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Functional interaction and colocalization of the herpes simplex virus 1 major regulatory protein ICP4 with EAP, a nucleolar–ribosomal protein

(EBER-associated protein/DNA–protein interaction)

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ABSTRACT The herpes simplex virus 1 infected cell protein 4 (ICP4) binds to DNA and regulates gene expression both positively and negatively. EAP (Epstein–Barr virus-encoded small nuclear RNA-associated protein) binds to small nonpolyadenylylated nuclear RNAs and is found in nucleoli and in ribosomes, where it is also known as L22. We report that EAP interacts with a domain of ICP4 that is known to bind viral DNA response elements and transcriptional factors. In a gel-shift assay, a glutathione *S*-transferase (GST)–EAP fusion protein disrupted the binding of ICP4 to its cognate site on DNA in a dose-dependent manner. This effect appeared to be specifically due to EAP binding to ICP4 because (*i*) GST alone did not alter the binding of ICP4 to DNA, (*ii*) GST–EAP did not bind to the probe DNA, and (*iii*) GST–EAP did not influence the binding of the α gene trans-inducing factor (αTIF or VP16) to its DNA cognate site. Early in infection, ICP4 was dispersed throughout the nucleoplasm, whereas EAP was localized to the nucleoli. Late in infection, EAP was translocated from nucleoli and colocalized with ICP4 in small, dense nuclear structures. The formation of dense structures and the colocalization of EAP and ICP4 did not occur if virus DNA synthesis and late gene expression were prevented by the infection of cells at the nonpermissive temperature with a mutant virus defective in DNA synthesis, or in cells infected and maintained in the presence of phosphonoacetate, which is an inhibitor of viral DNA synthesis. These results suggest that the translocation of EAP from the nucleolus to the nucleoplasm is a viral function and that EAP plays a role in the regulatory functions expressed by ICP4.

Infected cell protein 4 (ICP4) (1, 2), the major herpes simplex virus 1 (HSV-1) regulatory protein, is encoded by one of a set of genes (α) expressed immediately after infection; genes (β and γ) expressed later in infection require a functional ICP4 for their expression (3–5). ICP4 deletion mutants and temperature-sensitive mutants at nonpermissive temperature display a grossly reduced expression of β and γ genes (3–5).

ICP4 binds to DNA sites present in a number of HSV-1 gene promoters and has been reported to act as both a transactivator and a repressor (ref. 2 and references therein). ICP4 binds to consensus sites with high affinity and to sites without a recognizable consensus with low affinity (2). ICP4 repressor activity is associated with binding to high-affinity consensus sites located at the transcription initiation site of its own gene (α4) and of at least one more gene (ORF P), inasmuch as destruction of the DNA-binding sites by mutagenesis results in a significant increase in the accumulation of transcripts and protein (6, 7). However, the correlation between DNA binding and a detectable function holds only for the trans-repression but not for the trans-activating functions associated with ICP4. Other sites are present on β and γ gene promoters, but the role of these sites, which are recognized by ICP4 with high or low binding affinity but are not positioned at the gene transcription initiation site, is not well understood. The deletion of some of these sites does not have a significant effect on gene expression (2).

The central role of ICP4 in HSV-1 replication is not readily explained by its interaction with binding sites on the DNA, inasmuch as specific HSV-1 ICP4 mutants are able to trans-activate gene expression in the absence of DNA-binding activity (8). The repression of gene transcription by ICP4 has been variously attributed to its ability to cause DNA to bend at the binding site (9) or to the binding of the transcription factors TBP and TFIIB (10, 11). The mechanism by which ICP4 trans-activates gene expression is even less clear and leaves tenable the hypothesis that the trans-activating functions of ICP4 result from mechanisms that do not require direct, stable association of ICP4 with viral DNA. This hypothesis predicts that ICP4 may interact with additional proteins, different from those reported to date. In an attempt to identify such proteins, we used a yeast-based two-hybrid system to screen a HeLa cell cDNA library for cellular genes whose products interact with ICP4 (12).

We report here that ICP4 interacts with the EBER-associated protein (EAP), which was first identified because of its tight association with EBER, the small nonpolyadenylylated RNA produced in high amounts by Epstein–Barr virus (EBV) in infected cells (13). EAP was found to bind specifically a stem-loop structure within EBERs (14). The protein was later found to be L22, a ribosomal component whose primary sequence had not been defined (15). EAP–L22 normally resides in nucleoli and is associated with ribosomes. A recent study (16) on the RNA-binding properties of L22 suggests that it could bind the 28S ribosomal RNA in a highly conserved stem-loop structure resembling the binding site on EBERs. During EBV infection, a substantial portion of the nucleolar contingent of the protein relocates into the nucleoplasm and colocalizes with EBERs.

We also report that EAP interacts with a specific amino acid sequence of ICP4, that the interaction specifically displaces the binding of ICP4 to its cognate DNA sequence, and that late in infection EAP and ICP4 colocalize in discrete, clustered structures within the nucleus of the infected cell.

MATERIALS AND METHODS

The HSV-1(F) DNA sequence corresponding to amino acids 138–490 of ICP4 was cloned into the polylinker of pAS2 expression plasmid, a pGBT9 derivative (Clontech), between

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Abbreviations: HSV-1, herpes simplex virus 1; EBV, Epstein–Barr virus; EBER, EBV-encoded small nuclear RNA; EAP, EBER-associated protein; ICP4, infected cell protein 4; GST, glutathione *S*-transferase; αTIF, α gene trans-inducing factor.

the sites for *Bam*HI and *Sal*I. The DNA fragment was obtained by PCR amplification of a plasmid containing the full-length ICP4 gene (pRB3611) and was cloned in frame with the GAL4 DNA-binding domain as confirmed by DNA sequence analysis. The yeast strain HF7c was transformed according to the manufacturer's protocol (Matchmaker library, Clontech). Transformants were screened for interaction as determined by their ability to grow in minimal synthetic medium lacking histidine. About 150 colonies grew in the absence of histidine; of these colonies, 108 turned blue in the presence of 5-bromo-4-chloro-3-indolyl β-D-galactoside. Retransformation of the 108 plasmids yielded 47 positive clones, which tested negative for interaction with lamin or SNF1 genes fused to the GAL4 DNA-binding domain. None of the ICP4 constructs used in this study exhibited intrinsic trans-activating ability in the yeast-based two-hybrid system.

RESULTS

ICP4 Interacts with EAP in a Yeast-Based Two-Hybrid System. Evidence that ICP4 interacts with EAP emerged from analyses done in a yeast-based two-hybrid system. Transformants ($\approx 7 \times 10^5$) of a HeLa expression library were screened for interaction with the GAL4–ICP4 fusion after cotransformation in yeast. Of the 47 plasmids whose products interacted specifically with the ICP4 bait, 13 plasmids were partially sequenced and all of these were found to match completely the published sequence of EAP (13). The remaining 34 plasmids contained EAP DNA sequences as shown by hybridization with EAP DNA probe. Given this unexpected high number of EAP clones whose products interacted with ICP4, we investigated further the nature of the association between EAP and ICP4.

The ICP4 sequence used as bait in the yeast-based two-hybrid system was chosen because it contains a stretch of serines thought to be involved in protein–protein interactions and, adjacent to the stretch, the amino acids required for binding of ICP4 to its cognate DNA sequence (refs. 17 and 18; Fig. 1). To map more precisely the ICP4 domain interacting with EAP, DNA sequences encoding amino acids 138–490 were divided into three fragments (Fig. 1) and each fragment was cloned in frame with the GAL4 DNA-binding domain and transformed into yeast containing the plasmid expressing EAP. In this series of experiments, interaction of ICP4 sequences with EAP was observed only in yeast colonies transformed with the parent plasmid encoding amino acids 138–490 or the plasmid encoding amino acids 138–246 (Fig. 1).

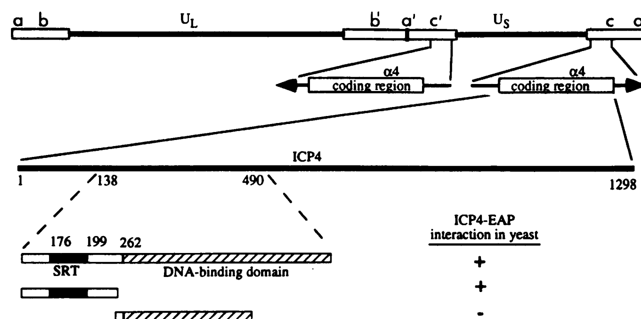

FIG. 1. Diagrammatic representation of the HSV-1 genome, α4 gene, and the ICP4 domains interacting with EAP in a yeast-based two-hybrid system. The thick lines represent unique sequences U_L and U_S. The rectangles represent the inverted repeat sequences *ab* and *b′a′* flanking U_L and *a′c′* and *ca* flanking U_S. The location and direction of transcription of the α4 gene are shown. Domains within amino acids 138–490 of ICP4 are indicated (SRT, serine-rich tract). A summary of the results of interactions between ICP4 sequences and EAP in the two-hybrid system is shown.

EAP Disrupts the Interaction of ICP4 with Its Cognate Site in HSV-1 DNA *in Vitro*. Evidence for direct physical interaction between ICP4 and EAP emerged from gel retardation studies. In this series of experiments, nuclear extracts of infected cells containing ICP4 were added to a DNA fragment containing the ICP4 cognate site, in the presence of glutathione *S*-transferase (GST)–EAP fusion protein or in the presence of

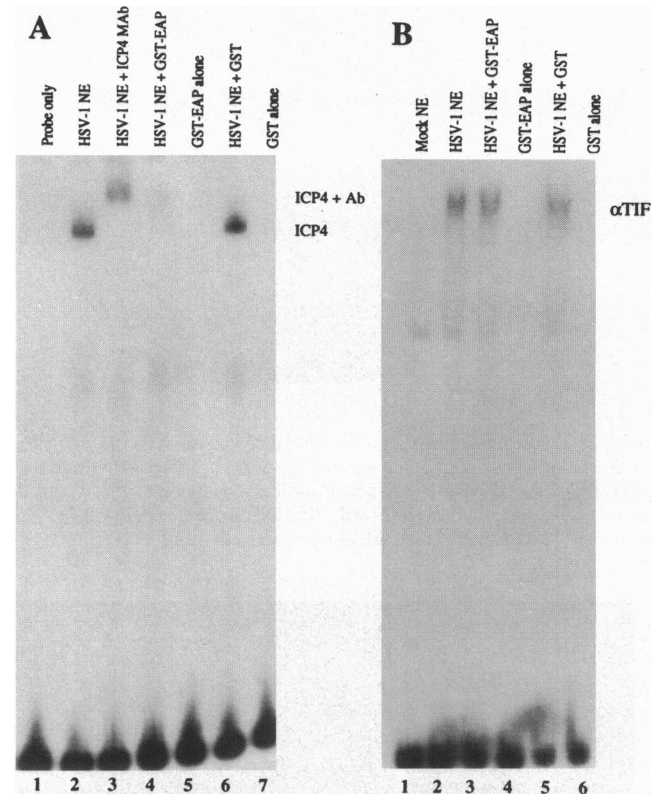

FIG. 2. Autoradiographic image of a gel retardation assay showing the interaction of ICP4 (*A*) or of αTIF (*B*) with their cognate sites in HSV-1 DNA in the presence and absence of EAP. Nuclear extracts (1 μg) from mock- or HSV-1(F)-infected cells were reacted with 2×10^4 cpm of probe DNA in 20 μl of a solution containing 20 mM Tris (pH 7.6), 50 mM KCl, 0.05% Nonidet P-40, 5% glycerol, 1 mM EDTA, 1 mg of bovine serum albumin (BSA) per ml, and 10 mM 2-mercaptoethanol for the ICP4 binding experiments or 25 μl of the same solution without BSA for αTIF binding experiments. The DNA probes were: a cloned 135-bp *Eco*RI–*Bam*HI fragment (pRB4018) that spans nucleotides −108 to +27 relative to the transcription initiation site of the α4 gene (19), dephosphorylated by alkaline phosphatase, and 5′-end-labeled with [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase; and a cloned 48-bp *Eco*RI–*Hin*dIII fragment (pRB606) of the α27 gene promoter containing an αTIF response element (20) and labeled with [α-^{32}P]dCTP in a Klenow enzyme fill-in reaction. Poly(dI·dC) or poly(dI)-poly(dC) (Pharmacia) was added for the ICP4 or αTIF binding assays, respectively, in 3-μg amounts to each reaction mixture as the competitor nucleic acid. The ICP4–DNA complexes were supershifted as originally described by Kristie and Roizman (19) by the addition to the reaction mixtures of the monoclonal antibody H943. Complexes were electrophoretically separated on nondenaturing acrylamide gels. (*A*) Lane 1, probe only; lane 2, probe plus 1 μg of infected cell nuclear extract; lane 3, same as lane 2 plus monoclonal antibody; lanes 4 and 6, probe, infected cell nuclear extract, plus GST–EAP (50 ng; lane 4) or GST (2 μg; lane 6), respectively; lanes 5 and 7, probe plus GST–EAP (50 ng; lane 5) or GST (2 μg; lane 7), respectively. Bands formed by ICP4–DNA complex or by ICP4–DNA complex plus the ICP4-specific monoclonal antibody are indicated. (*B*) Lane 1, probe plus 1 μg of mock-infected cell nuclear extract; lane 2, probe plus 1 μg of HSV-1-infected cell nuclear extract; lane 3, same as lane 2 plus GST–EAP (50 ng); lane 4, probe plus GST–EAP (50 ng) alone; lane 5, same as lane 2 plus GST (2 μg); lane 6, probe plus GST (2 μg) alone.

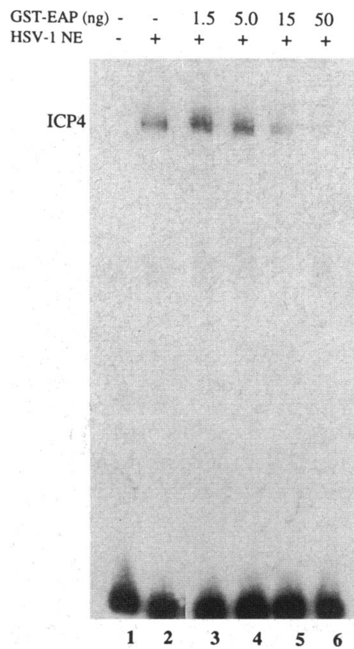

FIG. 3. Autoradiogram of a gel retardation assay. The same probe used in the experiments shown in Fig. 2*A* was reacted with HSV-1(F)-infected cell nuclear extracts (lane 2), plus increasing concentrations (1.5–50 ng; lanes 3–6) of affinity-purified GST–EAP. The mixtures were subjected to electrophoresis in nondenaturing polyacrylamide gels.

GST. The probe was a 135-bp *Eco*RI–*Bam*HI fragment extending from nucleotide −108 to +27 relative to the transcription start site of the gene and containing a high-affinity binding site for ICP4 (19). The identity of the ICP4–DNA complexes was verified by supershift of the complex by the addition of the ICP4-specific monoclonal antibody H640 (Fig. 2*A*, lane 3). The GST–EAP fusion protein produced according to published procedures (14) in *Escherichia coli* BL21 transformed with a pGEX-3X-EAP plasmid, extensively characterized (14) and generously provided by J. Steitz (Yale University, New Haven, CT), prevented the ICP4–DNA complex formation (Fig. 2*A*, lane 4) whereas GST alone had no effect on the complex (Fig. 2*A*, lane 6). Neither GST nor GST–EAP bound to the probe DNA in the absence of nuclear extracts from infected cells (Fig. 2*A*, lanes 5 and 7).

To study the specificity of this interaction, the effect of GST–EAP was tested on the binding of α gene trans-inducing factor (αTIF) to its DNA cognate site. αTIF requires a host factor, Oct-1, to bind to its high affinity cognate sites with the consensus GyATGnTAATGArATTCyTTGnGGG (20). The assay was set up as described (20) except that GST–EAP was added to the binding reaction in amounts known to displace completely the ICP4 binding to its cognate DNA site. GST–EAP did not affect the binding of αTIF and the host factors to DNA (Fig. 2*B*).

The Effect of EAP on the Binding of ICP4 to DNA Is Concentration Dependent. In this series of experiments, we tested the effect of different concentrations of EAP on the interaction of ICP4 with its DNA cognate site. As shown in Fig. 3, disruption of the interaction of ICP4 with its DNA-binding

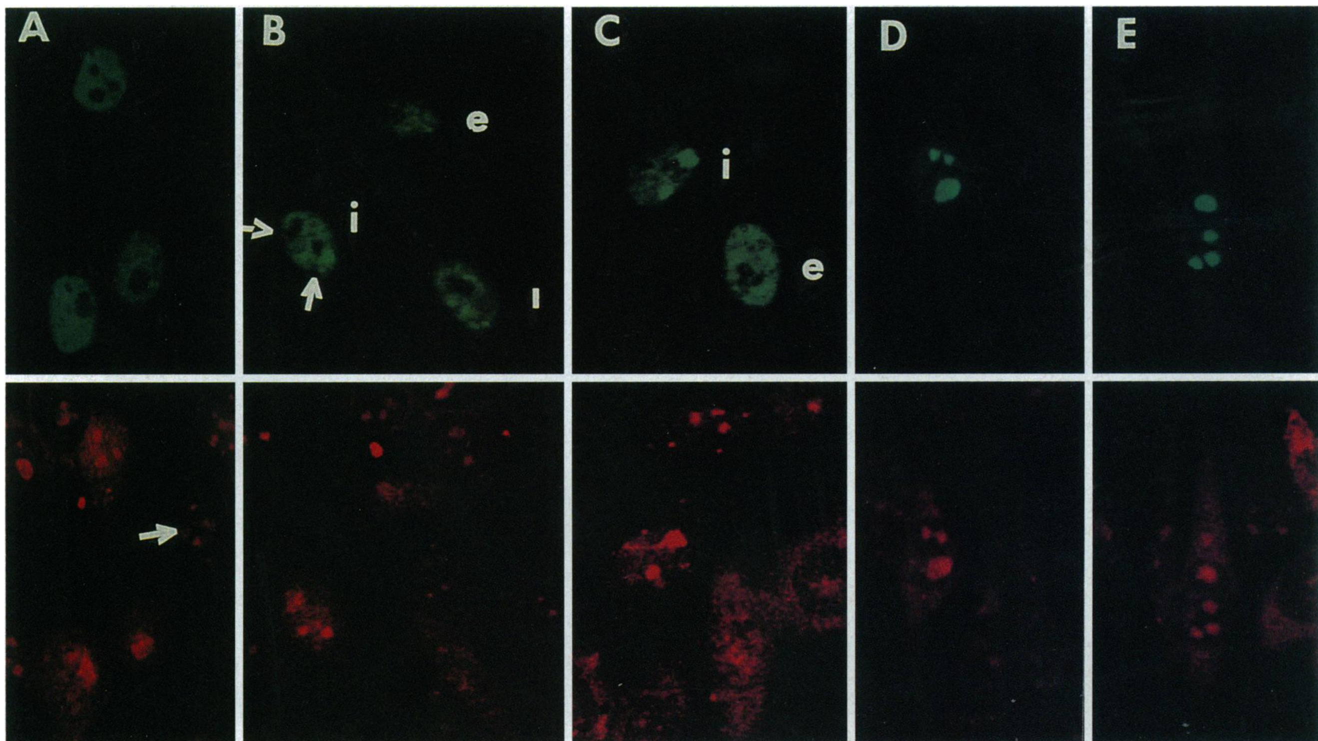

FIG. 4. Photomicrographs of infected HEp-2 cell cultures reacted with antibodies to GST–EAP and ICP4 and stained with secondary antibodies conjugated with Texas red and fluorescein isothiocyanate (FITC) (green). HEp-2 cells were seeded on microscope slides and infected with a HSV-1(F) mutant (R7032) deleted in the gene encoding the Fc receptor (gE). At appropriate times after infection, cells were fixed to the slides in methanol at −20°C for 20 min, blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h, and reacted first with a 1:500 dilution of a monoclonal antibody to ICP4 (H640; Goodwin Institute, St. Petersburg, FL) and a 1:100 dilution of a rabbit antiserum to EAP (a gift of J. Steitz) in PBS/0.1% BSA for 2–3 h. After rinsing, cells were reacted with a FITC-conjugated anti-mouse IgG and a Texas red-conjugated anti-rabbit IgG for 1 h. Slides were mounted in 10% PBS in glycerol and examined under a Zeiss confocal microscope. Images were processed with Zeiss software and printed in a CP210 Codonics (Middleburg Heights, OH) digital printer. (*A*) The arrow points to EAP localized in the nucleoli of a noninfected cell. (*B*) The upper arrow points to a cell with an intermediate phenotype, i.e., a nucleolus containing EAP located within a portion of the nucleus containing diffuse ICP4 whereas the lower arrow points to a site in the same nucleus in which both ICP4 and EAP colocalized in a dense structure. e, Early pattern; i, intermediate pattern. (Original magnification, ×1000.)

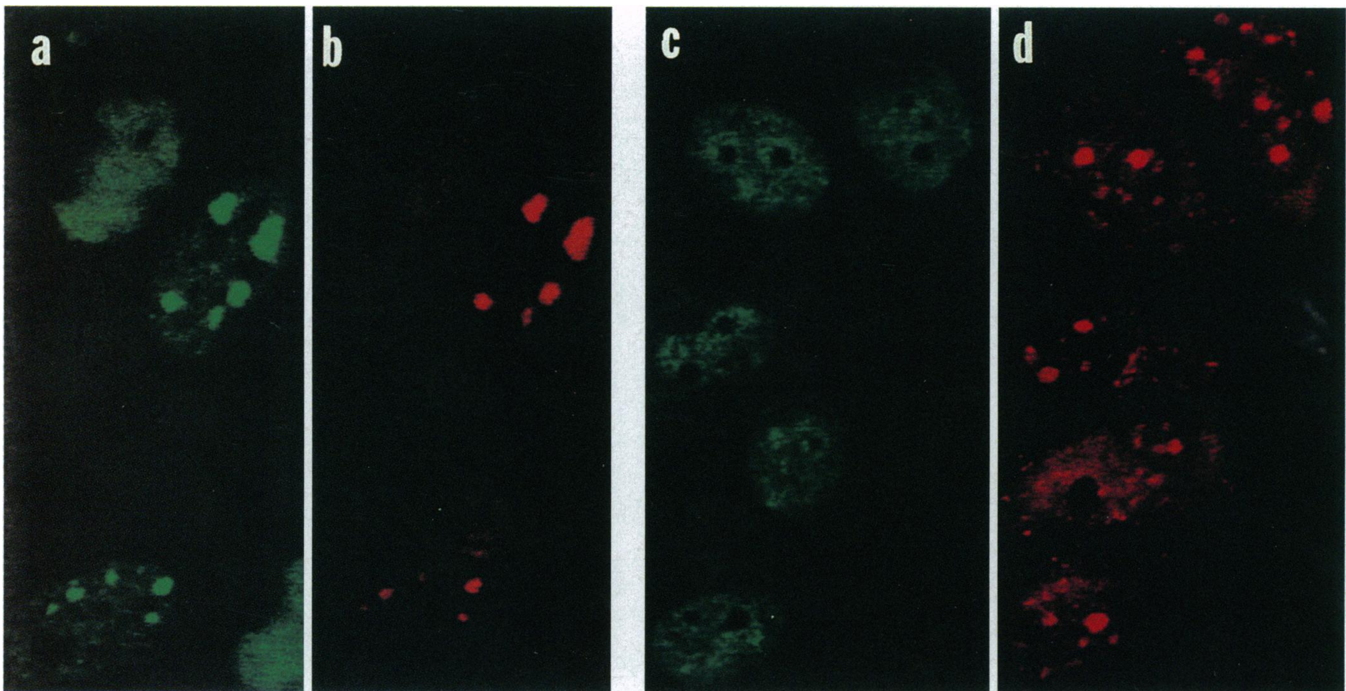

FIG. 5. Photomicrographs of HEp-2 cells analyzed by immunofluorescence. Cells were infected with a mutant (HSV-1 *ts*HA1) that carries a ts lesion in the gene encoding the single-stranded DNA-binding protein ICP8 and is DNA^- at 39.5°C, the nonpermissive temperature (22). HEp-2 cells were seeded on microscopy slides, infected, and maintained at either 37°C or 39.5°C for 18 h, and then reacted as described in the legend to Fig. 4 with the monoclonal antibody to ICP4 and the polyclonal antibody to EAP, except that the reaction was done in the presence of 10% pooled human serum to block the nonspecific background fluorescence caused by the viral Fc receptors. (Original magnification, ×2000.)

site by GST–EAP was found to be concentration dependent, inasmuch as addition of increasing amounts of GST–EAP gradually disrupted the interaction of ICP4 with its cognate DNA site (Fig. 3).

We conclude that effects of EAP are specifically due to its binding to ICP4 inasmuch as (*i*) GST alone did not alter the binding of ICP4 to DNA (Fig. 2*A*), (*ii*) GST–EAP did not bind to the probe DNA (Fig. 2*A*), and (*iii*) GST–EAP did not affect the formation of αTIF/host protein–DNA complexes (Fig. 2*B*).

ICP4 and EAP Colocalize in the Nucleus of Infected Cells. The purpose of these experiments was to determine whether ICP4 and EAP colocalize in infected cells. As mentioned, EAP is found in the cytoplasm and in nucleoli of uninfected cells whereas ICP4 localizes in nuclei but its distribution changes during the course of infection. A previous study (21) has shown that during early stages of infection ICP4 is dispersed throughout the nucleoplasm, whereas at later stages of infection ICP4 is tightly aggregated in dense nuclear structures within the nucleoplasm. To determine whether ICP4 and EAP colocalize, HSV-1-infected HEp-2 cells were reacted for immunofluorescence studies with an ICP4-specific monoclonal antibody and a polyclonal antibody to the GST–EAP fusion protein and then examined with the aid of a Zeiss confocal microscope. The results follow.

In uninfected cells, EAP was localized in structures previously shown to be nucleoli (ref. 15; arrow in Fig. 4*A*). At early stages of infection (Fig. 4*A* and cells labeled "e" in *B* and *C*), ICP4 was dispersed throughout the nucleus but excluded from structures containing aggregates of EAP and resembling the nucleoli of uninfected cells. Late in infection (Fig. 4 *D* and *E*), ICP4 and EAP colocalized in dense structures occupying only a portion of the infected cell nucleus. Occasionally both early and late patterns could be seen in the same cell (Fig. 4 *B* and *C*, cells labeled "i"). In these intermediate stage cells, EAP remained partially localized in discrete structures distinct from the dispersed ICP4 and partially colocalized with aggregated ICP4.

A Late Viral Function Is Required for ICP4–EAP Association. The purpose of this study was to determine the timing and requirements for the colocalization of ICP4 with EAP. HEp-2 cells were infected with an HSV-1 mutant (*ts*HA1) carrying a temperature sensitive ICP8 and thus defective for viral DNA synthesis at the nonpermissive temperature (39.5°C). Earlier studies (22) have shown that cells infected with this mutant and maintained at the nonpermissive temperature do not express γ_2 proteins whose synthesis is dependent upon the replication of viral DNA. In these experiments, infected cells were examined for the localization of ICP4 and EAP after 18 h of incubation at either the permissive (37°C) or nonpermissive (39.5°C) temperature. At this late time after infection and incubation at the permissive temperature, virtually all infected cells exhibited the "late" pattern shown in Fig. 4; that is, EAP and ICP4 colocalized in discrete, dense nuclear structures (Fig. 5 *a* and *b*). The distribution of ICP4 and EAP in cells incubated at the nonpermissive temperature was that of the "early" pattern; in most cells, EAP was localized in discrete structures whereas ICP4 was dispersed throughout the nucleus (Fig. 5 *c* and *d*). A few cells exhibited an "intermediate" pattern (Fig. 5) described above, but virtually none of the cells exhibited the late pattern seen in cells incubated at the permissive temperature (Fig. 5 *a* and *b*). The late pattern was not observed in cells infected and maintained in the presence of inhibitory concentrations of phosphonoacetic acid (a gift of Abbott, 300 μg/ml; data not shown), an inhibitor of HSV-1 DNA synthesis and late gene expression (reviewed in ref. 2).

From these results, we conclude that the relocation of EAP from the nucleolus to the dense nuclear structures is the function of one or more late (γ_2) genes whose expression requires viral DNA synthesis. It is conceivable that the formation of ICP4 clusters late in infection is also dependent on this function.

DISCUSSION

In this report, we show that EAP, a protein known to bind small RNA molecules, interacts with ICP4. The interaction of

EAP with a protein has not been previously reported. The salient points of this report are as follows:

(*i*) The colocalization of ICP4 and EAP supports the biochemical and genetic evidence for the interaction of these two proteins. Although the association of ICP4 and EAP in infected cells does not constitute evidence that the two proteins participate in a common function, the fact that they colocalize exclusively after the onset of virus DNA synthesis suggests that both proteins are involved in a series of functionally related events. The late function(s) required for the two partners to colocalize may involve (*a*) a posttranslational modification of ICP4 that renders it capable of binding EAP, (*b*) interaction of another viral gene product with EAP and/or ICP4, or (*c*) reorganization of nuclear compartments by viral products made late in infection.

(*ii*) EBERs were first detected by coimmunoprecipitation with La autoantigen by antibody from patients with lupus erythematosus (23). EBERs, by far the most abundant RNAs encoded by EBV (24), are small, nonpolyadenylylated nuclear RNAs that display extensive intramolecular base pairing, and share their La-binding property, as well as extensive portions of primary sequence, with virus-associated (VA) RNA of adenoviruses and U6 small cellular RNA (24). The function of EBERs and of EAP–EBER complexes is not known. EAP binds EBERs directly (13, 14). It has been suggested that EAP may transport EBERs to ribosomes, and it has been reported (25, 26) that EBERs substitute for adenovirus VA1 RNA in blocking the activation of interferon-induced PKR kinase. However, EBV lacking EBERs was shown to be no more sensitive to interferon than wild-type EBV (27). It has also been suggested that because U6 RNA base pairs with U4 RNA in particles required for *in vitro* splicing, EBERs could also play a role in this process (24). We consider it unlikely that EAP–ICP4 interaction is required for any of these functions in HSV-1-infected cells. This conclusion stems from the fact that in HSV-1-infected cells, phosphorylation of eukaryotic initiation factor 2α protein resulting from activation of PKR kinase is precluded by the viral protein $\gamma_1$34.5 (28), and splicing is specifically repressed by ICP27, a product of the gene α27 (29).

(iii) Two alternative hypotheses could account for the observed interaction of ICP4 with EAP. Late in infection transcription takes place from a relatively small number of concatemeric DNAs synthesized with a rolling circle mechanism (2). This would be consistent with the formation of a small number of tight aggregates of ICP4 within nuclei late in infection. The function of EAP may be to bind, through its interaction with ICP4, to nascent RNA and to expedite its transport from the transcription complex to the cytoplasm. Another hypothesis stems from the possibility that expression of late, or γ, genes after the onset of DNA synthesis may result from removal of repressors associated with viral DNA early in infection. It has been suggested that ICP4 could act as a repressor of γ gene expression since the ICP4 binding sites and *cis*-acting elements that control both repression and activation have been found in 5′-transcribed noncoding domains of late genes (reviewed in ref. 2). The translocation of EAP from nucleoli to transcriptional complexes containing ICP4 may initiate and account for the derepression of late gene expression.

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