Expression of the gene for the POU domain transcription factor Tst-1/Oct6 is regulated by an estrogen-dependent enhancer

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INTRODUCTION

POU domain proteins, in particular members of the class III and IV subfamilies, are expressed in very distinct spatio-temporal patterns in the developing and adult nervous system (1-4). It is believed that a combinatorial code of these proteins is involved in specifying the fate and identity of neuronal and glial cell populations. One of these POU domain proteins is the class III protein Tst-1/Oct6 which is also known as SCIP (5-9). In line with its supposed role during differentiation of glial and neuronal cells (10,11), targeted disruption of the Tst-1/Oct6 gene in mice led to severe disturbances of the normal myelination program in Schwann cells (12,13).

If Tst-1/Oct6 is a key regulator of neural differentiation, its expression should be under tight control. Indeed, it was shown that axonal contact stimulates expression of the Tst-1/Oct6 gene in Schwann cells by an increase in the intracellular level of cyclic-AMP (14). Induction of Tst-1/Oct6 gene expression through elevation of cyclic-AMP levels could also be obtained by application of forskolin to Schwann cell cultures (8,11).

ABSTRACT

Expression of the POU domain protein Tst-1/Oct6 during development of glia and neurons is subject to a tight multifactorial control. Here we show that 17β-estradiol increases the level of endogenous Tst-1/Oct6 in glial cells. This effect was mediated at the level of gene expression by an enhancer present in the 5′ flanking region of the mouse gene for Tst-1/Oct6, –5 kb upstream of the transcriptional start site. The enhancer contained as the functional element a sequence motif that closely resembled a classical estrogen response element. It consisted of an imperfect palindrome with a spacing of 3 bp, and was bound in vitro by activated estrogen receptor. Furthermore, this element was able to confer estrogen responsiveness when introduced into a heterologous promoter. In the Tst-1/Oct6 gene enhancer, a TPA response element was found in close proximity to the estrogen receptor binding site. As a consequence, TPA and estrogen activated transcription of the Tst-1/Oct6 gene in a synergistic manner.

MATERIALS AND METHODS

Plasmids

Approximately 10 kb of 5' flanking region from the mouse gene for Tst-1/Oct6 were isolated from a mouse genomic library derived from J1 ES cells (12). A BglII site was introduced at the translational start site of Tst-1/Oct6 (defined as +49 according to (21)). Using SalI and BglII restriction endonucleases, this fragment was cloned into the luciferase plasmid pGL2basic (Promega), yielding the reporter plasmid p10Kluc. Successively shortened versions of p10Kluc were generated by using the following restriction sites: NheI (p6.5Kluc, containing sequences from approximately –6.5 K to +49), AvrII (p3Kluc, containing sequences from approximately –3 K to +49), HincIII (p2Kluc, containing sequences from approximately –2 K to +49), BamHI (p0.6Kluc, containing sequences from –538 to +49), NolI (p0.3Kluc, containing sequences from –286 to +49), SacII (pminluc, containing sequences from –93 to +49) and NgoA IV (p0.1Kluc, containing sequences from –27 to +49). As shown in Figure 3, several fragments from the region between the NheI and AvrII sites (sequences from approximately –6.5 to –3 K) were also inserted immediately in front of the minimal promoter of the Tst-1/Oct6 gene present in pminluc. The resulting derivatives of pminluc were designated as pNAmiuluc, pXAmiuluc, pNXamiuluc,

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pDSminluc, pDXminluc, pBSminluc and pXSminluc. Plasmid pBXminluc contained juxtaposed to the Tst-1/Oct6 minimal promoter a 397 bp BstEII–XhoI fragment (DDBJ/EMBL/GenBank accession no. X99229) which is naturally situated ∼5 kb further upstream. A mutant version of this 397 bp fragment was derived by introducing an SpeI site into the first half-site of an identified estrogen response element, and cloned in an identical manner, yielding pBXminluc mut. The estrogen response element was also inserted in single copy immediately in front of the rat prolactin minimal promoter present in p36luc (22) using oligonucleotide M1 5′-AATCCATCAGGGTATCCTGCCCCA-3′, which bold letters represent the identified estrogen response element. The resulting plasmid M1p36luc, as well as its mutant versions M2p36luc–M5p36luc, are shown in Figure 4. Plasmids pRSV-ER and pCMV/Tst-1 have been described before (22,23).

**Cell culture, transfections and luciferase assays**

U87-MG glioblastoma. 33B oligodendroglioma and COS-7 cells were propagated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS). One day before transfection, U87-MG cells were plated at a density of 5x10^4 per 60 mm plate. U87-MG cells were transfected by the calcium phosphate technique (24) with 2 μg of luciferase reporter plasmid and 0.5 μg of expression plasmid for the estrogen receptor. The total amount of plasmid was kept constant. At 3 h post-transfection, cells were treated for 1 min with 30% (v/v) glycerol in phosphate buffered saline (PBS) and placed in fresh medium. Estrogen and estrogens, and 0.5% untreated FCS with comparable results. Cells were harvested 48 h after transfection, and extracts were assayed for luciferase activity (22). For protein extracts COS-7 cells were transfected in DMEM/10% FCS at a density of 2 x 10^5 per 100 mm plate using DEAE–dextran (25).

**Preparation of nuclear extracts and recombinant proteins**

Nuclear extracts were prepared from 33B oligodendroglioma cells, and transiently transfected COS-7 cells as described (26). Shortly, cells from two 100 mm plates were washed twice with PBS, scraped from the plates in hypotonic buffer, swollen on ice and lysed by the addition of 1% Nonidet P-40 and vortexing. Nuclei were pelleted and extracted for 15 min at 4°C under constant rotation in 200 μl ice cold 10 mM HEPES pH 7.9, 400 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM DTT, 1% Nonidet P-40, 2 μg/ml pepstatin, 2 μg/ml leupeptin and 1 μg/ml aprotinin. Jun-D was expressed as a glutathione-S-transferase fusion protein in the bacterial strain DH5α, and purified as described (22).

**Electrophoretic mobility shift assay**

In general, 0.5 ng of 32P-labeled probe (oligonucleotides M1–M6, for sequence see Figs 4 and 6A) was incubated for 20 min at room temperature with 2.5 μg of nuclear extract from COS-7 cells, 50 fmol of recombinant human estrogen receptor or 50 fmol of purified Jun-D in a 20 μl reaction mixture containing 10 mM HEPES (pH 7.9), 5% glycerol, 25 mM NaCl, 2 mM DTT, 0.1 mM EDTA and 1 μg of poly(dI–dC) as unspecific competitor.
flanking region from the mouse Tst-1/Oct6 gene. When transfected into U87-MG glioblastoma cells, the activity of this reporter construct could be stimulated ~10-fold in the presence of both estrogen and its receptor (p10Kluc in Fig. 2). No stimulation was observed, however, when only the hormone or the receptor were present.

To map the region responsible for mediating this estrogen-dependent transcriptional activation, we deleted various parts from the distal end of the Tst-1/Oct6 5' flanking region, thus generating a series of reporter plasmids carrying successively shortened fragments. With the exception of the shortest (p0.1Kluc), all fragments conferred similar basal levels of luciferase expression (data not shown). This indicates that a region of 142 bp immediately preceding the Tst-1/Oct6 coding region is fully sufficient to mediate basal transcription. We will refer to this region as the minimal Tst-1/Oct6 gene promoter.

Deletion of ~3.5 kb from the most distal part of the 5’ flanking region led to no significant alteration in the estrogen response (p6.5Kluc in Fig. 2). However, when the 5' flanking region was further shortened by additional 3.5 kb, estrogen responsiveness was lost almost completely dropping from a 13-fold induction for p6.5Kluc to a mere 2.5-fold stimulation for p3Kluc. None of the reporter plasmids carrying even shorter 5' flanking regions was inducible by estrogen, with luciferase activities being indistinguishable in the presence and absence of 17β-estradiol and its receptor.
observed 6.5-fold stimulation was comparable with the one observed for most responsive fragments and approximately half as high as the 11-fold stimulation obtained with the whole 3.5 kb fragment (compare pNAminluc with pBXminluc in Fig. 3).

Sequence analysis of the 3′ flanking region of the Tst-1/Oct6 gene led to a robust 11-fold estrogen-dependent stimulation of luciferase activity (M5p36luc). However, when the second half site was mutated in such a way that a perfect palindrome similar to the ERE from the vitellogenin gene was generated, estrogen response increased from an 11- to a 20-fold induction (compare M1p36luc with M4p36luc). As expected, mutation of two bases in the spacer region between both half-sites did not alter the responsiveness of this element toward estrogen (M3p36luc).

The ERE from the Tst-1/Oct6 gene was cloned in front of the rat prolactin minimal promoter of the luciferase reporter plasmid p36luc in its authentic sequence (M1) or after various base substitutions (M2–M5). The resulting luciferase plasmids were transfected into U87-MG glioblastoma cells with (+ER) or without (–ER) the expression plasmid for estrogen receptor pRSV/ER. Where indicated, 17β-estradiol (E2) was added to the media 24 h before harvesting. Luciferase activities in extracts from transfected cells were determined in three independent experiments, each performed in duplicate. Data are presented as fold inductions which were calculated for each reporter plasmid by comparing luciferase activities with values from cells which were transfected with reporter plasmid and empty RSV expression plasmid and were kept in the absence of added 17β-estradiol.

In another set of experiments, we transferred the motif in its naturally occurring sequence, orientation and context to the rat prolactin minimal promoter, placing it directly in front of the TATA box of the luciferase reporter p36luc. As shown in Figure 4, the presence of a single copy of the motif from the 5′ flanking region of the Tst-1/Oct6 gene led to a robust 11-fold estrogen-dependent stimulation of luciferase gene expression which could not be detected in its absence. Therefore the identified motif is not only necessary, but also sufficient to mediate the estrogen responsiveness to the Tst-1/Oct6 gene promoter (Fig. 3B).

Next we attempted a detailed analysis of the ERE from the 5′ flanking region of the Tst-1/Oct6 gene and the prototypic ERE from the vitellogenin gene it seemed reasonable to assume that the estrogen effect could be mediated by direct binding of the estrogen receptor to this sequence motif. To analyze this possibility we prepared natural nuclear extracts from 17β-estradiol treated COS cells which were either mock-transfected or transiently transfected with an expression vector for the estrogen receptor. These nuclear extracts were used in gel shift analyses with the ERE from the Tst-1/Oct6 gene in its natural context serving as a probe. Proteins from mock-transfected COS cell nuclear extracts were able to interact with the probe despite the absence of estrogen receptor in these extracts (Fig. 5A).

As expected, the resulting complex proved to be refractory to the presence of a monoclonal antibody directed against the estrogen receptor (Fig. 5A) and was found to be non-specific in competition studies (data not shown).

Transfection of COS cells with an expression plasmid for the estrogen receptor led to the appearance of a second complex which had a significantly lower mobility than the complex from mock-transfected COS cells. This complex was specifically shifted to an even lower mobility in the presence of antibodies directed against the estrogen receptor. This finding not only shows the presence of estrogen receptor in the complex, but also proves that the ERE from the 5′ flanking region of the Tst-1/Oct6 gene can indeed be recognized by estrogen receptor. In a series of competition experiments we compared the relative affinity of the estrogen receptor with the various mutant versions of the ERE used in previous transfection studies (Fig. 5B). Competition efficiencies correlated well with the ability of the respective site to confer estrogen responsiveness to a heterologous minimal promoter. M3 which contained base substitutions in the variable spacer region proved to be almost as good a competitor as the wild-type M1.
sequence, while M4 which contained a fully palindromic ERE was an even better one. Mutations in the second half-site (M5), and especially in the first half-site (M2), on the other hand, were highly effective in obliterating competitor function. Similar results were also obtained in experiments in which the various versions of the ERE from the Tst-1/Oct6 gene were used as probe instead of competitor (Fig. 5C). Whereas M3 and M4 bound the sequences of the M6 oligonucleotide. (B) Luciferase plasmids pNAm
 luc, pBXminluc and pBXminluc mut were transfected into U87-MG glioblastoma cells with or without the expression plasmid for estrogen receptor pSV/ER, 17β-estradiol (E2) or TPA were added to the media 24 h before harvesting as indicated. Luciferase activities in extracts from transfected cells were determined in two independent experiments, each performed in duplicate. Data are presented as fold inductions which were calculated for each reporter plasmid by comparing luciferase activities with values from cells which were transfected with reporter plasmid and empty RSV expression plasmid and were kept in the absence of 17β-estradiol and TPA. reporter plasmids which contained both the ERE and TRE were added to the media 24 h before harvesting as indicated. Luciferase activities in extracts from transfected cells were determined in two independent experiments, each performed in duplicate. Data are presented as fold inductions which were calculated for each reporter plasmid by comparing luciferase activities with values from cells which were transfected with reporter plasmid and empty RSV expression plasmid and were kept in the absence of 17β-estradiol and TPA. reporter plasmids which contained both the ERE and TRE were added to the media 24 h before harvesting as indicated. Luciferase activities in extracts from transfected cells were determined in two independent experiments, each performed in duplicate. Data are presented as fold inductions which were calculated for each reporter plasmid by comparing luciferase activities with values from cells which were transfected with reporter plasmid and empty RSV expression plasmid and were kept in the absence of 17β-estradiol and TPA. reporter plasmids which contained both the ERE and TRE were added to the media 24 h before harvesting as indicated. Luciferase activities in extracts from transfected cells were determined in two independent experiments, each performed in duplicate. Data are presented as fold inductions which were calculated for each reporter plasmid by comparing luciferase activities with values from cells which were transfected with reporter plasmid and empty RSV expression plasmid and were kept in the absence of 17β-estradiol and TPA.
however, synergistic activation by estrogen and TPA was no longer observed in this construct. These data clearly show that synergism is strictly dependent on the presence of the ERE.

Thus, the observed synergism between estrogen and TPA is mechanistically different from other cases described in the literature. For the ovalbumin gene promoter synergistic activation by TPA and estrogen is mediated by a single site which is different from both a classical TRE or ERE, and is bound by a complex of both AP-1 and estrogen receptor (29). In the human c-fos gene, both a classical TRE or ERE, and is bound by a complex of both and estrogen is mediated by a single site which is different from For the ovalbumin gene promoter synergistic activation by TPA and estrogen receptor has been shown to exist in both Schwann cells and oligodendrocytes (33–35). In agreement with these functions for estrogen in glial cells, however, synergistic activation by estrogen and TPA was no longer observed in this construct. These data clearly show that synergism is strictly dependent on the presence of the ERE.

Synergism with other signal transducers such as TPA seems to be typical for estrogen function in glial cells. For instance, estrogen-dependent stimulation of Schwann cell proliferation requires a simultaneous increase in intracellular cAMP levels (33). In addition to its influence on proliferation, estrogen also has a number of other POU domain proteins such as Pit-1 and Oct-3/4 (36,37), and provides a way of communication synergism is strictly dependent on the presence of the ERE.

Synergism rather seems to stem from both AP-1 and estrogen receptor binding to adjacent sites. This, of course, does not exclude the possibility that both factors also interact with each other directly.

REFERENCES

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