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The overlapping octamer/TAATGARAT motif is a high-affinity binding site for the cellular transcription factors Oct-1 and Oct-2

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The octamer motif in cellular promoters and the related TAATGARAT element in the herpes simplex virus (HSV) immediate-early promoters can both bind cellular octamer-binding proteins. The overlapping octamer/TAATGARAT elements (consensus ATGCTAATGARAT) found in the HSV-1 IE1 promoter thus represent a composite motif, each portion of which can independently bind octamer-binding protein. By comparing the binding characteristics of this composite motif with its individual elements, we show that it binds a single molecule of either Oct-1 or Oct-2 with much higher affinity than does either an octamer or TAATGARAT motif alone. This strong binding allows this element to direct a much higher level of gene expression when linked to a heterologous promoter than that observed with each of its individual components.

INTRODUCTION

The octamer motif (consensus ATGCAAATNA) is found in the promoters of a number of cellular genes, including those encoding histone H2B, the immunoglobulins and the small nuclear RNAs (reviewed in Falkner *et al.*, 1986). This motif binds a number of different cellular transcription factors, including the ubiquitous factor Oct-1 (Fletcher *et al.*, 1987) and the B cell- and neuronal-specific factor Oct-2 (Scheidereit *et al.*, 1987; He *et al.*, 1989), as well as a number of other embryonic and neuronal proteins (He *et al.*, 1989; Scholar *et al.*, 1989; Okamoto *et al.*, 1990). The motif plays a critical role in the expression of cellular genes that contain it (reviewed in Falkner *et al.*, 1986; Schaffner, 1990).

Similarly, the upstream regions of the herpes simplex virus (HSV) immediate-early genes contain multiple copies of the sequence TAATGARAT which is essential for their trans-activation by the virion protein Vmw65 (Batterson & Roizman, 1983; Campbell *et al.*, 1984; Preston *et al.*, 1984). As shown in Fig. 1, this sequence is related to the octamer consensus sequence, the right-hand half of the octamer motif showing close sequence similarity with the left-hand half of the TAATGARAT element. This similarity allows the TAATGARAT element to exhibit weak binding to Oct-1 and Oct-2 (Baumruker *et al.*, 1988; ap Rhys *et al.*, 1989). Following viral infection, however, Vmw65 forms a complex with Oct-1 which binds to the TAATGARAT motif with high affinity and mediates trans-activation of the immediate-early genes (McKnight *et al.*, 1987; Gerster & Roeder, 1988; O'Hare & Goding, 1988; Preston *et al.*, 1988; Triezenberg *et al.*, 1988; Stern *et al.*, 1989).

Although simple TAATGARAT motifs, such as those found in the majority of HSV-1 immediate-early genes, are sufficient for such trans-activation to occur (Bzik & Preston, 1986; Gelman & Silverstein, 1987), the upstream region of the HSV-1 IE1 gene contains five overlapping octamer/TAATGARAT motifs of the form ATGCATAATGARAT (Perry *et al.*, 1986), raising questions as to the function of such overlapping motifs. If the octamer-specific portion of this element (ATGC) or the TAATGARAT-specific portion (RAT) are destroyed by mutation, the remaining sequence continues to bind octamer-binding proteins (ap Rhys *et al.*, 1989). Hence both the octamer and TAATGARAT portions of this motif can function independently as octamer-binding elements.

To further characterize these overlapping motifs, we have studied the binding characteristics and function of an intact overlapping octamer/TAATGARAT motif. We show that this element binds a single molecule of Oct-1 or Oct-2 with very high affinity compared with single octamer or TAATGARAT elements, and directs a higher level of gene expression when linked to a heterologous promoter.

MATERIALS AND METHODS

Oligonucleotides

Complementary pairs of oligonucleotides with the sequences indicated in Fig. 1 were synthesized on an Applied Biosystems model 381A oligonucleotide synthesizer. All oligonucleotides were synthesized so that, when annealed, the double-stranded molecule would have a 5'-GATC single-stranded extension at either end to facilitate cloning. After annealing, the oligonucleotides were labelled by phosphorylation with [γ-^{32}P]ATP and T4 polynucleotide kinase.

DNA mobility shift assay

Nuclear extracts were made from 5×10^7 cells according to the method of Dignam *et al.* (1983). For the binding assay, 10 fmol

Octamer consensus	A	T	G	C	A	A	A	T	N	A		
HSV IE consensus	R	Y	G	N	T	A	A	T	G	A	R	A	T
O+/T+	A	T	G	C	T	A	A	T	G	A	G	A	T
O+/T−	A	T	G	C	T	A	A	T	G	A	T	A	T
O−/T+	G	C	G	G	T	A	A	T	G	A	G	A	T
O+	A	T	G	C	A	A	A	T	A	A			
Om	A	T	A	A	T	A	A	T	A	A			

Fig. 1. Comparison of the octamer motif consensus (Falkner *et al.*, 1986) and the viral TAATGARAT consensus (Whitton & Clements, 1984)

Also shown are the sequences of the oligonucleotides used in this study, comprising an overlapping octamer/TAATGARAT (O+/T+), a similar motif in which a single base of the TAATGARAT portion has been mutated (O+/T−), a similar motif with mutations in the octamer portion (O−/T+), a simple octamer motif (O+), and a mutant octamer (Om) which will not bind octamer-binding proteins (Lenardo *et al.*, 1987).

Abbreviations used: HSV, herpes simplex virus; CAT, chloramphenicol acetyltransferase.

of [^{32}P]ATP-labelled oligonucleotide probe was mixed with 1 μl of nuclear extract in the presence of 20 mM-Hepes, 5 mM-$MgCl_2$, 50 mM-KCl, 0.5 mM-dithiothreitol, 4% Ficoll and 2 μg of poly(dI,dC) per 20 μl reaction volume. Competitor DNA was added at a 1-, 10- or 100-fold molar excess at this stage, as required. The binding reaction was incubated for 40 min on ice, before separation of DNA/protein complexes by electrophoresis on a 4% polyacrylamide gel in 0.25 × TBE (TBE = 100 mM-Tris/100 mM-boric acid/2 mM-EDTA, pH 8.3). Gels were run for 2–3 h at 150 V and 4 °C, following pre-electrophoresis for about 2 h before use. Complexes were visualized by autoradiography of the dried gel. Autoradiographs were scanned on a Bio-Rad model 620 video densitometer.

Transfection

Annealed oligonucleotides were cloned into the *Bam*H1 site of pBL2 CAT (Luckow & Schutz, 1987), and recombinants containing the oligonucleotide were isolated by Grunstein–Hogness screening with labelled oligonucleotide (Grunstein & Hogness, 1981) and subsequent restriction mapping. Recombinant plasmids were transfected into BHK-21 cells (Macpherson & Stoker, 1962) as previously described (Kemp & Latchman, 1988) using 5 μg of DNA per 2×10^6 cells on a 90 mm plate. After transfection, cells were harvested, and the protein content was determined by the method of Bradford (1976). The chloramphenicol acetyltransferase (CAT) activity of samples equalized for protein content was then determined by the method of Gorman (1985).

RESULTS

To investigate the binding of octamer-binding proteins to an overlapping octamer/TAATGARAT motif, we synthesized an oligonucleotide bearing a perfect consensus of this motif (ATGCTAATGAGAT; Fig. 1) and used it in DNA mobility-shift experiments (Fried & Crothers, 1981) with a HeLa cell nuclear extract (Dignam *et al.*, 1983). In these experiments (Fig. 2), this oligonucleotide (O+/T+) produced a strong retarded band of similar mobility to that produced by an oligonucleotide with the consensus octamer motif alone (O+; Fig. 1). No additional slower-mobility band was detected with the O+/T+ oligonucleotide. This indicates that, like a single octamer motif, an overlapping octamer/TAATGARAT motif binds a single molecule of octamer-binding protein.

Fig. 2. DNA-mobility-shift assay using labelled O+/T+ (track 1) or O+ (track 2) oligonucleotides plus HeLa cell extract

In these initial experiments, the retarded band obtained with the O+/T+ oligonucleotide was considerably stronger than that obtained with the O+ oligonucleotide, suggesting that the overlapping octamer/TAATGARAT motif may constitute a high-affinity binding site for octamer-binding proteins. To investigate this possibility further, we carried out a band-shift experiment with labelled O+/T+ oligonucleotide and investigated the effects of various competitor oligonucleotides on the binding to O+/T+.

In these experiments (Fig. 3 and 4), the identical O+/T+ oligonucleotide was the most effective competitor for binding to itself, with binding to labelled oligonucleotide dramatically decreased at a 10-fold excess of competitor. In contrast, the O+ oligonucleotide competed less well, and an oligonucleotide containing a TAATGARAT motif (O+/T+; Fig. 1) identical with that found in the HSV-1 IE4/5 promoter (Whitton & Clements, 1984), was an even poorer competitor, as expected from the previously reported low affinity for octamer-binding proteins of this motif alone in the absence of Vmw65 (Baumruker *et al.*, 1988; ap Rhys *et al.*, 1989). The ability to compete for the protein binding to the O+/T+ sequence was specific for octamer or octamer-related motifs. Thus both a mutant octamer motif (Om; Fig. 1) which has been previously reported not to bind octamer-binding proteins (Lenardo *et al.*, 1987), and the binding site for the unrelated transcription factor Sp1 (Dynan & Tjian, 1983) failed to compete for binding. In these experiments, the O+/T+ oligonucleotide is, therefore, specifically binding to the ubiquitous octamer-binding protein Oct-1 with high affinity.

Thus, the O+/T+ motif appears to bind octamer-binding protein with a higher affinity than does either octamer or TAATGARAT motifs alone. Similar results to those obtained in HeLa cells were also obtained in BHK-21 cells (clone 13; (Macpherson & Stoker, 1962), suggesting that these results are not specific to one particular cell line (Fig. 4).

Both previous studies (Baumruker *et al.*, 1988; ap Rhys *et al.*, 1989) and our results indicate that the octamer motif alone has a higher affinity for octamer-binding protein than does an isolated TAATGARAT motif. It is likely, therefore, that the octamer portion of the overlapping octamer/TAATGARAT motif plays the major role in the ability of the motif to bind octamer-binding proteins with high affinity. To investigate the contribution of the TAATGARAT part of the motif, we synthesized an oligonucleotide (O+/T−; Fig. 1) in which a single base change at the seventh position of the TAATGARAT portion replaced a G with a T, making this oligonucleotide different from the consensus TAATGARAT motif but leaving the octamer sequence intact. As shown in Figs. 3 and 4, this resulted in a considerable decrease in the ability of this oligonucleotide to compete for binding to the O+/T+ oligonucleotide. When taken together with the results using the O−/T+ oligonucleotide, in which the octamer-specific bases have been mutated, this indicates that both halves of the overlapping octamer/TAATGARAT motif play an important role in its ability to bind octamer-binding protein with high affinity.

In mammalian B cells, an additional octamer-binding protein, Oct-2, is present in addition to the ubiquitous protein Oct-1 (Scheidereit *et al.*, 1987). To investigate the binding of this protein to the O+/T+ oligonucleotide, we carried out a DNA-mobility-shift assay using an extract prepared from IARC BL41 human B cells. In these experiments (Fig. 5 and 6), two major retarded bands were detected, the lower-mobility band representing binding of Oct-1 and the higher-mobility band being due to

binding of Oct-2. Both bands displayed competitive behaviour identical to that observed for Oct-1 in non-B cells, with the O+/T+ oligonucleotide competing much more effectively than any other oligonucleotide for binding to itself. Hence the overlapping octamer/TAATGARAT motif is a high-affinity binding site for both Oct-1 and Oct-2.

From these experiments it remained possible, however, that the O+/T+ oligonucleotide was binding with high-affinity specific forms of octamer-binding proteins which were poorly bound and hence competed for by the O+/T− or O−/T+ oligonucleotides. Conversely, one or both of these oligonucleotides might bind strongly to other forms of these proteins which were poorly bound by the O+/T+ oligonucleotide. To test this possibility, we carried out DNA-mobility-shift assays using either the O+/T− or O−/T+ labelled oligonucleotides, and competed for binding with our panel of octamer oligonucleotides.

In these experiments (Fig. 7), the O+/T+ oligonucleotide competed more effectively for binding to the O+/T− or O−/T+ oligonucleotides than did the homologous oligonucleotide itself or any other octamer oligonucleotide. Similar results were also obtained for Oct-1 and Oct-2 in B cell extracts (results not shown). Hence the octamer oligonucleotides do not appear to differ in their specificity for different forms of octamer-binding protein, but only in their affinity for Oct-1 and Oct-2, with the overlapping octamer/TAATGARAT motif forming the highest-affinity binding site.

The use of the different oligonucleotides labelled to the same

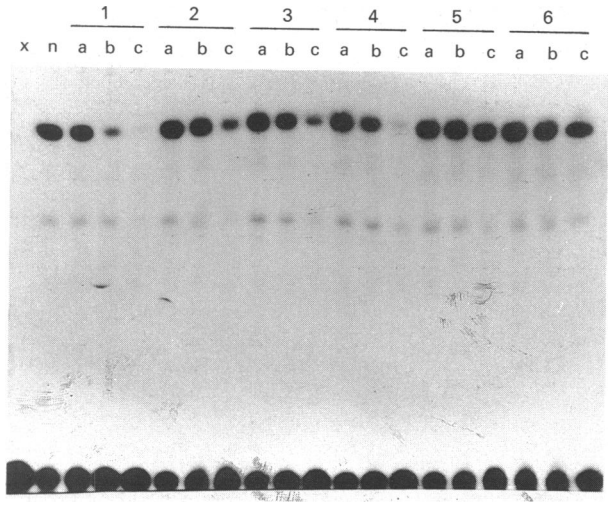

Fig. 3. DNA-mobility-shift assay using a labelled O+/T+ oligonucleotide plus HeLa cell extract in the presence of an excess of competitor oligonucleotide

Track x indicates the result with no extract added, and track n indicates the results obtained with HeLa extract in the absence of competing oligonucleotide. Tracks labelled a, b and c indicate respectively the results obtained with a 1-fold, 10-fold and 100-fold excess of the unlabelled competitor. Key to competitor oligonucleotides: 1, O+/T+; 2, O−/T+; 3, O+/T−; 4, O+; 5, Om; 6, Sp1.

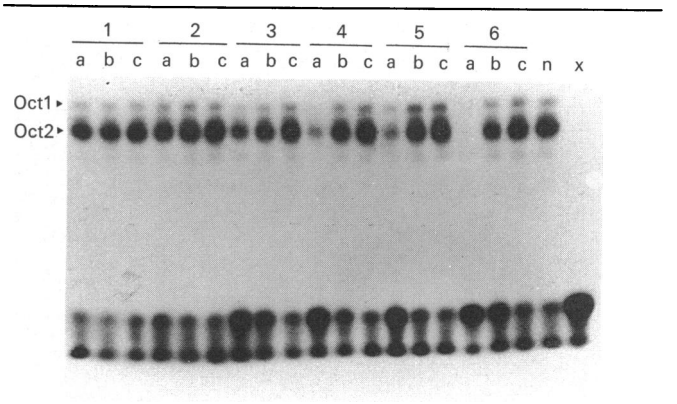

Fig. 5. DNA-mobility-shift assay using labelled O+/T+ oligonucleotide and IARC BL41 B cell extract

Note the two retarded bands produced by Oct-1 and Oct-2 binding. Track x indicates the result with no extract, and track n indicates the result with the B cell extract in the absence of competing oligonucleotide. Tracks labelled a, b and c indicate respectively the results obtained with a 100-fold, 10-fold and 1-fold excess of the unlabelled competitor. Key to competitor oligonucleotide: 1, Sp1; 2, Om; 3, O+; 4, O+/T−; 5, O−/T+; 6, O+/T+. The Oct 1 and Oct 2 bands are indicated.

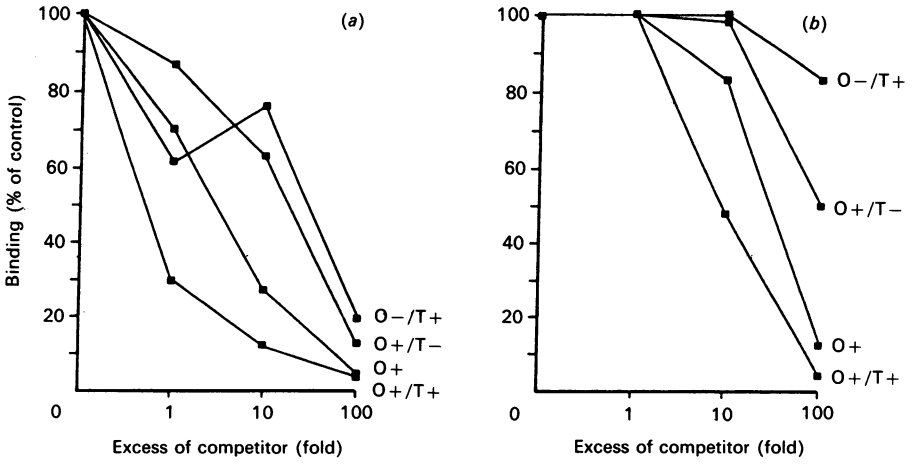

Fig. 4. Quantitative analysis of the competition data obtained by densitometric screening of the retarded band obtained in two replicate experiments similar to that shown in Fig. 2

The percentage decrease in binding to the O+/T+ oligonucleotide in the presence of each competitor is plotted against the excess of competitor used. (*a*) Results obtained using HeLa cell extracts; (*b*) results obtained with BHK-21 cell extracts.

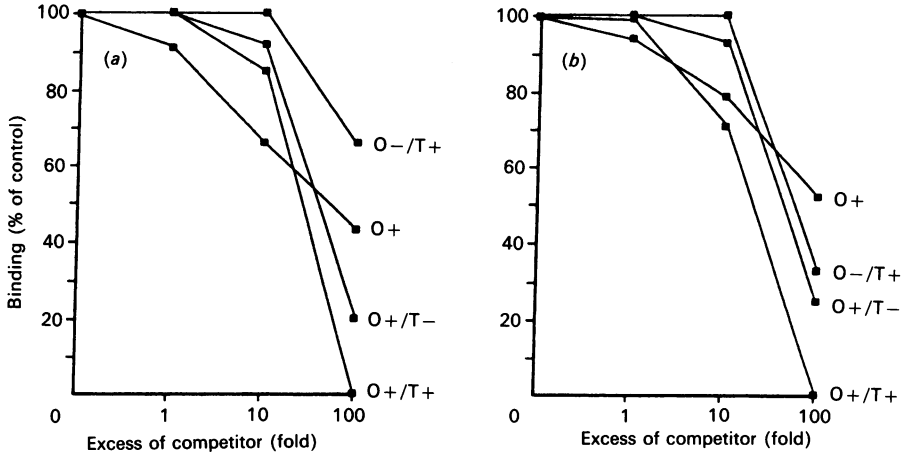

Fig. 6. Quantitative analysis of the B cell competition data for Oct-1 (*a*) and Oct-2 (*b*)

Percentage decreases in binding to the O+/T+ oligonucleotide in two replicate experiments is plotted against the excess of each competitor used.

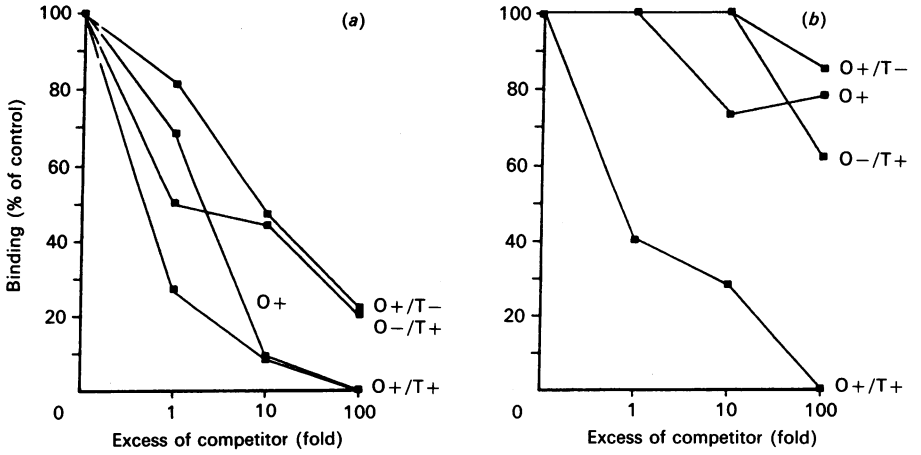

Fig. 7. Competition of various unlabelled oligonucleotides for the binding of Oct-1 to labelled O+/T− (*a*) or labelled O−/T+ (*b*) oligonucleotides using a BHK-21 extract

The percentage decrease in binding to each labelled oligonucleotide is plotted against the excess of unlabelled oligonucleotide used.

Table 1. Relative binding of octamer-proteins to octamer and TAATGARAT oligonucleotides

Values indicate the relative amounts binding to an equal amount of each labelled oligonucleotide with an identical amount of cell extract. In each case, the amount of binding to the O+/T+ oligonucleotide has been set at 100, and other values are expressed relative to this value.

	Relative binding			
Oligonucleotide	BHK-21	HeLa	B cell Oct-1	B cell Oct-2
O+/T+	100	100	100	100
O+/T−	11	17	40	55
O−/T+	6	2	8	13
O+	18	8	25	17
Om	1	0	0	6

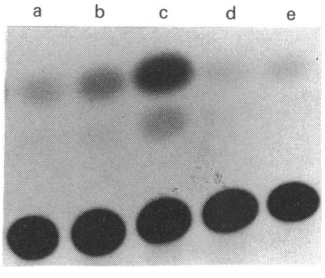

Fig. 8. Assay of CAT activity following transfection into BHK cells of recombinant plasmids obtained by cloning octamer oligonucleotides into the *Bam*H1 site of pBL2 CAT

Track e shows the CAT activity of pBL2 CAT alone; other tracks show the CAT activity of pBL2 CAT containing cloned O−/T+ oligonucleotide (*a*), O+/T− oligonucleotide (*b*), O+/T+ oligonucleotide (*c*) and Om oligonucleotide (*d*).

degree in a single DNA-mobility-shift experiment allowed an assessment of the relative extent of binding to each oligonucleotide in isolation, as opposed to competition experiments. Quantification of the amount of material retarded when an identical amount of a particular cell extract was added to each oligonucleotide (Table 1) showed strong binding to the O+/T+ oligonucleotide, with the O+/T− sequence being somewhat weaker and the O−/T+ sequence showing considerably lower binding. Hence, as before, the overlapping octamer/TAATGARAT motif showed the highest level of binding.

To assess whether this high level binding of octamer-binding protein by this motif has any functional consequences, we cloned single copies of each oligonucleotide into the upstream regulatory region of the plasmid pBL2 CAT (Luckow & Schutz, 1987). In this plasmid, a truncated HSV thymidine kinase promoter drives expression of the readily assayable CAT gene product (Gorman, 1985). As the truncated tK promoter is relatively weak in isolation, the effect of adding additional regulatory sequences can be readily assessed by their effect on the CAT activity of the recombinant plasmid. The constructs containing the various oligonucleotides were therefore introduced into BHK-21 cells by transfection and the level of CAT activity was assayed and compared with that produced by the vector alone.

As shown in Fig. 8, all of the oligonucleotides, with the exception of Om, which cannot bind octamer-binding proteins (Lenardo *et al.*, 1987), produced an increase in CAT activity over that observed with the parental vector. The greatest increase was observed, however, with the O+/T+ oligonucleotide, with the O+/T− sequence producing less than O+/T+ but more than O−/T+. Hence the different binding affinities of these sequences have functional consequences on their ability to enhance gene expression, the overlapping octamer/TAATGARAT motif having a higher affinity for octamer-binding proteins and directing a correspondingly higher level of gene expression than either an octamer motif or a TAATGARAT motif alone.

DISCUSSION

A previous study of the overlapping octamer/TAATGARAT motif (ap Rhys *et al.*, 1988) showed that when specific bases of either the octamer or TAATGARAT were destroyed by mutation, each of the individual elements could bind a single molecule of octamer-binding protein. The experiments described here show that a complex of identical mobility is obtained in DNA-mobility-shift assays carried out with the individual elements and the composite motif. Hence, like the individual elements, the composite motif also binds only a single molecule of octamer-binding protein, presumably because the fact that the central six bases (TAATGA) are common to both motifs causes steric hindrance to the binding of two molecules to the two overlapping motifs.

The binding to the composite motif is of high affinity; however, mutations in either the octamer or the TAATGARAT portions dramatically decrease binding affinity or the levels of gene expression driven by the motif. Such high-affinity binding raises the question of the reasons for the occurrences of these motifs in the HSV-1 IE1 promoter, whereas cellular genes have simple octamer motifs and the other HSV immediate-early genes have simple TAATGARAT motifs (Mackem & Roizman, 1980; Perry *et al.*, 1986; ap Rhys *et al.*, 1989).

Like the other immediate-early genes, the IE1 genes are trans-activated in lytic infection by the virion protein Vmw65, which acts via the TAATGARAT motif. In agreement with this, the overlapping octamer/TAATGARAT elements in the IE1 promoter can be trans-activated by Vmw65 in a similar manner to the TAATGARAT elements in the other immediate-early promoters (Gelman & Silverstein, 1987; O'Hare & Hayward, 1987). Interestingly, however, expression of IE1 also appears to be critically important for re-activation from latent infection. Thus viral mutants lacking this gene fail to re-activate both in latency models *in vitro* and after latent infection of animals (Leib *et al.*, 1989; Russell *et al.*, 1987). In this situation, the IE1 gene of the latent virus must be switched on at a high level, in response to a cellular signal, in the absence of Vmw65. It is possible, therefore, that such expression requires the presence of high-affinity binding sites for cellular octamer-binding proteins, rather than the low-affinity site provided by the TAATGARAT motif alone. Hence the overlapping octamer/TAATGARAT motif may represent a response to the need for the IE1 gene to be expressed at high levels in both the presence and the absence of Vmw65.

Whatever its precise role, it is clear that this motif represents a high-affinity binding site for cellular octamer-binding proteins, and offers a unique opportunity to study a composite motif, each half of which can bind the same transcription factor under different conditions.

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