Structural Requirements in the Herpes Simplex Virus Type 1 Transactivator Vmw65 for Interaction with the Cellular Octamer-Binding Protein and Target TAATGARAT Sequences

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Herpes simplex virus type 1 virion protein Vmw65 forms a complex (TRF.C) with TAATGARAT sequences and the cellular transcription factor oct-1, which has been implicated as an intermediate in the activation of gene expression by Vmw65. To examine structural requirements within Vmw65 for this interaction, we analyzed extracts of transfected cells that express mutant Vmw65 proteins by gel retardation assay and identified two regions in the primary sequence of Vmw65 which are necessary for *in vitro* assembly of TRF.C. The amino-terminal boundary for complex assembly and *trans* activation mapped between residues 49 and 75. At the carboxyl terminus, deletion as far as residue 388 did not affect *in vitro* TRF.C assembly, although *trans*-activating activity was abolished. Deletion beyond residue 388 rapidly impaired the ability of the protein to participate in the TRF.C complex, such that a truncated mutant of 380 residues was completely inactive. These requirements towards the carboxyl terminus overlap a region of strong local sequence similarity between Vmw65 and terminal protein p3 of bacteriophage ϕ 29. Although substitution of corresponding p3 residues into Vmw65 failed to produce a functional chimera, site-directed mutagenesis within the region of similarity identified a number of single-point mutant proteins which were completely deficient for TRF.C formation. These mutant proteins were also unable to *trans* activate expression from immediate-early promoters, despite the integrity of the acidic carboxyl terminus. The extreme sensitivity of both TRF.C formation and *trans* activation to single-residue substitutions within this region of Vmw65 suggests that it is directly involved in the protein-protein or protein-DNA interactions required for assembly of a transcriptional complex containing oct-1.

Expression of the immediate-early (IE) genes of herpes simplex virus (HSV) is necessary for the progression to delayed-early and late gene expression during lytic infection (23). Transcription from IE promoters is itself stimulated upon HSV infection by a virion tegument component identified as the major late phosphorylated protein Vmw65, known also as VP16 and α -TIF (3, 8, 38). Regulatory sequences in IE promoters which specify an IE pattern of expression have been identified upstream of constitutive promoter elements (24, 27, 49). Sequencing of the upstream regions led to identification of a consensus element, TAATGARAT, homologs of which are present in all IE promoters (32, 49) and are required for Vmw65-mediated induction (7, 17, 24, 36, 39, 47).

Despite identification of the virion *trans*-activating protein and its target sequences, Vmw65 has not been demonstrated to bind independently to DNA (28, 29), and investigators at several laboratories have sought to identify cellular factors which may bind the IE consensus and thus mediate *trans* activation by Vmw65. Oligonucleotide probes containing TAATGARAT elements show sequence-specific binding in gel retardation assays to a factor (TRF) from uninfected HeLa cell nuclei (33). It is likely that TRF is related to the α -H1 and HC3 TAATGARAT-binding proteins identified in similar studies (25, 40). The presence of a good octamer motif consensus overlapping many TAATGARAT elements, the identical gel mobility of a complex formed using octamer domain probes, and the ability of such probes to compete for TRF led us to propose that TRF is the ubiquitous octamer-binding protein. This transcription factor, known variously

as oct-1, OTF-I and OBP100 (5, 44–46), is indistinguishable from cellular factor NFIII, which is involved in adenovirus replication (37). Subsequent experiments have demonstrated that purified oct-1–NFIII does indeed bind to TAATGARAT elements (2, 4).

With either infected-cell nuclear extracts or uninfected nuclear extracts supplemented with Vmw65, a novel complex is formed by using TAATGARAT probes in gel retardation assays (29, 34, 40). We term this complex TRF.C, and it is probably identical to the complex termed α -TIF–DNA, VIC, or IEC by other researchers (19, 29, 40). The presence of Vmw65 in the complex has been demonstrated by antibody binding, and the presence of octamer-binding protein has been inferred by competition experiments (19, 29, 33, 34, 40). In studies using wild-type and mutant TAATGARAT elements placed upstream of reporter genes, we showed a correlation between TRF.C formation and Vmw65-mediated inducibility (33, 34). The TRF.C complex is therefore likely to be a functional intermediate in *trans* activation by Vmw65.

There are several published studies which have investigated the relationship of the primary structure of the 490-residue Vmw65 protein to its functions in *trans* activation. An initial result of these was to identify an acidic carboxyl-terminal domain required for *trans* activation (21, 47). This domain can function independently of the remainder of the Vmw65 molecule, as demonstrated by its ability to activate gene expression when fused to the DNA-binding domain of the yeast *trans* activator GAL4 (10, 44). A minimal region active in such chimeras maps to residues 413 to 453 (R. Greaves, unpublished data). That the acidic domain is dispensable for interactions with the octamer-

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binding protein and TAATGARAT elements was demonstrated directly by the ability of a 403-residue amino-terminal fragment of Vmw65 to form TRF.C (21). A similar truncated protein dominantly interferes with *trans* activation by wild-type Vmw65 (47), presumably by formation of nonfunctional complexes which lack an activatory domain, and expression of such a mutant protein in a stably transformed cell line can interfere with HSV replication (16). We have reported mapping of a boundary of requirements for TRF.C formation to the region between residues 316 and 403 of Vmw65 (21), and Triezenberg and colleagues mapped the boundary of requirements for interference with wild-type *trans* activation to residues 380 to 393 (47). With respect to the amino terminus, the same group has also reported mapping the limit of requirements for *trans* activation and for interference (by molecules lacking the acidic domain) to the region between residues 41 and 74. Expression of the more extensively deleted mutant proteins in this study could not, however, be demonstrated, and so the possibility remained that lack of activity was simply due to lack of a mutant protein. A further deletion study by Werstuck and Capone (48) used a similar dominant-interference assay and produced conflicting results. They described interference activity for a much more minimal fragment of the protein than that found by Triezenberg and colleagues. Specifically, the interfering fragment included only residues 1 to 25 and 141 to 189 of Vmw65.

In this study, we used a gel retardation assay to determine directly which regions of the Vmw65 protein are required for formation of the TRF.C complex. The amino-terminal boundary of a functional Vmw65 fragment was mapped to the region between residues 49 and 75, while the boundary towards the carboxyl terminus of the protein was mapped tightly to the region between residues 380 and 388. We noted with interest that this carboxyl-terminal boundary was located within a region of strong local sequence similarity to bacteriophage ϕ 29 protein p3. This region was therefore investigated more thoroughly by the introduction of single-point mutations and by the replacement of Vmw65 residues with corresponding residues from the bacteriophage protein. We identified point mutations in the region which completely abolish the ability of mutant Vmw65 proteins to participate in TRF.C, and we propose that this region is intimately involved in protein-protein or protein-DNA contacts within TRF.C.

MATERIALS AND METHODS

Plasmid construction. Vectors expressing mutant forms of Vmw65 truncated at the carboxyl terminus were generated by digestion of plasmid pRG14 (21) with *Sst*II, followed by limited digestion with *Bal* 31 exonuclease. Subsequent treatment with mung bean nuclease and blunt-end ligation to the triple frame stop linker

5'TAGCTAGCTAG 3'
3'ATCGATCGATCCTAGS'

was followed by digestion with *Hind*III. This procedure generates fragments of the gene for Vmw65 spanning from a *Hind*III linker at the *Eco*RV site in the leader to a *Bam*HI end after stop codons in all three frames at the truncated 3' terminus of the gene. These fragments were then introduced between *Hind*III and *Bam*HI sites of vector pCMV-IL2 (11) to give plasmids pRG39 to pRG49. Deletion endpoints were determined by plasmid sequence analysis.

Deletions close to the amino terminus of the protein were generated in plasmid pRG14 or pRG21 (21). Deletions were made from the unique *Sal*I site in these plasmids to other

unique restriction endonuclease sites by using bacteriophage T4 polymerase or mung bean nuclease, as appropriate, to preserve the reading frame, followed by blunt-end ligation. The *Sal*I-*Eco*NI deletion in pRG56 and pRG66 does not preserve the reading frame. *Hind*III-*Pvu*II or *Hind*III-*Sst*II fragments from these deleted constructs were then introduced into pRG50, which otherwise directs the expression of full-length Vmw65, to give plasmids pRG65 to pRG69. The coding sequence deletions present in each of these plasmids were verified by sequence analysis. pRG50 is identical to pRG4 (21), except for inclusion of a *Bam*HI linker downstream of the gene for Vmw65. Plasmids pRG55 and pRG56 are the pRG14-derived precursors of pRG65 and pRG66, respectively, and in addition to deletions at the amino terminus of the protein, the proteins encoded also have a 78-residue truncation at the carboxyl terminus.

Oligonucleotide-directed mutagenesis was performed on the *Hind*III-*Bam*HI fragment of pRG14 subcloned into vector pTZ18U (31) by using the Muta-Gene system (Bio-Rad Laboratories). Hence, *Sph*I and *Bgl*II sites were introduced at codons 361 to 363 and 381 to 383, respectively, by changes which do not affect the coded Vmw65 peptide sequence. Excision of the *Sph*I-*Bgl*II fragment and replacement by the oligonucleotide pair

5'CCGCTAAGATAGCGAGGACTAAAAAGTACGGGGTAGATCTAACTGCAGAGATA3'
3'GTACGGCGATTCTATCGCTCCTGATTTTCTTCATGCCCGAGCTAGATTGACGCTCTCTATCTAGS'

resulted in replacement of coding sequences for amino acids 364 to 380 by corresponding sequences from the bacteriophage ϕ 29 DNA terminal protein p3. *Hind*III-*Sst*II fragments from the modified plasmids described above were then inserted into either pRG14 or pRG50 to give pRG64 (pRG14 plus new *Sph*I and *Bgl*II sites), pRG71 (pRG64 plus the ϕ 29 switch), pRG70 (pRG50 plus new *Sph*I and *Bgl*II sites), and pRG72 (pRG70 plus the ϕ 29 switch).

The 160-base-pair *Sph*I-*Bam*HI fragment from pRG64 was subcloned into pTZ18U to give the parent vector, pRG73, for oligonucleotide-directed mutagenesis. Most mutations in the Vmw65-coding sequence were generated with the Muta-Gene system by using 15-mer oligonucleotides mismatched at the central nucleotide. Mutant clones were identified by sequencing and *Sph*I-*Bam*HI fragments reintroduced into *Sph*I- and *Bam*HI-cut pRG64. The *Sst*II-*Bam*HI fragment of pRG70 was subsequently introduced to restore the carboxyl-terminal activatory domain and so produce the mutants in full-length form. Mutations in serine 375 were generated differently, by insertion of the oligonucleotide pair

5'CGTACGACCGCGCGCGCTACGAAAAACAATTACGGGNGCTACCATCGAAGGCGCTGCTA3'
3'GTACGATGTGCGCGCGCGCATGCTTTTGTGTAATGCCCGATGGTAGCTTCGCGACGATCTAGS'

into *Sph*I- and *Bam*HI-cut pRG64. Mutations were identified by sequence analysis of the resulting plasmids. Introduction of the *Sst*II-*Bam*HI fragment of pRG70 was used to restore the carboxyl-terminal activatory domain to these mutant proteins.

Plasmid pRG11, which directs expression of a β -galactosidase-Vmw65 fusion protein in *Escherichia coli*, was constructed by insertion of the *Eco*RV-*Pst*I fragment containing the entire Vmw65-coding sequence into the *Bam*HI and *Pst*I sites of pUR290 (42) by using a *Bam*HI linker at the 5' end of the Vmw65 sequence to preserve the reading frame. The resulting fusion protein includes the entire Vmw65 primary sequence.

Plasmid pAB5, which contains the promoter-regulatory sequences of the gene for the HSV type 1 (HSV-1) IE110K protein fused to coding sequences for chloramphenicol acetyltransferase (CAT) has been previously described (33).

All oligonucleotides were synthesized on an Applied Biosystems 381A synthesizer. Oligonucleotides for the Vmw65-p3 switch and for mutation of serine 375 were purified on a urea-acrylamide gel and eluted by soaking in distilled water. Complementary oligonucleotide pairs were annealed by being cooled together from 70°C to 30°C over 60 min.

Fusion protein and antiserum production. *E. coli* BMH71-18 containing plasmid pRG11 was grown with aeration in L broth containing 100 µg of ampicillin per ml at 37°C to an optical density at 550 nm of 0.5 and then induced for 2 h with 1 mM isopropyl-β-D-thiogalactopyranoside. The fusion protein was then purified on Sepharose Cl-4B as described by Doorbar et al. (14). Fractions from the gel filtration column containing purified fusion protein were dialyzed against Tris-buffered saline to remove sodium dodecyl sulfate (SDS) and then against phosphate-buffered saline. Pooled dialyzed fractions were used to immunize rabbits at Serotec Laboratories Ltd. The resultant polyclonal sera (MC/2-1, MC/2-2, and MC/2-3) showed strong reactivity against Vmw65 produced in eucaryotic cells both by indirect immunofluorescence of fixed COS cell layers transfected with pRG50 and by Western blotting (immunoblotting) of transfected COS cell extracts.

Analysis of expression vector products. The protein products produced by expression vectors in COS cells were analyzed by SDS-polyacrylamide gel electrophoresis (26), followed by Western blotting (6). Antiserum MC/2-3 was used together with monoclonal antibody LP-1 (30), kindly supplied by T. Minson.

Western blotting on nitrocellulose sheets was performed as already described (21), except that the soluble extracts used for gel retardation analysis were used as samples. The extract from approximately 2×10^5 transfected cells was used for each sample. Incubation with antibody LP-1 ascites (1:5,000) or antiserum MC/2-3 (1:250) was followed by incubation with a goat anti-mouse (or anti-rabbit) immunoglobulin-horseradish peroxidase conjugate (Bio-Rad), and nickel-enhanced diaminobenzidine was then used to detect bound peroxidase activity.

Extract preparation and gel retardation analysis. COS cells transfected with expression vectors were prepared and harvested as already described (21), and whole-cell extracts were prepared by the method of Wu (50). Nuclear extracts of uninfected and HSV-1-infected HeLa cells were prepared as previously described (33). Incubations for gel retardation analysis were performed in 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.9)–50 mM KCl–5 mM dithiothreitol–1 mM sodium EDTA–0.05% Nonidet P-40–10% glycerol in 20-µl volumes. End-labeled oligonucleotide probe TAAT24 was added after preincubation of extracts and nonspecific competitor DNA (10 µg of sheared salmon sperm DNA) for 5 min at 20°C. Incubations were continued for 30 min at 20°C after addition of TAAT24, and samples were then loaded onto 4% nondenaturing polyacrylamide gels with a 19:1 acrylamide-bisacrylamide ratio. Electrophoresis was for 150 min at 200V. The gels were then processed as previously described (33).

Oligonucleotide probe TAAT24 has been previously described (33, 34). It contains a TAATGARAT element from the HSV IE110K promoter which has a good overlapping consensus octamer sequence (ATGCTAAT). The TAAT24 probe was end labeled with [α -³²P]dATP by using the Klenow fragment of DNA polymerase I.

Cells, transfection procedures, and transient expression assays. COS cells (20) were transfected for extract preparation and transient expression analysis by the N,N-bis(2-

hydroxyethyl)-2-aminoethanesulfonic acid-buffered saline method (9). The details of the cell culture and transfection procedures used were previously described (21). Transient expression assays were performed by the method of O'Hare and Hayward (35), with total input DNA per 4×10^5 cells raised to 2 µg by addition of pUC19 DNA. Quantitative CAT activity estimates were obtained by liquid scintillation counting of the labeled substrate and products excised from thin-layer chromatography plates.

RESULTS

Residues 46 to 75 of Vmw65 are required for TRF.C formation and for *trans* activation. Mutant Vmw65 proteins were expressed from plasmids containing the simian virus 40 origin of replication, in which the altered coding sequences were linked to the human cytomegalovirus IE promoter-regulatory region. The simian virus 40 origin allows amplification of the plasmids in transfected COS cells which, together with the very efficient transcription from the human cytomegalovirus IE promoter, results in expression of high levels of protein from introduced coding sequences (11, 21).

Five deletions of increasing sizes were generated from the *Sall* site just inside the 5' end of the coding sequence of Vmw65 to other restriction enzyme sites within the coding sequence (summarized in Fig. 1a). Deletions to *Xma*III, *Apa*I, *Mlu*I, and *Bal*I sites were made in frame to give, respectively, the constructs pRG65 (codons 6 to 24 deleted), pRG67 (6 to 75 deleted), pRG68 (6 to 116 deleted), and pRG69 (6 to 172 deleted). In addition, to refine the amino-terminal analysis, a *Sall*-*Eco*NI deletion was used. Although this deletion did not preserve the reading frame, it was used since previous work by Campbell et al. (8) indicated that internal initiation may occur after frameshift at the *Sall* site. The *Sall*-*Eco*NI deletion pRG66 does indeed express a protein of the predicted size in Western blots (see below), and so it was included in the analysis.

The ability of mutant proteins to form TRF.C was measured by gel retardation analysis using an octamer-GARAT probe (TAAT24) derived from the upstream region of the IE110K promoter. The assays described in this report were conducted with the mutant DNA-transfected COS cell extract supplemented with a nuclear extract of HeLa cells, which produces clearer results, presumably because of limiting TRF in the whole-cell extract (21). All assays were, however, repeated with COS cell extracts alone, giving essentially similar results. The results (Fig. 2a) demonstrate that the products of full-length pRG50 and mutants pRG65 and pRG66 were all competent for assembly of the TRF.C complex, identical to that observed when a nuclear extract of HSV-1-infected HeLa cells was used (inf). The pRG66 product has at least residues 1 to 48 deleted, and therefore, these residues are not required for TRF.C formation. In contrast, TRF.C formation was completely undetectable with extracts from cells transfected with pRG67, pRG68, and pRG69. The product of pRG67 has residues 6 to 75 deleted, and we therefore conclude that residues within the region from 49 to 75 are required for participation of Vmw65 in TRF.C. This may reflect either a requirement for correct protein folding or a more specific involvement in an interactive domain of Vmw65 which is required for TRF.C formation.

Western blots of the transfected cell extracts used in gel retardation analysis (Fig. 1b, left panel) showed the presence of all of the mutant proteins, as detected by polyclonal antiserum MC/2 3. The similar expression levels of the

FIG. 1. (a) Mutant Vmw65 proteins expressed by vectors pRG65 to pRG69, pRG55 and pRG56. Shaded bars represent residues expected in the expressed product, and gaps relative to the full-length pRG50 product represent residues deleted by removal of coding sequences. Plasmids pRG56 and pRG66 have an out-of-frame deletion after codon 6, and translation of their products probably reinitiates at either methionine 49 or methionine 54. (b) Western blots of the Vmw65 mutants summarized in panel a after separation of transfected COS cell extracts in SDS-polyacrylamide gels. The left panel shows proteins recognized by a polyclonal anti-Vmw65 serum, the right panel shows proteins recognized by monoclonal antibody LP-1. Molecular size standards are shown between the panels (kd, kilodaltons).

pRG66 and pRG67 products demonstrated that failure of the pRG67 product to form TRF.C was not due to a low level of mutant protein in the extract. The size of the pRG66 product, intermediate between those of pRG65 and pRG67, is consistent with initiation at methionine 49 or 54, the first available AUG codons after frameshift. Translation initiation of wild-type Vmw65 is thought to occur at methionine 1 or 12, and deletion of codon 12 in pRG66 could account for initiation at a later AUG codon. Monoclonal antibody LP-1 does not recognize even the product of pRG65 (Fig. 1b, right panel), and therefore, components of its epitope map within residues 6 to 24 which are deleted in this mutant.

The carboxyl-terminal activatory domain of Vmw65 has potential function if it can become attached to sequences upstream of a promoter. This has been demonstrated by production of a functional chimeric *trans* activator by fusion of this acidic domain to the otherwise nonactivating DNA-binding domain of *Saccharomyces cerevisiae* protein GAL4 (10, 43). We predicted that Vmw65 mutant proteins unable to form TRF.C, and thus unable to bind their IE promoter targets, should be incapable of *trans* activating via these targets, despite the integrity of their acidic activatory domains. This was indeed the case (Fig. 2b). Plasmids pRG65 and pRG66, which encode mutant proteins still competent for TRF.C formation, *trans* activated similarly to wild-type pRG50. In contrast, pRG67, whose product did not form TRF.C, completely failed to *trans* activate expression from pAB5. The activity reported here resulted from a single approximately optimal dose of 10 ng of the effector plasmid. All effectors were also investigated in dose-response exper-

FIG. 2. (a) Gel retardation analysis of TRF.C formation by the Vmw65 mutant protein containing extracts analyzed in Fig. 1b. Lane HeLa shows the analysis of a nuclear extract of uninfected HeLa cells. In the remaining lanes, a nuclear extract of HSV-1-infected HeLa cells (inf) or extracts of COS cells transfected by the indicated vectors were supplemented with the nuclear extract of uninfected HeLa cells. The positions of the TRF and TRF.C complexes are indicated. The unbound TAAT24 probe was run off the bottom of the gel. The pCMV19 plasmid should express no product and was used as a negative control. (b) *trans* activation of CAT expression from IE110K-CAT hybrid pAB5 upon cotransfection with the expression vectors whose products were analyzed in panel a. pAB5 (50 ng) was cotransfected into COS cells with 10 ng of each expression vector. Expression of the gene for CAT was estimated by CAT activity in a soluble extract of the transfected cells, and the amount of the labeled acetylated product is shown. The lane labeled with a minus sign shows basal CAT activity expressed by 50 ng of pAB5 without cotransfected Vmw65 expression plasmids.

iments (data not shown), and *trans* activation by pRG67 to pRG69 was never seen.

trans activation by pRG65 and pRG66 was dependent upon the integrity of the carboxyl-terminal acidic domain. This was shown by the failure of the equivalent constructs with stop linkers after codon 412 (Fig. 1a, pRG55 and pRG56) to *trans* activate (Fig. 2b), although as expected, their products were still able to form TRF.C (data not shown).

Requirement of residues 381 to 388 of Vmw65 for TRF.C complex formation. Previous results had demonstrated sequence requirements between amino acid residues 316 (pRG21) and 403 (pRG17) of Vmw65 for the formation of a TRF.C complex. Our strategy was therefore to generate

mutant vectors which express proteins truncated at points between these two limits. Clones with suitable-size inserts were selected and used to transfect COS cells, and soluble extracts of transfected cells were prepared.

Extracts of COS cells transfected with mutant protein-producing vectors were analyzed by gel retardation assay (Fig. 3c). TRF.C formation by the 403-residue protein expressed by pRG17 is shown next to the infected-cell extract (inf). Of the new mutant proteins, only the 388-residue product of pRG39 showed wild-type activity for TRF.C formation. The 384-residue products of pRG46 and pRG48 had the ability to form TRF.C virtually abolished, while 380-residue and shorter products (pRG43, pRG49, pRG40, and pRG21) were completely inactive. These results, therefore, revealed a very sharp cutoff in the ability of truncated Vmw65 proteins to participate in TRF.C formation. This function was impaired by removal of residues 385 to 388 and undetectable in mutants lacking residues 381 onward. A Western blot of the extracts used in gel retardation experiments (Fig. 3b) showed that all of the mutant proteins were expressed at levels similar to that of the wild-type protein expressed by pRG4. We can therefore conclude that there are requirements within the short block from residues 381 to 388 of Vmw65 for TRF.C formation. As with sequences towards the amino terminus, this requirement may result from specific involvement of these residues in a domain which interacts with other components of the TRF.C complex, or alternatively, it could reflect a broader requirement for these residues for correct folding of the protein.

Point mutations within a region of similarity to a phage terminal protein abolish the *trans* activating function of Vmw65. When the primary sequence of the HSV-1 Vmw65 protein (12) was used to search for homologs in the available protein data bases, the only protein detected with significant extended homology was the varicella-zoster virus Vmw65 homolog ORF10 (13). We detected a number of local sequence similarities to other proteins, and one of these is to three related DNA terminal proteins from bacteriophages ϕ 29 (15), nf, and pza. The region of similarity is located from residues 366 to 390 of Vmw65, and thus it shares its carboxyl-terminal boundary with our demonstrated requirements for TRF.C formation. The best alignment between Vmw65 and phage ϕ 29 protein p3 (Fig. 4) has 11 identities and 5 semiconservative changes in a continuous sequence of 25 residues. The similar residues are grouped into two regions, a basic stretch of 8 residues from residues 366 to 373 of Vmw65 and an acidic stretch of 10 residues from 381 to 390. In addition to primary sequence similarity, predictions of the secondary structures of the regions (18) revealed potential similarity at this level (Fig. 4).

To address the possibility of the functional importance of this region, residues within it were selected for point mutagenesis. Identical and nonidentical residues between Vmw65 and p3 were chosen, and both conservative and semiconservative changes were included to test the stringency of the primary sequence requirements. Mutations were introduced by oligonucleotide-directed mutagenesis of a single-strand phagemid, sequenced in the phagemid, and reintroduced into a vector that expresses a 412-residue amino-terminal fragment of Vmw65. Truncated 412-residue mutant proteins bear the suffix Δ 413. Vectors that express full-length mutant proteins were generated by replacement of carboxyl-terminal coding sequences. Mutant proteins were named by residue number, followed by the wild-type residue code, followed by the mutant residue code. We investigated the phenotypes of 11 such single-point mutant proteins both for

FIG. 3. (a) Endpoints of the truncated products of vectors with stop codons inserted towards the carboxyl terminus. The residue number given for each mutant protein refers to the last Vmw65 residue encoded by the vector. (b) Western blots of the truncated proteins summarized in panel a after separation of transfected COS cell extracts in SDS-polyacrylamide gels. Monoclonal antibody LP-1 was used to detect the expression products. pRG4 expresses full-length Vmw65. Molecular sizes of standards are shown to the left (kd, kilodaltons). (c) Gel retardation analysis of TRF.C formation by the extracts containing truncated proteins which were analyzed in panel b. Lane HeLa shows TRF formation by a nuclear extract of uninfected HeLa cells. In the remaining lanes, a nuclear extract of HSV-1-infected HeLa cells (inf) or extracts of COS cells transfected by the indicated vectors were supplemented with the nuclear extract of uninfected HeLa cells. The positions of the TRF and TRF.C complexes are indicated. The unbound TAAT24 probe was run off the bottom of the gel. Plasmid pRG21 expresses a 316-residue product deficient for TRF.C formation, and plasmid pCMV19 should express no product. Extracts transfected with these plasmids were used as negative controls.

TRF.C formation and for *trans* activation of the IE 110K promoter. The mutations were clustered within residues 373 to 379 (Fig. 5a). Figure 6a shows a gel retardation assay in which soluble extracts of transfected COS cells were used to investigate the ability of each 412-residue mutant protein to form TRF.C on a TAAT24 probe. The mutant proteins can be broadly divided into three classes, those with a phenotype indistinguishable from that of the parent, pRG64, those for which TRF.C formation was impaired but still significant, and those whose ability to form TRF.C had been nearly or absolutely abolished. Mutant proteins 373YF, 379GC, and 375ST had a normal phenotype by this assay, while 373YS and 373YC were partially impaired for TRF.C formation. The mutant proteins for which TRF.C formation was nearly or absolutely abolished were 375SP, 378EA, 374GA, 374GE, 378EG, and 375SA. A Western blot (Fig. 5b) of the extracts used in the gel retardation assay showed that each of the

FIG. 4. Alignment of the primary sequences of Vmw65 (12) and bacteriophage ϕ 29 p3 (15) within the region of similarity. The carboxyl-terminal limit of a region required for TRF.C formation by truncated Vmw65 proteins is shown. Solid vertical lines indicate identity, and broken lines indicate permitted substitutions. A prediction for secondary structure (18) is shown outside each peptide sequence. X indicates α -helical conformation, > indicates a turn, * indicates a random coil, and - indicates an open structure. Residue numbers are given for each polypeptide.

point mutant proteins was expressed at a level similar to that of the normal equivalent expressed by pRG64.

Each mutant protein was also tested in full-length form for its ability to *trans* activate expression from the IE 110K promoter. The resulting levels of chloramphenicol acetyltransferase activity in COS cells cotransfected with a single dose of 10 ng of each mutant expression plasmid together with 10 ng of pAB5 are shown in Fig. 6b. *Trans*-activating ability correlated well with the ability of the mutant proteins to form TRF.C in gel retention assays, with the wild-type phenotype shown only by mutant proteins 373YF and 379GC, which were fully competent for TRF.C formation. *trans* activation by mutant protein 375ST, wild type by gel retardation assay, was slightly impaired (activity reduced

FIG. 5. (a) Residues which were subjected to mutagenesis. The extent of the similarity to phage protein p3, and the determined limit for TRF.C formation are shown. (b) Western blot of Vmw65 point mutant proteins and the product of parent vector pRG64. Extracts of transfected COS cells were separated on SDS-polyacrylamide gels, and the products were detected after transfer with monoclonal antibody LP-1. The nomenclature used for the mutant proteins is explained in the text. Molecular sizes of standards are shown to the left (kd, kilodaltons).

FIG. 6. (a) Gel retardation analysis using probe TAAT24 of TRF.C formation by extracts containing the truncated Vmw65 point mutant proteins which were analyzed as shown in Fig. 5b. Lane HeLa shows TRF formation by a nuclear extract of uninfected HeLa cells. In the remaining lanes, a nuclear extract of HSV-1-infected HeLa cells (inf) or extracts of COS cells expressing the mutations named were supplemented with the nuclear extract of uninfected HeLa cells. The positions of the TRF and TRF.C complexes are indicated. The unbound TAAT24 probe was run off the bottom of the gel. Plasmid pCMV19 should express no product and was used as a negative control; the pRG64 product is active for TRF.C formation and was included as a wild-type control. (b) *trans* activation of CAT expression from IE110K-CAT hybrid pAB5 upon cotransfection with vectors that express the full-length point mutant proteins named. pRG70 is the parent of these plasmids and was included as a wild-type control. pAB5 (10 ng) was cotransfected into COS cells with 10 ng of each expression vector. Expression of the genes for CAT was estimated by CAT activity in a soluble extract of the transfected cells, and the amount of the labeled acetylated product is shown. The lane labeled with a minus sign shows the basal CAT activity expressed by 50 ng of pAB5 without cotransfected Vmw65 expression plasmids.

twofold). Mutant proteins 373YS and 373YC, designated as partial TRF.C formers, showed partial *trans*-activating activities which, although approximately 12-fold lower than that of the wild type, were still significantly above the background. All of the mutant proteins unable to form TRF.C were also virtually inactive as *trans* activators. The mutant proteins were also tested for *trans*-activating activity in dose-response experiments (data not shown). Optimal *trans*-activating doses for the mutant vectors did not differ significantly from that for the wild-type vector, with no increase in *trans* activation at higher doses. The single-dose results presented are therefore representative of the best activities of the mutant proteins relative to the wild-type protein.

FIG. 7. Linear map of the primary sequence of Vmw65 showing the acidic region required for transcription activation and the regions required for formation of TRF.C. Beneath, on the left, are mutant proteins containing deletions towards the amino terminus, together with their phenotypes for TRF.C formation and *trans* activation (+, wild-type activity; -, lack of activity). To the right are the endpoints of mutant proteins truncated at the carboxyl terminus, together with their phenotypes for TRF.C formation. Notation is the same, except that -/+ mutant proteins showed very low levels of TRF.C formation. The residues at which site-directed point mutations produced mutant proteins incapable of TRF.C formation and *trans* activation are marked with asterisks.

These experiments demonstrate that single amino acid substitutions within residues 373 to 378 can completely abolish functional activity of Vmw65 for TRF.C formation and *trans* activation, and they reinforce our proposal that this region may be directly involved in interactions required for TRF.C formation.

DISCUSSION

In this work, we have identified two regions of the Vmw65 protein which are required for its participation in a nucleoprotein complex (TRF.C) which includes its target TAATGA RAT sequences and the cellular octamer-binding protein.

The boundary of a required region close to the amino terminus of the Vmw65 protein maps between residues 49 and 75. Deletion of these residues results in proteins which are no longer able to form TRF.C and cannot transactivate expression from IE promoters (Fig. 7). Deletions at the amino terminus of the protein were also used to map components of the epitope for monoclonal antibody LP-1 (30) to residues 6 to 24.

The boundary of a required region for TRF.C formation previously identified (21) towards the carboxyl terminus of Vmw65 was mapped very tightly to residues 381 to 388 (Fig. 7). A short region of strong similarity between the Vmw65 primary sequence and that of DNA terminal protein p3 of bacteriophage ϕ 29 shares the same carboxyl-terminal boundary.

In the double-stranded linear genome of ϕ 29, p3 is covalently linked from serine 232 to a dAMP residue at the 5' end of each strand (22). The p3 protein interacts with both the DNA-p3 template and phage DNA polymerase p2 in the initiation step of ϕ 29 replication. Primary sequence requirements in the free p3 substrate for this reaction in vitro include a requirement for residues 14 onward (51). The region of homology to Vmw65 lies between residues 29 and 52 and may therefore be included in these requirements. There could be a related function, as either a protein-protein or a protein-DNA interactive domain, for the similar regions of p3 and Vmw65. In this respect, it is interesting that TRF is probably identical to the host cell factor NFIII, which is involved in the formation of an initiation complex during adenovirus DNA replication (37, 41). This process also

requires a viral DNA terminal protein, which has a function similar to that of protein p3 of ϕ 29. We have not, however, been able to identify any similarities to Vmw65 in the primary sequence of the adenovirus DNA terminal protein. A chimeric protein in which 16 p3 residues replaced similar residues in Vmw65 was constructed, but was it not competent for TRF.C formation. Although the two regions are therefore not functionally interchangeable, we propose that they may adopt similar conformations and may perform related functions.

Directed mutagenesis of Vmw65 within the region of similarity demonstrated the extreme sensitivity of several residues to point mutation. TRF.C formation and *trans* activation were abolished in 6 of 11 mutants tested. Single mutations at four separate residues produced proteins whose function in both assays was strongly impaired or abolished (Fig. 7). *trans* activation was reduced to less than 10% of the wild-type activity, and TRF.C formation was substantially impaired when tyrosine 373 was changed to cysteine or serine. Phenylalanine could substitute functionally for tyrosine at this position, and so the hydroxyl group of tyrosine 373 is unlikely to be important for TRF.C formation, for instance, as a phosphorylation site. In contrast, replacement of serine 375 with threonine preserved activity, whereas alanine or proline could not substitute. The tolerance of threonine but not alanine at this position could indicate a requirement for the phosphorylation of residue 375 before TRF.C formation. This hypothesis will be tested both biochemically and by further mutagenesis. It is interesting that the ϕ 29 residues introduced into the chimeric protein included a valine residue rather than a serine residue at this position and that this alone could account for the lack of activity of the chimera in TRF.C formation. Changing glycine 374 even to the relatively small residue alanine completely abolished function in both assays, and so there may be an absolute requirement for glycine in this position. Finally, the replacement of glutamic acid 378 by either alanine or glycine abolished activity.

The limits mapped in this study for sequences which are required for both TRF.C formation and *trans* activation are in broad agreement with those mapped by Triezenberg et al. (47) for the ability of truncated proteins lacking an activatory

domain to interfere with *trans* activation. Two studies have detailed in-frame insertional mutagenesis of Vmw65 with short linkers. Ace et al. (1) reported two regions of sensitivity to insertional mutagenesis. The sensitive insertions were at residues 172 and 177 and at residue 379, and in both regions they led to abolition of IEC formation and *trans*-activating activity. Insertion at residue 379 may disrupt the same required region that we characterized by point mutagenesis. Werstuck and Capone (48) reported sensitivity of *trans*-activating activity to in-frame insertions at residues 178, 215, 335, 369, 379, and 471. The insertions at residues 369 and 379 may also disrupt the same region which we investigated in this study.

While Vmw65 has a clear homolog in the ORF10 protein of varicella-zoster virus (13), the best alignment of the two primary sequences introduces a gap in ORF10 corresponding to residues 372 to 393 of Vmw65. Since ORF10 lacks a carboxyl-terminal acidic domain, it would not be expected to *trans* activate gene expression. The additional lack of residues homologous to those required for TRF.C formation in Vmw65 suggests that it may also not form a DNA-binding complex with oct-1. Recent results obtained by using a varicella-zoster virus ORF10 expression system identical to that used for Vmw65 indicate that ORF10 is indeed deficient in both of these functions (R. F. Greaves, unpublished data).

The identification of widely spaced requirements for TRF.C formation suggests that the domain(s) of Vmw65 responsible for this function may not be as simple to dissect as was the activatory domain. The site-directed mutagenesis experiments described here imply strongly that residues in the p3-homologous region are directly involved in interactions necessary for TRF.C formation. This conclusion is further supported by our demonstration that a small peptide derived from this region can interfere with TRF.C assembly in vitro (21a). Although the relatively crude deletion analysis presented does not provide such direct evidence, a region towards the amino terminus of the protein may also be directly involved in TRF.C. The two insertional-mutagenesis studies (1, 48) indicate a further region (residues 172 to 215) which could be directly involved in TRF.C formation. If such multiple regions exist in the primary sequence, it may be either that one large interactive site is formed by the juxtaposition of these regions in the folded protein or that separate, multiple interactions occur. In this latter respect, it is interesting that several studies (2, 19; O'Hare, unpublished data) indicate that further cellular factors, in addition to the octamer-binding protein, may be required for TRF.C formation. It is possible that the various regions required of Vmw65 interact separately with the various components of the TRF.C complex. This complex is one of the best-understood examples of selective gene activation by modification of an existing DNA-transcription factor complex by a further component(s). Identification of the nature of the defects in the Vmw65 mutant proteins described will be of particular value of understanding the mechanism of protein-protein interactions involved in the combinatorial control of gene expression.

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