Transcription factor USF, expressed during the entire phase of varicella-zoster virus infection, interacts physically with the major viral transactivator IE62 and plays a significant role in virus replication

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The expression of the genes of varicella-zoster virus (VZV) is regulated by self-encoded viral as well as cellular transcription factors. A potential candidate with an ability to influence the transcription of VZV genes is USF (upstream stimulatory factor), which recognizes the consensus E-box motif. Quantitative RT-PCR and immunoblot assays indicate stable expression of both USF1 and USF2 throughout infection. It was also found that USF binds to a variety of E-boxes (consensus and closely related motifs) within the promoters of ORF 8/9 (two elements), ORF 22 and ORF 67. Co-immunoprecipitation experiments and His-tag protein affinity pull-down assays indicate that a direct physical interaction occurs between USF and the major virus transactivator IE62. To study the general effects of USF in the replication of VZV, a cell line expressing a dominant–negative form of USF (A-USF), which inhibits binding of USF to its recognition sites, was created. A significant decrease in virus replication was detected when this cell line was infected with cell-free virus, indicating that USF is an important cellular factor that regulates the transcription of VZV genes.

INTRODUCTION

Varicella-zoster virus (VZV), a neurotropic alphaherpesvirus, is the aetiological agent of two distinct clinical exanthemas: varicella (chicken pox) and herpes zoster (shingles). While the general clinical picture of the disease is well defined, our knowledge on the molecular biology of VZV, especially in the fields of gene expression and regulation of transcription, is still limited.

VZV is the smallest of the alphaherpesviruses; its genome consists of approximately 125 kbp and contains at least 70 unique ORFs and three duplicated genes (ORFs 62–71, 63–70 and 64–69) (for an overview see Ruyechan & Hay, 2000). The VZV genome is packaged in a capsid of icosahedral shape, which is surrounded by the tegument. The tegument contains viral proteins that can initiate viral gene expression and DNA replication when the capsid enters the host cell. Capsid and tegument are enclosed by a lipid membrane envelope containing virus-encoded glycoproteins that are presumed to mediate cell entry. Following entry of the virus, uncoating, transport of the capsid to the nucleus and release of the genome into the nucleus, immediate–early (IE) genes are transcribed. Their products act as transregulatory proteins to orchestrate the expression of early (E) and late (L) genes as well as the replication of the genome (for an overview see Ruyechan & Hay, 2000; Sadzot-Delvaux et al., 1999).

The ORF 62 protein (IE62) is the predominant IE transactivator (175 kDa) in infected cells and is functionally similar to herpes simplex virus type 1 (HSV-1) ICP4 (Arvin et al., 1986; Forghani et al., 1990; Kinchington et al., 1992; Ng et al., 1994). IE62 can stimulate all VZV genes studied to date (Inchauspe & Ostrove, 1989; Moriuchi et al., 1994; Perera et al., 1992) as well as silencing its own transcription (Disney et al., 1990). Additionally, IE62 interacts directly with other viral and cellular factors, including VZV IE4 (Spengler et al., 2000), IE63 (Lynch et al., 2002), TBP (TATA-box-binding protein), TFIIB (Perera, 2000) and Sp1 (Ruyechan et al., 2003). Several mechanisms can be proposed by which IE62 recognizes and functions at different promoter elements. One of these potential mechanisms is to alter promoter binding capacities by interaction with other viral regulatory proteins, such as the products of...
ORFs 4, 10, 61 and 63. A second possibility could involve post-translational modification, such as phosphorylation by viral and cellular kinases (Ng et al., 1994; Kenyon et al., 2001). A third would involve cellular transcription factors as carriers or recruiters, which would allow IE62 to bind promoters indirectly at or near the cis elements corresponding to the binding sites of those factors.

In support of this last hypothesis, there is an increasing number of reports that demonstrate the participation of common cellular transcription factors in the regulation of VZV-encoded genes. The GC-box binding transcription factor Sp1 is implicated in the regulation of the expression of the viral glycoprotein E (gE) by substituting for TBP to initiate transcription (Rahaus & Wolff, 2000), as well as its physical interaction with the viral protein IE62 (Ruyechan et al., 2003). Kinchington et al. (1994) described the presence of GC-rich cis elements within the ORF 4 and ORF 63 promoters as well. A further cellular transcription factor of importance for the regulation of VZV genes is the activator protein 1 (Ap1). Ap1 is composed of members of the Jun, Fos and ATF family, forming hetero- and homodimers (Angel & Karin, 1991), and binds Ap1/TRE or ATF/CRE elements localized in a large number of VZV promoters. A knockout of Ap1 results in a significant decrease in VZV replication (Rahaus & Wolff, 2003).

Another factor of potential importance for the regulation of VZV genes is the transcription factor USF (upstream stimulatory factor). USF, a helix–loop–helix (HLH) transcription factor, was first identified because of its participation in the transcription of the adenosivirus major late gene (Carthew et al., 1985; Miyamoto et al., 1985; Sawadogo & Roeder, 1985). USF recognizes a CACGTG motif (E-box) (Sawadogo & Roeder, 1985), which includes the canonical CANNTG recognition sequence of HLH transcription factors (Murre et al., 1989). Two different polypeptides, with apparent molecular masses of 43 and 44 kDa – USF1 and USF2, respectively – have been shown to be responsible for USF activity. USF1 and USF2 derive from differential splicing as well as alternative utilization of translation start sites and polyadenylation signals (Sirito et al., 1994) and showed identical DNA-binding properties (Sawadogo, 1988; Sawadogo et al., 1988). In mammals, USF plays an essential role during embryonic development. Binding sites have been found in the promoters of a variety of cellular and viral genes, including VZV. Meier et al. (1994) reported that USF co-operates with VZV IE62 to activate the bi-directional promoter of ORF 28/29, and Ito et al. (2003) showed that USF and Sp1 are essential factors for the transregulation of the AUS element within the gI promoter.

In this report, we provide evidence that mRNA as well as proteins of both forms of USF, USF1 and USF2, remain present in VZV-infected cells during the entire phase of infection. We show that USF recognized various subtypes of E-boxes present in VZV promoters and demonstrate a direct interaction of USF with IE62. Additionally, inhibition of USF DNA-binding activity resulted in a significant decrease in virus replication, indicating that USF is one of the key players in the regulation of VZV-encoded genes.

**METHODS**

**Cells and viruses.** Mewo cells (ECACC, 93082609) were grown at 37°C in a 5% CO2 atmosphere in Earle’s modified Eagle’s medium supplemented with 10% foetal calf serum, 1% penicillin/streptomycin and 1% non-essential amino acids. VZV strain Ellen (ATCC, VR-386) was propagated by passage of infected cells showing 70–80% cytopathic effect (CPE) onto uninfected monolayers. Cell-free virus was prepared as described previously (Rahaus & Wolff, 2003).

**Development of a dominant-negative USF cell line.** The plasmids pRC/CMV-A-USF566 and pRC/CMV566 (kindly provided by C. Vinson) (see also Krylov et al., 1994, 1997; Qyang et al., 1999) were used to develop a stable cell line expressing a dominant-negative USF mutant as well as the corresponding cell line control. Mewo cell monolayers at 80% confluence in 12-well plates were transfected with 1.5 µg Poul-linearized plasmids using Metafectene (Biontex), according to the supplier’s instructions. At 48 h post-transfection, cells were trypsinized, seeded in 60 mm dishes and grown in culture medium supplemented with 1200 µg G418 ml–1. After 7 days, the concentration of G418 was reduced to 250 µg ml–1. Individual colonies were isolated and amplified 2 weeks after transfection. The transcriptional activity of the newly inserted plasmid was analysed by PCR amplification of a 738 bp fragment of the neomycin gene.

**RNA isolation and reverse transcription.** Confluent monolayers of Mewo cells were infected with VZV and harvested at 0, 2, 4, 6, 8, 10, 12, 24 and 48 h post-infection (p.i.). Isolation of total RNA as well as RT-PCR were performed as described previously (Rahaus & Wolff, 2003).

**Standard and quantitative PCR analyses.** cDNA obtained by reverse transcription was used for PCR amplification of USF1, USF2, VZV ORF 63 and cellular pyruvate dehydrogenase (PDH). Primer sequences are shown in Table 1. The PCR mixture contained 500 ng template DNA, 50 pmol each of primer, 100 µM dNTP, 3 mM MgCl2, 16 mM (NH4)2SO4, 67 mM Tris/HCl (pH 8.8), 0.1% Tween-20 and 2.5 U BioTherm DNA polymerase (Genecraft). The thermocycler conditions for amplification of ORF 63 were as described previously (Rahaus & Wolff, 2003). The resulting product of 326 bp (ORF 63) was separated on a 1.6% agarose gel.

For quantitative PCR analysis of transcript levels of USF1 and USF2, different amounts of the respective internal standard (1 × 105–1 × 108 molecules) were used. One µg cDNA was used for the amplification of the USF1 and USF2 fragments in 30 and 28 cycles, respectively; the reaction mixture was identical to that given above and the primer sequences used are shown in Table 1. The thermocycler conditions were: 4 min at 94°C (initial denaturation), followed by 30 × at 94°C, 30 s at 58.1°C (USF1) and 61.5°C (USF2) and 1 min at 72°C. Resulting products were 415 bp (USF1) and 405 bp (USF2) in length. Internal standards were designed using the hybrid primer technique (Forster, 1994) and resulted in truncated fragments of 325 bp (USF1) and 300 bp (USF2). Both internal standards differ from their origin gene fragments in length only and were re-amplified using the respective primer pairs shown in Table 1. PCR products were separated on 1.6% agarose gels. The densitometric evaluation of the resulting signals were carried out using the MOLECULAR ANALYST software, version 1.5 (Bio-Rad).

As a control, a part of the gene encoding cellular PDH was amplified as described earlier (Rahaus & Wolff, 2003).
Table 1. Primers and oligonucleotides used for PCR and as EMSA probes

<table>
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<th>Target</th>
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<td>GGGGGCCAGGTGATGTA</td>
<td>5'→3'</td>
<td>PCR</td>
</tr>
<tr>
<td></td>
<td>TCCGGGGAGCTCGTAcTTC</td>
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<td>PCR</td>
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<td>GGAGCAGGGACCCAGAAAGAAGAC</td>
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<td>PCR, internal standard</td>
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<td>EMSA probe</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>E-box 67–AUS</td>
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<td></td>
<td>ATGTCTCAGTCGGGTAATGTGTT</td>
<td>3'→5'</td>
<td>EMSA probe</td>
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Immunoblotting and immunofluorescence studies. Immunoblotting of protein extracts of uninfected and infected (48 h p.i.) Mewo cells was done as described previously (Rahaus & Wolff, 2000). Detection of USF1 or USF2 was carried out using polyclonal antibodies (dilution 1:500 or 1:1000, respectively, in PBS) (Santa Cruz). Polyclonal antibodies directed against VZV IE4 and p29 were diluted 1:1000 and 1:7000, respectively. Detection of gE was done using a monoclonal antibody (dilution 1:1000 in PBS) (Biosdesign International), followed by reaction with an alkaline phosphatase-conjugated secondary antibody (dilution 1:1000 in PBS) (Santa Cruz) and NBT/BCIP (ICN). Immunofluorescence studies were performed as described by Rahaus & Wolff, (2000).

Electrophoretic mobility shift assay (EMSA). Preparation of nuclear extracts was done as described by Andrews & Faller (1991). EMSAs were performed in a total volume of 20 µl with 10 µg of nuclear extract, which was incubated in a reaction mixture containing 5% glycerol, 50 mM NaCl, 10 mM Tris/HCl (pH 7.5), 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM DTT, 1 µg poly(dI: dC) as a non-specific competitor and 2 x 106 cpm. [32P]ATP-labelled probes (double-stranded oligonucleotides, Table 1). Supershifts were performed by the addition of 2 µg USF-specific antibody subsequent to the addition of the probe. After incubation for 25 min, DNA-protein complexes were resolved on a 4-7% polyacrylamide gel in 0-5 x TBE and detected by autoradiography.

Co-immunoprecipitation. Protein extracts for immunoprecipitations were prepared by lysing confluent monolayers of cells with RIPA (1 x PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0-1% SDS, 100 µM PMSF ml⁻¹, 1 mM orthovanadate and 20 µg leupeptine ml⁻¹). Lysates were passed through a 22-gauge needle, incubated on ice for 45 min and centrifuged for 10 min at 10000 g at 4°C. Supernatants were used for immunoprecipitations. Protein lysates (~500 µg protein) were pre-cleaned using 0.25 µg rabbit IgG ml⁻¹ and subsequently incubated with 10 µg rabbit polyclonal USF2 antibody for 2 h. Twenty µl of protein-A/G-plus agarose (Santa Cruz) was added and incubation was continued overnight. Following incubation, the reaction mixtures were centrifuged and the beads were washed three times with PBS and 1% Tween-80. Afterwards, samples were resuspended in 4 x SDS-PAGE loading buffer and boiled for 6 min. Samples were analysed on 6 and 12-5% SDS-PAGE gels and transferred to nitrocellulose membranes to detect IE62 and USF, respectively. The antibodies used for Western blot analyses were rabbit polyclonal α-USF2 antibody and rabbit polyclonal anti-USF2 antibody (Spengler et al., 2000).

His-tag protein affinity pull-down assay. Recombinant IE62 was expressed from a recombinant baculovirus and purified from infected S21 cells as described previously (Spengler et al., 2000). Cultures of untransformed *Escherichia coli* (DH5α), *E. coli* DH5α transformed with pH2F plasmid, expressing His-AUSF1 (aa 106–310), and *E. coli* BL21(DE3) pLYsS transformed with pET16b/His-RPA plasmid, expressing the His-tagged RPA14 subunit and untagged RPA32 subunit, were induced with 0.1 mM IPTG. After a growth period of 3 h, cells were pelleted and resuspended in 20 ml lysis buffer (20 mM HEPES, pH 7.9, 500 mM KCl and 10 mM imidazole). Cells were lysed on ice by sonication and subjected to centrifugation at 10000 g for 10 min at 4°C. Supernatants were aliquoted and stored at −80°C.

In the affinity pull-down assays, 100 µl 50% ProBond resin (Invitrogen) was transferred to Eppendorf tubes and washed with 400 µl cold, sterile dH2O. After equilibration with 400 µl lysis buffer, the ProBond beads were mixed with 400 µl of one of the *E. coli* lysates for 1 h with gentle shaking at 4°C. The beads were then washed twice with 800 µl wash buffer I (20 mM HEPES, pH 7.9, 500 mM KCl and 20 mM imidazole) and twice with 800 µl wash buffer II (20 mM HEPES, pH 7.9, 100 mM KCl and 20 mM imidazole). Next, 50 µg recombinant IE62 was incubated with the washed beads in 200 µl wash buffer II for 2 h at 4°C with gentle shaking. After washing twice with 800 µl wash buffer II and twice with 800 µl wash buffer III (20 mM HEPES, pH 7.9, 100 mM KCl, 40 mM imidazole and 0-1% Tween-20), beads were boiled in 60 µl 2 x SDS-PAGE sample buffer. The supernatants were then collected by low-speed centrifugation. Equivalent amounts of the supernatants were separated on 10 and 15% SDS-PAGE gels and either probed for the presence of IE62 by Western blot analysis using a polyclonal anti-IE62 antibody or stained with Coomassie blue to assess the levels of recombinant His-tagged proteins present as well as background levels of non-specifically bound bacterial proteins.
Haematoxylin and eosin staining. Cells were fixed in 4% paraformaldehyde for 30 min and stained with Mayer’s haemalum solution (Merck) and eosin Y (Merck), as described previously (Rahaus & Wolff, 2003).

RESULTS

Quantification of USF1 and USF2 mRNA in VZV-infected cells

Numerous VZV promoters contain consensus E-boxes and closely related motifs, to which the cellular transcription factors USF1 and USF2 can bind (Meier et al., 1994; Ruyechan et al., 2003; P. R. Kinchington, personal communication), suggesting that USF plays a significant role in VZV gene regulation. To clarify whether the transcription of USF1 or USF2 was affected by VZV infection, we performed quantitative RT-PCR studies. Quantification of total RNA isolated from Mewo cells at 0, 2, 4, 6, 8, 10, 12, 24 and 48 h p.i. was done by co-amplification of the original USF1 or USF2 fragment and $1 \times 10^4$–$1 \times 10^8$ molecules of the corresponding internal standards. Data were obtained by densitometric evaluation of three independent time-courses.

Transcription of both USF1 and USF2 was found to remain relatively stable during the entire infection. In the case of USF1, mRNA levels decreased slightly at 2 h p.i. but at 8 h p.i. returned to the levels found at 0 h p.i. (Fig. 1A). No significant changes were observed for the USF2 transcript (Fig. 1B). VZV ORF 63 mRNA was also amplified to monitor infection. To verify that the RNA samples did not contain any DNA contamination, all samples were analysed by PCR amplifying cellular PDH. PDH PCR was designed such that the region amplified contained an intron in the case of DNA, resulting in a fragment of 180 bp, whereas an amplification of mRNA, in which the intron is removed after processing, would result in a product of 104 bp (Rolfs et al., 1992). As shown in Fig. 1(C), only the 104 bp fragment was observed. As an additional control, PCRs on all samples were performed without RT and showed no evidence of DNA contamination (data not shown).

Detection of the USF1 and USF2 proteins and binding activity to a consensus E-box during infection

To verify the production of USF proteins in VZV-infected cells, protein extracts of mock-infected and infected Mewo cells (48 h p.i.) were used for Western blotting. Specific bands for both forms of USF, USF1 (43 kDa) and USF2 (44 kDa), were detected (Fig. 2A). As a control, the presence of VZV gE was also determined and the corresponding band of 85 kDa was found only in infected cell extracts. Immunofluorescence studies showed that the USF proteins were not present solely in uninfected areas of the monolayer cell cultures used to prepare protein extracts. In areas characterized by clearly visible CPE discovered by light

![Fig. 1. Quantitative RT-PCR analyses of USF1 (A) and USF2 (B) mRNA levels in cells at 0, 2, 4, 6, 8, 10, 12, 24 and 48 h p.i. with VZV. For each time-point, a series of PCR analyses was performed using increasing molecule numbers of the respective internal standard ($1 \times 10^4$–$1 \times 10^8$ molecules). Intensities of resulting bands after gel electrophoresis were evaluated densitometrically followed by calculations using MOLECULAR ANALYST software (Bio-Rad). All data resulted from an evaluation of three independent series. (C) Control experiments: analyses of VZV ORF 63 mRNA confirmed the infection of the cells and RT-PCR of cellular PDH confirmed the quality and purity of the RNA used. PDH PCR was designed in a way that the amplified region contained an intron in the case of DNA, resulting in a fragment of 180 bp (lane C2), whereas an amplification of purified mRNA, in which the intron was removed after processing, resulted in a product of 104 bp (lane C1).](image-url)
microscopy, both VZV gE and USF1 (or USF2) were observed in the same cells (data not shown). These results provide evidence for the availability of USF during VZV infection. To confirm these findings, Western blot analyses were done to detect the presence of USF1 and USF2 proteins at the same time-points analysed for the presence of the corresponding mRNAs. At all time-points tested, USF1 as well as USF2 were clearly detectable without any significant variation. Virus infection was confirmed by analysis for the presence VZV ORF 63 was determined as an infection control.

Fig. 2. Detection of the USF1 and USF2 proteins in infected cells by Western blotting. (A) The specific bands of USF1 (43 kDa) and USF2 (44 kDa) were found in the nuclear extracts of uninfected and VZV-infected Mewo cells. As a control, VZV gE (85 kDa) was also revealed in the protein extract of infected cells. (B) Both USF1 and USF2 were detected in infected cells during the entire phase of infection tested (0–48 h p.i.); the presence VZV ORF 63 was determined as an infection control.

DNA-binding activity was detected with all binding sites (Fig. 3B). As deduced from the intensity of the signals detected, the consensus element B in the ORF 8/9 promoter showed the strongest binding reaction. In contrast to this, the binding activity of element A was low. Band intensities observed with the consensus element (positive control), and the ORF 22 and ORF 67 promoter E-boxes, were relatively similar. The additional complex observed with the ORF 67–AUS probe is likely to be due to binding of Sp1 or Ap1 in addition to USF. To demonstrate that the complexes detected were made up of USF, supershifts were performed using a probe derived from the ORF 28/29 promoter, whose consensus E-box is known to bind USF (Meier et al., 1994). A clear shifting of the signal was detected (Fig. 3C).

Physical interaction between USF and IE62

Meier et al. (1994) showed that the major viral transactivator protein IE62 co-operates with USF to transactivate the ORF 28/29 promoter. Additional data describing a reduction in IE62-mediated transactivity in HeLa cells by a dominant–negative form of USF (Qyang et al., 1999) lead us to the hypothesis that IE62 and USF interact physically with each other. To investigate this, co-immunoprecipitation experiments were performed.

We immunoprecipitated USF from a VZV-infected Mewo
cell lysate using an USF-specific antibody. Western blot analysis showed that both USF and IE62 were present in the immunoprecipitates, implying that IE62 and USF are also associated in a complex inside infected cells (Fig. 4A, B). No binding of either protein was observed in control experiments using beads alone.

To investigate the possibility of a direct physical interaction between USF and IE62, His-Δ-USF (aa 106–310) present in transformed E. coli lysates was bound to ProBond beads and purified recombinant IE62 (Spengler et al., 2000) was added to the beads, which were then washed extensively and eluted by boiling. As controls, eluates from beads adsorbed with control E. coli lysates and E. coli lysates containing His-tagged RPA subunits were also assessed for their ability to interact with IE62. An IE62-specific band of 175 kDa was detected in the eluate of the His-Δ-USF1-bound beads (Fig. 4C, lane 3), whereas no such band was detected in eluates from the control lysate-bound beads and the one containing the RPA subunits (Fig. 4C, D, lanes 1 and 2). Coomassie blue staining following SDS-PAGE confirmed the presence of the RPA32 and RPA14 subunits and the tagged USF in the respective eluates (Fig. 4D). In summary, these results evidently demonstrate that VZV IE62 associates directly with USF both in vitro and in infected cells.

**VZV replication in infected cells expressing a dominant-negative USF mutant**

Having shown a direct interaction between IE62 and cellular USF, we investigated the general importance of USF for the replication of VZV. We created a Mewo-derived cell line that expressed A-USF, a dominant–negative form of this transcription factor. In this dominant–negative form, the DNA-binding domain is deleted and, consequently, after heterodimerization with endogenous USF, DNA binding is inhibited (Kim & Spiegelman, 1996; Krylov et al., 1994; MacGregor et al., 1996; Qyang et al., 1999). As a control, we created a cell line stably expressing the empty plasmid pRc/CMV566.
Confluent monolayers of normal Mewo, Mewo/566A-USF and Mewo/566-control cells were infected with cell-free VZV using an m.o.i. of 1 to avoid over infection that could potentially obscure knockout effects. Nuclear extracts prepared from these cells were used in EMSAs to monitor DNA binding to a consensus E-box (Table 1). Competition experiments to prove the specificity of the binding reaction were done using a 100-fold excess of unlabelled probe. With Mewo/566A-USF extracts, only an extremely weak binding signal was detected, indicating that the inhibition of DNA binding was almost complete, whereas with nuclear extracts of normal Mewo cells and Mewo/566-control cells, clear, readily detectable binding was observed (Fig. 5A). To investigate if this knockout of USF DNA-binding activity had any influence on VZV replication, normal Mewo, Mewo/566A-USF and Mewo/566A-control cells were infected with cell-free VZV as described above. At 30 h p.i., the presence of ORF 4 and ORF 29 proteins were determined by Western blotting. Since the IE4 promoter does not contain any USF-binding motif, no change in its expression was found. In contrast to this, ORF 29 expression is dependent on USF (Meier et al., 1994). As expected, only a very low amount of ORF 29 protein was detected (Fig. 5B). An additional control experiment, the expression of gE, as an example for the late steps in the VZV replication cycle, was examined by immunofluorescence microscopy. Quantification was achieved by counting gE-expressing cells. All experiments were repeated four times and the number of normal Mewo cells expressing gE after infection was set as 100 %. A significant reduction (to 52.9 %) in gE-expressing cells was observed in the Mewo/566A-USF cell line, whereas expression of gE in Mewo/566A-control cells was similar to that of the normal Mewo control (96.8 %) (Fig. 5C). To investigate the importance of USF for virus progeny, we analysed the formation of syncytia in newly infected cells to make sure that infectious virions were actually produced. Normal Mewo, Mewo/566A-USF and Mewo/566-control cells were infected with VZV. At 24 h p.i., cells were passaged onto the respective uninfected cells. Following incubation for 24 h, these cells were fixed and the formation of syncytia was visualized by haematoxylin and eosin staining and quantified. Experiments were done in triplicate; the number of syncytia present in the normal Mewo cells was set as 100 % (Fig. 5D). In Mewo/566A-USF cells, a 4-fold decrease in syncytia formation was found (21.1 %), whereas in the control cell line no significant change was obvious (95.2 %). Thus, inhibition of USF DNA-binding activity resulted in a dramatic reduction in virus replication, indicating that USF plays a significant role in the life cycle of VZV.

**DISCUSSION**

In this study, we analysed the effect of VZV infection on the expression of both isoforms of USF (USF1 and USF2). Data presented here provide clear evidence for an important function of USF in the virus life cycle.

A slight decrease in USF1 mRNA levels was observed in the very early phase of infection. We assume that this decline is a consequence of a cellular stress reaction to VZV infection. The fact that the mRNA level recovered to the level of that seen in uninfected cells, and that the mRNA level of USF2 was not influenced significantly, showed that USF is not affected directly by VZV-mediated vhs effects (Waterboer et al., 2002). Similar observations of uninfluenced or even slightly increased mRNA levels of the ubiquitous cellular transcription factors Sp1 and Ap1 in VZV-, HSV- or cytomegalovirus-infected cells have also been reported (Boldogh et al., 1990; Jang et al., 1991; Rahaus & Wolff, 2000, 2003). Both USF1 and USF2 were also present.

![Fig. 4. (A) Co-immunoprecipitation of USF and IE62 from protein lysates of infected Mewo cells. Detection of IE62 as a component of the complex precipitated with an USF antibody by Western blot. Lanes: 1, proteins derived from co-immunoprecipitation; 2, immunoprecipitation without primary antibody as control; 3, detection of IE62 from a protein extract at 10 h p.i. (B) Control experiment to detect USF in the precipitated complex; lanes were identical to those described in (A). (C) Direct physical interaction between VZV IE62 and USF. Western blot analysis for the presence of IE62 bound to HIS-tagged USF fragment and His-tagged control proteins (10 % SDS-PAGE). (D) Coomassie stained 15 % SDS-PAGE gel of material bound to the ProBond nicle resin. (C, D) Lanes: 1, eluate from control E. coli lysate; 2, eluate from E. coli lysate containing His-tagged RPA sub-units; 3, eluate from E. coli lysate containing His-tagged USF fragment.]
throughout infection without any significant variation on protein levels.

It is noteworthy that USF is not the only factor binding to the E-box sequence. This motif is also recognized by other transcription factors, such as c-Myc (Blackwell et al., 1990; Halazonetis & Kandil, 1991; Prendergast & Ziff, 1991) and Max/Myn (Blackwood & Eisenman, 1991; Prendergast et al., 1991). Additional analyses in our laboratory showed that c-Myc, which forms a heterodimer with Max, is no longer detectable in VZV-infected Mewo cells after a few h p.i., either at the level of mRNA or protein (data not shown), indicating (i) that c-Myc does not evade VZV-mediated vhs effects and (ii) that the functional complex of Myc/Max is not available in VZV-infected cells. Another E-box-binding complex, TFE3/TFEB (Beckmann et al., 1990; Carr & Sharp, 1990), was not detectable at transcript level in Mewo cells (data not shown). Data concerning the availability of Mad/Mxi heterodimers (Ayer et al., 1993; Zervos et al., 1993) in VZV-infected cells did not exist until now. Here, however, we demonstrated that USF evades VZV-mediated vhs effects and is present in infected cells throughout the entire phase of infection. Consequently, USF is available for the regulation of VZV-encoded genes. This hypothesis is substantiated firstly by our findings that USF is able to bind to various E-boxes identified within VZV promoters and

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**Fig. 5.** VZV protein expression and virus replication after inhibition of DNA-binding activity of USF. (A) Control experiment to prove the inhibition of USF DNA-binding activity in the Mewo/566A-USF cell line by EMSA. Competition experiments demonstrating the specificity of the binding reaction were done using a 100-fold excess of unlabelled probes. (B) In-system control experiments showing the unchanged expression of VZV IE4, which does not contain an USF-binding site in its promoter, and the strong reduction in the ORF 29 protein, whose expression depends on USF. (C) Relative amounts of VZV gE-expressing cells after infection of normal Mewo, Mewo/566A-USF and Mewo/566-control cells. In Mewo/566A-USF cells, expression of gE was reduced significantly. (D) Relative amounts of syncytia formation as a marker for virus progeny in VZV-infected normal Mewo, Mewo/566A-USF and Mewo/566-control cells. A dramatic reduction in syncytia formation was found in Mewo/566A-USF cells. Data from normal Mewo cells were set as 100%; error bars mean ± s, n = 4 (C) and n = 3 (D).
secondly by data from Meier et al. (1994) who showed that USF binds to an E-box element inside the bi-directional promoter of ORF 28/29 and stimulates its activity in co-operation with VZV IE62.

These results, together with the fact that co-transfection of Saos-2 cells with IE62 and a dominant–negative form of USF (A-USF) resulted in a strong reduction in IE62-mediated transactivation (Qyang et al., 1999), indicate a functional and possibly physical interaction between IE62 and USF. We investigated this latter possibility by immuno-precipitation using lysates of infected cells and found that USF and IE62 co-precipitate. Affinity pull-down assays with recombinant IE62 and USF demonstrated a direct interaction of these two factors in the absence of other viral and cellular proteins.

The inhibition of the DNA-binding activity of USF in a Mewo-derived cell line expressing a dominant–negative form of USF resulted in a dramatic decrease in the number of virus progeny. These data suggest that USF seems to be a key player in the regulation of expression of VZV-encoded genes and confirm reports from Ito et al. (2003) and Meier et al. (1994) investigating the promoters of gl and ORF 28/29, respectively. In both cases, it was shown that USF is involved in activation of these promoters. The fact that IE62 interacts physically with USF suggests that it may act as a carrier or recruiter of IE62.

IE62 is able to activate expression of all VZV genes investigated so far. However, it is not certain if IE62 recognizes a specific DNA sequence. Some studies suggest that IE62 can interact with multiple sequences and alters the structure of the bound DNA in a manner different from its HSV homologue, ICP4 (McKee et al., 1990; Tyler & Everett, 1993; Moriuchi et al., 1995). In contrast to this, at least 21 VZV promoters contain E-box motifs (Ruyechan et al., 2003; P. R. Kitchensgton, personal communication), at least two of which, the gl and ORF 28/29 promoters, are known to require both IE62 and USF for full activation.

In the experiments shown, there was still virus replication at a low level despite inhibition of USF DNA-binding activity. There are three possible explanations for this result. First, inhibition of USF DNA-binding activity seems to be not complete and a small amount of an active form of USF is left to participate in transactivation. Second, other E-box-binding proteins available in the infected cells are involved in transactivation. However, the reduction in syncytium formation using the Mewo/566A-USF cell line demonstrates clearly that USF is a major factor in trans-activation of VZV-encoded genes, even if other E-box-binding factors are present. Third, USF is not the only carrier/recruiter of IE62. Other cellular factors likely to be involved in these processes include Sp1, Ap1 and TBP. A large number of VZV promoters contain binding sites for Sp1 and Ap1, and TATA-boxes and TATA-like elements are present in all promoters examined thus far. Furthermore, reports describing the physical interaction of IE62 and Sp1 (Ruyechan et al., 2003), an inhibition of virus replication after an Ap1 knockout (Rahaus & Wolff, 2003) and an activation of VZV minimal promoters by IE62 based on TATA motifs (Perera, 2000) also support this theory.

Further studies have to be performed to elucidate these potential recruitment mechanisms to achieve a better understanding of the interaction of viral and cellular transcription factors and their orchestrated activation of VZV-encoded genes.

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Cellular USF plays a significant role in VZV replication.