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Identification of the functional domains of the essential human cytomegalovirus UL34 proteins

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Abstract

The human cytomegalovirus UL34 gene represses expression of the US3 immune evasion gene and is essential for viral replication in cell culture. Two highly related proteins, an early and a late protein, are synthesized at different times during infection from the UL34 gene. The late protein differs from the early protein only by the omission of 21 amino terminal amino acids. Both UL34 proteins repress expression of the US3 promoter and bind specifically to a DNA element in the US3 gene. We have localized the DNA-binding domain of the UL34 open reading frame to amino acids 22 to 243. Surprisingly, this same region of the UL34 open reading frame was also sufficient for transcriptional repression of US3 expression. The UL34 gene is unusual in encoding proteins that have extensively overlapping DNA-binding and transcriptional regulatory domains.

Keywords: Human cytomegalovirus; UL34; DNA binding; Transcriptional repression

Introduction

Human cytomegalovirus (HCMV) has a large genome with the potential to encode over 220 proteins (Chee et al., 1990; Davison et al., 2003). Expression of the viral genome occurs in a coordinately regulated pattern throughout the five-day replication cycle. Of the identified open reading frames in the HCMV genome, a relatively small number (36 to 45 genes) are essential for viral replication in cell culture (Dunn et al., 2003; Murphy et al., 2004). The essential viral genes encode transcription factors such as IE2, proteins needed for replication of the viral genome, proteins that contribute to the structure of the virion and proteins needed for virion egress. The functions of other essential genes remain unknown.

The UL34 gene is essential for HCMV replication in cell culture (Biegalke et al., 2004; Dunn et al., 2003; Murphy et al., 2004). Although the role of UL34 in viral replication is not well understood, its expression represses transcription of the HCMV US3 immune evasion gene (LaPierre and Biegalke, 2001). The transcriptional repressive activity of UL34 proteins is mediated through interactions with a specific DNA sequence element (the transcriptional repressive element or *tre*) located between the TATA box and the transcription start site in the US3 gene (Biegalke, 1998; Biegalke et al., 2004).

The UL34 gene is expressed throughout the viral replication cycle and encodes two highly related, yet distinct, proteins (Biegalke et al., 2004). Early in infection, a 43 kDa UL34 protein is synthesized followed by a switch to synthesis of a 40 kDa UL34 protein later in infection. The late UL34 protein ($pUL34^{40kDa}$) lacks 21 residues that are present at the amino terminus of the early protein; otherwise, the proteins are identical. Both UL34 proteins localize to the nucleus and repress US3 expression. The studies described here identify the DNA-binding and transcriptional repressive domains of the UL34 open reading frame.

Results

Computer analysis of the UL34 open reading frame

The UL34 open reading frame was analyzed for amino acid content and predicted p*I* (isoelectric focusing point). As

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diagramed in Fig. 1B, the amino terminus (residues 1–220; Fig. 1B, white rectangle) is very basic in nature, with a predicted p*I* of 9.48, while the carboxyl terminus is acidic, with a predicted p*I* of 4.54, due in part to the presence of 3 short acidic amino acid clusters (Fig. 1B). Twenty percent of the carboxyl terminal 34 amino acids is comprised of proline residues. Although two nuclear localization signals were predicted to be present in the UL34 open reading frame, with one located in each half of the open reading frame (Fig. 1B; PSORTII; http://psort.ims.u-tokyo.ac.jp/), immunofluorescent UL34 antibody staining demonstrated that no nuclear localization signal was present in the amino terminal half of the protein (data not show). Analogy to other transcription factors suggested that the amino terminal half of the UL34 open reading frame should comprise the DNA-binding domain of the protein while the carboxyl terminal half was predicted to contain the transcriptional regulatory domain.

Localization of the DNA-binding domain of pUL34

The DNA-binding domain of the UL34 open reading frame was identified using electrophoretic mobility shift assays (EMSAs) and in vitro synthesized UL34 proteins. Plasmids were constructed so that portions of the UL34 open reading frame could be expressed as proteins, using in vitro transcription/

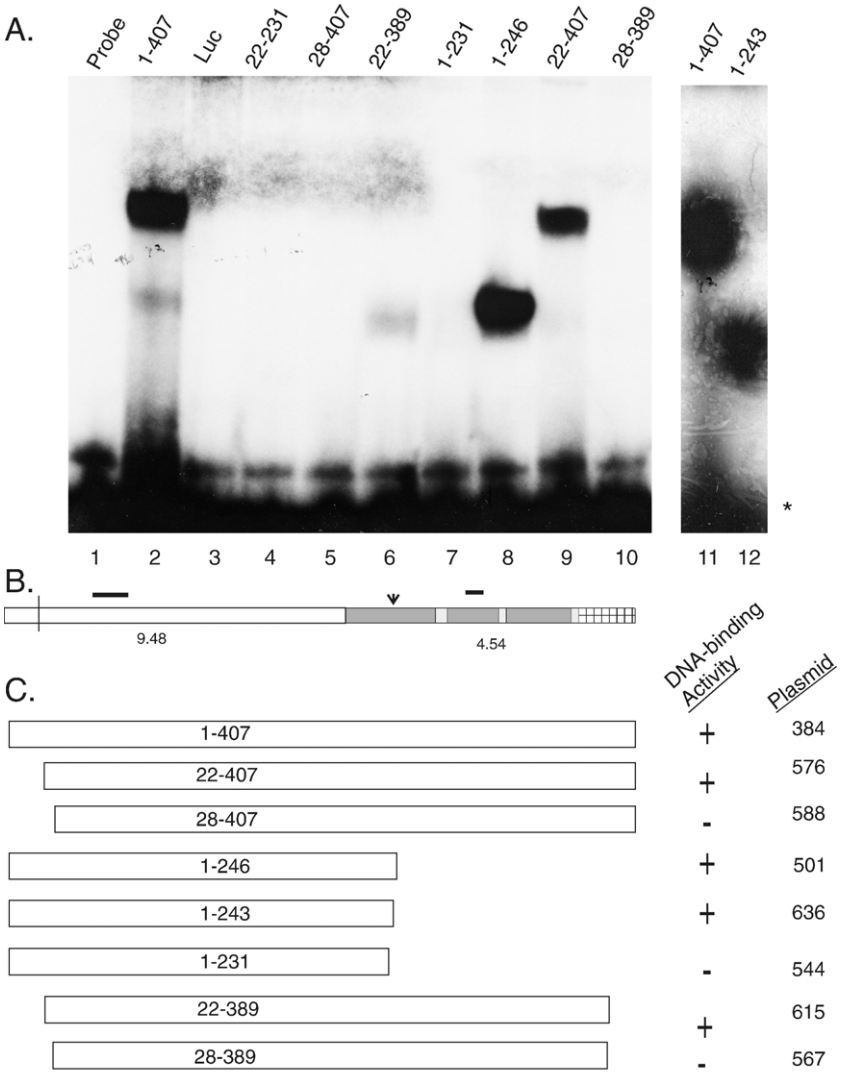

Fig. 1. Identification of the DNA-binding domain of pUL34. (A) Electrophoretic mobility shift analysis of UL34 peptides. Plasmids expressing portions of the UL34 open reading frame were constructed, and proteins synthesized using in vitro transcription/translation reactions. The ability of the proteins to interact with pUL34 binding site in the US3 gene was assayed by non-denaturing gel electrophoresis. The proteins assayed are: lane 1, probe alone; lanes 2 and 11, full-length pUL34; lane 3, luciferase; lane 4, UL34 amino acids 22–231; lane 5, UL34 amino acids 28–407; lane 6, UL34 amino acids 22–389; lane 7, UL34 amino acids 1–231; lane 8, UL34 amino acids 1–246; lane 9, UL34 amino acids 22–407; lane 10, UL34 amino acids 28–389; and lane 12, UL34 amino acids 1–243. The unbound probe is indicated, *. (B) Diagram of the UL34 open reading frame. The amino terminal half of the protein is very basic (as indicated by the predicted p*I* value of 9.48, white rectangle); the carboxyl terminal half is very acidic (predicted p*I* value of 4.54, medium gray rectangle). The predicted nuclear localization signals are indicated (black horizontal lines), as are the acidic amino acid clusters (light gray rectangles) and the proline-rich carboxyl terminal end (cross-hatched rectangle). The vertical line and the arrow indicate the amino and the carboxyl terminal boundaries of the DNA binding domain, respectively. (C) Diagram of the different forms of the UL34 protein with the amino acid residues in each indicated by the numbers. The ability of the protein to bind DNA is indicated as is the plasmid number.

translation reactions. In constructs where the ATG initiating the open reading frame was deleted, a consensus ATG was added during plasmid construction to provide a translation initiation codon. Protein synthesis was monitored by including ^{35}S-methionine in the translation reactions followed by polyacrylamide gel electrophoresis. Although the number of methionine residues and the translation efficiencies for the different portions of the UL34 open reading frame varied, translation products corresponding to the predicted sizes of the truncated UL34 proteins were detected following autoradiography of the protein gels (data not shown). The DNA probe used in the EMSAs was comprised of the US3 transcriptional repressive element (*tre*) which was previously shown to interact specifically with pUL34 (LaPierre and Biegalke, 2001). In vitro synthesized UL34 proteins or the control protein luciferase were incubated with the ^{32}P-labeled *tre* followed by electrophoresis on native polyacrylamide gels.

Both the early (amino acids 1–407) and late UL34 proteins (amino acids 22 to 407) interacted specifically with the *tre* in EMSAs as evidenced by the decrease in the migration rate of the radiolabeled DNA probe, as previously shown (Fig. 1A; Biegalke et al., 2004). Thus, the interaction of the late protein with the *tre* demonstrates that the first 21 amino acids of the UL34 open reading frame are not required for DNA binding activity. Additional truncated versions of the UL34 proteins were tested to localize the region necessary for DNA-binding activity. A protein comprised of amino acids 28 to 407 was unable to bind to the DNA probe (Fig. 1A); consequently, the amino terminal boundary for DNA-binding activity resides between residues 22 and 28. Pending additional mapping of the contributions of amino acids 22 to 30 to DNA-binding activity, amino acid 22 is defined as the amino terminal boundary of the DNA-binding domain.

The carboxyl terminal boundary of the DNA-binding domain of pUL34 was localized in a similar manner. Since a truncated protein comprised of amino acids 1 to 243 was able to bind the DNA probe while a protein comprised of amino acids 1 to 231 lacked DNA-binding activity (Fig. 1A), the carboxyl terminal boundary of the UL34 DNA-binding domain resides between residues 231 and 243.

The localization of the DNA-binding domain of UL34 between amino acids 22 and 243 is supported by the observation that a protein comprised of amino acids 22–389 was also able to bind the DNA probe (Fig. 1A, lane 6). The decrease in intensity of the DNA–protein complexes seen in lane 6 is a result of a reduction in the amount of protein present in the binding reaction (data not shown). UL34 proteins comprised of amino acid 22 to 231 or 28 to 389 were unable to bind DNA (Fig. 1A, lanes 4 and 10). The negative control protein luciferase was also synthesized using in vitro transcription/translation reactions and did not form a DNA–protein complex (luc, Fig. 1A, lane 3). The results of the experiments identifying the DNA-binding domain of the UL34 open reading frame are summarized in Fig. 1C. In summary, UL34 amino acids 22 to 243 are sufficient for specific DNA-binding activity, corresponding to the predicted role of the basic amino terminal portion of the UL34 open reading frame.

Transcriptional repressive activity of UL34

Because many transcription factors are comprised of discrete domains, the amino acid analysis of the UL34 open reading frame and the identification of the amino terminal DNA-binding domain suggested that the carboxyl terminal half of the protein would likely comprise the transcriptional regulatory domain. Further analyses of the UL34 protein, however, were complicated because of the necessity of retaining an intact DNA-binding domain and a nuclear localization signal. To surmount this problem, we constructed plasmids that expressed portions of the UL34 open reading frame as in-frame fusion proteins with the DNA-binding domain (DBD) of the yeast transcription factor *GAL4*. To assess the function of *GAL4*-DBD-UL34 fusion proteins, we also constructed a new reporter plasmid, substituting the *GAL4*-binding site for the UL34 binding site in the US3 regulatory region in the *lacZ* reporter gene plasmid pBJ171 (Fig. 2A; Biegalke, 1998). The plasmids expressing *GAL4*-DBD-UL34 fusion proteins were used in transient transfection assays, in combination with the reporter gene plasmid containing a *GAL4*-binding site, to identify the transcriptional regulatory domain of the UL34 open reading frame. The plasmids expressing the UL34 open reading frame were transfected into human fibroblasts along with a plasmid expressing the HCMV IE1 and IE2 proteins. The relative amounts of β-galactosidase produced were determined by measuring the fluorescence of the cleavage product of methylumbelliferyl β-D-galactoside (MUG). Immunofluorescence experiments demonstrated that all of the *GAL4*-DBD-UL34 fusion proteins localized to nuclei of transfected cells (data not shown).

The initial experiments examined the functionality of the *GAL4*-DBD-UL34 fusion proteins. The activity of pUL34 was compared with that of the *GAL4*-DBD-UL34 fusion protein. As depicted in Fig. 2B, expression of the *GAL4*-DBD alone had no repressive activity on the US3 promoter in the context of a UL34 binding site. In contrast, the *GAL4*-DBD-UL34 fusion protein was as efficient as pUL34 alone in repressing the US3 promoter (Fig. 2B), demonstrating that the addition of the *GAL4*-DBD to UL34 did not interfere with the repressive activity of UL34.

The UL34- and the *GAL4*-DBD-UL34 expression constructs were then assayed for activity on the reporter plasmid containing a *GAL4*-binding site substituted for the pUL34 binding site. Neither pUL34 nor the *GAL4*-DBD alone was able to repress expression of the plasmid containing the *GAL4*-binding site (Fig. 2B); however, the *GAL4*-DBD-UL34 fusion protein was very efficient at repressing transcription of the US3 promoter in the context of a *GAL4*-binding site (Fig. 2B). This observation demonstrated that the *GAL4* system could be used to define the transcriptional repression domain of pUL34. The negative control, a plasmid transcribing the UL34 gene in an anti-sense direction, had no effect on gene expression.

In the course of analyzing the effects of the *GAL4*-DBD-UL34 fusion protein on reporter gene activity, we observed that mutating the UL34 binding site to a *GAL4*-binding site significantly reduced the level of reporter gene activity seen

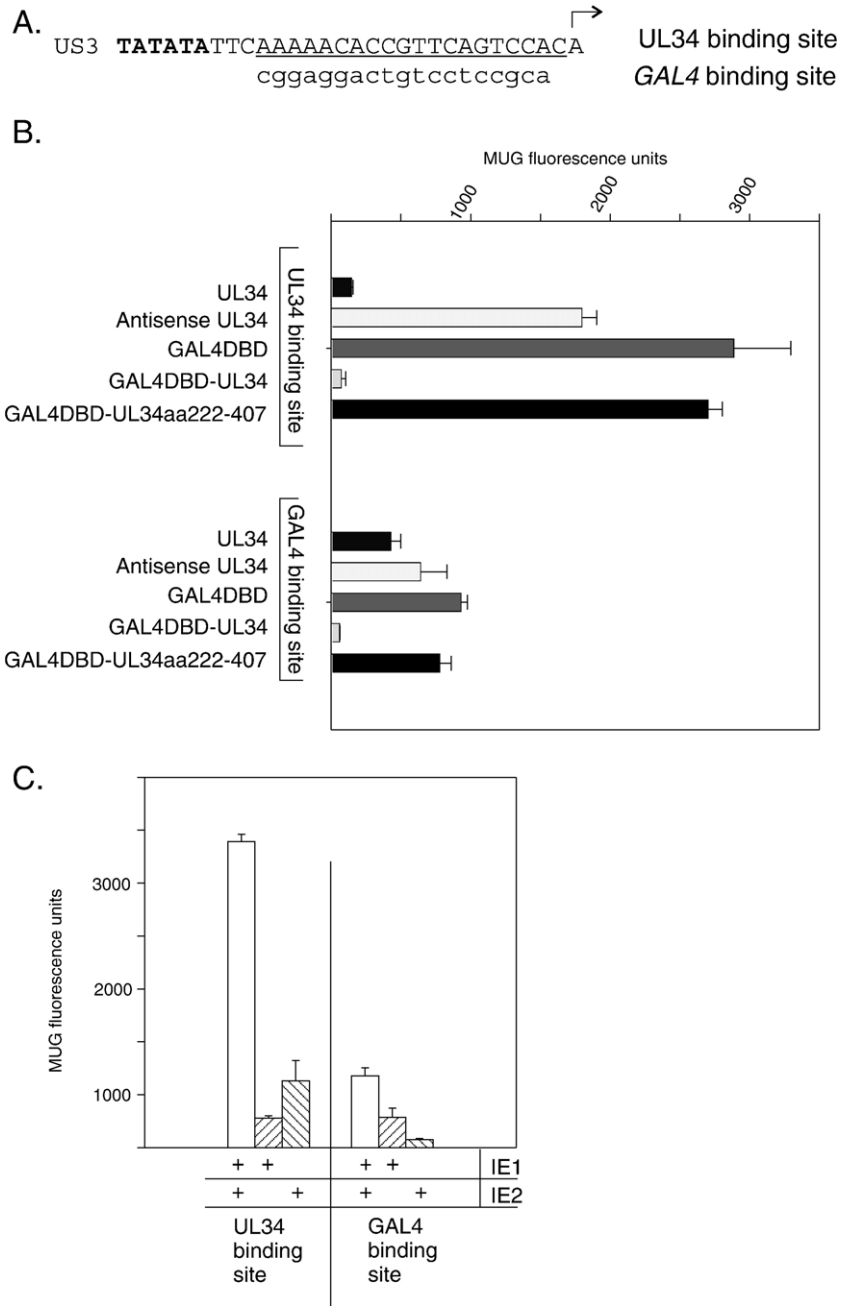

Fig. 2. Analysis of *GAL4* DBD-UL34 fusion proteins. (A) Sequence of the US3 TATA box and the UL34 binding site (underlined); the *GAL4*-binding site substituted for the UL34 binding site in the *lacZ* reporter gene plasmid is shown below the UL34 binding site (lowercase letters). (B) Analysis of the activity of the *GAL4* DBD-UL34 fusion proteins. The binding site for the *GAL4* DBD or pUL34 present in the reporter constructs is indicated. Plasmids encoding the indicated proteins were transfected into primary human diploid fibroblasts, along with either pBJ171, the US3-*tre* reporter gene plasmid (containing a UL34 binding site), or with BJ555, the US3-*GAL4*-binding site-reporter gene plasmid, in the presence of the HCMV major immediate early proteins, IE1 and IE1. The fusion protein comprised of the *GAL4*-DBD fused to UL34 amino acids 222–407 is encoded by pBJ539. (C) Analysis of the effects of IE1, IE2 or IE1 and IE2 on the relative levels of expression from the reporter constructs that contain a UL34 or a *GAL4*-binding site. The fluorescence of media containing the cleavage product of 5 methylumbelliferyl-β-D-galactoside (MUG) was measured as an indication of reporter gene transcription. The transfection assays were repeated multiple times; the results depicted are the average of two experiments, with one standard deviation. Background levels of fluorescence obtained with the promoterless control plasmid pEQ3 were subtracted from the values obtained with the US3 promoter–reporter plasmid.

in the presence of the HCMV major immediate early proteins IE1 and IE2 (Fig. 2B). To determine the basis of the reduced level of reporter gene activity, we compared the responses of the reporter plasmids which contained either a UL34 binding site or a *GAL4*-binding site to the major immediate early proteins, both singly and in combination. As seen in Fig. 2C, substitution of a *GAL4*-binding site for the UL34 binding site significantly decreased IE2 activation of the US3 promoter. In contrast, levels of expression seen with IE1 alone were similar for both reporter constructs. The marked decrease in IE2 activation of the US3 promoter following mutation of the UL34 binding site is of note as previous studies suggested that IE2 would bind to this region

(Biegalke, 1997, 1998; Thrower et al., 1996). These results support the idea that IE2 activates US3 expression by interacting with sequences located between the transcription start site and the TATA box.

Using *GAL4*-DBD-UL34 fusion constructs, we asked whether the carboxyl terminal half of pUL34 alone, when fused to a heterologous DNA-binding domain, was sufficient to mediate transcriptional repression by binding to the corresponding DNA element. A construct expressing UL34 amino acids 222 to 407 as a *GAL4*-DBD fusion protein (pBJ539; Fig. 2B) was analyzed for effects on expression of the US3 promoter paired with a *GAL4*-binding site. The fusion protein showed no repressive activity (Fig. 2B), contradicting the prediction that the carboxyl terminal half of pUL34 alone would be sufficient for transcriptional regulatory activity.

The region of the UL34 open reading frame necessary and sufficient for transcriptional repression was identified through the analysis of additional UL34 expression plasmids. Plasmids coding for *GAL4*-DBD-UL34 fusion proteins containing deletions or mutations were used to localize the transcriptional regulatory domain of pUL34. We first mapped the carboxyl terminal end of the transcriptional repressive domain using transient transfection assays as described above. Expression of UL34 amino acids 1 to 311 as well as amino acids 1 to 243 was sufficient, when fused to the *GAL4*-DBD, to repress expression from the US3 promoter (Fig. 3B). Deletion of an additional 11 amino acids, expressing only amino acids 1 to 235 as a fusion protein, abolished the transcriptional regulatory activity of the protein (Fig. 3B).

We also utilized the *GAL4*-DBD-UL34 fusion protein system to probe the amino terminal end of the UL34 transcriptional repressive domain. *GAL4*-DBD fusion proteins containing UL34 amino acids 1 to 407, 10 to 407, or 22 to 374 fully repressed US3 expression (Fig. 3C). In contrast, a fusion protein containing UL34 amino acids 61–407 was unable to mediate US3 repression (Fig. 3C).

These results mapped the amino and carboxyl terminal boundaries of the transcriptional repressive domain to amino acids 22 and 243, respectively, demonstrating that the amino terminal 243 amino acids of the protein is sufficient for transcriptional repression. Thus, when expressed as a *GAL4*-DBD-UL34 fusion, the transcriptional repressive domain of pUL34 overlaps extensively with the DNA-binding domain of the protein. The observation that the *GAL4*-DBD did not repress US3 expression (Figs. 3B and C) rules out physical protein–DNA interactions alone as mediating repression.

Results of site-directed mutagenesis experiments confirmed the contribution of the amino terminal half of the protein to DNA binding and transcriptional repression. As shown in Table 1, two UL34 mutant proteins, in which amino acids $G^{42}K^{43}$ were mutated to $V^{42}A^{43}$, or where amino acids $K^{45}L^{46}$ were mutated to $N^{45}A^{46}$, were generated to assess the contributions

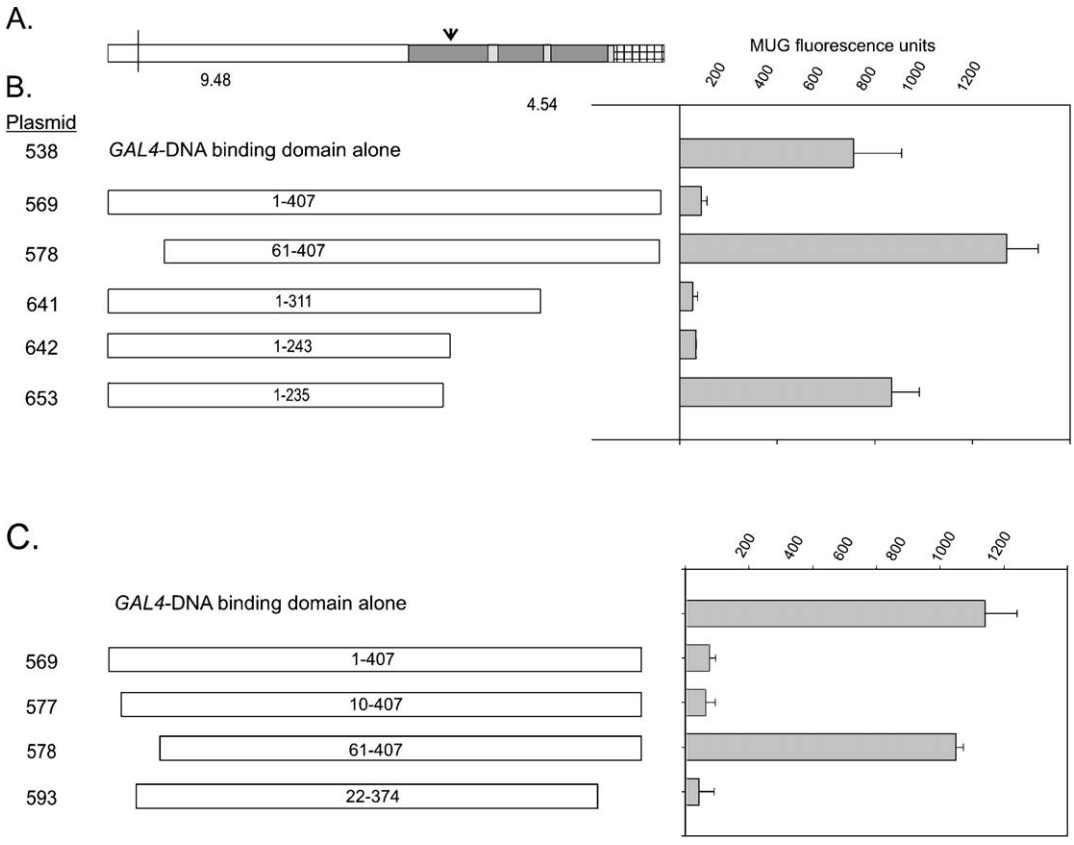

Fig. 3. Analysis of the transcriptional repressive activity of *GAL4*-UL34 fusion proteins. (A) Diagram of the UL34 coding region (see Fig. 1B). (B) Deletion analysis of the carboxyl terminal half of pUL34. (C) Deletion analysis of the amino terminal half of pUL34. The numbers indicate the amino acids present in each of the truncated proteins; reporter gene activity was measured as described in Fig. 2.

Table 1
Amino acid substitutions in the pUL34 mutants

Plasmid	Mutations
623	V^{312} to A; P^{313} to A; P^{314} to A; H^{315} to S
627	G^{42} to V; K^{43} to A
629	K^{45} to N; L^{46} to A
630	E^{334} to A; E^{335} to A

of the conserved basic residues to pUL34-DNA-binding activity (Fig. 4A). EMSA demonstrated that the point mutations abolished DNA binding activity (Fig. 4B). The ability of the mutated proteins to repress US3 expression when expressed as *GAL4*-fusion proteins was assayed; neither protein was able to repress transcription from the US3 promoter in the context of a *GAL4*-binding site (Fig. 4C). These experiments demonstrated that the amino terminal region of the protein is required for the DNA-binding activity as well as for transcriptional repression. In contrast, mutation of amino acids in the carboxyl terminal half of the protein had no effect on the repressive activity of the protein as expected, as shown for plasmids pBJ623 and pBJ630, where proline and glutamic acid residues were mutated, respectively (Fig. 4C and Table 1).

Discussion

The HCMV UL34 gene is intriguing, not only because of its role in regulating expression of a viral immune evasion gene (LaPierre and Biegalke, 2001) but also because expression of the open reading frame is required for viral replication in vitro (Murphy et al., 2004; Dunn et al., 2003; Biegalke et al., 2004). We have identified the DNA-binding and transcriptional regulatory domains of the UL34 open reading frame, localizing both functions to amino acids 22 to 243. The UL34 proteins are unusual among transcription factors, with the extensive overlap of the DNA-binding and transcriptional regulatory domains. The majority of transcription factors have discreet DNA-binding and regulatory domains. Overlap of regulatory and DNA-binding domains does occur in other proteins, however, including proteins such as nuclear factor 90 (Reichman et al., 2002) and v-erbA (Boucher and Privalsky, 1990). The extensive overlap of UL34 DNA-binding and transcriptional regulatory domains places the protein into a small group of unique proteins.

Direct physical interaction of pUL34 with the target DNA sequence is not required for transcriptional repression since a protein expressing a heterologous DNA-binding domain

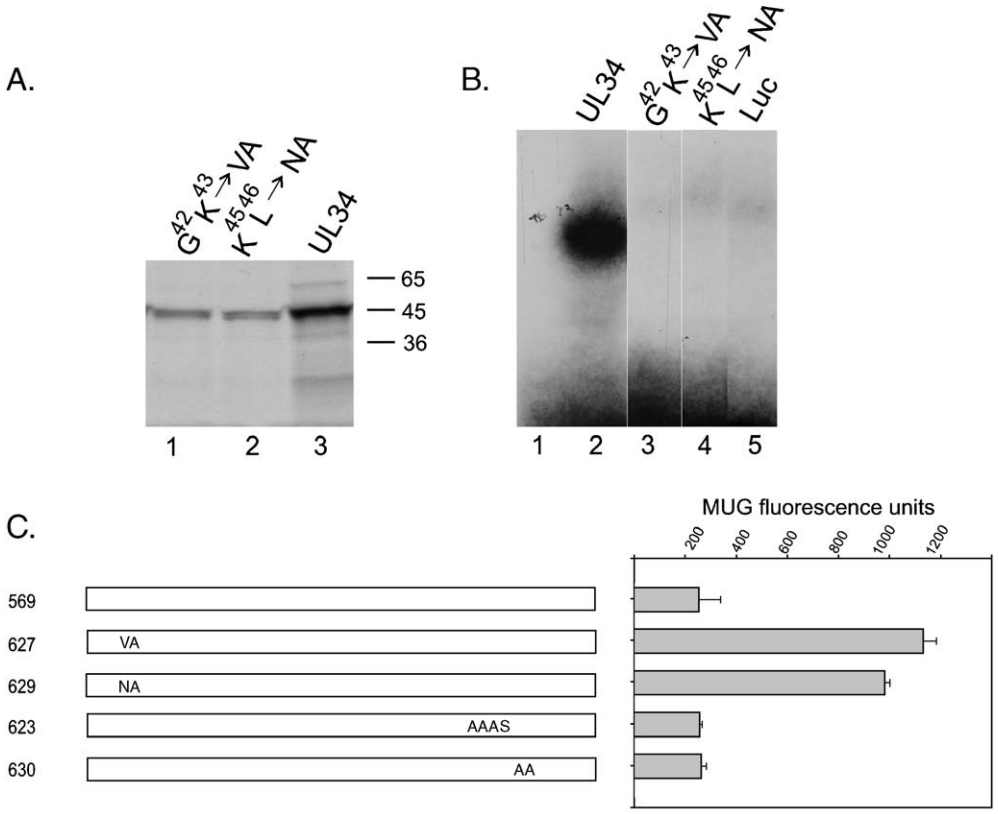

Fig. 4. Effects of clustered site-specific mutations on activity of pUL34. (A) Wild type and mutant proteins were generated using in vitro transcription/translation reactions, and the resulting products analyzed by denaturing polyacrylamide gel electrophoresis. The amino acids mutated are indicated above lanes 1 and 2, lane 3 contains the wild type pUL34; the positions of the molecular weight markers are indicated. (B) Electrophoretic mobility shift analysis of the DNA-binding activity of the mutant proteins. Lane 1, probe alone; lane 2, pUL34; lanes 3 and 4, mutant proteins, and lane 5, luciferase. (C) Transcriptional repressive activity of the mutant proteins. Plasmids encoding *GAL4*-DBD-UL34 fusion proteins were transfected into primary human diploid fibroblasts along with the US3 reporter gene plasmid that has a *GAL4*-binding site substituted for the UL34 binding site. Reporter gene activity was measured as described in the legend to Fig. 2. The amino acid changes in the mutant proteins are indicated by the single letter code.

(*GAL4*-DBD) fused to the UL34 open reading frame was efficient at repressing expression of a reporter gene that contained a *GAL4*-binding site in place of the pUL34 binding site. Similarly, simple binding of a protein to the transcriptional regulatory region was not sufficient for transcriptional repression since binding of the *GAL4*-DBD to a *GAL4*-binding site in this region had no effect on transcription. These results suggest that the transcriptional regulatory activity exhibited by pUL34 is most likely mediated through protein–protein interactions rather than through steric interference with the assembly of the pol II transcription initiation complex.

The amino terminal half of pUL34 is conserved among the cytomegaloviruses (Liu and Biegalke, 2001). Computer analysis predicts that the amino terminal half of the protein will adopt a structure composed of 4 helical regions separated by loops with disulfide bonds linking conserved cysteine residues (Rost et al., 2004). In contrast to the conservation seen in the amino terminal half of the UL34 homologs, the carboxyl terminal half is quite divergent and for HCMV, pUL34 is predicted to contain intrinsically unstructured regions (Dyson and Wright, 2005). The conservation of the amino terminal half of pUL34 and its homologs, coupled with the identification of predicted multiple pUL34 binding sites within the genomes of cytomegaloviruses, suggests that pUL34 may play additional roles in viral replication, in addition to its role in regulating US3 expression. Determination of the structure of the protein, coupled with additional site-directed mutagenesis, will be critical for advancing our understanding of the mechanism of action of pUL34.

Materials and methods

Tissue culture

Primary human diploid fibroblasts (HDFs) were propagated in Dulbecco's minimal essential medium supplemented with penicillin, streptomycin, glutamine and 10% NuSerum I (Becton Dickinson, Bedford, MA) as described (Biegalke and Geballe, 1990). Transient transfections were performed using DEA–dextran, assaying the level of β-galactosidase activity by measuring the fluorescence of the cleavage product of methylumbelliferyl-β-D-galactoside (Biegalke and Geballe, 1991).

Plasmids

The plasmids described below were constructed for the identification of the DNA domain of the UL34 proteins. Various portions of the UL34 open reading frame were inserted into in pBS$^+$ (Stratagene, La Jolla, CA), with expression of the UL34 sequences transcribed under the control of the T7 promoter. pBJ384 contains the entire early UL34 open reading frame (LaPierre and Biegalke, 2001). The following plasmids were used to express portions of the UL34 open reading frame using in vitro transcription/translation reactions. pBJ501 was constructed from pBJ384 by digestion with *Sph*I followed by recircularization. pBJ544 and pBJ236 were constructed from pBJ384 by digestion with *Eco*RV and *Hin*dIII or *Stu*I and *Sal*I, respectively, followed by Klenow treatment and recircularization. The following plasmids were generated by polymerase chain amplification of the UL34 open reading frame using the oligonucleotides listed in Table 2: pBJ567, oligonucleotides

Table 2
Oligonucleotides used for plasmid construction

Oligonucleotides	
219	5′ CTCGTCGACTTAAATACACAACGGGGTTATGG 3′
269	5′ AAGGATCCTCATGAACTTCATCATCACC 3′
270	5′ AAGGATCCTCATGAACTTCATCATCACC 3′
297	5′ CGTCACCAAGAAACGCTATATATTCCGGAGGACCGTGCAGTCCACACGC 3′
298	5′ GCGTGTGGACTGCACGGTCCTCCGGAATATATAGCGTTTCTTGGTGACG 3′
299	5′ CGCTATATATTCCGGAGGACTGTCCTCCGCACACGCTACTTCTCAGCG 3′
300	5′ CGCTGAGAAGTAGCGTGTGCGGAGGACAGTCCTCCGGAATATATAGCG 3′
303	5′ AAGTCGACTGGAAGAGCATGCGGTAG 3′
304	5′ GGGAATTCATCATGGGCTCGATTTCCAAAGCG 3′
311	5′ CGTCTAGAGGATCCACTTCTCCAACGACGATTC 3′
314	5′ AAGGATCCGCAACAGCAACCTCTTCG 3′
316	5′ CCGGAATTCGAGATGCGTGACAACGTG 3′
319	5′ CGCGTCGACTTAGAGGTCCGTCTTCTTC 3′
320	5′ CGCGTCGACAAACAGCTCCTCTTCTTCC 3′
321	5′ CGCGTCGACATCCAAACCTGAGTTCATC 3′
326	5′ AAGGATCCAGATGCGTGACAACGTGG 3′
346	5′ ACGGTACGCGCCGAAGTCGCGAAGAAGCTG 3′
347	5′ CTTCGGCGCGTACCGTACCCTTGTAC 3′
348	5′ GAAGGCAAGAAGAACGCGTTGCTGAAGC 3′
349	5′ TTCTTCTTGCCTTCGGCGCGTACCG 3′
354	5′ CGTCCCCGCACACGCGCCGCGGCTAGCGAGCAAAAGCCC 3′
355	5′ CGCGTGTGCGGGGACGACGGGCGTTGC 3′
356	5′ CCGAAGAAAACGAGGCTGCAGAAGAGGAGCTG 3′
357	5′ CCTCGTTTTCTTCGGGCTTTTGCTCGTG 3′

Underlined letters indicate the position of restriction enzyme sites.

303 and 304; pBJ576, oligonucleotides 219 and 311; pBJ587, oligonucleotides 316 and 321, pBJ588, oligonucleotides 219 and 304; and pBJ615, oligonucleotides 316 and 303. The resulting amplimers were digested with *Eco*RI and *Sal*I and inserted into *Eco*RI/*Sal*I digested pBS$^+$ (Stratagene).

The following reporter gene plasmids were used in transient transfection assays. pEQ3 is a promoterless plasmid that contains the *lacZ* gene (Biegalke and Geballe, 1990), pBJ171 expresses the *lacZ* gene under the control of the US3 promoter and transcriptional repressive element (tre; Biegalke, 1995) and pBJ555 expresses the *lacZ* gene under the control of the US3 promoter and a *GAL4* DNA-binding site. pBJ555 was constructed by mutating the *tre* in pBJ171 using the QuickChange XL site-directed mutagenesis kit (Stratagene). Two rounds of mutagenesis were used to generate pBJ555; the first round used oligonucleotides 297 and 298 resulting in intermediate plasmid pBJ554; the second round of mutagenesis used pBJ554 as a template and oligonucleotides 299 and 300 to generate pBJ555.

The following protein expression plasmids were used in transfection assays to measure the effects of UL34 on reporter gene expression. pEQ276 expresses IE1 and IE2, pEQ273 expresses IE1 and pEQ326 expresses IE2 (Biegalke and Geballe, 1991). pBJ503 transcribes the UL34 open reading frame in an anti-sense direction (LaPierre and Biegalke, 2001).

Portions of the UL34 open reading frame were expressed as fusion proteins with the *GAL4* DNA binding domain. pBJ538 expresses the *GAL4* DNA-binding domain under the control of the mIE promoter and was constructed from pBJ201 (Biegalke, 1997). pBJ201 was modified to remove the unique *Bam*HI site by digestion, Klenow treatment and ligation, resulting in pBJ531. A DNA fragment containing the *GAL4* DNA-binding domain was isolated from pGBT9 (Clontech Laboratories, Palo Alto, CA) using *Hin*dIII and inserted into the *Hin*dIII site of pBJ531. UL34 fragments were prepared by polymerase chain reaction (see Table 2 for sequences of the oligonucleotides); the resulting amplimers were digested with *Bam*HI and *Sal*I and inserted into the *Bam*HI and *Sal*I sites of pBJ538. pBJ569 was constructed using oligonucleotides 269 and 219; pBJ577 with 311 and 219; pBJ578 with 314 and 219, and pBJ593 with 326 and 319. pBJ641, pBJ642 and pBJ653 were constructed by *Mlu*I/*Sal*I, *Stu*I/*Sal*I or *Eco*RV/*Sal*I digestion, respectively, of pBJ569 followed by Klenow treatment and ligation. pBJ623, 627, 629 and 630 contain specific point mutations inserted using the Gene Tailor Site-Directed mutagenesis kit (Invitrogen, Carlsbad, CA) and the indicated oligonucleotides: pBJ623, oligonucleotides 354 and 355; pBJ627, oligonucleotides 346 and 347; pBJ629, oligonucleotides 348 and 349; and pBJ630, oligonucleotides 356 and 357. The open reading frames of the UL34 expression plasmids were sequenced.

Electrophoretic mobility shift assays (EMSAs)

EMSAs were performed essentially as described, synthesizing UL34 proteins by in vitro transcription/translation reactions and assaying the ability of the resulting proteins to retard the migration of a radiolabeled DNA fragment containing the UL34 binding site in the US3 gene, the *tre* (LaPierre and Biegalke, 2001).

Acknowledgments

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