JOURNAL OF VIROLOGY, June 1989, p. 2798–2812
0022-538X/89/062798-15$02.00/0

Vol. 63, No. 6

Overlapping Octamer and TAATGARAT Motifs in the VF65-Response Elements in Herpes Simplex Virus Immediate-Early Promoters Represent Independent Binding Sites for Cellular Nuclear Factor III

COLETTE M. J. APRHYS,[1] DOLORES M. CIUFO,[1] EDWARD A. O'NEILL,[2] THOMAS J. KELLY,[2] AND GARY S. HAYWARD[1]*

The Virology Laboratories, Department of Pharmacology & Molecular Sciences,[1] and Department of Molecular Biology and Genetics,[2] the Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, Maryland 21205

Received 19 December 1988/Accepted 3 March 1989

Expression of the immediate-early (IE) genes of herpes simplex virus (HSV) is specifically stimulated by a 65-kilodalton virion transcription factor (VF65 or VP16) that is introduced as a component of infecting virions. In both the IE175(ICP4) and IE110(ICP0) promoters, this activation requires an upstream *cis*-acting target response element that contains a single TAATGARAT consensus element. Furthermore, many HSV IE TAATGARAT elements overlap with ATGCTAAT octamer motifs that are similar to the OTF-1-binding sites found in both immunoglobulin and histone H2b genes and to the nuclear factor III (NFIII)-binding site within the adenovirus type 2 origin of DNA replication. Purified HeLa cell NFIII protein proved to form specific DNA-protein complexes with several upstream regions from both the IE110 and IE175 promoters, and this interaction was subject to efficient competition with an adenovirus type 2 DNA fragment containing an intact NFIII-binding site. Surprisingly, the NFIII protein bound to synthetic oligonucleotides containing only the TAATGARAT consensus elements as well as to those containing the ATGCTAAT octamer sequence, although the former exhibited lower affinity and gave complexes with slightly different electrophoretic mobility. The ATGCTAAT oligonucleotide also competed more efficiently than the TAATGARAT sequence itself for binding to a TAATGARAT probe, indicating that the same protein species binds to both sites. The oligonucleotides also formed novel supershifted complexes with lysed virion proteins, but only in the presence of a crude nuclear extract and not with affinity-purified NFIII alone. We conclude that the cellular NFIII protein can recognize both the ATGCTAAT and TAATGARAT elements independently but that only the interaction with TAATGARAT represents an intermediate step in the transcriptional stimulation of IE genes by the HSV virion factor.

The genome of herpes simplex virus (HSV) contains nearly 70 distinct genes and promoter elements that are expressed in a well-defined three-step cascade representing the immediate-early (IE), delayed-early (DE), and late (L) phases of lytic cycle infection. The HSV IE genes are defined as those that are transcribed when infection is initiated in the presence of cycloheximide or anisomycin to block de novo protein synthesis. However, unlike the T-Ag and E1A promoters of papovaviruses and adenoviruses, whose initial expression is solely dependent upon recruiting preexisting cellular transcription factors, the HSV IE promoters respond to transcriptional activation by a virus-encoded factor (referred to here as VF65, but also known as Vmw65, VP16, or α-TIF) that is introduced as a component of the incoming virions. Transcription of most DE and L genes requires the presence of the viral nuclear phosphoprotein IE175 or ICP4 (7, 26, 44, 46, 65), which behaves as a specific *trans*-activator of DE promoters and also negatively autoregulates expression from its own promoter (8, 10, 15, 16, 37–39, 51, 52). The IE110 (ICP0) gene product also stimulates expression from many HSV and heterologous viral and cellular promoters in transient assays (10, 15, 32, 37, 40, 51), but unlike IE175, it is not essential for lytic cycle growth at high multiplicities of infection (55, 61), and its physiological role may be more significant for reactivation from the latent state.

Four distinct IE promoter-regulatory regions that may be targets for VF65 have been defined in both HSV-1 and HSV-2 (27, 29, 47, 68). Two of them occupy a 700-base-pair (bp) divergent region surrounding the *ori*-S replication origin within both copies of the S-segment inverted repeats. One direction drives expression of the mRNA encoding the IE175 protein, and the other gives rise to mRNA species encoding the IE68 and IE12 proteins. The third and fourth IE upstream regulatory domains are between 400 and 600 bp in length and contain unidirectional promoters driving expression of either the IE110 protein (within both copies of the L-segment inverted repeats) or that of the IE63 protein in U_L. All have been recognized to contain multiple copies of a consensus sequence commonly referred to as the TAATGA RAT element (3, 20, 25, 28). Eleven CCCGCCC motifs that bind to the purified cellular SP-1 factor and may be associated with basal expression properties have also been described in the IE175-IE68 region (19).

Evidence for the existence of the virion factor came from experiments in which the promoters from the IE175 and IE68 genes of HSV-1 were isolated and fused to reporter genes encoding thymidine kinase, interferon, or chloramphenicol acetyl transferase (CAT) and introduced into permanent cell lines by DNA transfection procedures. Each responded at the transcriptional level to stimulation by HSV

* Corresponding author.

infection even in the presence of cycloheximide or after infection with either inactivated virus particles or with the HSV *ts*B7 uncoating mutant at a nonpermissive temperature (1, 33, 36, 45, 47). This effect is specific for HSV IE promoters and appears to correlate with the presence of at least one TAATGARAT consensus sequence in each of the responsive target gene constructions (13, 20, 39, 47). The *trans*-activation is mediated by a 65-kilodalton (kDa) phosphoprotein located between the envelope and capsid portions of intact assembled virions. Cotransfection with DNA containing the L-class viral gene encoding the virion factor (referred to here as VF65) also specifically stimulates expression from HSV IE target promoters, but not from a variety of heterologous genes in transient assays (4, 16, 38–40; D. M. Ciufo, C. M. J. apRhys, M. C. Pizzorno, and G. S. Hayward, manuscript in preparation). A second IE promoter element which may contribute to the virion factor response, GCGGAAC, has also been described (3, 64). Several groups have shown that the 490-amino-acid VF65 protein does not act directly by specific DNA binding but may instead interact with a host cell factor that recognizes and binds to the TAATGARAT response elements (22, 30, 31, 35, 48, 63). Triezenberg et al. (64) reported that an 80-residue acidic domain at the COOH end of VF65 is essential for the activator functions and that the remainder of the protein can produce transdominant interference, presumably through specific protein-protein interactions with target transcription factors.

We have previously (18) pointed out similarities between the consensus TAATGARAT elements that are believed to be targets for the HSV virion factor response (especially those in the IE110 and IE63 promoters) and a sequence between nucleotides 40 and 52 near the replication origin within the left end of adenovirus type 2 (Ad2) DNA. This adenovirus DNA sequence includes the binding site for a 92-kDa cellular factor referred to as nuclear factor III (NFIII) (or ORP-C), which is functionally important for efficient initiation of adenovirus DNA replication in vitro (42, 49, 54, 69). This protein also appears to be identical to the ubiquitious cellular octamer transcription factor OTF-1 (11, 41, 42). Therefore, we were interested in determining whether or not the consensus elements in IE110 are in fact binding sites for the cellular NFIII protein and whether they may also represent targets for VF65 responses. Surprisingly, our results indicate that the IE110 VF65 response sites contain octamerlike ATGCTAAT motifs that overlap with the TAATGARAT motifs and that both of these elements represent independent binding sites for affinity-purified NFIII.

MATERIALS AND METHODS

Plasmids containing HSV IE promoter sequences. DNA fragments containing IE175 promoter sequences were prepared from plasmids pPOH2 and pPOH13 containing the HSV-1 IE175(−1900/+30)-CAT and IE175(−375/+30)-CAT reporter genes inserted into pBR322-derived vectors (36, 39, 40). DNA fragments from across the HSV-1 IE110 promoter were similarly derived from plasmids pGH83 and pGH84 containing the HSV-1 IE110(−800/+120)-CAT and IE110 (−800/−30)-CAT reporter genes (52). The sizes and map locations of HSV IE promoter fragments derived from these plasmids are shown in Fig. 1. A smaller version of the active HSV-1 IE110(−800/+120)-CAT reporter gene containing only one copy of the consensus VF65 response element within the sequences from −165 to +120 was produced by first inserting the 285-bp *Sph*I-to-*Bam*HI fragment from pGH83 into a pUC18 vector and then moving the same upstream region as a *Hin*dIII-to-*Bam*HI fragment in pCATB′ to create the IE110(−165/+120)-CAT gene in plasmid pGH135. Removal of the last copy of the consensus element from position −167 to −152 was accomplished by insertion of a *Hin*dIII linker at the *Sma*I site at −128 followed by deletion between the *Hin*dIII sites at −800 and −128 to give plasmid pCA61 containing the IE110(−128/+120)-CAT gene.

A set of pKP54-derived plasmids containing single and multiple tandemly repeated copies of the left-hand terminal 67 bp of Ad2 DNA were described previously by Rosenfeld and Kelly (53). Point mutations (pU*pm*42 and pU*pm*46) in the NFIII recognition motif of a single-copy version of the Ad2 67-bp fragment were also described by Rosenfeld et al. (54). The *pm*42 mutation contains a G-to-A substitution at position 42, and *pm*46 contains an A-to-T substitution at position 46, both of which decrease the binding affinity for NFIII by 10- to 20-fold.

Transient DNA transfection and CAT assays. All short-term expression assays were carried out with CsCl-purified plasmid DNA samples by the calcium phosphate transfection procedure with a glycerol boost into subconfluent monolayers of Vero cells in 6-well culture dishes (35-mm diameter) (36, 37). In each experiment, the input DNA samples were all brought up to the same total DNA concentration by addition of pBR322 carrier DNA. Percent conversion of [^{14}C]chloramphenicol to 1′- and 3′-acetyl chloramphenicol was measured by scintillation counting of the radioactivity in appropriate isolated spots from the silica gel plate. The source of VF65 transactivator protein in cotransfection assays was the effector plasmid pGH62, which was derived from the previously described plasmid pGR212b (38) and contained the 4.7-kilobase *Bam*HI F fragment from HSV-1(MP) in a pKP54 vector background.

Labeling of isolated DNA fragments. DNA restriction fragments were purified by electroelution through a 2% agarose gel. The correct DNA fragment was excised, placed at −70°C for 5 min, and centrifuged through a Costar Spin-X column for 15 min. The elutant was extracted twice with phenol and once with chloroform and then ethanol precipitated. The fragment was dephosphorylated with calf alkaline phosphatase and end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. Unincorporated radionucleotides were removed by adding 2 ml of 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA–100 mM NaCl and centrifuging the sample through an Amicon 30 column.

Synthetic oligonucleotides. A number of 26-bp-long single-stranded oligonucleotides (see Table 1) were synthesized by Scott Morrow (Department of Biochemistry, Johns Hopkins School of Hygiene) and purified by high-pressure liquid chromatography procedures. Each sequence contained an 8-bp palindrome at the 3′ end which was used to produce self-annealed forms of the oligomers which were extended into 44-bp double-stranded head-to-head dimers. Each of the 44-bp dimers had the potential to be cleaved into 22-bp monomers with *Pvu*II. To prepare labeled oligonucleotides, 1 μg of each 26-bp single-stranded oligonucleotide was 5′ end labeled at 37°C for 30 min with 1 U of T4 polynucleotide kinase and 150 μCi of [γ-^{32}P]ATP (10 mCi/mmol) in a total volume of 25 μl of 25 mM Tris hydrochloride (pH 8.0)–5 mM $MgCl_2$–7.5 mM dithiothreitol. After radiolabeling, the sample was dried and suspended in 10 μl of 150 mM NaCl. The oligonucleotides were allowed to self-anneal by incubation at 37°C for 30 min followed by slow cooling to room tempera-

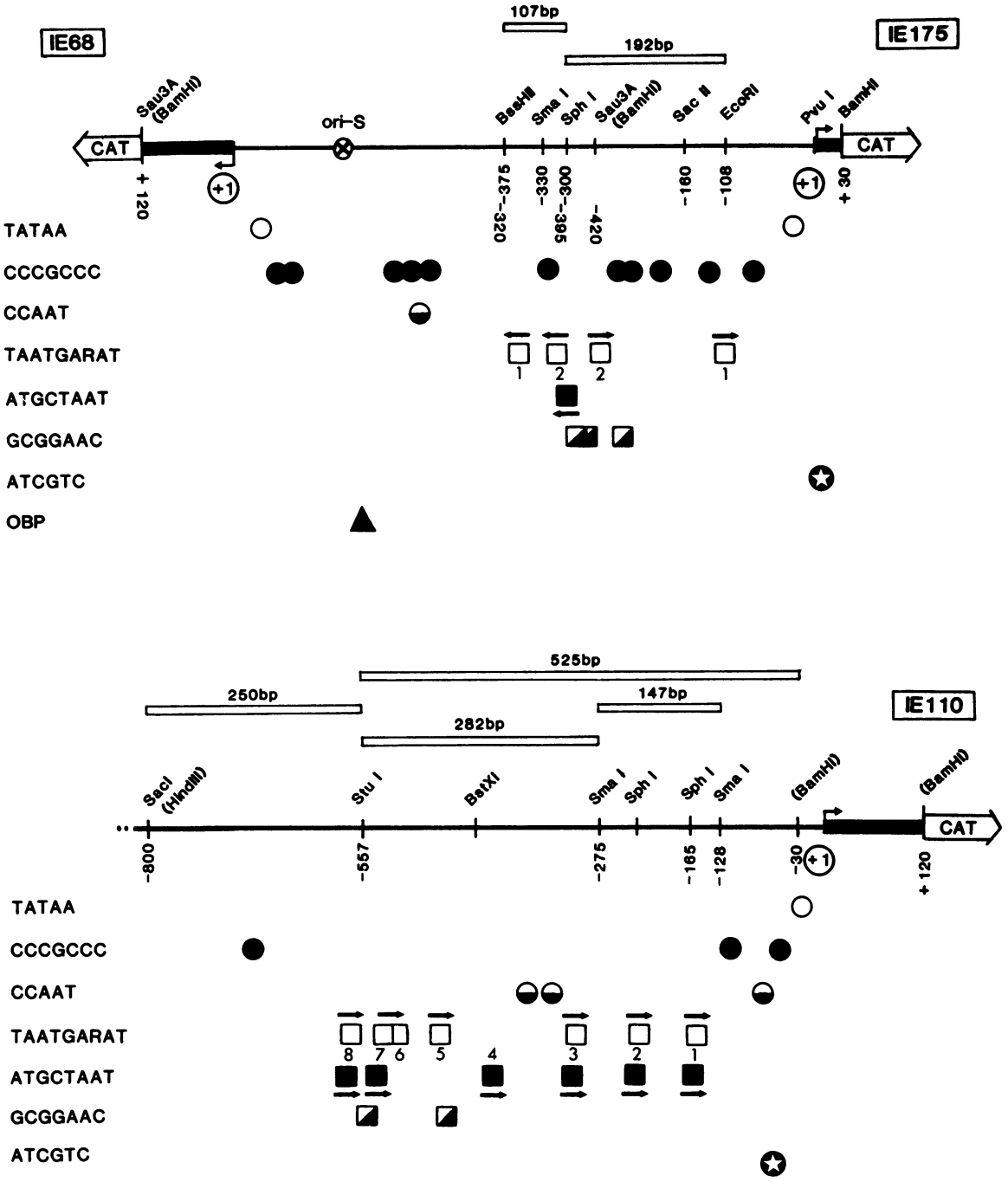

FIG. 1. Map location of DNA probes in the HSV-1 IE175, IE110, and IE68 promoter/regulatory regions relative to known or putative consensus elements and transcription factor-binding sites. The upper diagram represents the 700-bp divergent IE68/*ori*-S/IE175 control region present in each of the S-segment inverted repeats, and the lower diagram represents the unidirectional IE110 5′-upstream region present in each of the L-segment inverted repeats. ▬, Leader sequences included in the CAT gene constructions. Locations of consensus TATAA boxes (○), CCAAT boxes (◒) SP1-binding sites (●), ATGCTAAT octamer-like motifs (■), consensus TAATGARAT elements (□), and consensus GCGGAAC motifs (◪) are also indicated. The orientation and numbering system used for the proposed group O and group O/T NFIII-binding sites in this paper is indicated. The sizes and boundaries of the various DNA fragment probes used in Fig. 3 are illustrated (▭). Restriction sites introduced as linkers are indicated in parentheses.

ture. Subsequently, they were extended by the addition of 3 μl of 2.5 mM dinucleotide triphosphates and 1 U of T4 DNA polymerase in a volume of 30 μl. After incubation at 23°C for 30 min, the reaction mixture was heated to 65°C for 15 min and the remaining unincorporated nucleotides and single-stranded oligonucleotides were separated from double-stranded extended oligonucleotides by passage through a Schleicher & Schuell Elutip-d column. Unlabeled 26-bp oligonucleotides (10 μg) for competition experiments were self-annealed directly in 10 μl of 150 mM NaCl. The volume

was brought to 30 μl with buffer and adjusted to 25 mM Tris hydrochloride (pH 7.8)–10 mM $MgCl_2$–2.5 mM nucleotide triphosphate. Extension was accomplished with 10 U of T4 polymerase at 23°C as described above.

Gel electrophoresis DNA-binding assays. Our standard NFIII-binding reaction conditions were those developed by Rosenfeld et al. (54) and modified by O'Neill and Kelly (42). Binding assay buffer contained 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.5), 75 mM NaCl, 10% glycerol, 0.05% Nonidet P-40, 400 μg of bovine serum albumin per ml, 1 mM dithiothreitol, 1 mM EDTA, and 40 μg of the alternating copolymer poly(dI-dC) · poly(dI-dC). ^{32}P-labeled DNA fragments or oligonucleotides (5 fmol) were incubated in 12.5 μl of binding buffer with between 1 and 4 μl of cellular extract or purified protein sample for 30 min at 23°C. After reaching equilibrium, the samples were loaded onto a 2% agarose nondenaturing gel prepared in 12 mM Tris-acetate (pH 7.5)–1 mM EDTA–0.05% Nonidet P-40 for mobility retardation assays (5, 12, 14). Electrophoresis was carried out for 90 to 120 min at 10 V/cm in 12 mM Tris-acetate (pH 7.5)–1 mM EDTA–0.01% Nonidet P-40 buffer with continuous buffer recirculation.

NFIII purification procedure. Crude HeLa cell nuclear extracts were prepared by high-salt lysis of isolated nuclei (9) essentially as described by Rosenfeld and Kelly (53). Chromatography on BioRex 70 removed the bulk of the NFI DNA-binding activity, leaving the NFIII activity in the flowthrough fraction. A more highly purified fraction of NFIII was prepared by additional phosphocellulose and hydroxylapatite chromatography followed by *Escherichia coli* DNA-cellulose and DNA recognition site affinity chromatography. The latter step involved binding to pKB67-88 plasmid DNA affixed to a cellulose matrix (42, 53). Purification of NFIII was monitored by its stimulatory activity for pTP-dCMP complex formation in vitro and by gel mobility shift-up assays with a DNA fragment containing only domain C (bp 38 to 67) from the replication origin region at the left end of Ad2 DNA (42, 54). NFIII was shown to be a single 92-kDa polypeptide by recovery of domain C-specific binding activity after isolation and renaturation from a sodium dodecyl sulfate–polyacrylamide gel fractionation step (42).

Virion factor preparation. Enveloped virions were purified from the cytoplasm of HSV-1(MP)-infected Vero cells by banding in 10 to 50% sucrose gradients and then were pelleted and suspended in 50 mM NaCl–10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA at 10^{10} PFU/ml. The samples were then incubated in 0.03% Nonidet P-40 at 4°C for 1 h followed by pelleting of the capsid core and DNA at 100,000 × *g* for 1 h. The recovered supernatant contained envelope proteins and some released 65-kDa tegument phosphoprotein as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Binding reactions in the presence of virion extract were carried out in 20 mM HEPES (pH 7.9)–0.6 mM dithiothreitol–2.3 mM $MgCl_2$–94 mM NaCl–1.24 mg of bovine serum albumin per ml–100 μg of poly(dI-dC) · poly(dI-dC) per ml–5 fmol of ^{32}P-labeled oligonucleotide probe plus HeLa cell nuclear extract or affinity-purified NFIII. The reaction products were analyzed in 1.6% agarose gels prepared in 45 mM Tris–45 mM boric acid–1.25 mM EDTA.

RESULTS

Similarities between the HSV IE TAATGARAT consensus sequences, adenovirus NFIII-binding sites, and immunoglobulin octamer consensus elements. The resemblance between the TAATGARAT elements present in the IE175 promoter region and the TAATGAGGG sequence found between bases 44 and 52 of the Ad2 origin of DNA replication (*ori*) extends over several additional bases on the 5′ side in many of the consensus elements present in the HSV IE110 and IE63 promoters. The extended homology encompasses an HSV IE consensus element YATGCTAAT, which is similar to both the Ad2 NFIII-binding site (TATGATAAT) and the canonical ATGCAAAT octamer motif found in immunoglobulin and H2b gene control regions, etc. (23, 24, 57–60). The sequences of several adenovirus NFIII-binding sites and cellular octamer regulatory elements together with 23 potential TAATGARAT signal sequences found in the available upstream sequence information for HSV-1 and HSV-2 IE promoters (6, 34, 43, 67, 68) are compared in Fig. 2. The HSV sequences can be divided into two distinct groups: those that include the 5′-YATGC or YATGN portion of the octamerlike sequence in front of the TAATGARATNC consensus (referred to as class O/T sites) and those that do not (referred to as class T sites). Note that both proximal sites in the HSV-1 and HSV-2 IE175 promoters fall into the T group, whereas five of the seven TAATGARAT sites in HSV-1 IE110 fit the O/T classification and an eighth site in IE110 (at position −397 to −404) resembles just a single octamer motif (O class). Interestingly, if just the two proximal TAATGARAT elements upstream of IE175 are considered relevant to that gene and the two distal ones are considered as part of the divergent IE68/12 promoter, then all of the HSV IE octamer and TAATGARAT elements are oriented in the same direction relative to their respective transcription st sites (Fig. 1 and 2).

Direct evidence for NFIII-binding sites in the IE110 promoter region. For further evaluation of the similarity to the Ad2 sequences, isolated DNA fragments encompassing some of the octamer and TAATGARAT consensus signals in the upstream promoter-regulatory regions of the HSV-1 IE175 and IE110 gene sites (Fig. 1) were tested for the ability to form stable DNA-protein complexes with purified NFIII as measured by agarose gel retardation (or shift-up) assays (5, 12, 14). In this experiment, a control end-labeled 170-bp probe DNA fragment containing the 67-bp Ad2 sequence in a pUC9 DNA background gave almost complete incorporation into a shifted DNA-protein complex in the presence of increasing amounts of an affinity-purified NFIII protein fraction (Fig. 3a). Under the same conditions, a 100-bp pUC9 control DNA fragment did not bind, but a 250-bp portion of the IE110 promoter sequences (*Hin*dIII to *Stu*I) containing the eighth (or farthest upstream) consensus element (ATGGTAATTAAAA) produced a 50% shift into a complex migrating at almost the same location in the gel as that for the Ad2 site. A 525-bp DNA fragment encompassing sites 1 through 7 from IE110 (between *Stu*I at −555 and the *Bam*HI linker at −30 in pGH84) produced a similar prominent band, together with several slower-mobility species, which would be consistent with NFIII protein interactions at multiple binding sites in some of the DNA molecules.

Similar DNA-protein complexes were also obtained with three other regions from the HSV IE110 or IE175 promoters that were tested (Fig. 3b), namely, the 147-bp IE110 fragment (sites 1 and 2), the 282-bp IE110 fragment (sites 3 to 7) and the 192-bp IE175 fragment (site 2), and also with a 107-bp *Sph*I-to-*Bam*HI fragment of pPOH13 containing sequences from −395 to −320 in the HSV-1 IE68 promoter (not shown). We have consistently observed that the shifted NFIII protein-DNA complexes migrate faster when associated with relatively large probe DNA fragments and slower

I. HSV TAATGARAT Elements (Group T)

HSV-1			
IE175(1)	−119	GTGCATAATGGAATTC	−104
IE175(2)	−268	GGCGGTAATGAGATGC	−253
IE110(5)	−476	GCCCTTAATGGGCAAC	−461
IE110(6)	−523	CTCATTAATGGGCGGC	−508
IE68(1)	−344	GGCGGTAATGAGATAC	−329
HSV-2			
IE175(1)	−141	GCGCATAATGCGGTTC	−126
IE175(2)	−234	GGCGCTAATGAGATCC	−219
Consensus	−	TAATGRRATNC−	

II. HSV octamer-TAATGARAT elements (Group O/T)

HSV-1			
IE110(1)	−167	CATGCTAATGATATTC	−152
IE110(2)	−279	CATGCTAATGGGGTTC	−214
IE110(3)	−311	TATGGTAATGAGTTTC	−296
IE110(4)	−405	TATGCTAATTGCTTTT	−390
IE110(7)	−538	TATGGTAATTAGAAAC	−523
IE110(8)	−582	TATGGTAATTAAAAAC	−567
IE68(2)	−398	CATGCTAACGAGGAAC	−383
IE63(1)	−156	TATGCTAATTAAATAC	−141
IE63(2)	−315	TATGCAAATGAAAATC	−300
IE63(3)	−366	TATGCAAATTGGAAAC	−351
RR-A	−135	AATGCAAATGGGATAC	−120
HSV-2			
IE68(1)	−443	CGTGGTAATGAGATNC	−428
IE68(2)	−495	GATGCTAATGAGACCA	−480
IE63(1)	−157	TATGTCAATTAAATAC	−142
IE63(2)	−319	CATGCAAATTAAAATC	−304
IE63(3)	−370	CATACAAATTAAATAC	−355
RR-A	−137	CATGCAAATGGGATTC	−122
Consensus		−YATGCTAATGRRATNC−	

III. Adenovirus ORP-C NFIII-binding sites

Ad2/5	(39)	TATGATAATGAGGGGG	(54)
Ad3/7	(41)	CATGTAAATGAGGTAA	(56)
Ad4		TATGCAAATAAGGCGT	
Ad12/18/31	(39)	CATTAAAATGAAGTGG	(54)
SA7		TATGCTAATGAGGTGG	
Consensus		−YATGCAAATGARGT−	

IV. Immunoglobulin-enhancer octamer elements

IgH ENH	(549)	AATGCAAATACCCAGG	(533)
Ig V-LK	−58	TATGCAAATTATTAAG	−73
Ig V-H	−87	TATGCAAATCAGAGGT	−72
H2B HU	−41	TATGCAAATAAGGTGA	−56
H2B PM	−57	TATGCAAATGTTAGCC	−72
U2 HU	−222	CATGCAAATTCGAAAT	−207
U2 RA	−232	CATGTAAATCAGAGTC	−217
U2 XL	−270	TATGCAAATAGGGTGT	−255
SV40 ENH	(141)	TATGTAAAGCATGCAT	(126)
HSV-1 TK	−132	CATGCAAATATATTTT	−145
Consensus		−YATGCAAAT−	

FIG. 2. Comparison of HSV IE consensus sequences with Ad2 NFIII sites and cellular octamer motifs. RR-A, A subunit of ribonucleotide reductase (ICP6) (the DE promoter for RR-A is IE175 independent and responds positively to VF65 *trans*-activation [71]); Ig, immunoglobulin; ENH, enhancer; SV40, simian virus 40; TK, thymidine kinase; HU, human; PM, sea urchin; RA, rat; XL, *Xenopus laevis*.

when associated with relatively small DNA fragments, implying that the protein has a net positive charge under the electrophoresis conditions employed. Essentially identical shift-up patterns were obtained with NFIII-positive fractions from earlier stages in the purification of the Ad2 replication factor, including both the BioRex-70 flowthrough fraction and the crude unfractionated HeLa cell nuclear extracts (not shown). Thus, NFIII appears to be the predominant IE promoter-binding activity found in crude HeLa cell nuclear extracts under the conditions used in our assays. A similar prominent binding activity giving the same retardation coefficient with the 147-bp IE110 fragment was also found in crude nuclear extracts from uninfected Vero cells. Note that binding to the IE110 fragments containing O/T consensus elements was expected because of the similarity of the ATGCTAAT motif to the canonical NFIII-OTF-1 octamer motif but that binding to the IE175 site 2 fragment, which lacks any obvious octamerlike motifs, represented a surprising result.

Competition with the Ad2 left-end NFIII-binding site. Competition experiments were used to determine whether the same protein binds to both the adenovirus *ori* and IE promoter sequences. The addition of an excess of unlabeled wild-type Ad2 NFIII-binding site DNA clearly demonstrated competition for formation of protein-DNA complexes between NFIII and the control Ad2 170-bp fragment (Fig. 3b). All binding was subject to competition with a 100-fold molar excess of the unlabeled Ad2 wild-type 67-bp DNA fragment but not by a 100-fold excess of the pm42 Ad2 mutated binding-site fragment. Similarly, the protein-DNA complexes formed between affinity-purified NFIII and the isolated HSV IE110 promoter fragments of 147 and 282 bp and the IE175 fragment of 192 bp were also subject to specific competition (Fig. 3b).

The relative affinities of NFIII for the HSV IE110 promoter and the adenovirus *ori* sequence were measured in a dose-response competition experiment (Fig. 3c). An excess of ^{32}P-labeled Ad2 67-bp DNA together with increasing amounts of unlabeled potential competitor DNAs was allowed to form complexes by incubation with a limiting amount of affinity-purified NFIII protein and then subjected to gel retardation analysis to measure alterations in the fraction of labeled 67-bp DNA bound. The results showed that the 147-bp IE110 DNA fragment (containing O/T consensus sites 1 and 2) bound NFIII with twofold higher affinity than did the wild-type Ad2 67-bp fragment and with at least 300-fold higher affinity than did a control pUC18 vector DNA fragment. In similar experiments, a 170-bp *Bam*HI-P*vu*II fragment derived from plasmid pU*pm*45 and containing a point mutation in the Ad2 octamer motif (ATG ATATT) bound NFIII with 20-fold-lower affinity than did either the wild-type Ad2 170-bp fragment or a DNA fragment containing the canonical H2b octamer (41).

A single IE110 overlapping octamer-TAATGARAT element is sufficient for VF65 responses. We showed previously that an IE175(−160/+30)-CAT reporter gene containing the single proximal TAATGARAT consensus element (site 1) responded up to 40-fold to cotransfection with the VF65 gene but that removal of a 52-bp region containing that site in IE175(−108/+30)-CAT abolished the response (39). We similarly wished to address whether the overlapping octamer-TAATGARAT element from the IE110 promoter would respond to VF65 cotransfection. Therefore, an intact IE110(−800/+120)-CAT reporter gene (52) was transfected into Vero cells together with increasing amounts of VF65 effector DNA (Fig. 4a). This construction gave higher basal expression than the IE175-CAT control DNA but was still stimulated up to 18-fold in the assay. Two deletion variants of this target gene were also examined. The construction in which sites 2 to 8 were removed, IE110(−165/+120)-CAT, was still activated up to 17-fold in an experiment in which parallel samples of IE110(−128/+120)-CAT failed to respond at all (Fig. 4b). Note that the latter construction lacks an

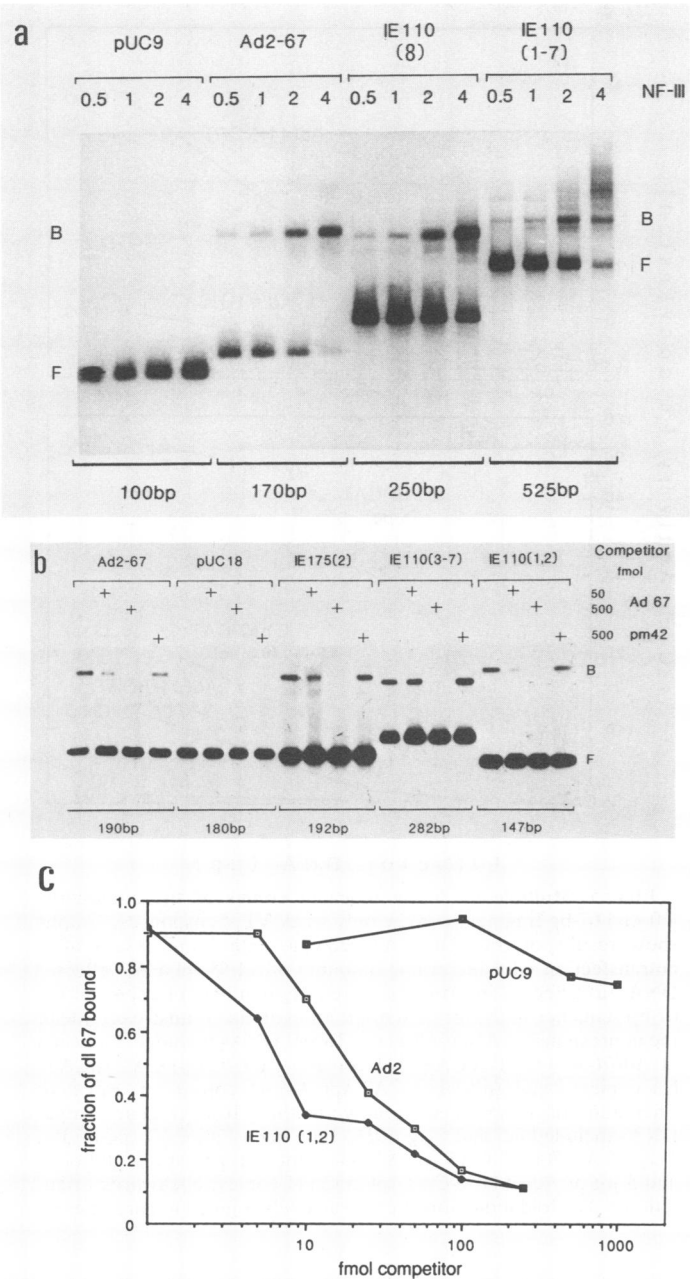

FIG. 3. (a) Affinity-purified NFIII protein from uninfected HeLa cells binds to multiple sites in the HSV-1 IE110 and IE175 upstream promoter regions. Autoradiographs show mobility shift assays with various isolated ^{32}P-labeled DNA species (5 fmol each). The four tested DNA fragments were incubated either in the presence or absence of increasing volumes (indicated in microliters above the lanes) of NFIII/ORP-C purified as described by O'Neill and Kelly (42). DNA samples used included a 100-bp *Hin*dIII-to-*Pvu*II negative control fragment from pUC9 DNA, a 170-bp *Hin*dIII-to-*Pvu*II positive control fragment from pUd167 containing the Ad2 left-end 67-bp sequence, a 250-bp *Hin*dIII-to-*Stu*I fragment of pGH84 containing the distal consensus site 8 from −800 to −557 in the HSV-1 IE110 promoter, and a 525-bp *Stu*I-to-*Bam*HI fragment from pGH84 containing consensus sites 1 to 7 from positions −557 to −30 in the IE110 promoter. F, Free unbound DNA; B, bound DNA. (b) Demonstration of the binding specificity by competition with DNA fragments containing wild-type and point mutant forms of the Ad2 NFIII-binding site. Mobility shift-up competition assays were carried out with test DNA probes consisting of a 190-bp *Pvu*I-*Bam*HI positive control fragment from pUd167 (Ad2 67-bp wild type), a 180-bp *Pvu*I-*Eco*RI negative control fragment from pUC18, a 192-bp *Sph*I-*Eco*RI fragment of pPOH2 from −301 to −108 in the HSV-1 IE175 promoter (site 2), the 282-bp *Stu*I-*Sph*I fragment of pGH83 from −557 to −275 in the IE110 promoter (sites 3 to 7), and the 147-bp *Sma*I-to-*Sma*I fragment from −275 to −128 in the IE110 promoter (sites 1 and 2). All labeled DNA samples (5 fmol) were incubated with 1 μl of the affinity-purified NFIII fraction in the presence or absence of excess Ad2 67-bp wild-type or mutant (*pm*42) competitor DNA. (c) Competition by the IE110 O/T consensus sites 1 and 2 for NFIII binding to the Ad2 *ori* DNA fragment. A 170-bp *Pvu*I-*Bam*HI ^{32}P-labeled Ad2 DNA fragment (10 fmol) containing one copy of the 67-bp replication origin region was incubated with a limiting amount of affinity-purified NFIII in the presence of increasing amounts of excess unlabeled competitor DNAs. The residual fraction of labeled Ad2-67 DNA fragment bound was determined by scintillation counting of shifted bands cut out after gel mobility retardation assays. The competitor DNAs included the 170-bp *Pvu*I-*Bam*HI fragment from pUd167 (Ad2), the 147-bp *Sma*I-*Sma*I fragment for pGH83 (IE110), and the 100-bp *Hin*dIII-*Bam*HI fragment from pUC9 as a negative control.

additional 37-bp sequence which includes the final and most proximal consensus octamer-TAATGARAT element (site 1).

The Ad2 left-end 67-bp sequence conveys weak VF65 responsiveness. Because of the similarity of the HSV IE110 O/T class consensus sequences to the NFIII-binding site motif at the left terminus of Ad2 DNA, we also examined several target promoter constructions containing tandemly repeated copies of the terminal Ad2 67-bp fragment placed upstream from the minimal simian virus 40 early promoter in A10-CAT. Multicopy inserts containing 11 and 44 tandem repeats consistently boosted basal expression levels in Vero cells up to 4- to 12-fold compared with either a single-copy element or the parent A10-CAT DNA (not shown). Furthermore, the reporter genes containing 11 and 44 copies of the adenovirus sequence in the forward orientation [Ad(11+)-CAT and Ad(44+)-CAT] were stimulated 11- and 16-fold, respectively, by cotransfection with VF65 effector DNA (Fig. 5a). However, their overall activity proved to be relatively small compared with that from even one-tenth as much input IE110(−165/+120)-CAT control target DNA (pGR135), and similar inserts oriented in the backward direction [Ad(11−)-CAT and Ad(44−)-CAT] gave 5- to 10-fold-lower basal expression (not shown) and yielded reduced responses [e.g., 8-fold compared with 20-fold for Ad(44−)-CAT in Fig. 5b]. A single copy of the forward-oriented Ad2 67-bp fragment in an A10-CAT background [Ad(1+)-CAT] produced only a minimum *trans*-activation effect in Vero cells (i.e., threefold [Fig. 5a]). We conclude that the Ad2 67-bp DNA sequence containing the ATGATAATGAGGG motif represents only a relatively weak and perhaps orientation-dependent target element for HSV VF65.

Binding of NFIII in crude extracts to oligonucleotides containing wild-type and mutated HSV consensus motifs. To more directly assess the relative roles of the ATGCTAAT and TAATGARAT elements in the HSV group O/T consensus sequences, we synthesized a set of 26-bp-long, single-stranded oligonucleotides containing various combinations of these sequences (Table 1). These synthetic oligonucleotides included self-complementary 8-bp palindromic sequences at the 3′ end to allow formation of 44-bp double-stranded molecules after self-annealing and extension with T4 DNA polymerase. Therefore, each ^{32}P-labeled probe oligonucleotide contained two inverted copies of the wild-type or mutated consensus sequences. In the case of the prototype IE110 O^+/T^+ consensus oligonucleotide (no. 41),

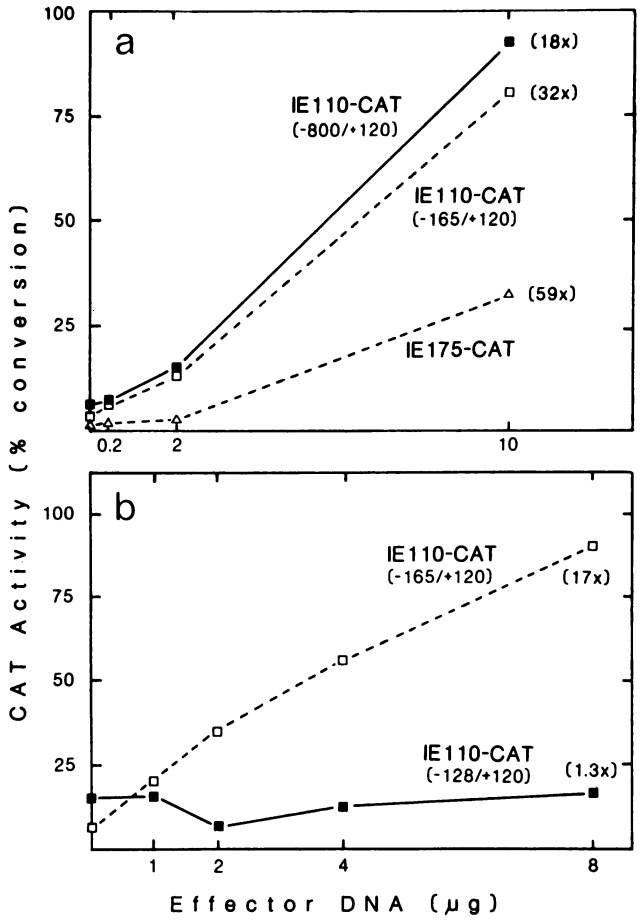

FIG. 4. The 38-bp upstream region encompassing a single overlapping octamer-TAATGARAT element within the IE110 promoter is necessary for a strong VF65 response. Graphs show results of transient CAT enzyme expression assays after cotransfection with increasing amounts of VF65 (pGH62) effector DNA. (a) Two micrograms each of IE110(−800/+120)-CAT (pGH83), IE110 (−165/+120)-CAT (pGH135), and IE175(−1900/+30)-CAT (pPOH2) target DNAs were used. (b) Five micrograms each of IE110(−165/+120)-CAT (pGH135) and IE110(−128/+120)-CAT (pCA61) target DNAs were used. All samples were brought up to the same levels of total input DNA by addition of pBR322 carrier DNA. The final fold induction over basal expression (i.e., cotransfection with pBR322 DNA only) is given in parentheses for each target DNA.

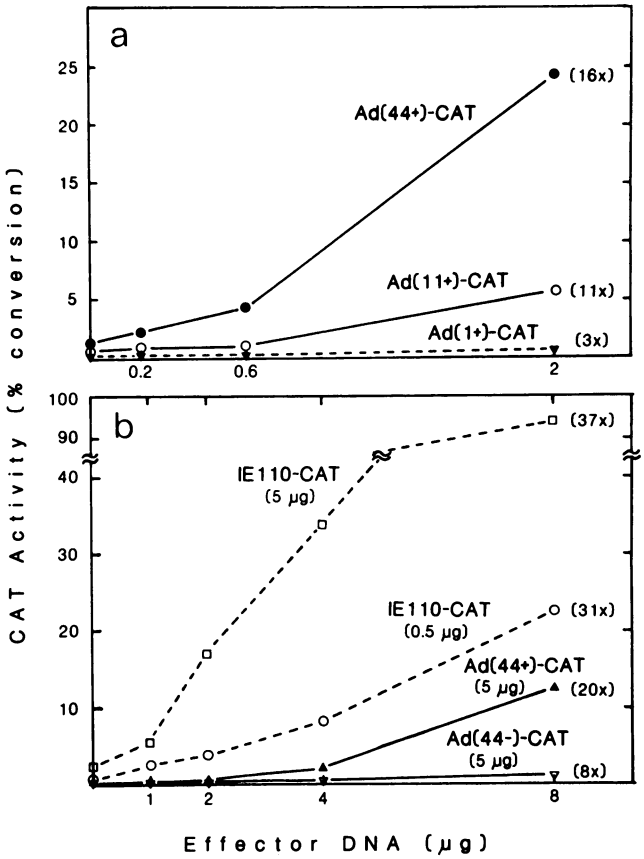

FIG. 5. Multiple tandemly repeated copies of the adenovirus left-end 67-bp fragment convey only weak VF65 responses. Graphs show results of transient CAT enzyme expression assays after cotransfection with increasing amounts of VF65 (pGH62) effector DNA. (a) Effect of insertion of increasing numbers of copies of the 67-bp sequence in the forward orientation on the 5′-upstream side of the nonresponsive A10-CAT gene. Target DNAs included 3 μg each of Ad67(44+)-CAT (pCA33 [44 copies]), Ad67(11+)-CAT (pCA31 [11 copies]), and Ad67(1+)-CAT (pCA49 [1 copy]). (b) Effect of orientation of 67-bp repeats relative to the A10-CAT gene. Target DNAs included 0.5 and 5 μg of IE110(−165/+120)-CAT (pGH135), 5 μg of Ad67(44+)-CAT (pCA33 [44 copies, forward orientation]), and 5 μg of Ad67(44−)-CAT (pCA32 [44 copies, backwards orientation]). The fold induction over basal levels is given in parentheses.

the test binding sites were spaced with their centers 26 bp apart (as shown below).

5′-CAAGAATCTCATTAGCATGCAGCTGCATGCTAATGAGATTCTTG-3′
3′-GTTCTTAGAGTAATCGTACGTCGACGTACGATTACTCTAAGAAC-5′

With the crude HeLa cell nuclear extract sample, this wild-type O^+/T^+ oligonucleotide (no. 41) gave efficient complex formation in the presence of 40 μg of poly(dI-dC) · poly(dI-dC) per ml and formed a double-band pattern, presumably representing interactions with either one or both copies of the identical binding sites present in the dimer oligonucleotide (Fig. 6a, lane 2). Cleavage of the 44-bp wild-type oligonucleotide (no. 41) into two identical 22-bp fragments with *Pvu*II altered the pattern to one with a single shift-up species (Fig. 6a, lane 4), providing evidence in support of the notion that the upper band represented occupation of both consensus sites on some DNA molecules rather than interactions with two separate proteins. Two mutated IE110 O^+/T^- oligonucleotides, with substitutions either at each of the last three bases (positions 11, 12, and 13) in the ATGCTAATGAGAT consensus (no. 42; Fig. 6b) or at positions 9 and 10 (no. 56; not shown), produced a binding pattern similar to that displayed by the wild-type sequence. In contrast, the IE110 O^-/T^+ oligonucleotides with alterations at positions 2 and 3 (no. 44; Fig. 6b) or at positions 1, 2, and 4 (no. 54; not shown) gave only a single band indicating a markedly reduced efficiency of complex formation, while the O^-/T^- oligonucleotide with transversions at positions 6 and 7 (no. 43; Fig. 6b) had totally lost the ability to form even the faster-migrating complex. The use of increasing amounts of nuclear extract revealed that the IE110 O^+/T^- oligonucleotide (no. 42) initially entered the faster-migrating complex only but gave both types of bands when a larger amount of extract was used (Fig. 6c). Even

TABLE 1. Summary and properties of mutant synthetic oligonucleotides

Oligonucleotide type and no.	Sequence[a]	Binding-site type	Mutation positions	Relative NFIII binding	Mobility type[b]
IE110 site 1 plus 2 consensus					
41	CTGCATGCTAATGAGATTCTTG	O^+/T^+	None	++++	F
42	CTGCATGCTAATGACCGTCTTG	O^+/T^-	11, 12, 13	++++	F
56	CTGCATGCTAATTCGATTCTTG	O^+/T^-	9, 10	++++	F
87	CTGCATGCTAATTCCCGTCTTG	O^+	9, 10, 11, 12, 13	++++	F
44	CTGCAGCCTAATGAGATTCTTG	O^-/T^+	2, 3	++	S
54	CTGCGAGGTAATGAGATTCTTG	O^-/T^+	1, 2, 4	++	S
43	CTGCATGCTCCTGAGATTCTTG	O^-/T^-	6, 7	−[c]	−
150	CTGCAGCGTAATGACCGTCTTG	T^-	2, 3, 4, 11, 12, 13	(+)[d]	(S)
149	CTGCAGCGTAATACCCGTCTTG	T^-	2, 3, 4, 9, 10, 11, 12, 13	−	−
151	CGACTAATGAGATTCAGTCTTG	T^+	1, 2, 3	++++	S
152	CTGCGACTCATGCTAATTCTTG	O^+	9, 10, 11, 12, 13	++++	F
IE175 site 1 consensus					
55	CTGCTGCATAATGGAATTCTTG	T^+	None	++	S
86	CTGCTGCATAATGGCCGTCTTG	T^-	11, 12, 13	+	S

[a] Only the right hand 22 bp from the top strand of the 44-bp double-stranded dimers is shown.
[b] F, Fast; S, slow; (S), barely detectable.
[c] −, None.
[d] Barely detectable binding.

with the IE110 O^-/T^+ oligonucleotide (no. 44), a small fraction of the DNA migrated at the doubly occupied position when more extract was added.

These results confirmed that the 5′-ATGCTAAT octamer portion of the IE110 O/T consensus sequence possesses high affinity for binding to a protein factor detectable in crude nuclear extracts of HeLa cells. Nevertheless, all IE110 oligonucleotides that retained an intact T^+ element only were also capable of binding to some factor in these extracts, although with lower affinity than that displayed by any of the oligonucleotides with an intact O^+ target site. Interestingly, in parallel experiments, an additional 44-bp oligonucleotide (no. 55, Table 1) representing the proximal TAATGGAAT element in the IE175 promoter region (T^+) gave a similar single-band binding pattern to that observed with the O^-/T^+ mutant IE110 oligonucleotides.

Distinctive characteristics of the ATGCTAAT and TAAT GARAT complexes. In the experiments described above, the mutated IE110 oligonucleotide that lacked the ATGC portion of the O/T consensus (no. 44) appeared to produce slightly slower-migrating T forms of both the singly occupied and doubly occupied complexes (Fig. 6b). In contrast, all IE110-based oligonucleotides tested that contained an intact ATGCTAAT octamer consensus motif (no. 41, 42, and 56) produced slightly faster-migrating O forms of the complex, irrespective of whether they contained an intact TAATGA RAT sequence as well.

To investigate whether the complexes formed with the synthetic TAATGARAT oligonucleotides contain proteins of the NFIII type, a highly concentrated, affinity-purified preparation of NFIII was incubated with the IE175 TAAT GARAT consensus oligonucleotide (no. 55) and compared with an equivalent amount of crude unfractionated extract under conditions in which only a single site was occupied (Fig. 7a). Although several minor faster-migrating species (labeled X) were seen in the crude extract (lane 2), the major T complex formed with purified NFIII (lane 3) proved to have mobility identical to that from the crude extract. The broad band of slightly faster-migrating material in lane 3 probably represents some degradation of the affinity-purified 92-kDa NFIII protein (42). The mobility of the IE175 TAAT GARAT complex formed with purified NFIII was also compared with that of another IE110 (O^+/T^-) variant 44-bp oligonucleotide (no. 87) which lacks the entire GAGAT portion of the consensus sequence beyond the intact octamer motif. The results revealed that the singly occupied O and T complexes formed between these two oligonucleotides and the crude nuclear extract also displayed the characteristic differences in mobility (Fig. 7b, lanes 1 and 4). Furthermore, these differences persisted even with affinity-purified NFIII (lanes 2 and 3). Note that in this experiment the O^+ probe was mixed with an eightfold-greater dilution of NFIII than was the T^+ probe to compensate for its higher affinity.

To ensure that the different gel electrophoretic mobilities of the O and T complexes were not caused by positional effects, two additional oligonucleotides were prepared (no. 151 and 152) in which the locations of the IE110 octamer and TAATGAGAT elements relative to the physical ends of the 44-bp dimer were exchanged (Table 1). Nevertheless, the complexes formed with these new variants exhibited the same characteristic sequence-specific differences in gel retardation properties (Fig. 7c, lanes 1 and 2). Intriguingly, the amount of T complex formed with oligonucleotide no. 151 was found to be markedly higher than that with no. 55 and equal to that with the O^+ oligonucleotides no. 41, 87, and 152 (summarized in Table 1). Therefore, in some flanking-sequence contexts the affinity of NFIII for the TAATGAGAT element may be almost as high as for the ATGCTAAT element. Three additional probes that retained only the TAAT or TAATGA portion of the IE110 consensus element or only the TAATGG portion of the IE175 consensus element were also tested for binding activity with the crude nuclear extract in this experiment (Fig. 7c, lanes 3, 4, and 5). Neither TAAT (no. 150) nor TAATGA (no. 149) gave any significant bands characteristic of the NFIII complex, although TAATGG (no. 86) produced a small amount of the slower-migrating T type of complex. Therefore, the 3′-AAT, 3′-GAT, and 3′-GAGAT portions of the IE175 and IE110 TAATGARAT motifs are critical components of the T-complex-binding site. The TAATGG oligonucleotide (no. 86) also yielded three strong, fast-migrating, DNA-protein complexes of the X type similar to those seen previously with the parent IE175 TAATGGAAT sequence (no. 55).

The ATGCTAAT and TAATGARAT motifs bind to the

same protein. Since the Ad2 67-bp DNA fragment used for affinity purification of NFIII contains a sequence that closely resembles a combined IE110 O/T site (Fig. 2), the possibility arose that two distinct but similar-sized DNA-binding proteins might be involved. Therefore, a series of experiments were carried out in which various unlabeled IE110- or IE175-derived oligonucleotides were used to compete with the labeled IE175 proximal TAATGGAAT site oligonucleotide (no. 55) for binding to crude nuclear extracts (Fig. 8). We reasoned that binding to the lower-affinity TAATGA RAT site should not be subject to competition by an ATG CTAAT sequence if two separate proteins were involved but would be subject to very efficient competition if the same protein were involved. The results showed that two oligonucleotides with intact octamer motifs (no. 41 [O^+/T^+] and no. 42, [O^+/T^-]) competed for binding to the TAATGGAAT site extremely effectively and did so at 5- to 10-fold-lower molar excess than that at which even oligonucleotide no. 55 competed with itself. In contrast, the control nonbinding O^-/T^- mutant oligonucleotide (no. 43) failed to compete significantly even at 100-fold molar excess. An IE110 O^-/T^+ oligonucleotide (no. 44) competed with the same efficiency as the IE175 TAATGGAAT oligonucleotide. This result could only be obtained with separate protein moieties if they formed a stable protein-protein complex or if they could both independently recognize the octamer target sequence. Therefore, the most reasonable assumption is that the NFIII protein itself can independently recognize and bind to TAATGARAT elements without invoking the need for a second protein.

To further characterize the relative stability of the NFIII: DNA interactions at the O and T sites and to screen for other DNA-binding proteins in the crude extracts that might recognize the TAATGARAT elements, we carried out mobility shift assays in the presence of a range of increasing concentrations of poly(dI-dC) · poly(dI-dC) or poly(dA-dT) · poly(dA-dT) alternating copolymer competitor DNAs (Fig. 9). In this experiment, the affinity of proteins in HeLa cell crude nuclear extracts for the IE175 T^+ probe (no. 55) was compared with their affinity for the IE110 O^+ probe (no. 87). Note that only the singly occupied forms of the complexes were observed on this occasion. The results showed the following. (i) Both oligonucleotides formed a discrete but presumably nonspecific complex with intermediate mobility (NS) in the absence of any competitor DNA. (ii) The IE110 O complex formed even in the absence of competitor and was significantly more resistent to both poly(dI-dC) · poly (dI-dC) and poly(dA-dT) · poly(dA-dT) competition than was the equivalent IE175 T complex. (iii) Formation of the T-site complex required that some poly(dI-dC) · poly(dI-dC) be present but was totally abolished in the presence of poly(dA-dT) · poly(dA-dT). (iv) A second, faster-migrating complex (X) was again detected, but this protein species was bound stably only by the TAATGGAAT oligonucleotide and was subject to efficient competition by poly(dI-dC) · poly(dI-dC) but not by poly(dA-dT) · poly(dA-dT). These properties of both the O and T forms of NFIII complexes argue against the X species being degradation products of NFIII and are

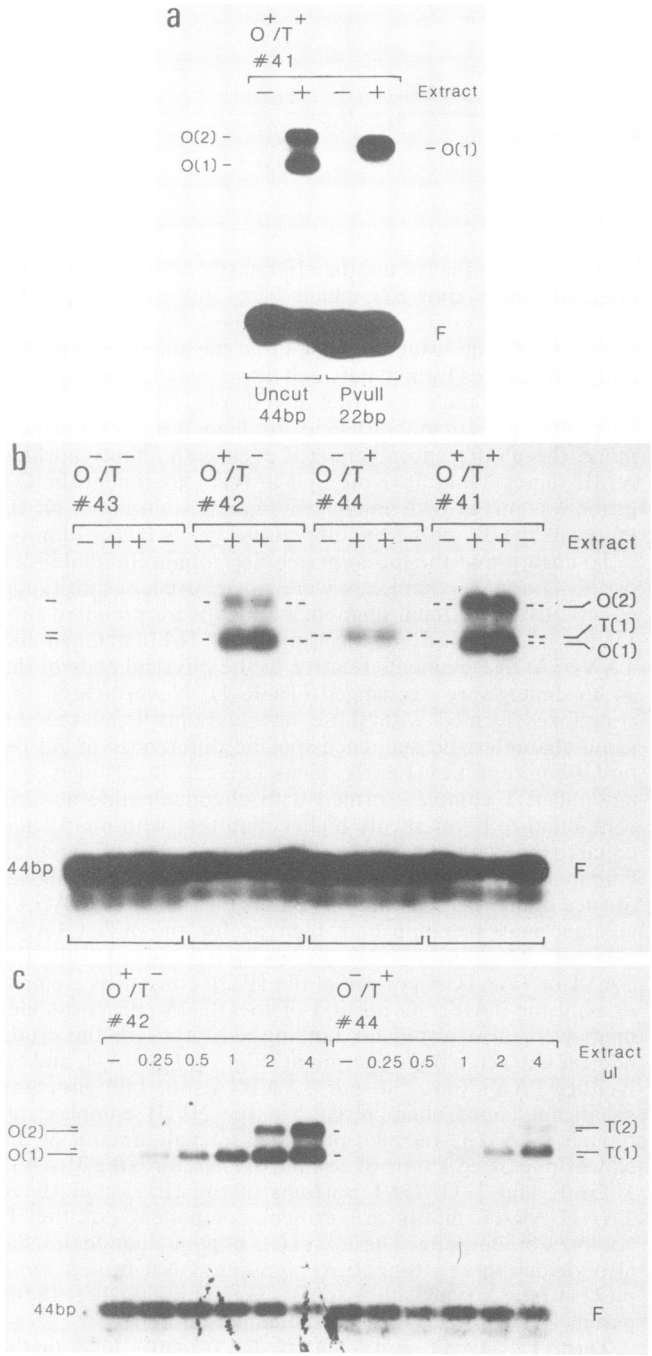

FIG. 6. Mobility shift-up assays with synthetic oligonucleotides containing consensus octamer or TAATGARAT motifs. The autoradiographs show binding of proteins in crude HeLa cell nuclear extracts to various ^{32}P-labeled dimeric 44-bp double-stranded oligonucleotides (Table 1) in an agarose gel retardation assay. (a) Effect of cleavage of the wild-type O^+/T^+ 44-bp IE110 oligonucleotide (no. 41) into 22-bp monomers with *Pvu*II. O(1), 44-bp complex with a single site occupied; O(2), 44-bp complex with both sites occupied; F, free (unbound) oligonucleotides. (b) Comparison of wild-type and mutated forms of the IE110-binding site. No. 41, intact IE110 combined octamer-TAATGAGAT consensus; no. 42, IE110 point mutations at positions 11, 12, and 13; no. 43, IE110 point mutations at positions 6 and 7; no. 44, IE110 point mutations at positions 2 and 3 in the overlapping consensus sequences. O(1), O(2), T(1), bound complexes; F, free (unbound) oligonucleotides. (c) Relative amounts of singly and doubly occupied forms of the O and T complexes produced with oligonucleotides no. 42 and 44 by using increasing amounts of crude nuclear extract. Bound complexes and free oligonucleotides are indicated.

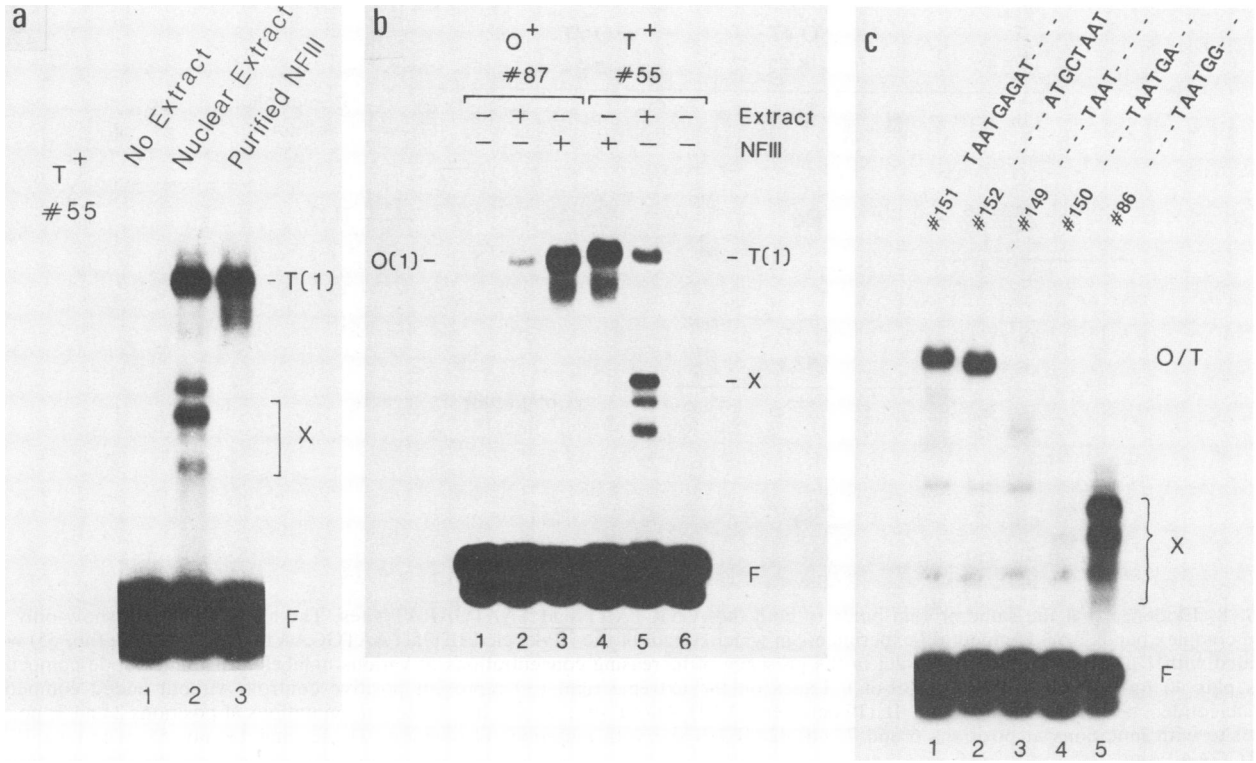

FIG. 7. Binding of affinity-purified NFIII to TAATGARAT but not TAATGA oligonucleotides and differences in mobility of the O and T complexes. Autoradiographs show comparisons of binding in mobility shift assays of crude nuclear extracts or affinity-purified NFIII to different ^{32}P-labeled 44-bp dimer oligonucleotides (5 fmol each). (a) Confirmation that the major binding activity for the IE175 TAATGGAAT (oligonucleotide no. 55) in crude extracts represents NFIII. Lanes: 1, no extract; 2, crude HeLa cell nuclear extract; 3, affinity-purified NFIII. T(1), Singly occupied NFIII complex; X, unidentified faster-migrating minor complexes. (b) Differences in relative mobility between complexes formed with the IE175 TAATGGAAT oligonucleotide (no. 55, T^+) and those formed with an IE110 octamer-only oligonucleotide (no. 87, O^+). Lanes: 1 and 6, no extract; 2, 1 μl of an eightfold dilution of crude HeLa cell nuclear extract; 3, 5 μl of a 1:200 dilution of affinity-purified NFIII fraction; 4, 5 μl of a 1:25 dilution of affinity-purified NFIII fraction; 5, 1 μl of undiluted HeLa cell crude nuclear extract. O(1), singly occupied monomeric form of the ATGCTAAT complex; T(1), singly occupied form of the TAATGGAAT complex. X and F are as in panel A. (c) Improved binding to TAATGARAT in a different flanking-sequence context and lack of binding to mutated TAATGARAT elements. The relative positions of the IE110 ATGCTAAT and TAATGAGAT elements were exchanged in probes no. 151 and 152, and the 3′ GAGAT, GAT, or AAT sequences were mutated in probes 149, 150, and 86 (Table 1). Crude HeLa cell nuclear extract was used. O/T, Positions of singly-occupied NFIII complexes; X, unidentified cellular binding factor; F, free (unbound) oligonucleotides.

similar to those of the α-H1 TAATGARAT-binding protein from HSV-infected cell extracts that was described by Kristie and Roizman (21).

Interactions with the HSV virion factor. McKnight et al. (31) and Preston et al. (48) described preliminary in vitro evidence for complex formation between the VF65 protein component of HSV virions and a cellular factor that binds to HSV IE promoter region DNA fragments. The supershifted bands (referred to as IEC) were formed either directly with infected-cell extracts or after addition of lysed virions to uninfected-cell extracts and migrated slower than the α-H1 DNA-protein complexes formed with the uninfected-cell extracts. An example of a similar phenomenon detected with our T^+ oligonucleotide probe (no. 55) under the electrophoresis conditions described by Preston et al. (48) and after cleavage to monomers with *Pvu*II is shown in Fig. 10. Although very little of the normal NFIII T complex was formed in the control sample on this occasion (lane 2), addition of a lysed virion preparation to the crude HeLa cell nuclear extract produced an abundant new shifted species (C) migrating considerably more slowly than the direct NFIII complex (lane 3). Interestingly, under these circumstances the minor X bands disappeared, although the nonspecific bands were unaffected. The O^+/T^+ oligonucleotide (no. 41) also produced a similar C supercomplex (lane 8), but no complexes of any kind were formed by adding virion components alone in the absence of the nuclear extract (not shown). Surprisingly, although the T^+ probe formed the supershifted complex when the virion proteins were added to the crude nuclear extract, it did not do so when purified NFIII was substituted for the crude extract. Instead, the normal NFIII T complexes appeared to be increased in abundance but not supershifted. In contrast, with the crude extract the normal NFIII complex was reduced in abundance when the supershifted complex formed. These results indicate that purified NFIII alone is insufficient for formation of the C complex and that some other factor present in the crude nuclear extract is required as well (or instead) and that formation of the C complex interferes with both the O/T and X complexes, either by including these same cellular factors in the supercomplexes or by competing with them for the probe DNA.

DISCUSSION

The 13-bp overlapping O/T consensus elements from the HSV IE110 gene are remarkably similar to the well-defined NFIII/ORP-C binding site near the Ad2 DNA replication

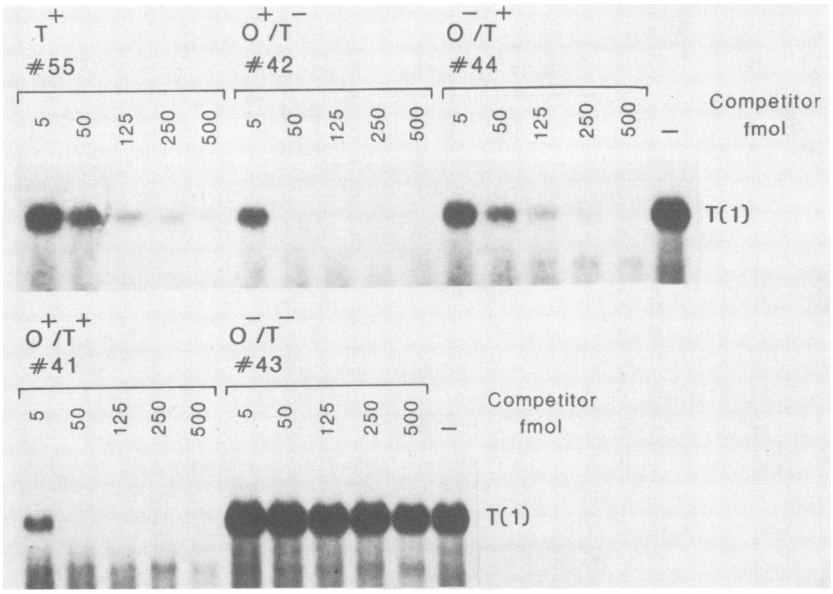

FIG. 8. Evidence that the same protein binds to both the ATGCTAAT and TAATGARAT sites. The autoradiographs show only the shifted complex bands from competition experiments in which 3 fmol of the ^{32}P-labeled IE175 TAATGGAAT oligonucleotide (no. 55) were incubated with 1 μl of crude nuclear extract in the presence of increasing concentrations of various unlabeled oligonucleotide competitor DNAs plus 40 μg of poly(dI-dC) · poly(dI-dC). Lanes on the extreme right (−) represent positive controls without added competitor oligonucleotides. No. 41 (#41), Wild-type IE110 octamer-TAATGAGAT site; no. 44, IE110 site with mutations at positions 2 and 3; no. 43, IE110 site with mutations at positions 6 and 7; no. 42, IE110 site with mutations at positions 11, 12, and 13; no. 55, wild-type IE175 TAATGGAAT site.

origin. Furthermore, the ATGCTAAT portion of the O/T VF65 response motif matches closely with the ATGCAAAT octamer sequence known to be an important transcriptional regulatory element in immunoglobulin promoters and enhancers and in the U2 small nuclear RNA, histone H2b, and mouse mammary tumor virus long terminal repeat promoters. Using gel mobility retardation assays, we have shown here that at least three independent DNA fragments from the IE110 5′-upstream promoter region, plus at least one fragment each from the IE175 and IE68 promoters, all bind directly and specifically to affinity-purified NFIII isolated from uninfected HeLa cells. Overall, the proximal 600 bp of the IE110 upstream region may contain as many as eight single or overlapping ATGCTAAT and TAATGARAT motifs, and the divergent IE175/*ori*-S/IE68 control region contains four such elements.

Extensive analysis by numerous groups interested in immunoglobulin and histone gene regulation have shown that two distinct cellular DNA-binding proteins recognize the standard canonical ATGCAAAT octamer consensus element (11, 56, 66). One is found in all cell types (OTF-1) and is apparently not involved in immunoglobulin gene activation (10), whereas the other (OTF-2) is found only after stimulation of B cells and is directly involved in activation of immunoglobulin gene expression (56, 70). Interaction of OTF-1 with an upstream octamer element present at −60 to −50 in the histone H2b promoter imparts S-phase-specific cell cycle regulation to that gene (11, 60). Recent evidence shows that the 92-kDa NFIII protein involved in adenovirus DNA replication binds efficiently to the canonical H2b and immunoglobulin octamer signals (42) and that the ubiquitous octamer-binding protein (OTF-1) purified in the laboratory of Roeder has the same electrophoretic mobility as NFIII and can functionally substitute for NFIII in the in vitro replication assay (41). Therefore, even if NFIII or OTF-1 are not involved in virion factor activation, the predominant localization of multiple octamer-binding sites overlapping with the TAATGARAT elements in the IE110 (and IE63) promoter regions and their absence in the IE175 promoter region could have profound and intriguing consequences for differential control of IE110 gene expression, especially in the absence of VF65 during the establishment of and reactivation from the latent state in neuronal cells in vivo.

A crucial question concerns the relationship between NFIII binding and the phenomenon of VF65 responsiveness. We have shown that the 5′-upstream regions of the IE110, IE175, and IE68 genes from HSV-1 all contain target sites for strong and very specific responses to *trans*-activation by cotransfection with the isolated HSV VF65 gene in transient assays in Vero cells (38, 52; Ciufo et al., unpublished data). Single copies of the O/T-class ATGCTAATGATAT consensus element at −165 to −153 within its natural IE110 promoter context or of the T-class TAATGGAAT consensus element at −114 to −105 in the IE175 promoter both appear to be sufficient to act as target signals, although other nearby viral sequences may also be required. In contrast, the human cytomegalovirus major IE promoter, several HSV DE promoters, and all heterologous viral and cellular promoters that we have tested failed to respond (39, 40). The unresponsive target promoters include both the immunoglobulin κ and λ region enhancers and the HSV thymidine kinase promoter, which all possess single canonical (but backward-oriented) ATGCAAAT motifs (Ciufo et al., unpublished data). Similarly, even the Ad2 NFIII consensus elements proved to be only very weak targets for VF65 activation in an A10-CAT background. Therefore, the TAATGARAT and not the ATGCTAAT motifs appear to be essential.

Our initial puzzling observation that the isolated DNA fragment containing IE175 TAATGAGAT site 2 (which lacks any associated octamer consensus motif) bound to a

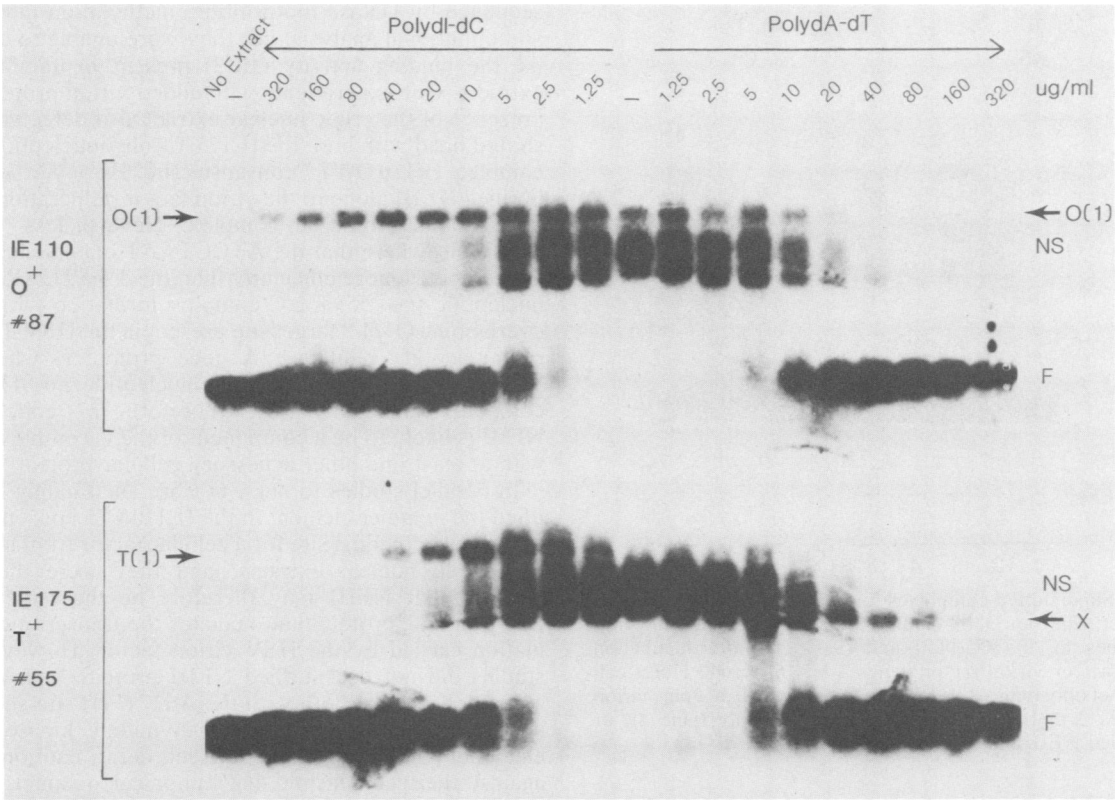

FIG. 9. Comparison of the stability of ATGCTAAT and TAATGARAT complexes to competition with alternating copolymers of poly(dI-dC) · poly(dI-dC) and poly(dA-dT) · poly(dA-dT). The autoradiographs show binding of crude HeLa cell nuclear extract protein to 44-bp synthetic oligonucleotides containing the consensus IE110 ATGCTAAT motif only (O^+/T^-, no. 87 [#87]; top) or the IE175 TAATGGAAT motif only (T^+, #55; bottom). The center lane (–) in each panel shows binding in the absence of competitor DNA. Lanes to the left show the effects of increasing concentrations (indicated above the lanes) of double-stranded poly(dI-dC) · poly(dI-dC) (in micrograms per milliliter) and the lanes to the right show the effects of increasing concentrations of double-stranded poly(dA-dT) · poly(dA-dT). O(1), Singly occupied ATCGTAAT site NFIII complex; T(1), singly occupied TAATGGAAT site NFIII complex; X, unidentified poly(dI-dC) · poly(dI-dC)-sensitive IE175 site complex; NS, nonspecific binding activity; F, free (unbound) oligonucleotides.

protein that copurified with NFIII activity through the DNA recognition site affinity column was confirmed by the use of short defined-site synthetic oligonucleotide probes. Although the GARAT portion of the IE110 O/T consensus sequence was dispensable for efficient NFIII binding in the presence of an intact ATGCTAAT octamer element, in the absence of an intact ATGCTAAT motif both the IE175 TAATGGAAT oligonucleotide and several O^-/T^+ mutated IE110 oligonucleotides were still capable of binding to NFIII. Considering that neither the TAAT nor TAATGA sequences alone were sufficient for binding, we concluded that the O and T motifs must represent two separate and distinct protein-binding sites with overlapping recognition elements.

In an attempt to resolve whether two different proteins of nearly the same size were involved or whether NFIII itself could independently recognize both elements, we discovered that the ATGCTAAT and TAATGARAT oligonucleotides produced distinct forms of DNA-protein complexes even with affinity-purified NFIII. In most cases, the O complexes consistently displayed an eightfold-higher binding affinity and greater stability to poly(dI-dC) · poly(dI-dC) and poly(dA-dT) · poly(dA-dT) competition than did the T complexes. Although the TAATGARAT site-binding interaction proved to be extremely sensitive to poly(dA-dT) · poly(dA-dT) competition, both binding activities showed the same preferential affinity for poly(dA-dT) · poly(dA-dT) over poly(dI-dC) · poly(dI-dC). Furthermore, the two types of protein-DNA complexes exhibited slight but significant differences in their gel electrophoretic mobility, even though the oligonucleotide probes were of identical length. This latter result may reflect the fact that the bound NFIII proteins assume different conformations at the two types of binding sites. Possible positional effects caused by the bound protein molecules being nearer to the physical ends of the oligonucleotide in the case of the TAATGARAT sites compared with the octamer sites were ruled out by reversal of the positions of the elements within the oligonucleotides. Moreover, the results of our oligonucleotide competition experiments argue strongly against the idea that ATG CTAAT and TAATGARAT shift-up complexes contain two different proteins. Indeed, the octamer sequence alone competed 5- to 10-fold more effectively for binding of NFIII with the labeled IE175 TAATGARAT probe than did the IE175 TAATGARAT DNA sequence itself. A distinct octamer-binding protein would not have been expected to compete significantly for binding to the TAATGGAAT recognition site in such an experiment.

On the basis of these results, we propose a model whereby the NFIII/OTF-1 protein itself might have flexible sequence recognition characteristics such that a conformational change allows binding to the TAATGARAT target sequence as well as to the ATGCTAAT type of octamer sequence. Potentially, the T complexes may be stabilized in the pres-

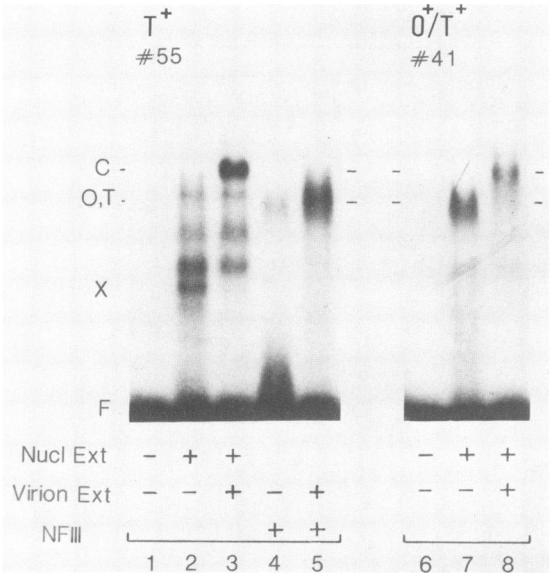

FIG. 10. Supershifted complexes formed by addition of an HSV virion extract. Shift-up DNA-binding assays with labeled oligonucleotide probes no. 55 (#55 [T^+]) and 41 (O^+/T^+) after incubation with no extract or virion factor (lanes 1 and 6), crude HeLa cell nuclear extract only (lanes 2 and 7), crude nuclear extract plus virion extract (lanes 3 and 8), affinity-purified NFIII only (lane 4), or affinity-purified NFIII plus virion extract (lane 5).

ence of the HSV VF65 protein to produce both more efficient TAATGARAT binding and the *trans*-activation responses. Thus, the TAATGARAT elements and the overlapping octamer elements together may represent a single complex target for *trans*-activation through NFIII-VF65 protein-protein interactions or VF65-mediated modification of NFIII. The overlap between the two types of sites may have the advantage of allowing initial occupancy of the higher-affinity octamer site by NFIII alone followed by translocation into the lower-affinity TAATGARAT site in the presence of VF65. Interestingly, the notion of flexibility in octamer-binding-site recognition has also been forwarded by Sturm et al. (62), who worked with a similar octamer-binding protein (OBF-100) and divergent octamerlike sequences in the simian virus 40 enhancer. One could imagine that a shift from functional recognition of the conserved octamer motifs in cellular promoters to degenerate octamerlike TAATGARAT motifs in viral promoters represents an excellent and subtle mechanism for a virus to rapidly usurp control of preexisting cellular transcription machinery.

The properties that we have described for NFIII-TAATGARAT complexes, especially the poly(dA-dT) · poly(dA-dT) sensitivity, are similar to those described by Kristie and Roizman (21) and McKnight et al. (31) for a 110-kDa cellular protein referred to as α-H1. In shift-up studies using crude nuclear extracts of HeLa cells, they showed that this protein bound to isolated HSV-1 DNA fragments containing O/T or T consensus motifs derived from various HSV IE promoters. Both DNase I footprinting and methylation interference studies with α-H1 complexes formed at the IE63 O/T site 1 (22) closely resemble those for NFIII and OTF-1 bound to the Ad2 *ori* or H2b sites (42, 50). Preston et al. (48) reported the presence of VF65 in a complex with an unidentified cellular protein, using the HSV IE68 promoter site 1 as the target DNA sequence (which represents a T-type element only). This complex was centered over the TAATGARAT sequence by DNase footprinting, methylation interference, and mutational analysis, but they were unable to characterize the binding activity (HC3) present in uninfected cell extracts. Our experiments with added virion proteins in the presence of the crude nuclear extract also detected a supershifted band with both the IE175 T^+ oligonucleotide and the complete IE110 O^+/T^+ consensus site. However, addition of purified NFIII alone to the virion factor preparation failed to form this supershifted complex. Nevertheless, we have shown elsewhere that the ATGCTAAT oligonucleotide (no. 87) competes more efficiently than the TAATGARAT oligonucleotide (no. 55) for C-complex formation with another overlapping O^+/T^+ target site present in the HSV-2 ICP10 or ribonucleotide reductase A gene promoter, whereas the TAAT-to-TCCT mutation (oligonucleotide no. 43) fails to compete significantly (71). Therefore, we conclude that NFIII is likely to be a component of the C complex together with at least one other accessory cellular protein.

In parallel studies to ours, O'Hare and Goding (35) identified the octamer element in the IE110 O/T consensus site 1 as a specific binding site for a cellular factor from uninfected HeLa cell nuclear extracts, and they were the first to propose that NFIII may therefore be the target cellular DNA-binding protein that mediates the transcriptional stimulation caused by the HSV virion factor. However, those studies did not use purified NFIII protein, and our *trans*-activation results, both with the Ad2 NFIII site shown here and with immunoglobulin and thymidine kinase octamer elements (Ciufo et al., unpublished data), caution strongly against their conclusion that canonical octamer elements alone can give VF65 responses. Although it is tempting to speculate that the NFIII complexes represent essential cellular intermediates required for mediating VF65 stimulatory activity, it is still necessary to demonstrate that point mutations which abolish NFIII binding to single ATGCTAAT GARAT or TAATGARAT elements also eliminate VF65 responsiveness.

After our work was completed, Gerster and Roeder (17) reported that affinity-purified OTF-1 and OTF-2 both bind to the overlapping octamer-TAATGARAT elements in HSV IE promoters and that OTF-1 also appears to form a complex with the VF65 *trans*-activator protein only in the presence of another unidentified factor present in crude nuclear extracts. Furthermore, Baumruker et al. (2) have recently presented conclusions similar to ours about their OBP100 protein being able to recognize TAATGARAT elements as well as octamer elements.

ACKNOWLEDGMENTS

This work was funded by Public Health Service grants R01 CA22130 and R01 CA28473 to G.S.H. and P01 CA16519 to T.J.K. from the National Institutes of Health. E.A.O. was supported by a Postdoctoral Fellowship from the Monsanto Co. and by Public Health Service grant G32 CA09139 from the National Institutes of Health. G.S.H. was supported by American Cancer Society Faculty Research Award no. 247.

We thank Mabel Chiu and Barbara Daily for excellent technical assistance and Pamela Wright and Sarah Heaggans for photography and typing of the manuscript.

LITERATURE CITED

1. **Batterson, W., and B. Roizman.** 1983. Characterization of the herpes simplex virion-associated factor responsible for the induction of α genes. J. Virol. **46**:371–377.
2. **Baumruker, T., R. Sturm, and W. Herr.** 1988. OBP100 binds remarkably degenerate octamer motifs through specific interactions with flanking sequences. Genes Dev. **2**:1400–1413.

3. **Bzik, D. J., and C. M. Preston.** 1986. Analysis of DNA sequences which regulate the transcription of herpes simplex virus immediate early gene 3: DNA sequences required for enhancer-like activity and response to trans-activation by a virion polypeptide. Nucleic Acids Res. **14**:929–943.
4. **Campbell, M. E. M., J. W. Palfreyman, and C. M. Preston.** 1984. Identification of herpes simplex virus DNA sequences which encode a *trans*-acting polypeptide responsible for stimulation of immediate early transcription. J. Mol. Biol. **180**:1–19.
5. **Carthew, R. W., L. A. Chodosh, and P. A. Sharp.** 1985. An RNA polymerase II transcription factor binds to an upstream element in the adenovirus major late promoter. Cell **43**:439–448.
6. **Chou, J., and B. Roizman.** 1986. The terminal *a* sequence of the herpes simplex virus genome contains the promoter of a gene located in the repeat sequences of the L component. J. Virol. **57**:629–637.
7. **DeLuca, D., A. M. McCarthy, and P. Schaffer.** 1987. Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4. J. Virol. **56**:558–570.
8. **DeLuca, N. A., and P. A. Schaffer.** 1985. Activation of immediate-early, early, and late promoters by temperature-sensitive and wild-type forms of herpes simplex virus type 1 protein ICP4. Mol. Cell. Biol. **5**:1997–2008.
9. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**:1475–1489.
10. **Everett, R. D.** 1984. *Trans*-activation of transcription by herpes virus products: requirement for two HSV-1 immediate-early polypeptides for maximum activity. EMBO J. **3**:3135–3141.
11. **Fletcher, C., N. Heintz, and R. G. Roeder.** 1987. Purification and characterization of OTF-1, a transcription factor regulating cell cycle expression of a human histone H2b gene. Cell **51**:773–781.
12. **Fried, M., and D. Crothers.** 1981. Equilibria and kinetics of *lac* repressor-operator interactions by polyacrylamide gel electrophoresis. Nucleic Acids Res. **9**:6505–6525.
13. **Gaffney, D. F., J. McLauchlan, J. L. Whitton, and J. B. Clements.** 1985. A modular system for the assay of transcription regulatory signals: the sequence TAATGARAT is required for herpes simplex virus immediate early gene activation. Nucleic Acids Res. **13**:7847–7863.
14. **Garner, M. M., and A. Revzin.** 1981. A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the Escherichia coli lactose operon regulatory system. Nucleic Acids Res. **9**:3047–3060.
15. **Gelman, I. H., and S. Silverstein.** 1985. Identification of immediate early genes from herpes simplex virus that transactivate the virus thymidine kinase gene. Proc. Natl. Acad. Sci. USA **82**:5265–5269.
16. **Gelman, I. H., and S. Silverstein.** 1987. Herpes simplex virus immediate-early promoters are responsive to virus and cell *trans*-acting factors. J. Virol. **61**:2286–2296.
17. **Gerster, T., and R. G. Roeder.** 1988. A herpesvirus trans-activating protein interacts with transcription factor OTF-1 and other cellular proteins. Proc. Natl. Acad. Sci. USA **85**:6347–6351.
18. **Hayward, G. S.** 1986. Herpesviruses. I. Genome structure and regulation. Cancer Cells **4**:59–93.
19. **Jones, K. A., and R. Tjian.** 1985. Sp1 binds to promoter sequences and activates herpes simplex virus "immediate-early" gene transcription *in vitro*. Science **317**:179–182.
20. **Kristie, T. M., and B. Roizman.** 1984. Separation of sequences defining basal expression from those conferring α gene recognition within the regulatory domains of herpes simplex virus 1 α genes. Proc. Natl. Acad. Sci. USA **81**:4065–4069.
21. **Kristie, T. M., and B. Roizman.** 1987. Host cell proteins bind to the cis-acting site required for virion-mediated induction of herpes simplex virus 1 α genes. Proc. Natl. Acad. Sci. USA **84**:71–75.
22. **Kristie, T. M., and B. Roizman.** 1988. Differentiation and DNA contact points of host proteins binding at the *cis* site for virion-mediated induction of α genes of herpes simplex virus 1. J. Virol. **62**:1145–1157.
23. **Landolfi, N. F., J. D. Capra, and P. W. Tucker.** 1986. Interaction of cell-type-specific nuclear proteins with immuno-globulin V_H promoter region sequences. Nature (London) **323**:548–551.
24. **Landolfi, N. F., J. D. Capra, and P. W. Tucker.** 1987. Protein-nucleotide contacts in the immunoglobulin heavy-chain promoter region. Proc. Natl. Acad. Sci. USA **84**:3851–3855.
25. **Lang, J. C., D. A. Spandidos, and N. M. Wilkie.** 1984. Transcriptional regulation of a herpes simplex virus immediate early gene is mediated through an enhancer-type sequence. EMBO J. **3**:389–395.
26. **Leung, W.-C., K. Dimock, J. R. Smiley, and S. Bacchetti.** 1980. Herpes simplex virus thymidine kinase transcripts are absent from both nucleus and cytoplasm during infection in the presence of cycloheximide. J. Virol. **36**:361–365.
27. **Mackem, S., and B. Roizman.** 1980. Regulation of herpesvirus macromolecular synthesis: transcription-initiation sites and domains of α genes. Proc. Natl. Acad. Sci. USA **77**:7122–7126.
28. **Mackem, S., and B. Roizman.** 1982. Differentiation between α promoter and regulator regions of herpes simplex virus 1: the functional domains and sequence of a movable α regulator. Proc. Natl. Acad. Sci. USA **79**:4917–4921.
29. **Mackem, S., and B. Roizman.** 1982. Structural features of the herpes simplex virus α gene 4, 0, and 27 promoter-regulatory sequences which confer α regulation on chimeric thymidine kinase genes. J. Virol. **44**:939–949.
30. **Marsden, H. S., M. E. M. Campbell, L. Haarr, M. C. Frame, D. S. Parris, M. Murphy, R. G. Hope, and C. M. Preston.** 1987. The 65,000-M_r DNA-binding and virion *trans*-inducing proteins of herpes simplex virus type 1. J. Virol. **61**:2428–2437.
31. **McKnight, J. L. C., T. M. Kristie, and B. Roizman.** 1987. Binding of the virion protein mediating α gene induction in herpes simplex virus 1-infected cells to its cis site requires cellular proteins. Proc. Natl. Acad. Sci. USA **84**:7061–7065.
32. **Mosca, J. D., D. P. Bednarik, C. A. Rosen, J. G. Sodrowski, W. A. Haseltine, G. S. Hayward, and P. M. Pitha.** 1987. Identification of the viral gene product and target sequences responsible for herpes simplex virus induction of HIV LTR expression. Proc. Natl. Acad. Sci. USA **84**:7408–7412.
33. **Mosca, J. D., G. R. Reyes, P. M. Pitha, and G. S. Hayward.** 1985. Differential activation of hybrid genes containing herpes simplex virus immediate-early or delayed-early promoters after superinfection of stable DNA-transfected cell lines. J. Virol. **56**:867–878.
34. **Murchie, M. J., and D. J. McGeoch.** 1982. DNA sequence analysis of an immediate-early gene region of the herpes simplex virus type 1 genome (map coordinates 0.950 to 0.978). J. Gen. Virol. **62**:1–15.
35. **O'Hare, P., and C. R. Goding.** 1988. Herpes simplex virus regulatory elements and the immunoglobulin octamer domain bind a common factor and are both targets for virion transactivation. Cell **52**:435–445.
36. **O'Hare, P., and G. S. Hayward.** 1984. Expression of recombinant genes containing herpes simplex virus delayed-early and immediate-early regulatory regions and *trans*-activation by herpesvirus infection. J. Virol. **52**:522–531.
37. **O'Hare, P., and G. S. Hayward.** 1985. Evidence for a direct role for both the 175,000- and 110,000-molecular-weight immediate-early proteins of herpes simplex virus in the transactivation of delayed-early promoters. J. Virol. **53**:751–760.
38. **O'Hare, P., and G. S. Hayward.** 1985. Three *trans*-acting regulatory proteins of herpes simplex virus modulate immediate-early gene expression in a pathway involving positive and negative feedback regulation. J. Virol. **56**:723–733.
39. **O'Hare, P., and G. S. Hayward.** 1987. Comparison of upstream sequence requirements for positive and negative regulation of a herpes simplex virus immediate-early gene by three virus-encoded *trans*-acting factors. J. Virol. **61**:190–199.
40. **O'Hare, P., J. D. Mosca, and G. S. Hayward.** 1986. Multiple trans-activating proteins of herpes simplex virus that have different target promoter specificities and exhibit both positive and negative regulatory functions. Cancer Cells **4**:175–188.

41. **O'Neill, E. A., C. Fletcher, C. R. Burrow, N. Heintz, R. G. Roeder, and T. J. Kelly.** 1988. Transcription factor OTF-1 is functionally identical to the DNA replication factor NF-III. Science **241**:1210–1213.
42. **O'Neill, E. A., and T. J. Kelly.** 1988. Purification and characterization of nuclear factor III (ORP-C), a sequence specific DNA binding protein required for efficient initiation of adenovirus DNA replication. J. Biol. Chem. **263**:931–937.
43. **Perry, L. J., F. J. Rixon, R. D. Everett, M. C. Frame, and D. J. McGeoch.** 1986. Characterization of the IE110 gene of herpes simplex virus type 1. J. Gen. Virol. **67**:2365–2380.
44. **Persson, R. H., S. Bacchetti, and J. R. Smiley.** 1985. Cells that constitutively express the herpes simplex virus immediate-early protein ICP4 allow efficient activation of viral delayed-early genes in *trans*. J. Virol. **54**:414–421.
45. **Post, L. E., S. Mackem, and B. Roizman.** 1981. Regulation of genes of herpes simplex virus: expression of chimeric genes produced by fusion of thymidine kinase with gene promoters. Cell **24**:555–565.
46. **Preston, C. M.** 1979. Control of herpes simplex virus type 1 mRNA synthesis in cells infected with wild-type virus or the temperature-sensitive mutant *tsK*. J. Virol. **29**:275–284.
47. **Preston, C. M., M. G. Cordingley, and N. D. Stow.** 1984. Analysis of DNA sequences which regulate the transcription of a herpes simplex virus immediate-early gene. J. Virol. **50**: 708–716.
48. **Preston, C. M., M. C. Frame, and M. E. M. Campbell.** 1988. A complex formed between cell components and an HSV structural polypeptide binds to a viral immediate-early gene regulatory DNA sequence. Cell **52**:425–434.
49. **Pruijn, G. J. M., W. van Driel, and P. C. van der Vliet.** 1986. Nuclear factor III, a novel sequence-specific DNA-binding protein from HeLa cells stimulating adenovirus DNA replication. Nature (London) **322**:656–659.
50. **Pruijn, G. J. M., R. T. van Miltenberg, J. A. J. Claessens, and P. van der Vliet.** 1988. Interaction between the octamer-binding protein nuclear factor III and the adenovirus origin of DNA replication. J. Virol. **62**:3092–3102.
51. **Quinlan, M. P., and D. M. Knipe.** 1985. Stimulation of expression of a herpes simplex virus DNA-binding protein by two viral functions. Mol. Cell. Biol. **5**:957–963.
52. **Roberts, S. M., A. Boundy, P. O'Hare, M. C. Pizzorno, D. C. Cuifo, and G. S. Hayward.** 1988. Direct correlation between a negative autoregulatory response element at the cap site of the herpes simplex virus type 1 IE175(α4) promoter and a specific binding site for the IE175(ICP4) protein. J. Virol. **62**:4307–4320.
53. **Rosenfeld, P. J., and T. J. Kelly.** 1986. Purification of nuclear factor I by DNA recognition site affinity chromatography. J. Biol. Chem. **261**:1390–1408.
54. **Rosenfeld, P. J., E. A. O'Neill, R. J. Wides, and T. J. Kelly.** 1987. Sequence-specific interactions between cellular DNA-binding proteins and the adenovirus origin of DNA replication. Mol. Cell. Biol. **7**:875–886.
55. **Sacks, W. R., and P. A. Schaffer.** 1987. Deletion mutants in the gene encoding the herpes simplex virus type 1 immediate-early protein ICPO exhibit impaired growth in cell culture. J. Virol. **61**:829–839.
56. **Scheidereit, C., A. Heguy, and R. G. Roeder.** 1987. Identification and purification of a human lymphoid-specific octamer-binding protein (OTF-2) that activates transcription of an immunoglobulin promoter *in vitro*. Cell **51**:783–793.
57. **Sen, R., and D. Baltimore.** 1986. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell **46**:705–716.
58. **Sen, R., and D. Baltimore.** 1987. In vitro transcription of immunoglobulin genes in a B-cell extract: effects of enhancer and promoter sequences. Mol. Cell. Biol. **7**:1989–1994.
59. **Singh, H., R. Sen, D. Baltimore, and P. A. Sharp.** 1986. A nuclear factor that binds to a conserved sequence motif in transcriptional control elements of immunoglobulin genes. Nature (London) **319**:154–158.
60. **Sive, H. L., N. Heintz, and R. G. Roeder.** 1986. Multiple sequence elements are required for maximal *in vitro* transcription of a human histone H2B gene. Mol. Cell. Biol. **6**:3329–3340.
61. **Stow, N. D., and E. C. Stow.** 1986. Isolation and characterization of a herpes simplex virus type 1 mutant containing a deletion within the gene encoding the immediate-early polypeptide Vmw110. J. Gen. Virol. **67**:2571–2585.
62. **Sturm, R., T. Baumraker, B. R. Franza, and W. Herr.** 1987. A 100kD HeLa cell octamer binding protein (OBP100) interacts differently with two separate octamer-related sequences within the SV40 enhancer. Genes Dev. **1**:1147–1160.
63. **Triezenberg, S. J., R. C. Kingsbury, and S. L. McKnight.** 1988. Functional dissection of VP16, the transactivator of herpes simplex virus immediate-early gene expression. Genes Dev. **2**:718–729.
64. **Triezenberg, S. J., K. L. LaMarco, and S. L. McKnight.** 1988. Evidence of DNA:protein interactions that mediate HSV-1 immediate-early gene activation by VP16. Genes Dev. **2**:730–742.
65. **Watson, R. J., and J. B. Clements.** 1980. A herpes simplex virus type 1 function continuously required for early and late virus RNA synthesis. Nature (London) **285**:329–330.
66. **Weinberger, J., D. Baltimore, and P. A. Sharp.** 1986. Distinct factors bind to apparently homologous sequences in the immunoglobulin heavy-chain enhancer. Nature (London) **322**:846–847.
67. **Whitton, J. L., and J. B. Clements.** 1984. Replication origins and a sequence involved in coordinate induction of the immediate-early gene family are conserved in an intergenic region of herpes simplex virus. Nucleic Acids Res. **12**:2061–2079.
68. **Whitton, J. L., F. J. Rixon, A. E. Easton, and J. B. Clements.** 1983. Immediate-early mRNA-2 of herpes simplex virus types 1 and 2 is unspliced; conserved sequences around the 5′ and 3′ termini correspond to transcription regulatory signals. Nucleic Acids Res. **11**:6271–6287.
69. **Wides, R. J., M. D. Challberg, D. R. Rawlins, and T. J. Kelly.** 1987. Adenovirus origin of DNA replication: sequence requirements for replication in vitro. Mol. Cell. Biol. **7**:864–874.
70. **Wirth, T., L. Staudt, and D. Baltimore.** 1987. An octamer oligonucleotide upstream of a TATA motif is sufficient for lymphoid-specific promoter activity. Nature (London) **329**:174–178.
71. **Wymer, J. P., T. D. Cheung, Y.-N. Chang, G. S. Hayward, and L. Aurelian.** 1989. Identification of immediate-early-type *cis*-response elements in the promoter for the ribonucleotide reductase large subunit from herpes simplex virus type 2. J. Virol. **63**:2773–2784.