Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 6347–6351, September 1988
Biochemistry

A herpesvirus trans-activating protein interacts with transcription factor OTF-1 and other cellular proteins

(transcription regulation/octamer transcription factors/protein–protein interactions)

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ABSTRACT **Immediate early genes of herpes simplex viruses contain one or more copies of the conserved TAATGARAT (where R is purine) DNA motif. A virus-encoded regulatory protein (Vmw65) is believed to stimulate transcription via this element, although the protein does not bind directly to DNA. Overlapping the TAATGARAT element in many cases is an octamer sequence (ATGCAAAT) that is involved both in transcription by RNA polymerases II and III and in adenovirus DNA replication. So far at least two proteins (OTF-1 and OTF-2) have been identified that bind to the octamer. We show that both affinity-purified OTF-1 and OTF-2 bind to the TAATGARAT sequence and that Vmw65 induces the formation of an additional complex that involves OTF-1 and that is further retarded in a band-shift gel assay. Complementation experiments involving addition of purified OTF-1 to nuclear extracts that have been depleted of endogenous OTF-1 show that at least one other cellular factor(s) is required for complex formation. This cellular factor may be involved in recognition of the GARAT sequence.**

Many promoters and enhancers contain the conserved sequence motif ATGCAAATNA commonly referred to as octamer or decanucleotide (1–3). Two transcription factors have been purified that both interact with this element and stimulate *in vitro* transcription through it. OTF-1 (ref. 4 and references therein) occurs in a wide variety of different cell types. It appears to be involved not only in the regulation of genes transcribed by RNA polymerase II but also in gene-specific transcription by RNA polymerase III (reviewed in ref. 5) and in adenovirus DNA replication (E. O. O'Neill, C. F. Fletcher, C. R. Burrows, N. Heintz, R.G.R., and T. J. Kelly, unpublished data). The factor OTF-2 (ref. 6 and references therein) has been found so far only in cells of the lymphoid lineage and is presumed to play a major role in the cell type-specific transcription of immunoglobulin genes.

The promoters of immediate early genes of herpes simplex viruses (HSV) contain one or several copies of a sequence element whose consensus reads TAATGARAT (where R is purine) (reviewed in ref. 7). This sequence motif has been implicated in the stimulatory response to a viral protein (8–14). The viral component has been identified as the tegument protein Vmw65 (also called VP16) (15) located between the viral membrane and the capsid structure. Despite its involvement in the transcriptional activation of TAATGARAT-containing promoters, Vmw65 itself does not appear to bind to DNA (16). However, earlier studies reported the binding of an unidentified cellular factor from uninfected HeLa cells to TAATGARAT (17, 18).

A similarity between the octamer motif and sequences overlapping many TAATGARAT elements (in the TAAT region) has been noted (18, 19). For example, the proximal element of the HSV-1 ICP0 gene has the sequence ATGCTAATGATAT matching in seven of eight the octamer consensus of ATGCAAAT. Therefore, the attractive possibility existed that the Vmw65 protein could be interacting with one of the OTF factors. While this work was in progress, promoter interactions of Vmw65 in conjunction with unidentified cellular factors were reported (20, 21). We demonstrate here that both affinity-purified OTF-1 and OTF-2 can bind to the TAATGARAT element and that OTF-1 (but not OTF-2) is capable of forming a DNA–protein complex with Vmw65. We also provide evidence that another cellular factor(s) is involved in the formation of this complex.

MATERIALS AND METHODS

Band-Shift Assays. Reaction mixtures (15 μl) contained, together with the protein samples, 2 fmol of ^{32}P-end-labeled probe, 1.5 μg of poly(dI-dC) in 8 mM Hepes, pH 7.9/60 mM KCl/2 mM EDTA/0.2 mM dithiothreitol/4 mM spermidine/100 μg of bovine serum albumin per ml/0.03% Nonidet P-40/4% Ficoll 400. After incubation for 30 min at 30°C reaction mixtures were applied to 4% polyacrylamide gels (acrylamide/bisacrylamide, 39:1; 0.25× TBE).

Nuclear extracts of HeLa cells were prepared as described (22). Usually 4 μg of nuclear extract protein was used per assay. Nuclear extracts of HSV-1-infected HeLa cells (a kind gift from S. Silverstein, Columbia University, New York) were prepared 5 hr after infecting cells at a multiplicity of infection of 20 plaque-forming units.

DNA Probes and Competitor Oligonucleotides. For most experiments, an oligonucleotide spanning positions −170 to −143 of the ICP0 gene of HSV-1 was used as a probe. The oligonucleotide ICP0 with the sequence

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GATCCCGTGCATGCTAATGATATTCTTTGGG
    GGCACGTACGATTACTATAAGAAACCCCTAG
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was cloned into the *Bam*HI site of pUC18, excised as an *Eco*RI/*Hin*dIII fragment, and labeled like all other probes at the 3′ ends with Klenow polymerase. Probe IgH contained the *Dde*I/*Hin*fI fragment of the murine immunoglobulin heavy-chain gene enhancer subcloned into pUC7 and excised as an *Eco*RI fragment. The H2B probe was a fragment spanning positions −60 to −19 of the human histone H2B promoter and flanked by *Hin*dIII linkers used to excise the fragment. For the experiment shown in Fig. 3, the following oligonucleotides were synthesized and annealed as indicated and either cloned into the *Sac* I and *Sal* I sites of pUC18 for use as probes or used directly as competitors in band-shift assays:

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Abbreviations: VIC, Vmw65-induced complex; HSV, herpes simplex virus.

WT, CGAGCCGTGCATGCTAATGATATTCTTTGGG
TCGAGCTCGGCACGTACGATTACTATAAGAAACCCAGCT;

OC⁻, CGAGCCGTGCcTGaTcATGATATTCTTTGGG
TCGAGCTCGGCACGgACtAgTACTATAAGAAACCCAGCT;

GA⁻, CGAGCCGTGCATGCTAATtAccgTCTTTGGG
TCGAGCTCGGCACGTACGATTAaTggcAGAAACCCAGCT

(lowercase letters indicate position of mutated nucleotides). The sequence of the octamer-containing H2B oligonucleotide used for competitions was

CTTCACCTTATTTGCATAAGC
GAAGTGGAATAAACGTATTCG.

The oligonucleotide

GGGTGAGACCCTCTTGC
CCCACTCTGGGAGAACG

from the human histone H4 gene was used as a heterologous competitor fragment.

***In Vitro* Translation.** The coding sequence of the Vmw65 gene was excised by *Eco*RV and *Mae* III from the *Bam*HI F fragment of HSV-1 strain 17 and inserted into the *Sma* I site just 3′ of the T7 RNA polymerase promoter of plasmid pGem-1. This DNA was transcribed by T7 RNA polymerase *in vitro* into RNA that was used to program a rabbit reticulocyte lysate (Promega) as described (23). For band-shift reactions with *in vitro* translated Vmw65 protein, 100 μg of rabbit reticulocyte lysate proteins containing ≈10 fmol of Vmw65 was mixed with the other components of the band-shift reaction mixture and incubated for 30 min at 30°C before loading on the gel.

Photoaffinity Labeling. The lower strand of the WT oligonucleotide was hybridized to the primer AGCTCGAGCC and double-stranded 5-bromodeoxyuridine-substituted DNA was synthesized by Klenow polymerase in the presence of 50 μM BrdUTP, dGTP, dCTP, and 2 μM [α-^{32}P]dATP (24). Unincorporated nucleotides were removed by gel filtration and 50 fmol of DNA was incubated in band-shift buffer with 50 times the amount of OTF-1 usually used for a single band-shift assay at 30°C for 30 min. The samples were then cross-linked for 30 min at 4°C under a UV lamp (1600 μW/cm² at 260 nm), brought to 10 mM $CaCl_2$/2 mM $MgCl_2$, and digested with 5 units of micrococcal nuclease and 8 units of DNase I for 15 min at 37°C. Digestion was stopped by addition of EDTA and the samples were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography.

RESULTS

Vmw65 Alters the Mobility of TAATGARAT-Binding Proteins. A fragment containing the TAATGARAT sequence of the ICP0 gene of HSV-1 was used as a probe in band-shift assays. When the probe was incubated with a nuclear extract from noninfected HeLa cells, one retarded species was observed (Fig. 1, lane 2). Based on homology between sequences overlapping the TAATGARAT motif and other octamer sequence elements, it seemed probable that the binding activity might be OTF-1 (18, 19). Using affinity-purified OTF protein and photoaffinity labeling, we demonstrate that this is indeed the case (see below). When the same kind of assay was performed with an extract prepared from HSV-1-infected HeLa cells, a very strong retarded band was observed in addition to the OTF-1 band (lane 3). On the presumption that this newly discovered species is dependent on the presence of the viral trans-activating Vmw65 protein in the extract, we refer to it as VIC (Vmw65-induced complex). Competition experiments indicated that binding of OTF-1 was required for VIC formation. Thus, unlabeled oligonucleotides containing either the octamer element of the TAATGARAT sequence (lane 4) or the human histone H2B promoter (lane 5) abolished VIC, as well as the OTF-1 complex, whereas an oligonucleotide with heterologous sequence derived from the human histone H4 gene did not compete (lane 6).

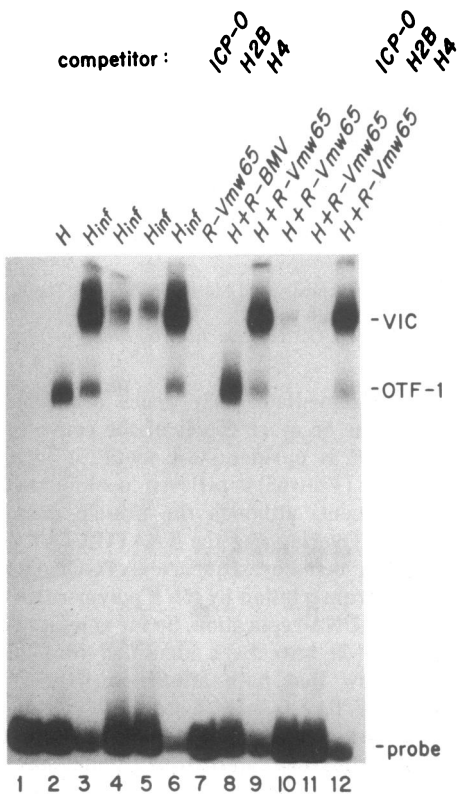

FIG. 1. Vmw65-dependent complexes on the TAATGARAT element. In a band-shift experiment, a 3′-end-labeled probe containing a TAATGARAT element of the ICP0 promoter was incubated with extract from uninfected HeLa cells (lane 2), extract from HSV-1-infected cells (lanes 3–6), rabbit reticulocyte lysate programmed with Vmw65 RNA (lane 7), extract from uninfected cells mixed with reticulocyte lysate programmed with either heterologous mRNA from brome mosaic virus (BMV) (lane 8) or synthetic Vmw65 mRNA (lanes 9–12). To assess the specificity of the shifts, a 150-fold molar exess of three different oligonucleotides was used (see *Materials and Methods* for their exact sequences): ICP0 containing the TAATGARAT element (lanes 4 and 10), H2B containing an octamer sequence (lanes 5 and 11), and H4 carrying no related sequence motif (lanes 6 and 12).

To prove that VIC formation was dependent on the presence of Vmw65 in the infected extract, mRNA derived from the cloned gene was translated *in vitro* in a rabbit reticulocyte lysate. The newly translated protein was equivalent in size to Vmw65 isolated from virions and was recognized by the monoclonal antibody LP1 raised against Vmw65 (25), both on immunoblots and in immunoprecipitation assays (data not shown), and therefore represented *bona fide* Vmw65.

When the Vmw65-containing translation reaction mixture was mixed with extract from noninfected HeLa cells, a band at the same position as VIC in extracts from herpes-infected cells was observed (lane 9). A control lysate programmed with a heterologous (brome mosaic virus) mRNA did not give rise to the second shift after incubation with HeLa extract (lane 8). The additional band showed the same competition pattern as did the VIC band (compare lanes 10–12 with lanes 4–6). We therefore conclude that Vmw65 is the viral agent

responsible for forming the slower-migrating complex in infected cell extracts. In agreement with the finding of others (20, 21), control experiments failed to show any specific complexes of Vmw65 with the ICP0 probe in the absence of other factors (Fig. 1, lane 7; see also Figs. 2–4 below).

Both the Octamer and the GARAT Sequence Are Required for VIC Formation. Competition experiments with octamer-containing sequences and the use of purified OTF-1 protein (see below) show that the octamer element is required for the formation of VIC. Nevertheless, comparative band-shift experiments with octamer-containing probes from the murine IgH enhancer or the human histone H2B promoter have revealed that additional sequences are needed to form a VIC complex. As shown in Fig. 2, no formation of VIC was detected after incubation of these probes in the presence of nuclear extracts and Vmw65-containing lysate (lanes 6 and 12), in contrast to the results obtained with the ICP0 probe (lane 18). Similar results were obtained with a probe containing the octamer of a human Igκ light-chain promoter (data not shown).

To localize the sequences required in addition to the octamer for VIC formation, oligonucleotides with clustered point mutations were synthesized. Since the GARAT part of the TAATGARAT motif is strongly conserved it appeared probable that this sequence plays an important role. Therefore, a mutant (GA^-) was generated with four transversions in this region, sparing only the A that is part of the decanucleotide consensus sequence. Another mutant (OC^-) contained three transversions in the octamer element and had been shown earlier to abolish OTF binding and transcription activation *in vivo* (26). These oligonucleotides were cloned into pUC18 together with an oligonucleotide with the wild-type sequence (WT) and used as probes for band-shift experiments.

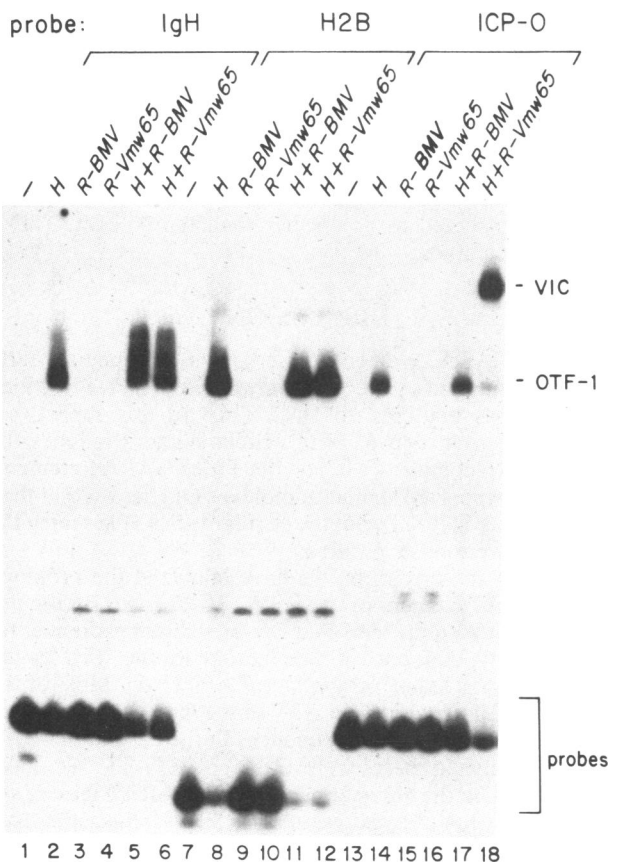

FIG. 2. Specificity of VIC formation on octamer-containing promoter and enhancer sequences. Three probes containing different octamer sequences were used for band shifting. The radioactively labeled DNA fragments were incubated with extract of uninfected HeLa cells alone (lanes 2, 8, and 14), with reticulocyte lysate programmed with brome mosaic virus (BMV) RNA (lanes 3, 9, and 15), with reticulocyte lysate programmed with Vmw65 mRNA (lanes 4, 10, and 16), and with mixtures of HeLa extract with BMV lysate (lanes 5, 11, and 17) and Vmw65 lysate, respectively (lanes 6, 12, and 18).

In band-shift assays with HeLa nuclear extract and Vmw65-containing lysates (Fig. 3), VIC formation was observed only with the WT (lane 3) but not with the GA^- (lane 9) or the OC^- probes (lane 15). The GA^- probe with its unaltered octamer sequence was still capable of binding OTF-1, whereas the OC^- probe showed no specific binding to any protein. Use of the three different oligonucleotides as competitors corroborates this picture. Both WT and GA^- oligonucleotides were efficient competitors for formation of both the OTF-1 and VIC complexes, whereas the OC^- oligonucleotide did not block formation of either (lanes 4–6 and 10–12).

OTF-1 and an Additional Cellular Factor(s) Are Required for VIC Formation. To verify that the previously defined OTF factors bind to the TAATGARAT motif, as was predicted by sequence homology (18, 19) and by our competition experiments, we performed band-shift experiments with affinity-purified OTF-1 and OTF-2 proteins (4, 6) (Fig. 4). In the absence of any other proteins, purified OTF-1 (lane 7) gave rise to a band whose position was the same as that of the complex in crude nuclear extracts (lane 1). As expected from previous demonstrations of the inability of OTF-1 and OTF-2 to discriminate between different octamer sites in binding assays, purified OTF-2 protein also was able to recognize the octamer in the TAATGARAT sequence (lane 13).

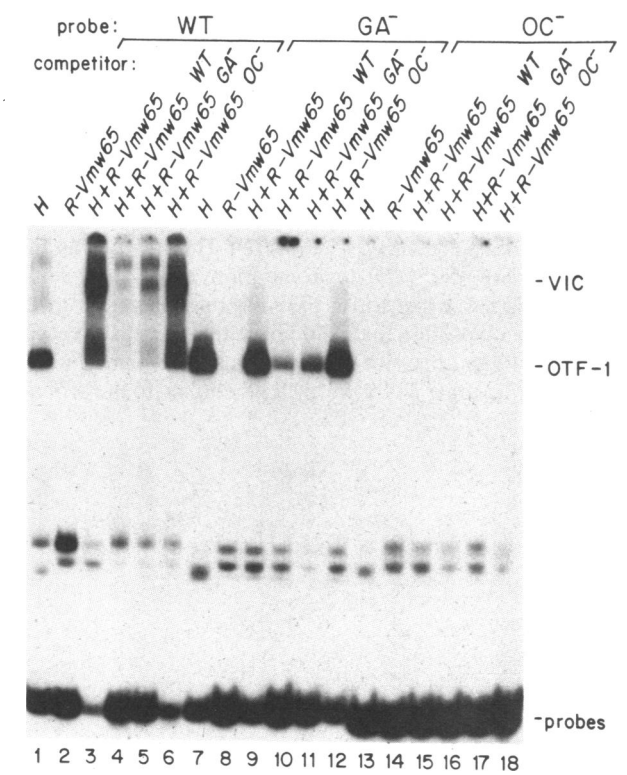

FIG. 3. The GARAT sequence is required for VIC formation. The three probes WT, GA^-, and OC^- were used for band-shift experiments with HeLa nuclear extract (lanes 1, 7, and 13), with reticulocyte lysate programmed with Vmw65 RNA (lanes 2, 8, and 14), and with a mixture of HeLa extract and Vmw65 lysate (lanes 3–6, 9–12, and 15–18). A 150-fold molar excess of unlabeled oligonucleotide was added to some incubation mixtures as indicated.

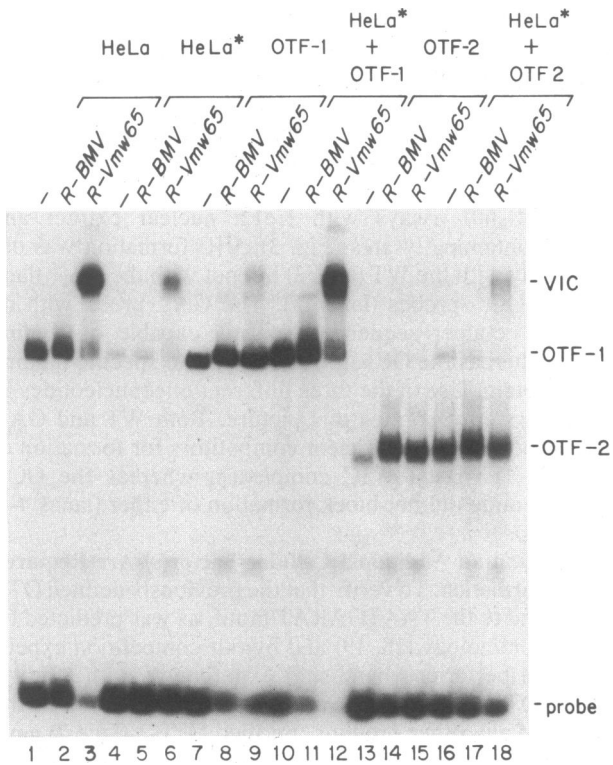

FIG. 4. Cellular factors in addition to OTF-1 are required for VIC formation. The ICP0 probe was used in band-shift experiments with crude nuclear HeLa extract (lanes 1–3), HeLa extract depleted for OTF-1 (6) (lanes 4–6), affinity-purified OTF-1 protein (lanes 7–9), depleted HeLa extract and purified OTF-1 (lanes 10–12), affinity-purified OTF-2 (lanes 13–15), and depleted HeLa extract and purified OTF-2 (lanes 16–18). To these incubation mixtures reticulocyte lysate programmed with either brome mosaic virus (BMV) RNA (lanes 2, 5, 8, 11, 14, and 17) or Vmw65 RNA (lanes 3, 6, 9, 12, 15, and 18), respectively, was added.

Since some minor components contaminated the affinity-purified OTF-1 we used photoaffinity labeling to identify OTF-1 *per se* as the DNA binding protein in this preparation. The WT oligonucleotide was substituted with 5-bromodeoxyuridine and labeled with [α-^{32}P]dATP. This DNA was first incubated with the OTF-1 preparation and subsequently irradiated with UV light to crosslink bound proteins to the DNA. The only protein that could be labeled in this way (Fig. 5, lane 1) had an apparent molecular mass of slightly above 100 kDa. Since some DNA is covalently linked to the protein,

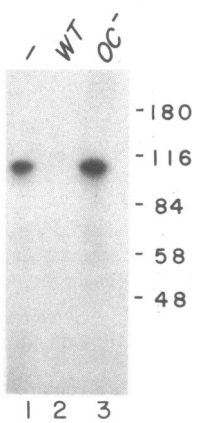

FIG. 5. Photoaffinity labeling of OTF-1. The ^{32}P-labeled WT oligonucleotide was incubated with affinity-purified OTF-1 in the absence of any competitor (lane 1) in the presence of a 2400-fold excess of unlabeled WT (lane 2) or OC$^-$ oligonucleotide (lane 3).

this fits well the observed molecular mass of free OTF-1 of ≈90 kDa (4). The binding was specific since it could be blocked by competition with unlabeled WT oligonucleotide (lane 2) but not OC$^-$ (lane 3).

When Vmw65-containing lysate was mixed with OTF-1, only very little VIC formation was observed (Fig. 4, lane 9). Therefore, we assumed that an auxiliary factor might be required besides OTF-1 and Vmw65. To test this, we used a HeLa nuclear extract that had been depleted of most of its OTF-1 by incubation with a DNA affinity matrix carrying an octamer sequence (6) (lanes 4–6). As expected, the addition to this extract of purified OTF-1 markedly raised the level of the OTF-1 complex (compare lane 10 with lane 4). Addition of both purified OTF-1 and Vmw65-containing lysate dramatically increased the level of VIC (compare lane 12 to lane 6) and resulted in a proportionally lower level of the OTF-1 complex (compared lane 12 to lane 10). This result provides direct proof that OTF-1 is required for VIC formation but further indicates the involvement of another cellular factor(s) that could be involved in recognition of the GARAT part of the TAATGARAT element. The very weak VIC band observed when Vmw65-containing lysate and purified OTF-1 were coincubated in the absence of HeLa extract (lane 9) most likely reflects traces of the rabbit homologue for the additional factor(s) in the reticulocyte lysate. This interpretation is supported by our observation of low amounts of other endogenous factors (e.g., OTF-1) in this lysate (data not shown).

In contrast, OTF-2 did not appear to be capable of mediating any significant level of VIC formation (Fig. 4, lanes 16–18), despite its ability to interact with the ICP0 probe (lanes 13–18). The low amount of VIC observed with OTF-2 and Vmw65 (lane 18) is best explained by the obvious residual level of OTF-1 (lanes 4–6) in the depleted HeLa extract (compare the VIC signal in lanes 6 and 18 of Fig. 4). Furthermore, a VIC complex containing the ≈60-kDa OTF-2 would be expected to be smaller than the 90-kDa OTF-1-containing complex.

DISCUSSION

The TAATGARAT elements of herpes virus immediate early genes are activated by the viral Vmw65 protein (7–15), which by itself does not bind to DNA (16). In this report, we demonstrate that Vmw65 with cellular factors induces the formation of a complex (VIC) on the TAATGARAT element. Others have reported similar complexes and shown that they contain Vmw65 (20, 21), but the cellular factors present in the complexes were not identified.

Extending these reports, we have analyzed the proximal TAATGARAT element of the HSV-1 ICP0 gene. By the use of affinity-purified proteins and UV-crosslinking studies, we show directly that one of the factors in the VIC is the octamer-binding transcription factor 1 (OTF-1). Most of the TAATGARAT sequences in HSV immediate early genes are overlapped by an octamer sequence. In the case of the ICP0 element analyzed here (ATGCTAATGATAT), we have shown that both the octamer part and the GARAT moiety are required for the formation of the VIC. Thus, the true functional element may properly be described as OCTA/GARAT. However, a few TAATGARAT elements show no obvious homology to the octamer 5′ of the TAAT sequence. Nevertheless, the motif around position −250 of the ICP4 promoter of HSV-1 (GGGCGGTAATGAGATGC) appears to compete for the same cellular factor as the OCTA/GARAT sequences (18). Moreover, given the observations that a single transcription factor may bind to very different DNA sequence elements independently (27) or in conjunction with a second stabilizing factor (28), it is quite possible that all TAATGARAT motifs can be recognized by OTF proteins.

Besides the TAATGARAT motif, another G+A-rich sequence element has been implicated in the Vmw65-mediated transcription stimulation of ICP4 (13, 14). The factor(s) interacting with the G+A-rich element seems biochemically distinct from the factor(s) binding to TAATGARAT (14). It has also been reported that the major immediate early gene of pseudorabies virus and the long terminal repeat of human immunodeficiency virus respond to Vmw65 (29, 30), but the DNA sequence motifs involved have not been analyzed. These results raise the possibility that OTF-1 is not the only cellular site-specific DNA binding protein with which Vmw65 may interact.

In contrast to the situation observed with an OTF-1–OCTA/GARAT complex, Vmw65 appeared not to recognize an OTF-2–OCTA/GARAT complex to form a VIC. This functional difference between OTF-1 and OTF-2 is interesting because these proteins bind virtually indistinguishably (qualitatively and quantitatively) to various octamer elements, even though they appear to differ with respect to their capabilities for stimulating transcription from an Igκ gene promoter (A. Heguy, C. Scheidereit, and R.G.R., unpublished data). These findings may best be explained by assuming differential interactions between the various OTF factors and other proteins required for transcription. Our study of the interactions of OTF-1 with the viral Vmw65 protein and another cellular factor(s) (see below) provides a model system that may allow us to understand the functions of OTF proteins in particular and protein–protein interactions involved in cell type-specific transcription of cellular genes in general.

In this work, we also demonstrate that at least one cellular factor in addition to OTF-1 and Vmw65 is necessary for efficient formation of the Vmw65-containing complex (VIC). Our initial purification studies have indicated that this protein(s) has a general affinity for DNA (unpublished results). On the other hand, Vmw65 has been reported not to have this property (16) and is shown here not to exhibit site-specific DNA binding in the absence of other factors. Therefore, we favor the idea that recognition of the GARAT element involves the additional cellular factor(s), although the possibility that this element is recognized by Vmw65, or even OTF-1, cannot yet be excluded. However, like VIC formation, GARAT recognition also appears to require interactions between all three factors (OTF-1, Vmw65, and the cellular factor) since no specific complex other than the OTF-1–promoter complex was observed when any single factor was omitted or when either the OCTA or the GARAT motifs were mutated. Although it seems probable that VIC formation requires stoichiometric participation of the cellular factor(s), it is formally possible that this factor acts catalytically to modify either OTF-1 or Vmw65.

Inspection of the predicted amino acid sequence of Vmw65 shows that its carboxyl terminus contains an unusually high density of negatively charged amino acids (31). This is reminiscent of the very acidic regions in the transcriptional activation domains of the yeast regulatory proteins GCN4 (32) and GAL4 (33). In fact, McKnight and colleagues have recently demonstrated (34) that this acidic region of Vmw65 is required for activating transcription from the herpes ICP-4 promoter. It is possible, therefore, that the primary role of OTF-1 and the GARAT-interacting factor(s) is to position the activation domain of Vmw65 on the herpes immediate early promoters. The requirement for the GARAT motif in addition to the octamer element for VIC formation may ensure that only the viral immediate early and not all other cellular octamer-containing genes become activated by Vmw65. Finally, these studies raise the possibility that OTF-1 and OTF-2 may function similarly on their respective cellular genes—i.e., to bind and position other regulated activators rather than activating directly.

We thank Dr. S. Silverstein (Columbia University) for a generous gift of extract from HSV-1-infected HeLa cells and for a plasmid containing the Vmw65 gene and Dr. A. Minson (Cambridge University, Cambridge, U.K.) for the gift of the LP1 antibody. We are indebted to Drs. C. Scheidereit and A. Heguy for their gift of purified OTF proteins and OTF-depleted HeLa extract, respectively, and to Drs. A. Pierani, I. Gelman, L. Poellinger, and C. Scheidereit for many helpful discussions. T.G. thanks the Schweizerischer Nationalfonds for support. This work was supported by National Institutes of Health Grant 42567 to R.G.R. and by general support from the PEW Trusts to the Rockefeller University.

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