The ability of POU family transcription factors to activate or repress gene expression is dependent on the spacing and context of their specific response elements

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The different forms of the Oct-2 and Brn-3 POU family transcription factors can have distinct effects on their target promoters involving both the activation and repression of gene expression. To investigate the requirements for these effects we have altered both the context and spacing of the two TAATGARAT binding sites for these factors within the herpes simplex virus immediate-early 3 gene promoter. We show that the activation of this

promoter by Brn-3a and its repression by Brn-3b is dependent on the correct spacing of these binding sites. In contrast, repression of the promoter by Oct-2.4 and Oct-2.5 is dependent on both the spacing and context of these sites with the requirements for repression by Oct-2.4 or Oct-2.5 being different. These effects are discussed in the context of the mechanisms by which POU factors activate or repress their target genes.

INTRODUCTION

The wide variety of specific patterns of gene expression which are required for normal embryonic development and the proper functioning of the adult organism are regulated by transcription factors which specifically activate or repress gene expression (for reviews see [1,2]). The ability of a relatively small number of such factors to regulate the expression of a vast array of different genes is enhanced by a number of processes which allow a single factor to have different effects in different situations. Thus in specific cases, a single transcription factor can effect gene expression either positively or negatively depending either on the nature of its binding site in the gene promoter (see, for example, [3]) or on the presence or absence of other sequences adjacent to its binding site constituting the context of the binding site (see, for example, [4,5]). Similarly, alternative splicing of a single gene transcript can produce mRNAs encoding different forms of a transcription factor which either activate or repress expression of the same promoter (see, for example, [6]).

These processes are well illustrated by the POU family of transcription factors which was originally defined on the basis of a conserved region of 150–160 amino acids shared by the Pit-1, Oct-1 and Unc-86 factors and which bind to sequences related to the consensus octamer motif ATGCAAAT (for reviews see [7,8]). Thus the Brn-3 subfamily of POU factors consists of three related proteins Brn-3a [9–11], Brn-3b [10,12–14] and Brn-3c [10,11,15] which are encoded by different genes [16]. Despite their close relationship, however, these factors differ in their effects on target genes. For example, Brn-3a activates a test promoter containing an octamer motif [17,18], the herpes simplex virus (HSV) immediate-early (IE) promoters which contain the octamer-related TAATGARAT motif [19] and the α -internexin promoter [20]. In contrast Brn-3b represses these promoters and can also interfere with their activation by Brn-3a [17–20]. However, when tested on the promoter of the neuronal nicotinic

acetylcholine receptor α 2 subunit gene, Brn-3b activates transcription whereas Brn-3a has no effect [21]. Such differences in activity are paralleled by differences in the expression patterns of the different Brn-3 forms [9–15] and are therefore likely to be of importance in the regulation of specific target genes.

In the case of Brn-3 the different effects on transcription are produced by three distinct, but related, factors which are encoded by three different genes [16]. In contrast, the Oct-2 POU family transcription factor is encoded by a single gene [22–24]. Interestingly, however, the primary transcript of this gene is alternatively spliced [25] producing mRNAs encoding isoforms with distinct effects on gene expression [26–28]. Thus the Oct-2.1 isoform, which is the predominant form in B lymphocytes, contains a C-terminal activation domain and is able to activate the HSV IE promoters upon co-transfection into fibroblasts [27]. In contrast the predominant forms in neuronal cells, Oct-2.4 and Oct-2.5, lack this C-terminal activation domain and therefore inhibit HSV IE gene expression [27]. However, all these isoforms are able to activate a simple promoter containing the octamer motif upstream of a TATA box [25,27] and all can repress the tyrosine hydroxylase promoter [29].

These findings suggest that the regulation of specific genes by different POU proteins is highly complex with different related factors or alternatively spliced forms of a single factor having distinct effects depending on parameters such as the sequence of the binding site in the promoter or its context relative to other sequence motifs. To begin to dissect these effects, we have focused on the HSV IE3 gene promoter which contains two TAATGARAT sequences [30–32] that can act as target sites for POU family transcription factors [32–34]. We have previously shown that this promoter is differentially modulated by the various forms of Oct-2 and Brn-3 [19,26]. We have therefore investigated the effects of altering the number, spacing and context of the TAATGARAT motifs in the IE promoter on the response to different forms of Oct-2 and Brn-3.

Abbreviations used: CAT, chloramphenicol acetyltransferase; HSV, herpes simplex virus; IE, immediate-early; tk, thymidine kinase.

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MATERIALS AND METHODS

Plasmid DNAs

The basic IE3 reporter construct contains the appropriate promoter (from -330 to $+33$ for IE3 [35]) driving expression of the chloramphenicol acetyltransferase (CAT) reporter gene. For further analysis appropriate fragments of the promoter were cloned upstream of the HSV thymidine kinase (tk) promoter in the vector pBLCat2 [36] (see Figure 1). The Oct-2.4 and Oct-2.5 expression vectors contain the appropriate full-length cDNAs cloned under the control of the cytomegalovirus IE promoter [25]. The Brn-3a and Brn-3b expression vectors contain full-length cDNA or genomic clones for each of these proteins cloned under the control of the Moloney murine leukaemia virus promoter in the vector pLTR poly, which has been modified by deletion of a cryptic splice site in the simian virus 40 (SV40) 3' untranslated region [10,17,18].

DNA transfection

BHK-21 cells [37] were transfected by the method of Gorman [38]. Transfections were carried out with 5×10^5 cells on a 90-mm-diameter plate transfected with $10 \mu\text{g}$ of the expression vector and $10 \mu\text{g}$ of target plasmid.

CAT assays

Assays of CAT activity in the transfected cells were carried out as described by Gorman [38], extracts having been previously equalized for protein content as described by Bradford [39]. The percentage conversion of chloramphenicol to the acetylated form was determined by scintillation counting of the regions of the TLC plate corresponding to the acetylated and non-acetylated forms, as detected by autoradiography. In all cases, the values obtained were equalized for differences in plasmid uptake between samples on the basis of the results of dot blot hybridization of an aliquot of the transfected cell extract with a vector DNA probe [40].

DNA mobility shift assays

DNA mobility shift assays were carried out as previously described [10], using *in vitro* transcribed and translated Brn-3 proteins and labelled oligonucleotides 5'-GATCGCGTAATGARAT-3' (representing a simple TAATGARAT motif), 5'-GCATGCTAATGATATTCTTT-3' (representing an overlapping octamer/TAATGARAT motif) and the sequence 5'-TGC-TCCTGCATAAATAATAGGG-3' from the corticotrophin-releasing hormone gene promoter [11].

RESULTS

In our previous experiments [19], both the Oct-2.4 and Oct-2.5 isoforms were able to repress the HSV IE3 promoter when co-transfected into BHK-21 fibroblast cells. To investigate these effects further we wished to manipulate the number and spacing of the TAATGARAT response elements in this promoter and determine the effect on the response to Oct-2. As many of these constructs would lack their own basal promoter elements and transcriptional start sites we cloned various fragments of the IE3 promoter upstream of the tk promoter in the vector pBLCat2 [36] (see Figure 1 for details of the constructs used in this study).

We initially prepared a construct in which the entire IE3 promoter fragment from -330 to $+33$ was cloned upstream of the tk promoter to use as a basis for further studies. This construct was, as expected, repressed by both Oct-2.4 and Oct-

Figure 1 Structure of the various constructs used in this study

In each case the indicated fragment of the IE3 promoter was cloned upstream of the tk promoter in the vector pBLCat2 [36].

Table 1 Effect of Oct-2.4 or Oct-2.5 on the activity of IE3 promoter

The values represent the CAT activity of each of the constructs (numbered as in Figure 1) when co-transfected in BHK-21 cells with either Oct-2.4 or Oct-2.5 expression vectors. All values are expressed relative to the value obtained upon co-transfecting each construct with the expression vector lacking any insert (set at 100%) and are the averages of three determinations whose range is indicated.

Construct	CAT activity (%)	
	Oct 2.4	Oct 2.5
1	11 $\pm$ 5	11 $\pm$ 6
2	102 $\pm$ 11	51 $\pm$ 5
3	105 $\pm$ 32	25 $\pm$ 5
4	83 $\pm$ 7	46 $\pm$ 11
5	37 $\pm$ 9	47 $\pm$ 16
6	239 $\pm$ 20	78 $\pm$ 16

2.5 when co-transfected in BHK-21 fibroblasts (Table 1). To manipulate the construct further we deleted the transcriptional start site and TATA box leaving an IE3 fragment from -330 to -104 containing both TAATGARAT motifs upstream of the tk promoter. This construct (construct 2 in Figure 1) was still repressed by Oct-2.5 but was no longer repressed by Oct-2.4 (Table 1). This indicates that repression by Oct-2.4 is likely to require basal elements in the IE3 promoter and/or its transcriptional start site to mediate its repressive effect even though it acts by binding to the TAATGARAT elements [26,33,34].

The fragment of the IE3 promoter in construct 2 still retains both TAATGARAT elements and additional constructs were therefore prepared containing only the proximal TAATGARAT (construct 3 containing the promoter fragment from -104 to -134 ; see Figure 1) or only the distal TAATGARAT (construct 4 containing the promoter fragment from -330 to -134 ; see Figure 1). Although the basal level of activity of these constructs was different, both were repressed by Oct-2.5 and not by Oct-2.4 (Table 1). This supports the conclusion that repression by Oct-

Figure 2 DNA mobility shift assay using Brn-3a protein produced by *in vitro* transcription and translation and labelled oligonucleotides containing either an overlapping octamer/TAATGARAT motif (track 1), the Brn-3 binding site from the corticotrophin-releasing hormone promoter (track 2), or the simple TAATGARAT motif (tracks 3–6)

Tracks 4 and 5 show the result with 10-fold or 100-fold excess of unlabelled TAATGARAT oligonucleotide respectively and track 6 that with a 100-fold excess of an unrelated oligonucleotide.

2.4 requires other elements not present in the enhancer fragments tested. It also indicates, however, that Oct-2.5 has much simpler requirements and can repress via a fragment containing only a single TAATGARAT motif.

We next investigated the effects of the spacing of the two TAATGARAT motifs on repression by Oct-2.4 and Oct-2.5. To do this a fragment between the two TAATGARAT motifs (from –134 to –205) in construct 2 was removed, reducing the spacing of the two motifs from 140 to 68 bp (construct 5 in Figure 1). Interestingly, this manipulation had no effect on the ability of Oct-2.5 to repress the promoter but it restored repression by Oct-2.4 (Table 1). Hence reduction in the spacing of the two TAATGARAT motifs can compensate for the absence of the IE3 basal promoter elements/transcriptional start site and restore repression by Oct-2.4.

In order to restore the spacing of the two TAATGARAT elements we inserted a 72 bp fragment containing an unrelated linker sequence into the restriction site created by the deletion in construct 5 in order to produce construct 6 (see Figure 1). This construct was no longer repressible by Oct-2.5 and was actually activated by Oct-2.4 (Table 1). Thus restoration of the spacing between the two TAATGARAT elements once again abolishes the repression by Oct-2.4 as was also observed in construct 2 compared with construct 5. Surprisingly, however, it also prevents repression by Oct-2.5 which is observed in constructs 2 and 5. This suggests that where two TAATGARAT elements are present repression by Oct-2.5 may require either close proximity of the two elements or a specific sequence between them which may, for example, bend to bring the two elements into close proximity.

Table 2 Effect of Brn-3a and Brn-3b in the IE3 promoter constructs

The values represent the CAT activity of each of the constructs (Figure 1) when co-transfected into the indicated cell type with Brn-3a or Brn-3b expression vectors. All values are expressed relative to the value obtained upon co-transfecting each construct with the expression vector lacking any insert (set at 100%) and are the averages of three determinations whose range is indicated.

Construct	CAT activity (%)			
	BHK-21 cells		ND-7 cells	
	Brn-3a	Brn-3b	Brn-3a	Brn-3b
1	151 ± 11	64 ± 40	400 ± 200	30 ± 10
2	211 ± 5	90 ± 5	203 ± 8	47 ± 30
3	71 ± 20	32 ± 8	140 ± 5	90 ± 10
4	262 ± 40	40 ± 5	183 ± 80	97 ± 50
5	79 ± 29	118 ± 20	65 ± 25	163 ± 67
6	224 ± 102	104 ± 5	300 ± 90	31 ± 9

Whatever the precise mechanisms of these effects, they clearly illustrate the importance of basal elements, and the spacing and context of the TAATGARAT elements in mediating the response to Oct-2.4 and Oct-2.5. Having studied the effects of these factors on the response to Oct-2.4 and Oct-2.5, we wished to investigate their effect on the response to the different forms of Brn-3, since we have previously shown that Brn-3a activates the IE3 promoter whereas Brn-3b represses it [19]. However, while Brn-3a has previously been shown to bind to various octamer-related motifs including the overlapping octamer/TAATGARAT motif (ATGCTAATGATAT) from the IE1 promoter [10] it has not previously been shown to bind to a simple TAATGARAT motif like those present in the IE3 promoter.

We therefore carried out DNA mobility-shift assays using this TAATGARAT motif as a probe. In this experiment (Figure 2), *in vitro* transcribed and translated Brn-3a bound specifically to the TAATGARAT probe and was removed by competition with excess unlabelled homologous competitor but not with unrelated competitor. Hence Brn-3a can bind specifically to this sequence although the binding was less strong than that observed with an overlapping octamer/TAATGARAT motif or the Brn-3 binding site from the corticotrophin-releasing hormone promoter [11].

We therefore tested the effects of Brn-3a and Brn-3b on our various constructs, in BHK-21 cells lacking any endogenous Brn-3. In these experiments (Table 2), Brn-3a activated construct 1 while Brn-3b repressed its activity, paralleling our previous results with the IE3 promoter [19]. These effects still occurred in construct 2 containing the upstream promoter fragment from –330 to –104; this indicates that they were not dependent on the presence of basal promoter elements downstream of –104 in the IE3 promoter. Interestingly, construct 4 was also strongly activated by Brn-3a and repressed by Brn-3b, indicating that the distal TAATGARAT in the IE3 promoter responds more strongly than the proximal TAATGARAT present in construct 3 which was not activated by Brn-3a although it was repressed by Brn-3b (Table 2).

In contrast to the activation of these constructs by Brn-3a and their repression by Brn-3b, construct 5, in which the two TAATGARAT motifs were moved close together (Figure 1), was weakly activated (rather than repressed) by Brn-3b, but was not activated by Brn-3a. When the natural spacing of the two TAATGARAT motifs was restored, however, in construct 6, activation by Brn-3a was restored. Hence, although Brn-3a can

Table 3 Effect of Vmw65 on the IE3 promoter constructs

The values represent the CAT activity of each of the constructs (Figure 1) when co-transfected into the indicated cell type with Vmw65 expression vector compared with the activity obtained when each construct was co-transfected with vector lacking any insert (set at 100%).

Construct	CAT activity (%)	
	BHK-21 cells	ND-7 cells
1	383	220
2	2066	553
3	626	2200
4	320	1350
5	450	240
6	500	1100

activate via a single TAATGARAT motif or a pair of spaced motifs, it cannot do so via two adjacent TAATGARAT motifs while this arrangement does allow weak activation by Brn-3b.

In order to extend these observations to cells which contain endogenous Brn-3, we also tested the effect of the various forms of Brn-3 when co-transfected with the promoter constructs into the ND7 neuronal cell line [41] which expresses all the forms of Brn-3 [12]. In these experiments (Table 2), similar effects were observed to those seen in BHK cells with all the constructs except construct 5 being activated by Brn-3a whereas construct 5 alone was activated by Brn-3b. Interestingly, in this case the activation of construct 5 by Brn-3b was much stronger than in BHK cells, whereas construct 6, in which the spacing of the TAATGARAT motifs has been restored, was strongly repressed by Brn-3b.

As well as acting as a target for cellular POU factors such as Oct-2 and Brn-3, the HSV TAATGARAT motifs also act as a target for the complex of the cellular POU factor Oct-1 and the viral transactivator Vmw65 [32,42,43]. This binding is essential for the strong activation of the IE promoters by Vmw65 during lytic infection. We therefore wished to test whether our constructs would be transactivated by Vmw65. To test this each construct was introduced into BHK or ND7 cells with or without Vmw65 expression vector [44]. In these experiments all the constructs were transactivated by Vmw65 in both BHK cells and ND7 cells (Table 3) although there was some variation in the degree of transactivation of each construct. Despite this variation these findings clearly indicate that transactivation by Vmw65 can occur with only a single TAATGARAT motif and regardless of the spacing or context of multiple TAATGARAT motifs.

DISCUSSION

The Brn-3a POU factor has been previously shown to activate a wide variety of different promoters bearing different binding sites for this factor [11,13,17–20]. In agreement with this we show here that this factor can activate via a single TAATGARAT motif in the HSV IE3 promoter and can also do so when basal promoter elements are removed or the context of these motifs in the promoter is altered provided that their spacing is maintained. Similarly all the constructs which are activated by Brn-3a are repressed by Brn-3b, paralleling the previously reported ability of Brn-3b to repress several Brn-3a-activated promoters and to inhibit their activation by Brn-3a [17–20]. Paradoxically, however, the results presented here show that Brn-3b can activate an IE3 promoter construct where the two TAATGARAT motifs are adjacent to one another, whereas such a construct is unaffected by Brn-3a. This effect parallels the activation of the

gene encoding the neuronal nicotinic acetylcholine receptor $\alpha 2$ subunit by Brn-3b but not Brn-3a [21]. In this case also activation by Brn-3b is dependent on the precise arrangement of the six copies of the octamer-related 11 bp CCCCATGCAAT motif in the promoter, since deletion of two of these copies prevents activation by Brn-3b. It is possible therefore that Brn-3b may bind to certain specific arrangements containing more than one copy of its binding site, allowing it to activate gene expression; whereas Brn-3a cannot bind, perhaps due to the steric constraints produced by adjacent binding sites. Further DNA binding studies involving these specific motifs will be necessary to confirm this possibility.

Whatever the case, however, it is clear that Brn-3a can activate and Brn-3b repress via a single copy of its binding site and in a manner independent of the context of such a site. However, a small number of specific arrangements of multiple binding sites do allow gene activation by Brn-3b and both the arrangements described thus far (construct 5 in this paper and the $\alpha 2$ receptor promoter) are not activated by Brn-3a, although it is possible that specific arrangements which can be activated by both Brn-3a and Brn-3b do exist.

In contrast to these relatively simple rules for predicting the effect of Brn-3a and Brn-3b, the parameters which control whether Oct-2.4 or Oct-2.5 can repress a particular promoter are more complex. Thus repression by Oct-2.4 is abolished by deletion of proximal promoter elements in the IE3 promoter but can be restored when the two TAATGARAT motifs in the upstream promoter are adjacent to one another. Conversely, Oct-2.5 can repress via a single TAATGARAT as well as in the absence of proximal promoter elements or when the two TAATGARAT elements are adjacent to one another. It does appear, however, to be sensitive to the context of the TAATGARAT motifs since replacing the fragment between them with a random sequence while maintaining the spacing abolishes repression by Oct-2.5 (construct 6).

Although the mechanisms mediating these effects are uncertain, they do focus attention on the differences between Oct-2.4 and Oct-2.5 which produce differences in the dependence of their inhibitory effects on the context and spacing of the binding site in the promoter. This is particularly so since Oct-2.4 and Oct-2.5 do differ in their effects on reporter constructs containing different octamer/TAATGARAT sequences cloned at the same position upstream of the tk promoter, indicating that they are also differentially affected by the sequence of their target binding site [26]. Although both Oct-2.4 and Oct-2.5 are produced by alternative splicing events which lead to the loss of the strong C-terminal activation domain present in Oct-2.1, they differ in the nature of their C-terminal sequences. Thus Oct-2.4 contains only a 17-amino-acid tail replacing the last 82 amino acids of Oct-2.1 whereas Oct-2.5 contains an extra 132 amino acids replacing the last 12 amino acids of Oct-2.1 [25].

We have previously shown [28] that the inhibitory effect of Oct-2 on the IE3 promoter requires the presence of a 40-amino-acid N-terminal inhibitory domain which interacts with other transcription factors leading to a reduction in the activity of the basal transcriptional complex. The effect of this region is overridden in Oct-2.1 by the strong stimulating activity of the C-terminal activation domain, whereas the inhibitory domain can function in the Oct-2.4 and Oct-2.5 forms where the activation domain is absent. It is possible therefore that the differences in the C-termini of Oct-2.4 and Oct-2.5 may indirectly affect the precise structure of this N-terminal inhibitory region and thereby affect its ability to interact with the transcriptional complexes bound at different basal promoter elements.

Whatever the exact mechanism of the effects we observe, it is

clear that the effects of Oct-2.4 and Oct-2.5 and of Brn-3a and Brn-3b on gene expression are affected by the spacing and context of their specific response elements. This will allow these factors, either alone or in combination, to have a variety of effects on the expression of target genes. In turn this will enable them to contribute to the complex patterns of gene expression seen in the nervous system where all these factors are expressed in some but not all neuronal cells.

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