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The Oct-2 transcription factor represses tyrosine hydroxylase expression via a heptamer TAATGARAT-like motif in the gene promoter

Sally J.Dawson[1], Sung Ok Yoon[2], Dona M.Chikaraishi[2], Karen A.Lillycrop[1,3] and David S.Latchman[1]*

[1]Department of Molecular Pathology, University College London Medical School, The Windeyer Building, Cleveland Street, London W1P 6DB, UK, [2]Department of Molecular Biology and Microbiology and [3]Neuroscience Program, Tufts University School of Medicine, Boston, MA 02111, USA

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ABSTRACT

The tyrosine hydroxylase (TH) gene promoter contains adjacent octamer and heptamer motifs which act as target sites for octamer binding transcription factors. Mutation of the heptamer motif but not the octamer motif enhances TH promoter activity in neuronal cells expressing Oct-2 but not in non-expressing fibroblasts. Similarly addition of the heptamer motif to a minimal TH promoter represses gene expression in neuronal cells but not in fibroblasts. These effects can be reproduced by the artificial expression of neuronal isoforms of Oct-2 in fibroblasts which results in the repression of transfected TH promoters containing an intact heptamer motif but not those in which this motif has been mutated or deleted. The TH promoter thus represents the first example of a cellular promoter which is repressed by Oct-2. The significance of this effect is discussed in terms of the cell type specificity of the TH promoter and its induction by different physiological stimuli.

INTRODUCTION

The octamer binding proteins bind to the octamer motif (ATGCAAAT) which is present in the regulatory regions of a large number of different genes such as those encoding the small nuclear RNAs, histone H2B and the immunoglobulin genes (for review see 1). In addition these proteins also bind to the unrelated heptamer motif (CTCATGA) which is located at a variable distance (2–22 bases) upstream of the octamer motif in the immunoglobulin heavy chain gene promoters (2).

The co-operative binding of octamer binding proteins to the adjacent octamer and heptamer motifs is necessary for the high level activity of the immunoglobulin promoters (3). In particular the octamer binding protein Oct-2 which is expressed at high levels in B cells but which is absent in most other cell types (4, 5) strongly transactivates the immunoglobulin promoters (6).

Interestingly the Oct-2 protein is also present in neuronal cells (7, 8). In this case however, it appears to play a predominantly inhibitory role repressing the expression of, for example, artificial promoters containing an inserted octamer motif (9). Similarly, the herpes simplex virus (HSV) immediate-early genes are also repressed by Oct-2 in neuronal cells (10), with this effect being mediated via the octamer-related-TAATGARAT motif in the HSV promoters which is known to bind octamer-binding proteins (11, 12).

Thus far however, the role played by Oct-2 in regulating cellular gene expression in neuronal cells remains unclear. In particular, whilst both viral and artificial octamer containing promoters have been shown to be inhibited by Oct-2, this effect has never been demonstrated for a cellular gene which is expressed in neuronal cells. One potential target for repression by Oct-2 in neuronal cells is the gene encoding tyrosine hydroxylase (TH) which is expressed in catecholaminergic neurons in both the peripheral and central nervous systems as well as in the chromaffin cells of the adrenal medulla (13). Thus the region of the TH promoter from −175 to −158 contains adjacent sequences with a one base pair difference (ATGCAATT) from the consensus octamer motif (ATGCAAAT) and two base pair differences (CTAATGG) from the consensus heptamer motif (CTCATGA) (Figure 1). Both these motifs also show good homology to the TAATGARAT (R = purine) motif in the HSV immediate-early genes with the heptamer motif showing a better match (seven out of nine bases compared to five out of nine bases—see Figure 1). In view of the relationship between the motifs in the TH promoter and those in the HSV promoters which can confer inhibition by Oct-2 we have investigated the role of these motifs in the regulation of the TH promoter and have examined their response to Oct-2.

*To whom correspondence should be addressed.

MATERIALS AND METHODS

Cell culture

BHK cells (14) were grown in DMEM supplemented with 10% foetal calf serum and the ND7 immortalized neuronal cell line (15) was grown in L15 medium supplemented with 10% foetal calf serum, 0.3% glucose and 0.37% sodium bicarbonate.

Plasmid constructs

The basic tyrosine hydroxylase construct contains the TH promoter from −272 to +27 driving expression of the chloramphenicol acetyl transferase (CAT) gene (16). The constructs with mutant octamer or heptamer motifs were derived from this construct by *in vitro* mutagenesis as previously described (17, see Figure 1). The truncated TH construct contains the TH promoter from −44 to +27 driving expression of the CAT gene (18). The heptamer motif was inserted into the minimal TH promoter by synthesizing oligonucleotides with the sequence 5′ CGAGGGATCTAATGGGACG 3′ and 5′ TCGACGT-CCCATTAGATCCCTCGAGTC 3′. These oligonucleotides were then annealed together and inserted into the 44TH construct which had been digested with *Sac*I and *Sal*I in order to generate single stranded ends compatible with those on the annealed oligonucleotide. The Oct-2 expression vectors contain cDNA encoding the appropriate isoform of Oct-2 driven by the

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          A T G C A A T T G A T C T A A T G G G A C
oct       A T G C A A A T N A
hep                         C T C A T G A
TAAT (1)          T A A T G A R A T
TAAT (2)                      T A A T G A R A T
oct       C G C G C C G G
hep                   T G C G G C C G C T
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Figure 1. Sequence of the rat tyrosine hydroxylase promoter from −175 to −158 showing the homologies to the consensus octamer (Oct) and heptamer (hep) motifs as well as the two matches to the octamer-related TAATGARAT (R = purine) sequence found in the HSV immediate-early gene promoters. The mutations used to inactivate either the octamer (oct-) or the heptamer (hep-) in the intact promoter are illustrated in each case (see reference 17 for further details).

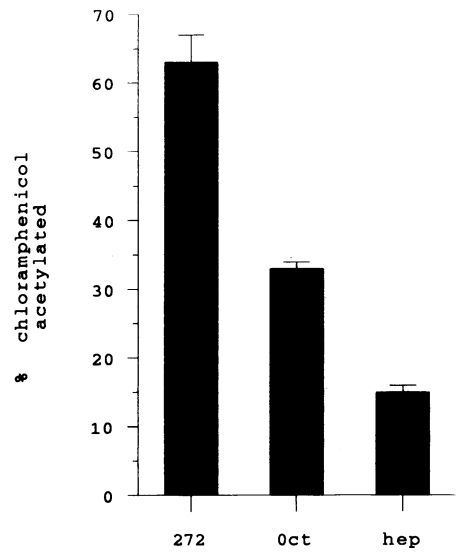

Figure 2. CAT activity of the intact tyrosine hydroxylase promoter (272) and the same promoter with mutations in either the octamer (oct-) or heptamer (hep-) motifs transfected into BHK cells. Values are the average of two replicate experiments whose standard deviation is shown by the bars.

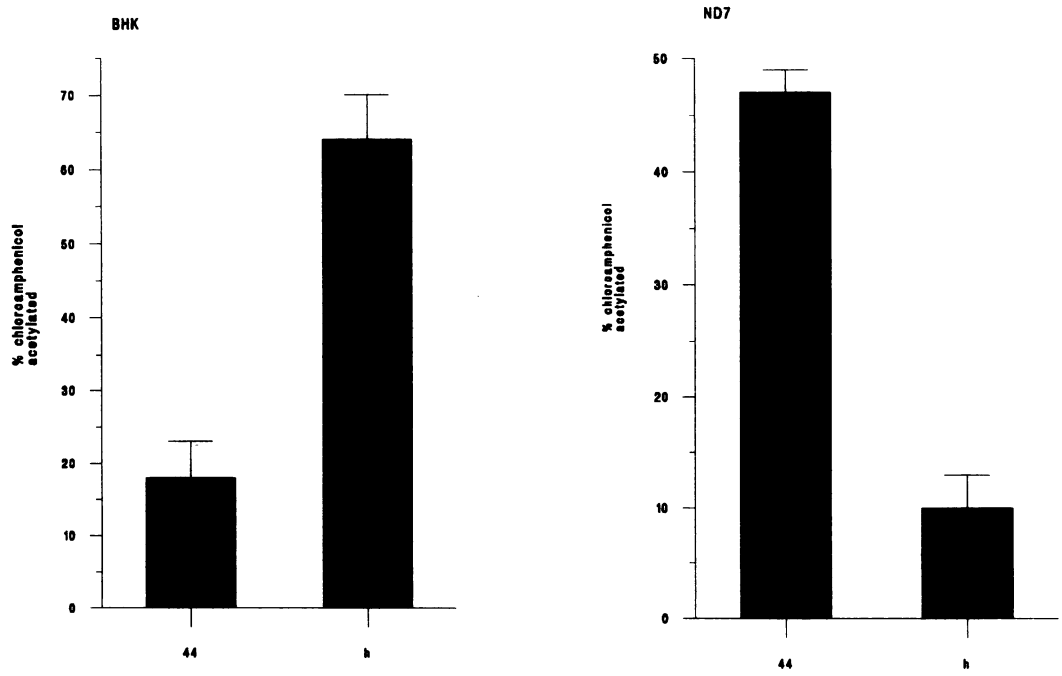

Figure 3. CAT activity in BHK cells or ND7 neuronal cells transfected with the minimal tyrosine hydroxylase promoter (44) and the same promoter containing an added heptamer motif (h). Values are the average of two replicate experiments whose standard deviation is shown by the bars.

constitutive immediate-early promoter of cytomegalovirus (CMV—19)).

Transfection

All transfections were carried out as described by Gorman (20) using 2×10^6 cells on a 90 mm plate. Except where indicated otherwise cells were transfected with 5 μg of the target plasmid with 10 μg of either the Oct-2 expression vector or PJ7 plasmid vector containing the CMV promoter alone (21). Following transfection cells were assayed for CAT activity as described by Gorman, (20), extracts having been equalized for protein content as determined by the method of Bradford (22). The values obtained in this way were equalized for any differences in plasmid uptake between the different samples as determined by dot blot hybridization of the DNA in an aliquot of the cell extract using a probe derived from the CAT gene (23).

RESULTS

The tyrosine hydroxylase (TH) promoter from −272 to the start site of transcription has been shown to contain all the information necessary for the neuronal specific expression of this gene (18, 24) and for the regulation of its expression by factors such as nerve growth factor (25). As this region contains both the octamer and heptamer TAATGARAT-like motifs it was used in our studies.

In previous experiments (17) mutation of the octamer motif had no effect on TH promoter activity in TH expressing PC8b cells (a cell line derived from PC12 cells). In contrast mutation of the adjacent heptamer motif resulted in a 2−3 fold increase in TH promoter activity in both PC8b cells and in non-expressing B103 neuroblastoma cells suggesting that the heptamer motif has an inhibitory effect on promoter activity. To investigate whether this effect was confined to cells of neuronal origin, we transfected BHK fibroblast cells (14) with constructs in which the expression of the CAT gene was driven by the intact TH promoter from −272 to +27 or by the same promoter in which either the octamer or the heptamer motif had been mutated (see Figure 1). In these experiments (Figure 2) no enhancement of promoter activity was observed with either mutant construct. Indeed mutation of the octamer motif resulted in an approximately two fold fall in gene activity whilst similar mutation of the heptamer led to a five fold decrease.

In order to confirm that the heptamer motif alone was able to specifically down regulate TH promoter activity in neuronal but not non-neuronal cells, the heptamer motif from the TH promoter was cloned upstream of a minimal TH promoter (−44 to +27) which contains the TH TATA box but lacks the regulatory region (18). This construct was then introduced into both BHK cells and ND7 neuronal cells (15). In these experiments (Figure 3) the presence of the heptamer motif enhanced activity of the minimal TH promoter in BHK cells. In contrast, promoter activity was down regulated approximately six fold in the ND7 cells when the heptamer motif was present.

It is clear therefore that the heptamer motif has an inhibitory effect on the TH promoter in neuronal but not in non-neuronal cells. In DNA mobility shift experiments, the isolated heptamer motif was able to bind two sequence specific octamer binding proteins in ND7 cell extracts which could be removed by competition with a consensus binding site for octamer binding proteins but not by an unrelated oligonucleotide containing the Spl binding site (Figure 4). Although the Oct-1 protein is expressed in all cell types, the Oct-2 factor is expressed in ND7

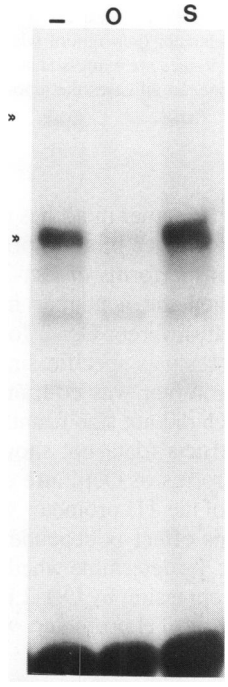

Figure 4. DNA mobility shift using ND7 cell extract and labelled heptamer motif from the TH promoter in the absence of competitor or in the presence of a tenfold excess of unlabelled oligonucleotide containing a consensus octamer motif (O) or an Spl binding site (S). The arrows indicate the positions of sequence specific octamer binding proteins with the mobilities of Oct-1 and Oct-2.

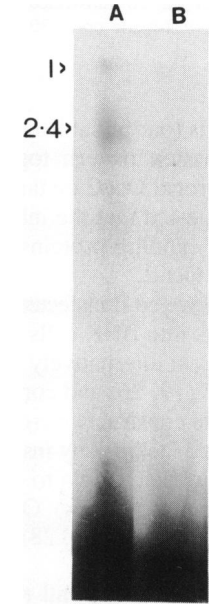

Figure 5. DNA mobility shift assay using a labelled octamer motif and extract from BHK cells transfected with an Oct 2.4 expression vector in the absence (**A**) or presence (**B**) of an excess of unlabelled competitor octamer oligonucleotide. The arrows indicate the positions of the endogenous Oct-1 protein (1) and the exogenously expressed Oct 2.4 protein (2.4).

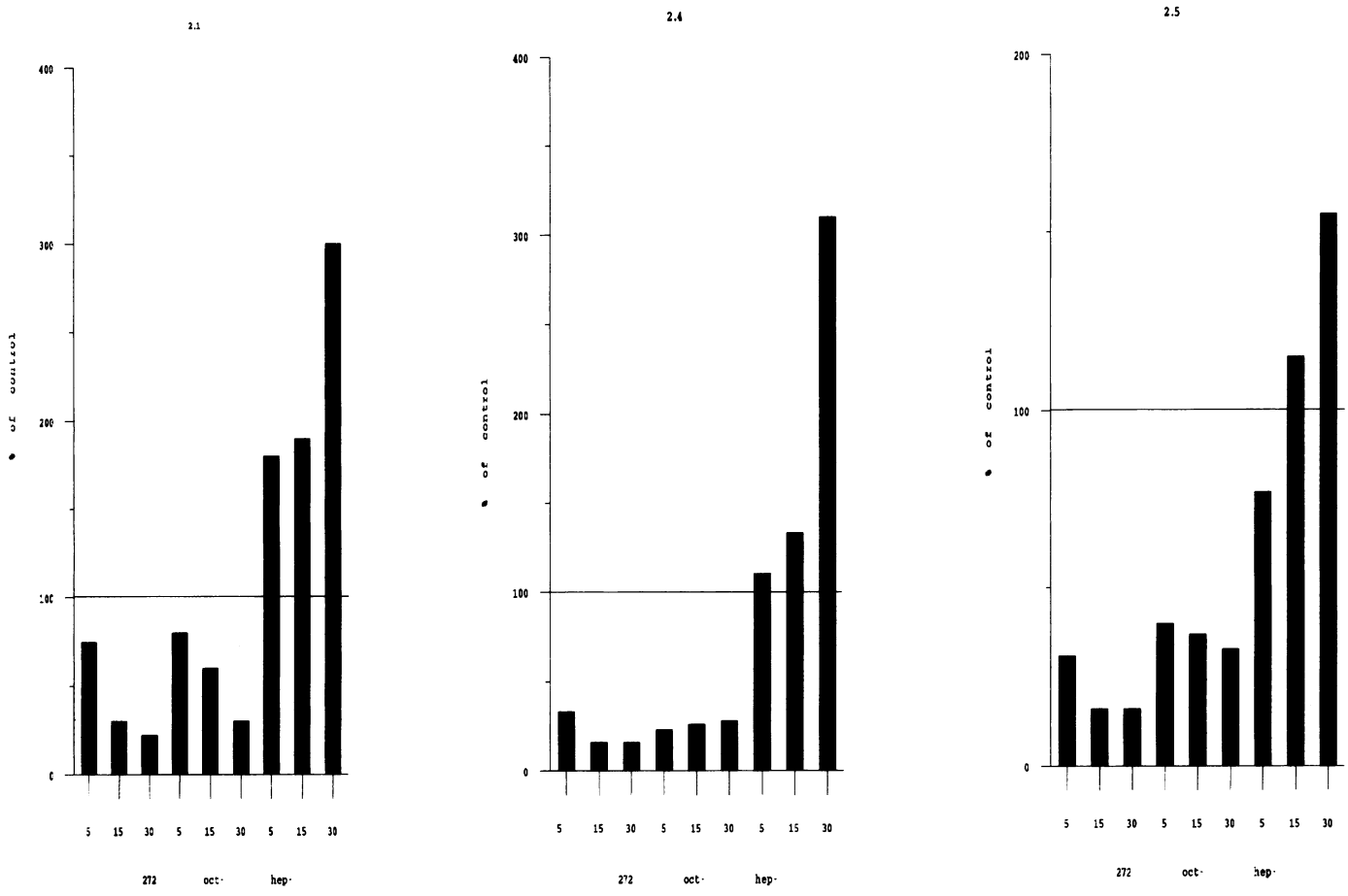

Figure 6. CAT activity of the intact TH promoter (272) or the same promoter with a mutant octamer (oct-) or heptamer (hep-) motif when co-transfected into BHK cells with the indicated amount (in micro-grams) of expression vectors expressing either Oct 2.1, Oct 2.4 or 2.5. Values are expressed as a percentage of the activity obtained when each promoter was co-transfected with the identical amount of the expression vector plasmid alone. In all cases the amount of DNA co-transfected with the promoter construct was made up to 30 μg with carrier DNA.

cells (26) and PC12 cells (our unpublished data) and not in BHK cells (10). This expression pattern together with the known inhibitory effect of neuronal Oct-2 on target promoters to which it can bind (9, 27) suggested that the inhibiting effect produced by binding of octamer binding proteins to the heptamer motif could be mediated by Oct-2.

To test this possibility we co-transfected the intact 272 construct or the mutant constructs into BHK cells with expression vectors encoding various different alternatively spliced forms of Oct-2; Oct 2.1, Oct 2.4 or 2.5 (19, 26) and compared the effect to that observed when the same constructs were co-transfected with the CMV expression plasmid lacking any insert. All these expression vectors have previously been shown to direct the expression of significant levels of the appropriate Oct-2 isoform following transfection of cultured cells (19, 26, 28) and this was confirmed in our experiments (see Figure 5).

In these experiments (Figure 6) all three different forms of Oct-2 were able to down regulate the intact TH promoter by up to six fold in a concentration dependent manner compared to the activity observed when similar amounts of plasmid vector were co-transfected. A similar, although less extensive down regulation was observed with the construct in which the octamer motif alone was mutated. In contrast, no repression was observed with the construct in which the heptamer motif has been mutated to a form which does not bind octamer binding proteins. Indeed at high concentrations each of the forms of Oct-2 can actually activate the TH promoter when the heptamer motif is mutated. The inhibitory effect of the different Oct-2 forms on the heptamer-containing TH promoter was specific since this effect was not observed when the promoter was co-transfected with an Oct-1 expression vector which did not significantly affect the expression of any of these constructs (data not shown).

Hence the various forms of Oct-2 are capable of specifically inhibiting the activity of the TH promoter when expressed in non-neuronal cells and this effect is dependent upon the heptamer motif in the promoter. To determine whether the heptamer motif alone could mediate repression by Oct-2 in the absence of other upstream sequences in the TH promoter, we co-transfected BHK cells with the minimal TH promoter (−44 to +27) with or without an additional cloned heptamer motif. In these experiments (Figure 7) Oct 2.1 had some stimulating effect on the minimal TH promoter but was able to strongly repress the promoter containing the added heptamer motif. Similarly although, Oct 2.4 had no effect on the activity of the minimal TH promoter it was able to produce an approximately twenty fold down regulation of the promoter containing the cloned heptamer motif.

The effect observed with Oct 2.5 was less clear cut however, since this factor had some inhibitory effect on the minimal TH promoter, although even in this case enhanced inhibition was observed with the construct containing the cloned heptamer motif.

In order to assess the specificity of these effects, we prepared minimal TH promoter constructs containing either an added TH octamer motif without the adjacent heptamer motif or the API site which is present at −207 to −199 in the intact TH promoter and plays a critical role in TH promoter activity (17). In these experiments (Figure 7) none of the forms of Oct-2 was able to repress these constructs compared to the effect observed with the minimal TH promoter alone confirming that this effect is specific to constructs containing the heptamer motif and that Oct-2 specifically inhibits expression via this motif. Similarly no effect was observed when any of the constructs with or without the heptamer motif was co-transfected with an Oct-1 expression vector (data not shown).

DISCUSSION

Previous studies (17) have defined a region from −202 to −185 as a minimal enhancer element directing high level TH gene expression specifically in TH expressing PC8b cells derived from PC12 cells and not in non-expressing neuroblastoma cells. In the experiments described here we have shown that the heptamer TAATGARAT-like motif located adjacent to this region at −164 to −158 can repress TH promoter activity in a cell type specific manner which operates only in neuronal cells and not in fibroblasts. In contrast the adjacent octamer motif does not appear to play a significant role in TH gene regulation since mutation of this motif alone does not affect promoter activity in neuronal cells whilst its presence or absence does not affect the ability of the heptamer motif to repress gene expression in such cells when linked to a truncated TH promoter.

Similarly only the heptamer motif and not the octamer or an API site can confer on the TH promoter the ability to be repressed by Oct-2. The inhibitory effect of Oct-2 on the TH heptamer motif but not on the octamer motif is likely to be dependent upon the much closer sequence relationship of the heptamer motif to the octamer-related TAATGARAT sequence (seven out of nine match compared to five out of nine—see Figure 1) which is present in the herpes simplex virus (HSV) immediate-early gene promoters and mediates their repression in neuronal cells (10, 27). Hence this motif is likely to be particularly efficient at mediating repression by octamer binding proteins.

In the case of the HSV immediate-early promoters the repression in neuronal cells is produced by the Oct-2 transcription factor (10). Similarly, the inhibitory effect of the heptamer motif on TH promoter activity in neuronal cells can be reproduced in fibroblasts by co-transfection of the TH promoter with different isoforms of Oct-2 indicating that these isoforms can inhibit the TH promoter via the heptamer motif, the first time such an inhibitory effect of Oct-2 on a natural cellular promoter expressed in neuronal cells has been demonstrated.

In constrast Oct-1 had no effect when co-transfected with the TH promoter. These findings therefore establish the relevance of our previous observations of the inhibitory effect of Oct-2 using viral and artificial promoter constructs (10, 26) to the regulation of a natural cellular promoter.

Interestingly in our previous experiments with the HSV promoter, the Oct 2.4 and 2.5 isoforms which predominate in neuronal cell repressed the promoter whereas the Oct 2.1 isoform

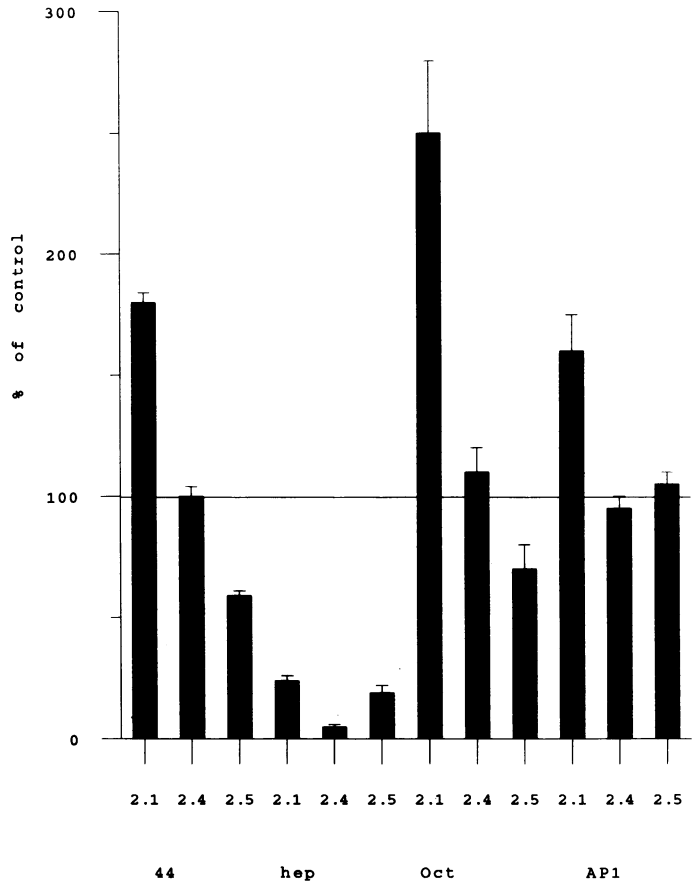

Figure 7. CAT activity of the minimal TH promoter (44) and of the same promoter with an added TH heptamer motif (hep), an added TH octamer motif (oct) or an added TH API site (API) when transfected into BHK cells with expression vectors encoding Oct 2.1, Oct 2.4 or 2.5. Values are expressed as a percentage of the activity obtained when each promoter was co-transfected with the expression vector plasmid alone. The values shown are the average of two replicate experiments whose standard deviation is shown by the bars.

which predominates in B cells actually activated it (26). In contrast all the isoforms were able to repress the TH promoter. Hence the activity of Oct-2.1 can vary depending on the promoter tested. Interestingly the ability of different forms of Oct-2 to activate various-containing promoters is known to be dependent upon the context of the octamer-motif in the promoter (28). Indeed in previous experiments (KL and DSL unpublished data) we have observed an inhibitory effect of Oct 2.1 on octamer-mediated gene expression in specific artificial promoter constructs with this effect being dependent on the context of the octamer motif in the promoter. It is probable therefore that the strong C-terminal activation domain present in Oct 2.1 (19) allows it to activate the IE promoter but not the TH promoter whereas Oct 2.4 and 2.5 which lack this activation domain are unable to activate either promoter. In the absence of any activation, an inhibitory domain which we have recently mapped to the N-terminal region common to all the forms of Oct-2 (Lillycrop *et al.*, submitted) would then repress transcription from the target promoters.

Whatever the precise mechanism of the differences in activity between the different forms of Oct-2, the fact that Oct 2.4 and 2.5 are the predominant forms of Oct-2 in neuronal cells where they are expressed at high levels (26) indicates that they are likely

to be responsible for the observed inhibitory effect of the heptamer motif on the TH gene promoter since this effect is not observed in BHK cells which express only the ubiquitous Oct-1 factor (10) but is observed in PC8b cells as well as in ND7 cells which express only Oct-1 and Oct-2 and no other octamer binding proteins (10, 29).

Although it is clear that Oct-2 can repress the TH promoter in neuronal cells, the significance of this effect remains uncertain. It is unlikely that it operates to restrict the expression of TH to the appropriate neuronal subset since repression by the heptamer motif is observed in PC8b cells and ND7 cells both of which naturally express TH (30–32) as well as in B103 neuroblastoma cells which do not. Moreover, the neuronal subtype specificity of the TH promoter has already been demonstrated to be dependent on the dyad motif in the −202 to −185 region which ispreferentially active in PC8b compared to B103 cells.

Rather it seems more probable that alterations in Oct-2 levels and thereby in heptamer motif-mediated repression, play some role in the modulation of TH expression in neuronal cells in response to numerous physiological stimuli. In this regard it is paradoxical that Oct-2 levels are elevated in neuronal cells in response to nerve growth factor (NGF) treatment (33) which also produces a transient increase in TH gene expression (25, 31). Interestingly however, whilst the binding of Fos/Jun proteins to an API site in the TH promoter has been shown to be responsible for its induction by NGF (25) the mechanisms responsible for the subsequent decrease in TH synthesis following prolonged NGF treatment (Leonard *et al.*, 1987) remain obscure and may involve Oct-2 mediated repression of the TH promoter.

It is also probable however, that some of the stimuli which enhance TH expression may do so by causing a decline in the level of Oct-2. In this regard it is of interest that a number of stimuli which induce TH synthesis including stress (33, 34), epidermal growth factor (16) cAMP and glucocorticoid hormones (35) also activate herpes simplex virus (HSV) immediate-early gene expression (36, 37) and induce reactivation of virus latent in neuronal cells (for reviews see 38, 39) suggesting that common neuronal signalling pathway(s) may regulate both TH expression and HSV latency. As HSV reactivation involves the induction of the viral immediate-early genes which are normally inhibited in neuronal cells by Oct-2 (10) at least some of these stimuli may act by lowering Oct-2 levels resulting in parallel reactivation of latent HSV and activation of the TH gene promoter.

Although further studies are required to test this possibility, it is already clear that the inhibitory effect of Oct-2 in neuronal cells is not confined to viral and artificial gene promoters but that it can also repress the expression of the cellular TH gene via the heptamer TAATGARAT-like motif in its promoter.

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