Differential Regulation of the Two Neuronal Nitric-oxide Synthase Gene Promoters by the Oct-2 Transcription Factor*

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The Oct-2 transcription factor has been shown previously to repress both the cellular tyrosine hydroxylase and the herpes simplex virus immediate-early genes in neuronal cells. Here we identify the gene encoding the neuronal nitric-oxide synthase (nNOS) as the first example of a gene activated in neuronal cells by Oct-2. The levels of the nNOS mRNA and protein are greatly reduced in neuronal cell lines in which Oct-2 levels have been reduced by an antisense method, although these cells have enhanced levels of tyrosine hydroxylase. Moreover, the nNOS gene regulatory region is activated by Oct-2 expression vectors upon cotransfection into both neuronal and non-neuronal cells, and this response is dependent upon a 20-amino acid region within the COOH-terminal activation domain of Oct-2. Of the two closely linked promoters that drive nNOS gene expression, only the downstream 5.1 promoter is activated by Oct-2, whereas the 5.2 promoter is unaffected. These effects are discussed in terms of the potential role of Oct-2 in regulating nNOS expression in the nervous system.

The Oct-2 transcription factor is a member of the POU (Pit-Oct-Unc) family of transcription factors that contain a bipartite DNA binding domain consisting of a POU-specific domain and a POU homeodomain (for reviews, see Refs. 1 and 2). This POU domain allows the Oct-2 factor to bind to the octamer sequence ATGCAAAT, which is found in the promoter and enhancer regions of several cellular genes including those encoding the heavy and light chains of immunoglobulin (for review, see Ref. 3). The original identification of the Oct-2 factor as being expressed in B lymphocytes and not in other cell types led to the suggestion that this factor plays a critical role in the regulation of immunoglobulin gene expression in B lymphocytes (4, 5). In agreement with this idea the immunoglobulin enhancer exhibits a dramatically reduced activity in B cells lacking Oct-2 (6), and knockout mice lacking a functional Oct-2 gene show defects in B cell maturation and response to antigen (7). Moreover, these knockout mice also exhibit a lack of CD36 expression in their B lymphocytes, identifying the gene encoding this factor as another target for activation by Oct-2 in B lymphocytes (8).

Although Oct-2 is absent in most non-B cells, it was subsequently shown by a number of different laboratories to be expressed in neuronal cells in the brain (9–13). Interestingly, however, the single gene encoding Oct-2 is subject to alternative splicing to generate mRNAs encoding different isoforms (14), and this alternative splicing is regulated in a tissue-specific manner (15). Thus, B lymphocytes contain predominantly the Oct-2.1 isoform, which contains a strong COOH-terminal activation domain and therefore has a predominantly stimulatory effect on gene expression (15, 16). In contrast, this isoform is present at a relatively lower level in neuronal cells where the Oct-2.4 and 2.5 isoforms predominate (15). Since these isoforms lack the COOH-terminal activation domain they have an inhibitory effect on the activity of a number of different promoters (15, 16).

Indeed, the available evidence suggests that in neuronal cells Oct-2 has a predominantly inhibitory effect on gene expression. Thus, the presence of Oct-2 in these cells is associated with an inhibition of the activity of the herpes simplex virus immediate-early gene promoters that contain the octamer-related TAATGARAT motif, which mediates repression by Oct-2 (11, 12). Moreover, we have shown previously that the neuronally expressed tyrosine hydroxylase gene promoter is repressed by cotransfection with Oct-2.4 and 2.5 expression vectors (17). This effect was dependent upon a specific Oct-2 binding site in the promoter (17) correlating with earlier studies in which mutation of this site within the transfected tyrosine hydroxylase promoter greatly enhanced its activity in neuronal cells (18).

To confirm the idea that the Oct-2 transcription factor can inhibit tyrosine hydroxylase gene expression in neuronal cells, we recently used an antisense approach to reduce greatly the expression of Oct-2 in the ND7 neuronal cell line (19) which expresses high levels of Oct-2 (12). These antisense cell lines (20) did indeed exhibit greatly enhanced expression of the tyrosine hydroxylase gene, confirming that Oct-2 does inhibit the expression of the endogenous tyrosine hydroxylase gene. In an attempt to identify other targets for regulation by Oct-2 we used immunohistochemistry to screen these cells for altered expression of a number of different neuronal expressed proteins compared with control ND7 cells. In these experiments most proteins showed no alteration in expression, but surprisingly the antisense cells showed decreased expression of the neuronal nitric-oxide synthase (nNOS) protein (20) which is expressed in specific cells within the central nervous system.

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In previous experiments (20) we obtained two cell lines 16e and 22f, which exhibited decreased levels of the Oct-2 transcription factor compared with the parental ND7 cells. The 22f and 16e cells were obtained by transfection with a construct containing a 5' region of the Oct-2 mRNA common to all of the different isoforms inserted in the antisense orientation in the expression vector pJS5 (30), which contains the dexamethasone-inducible promoter of mouse mammary tumor virus. They thus contain a lowered level of Oct-2 compared with the parental ND7 cells which is reduced further when increased expression of the antisense construct is induced with dexamethasone (20).

In initial experiments (20) both cell lines demonstrated a reduced proportion of cells staining with an antibody to nNOS compared with the parental ND7 cells. To quantitate the level of nNOS in the antisense cells, we performed quantitative Western blotting using an antibody specific to nNOS. The levels of nNOS in each cell line were then obtained by densitometric scanning and comparison with the level of the neuronal pGp9.5 protein, which we have shown previously to be present at similar levels in all of the cell lines (20). As illustrated in Fig. 1 both the 22f and 16e cell lines exhibited a dramatically reduced level of the nNOS protein when compared with the parental ND7 cells, which was observed both in the presence and absence of dexamethasone. Hence the antisense cells lines do exhibit a clear decrease in the level of the nNOS protein paralleling their decreased levels of Oct-2.

We next wished to investigate whether this fall in nNOS protein was paralleled by a decrease in the nNOS mRNA as would be expected if the Oct-2 factor regulates the transcription of the nNOS gene. We therefore used a reverse transcriptase/PCR method to measure the level of the nNOS mRNA in our cell lines. This semiquantitative method relies on a comparison of the amount of PCR product obtained by amplification with the amount of cDNA present in the starting material.
ification of the test mRNA (amplified with specific primers) with that obtained upon amplification of a control mRNA encoding ribosomal L27 protein, which is present at similar levels in all samples. We have used this method previously to measure successfully the level of a number of different mRNAs in neuronal cells (31, 32).

As illustrated in Fig. 2, using this method we were able to demonstrate a clear decrease in the level of the nNOS mRNA particularly in the 22f cell line where a considerable decrease in nNOS mRNA levels was noted upon dexamethasone treatment to induce enhanced expression of the antisense construct. The larger effect observed in the 22f cell line is consistent with the larger decrease in the Oct-2 mRNA in this cell line compared with the 16e cell line, which we observed in our previous experiments (20). Hence the lowered level of the nNOS protein in this cell line is indeed paralleled by a decreased level of the nNOS mRNA, suggesting that the Oct-2 factor may indeed affect transcription of the nNOS gene directly.

To investigate further the effect of Oct-2 on the nNOS gene, we utilized a construct in which 4.3 kilobases of the upstream regulatory region of the nNOS gene have been linked to a reporter gene encoding the readily assayable luciferase protein (22). This construct was cotransfected into both BHK fibroblast cells, which lack endogenous Oct-2 (12, 15), and the parental unmanipulated ND7 cells together with expression vectors encoding the various isoforms of Oct-2. In these experiments (Fig. 3) the nNOS promoter was clearly up-regulated in BHK cells by cotransfection with the Oct-2.1 vector and to a lesser extent with the vector expressing Oct-2.5, whereas the expression vector encoding Oct-2.4 did not appear to have a stimulatory effect. Hence the expression of at least one of the isoforms of Oct-2 can strongly activate the nNOS promoter in a non-neuronal cell line lacking endogenous Oct-2. Interestingly, activation of the nNOS promoter by Oct-2 was also observed in the ND7 cell line, which expresses endogenous Oct-2, although in this case all three of the different isoforms of Oct-2 were able to activate the nNOS promoter strongly. As expected, the promoterless vector containing the luciferase gene was unaffected by any of the forms of Oct-2, confirming that these effects were specific to the nNOS promoter and indicating that this promoter can be activated directly by Oct-2.

In previous experiments, it has been shown that different forms of the Oct-2 factor contain both an activation domain (23, 33) and an inhibitory domain (16) which are located NH2-terminal to the central DNA binding POU domain. In addition, the Oct-2.1 isoform contains a COOH-terminal activation domain that is not present in Oct-2.4 or 2.5. To determine which of the activation domains of Oct-2 was responsible for its ability to stimulate the nNOS promoter the promoter/reporter construct was cotransfected with deletion mutants of Oct-2.1 lacking either the NH2-terminal (construct 6) or COOH-terminal (construct 12) regions. Values are the average of six determinations whose standard deviations are shown by the bars.
moter than did Oct-2.1, whereas the COOH-terminal deletion exhibited virtually no stimulatory effect on the promoter. This suggests therefore that the ability of Oct-2 to stimulate the nNOS promoter is dependent primarily upon the COOH-terminal activation domain, whereas the presence of the NH2-terminal domain, if anything, reduces the ability to stimulate the promoter.

To determine the precise region of the COOH terminus involved in activation of the nNOS promoter we used a series of deletion mutants lacking progressively larger numbers of COOH-terminal amino acids (33). In these experiments (Fig. 4) deletion of the most COOH-terminal 18 amino acids produced some reduction in the activation of the nNOS promoter compared with the effect of intact Oct-2.1. However, deletion of an additional 20 amino acids resulting in a construct lacking the COOH-terminal 38 amino acids totally abolished activation of the nNOS promoter; the same effect was also observed with a construct lacking the COOH-terminal 87 amino acids. Hence the amino acids from 446 to 463 plays some role in the induction of the nNOS promoter, whereas loss of the 20 amino acids between positions 426 and 445 of Oct-2 abolishes its ability to activate the nNOS promoter.

It has been shown previously that the nNOS gene contains two closely linked promoters located within 300 base pairs of each other (22). To determine whether one or both of these promoters were inducible by Oct-2, we utilized constructs that contain either the 300 base pairs of 5' sequence specific to the nNOS 5.1 promoter (pNOS 51b) or 1.8 kilobases of upstream sequence specific to the nNOS 5.2 promoter. These promoter constructs were transfected into BHK and ND7 cells as before. As illustrated in Fig. 5 the nNOS 5.1 promoter was activated by Oct-2.1 more strongly than by Oct 2.5 in BHK cells, whereas it was unaffected by cotransfection with Oct-2.4. Moreover, this promoter was similarly activated by all of the different Oct-2 forms in ND7 cells. The behavior of this promoter thus mimics the behavior of the construct containing both the promoters (compare Figs. 3 and 5). In contrast, the nNOS 5.2 promoter (pNOS L) was unaffected by cotransfection with any of the forms of Oct-2 in BHK cells, although it was activated weakly by Oct-2.1 in ND7 cells. Hence, the effect of Oct-2 on the nNOS gene appears to be mediated by the 5.1 promoter, whereas it has no effect upon the 5.2 promoter.

**DISCUSSION**

The data presented here identify the nNOS gene as the first example of a neuronally expressed gene that is positively regulated by Oct-2. Thus, the levels of nNOS mRNA and protein fall in our cell lines expressing lowered levels of Oct-2 in contrast to the enhanced levels of tyrosine hydroxylase levels expressed by these cells. Moreover, one of the two promoters that drive the expression of the nNOS gene is strongly stimu-

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**Fig. 4.** Assay of luciferase activity in BHK cells transfected with the promoterless vector pXP2 or the 4.3-kilobase nNOS promoter (pNOS 4.3L) together with either expression vector lacking any insert (pJ7) or the same vector encoding Oct-2.1 or COOH-terminal truncations lacking the indicated amino acids. Values are the average of six determinations whose standard deviations are shown by the bars.

**Fig. 5.** Assay of luciferase activity in BHK (panel A) or ND7 (panel B) cells transfected with constructs containing either the nNOS 5.1 promoter-specific region (pNOS 51b) or the region upstream of the nNOS 5.2 promoter (pNOS 1842-L) together with either expression vector lacking any insert (pJ7) or the same vector containing cDNAs for Oct-2.1, 2.4, or 2.5. Values are the average of six determinations whose standard deviations are shown by the bars.
lated by Oct-2, whereas the other is relatively unaffected.

Activity of the two nNOS promoters produces two distinct mRNAs that encode the same protein but differ in their 5'-translated region (22). It is likely therefore that the use of these two promoters plays some role in the regulation of the nNOS gene in specific situations. In this regard, it is of interest that the reduction of Oct-2 expression in the antisense cell lines had a more severe effect on nNOS protein levels than on the corresponding mRNA levels (compare Figs. 1 and 2), suggesting that Oct-2 may regulate an nNOS mRNA that is translated preferentially compared with the non-regulated mRNA. Whether this is the case or not, it will be of particular interest to compare the expression of Oct-2 in different neuronal cells in vitro with that of the nNOS gene and its different transcripts. Thus, we have shown previously that the Oct-2 gene is expressed in sensory neurons within dorsal root ganglia (12, 32) which also express nNOS (34). Moreover, both Oct-2 (35) and nNOS (36) increase in abundance in dorsal root ganglia neurons following axotomy. Thus, changes in Oct-2 expression following exposure to various stimuli may play a key role in modulating nNOS expression following exposure to such stimuli as well as potentially regulating its basal expression in different neuronal cell types. In this manner, changes in Oct-2 would result in alterations in nNOS expression in response to specific stimuli allowing it to fulfill its important functional roles in the normal and damaged brain (for review, see Refs. 37 and 38). This would parallel the potential role of Oct-2 in regulating tyrosine hydroxylase expression in response to stressful stimuli, although in this case Oct-2 represses tyrosine hydroxylase gene expression rather than activating it.

The opposite effect of Oct-2 on the nNOS and tyrosine hydroxylase promoters raises the question of the mechanisms of this effect. In our experiments the strongest activation of the nNOS promoter was observed with the Oct-2.1 form of the factor which contains a functional activation domain. In agreement with the critical role of this domain, its deletion results in a large reduction in stimulation of the nNOS promoter. Previous studies using artificial octamer motif-containing promoters suggested that the 85 amino acids at the COOH terminus of Oct-2 (39) and in particular the extreme 18 COOH-terminal amino acids (33) were critical for promoter activation. Our studies using the natural nNOS promoter suggest only a relatively small role for the 18 COOH-terminal amino acids and indicate that the adjacent 20 amino acids within the COOH-terminal region are critical for activation of this promoter.

Oct-2.1, which contains these critical amino acids, constitutes approximately 40% of the mRNA in the ND7 cell line (15) and is likely therefore to play a key role in the positive effect of Oct-2 on nNOS gene expression, which we observed in this cell line. However, the majority of the mRNA in this cell line encodes the Oct-2.4 and 2.5 isoforms, which lack the COOH-terminal activation domain and in BHK cells seems to have either no or a much smaller stimulatory effect on the nNOS promoter. Interestingly, however, in ND7 cells these forms can also strongly stimulate the nNOS promoter, suggesting that other neuron-specific factors may potentiate the ability of Oct-2.4 and 2.5 to activate the nNOS promoter. Such cell type-specific differences in the effects of different Oct-2 isoforms are not without precedent. Thus, when cotransfected into BHK cells with the herpes simplex virus immediately-early gene promoter Oct-2.1 activates the promoter, whereas Oct-2.4 and 2.5 do not, producing a pattern of effects which is similar to the effects on the nNOS promoter in this cell type (15). In contrast, however, in ND7 cells all of the isoforms of Oct-2 repress the herpes simplex virus immediate-early promoter, accounting for the strong repression of this promoter which is observed in neuronal cells (15). We have shown previously that such an inhibitory effect of Oct-2.1 on the immediate-early promoter can also be produced in BHK cells artificially expressing Oct-2.4 or 2.5 (40). Thus, this effect is dependent upon an interaction between Oct-2.1 and either Oct-2.4 or 2.5, which converts Oct-2.1 from an activator to a repressor of the immediate-early promoter.

It is therefore possible that a similar interaction with endogenous Oct-2.1 in the ND7 cells confers upon Oct-2.4 or 2.5 the ability to activate the nNOS promoter in these cells. Alternatively, this effect may be dependent upon another factor expressed in the ND7 cells which allows Oct-2.4 or 2.5 to activate the nNOS promoter despite the absence of the COOH-terminal activation domain present in Oct-2.1. These ideas indicate, however, that the nNOS promoter is likely to have a so far unique architecture that allows it to respond to Oct-2.1 in a positive fashion in neuronal cells even in the presence of Oct-2.4 or 2.5. Upon examination of the 300-base pair region that is unique to the nNOS 5.1 promoter (22, 41) we did not identify any sequence closely related to the normal binding site of Oct-2 (ATGCAAT:3). It is possible therefore that the unique response pattern of the nNOS promoter is produced by a response element that is distinct or only distantly related in sequence to the consensus octamer motif found in most cellular promoters.

Similarly unique response may depend on the fact that unlike the tyrosine hydroxylase promoter, the nNOS 5.1 promoter lacks a TATA box (22). Thus, we have shown previously that the Oct-2 inhibitory domain that represses transcription of the tyrosine hydroxylase and immediate-early gene promoters can do so only when the target promoter contains a TATA box (42). Further studies of the nNOS promoter region will obviously be necessary to confirm these possibilities.

Whatever the case, it is clear that the data presented here identify the nNOS gene as the first example of a neuronal gene that is positively regulated by Oct-2 and suggest that the Oct-2 transcription factor may play an important role in regulating the vital function of nNOS within the nervous system.

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