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Characterization of bidirectional promoter of mouse endonuclease III (*Nthl1*) and tuberous sclerosis 2 (*Tsc2*) genes

Shogo IKEDA, Aiki MOCHIZUKI*, Yuichi SEKI** and Hiroaki AYABE*

*Department of Biochemistry, Faculty of Science, and *Graduate School of Science, Okayama University of Science, 1-1 Ridai-cho, Okayama 700-0005, Japan*

***Department of Physiology, Graduate School of Medicine and Dentistry, Okayama University, 2-5-1 Shikata-cho, Okayama 700-8558, Japan*

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The gene for the mammalian endonuclease III homolog (*Nthl1*) lies immediately adjacent to the *Tsc2* gene, a tuberous sclerosis disease-determining gene, in a head-to-head orientation. Using a luciferase assay, we previously identified a short spacer sequence between the mouse *Nthl1* and *Tsc2* genes that is essential for bidirectional transcription, and demonstrated that the expression of both of these 2 genes is regulated by a core promoter that contains Ets-transcription factor binding site. In the present study, we further characterized the *Nthl1/Tsc2* promoter, and detected a novel functional element, probably a Tst-1 binding site, that only exhibits positive regulation of *Tsc2* transcription. Overexpression of the Ets-family proteins c-Ets1 and p55-erg negatively affected the activity of the bidirectional promoter, suggesting that these Ets-family proteins are not responsible for the transcription of *Nthl* or *Tsc2*. The promoter's activities in the *Nthl1* and *Tsc2* directions were simultaneously measured in various cell lines using a plasmid that contains the core promoter sequence and 2 different reporter genes (one on each side of the promoter). The results show that the activity ratio of *Tsc2*- to *Nthl1*-transcription varies among the different cell types, indicating that transcription in each direction (*Tsc2* and *Nthl1*) is regulated in a separate manner.

1. Introduction

The mammalian endonuclease III homolog is a DNA glycosylase/AP lyase that recognizes oxidized pyrimidines, such as thymine glycol, in the first step of base excision repair [1,2]. Analysis of the gene (*NTHL1/Nthl1*) for this enzyme in humans and mice has shown that it lies immediately adjacent to a tuberous sclerosis disease-determining gene (*TSC2/Tsc2*) in a head-to-head orientation [2-4]. The *TSC2/Tsc2* gene, which has a length of approximately 45 kb, encodes the tuberin protein, which has a molecular mass of approximately 200 kDa [5]. Germ-line mutations of human *TSC2* cause tuberous sclerosis, a disease characterized by the development of hamartomas in various organs (reviewed in Ref. [6]). To clarify the regulation of these adjacent genes, we previously studied promotion of their transcription in a luciferase assay using HeLa cells [7]. We found that the short spacer sequence of 63 bp between the mouse *Nthl1* and *Tsc2* genes shows bidirectional promoter activity essential for the transcription of both genes. The 63-bp sequence is well conserved among several mammalian species, and contains 2 Ets-transcription factor binding sites facing in opposite directions. An Ets-family protein in HeLa nuclear extract specifically binds to both of these Ets binding sites. Experimental mutation of the core motif of the Ets binding sites demonstrated that each of these Ets-binding sites positively regulates transcription of

both the *Nthl1* and *Tsc2* genes. These Ets-binding sites had an additive effect on transcription, and each site functioned equally strongly in both directions. In this study, we defined important features of the bidirectional promoter. We detected a novel *cis*-element that positively regulates *Tsc2* transcription. We demonstrated suppression of the bidirectional promoter by expression of Ets-family proteins in HeLa cells, and differential expression of *Nthl1* and *Tsc2* genes in various cell lines.

2. Materials and Methods

2.1. Materials

The cell lines GI-1 (Cell No. RCB0763), A549 (RCB0098) and BALB/3T3 cone A31 (RCB0005) were purchased from RIKEN Cell Bank, Saitama, Japan. OUMS-24 cells were provided by Prof. M. Namba (Okayama University Medical School). HeLa cells were grown in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal calf serum. GI-1 and A549 cells were grown in Dulbecco's Modified EMEM supplemented with 10% fetal calf serum. BALB cells were grown in EMEM supplemented with 10% calf serum. These cells were incubated at 37°C in a humidified atmosphere of 5% CO2. Plasmids expressing chicken c-Ets1 (DEB-Ets1) and human p55-erg protein (PSG5-Hu-erg) in mammalian cells were kindly donated by Dr. D. Kerbiriou-Nabias (Hospital de Bicetre, France) [8]. These expression plasmids were constructed using ΔEB (for c-Ets1) and PSG5 (for p55-erg) as vector plasmids.

2.2. Plasmid construction for luciferase assay

The DNA fragments used for the luciferase assay, shown in Figures 1 and 2, were amplified by PCR using *pfu* DNA polymerase (Promega) and 20-mer specific primers according to the methods described in a previous report [7]. The fragments were then inserted into the firefly luciferase vector PGV-B (TOYO INK) in either orientation to measure the promoter activity in the *Nthl1*- and *Tsc2*-transcription directions.

2.3. DNA transfection and measurement of luciferase activity

Using FuGENE 6 transfection reagent (Roche Molecular Biochemicals), HeLa cells ($1x10^5$ cells in each well of a 24-well plate) were transfected with the expression plasmid (0.67 μg/well) and 2 ng of pRL-CMV, a plasmid containing the sea pansy luciferase gene under a cytomegalovirus promoter. Forty-eight hours after transfection, cell were lysed with the lysis reagent included in the Pica Gene Dual Sea Pansy Luminescence kit (TOYO INK). The activity of the firefly and sea pansy luciferase were measured using the kit and a Lumat LB9507 luminometer (EG & G BERTHOLD). All values of luciferase activity were normalized to the sea pansy luciferase in the same extract.

3. Results and Discussion

3.1. Characterization of the *cis*-element with positive effects on *Tsc2* transcription

Results of previous transcription promoter analysis of the mouse *Nthl1/Tsc2* gene indicated that the -161/ -1 region is both necessary and sufficient for transcription in both the *Nthl1* and *Tsc2* directions [7]. In the present study, we deleted 3' portions of -345/-1 region to determine what part of that region is involved in transcription (Fig. 1A). Deletion of the -32/-1 region did not affect the promoter activity in either direction. When the -98/-1 region was deleted, promoter activity in the *Tsc2* direction was about 40% of the activity of the full-length promoter, but the activity in the *Nthl1* direction was not affected by this deletion (Fig. 1B). Therefore, we concluded that the -98/ -33 region is necessary for normal trans-cription in the *Tsc2* direction. However, this region is not necessarily sufficient for the promoter activity in the *Nthl1* direction. In other words, the -98/-34 region does not affect transcription in the *Nthl*1 direction but has a positive effect on transcription in the *Tsc2* direction. Deletion of the -161/-1 region, where the 2 Ets-binding sites are located, abolished promoter activity in both directions. In order to more accurately define

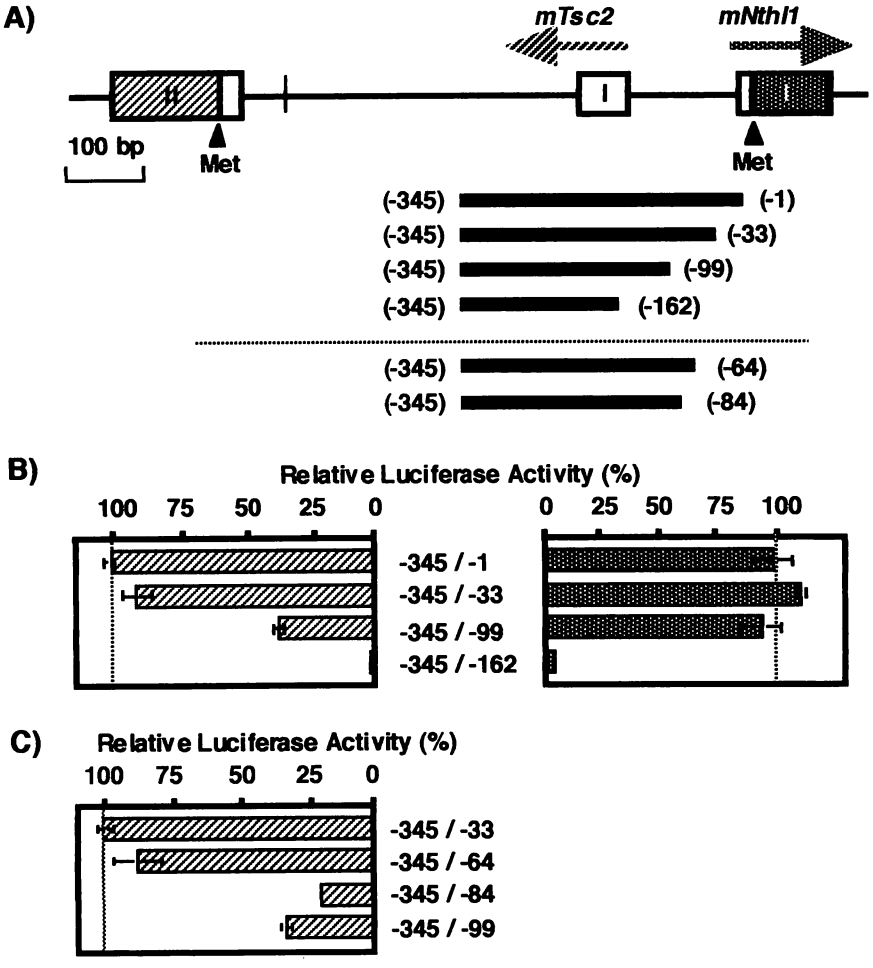

Fig. 1 Promoter activity of the DNA region between *Nthl1* and *Tsc2* genes. (A) Diagram of the 5' regions of the mouse *Nthl1* and *Tsc2* genes. Filled and open boxes represent translated and untranslated regions, respectively. DNA fragments used for luciferase assay are shown as bars. Numbers in parentheses indicate the base positions of the ends of the fragments. The DNA fragments were inserted into the PGV-B luciferase vector in both directions, as described in Materials and Methods. (B) Luciferase activities in HeLa cells containing the entire bidirectional promoter (-345/-1) and the fragments with deletions of the 3' region of the promoter. The activities of various constructs were normalized for equal transfection efficiency using the activity of CMV-sea pansy luciferase (pRL-CMV), which was cotransfected as an internal control. Left and right bar graphs show the luciferase activities in the *Tsc2* and *Nthl1* directions, respectively. The activity of the -345/-1 fragment was designated as 100%. Values from at least 2 independent experiments with triplicate samples were averaged. Error bars represent the standard error of the mean. (C) Luciferase activities of the detection constructs between -33 and -99. Experimental details were the same as described in (B), but only activity in the *Tsc2* direction was measured. The activity of the -345/-33 fragment was used as the 100% standard.

the region that is only necessary for *Tsc2* transcription, the luciferase constructs used for analysis of promoter activity in the *Tsc2* direction contained either the -345/-64 region or the -345/-84 region. The level of promoter activity of the plasmid containing the -345/-64 region was nearly normal, whereas the activity of the plasmid containing the -345/-84 region was very low (Fig. 1C). These results indicated that the *cis*-element that has a positive effect on *Tsc2* transcription is located between -83 and -64.

A computer search for transcription factor binding

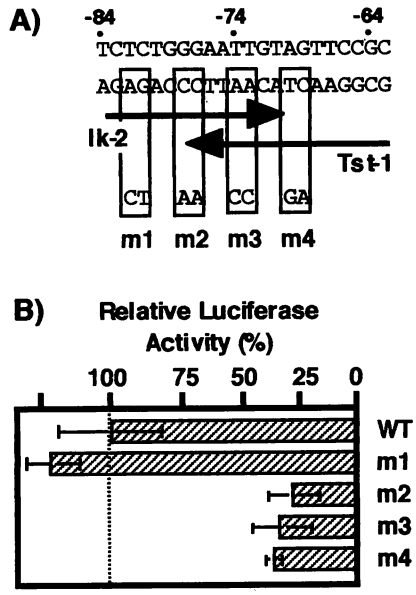

Fig. 2 The transcription factor binding sites in the -84/-64 region of the *Nthl1/Tsc2* promoter and the functional analysis of the mutations in the binding motifs. (A) The transcription factor binding sites indicated by arrows were found using TRANSFAC, the databases of Heinemeyer *et al.* [10], and the TFSEARCH program (http://www.rwcp.or.jp/papia/) written by Y. Akiyama. Mutations (m1 to m4) shown in boxes were introduced by PCR using primers that contain transversion mutations. (B) Effect of mutations m1 to m4 on promoter activity in the *Tsc2* direction. Experiment details were the same as those described in the legend for Fig. 1B. The activity was expressed relative to the activity of the wild type.

sites in the -83/-64 region yielded 2 candidate transcription factors: Ik-2 and Tst-1 (Fig. 2A). In order to further investigate the region, we constructed promoter plasmids with transversion mutations in the -82/-64 region. Sites of mutations were as follows: -82/-81 (m1), -78/-77 (m2), -74/-73 (m3), and -70/-69 (m4). In each mutation, adenine was replaced with cytosine (A to C), cytosine with adenine (C to A), guanine with thymine (G to T), and thymine with guanine (T to G). We performed the luciferase assay with these plasmids, and found that the wild type promoter and m1 promoter produced almost the same level of transcriptional activity, and that the m2, m3, and m4 mutations caused a large decrease in activity (Fig. 2B). These results suggest that mutations m2, m3 and m4 interfere with binding of a transcription factor to the -83/-64 region, and that Tst-1 is the factor that binds to this *cis*-element.

3.2. Effects of Ets-family proteins on the activity of the mouse *Nthl1/Tsc2* promoter

Results of previous studies indicate that there are 2 Ets-binding sites in the *Nthl1/Tsc2* core promoter that act positively on transcriptional activity in both the *Nthl1* and *Tsc2* directions when they bind to Ets-family proteins [7]. In order to determine which proteins bind to these sites, a plasmid that encodes an Ets-family protein was cotransfected with a reporter plasmid containing the *Nthl1/Tsc2* core promoter (-178/-1) into HeLa cells (Fig. 3). We used the plasmids ΔEB-Ets1 and PSG5-Hu-erg, which express chicken c-Ets1 and human p55-erg, respectively [8]. Cotransfection with either of these plasmids resulted in lower transcriptional activity, in both the *Nthl1* and *Tsc2* directions, than an empty vector. This indicates that c-Ets1 and p55-erg interacted with the *cis*-elements within the *Nthl1/Tsc2* core promoter but did not stimulate transcription activity. c-Ets1 and p55-erg are members of the Ets family of proteins which includes almost 20 proteins [9]. The results suggest that neither c-Ets1 nor p55-erg is the Ets-family protein responsible for the activation of the mouse *Nthl/Tsc2* core promoter.

3.3. Simultaneous measurement of *Nthl1/Tsc2* promoter activity in both directions in various cell lines

We constructed a plasmid that contains 2 reporter genes (dual reporter plasmid), in order to simultaneously assess transcription activity in the both *Nthl1* and *Tsc2* directions (Fig. 4A). The plasmid was constructed by inserting a sea pansy luciferase gene into a firefly luciferase plasmid that contains the *Nthl1/Tsc2* promoter region (-1178/-1). A 1430-bp DNA fragment containing sea pansy luciferase gene and SV40 late poly A signal was

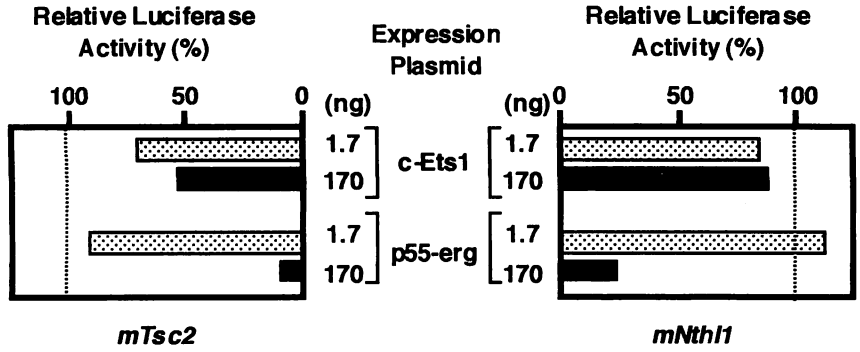

Fig. 3 Effect of Ets-family proteins on the activity of the *Nthl1/Tsc2* bidirectional promoter. A fragment (2 μg) containing the -178/-1 region of the *Nthl1/Tsc2* promoter was cotransfected with different amounts (1.7 ng or 170 ng) of ΔEB-Ets1 or PSG5-Hu-erg plasmids into HeLa cells. Identical cotransfections were performed with the empty expression plasmids ΔEB (for ΔEB-Ets1) and PSG-5 (for PSG5-Hu-erg). We also used pRL-CMV as an internal standard. Luciferase activity after cotransfection of the expression plasmids was expressed as percentage of the activity after cotransfection of the empty plasmids.

amplified from pRL-CMV by PCR with *pfu* DNA polymerase. This fragment was positioned next to the promoter region in the opposite orientation of the firefly luciferase gene. In the luciferase assay using this dual reporter plasmid, sea pansy luciferase activity represents transcription activity in the *Tsc2* direction, and firefly luciferase activity represents transcription activity in the *Nthl1* direction. Experimental results are shown as the ratio of sea pansy luciferase activity to firefly luciferase activity (Fig. 4B). Thus, a cell with a higher value of the ratio transcribes *Tsc2* more actively than *Nthl1*. The *Nthl1/Tsc2* promoter activities were simultaneously measured in several cell lines derived from various tissues, including BALB/3T3 (fibroblast-like cells from mouse embryo), Cos7 (African green monkey kidney cells), OUMS (human normal fibroblast cells), HeLa (epithelial-like cells from human cervix), Gl-1 (human glioma from gliosarcoma), and A549 (epithelial-like cells from human lung carcinoma). We found that the activity ratio of *Tsc2* to *Nthl1* varies among these different cell types. A549 (highest ratio) had a value 40 times higher than that of BALB/3T3 (lowest ratio). This indicates that

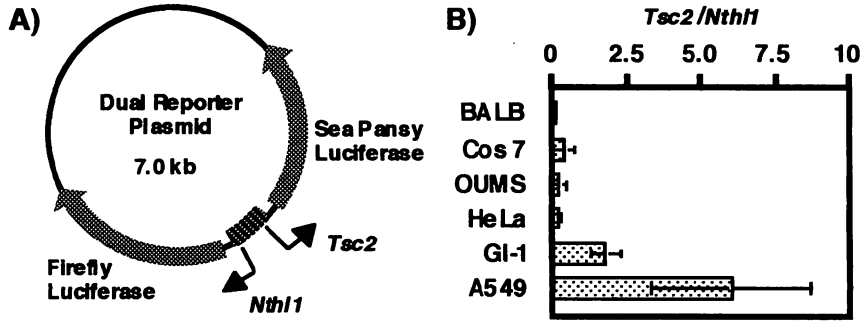

Fig. 4 Simultaneous measurement of the promoter activities in both *Nthl1* and *Tsc2* directions in various cell lines using a dual reporter plasmid. A) Structure of the dual reporter plasmid. Details of construction of the plasmid are described in the text. B) Transcriptional activity in the *Nthl1* and *Tsc2* directions are measured at the same time in various cell lines. The dual reporter plasmid (2 μg) was transfected into various cell lines, and the ratio of the promoter activity in the *Tsc2* direction to activity in the *Nthl1* direction was calculated.

transcription in each direction is regulated in a separate manner, and that different types of cells transcribe these 2 genes at different ratios of activity. Further analysis is needed to characterize the *cis*-element involved in the tissue-specific regulation of these genes, which exhibit markedly different patterns of transcription. Thus, 2 aspects of transcription regulation of *Nthl1* and *Tsc2* have so far been investigated: the previously reported regulation of both genes by Ets-binding sites, and the presently reported differences in rates of transcription of the 2 genes.

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