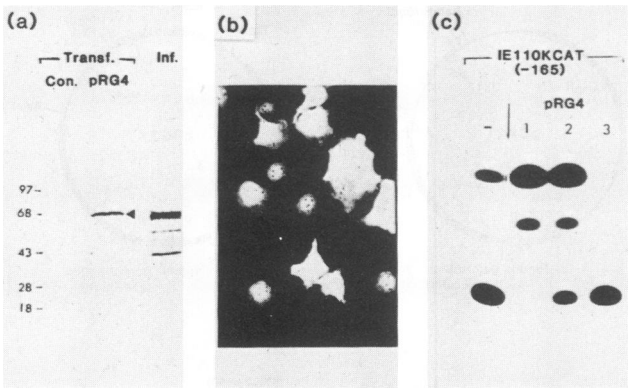

FIG. 2. Expression of functional Vmw65 from pRG4. (a) Equal amounts of COS cells transfected (Transf.) with the parent vector pCMV-IL2 (Con.) or pRG4 were solubilized at 44 h after transfection, electrophoresed in 12% polyacrylamide gels, transferred to nitrocellulose, and probed for Vmw65 with the primary antibody LP1 and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin as the secondary antibody. An equal amount of cells harvested at 16 h after infection with HSV-1 at a multiplicity of 5 PFU per cell was analyzed in parallel (Inf.). The numbers to the left indicate molecular sizes in kilodaltons. (b) Typical immunofluorescence pattern in a monolayer of COS cells fixed with 50% methanol-50% acetone 44 h after transfection with pRG4 and stained with LP1 as described in the text. Parallel monolayers transfected with a control vector were negative. (c) Monolayers of COS cells in six-well cluster dishes (4×10^5 cells per dish) were transfected with 50 ng of pAB5, which contains the upstream regulatory-promoter region of the IE110K gene (−165) linked to the CAT gene, together with 0 ng (−), 10 ng (lane 1), 50 ng (lane 2), or 1 μg (lane 3) of pRG4. The amount of cotransfected DNA was made up to 1 μg in all cases by inclusion of pUC19 DNA. Cells were harvested 44 h after transfection, and equal samples were assayed for CAT activity.

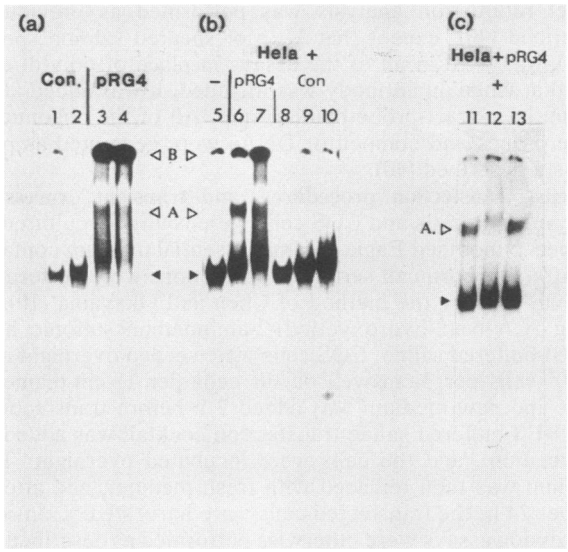

FIG. 3. Analysis of DNA-binding complex formation in extracts of pRG4-transfected cells. (a) Approximately 0.1 μg (lanes 1 and 3) or 0.5 μg (lanes 2 and 4) of protein prepared from a whole-cell extract of cultures of 10^7 cells transfected with parent vector pCMV-IL2 (Con.; lanes 1 and 2) or pRG4 (lanes 3 and 4) was incubated with probe TAAT24, which contains bases from −171 to −149 of the IE110K upstream-regulatory region and encompasses the TAATGARAT signal located at about −160. Binding reactions were performed in the presence of excess nonspecific competitor DNA, as indicated in Materials and Methods. (b) Approximately 1 μg of a nuclear extract of HeLa cells with no addition (lane 5) or supplemented with 0.05 or 0.1 μg of pRG4-transfected cells (lanes 6 and 7, respectively) or 0.05, 0.1, or 0.5 μg of pCMV-IL2-transfected cells (lanes 8, 9, and 10, respectively) was analyzed for complex formation with the TAAT24 probe as indicated. (c) Each reaction contained 1 μg of a nuclear extract of HeLa cells and 0.05 μg of pRG4-transfected cells with no antibody (lane 11) or with 0.1 μl of LP1 (lane 12) or 0.1 μl of 24S (lane 13), a monoclonal antibody directed against an unrelated virus protein. The specific shift in the upper complex A (open arrowhead) when it was incubated with LP1 was clearly observed, indicating that Vmw65 was directly bound in this complex. The closed arrowheads indicate TRF.

with amounts routinely used when analyzing induction by the intact Vmw65 gene (approximately 1 μg) resulted in little transactivation by the CMV-Vmw65 gene (Fig. 2c, lane 3). To examine whether this effect was due to a nonspecific inhibitory effect of the vector sequences alone, parallel analyses of the effects of the CMV promoter-regulatory region on SV40 expression and on IE CAT expression induced by an intact Vmw65 gene were performed. No significant inhibitory activity of cotransfection with the vector was observed in either case (data not shown). One possible explanation of the result is that the overexpression of Vmw65 which would result from the extremely strong promoter of CMV may be deleterious to functional activity. We have observed such a biphasic dose-response curve in analyses of other regulatory proteins of HSV (O'Hare et al., unpublished data).

Induction of the IE CAT target was specific, since no induction was observed with heterologous CAT target genes and a deletion in the IE promoter-regulatory region which removed the specific TAATGARAT signal resulted in loss of induction (data not shown).

Complex formation by Vmw65 in transfected cells. We have previously demonstrated by using infected-cell extracts or purified Vmw65 that it is the specific formation of a complex containing Vmw65 and the cellular factor TRF on the IE-specific *cis*-acting TAATGARAT signal which correlates with transactivation by Vmw65. To begin analyses of structure-function relationships in Vmw65, we wished to examine whether complex formation could be detected with extracts of cells transfected with the CMV-Vmw65 vector.

Confluent monolayers of COS cells in 140-mm-diameter dishes (10^7 cells per dish) were transfected with 20 μg of the parental vector or pRG4, and whole-cell extracts were made approximately 44 h later. Equal amounts of these extracts were then analyzed for binding complexes with or without supplementation by a sample of nuclear proteins from a large-scale extract of HeLa cells. Binding complexes were analyzed with the TAAT24 oligonucleotide probe, which encompasses the octamer-TAATGARAT signal at −165 of the IE110K gene. The results (Fig. 3) demonstrate that the extract from CMV-Vmw65 transfected cells produced a pattern of complex formation which was distinct from that observed in control transfected cells. Extracts from COS cells transfected with the control vector (Fig. 3a, lanes 1 and 2) contained a binding complex which comigrated with TRF from the HeLa cell nuclear preparation (Fig. 3b, lane 5). In comparison, extracts from pRG4-transfected cells contained additional complexes of lower mobility. A major complex (open arrowheads, A) comigrated with the Vmw65-containing complex from infected cells (see Fig. 7). In addition, a second novel complex which barely entered the gel (open arrowheads, B) was observed. The difference between pRG4- and control-vector-transfected cells was also ob-

served when the COS extracts were added to nuclear extracts of uninfected HeLa cells (Fig. 3b, lanes 5 to 10). Addition of 0.5 μl of the pRG4-transfected COS extract (a dose in which complex A was barely detectable when assayed independently) to the HeLa cell extract resulted in ready detection of complex A (Fig. 3b, lane 6). Higher amounts of the pRG4-transfected COS extract resulted in detection of complex B (Fig. 3b, lane 7). In several control dose-response experiments, addition of the pCMV19-transfected COS extract never resulted in the appearance of either complex A or B at any dose tested (Fig. 3b, lanes 8 to 10). While complex A was readily detected independently in the binding profile of small-scale extracts of the transfected cells, clearer results were generally obtained by supplementing the extract with 1 μg of protein from a large-scale HeLa cell nuclear extract. It is reasonable to propose that this was likely to be due to higher levels of a limiting cellular factor necessary for complex formation in the HeLa cell extract, and subsequent assays were performed by this method. However, throughout this work, analysis of complex formation in the COS extracts independently parallel those obtained when the extracts were supplemented with HeLa cell extract.

Further analyses demonstrated that both complexes A and B were specific and that at least complex A contained directly bound Vmw65. Thus, the complex A seen in pRG4-transfected extracts was shifted to lower mobility when LP1 was included in the binding reaction (Fig. 3c, lanes 11 and 12), while a control antibody had no effect (lane 13). This result parallels those observed for infected-cell extracts or uninfected-cell extracts supplemented with purified Vmw65 (40a) and indicated that Vmw65 was directly bound in complex A from pRG4-transfected cells. Since this experiment was not feasible with complex B, its specificity was demonstrated in binding and competition analyses. The results in Fig. 4a compare the binding profiles of two doses of control (lanes 1 and 2) and pRG4-transfected (lanes 3 and 4) cells with different labeled probes. A comparison of lanes 2 and 4 with the TAAT24 probe shows that complexes A and B were specific to the pRG4-transfected extracts. We have previously shown that probe TAAT22, which contains mutations in the GARAT region of the TAATGARAT signal, does not promote formation of the TRF-Vmw65 complex (40a). When this latter probe was used with the pRG4-transfected cells, it was clear that while TRF (solid arrowhead) was still observed, formation of complexes A and B was abolished. Further controls with the CCAAT-binding probe demonstrated no significant difference between control and pRG4-transfected cells. To further examine the specificity of complexes A and B, a competition analysis was performed with the wild-type probe and an unlabeled wild-type competitor or a mutant competitor containing transversions in each of the conserved TAAT bases of the TAAT GARAT domain of the IE175K gene (TAAT7). The wild-type oligonucleotide competed for binding of TRF and both complexes A and B, while the mutant sequence competed for none of the binding species (Fig. 4b). These results, together with those on the binding profiles obtained with probes with intact or mutated GARAT domains, demonstrate the specificity of the complexes observed in extracts of the transfected cells.

In summary, Vmw65 expressed in COS cells from the CMV-Vmw65 expression vector was functional in both transactivation of IE expression and complex formation with the host cell factor TRF. This system, therefore, enabled

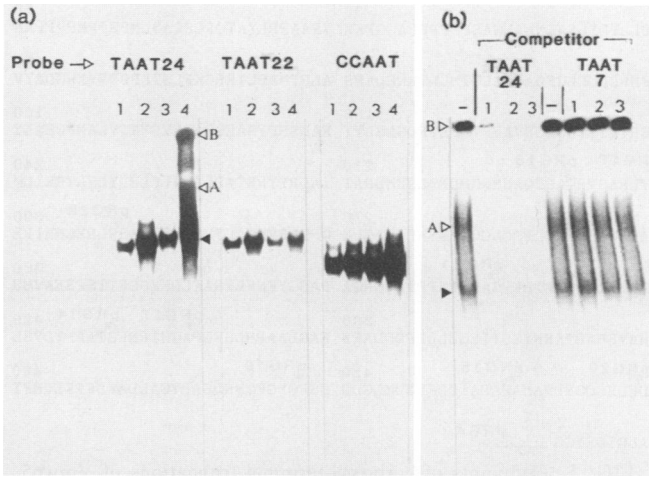

FIG. 4. Specific complex formation in pRG4-transfected cells. (a) Approximately 1 μg of a HeLa cell nuclear extract supplemented with 0.05 (lanes 1 and 3) or 0.1 (lanes 2 and 4) μg of pCMV-IL2-transfected cells (lanes 1 and 2) or pRG4-transfected cells (lanes 3 and 4) was incubated with the wild-type probe (TAAT24), a mutant version of TAAT24 containing transversions in each base of the GARAT domain (TAAT22), or a probe for the CCAAT transcription factor, as indicated. Although TRF binding (closed arrowhead) was observed with TAAT22, complexes A and B were only detected with the wild-type probe. (b) A 0.5-μg sample of the whole-cell extract from pRG4-transfected cells was incubated with the wild-type TAAT24 probe without (−) or with 5 min of preincubation with 10 (lane 1), 100 (lane 2), or 250 (lane 3) ng of unlabeled competitor DNA. The competitors were the homologous wild-type oligonucleotide TAAT24 and a mutant TAATGARAT, TAAT7, which shows little or no binding affinity for TRF (closed arrowhead).

analyses of the requirements within the Vmw65 protein for these activities.

Separation of requirements within Vmw65 for complex formation and transactivation. Comparison of the sequence of Vmw65 (13, 45) with the European Molecular Biology Organization data base has revealed no significant sequence homologies other than the homolog of Vmw65 present in the varicella-zoster virus (VZV) genome, UL10 (13, 14). Interestingly, while the amino acid sequences of Vmw65 and UL10 are well conserved, VZV UL10 has a predicted molecular mass of 46 kilodaltons and lacks sequences corresponding to the COOH-terminal 80 amino acids of Vmw65. Furthermore, this region of Vmw65 is notably enriched in negatively charged amino acids, containing 27 aspartic or glutamic acid residues in the COOH-terminal 110 amino acids. Transcriptional activation regions of a number of regulatory proteins have been shown to be enriched in negative charge (26, 31), and for this reason, together with the interesting divergence between Vmw65 and UL10 of VZV, we constructed a series of COOH-terminal truncation mutants of Vmw65 to begin analyzing requirements for transactivation and complex formation. The series of truncation mutants in the CMV-Vmw65 gene were made by insertion of an oligonucleotide containing termination codons in all three reading frames at a number of restriction enzyme sites within the protein-coding region. A summary of the endpoints of the deletion mutants in relation to the amino acid sequence of Vmw65 is illustrated in Fig. 5.

Each of the deletion mutants was transfected into COS cells, and extracts were made for analysis of complex formation in gel retardation assays and analysis of functional transactivation of cotransfected IE-CAT target genes.

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                                30                                  60
MDLLVDELFADMNADGASPPPPRPAGGPKN TPAAPPLYATGRLSQAQLMPSPPMPVPPAA

                                90                                 120
LFNRLLDDLGFSAGPALCTMLDTWNEDLFS ALPTNADLYRECKFLSTLPSDVVEWGDAYV

                               150                                 180
PERTQIDIRAHGDVAFPTLPATRDGLGLYY EALSRFFHAELRAREESYRTVLANFCSALY

pRG16 pRG18                    210                                 240
RYLRASVRQLHRQAHMRGRDRDLGEMLRAT IADRYYRETARLARVLFLHLYLFLTREILW

                               270                          pRG22  300
AAYAEQMMRPDLFDCLCCDLESWRQLAGLF QPFMFVNGALTVRGVPIEARRLRELNHIRE

                    pRG21      330                                 360
HLNLPLVRSAATEEPGAPLTTPPTLHGNQA RASGYFMVLIRAKLDSYSSFTTSPSEAVMR

                               390                   pRG17 pRG14   420
EHAYSRARTKNNYGSTIEGLLDLPDDDAPE EAGLAAPRLSFLPAGHTRRLSTAPPTDVSL

pRG20      pRG15               450   pRG19                         480
GDELHLDGEDVAMAHADALDDFDLDMLGDG DSPGPGFTPHDSAPYGALDMADFEFEQMFT

DALGIDEYGG ____ pRG4
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FIG. 5. Endpoints of carboxyl-terminal truncations of Vmw65. The sequence of Vmw65 of HSV-1 is taken from Dalrymple et al. (13). The restriction sites predicted by the sequence were used to create carboxy-terminal deletions in Vmw65, and a triple-frame stop linker was inserted at each blunt-ended site. The filled arrowheads indicate the terminal amino acids encoded by the Vmw65 gene in the constructs. A splice site and polyadenylation signals were supplied in all cases (except for the parent construct, which contains its own polyadenylation signal) from the rat preproinsulin gene (Fig. 1).

Typical results from an analysis of the binding profiles in extracts of cells transfected with wild-type and mutant Vmw65 constructs are shown in Fig. 6. TRF and the Vmw65-induced complexes are indicated by closed and open arrowheads, respectively. Formation of both complexes A and B was observed with each of the constructs, pRG4 (490 amino acids), pRG19 (453 amino acids), pRG15 (434 amino acids), pRG20 (422 amino acids), pRG14 (411 amino acids), and pRG17 (403 amino acids). Complexes formed with each of these constructs comigrated with those from infected cells (Fig. 6, lane inf.), and no significant differences in mobility were observed between complexes formed with the intact versus the truncated forms of Vmw65 (Fig. 6; data not shown). Over the course of this work, constructs pRG4 to pRG17 promoted complex formation with approximately equal efficiencies. In contrast, deletion mutant pRG21 (316 amino acids) failed to form either complex A or B, and complex formation was also undetected with the more extensively deleted constructs pRG22 (290 amino acids), pRG18 (189 amino acids), and pRG16 (181 amino acids). These results indicate that requirements for complex formation by Vmw65 reside with the N-terminal 403 amino acids and the highly acidic COOH-terminal residues are completely dispensable. In addition, the absence of detectable complex formation in pRG21 may indicate that amino acids between 317 and 403 are required for complex formation directly or indirectly. However, controls for the relative efficiency of expression indicated that while quantitatively similar amounts of Vmw65 were synthesized for each of the constructs pRG4 to pRG17, substantially reduced amounts were observed in cells transfected with pRG21 or further deletion constructs (Fig. 7). Nonetheless, experiments combining Western blot analysis and gel retardation experiments with dilutions of pRG17-transfected cells or increased amounts of pRG21-transfected cells demonstrated that complex formation with pRG17 was still detected at a 1/80 dilution of the extract, while it was never observed with any dose of pRG21. The results (data not shown) indicated that for complex formation the specific activity of the pRG17-expressed protein is at least 10-fold higher than that of the pRG21-expressed protein.

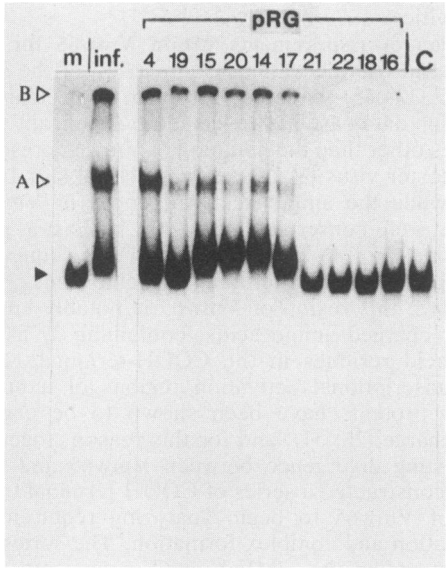

FIG. 6. Complex formation in extracts of cells transfected with constructs encoding intact or truncated Vmw65. Approximately equal portions (0.5 μg each) of whole-cell extracts of cultures transfected with 10 μg of pRG4, the vectors encoding truncated products, and the parental vector (C) were incubated with 2.5 μg of a HeLa cell nuclear extract and the wild-type probe TAAT24. Nuclear extracts from HeLa cells which were mock infected (lane m) or infected with HSV-1 at a multiplicity of 10 PFU per cell (inf.) were analyzed in parallel. TRF is indicated by the solid arrow, and complexes A and B are indicated by open arrows.

The experiments confirmed that the acidic COOH-terminal domain of Vmw65 is not required for complex formation and deletion of this region has no significant effect on overall expression of the polypeptide.

In striking contrast, deletion of this region had a severe effect on Vmw65 function. Analyses of the effects of the COOH-terminal truncations on the functional activity of Vmw65 were performed in dose-response experiments similar to that shown in Fig. 2, in which increasing amounts of each of the mutant constructs, or the parent vector as a control, were cotransfected with constant amounts of a CAT target gene containing the IE110K promoter-regulatory region. The data are expressed (Fig. 8a) as the maximum amounts of induced CAT activity observed for each mutant Vmw65.

The results indicate that pRG19, which lacks the COOH-terminal 37 amino acids, transactivates IE expression with an efficiency approximately equal to that of the intact protein. This result was confirmed in several independent assays, and a dose-response experiment comparing pRG4 and pRG19 is shown in Fig. 8b. Deletion of the terminal 37 amino acids had no significant quantitative effect on Vmw65 transactivation. However, deletion of a further 17 amino acids (pRG15) almost completely abolished activity. Controls for the relative levels of expression of the truncated forms of Vmw65 (Fig. 7a) demonstrated that the inactivity of the pRG15 construct was not due to reduction of the overall synthesis of the truncated versus the intact protein. Residual activity ($\leq$5% of that of the wild type) was infrequently observed for pRG15, but more consistently no transactiva-

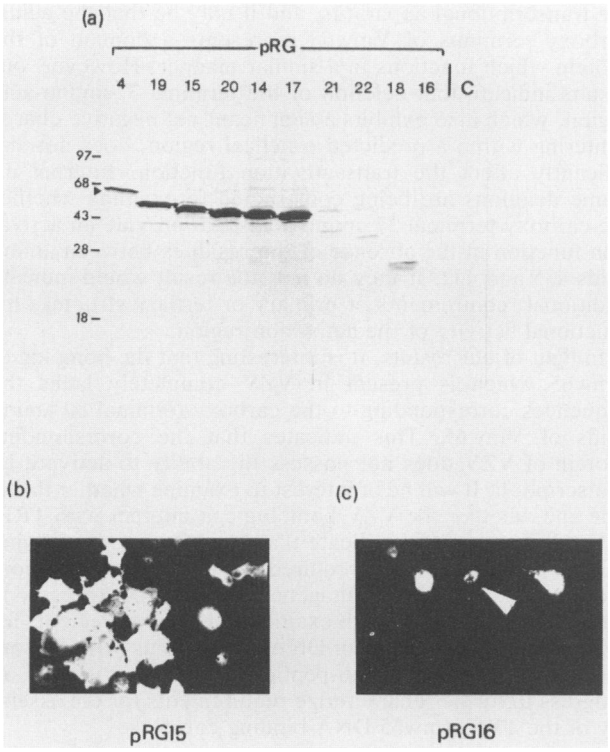

FIG. 7. Expression levels of intact and truncated forms of Vmw65 in whole-cell extracts of transfected cells. (a) Equal portions of whole-cell extracts of COS cells transfected with each of the expression plasmids, as indicated, or parental vector pCMV-IL2 (lane C) were electrophoresed in a sodium dodecyl sulfate–15% polyacrylamide gel, and the separated proteins were transferred to nitrocellulose. Vmw65 was detected by using LP1 and horseradish peroxidase-conjugated anti-mouse immunoglobulin, as indicated in Materials and Methods. Intact Vmw65 is indicated by the solid arrowhead. The numbers on the left indicate molecular sizes in kilodaltons. (b and c) Immunofluorescence patterns from pRG15 (b)- and pRG16 (c)-transfected cells. Patterns from pRG15-transfected cells were typical of pRG19, pRG15, pRG20, pRG14, and pRG17 in that no significant quantitative or qualitative differences were observed from those obtained with intact Vmw65 (pRG4). Approximately 0.5 to 2% of cells detectably expressed the truncated products from pRG21, pRG21, pRG18, and pRG16, consistent with the reduced amounts observed in Western blot analyses. However, in addition, the pattern produced by pRG18 and pRG16 was qualitatively altered, with expression detected predominantly in large nuclear aggregates. Monolayers were fixed 44 h after transfection and prepared as indicated in Materials and Methods. The white arrowhead indicates a cell exhibiting a pattern of distribution in which expression was detected mainly in nuclear aggregates.

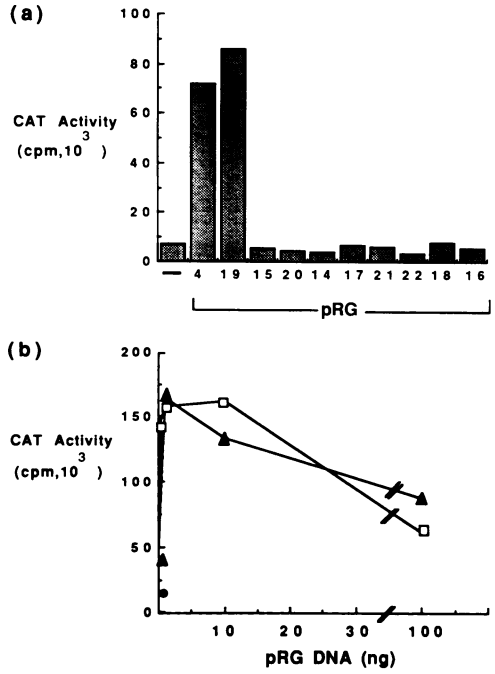

FIG. 8. Comparison of functional activities of intact and truncated forms of Vmw65. (a) Approximately 20 ng of pAB5 was transfected with 10 ng of each of the expression plasmids, as indicated. In each case, the total amount of transfected DNA was made 1 μg by addition of pUC19 DNA. The basal level of expression from pAB5 transfected only with pUC19 DNA is indicated (——). Cells were harvested 44 h after transfection, and equal amounts were assayed for CAT activity. Activity is expressed as the total number of counts appearing in the acetylated products, with approximately 160×10^3 cpm being supplied in each case as a ^{14}C-radiolabeled chloramphenicol substrate. (b) Dose-response comparison of pRG4 and pRG19. Cells were transfected with 20 ng of pAB5 together with 0, 0.1, 1, 10, and 100 ng and 1 μg of pRG4 (▲) or pRG19 (□). The total amount of transfected DNA was made to 1 μg in each case by addition of the appropriate amount of pUC19 DNA. The constitutive activity of the pAB5 target construct is indicated (●). The cells were harvested 44 h after transfection, and equal portions were assayed for CAT activity.

tion above the background was obtained and the protein from pRG15-transfected cells was essentially inactive. As expected, mutants with more extensive deletions (pRG20, pRG14, pRG17, pRG21, pRG22, pRG18, and pRG16) all failed to transactivate IE CAT gene expression, although with pRG21, pRG22, pRG18, and pRG16 the lack of activation was expected for the additional reason of low levels of the truncated products in the transfected cells (Fig. 7a). It is also noteworthy that the constructs pRG21, pRG22, pRG18, and pRG16 exhibited an altered pattern of distribution typified by that for pRG16 (Fig. 7c, open arrowhead), in which expression was detected in large nuclear aggregates. This pattern of distribution was reflected in an increased proportion of the truncated protein from these vectors partitioning in the insoluble fraction after extract preparation (data not shown). We anticipate that the lower levels of accumulated protein and solubility for these constructs resulted from synthesis of unstable aberrant polypeptides which did not tolerate the larger distortion from their native structure.

Clearly however, expression from the constructs pRG19, pRG15, pRG20, pRG14, and pRG17 was indistinguishable from that of the wild type with respect to total synthesis (Fig. 7a), solubility (data not shown), distribution (typified by pRG15, Fig. 7b), and functional ability to form a complex (Fig. 6). Thus, the result of retention of the transactivation function in pRG19 and its abolition in pRG15 (and the remainder of the constructs) indicates that the N-terminal 453 amino acids suffice for full activity of Vmw65 in inducing IE expression in transient assays and locates residues critical for activity (in the 17-amino-acid region) between amino acids 435 and 453. Importantly, the results allow the separation of requirements for functional activity from those for complex formation, since deletion mutant pRG15, containing 434 amino acids, promoted formation of the octamer-TAATGARAT-binding complex as efficiently as the intact protein while being virtually inactive in the induction of IE transcription. Together with our previous work, the results

reported here indicate that complex formation is required but not sufficient for transactivation of IE gene expression by Vmw65.

DISCUSSION

In this work, we establish a transient expression system for production of the Vmw65 protein of HSV which is functional both in the transactivation of IE gene expression and in the formation of specific DNA-binding complexes with the cellular octamer-binding factor TRF. At least one of these Vmw65-induced complexes contains directly bound Vmw65 and migrates with an identified mobility to the Vmw65-TRF complex observed in infected cells. The second Vmw65-induced complex exhibits an even lower mobility than the Vmw65-TRF complex, and while it may also contain directly bound Vmw65, we have no evidence for this or for the nature of any additional modification which further reduces its mobility. The formation of both complexes was specific in that no alteration was observed in the profile of a heterologous binding factor (e.g., CCAAT transcription factor) in Vmw65-transfected cells, and formation of both Vmw65-induced complexes required an intact GARAT domain flanking the octamer sequence in the oligonucleotide probe. Characteristics of Vmw65 complex formation in this system correctly reflect those observed in infected cells (40), and we therefore used this transient expression system to analyze structure-function relationships within Vmw65. The most important feature of our results is the demonstration that the ability of Vmw65 to promote Vmw65-TRF-TAAT GARAT complex assembly is not by itself sufficient for IE transactivation. Thus, carboxy-terminal truncated proteins synthesized in amounts similar to those of the intact protein and exhibiting wild-type properties with respect to complex formation are completely deficient in transactivation. Similar results for transactivation by Vmw65 have recently been published by Triezenberg et al. (57). No direct evidence for complex formation was reported, although these researchers assumed some such function for the N-terminal region of the protein by virtue of its ability to interfere with the activity of the intact protein. Our results locate requirements for complex formation entirely within the N-terminal 403 amino acids of Vmw65, while transactivation exhibits additional requirements within amino acids 434 to 453. This region of Vmw65 is highly negatively charged, containing 7 aspartic acids within the 19 amino acids, and is predicted to assume an α-helical conformation on the basis of secondary structure analysis. Furthermore, helical wheel analysis of this region indicates that the aspartic acid residues would be asymmetrically distributed on one face of this potential helix. This feature of Vmw65 is strikingly similar to that exhibited by two transacting regulatory proteins of *Saccharomyces cerevisiae*, GAL4 and GCN4. Results from several laboratories indicate that GAL4 and GCN4 consist of multiple domains, one of which contains intrinsic and specific DNA-binding properties (unlike Vmw65), while a distinct domain (consisting of an amphipathic helix, at least in GAL4) possessing a significant negative charge is required for transactivation. Although a negative charge may not be the only requirement within these latter domains (5, 26, 31), it is postulated to be the main requirement, since several short open reading frames with no similarity other than a negative charge can functionally substitute for at least one of the activation domains of GAL4 (32). These negatively charged activation domains may be involved in contacting or promoting the interaction of some common component of the transcriptional apparatus, and it may be that the acidic carboxy terminus of Vmw65 represents a domain of the protein which functions in a similar manner. However, our results indicate that deletion of the terminal 37-amino-acid region, which also exhibits a significant net negative charge centering within a predicted α-helical region, does not significantly affect the transactivation function. Internal in-frame deletions are being constructed to examine whether the carboxy-terminal 37 amino acids can provide an activation function in the absence of the residues between amino acids 435 and 452. If they do not, the result would indicate additional requirements of primary or tertiary structure for functional activity of the activation region.

In light of our results, it is interesting that the homolog of Vmw65 which is present in VZV completely lacks the sequences corresponding to the carboxy-terminal 80 amino acids of Vmw65. This indicates that the corresponding protein of VZV does not possess the ability to activate IE transcription. It will be of interest to examine whether this is true and whether the VZV homolog can interact with TRF.

Finally, our results indicate that residues between amino acids 317 and 404 are required for complex formation. More-refined site-direct mutagenesis of Vmw65 expressed in this system, combined with examination of requirements for protein-protein and protein-DNA interactions with region-specific peptides and anti-peptide antibodies, is now in progress to further characterize requirements for the assembly of the TRF-Vmw65 DNA-binding complex.

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