Genomic organization, chromosomal localization, and promoter of human gene for FK506-binding protein 12.6

ARTICLE *in* GENE · NOVEMBER 2005

Impact Factor: 2.14 · DOI: 10.1016/j.gene.2005.07.004 · Source: PubMed

Available online at www.sciencedirect.com
SCIENCE DIRECT
Gene 360 (2005) 55 – 64
GENE
SECTION
FUNCTIONAL GENOMICS
www.elsevier.com/locate/gene

Genomic organization, chromosomal localization, and promoter of human gene for FK506-binding protein 12.6☆

Tetsuya Nakazawa [a], Shin Takasawa [a], Naoya Noguchi [a], Koji Nata [a], Akira Tohgo [a], Mitsuko Mori [b], Kan-ichi Nakagawara [b], Takako Akiyama [c], Takayuki Ikeda [c], Akiyo Yamauchi [c], Iwao Takahashi [c], Takeo Yoshikawa [c], Hiroshi Okamoto [c,*]

[a] *Department of Biochemistry, Tohoku University Graduate School of Medicine, Sendai 980-8575, Miyagi, Japan*
[b] *Nihon Gene Research Laboratories, Inc., Miyagino-ku, Sendai 980-0012, Miyagi, Japan*
[c] *Department of Advanced Biological Sciences for Regeneration (Kotobiken Medical Laboratories), Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Miyagi, Japan*

Received 25 March 2005; received in revised form 29 June 2005; accepted 1 July 2005
Available online 24 August 2005
Received by R. Di Lauro

Abstract

Cyclic ADP-ribose (cADPR) induces the release of Ca^{2+} from microsomes of pancreatic islets for insulin secretion. It has been demonstrated that cADPR binds to FK506-binding protein 12.6 (FKBP 12.6) on rat islet ryanodine receptor and that the binding of cADPR to FKBP12.6 frees the ryanodine receptor from FKBP12.6, causing it to release Ca^{2+} [Noguchi, N., Takasawa, S., Nata, K., Tohgo, A., Kato, I., Ikehata, F., Yonekura, H., Okamoto, H., 1997. Cyclic ADP-ribose binds to FK506-binding protein to release Ca^{2+} from islet microsomes. J. Biol. Chem. 272, 3133–3136.]. In this study, we cloned, characterized the structural organization of the human *FKBP12.6*, which is highly homologous to human *FKBP12*, and analyzed the promoters for *FKBP12.6* and *FKBP12*. Human *FKBP12.6* gene spanned about 16 kb in length and consisted of four exons and three introns. The positions of exon–intron junction of the *FKBP12.6* gene were perfectly matched with those of *FKBP12* gene except that *FKBP12* has an additional exon, exon V, to code exclusively for 3′-*UTR*. Fluorescence in situ hybridization revealed that the *FKBP12.6* gene was located on chromosome 2 p21–23, which is different from the locus (chromosome 20 p13) of the *FKBP12* gene. Reporter gene analyses revealed that the regions of −58 ~ −24 of *FKBP12.6* and −106 ~ −79 of *FKBP12* are important for promoter activities. The promoters contain a consensus transcription factor binding sequence for Sp family in *FKBP12.6* and Ets-1 in *FKBP12*. Electrophoretic mobility shift assays showed that nuclear proteins bind to the promoters. The DNA/protein complex on *FKBP12.6* promoter was competed out by Sp1 consensus probe and the complex was supershifted by anti-Sp3 antibodies. On the other hand, the DNA/protein complex on *FKBP12* promoter was competed out by Ets-1 consensus probe but not by its mutant probe, indicating that Sp3 and Ets-1 play an essential role in transcription of *FKBP12.6* and *FKBP12*, respectively.

Keywords: FKBP12.6; FKBP12; Cyclic ADP-ribose; Ryanodine receptor; Gene structure; Promoter assay

1. Introduction

Cyclic ADP-ribose (cADPR), a specific metabolite of NAD^+, mobilizes intracellular Ca^{2+} by a mechanism completely independent of D-myo-inositol 1,4,5-trisphosphate. This cADPR-mediated Ca^{2+} signaling participates in the regulation of a variety of physiological functions in different tissue and cells via the activation of the ryanodine receptors (RyRs) of the endoplasmic reticulum including pancreatic β-cells (Takasawa et al., 1993, 1998; Okamoto and Takasawa,

Abbreviations: cADPR, cyclic ADP-ribose; cDNA, DNA complementary to RNA; EMSA, electrophoretic mobility shift assay; FISH, fluorescence in situ hybridization; FKBP12, FK506-binding protein 12; FKBP12.6, FK506-binding protein 12.6; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; RyR, ryanodine receptor.

☆ Sequence data from this study have been deposited in the GenBank database with accession numbers AB190793, and AB190794.

* Corresponding author. Tel.: +81 22 717 8079; fax: +81 22 717 8083.
E-mail address: okamotoh@mail.tains.tohoku.ac.jp (H. Okamoto).

0378-1119/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.gene.2005.07.004

2002; Lee, 2002). To date, three different RyRs encoded by separate genes have been identified, namely the skeletal-type *RyR1*, the cardiac-type *RyR2* and the brain-type *RyR3* (Rossi and Sorrentino, 2002). RyR1 and RyR2 function as Ca^{2+}-induced Ca^{2+} release channels for the contraction of skeletal and cardiac muscles, respectively, and RyR3 was first isolated from brain. These 3 channels are expressed in many tissues including skeletal muscle, heart, brain, and pancreatic islets (Takasawa et al., 1998). There is increasing evidence that FK-506 binding proteins (FKBP) play an important role in the regulation of the RyRs activation and the resulting Ca^{2+} release from the ER (Marks, 1996, 2001). *FKBP12* gene encodes a ubiquitous 12-kDa cytosolic protein. FKBP12 has been found to be tightly associated with the RyR1 calcium channel. As an accessory protein, each FKBP12 can bind to one RyR monomer and its activity can be inhibited by the immunosuppressants FK-506 and rapamycin (Marks, 1996, 2001). It has been demonstrated that the Ca^{2+} release from the endoplasmic reticulum was inhibited when FKBP12 bound to the RyR and vice versa. FK-506, as a ligand, binds to and consequently dissociates FKBP12 from the RyRs, resulting in RyRs activation and Ca^{2+} release (Marks, 1996; Valdivia, 1998; Marks, 2001). A second isoform of FKBP12, the FKBP12.6 protein, has been found to bind to RyR2, while RyR3 associates with both FKBP12 and FKBP12.6. Recent studies (Noguchi et al., 1997; Tang et al., 2002; Wang et al., 2004) indicated that cADPR is able to bind to FKBP12.6 like FK-506 and cause the activation of the RyRs. Despite the important roles of FKBP12.6/12, little is known about genomic organization, chromosomal localization, and promoter activity of the human FKBP12.6-encoding gene, *FKBP12.6*.

2. Materials and methods

2.1. Isolation of human FKBP12.6 gene

We amplified an intron containing a DNA fragment of the human *FKBP12.6* by PCR with two synthetic oligos corresponding to nt 151–176 and 368–393 of the human *FKBP12.6* cDNA (Lam et al., 1995). The resulting 3 kb fragment was sequenced using a Thermo Sequenase™ cycle sequencing kit (Perkin-Elmer, Norwalk, CT) and a 0.4 kb fragment containing intron III and exon IV was used for the initial screening. A λEMBL3 human genomic library (Nata et al., 1997) was screened first with the 0.4 kb fragment and then the 1.2 kb fragment of the 5′ end of the initially isolated λEMBL3 clone. The nt sequences were determined as described previously (Nata et al., 2004).

2.2. Chromosomal mapping of human FKBP12.6 gene by FISH (fluorescence in situ hybridization)

Plasmid containing a 14 kb fragment of human *FKBP12.6* gene (clone 12.6-2, which contains exons I–III and 5′-flanking region of the gene) or a 20 kb fragment of human *FKBP12* gene, which contains exons I–V along with 5′- and 3′-flanking region of the gene, was labeled with biotin-16-dUTP using a nick translation kit (Roche). FISH procedure was carried out as described previously (Nakagawara et al., 1995).

2.3. Determination of major tsp

Synthetic oligos of 30 nt complementary to the human *FKBP12.6* gene (+83 – +112) and the human *FKBP12* gene (+30 – +59) were labeled at 5′-ends with [γ-^{32}P]ATP (Amersham Pharmacia Biotech) and T4 polynucleotide kinase (Takara Shuzou, Otsu, Japan), and used as primers. The labeled primer for *FKBP12.6* or *FKBP12* was then hybridized to 5 μg of poly $(A)^+$ RNA isolated from human pancreas for 12 h at 50 °C in a 10 μl solution containing 5.5 mM Tris–HCl (pH 8.3), 0.55 mM EDTA and 0.9 M KCl. The annealed RNA/primer mixture was precipitated with ethanol and then subjected to the primer extension with SuperScript™ II (Invitrogen, Carlsbad, CA) at 42 °C for 60 min. The extended cDNA were extracted with phenol/chloroform and precipitated with ethanol. The precipitated products were resuspended in a sample buffer and electrophoresed on 6% polyacrylamide gel containing 8.3 M urea along with the appropriate dideoxy sequencing reactions as size markers as described (Nata et al., 2004).

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated (Noguchi et al., 1997) and RT was carried out (Takasawa et al., 1998; Sasamori et al.,

Table 1
Oligonucleotides used for EMSA

Name[a]	Sequence
Probes for EMSAs (sense strand)	
−58/−24	5′-CGCGCTCCGCCCCGCCGCCCCCTTGCCAGTGGCCC-3′
−106/−79	5′-GCTTCTGGGCTTCCGGTCCCTCTTCCGG-3′
Competitors (sense strand)	
Sp1	5′-ATTCGATCGGGGCGGGGCGAGC-3′
Ets-1	5′-GATCTCGAGCAGGAAGTTCGA-3′
mEts-1	5′-GATCTCGAGCAAGAAGTTCGA-3′

[a] The oligos were paired with their respective complements and are named according to their positions in the promoter or according to the affected binding element in competition assays.

2004) in a volume of 20 μl using 1 μg of total RNA as a template. The RT sample (0.5 μl) was used for PCR in a final volume of 20 μl as described (Takasawa et al., 1998). The PCR primers correspond to nt 92–111 and 459–479 for *FKBP12.6* (NM_004116), nt 103–122 and 469–492 for *FKBP12* (NM_000801), nt 1235–1254 and 1665–1684 for

```
ttctttaacaacacagccagaacaccttcagggatgcagacaacctcatctgggacctaagcatccgggagagtggggat  -1676
gaggaaatggaggaagaacacccttggggctcattctggaccagtttgccaggcttttctccccatgcctatcttccagt  -1596
gccactcaggagaatcatatcttcacatcagtttttatgtttttctttttctttttctttttttttaaaaataaagat  -1516
ggcatgcctgtaatcccagcactttgggaggctgaggcgggcggataacctgaggtcgggagttcgagaccagcctggcc  -1436
aacatggtgaaacctgtctctactaaaaatttaaaaattagccgaacgctggtgtctcacgcctgtaatcccagctactt  -1356
gggagactgaggcacgagaatcgcttgaacctgggaggcggaggttgcagtgagccgagattgtgccactgcactccatc  -1276
ctggcgacagagtgaagattctgtctcaaaaaaaaaaaaaaaaaaaaggagagagagagagacgggatctcgctatgtta  -1196
cccaggctggtcttgaacttctaggctcaagcaatcctcctgcctcggtctcctacagtgttgagattacaggtgtgagc  -1116
cattgttccaaaaattgatatacttggcctcacatcaatttttggaacaatgcaggtaataatgaggggggaaaaaatttt  -1036
aaaagtcccaattacccttaaacaaatattacctcctccaaaaaatatttttctagattcctgaatgtatttttggtg   -956
gaggaggaactcaaagtagaattcatttacatattcattcattcattccacagcactaatcactccatgcagtaactgtg   -876
tgtccccgtccctctcagacttagtacctggaagacagccaacctgtcttaattctattggtatccccagggcgcatttc   -796
tttgccttgaaaattgagtacccaataaatgtgcgtcgagcagaactacacgagatggagaacaggggtttaggggtatg   -716
ggggagagtcagcaattactggtgtcctccctccctcccccagctctgctcctacagatccaggacgcctagcgagggta   -636
ggaacctcttctgtgcttccgcaacgctctgaactgcacggcactgagcgcatctaattaaagtgatggtttcattagat   -556
agcaacacagcgagaccccgtttctacaaaaaatataaaaataaaaaatataaagattagctgggcgtggttgctcgcgcctc   -476
tagtcccagctacttgggtggaggctgaggaagaaggatcgcttgagcctaagagttcgaggttgctgtgagctatgatc   -396
gcgccactggactccagcctgggcgacagagcaagatcctctgtctaaaataaaaataaaaataaaatgatggtttaatc   -316
gccggcctttgccccttctgccccccaccccccacaagtctgtaggactgggttgacttcccagagcttactacagatta   -236
gatgctaaataaatgtctattggacggatgcgcgaatgaatggatggatggaggatgtggttgtgcttaccacgctccct   -156
ggtcagtgggttaatgtcgtcaggcggggccaggctcgctaacagccgggcggggtcccttcccccgggccccgccccgg    -76

ccgaccccacctttccgcgcgctccgccccgccgcccccttgccagtggccccctccccgcgcaggtcccgactccAGCCG     +5

                  EXON I
CACCTCCTCCGGCTCTGCAGTGGCGGCGAGGAGGCGAGCCGGAGCGACGGCGGGGCTGGGGCCGGAGCCGAGCCGGGGTC    +85

GGGCAGCAGCAGGGACCCCCCAGAGGCGGGGCCTGTGGGACCGCTATGGGCGTGGAGATCGAGACCATCTCCCCCGGAGA   +165

CGgtaccggctccctccggagccaggggaggggaggggtcccgggcggagcccggagggcgggggtctgattcggaggtg   +245
gcgtggagggtgctgaagggtc---3.8kbp---aggggaatgcaggctggagctcttggtcaggatcatacttaatgtc  +4126

                                                                    EXON II
ccaggtgtttttccaatatcctactccttcatctgccctcatgtctccctgcagGAAGGACATTCCCCAAGAAGGGCCAA  +4206

ACGTGTGTGGTGCACTACACAGgtaagtctcacccccctcagcccccacccagtccttcccctcccaaggcagggctgtcc  +4286
tctgagcttctctccagaggtgcctgcctctggatcagggcta--6.8kbp---ggtgtgtgcagaaaaggcattccatt +11018

ttagacatgtggaatctgaattgggagtttactcatgaccacagctctattgcacccgattcttgctttgacagGAATGC +11098

                                  EXON III
TCCAAAATGGGAAGAAGTTTGATTCATCCAGAGACAGAAACAAACCTTTCAAGTTCAGAATTGGCAAACAGGAAGTCATC +11178

AAAGGTTTTGAAGAGGGTGCAGCCCAGgtaggatgaggattcgcattaaaggggatctggggagttggggatctgggatc +11258
agcactctgccctccacctccaccttcacccactcacagaccactac---2.0kbp---tgaggatggtttgggaaaatg +13263

ccatagtcatctaacttttttctttaaatcaagctgggtcctctttctcctctccccatctgcccccatccctgctagA  +13343

                                  EXON IV
TGAGCTTGGGGCAGAGGGCGAAGCTGACCTGCACCCCTGATGTGGCATATGGAGCCACGGGCCACCCCGGTGTCATCCCT +13423

CCCAATGCCACCCTCATCTTTGACGTGGAGCTGCTCAACTTAGAGTGAAGGCAGGAAGGAACTCAAGGTGGCTGGAGATG +13503

GCTGCTGCTCACCCTCCTAGCCTGCTCTGCCACTGGGACGGCTCCTGCTTTTGGGGCTCTTGATCAGTGTGCTAACCTCA +13583

CTGCCTCATGGCATCATCCATTCTCTCTGCCCAAGTTGCTCTGTATGTGTTCGTCAGTGTTCATGCGAATTCTTGCTTGA +13663

GGAAACTTCGGTTGCAGATTGAAGCATTTCAGGTTGTGCATTTTGTGTGATGCATGTAGTAGCCTTTCCTGATGACAGAA +13743

CACAGATCTCTTGTTCGCACAATCTACACTGCCTTACCTTCACTTAAACCACACACACAAGGTGCTCAGACATGAAATGT +13823

ACATGGCGTACCGTACACAGAGGGACTTGAGCCAGTTACCTTTGCTGTCACTTTCTCTCTTATAAATTCTGTTAGCTGCT +13903

CACTTAAACAATGTCCTCTTTGAGAAAATGTAAAATAAAGGCTCTGTGCTTGACAaactcctgtccatccttatt      +13978
```

Fig. 1. Nucleotide sequence of the human *FKBP12.6* gene. Nucleotide residues are numbered in the 5′ to 3′ direction, beginning with *tsp*, and nt on the 5′ side of residue 1 are indicated by negative numbers. Capital letters indicate exons and lower case letters are used for introns and 5′ and 3′ flanking sequences. The translation start and stop codons and the polyadenylation signal are underlined. The promoter region (−58 ~ −24) essential for transcription is underscored with a wavy line and a consensus binding sequence for Sp1 in the region is shaded.

Sp3 (AY441957), nt 356–375 and 1330–1349 for *Ets-1* (NM_005238), and nt 112–132 and 928–948 for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (J02642).

2.5. Preparation of reporter constructs

The human *FKBP12.6* 5′-flanking region (−1691 ~ +109) and human *FKBP12* 5′-flanking region (−1706 ~ +64) were inserted into pGL3-Basic vector (Promega, Madison, WI) to construct reporter plasmids. Unidirectional deletions were made using Deletion Kit for Kilo-Sequencing (Takara Shuzo). The extent of deletion in each individual construct was determined by DNA sequence analysis. For construction of the promoter mutants used in this study, sequences of the FKBP12.6/luciferase construct "−58" and the FKBP12/luciferase construct "−106" (Fig. 5) were replaced as primers by using PCR.

2.6. Cell culturing and transfections

HEK-293 cells were grown in DMEM medium (Sigma) containing 10% (v/v) fetal calf serum (JRH Biosciences, Lenexa, KS), 100 units/ml penicillin G (Wako, Osaka, Japan), and 100 μg/ml streptomycin (Wako). LS-174T cells

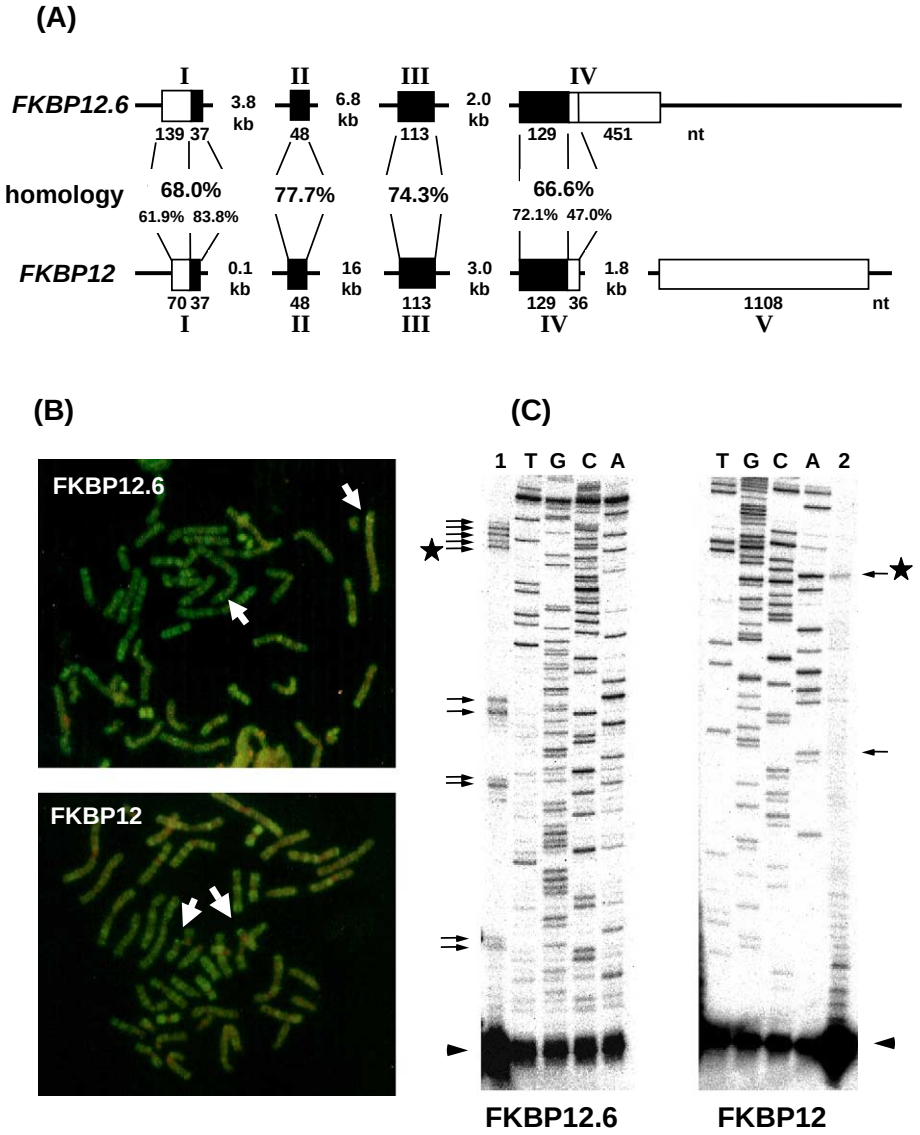


Fig. 2. (A) Nucleotide sequence homology within the corresponding exons between human *FKBP12.6* and human *FKBP12* gene. The exons are depicted as boxes numbered I–IV or V. Filled and open boxes represent ORFs and *UTR*, respectively. The exon size (nt) is represented under each exon. Identities are shown between each exon. (B) Chromosomal mapping of the human *FKBP12.6* and *FKBP12* genes by FISH. R-banded metaphase chromosome spreads from human lymphocytes hybridized with *FKBP12.6* and *FKBP12* genes, showing the localization of spots on both chromatids of chromosome 2 and 20, respectively. Arrows indicate fluorescent signals on 2p21–24 and 20p13. (C) Localization of *tsp* of the human *FKBP12.6* and *FKBP12* genes. Synthetic oligos of 30 nt complementary to the human *FKBP12.6* gene (+83 – +112) and the human *FKBP12* gene (+30 – +59) were labeled at 5′-ends with [γ-^{32}P]ATP and T4 polynucleotide kinase and used as primers. The labeled primer for *FKBP12.6* or *FKBP12* was then hybridized to 5 μg of poly (A)$^+$ RNA and the extended products were analyzed as described (Nata et al., 2004).

```
caggaatttttttttttttttttttgagactgttctcactctgtcacccagctgaatgcagagtgcaatcttgctcact -1618
acaacctccgcctcctggtttaagcaattcttatgcctcagcctctcaagtggctgggattacaagcctgtgccaccatg -1538
cctggctaatttttttgtatttttagtagaaactggtttcaccatgttggccaggctggtcttgaacgcctggcctcca -1458
agtgatctgcccgcctcagcttcccaaagtgctgggattacaggcatgagccaccgtgcccagcctcaggtattctttat -1378
agcagtgtgagaatcgactaatgcagaagcacacacacacacagatcagagaaagattagtcacatgtgcctccttatgt -1298
cctagaccatgtcacagtgttctcttgcgttacagtgcctctgcaggtactgctccttctgcctcccctaccccaacac -1218
atacacacaattggccttctaaccttggctaaacctggctaaacctggctaactcagggaaatatctgaccacattccag -1138
actaagtgaggcatctctgttatattaacacgcccctcacagcatgtattactgtctcataatattacagatttgaaact -1058
ttacatttattccgtatctttttttacttaaacaggttatttgattatctctcccacatcatacgtagattctgtgaggt  -978
cagggactaagtctgactccttcaccgctgtatcctcagcacctagcacagtacatgacatacagaaggtgcttgtcaaa  -898
gtcaaaataaaatatggagatgaatctctaaatgtaatgttttatttggggaagcgagaatcgcaactcggagcatacac  -818
acagatctggtggtctttggtatgtccgaaaaacaaagagaaggctgggcgttttattagaaagggaaatgcattggcac  -738
tagtaaagtttcagggagctggcaagctctgattggtgactgacagtggtaagtaaaactagtattaggttatggcgggt  -658
tgtttcagcagttactagctgaatctggtctcaggggacagcaggccatttccgcagcagttagtggaaatattggttc  -578
ttggagcaggcgctgcgtgcctccagtgcttttcccctggccctggattctgattccgttgggcataagaagagtgacc  -498
caattcgtattatcaactttcacatgctcaataagaatgtatgcatgtaagctatgggtgagtgaatgaatttggacaac  -418
tcctcctccagtgaagaaaagcagaacttacaaccttgagccacttataaaatactaacatactaccaccacctgtgcgc  -338
tgtcttctcagaaccatgctcttcccaggatgagtaactgtgtgcttctaagtggaaacctgatgacagtcacccagtca  -258
gtgactggcggacccccctattattactcccattctttgggcagggagatgcttaaccggctggaagcacagaggaaggggc  -178

aggagaacgaagcaagtcacccggcaaacacagctgtatccggaggcctccgggcttccggaggtctcggggcttctggg   -98

cttccggtccctcttccggaggcctgggtttccgggacgtcgcgcgccgtgtggggcgcgcacgcagggctgggcgtgag   -18

                                                EXON I
ggggcgtgcgcgtgcgcAGGCGACGCGCCGAGGTACTAGGCAGAGCCGTGGAACCGCCGCCAGGTCGCTGTTGGTCCACG   +63

CCGCCCGTCGCGCCGCCCGCCCGCTCAGCGTCCGCCGCCGCCATGGGAGTGCAGGTGGAAACCATCTCCCCAGGAGACG   +142
```

Fig. 3. Promoter sequence of human *FKBP12* gene. The nt sequence is numbered relative to position +1 representing the major *tsp*. Negative numbers indicate 5′-flanking sequence. The translation start codon is underlined. The promoter region is underscored with a wavy line and a consensus sequence of the transcription factor Ets-1 in the promoter is shaded.

were maintained in RPMI1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin. SW948 cells were maintained in L15 medium (Sigma) supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin. Cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C. The day before transfection, approximately 2×10^5 cells/well (HEK-293 cells) and 1×10^4 cells/well (LS-174T and SW948 cells) were transferred to a 24-well plate. The cells were transfected with 0.8 μg QIAGEN purified reporter vector and 0.1 μg pMCV-SPORT-βgal plasmid (Invitrogen) as a transfection control. Lipofectamine™ 2000 (Invitrogen) was used in a 25:1 ratio for HEK-293 cells, and Cellfectin™ (Invitrogen) was used in 250:7.5 ratio for LS-174T and SW948 cells according to the manufacturers' instructions.

2.7. Luciferase assays

After transfection and incubation for 48 h, cells were harvested as described (Akiyama et al., 2001). The luciferase activity was measured as described (Akiyama et al., 2001) using PicaGene Luciferase assay system (Toyo, Tokyo, Japan). βGal activity was determined as described (Akiyama et al., 2001) using Aurora GAL-XE (ICN).

2.8. Nuclear extract preparation and electrophoretic mobility shift assay (EMSA)

Nuclear extract were prepared as described previously (Akiyama et al., 2001) using Nu-CLEAR™ Extraction Kit (Sigma) and stored at −80 °C until use. DNA probes for EMSA were synthesized as oligos (Table 1) and end-labeled using T4 polynucleotide kinase in the presence of

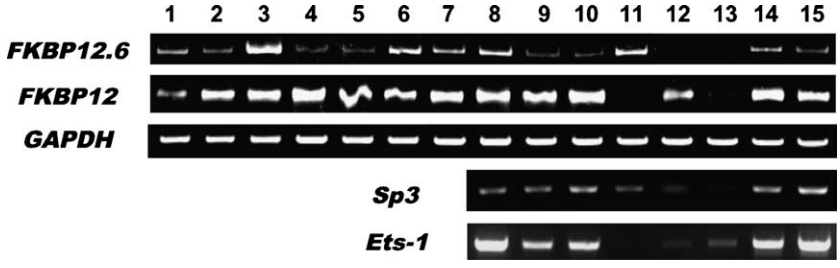
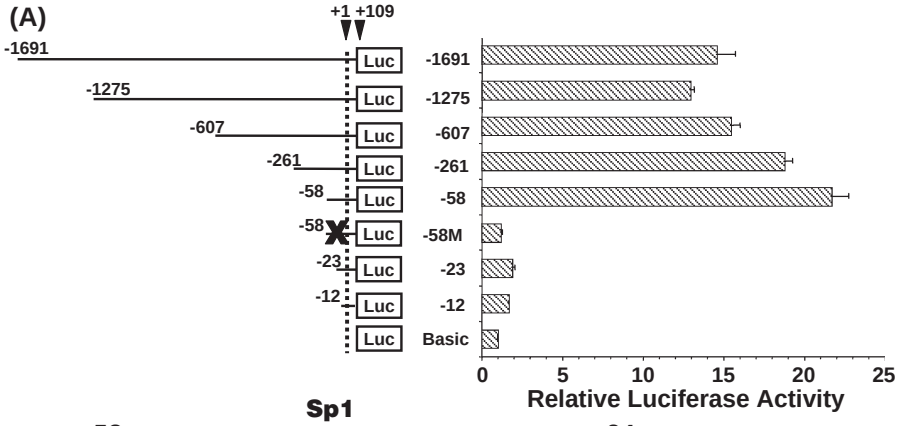
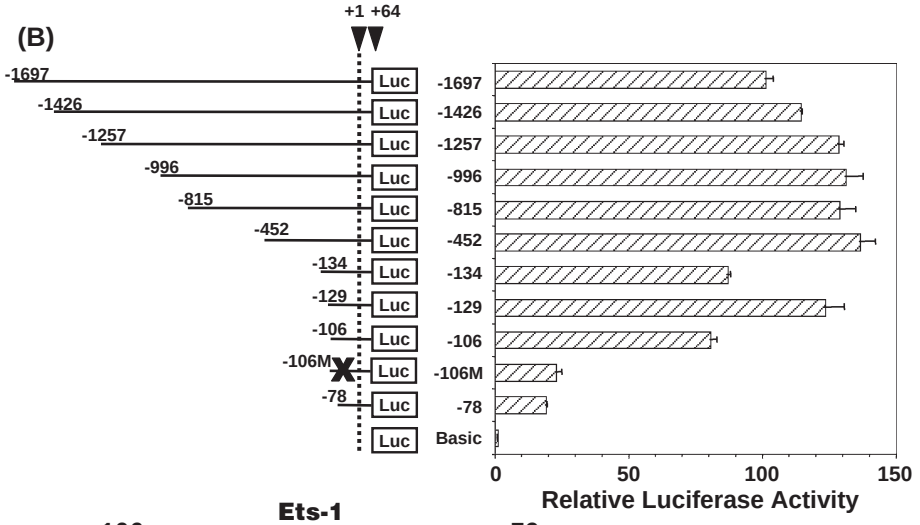


Fig. 4. Expression of *FKBP12.6*, *FKBP12*, *Sp3*, and *Ets-1* genes in human tissues and cell lines was analyzed by RT-PCR. Lane 1, lymphoma; Lane 2, islets; Lane 3, heart; Lane 4, brain; Lane 5, kidney; Lane 6, liver; Lane 7, placenta; Lane 8, NB-1 (a neuroblastoma cell line); Lane 9, HeLa (a cervical cancer cell line); Lane 10, HEK-293 (an embryonic kidney cell line); Lane 11, LS-174T (a colon cancer cell line); Lane 12, SW948 (a colon cancer cell line); Lane 13, Kato III (a gastric cancer cell line); Lane 14, Capan 1 (a pancreatic cancer cell line); Lane 15, PANC-1 (a pancreatic cancer cell line).



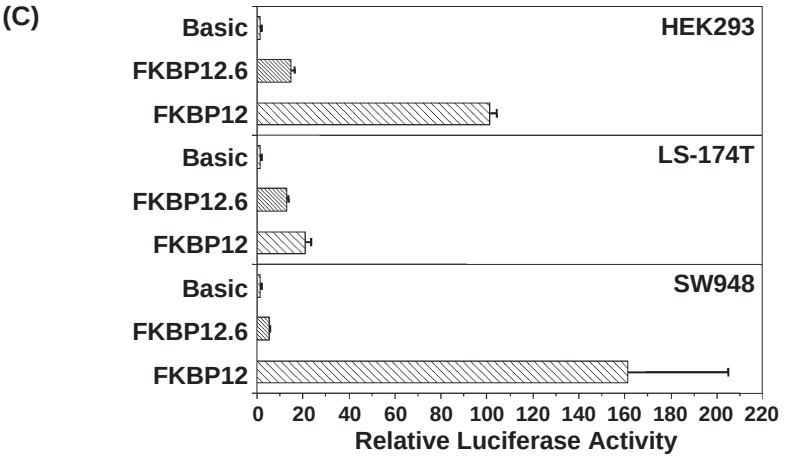
Sp1

```
      -58                                  -24
Human cgc-gctccgccccgccgccccttgccagtggccc
Mouse *a*a*****a**************c**g*c*****
Bovine ***c***************t*****c**t*******
-58M  **********AA***AA******************
```



Ets-1

```
      -106                          -79
Human gcttctgggcttccggtccctcttccgg
Mouse **c**c**a************cg**ta
Rat   **c**c**a************cg**ca
-106M ************T**************
```



[γ-^{32}P]ATP. EMSAs were performed as described (Akiyama et al., 2001). Briefly, aliquots of 10 μg of protein of the nuclear extract from HEK-293 cells were incubated at room temperature for 30 min in a final volume of 20 μl of buffer containing 20 mM Tris–HCl (pH 7.9), 2 mM $MgCl_2$, 50 mM NaCl, 2 mM EDTA, 10% glycerol, 0.1% Nonidet P-40, 1 mM DTT, 50 μg/ml bovine serum albumin, 1 μg of poly(dI-dC) and ^{32}P-labeled probe. For competition or supershift assays, nuclear extracts were pre-incubated for 10 min at room temperature with a 100-fold molar excess of unlabeled competitor oligo or appropriate antibody (Santa Cruz Biotechnology, Santa Cruz, CA), respectively. DNA-protein complexes were analyzed by electrophoresis on non-denaturing 4% polyacrylamide gel (acrylamide : bis-acrylamide, 58 : 1) in 0.5 × TBE (45 mM Tris, 45 mM borate, 1 mM EDTA; pH 8.3). The gels were dried and autoradiography was performed overnight with an intensifier screen.

3. Results and discussion

3.1. Isolation and sequence analysis of human FKBP12.6 gene

We amplified an intron containing a DNA fragment of the human *FKBP12.6* by PCR and the PCR fragment containing intron III and exon IV was used for the initial screening for *FKBP12.6* gene. A positively hybridized clone designated 12.6-1 with ~16 kb insert was isolated from a λEMBL3 human genomic library. Sequence analyses revealed that the clone contained exons III and IV of the human *FKBP12.6* gene. We re-screened the λEMBL3 human genomic library using the 1.2 kb fragment of the 5′ end of 12.6-1 and isolated a clone, 12.6-2 with ~14 kb insert. Sequence analyses revealed that two clones overlapped each other and covered the entire genomic region coding for the human *FKBP12.6* including its 5′- and 3′-flanking regions. We determined the nucleotide sequence of all the exons including exon–intron junctions and 5′- and 3′-flanking regions on both strands (Fig. 1). Comparison of the genomic sequence with the cDNA sequence (Lam et al., 1995) revealed that the coding region was divided into four exons and three introns and that all the exon–intron junctions conformed to the GT/AG rule.

We then compared the gene structure with that for FKBP12, which is composed of 108 aa and is highly homologous to FKBP12.6 (Noguchi et al., 1997; Lam et al., 1995). The ORFs of both genes were divided into 4 exons. The 4 exons of the *FKBP12.6* gene are identical in size to those of *FKBP12*, excluding the number of nt in the 5′- and 3′-*UTR* (Fig. 2A). In addition, the positions of introns were also conserved between the two and the exons of *FKBP12.6* and *FKBP12* align identically with regard to the aa that they encode (data not shown). In spite of the high degree of sequence homology and the conservation of the position of the exon–intron junction in the ORF, the organization of the two genes are different: *FKBP12.6* gene is composed of 4 exons, whereas *FKBP12* has an additional one encoding a 3′-*UTR*.

3.2. Chromosomal localization of FKBP12.6 gene

Following FISH with the 14 kb FKBP12.6 probe containing exons I–III and the 5′-flanking region (12.6-2), 50 metaphases showing typical R-bands were analyzed to determine the chromosomal localization of the human *FKBP12.6* gene. Of the total 100 samples of chromosome 2 analyzed, 72 had labeling on both chromatids and 24 had a signal on one chromatid, 2p21–24 (Fig. 2B). The remaining 4 signals were randomly distributed over chromosome 2. These results indicate that the gene is located at human chromosome 2p21–24. Based on the report of DiLella et al. (1992), *FKBP12* gene is localized on chromosome 20p13. As genes belonging to a gene family are frequently reported to be clustered in a restricted region of the same chromosome (Nata et al., 2004), we isolated a 20 kb DNA fragment containing exons I–V of human *FKBP12* and re-examined the assignment of *FKBP12* gene by the DNA as a probe. All the signals were distributed over chromosome 20, and 75 samples of chromosome 20 had signals on both chromatids and 16 had a signal on one chromatid, 20p13 (Fig. 2B). The remaining 9 signals were randomly distributed over chromosome 20. These results indicate that genes for *FKBP12.6* and *FKBP12* are not clustered in the same chromosomal region despite their highly conserved genomic structures.

3.3. Identification of tsp

The *tsp* of human *FKBP12.6* and *FKBP12* gene were mapped by primer extension. As shown in Fig. 2C, the *tsp* of human *FKBP12.6* and *FKBP12* gene were heterogeneous: 11 major bands were observed in the *FKBP12.6* primer extension and the longest one was 137 nt upstream

Fig. 5. (A) Transcriptional activity of constructs containing various 5′-deleted fragments and a site-directed mutant of the human *FKBP12.6* promoter. (B) Transcriptional activity of constructs containing various 5′-deleted fragments and a site-directed mutant of the human *FKBP12* promoter. A series of luciferase constructs containing promoter fragments with various 5′-ends and mutations in GC box (−58 M) and Ets-1 binding sequence (−106 M) were transfected into HEK-293 cells. The promoter activity is normalized for variations in transfection efficiency using βGal activity as an internal standard and is expressed relative to the activity of the promoterless construct pGL3-Basic. Reported values are means ± SE from 4–8 independent experiments. Conservation of the promoters of *FKBP12.6* and *FKBP12* is also shown by comparison with the corresponding regions of mouse *FKBP12.6* (CAAA01010937), bovine *FKBP12.6* (AAFC01085445), mouse *FKBP12* (CAAA01164414), and rat *FKBP12* (AABR03025068). (C) Transcriptional activities of *FKBP12.6* and *FKBP12* promoters in HEK-293 cells, LS-174T cells, and SW948 cells. Reporter constructs for *FKBP12.6* (−1691 to +109) and *FKBP12* (−1697 to +64) were transfected into HEK-293, LS-174T, and SW948 cells and measured promoter activities as described above.

from the translational start site (Fig. 1). Densitometric analysis revealed that a band located 130 nt upstream of the translation initiation codon, which was located in a cap consensus sequence (Bucher, 1990), was the most dense among the 11 bands and that the intensity of 5 bands (130–137 nt upstream from ATG) accounts for 45% of all the extended bands. Therefore, we assumed that the major *tsp* of the *FKBP12.6* gene was located about 130 nt upstream of the ATG codon. Using the *FKBP12* primer, several bands were extended and the longest one was 108 nt upstream from the ATG triplet (Fig. 2C). The 5′ end of human *FKBP12* cDNA was reported to be located 69 nt upstream from the ATG codon (Maki et al., 1990). From these results, we assumed that the major *tsp* of the *FKBP12* gene was located about 108 nt upstream of the translation start codon.

3.4. Sequences of the upstream region of FKBP12.6 and FKBP12 genes

We sequenced about 1.7 kb of DNA upstream from the *tsp* of the *FKBP12.6* and *FKBP12* genes (Figs. 1 and 3). As expected from the observation that there are multiple sites of transcription initiation, both of the 5′-flanking regions lack the canonical TATA box sequence that determines the specificity of mRNA synthesis initiation by RNA polymerase II. As shown in Figs. 1 and 3, the regions around the *tsp* of *FKBP12.6* (−138 to +129) and *FKBP12* (−173 to +70) genes had high G+C content (77.4% and 80.0%, respectively). In these regions, the cytosine-guanine dinucleotide (CpG) content is significantly greater than average, suggesting that these regions of the *FKBP12.6* and *FKBP12* genes are CpG islands. Typical GC boxes were found at −133 ~ −128, −107 ~ −102, −85 ~ −80, −48 ~ −43, +54 ~ +59, and +110 ~ +115 in the *FKBP12.6* gene and −664 ~ −659, +29 ~ +34, and +79 ~ +84 in *FKBP12* gene. Tissue specific cis-elements were found in the 5′-flanking region of neither the *FKBP12.6* nor *FKBP12* genes. These results are consistent with the fact that *FKBP12.6* and *FKBP12* mRNAs are expressed ubiquitously in a variety of tissues and cells (Fig. 4) although the expression patterns of mRNAs for FKBP12.6 and FKBP12 are different. FKBP12.6 is the most predominant FKBP isoform in LS-174T colon cancer cells. *FKBP12.6* and *FKBP12* mRNAs are expressed at similar levels in lymphoma and heart. In all other cases, *FKBP12* mRNA is the most predominant isoform. These differences in patterns of mRNA expression may reflect the fact that the 5′-flanking region of the *FKBP12.6* gene showed no significant homology with the corresponding region of the *FKBP12* gene.

3.5. Localization of promoter for FKBP12.6 and FKBP12

The constructs were then tested for their functional abilities as promoters in transient transfection assays in human embryonic kidney cells, HEK-293. As shown in Fig. 5A, the deletions down to position −58 showed no significant change in promoter activity in the *FKBP12.6* gene. Further deletion of 35 nt, down to position −23 abolished the promoter activity. On the other hand, we found that the region of −106 ~ −79 in the *FKBP12* gene was indispensably important for the promoter activity (Fig. 5B). Analyses of the regions (−58 to −24 of the *FKBP12.6* promoter and −106 to −79 of the *FKBP12* promoter) using TRANSFAC (Heinemeyer et al., 1999) revealed a potential transcription regulation element for Sp1 binding (−51 to −39) in the *FKBP12.6* promoter (Figs. 1 and 5A) and an element for Ets-1 binding (−99 to −90) in the *FKBP12* promoter (Figs. 3 and 5B).

In order to assess whether or not the active Sp1 binding sequence described in the *FKBP12.6* gene is essential for the activity of the promoters, the Sp1 binding sequence located at position −51 to −39 (Figs. 1 and 5) of the *FKBP12.6* promoter was mutated from CGCCCCGCCGCCC to CGAACCGAAGCCC. In a similar way, the Ets-1 binding sequence located at position −99 to −90 of the *FKBP12* promoter was mutated from GGCTTCCGGT to GGCTTCTGGT. The luciferase activity was measured as described above. The results indicated that the mutated promoters have no promoter activity (Fig. 5), indicating that the binding sequences for Sp1 and Ets-1 are essential for the promoters of *FKBP12.6* and *FKBP12*, respectively.

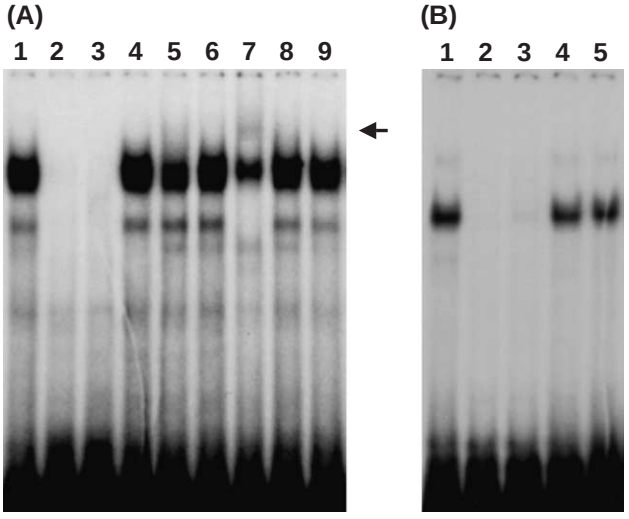


Fig. 6. Detection of nuclear protein binding with *FKBP* promoters. (A) EMSA for *FKBP12.6* promoter. The ^{32}P-labeled probe (−58/−24) was incubated with HEK-293 nuclear extracts. Lane 1, without competitor nor antibody; Lane 2, with cold probe (−58/−24); Lane 3, with Sp1 oligo; Lane 4, with cold FKBP12 probe (−106/−79); Lane 5, with anti-Sp1; Lane 6, with anti-Sp2; Lane 7, with anti-Sp3; Lane 8, with anti-Sp4; Lane 9, with anti-glucocorticoid receptor. The arrow indicates supershift. (B) EMSA for *FKBP12* promoter. The ^{32}P-labeled probe (−106/−79) was incubated with HEK-293 nuclear extracts. Lane 1, without competitor; Lane 2, with cold probe (−106/−79); Lane 3, with Ets-1 oligo; Lane 4, with mEts-1 (mutated Ets-1) oligo; Lane 5, with cold FKBP12.6 probe (−58/−24). All competitors were in 100-fold molar excess. For the supershift assays, 2 μg of antibodies were added.

To verify whether nuclear proteins interact with these sequences, EMSAs were performed using double stranded oligo corresponding to the regions and nuclear extracts isolated from HEK-293 cells (Fig. 6). The DNA/protein complex observed in the *FKBP12.6* promoter was completely competed out both by the unlabeled probe and by the Sp1 consensus oligo (Fig. 6A), suggesting that GC-box binding proteins were involved in the *FKBP12.6* transcription. Moreover, in supershift experiments, the complex was shifted by antibodies against Sp3 (lane 7) but not by anti-Sp1, anti-Sp2 nor anti-Sp4 (lanes 5, 6, and 8). These results indicate that Sp3 plays an essential role in *FKBP12.6* transcription through the GC-box sequence (−51 to −39) in the *FKBP12.6* promoter. In *FKBP12* promoter, the DNA/protein complex in EMSA was competed out by the unlabeled probe and by the Ets-1 binding consensus oligo but not by a mutated Ets-1 probe (Fig. 6B), indicating that Ets-1 is involved in *FKBP12* transcription through this binding site (−99 to −90 in the *FKBP12* promoter). This concept was further supported by the results that *Ets-1* mRNA was scarcely detected in LS-174T cells and the levels of *Sp3* mRNA were apparently reduced in SW948 cells (Fig. 4) and that the promoter activities for *FKBP12.6* and *FKBP12* were apparently reduced in SW948 cells and LS-174T cells, respectively (Fig. 5C).

Recently, the gene expression of *FKBP12.6* and *FKBP12* during chicken development was studied and the heart-selective expression of chicken *FKBP12.6* was demonstrated (Yazawa et al., 2003). In the present study, it was revealed that Sp3 transcription factor plays an essential role in the *FKBP12.6* transcription. As Sp transcription factors including Sp3 are recognized as being among the cardiac specific transcription factors (Suske, 1999; Flesch, 2001), Sp3 may be involved in the heart-specific expression of *FKBP12.6* during embryonic development. In contrast to *FKBP12.6* expression, the *FKBP12* mRNA was reported to be abundant throughout the embryo at an early stage of development and to subsequently undergo gradual down-regulation. Our results suggest the possible involvement of Ets-1 in *FKBP12* transcription. From several developmental studies, *Ets-1* mRNA is considered to be expressed in proliferating mesodermal- and neural-derivative cells (Remy and Baltzinger, 2000; Verger and Duterque-Coquillaud, 2002; Oikawa and Yamada, 2003). Therefore, it is reasonable to assume that Ets-1 may participate in the gene expression of *FKBP12* during embryogenesis.

Interestingly, the gene expression of some Ca^{2+} cycling proteins such as RyR2, sacro(endo)plasmic reticulum Ca^{2+}-ATPase type 3, and L-type Ca^{2+} channel α_{1c} subunit was reported to be regulated by Ets and/or Sp family transcription factors (Nishida et al., 1996; Hadri et al., 2002; Fan et al., 2002). In the present study, we added two additional examples (*FKBP12.6* and *FKBP12*) to the list. In this view, human *CD38* (ADP-ribosyl cyclase/cADPR hydrolase) gene has a potential binding site for Ets family at position −84 (Nata et al., 1997), suggesting that Ets family transcription factor may play an important role in *CD38* transcription.

Acknowledgments

We are grateful to Mr. Yuya Shichinohe for technical assistance and Mr. Brent Bell for critical reading of the manuscript. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan and is in partial fulfillment by T. N. of the degree of Doctor of Medical Science at Tohoku University. T. A. is the recipient of a fellowship from the Japan Society for Promotion of Science.

References

Akiyama, T., et al., 2001. Activation of *Reg* gene, a gene for insulin-producing β-cell regeneration: poly(ADP-ribose) polymerase binds *Reg* promoter and regulates the transcription by autopoly(ADP-ribosyl)ation. Proc. Natl. Acad. Sci. U. S. A. 98, 48–53.

Bucher, P., 1990. Weight matrix descriptions of four eukaryotic RNA polymerase II promoter elements derived from 502 unrelated promoter sequences. J. Mol. Biol. 212, 563–578.

DiLella, A.G., Hawkins, A., Craig, R.J., Schreiber, S.L., Griffin, C.A., 1992. Chromosomal band assignments of the genes encoding human FKBP12 and FKBP13. Biochem. Biophys. Res Commun. 189, 819–823.

Fan, Q.I., Vanderpool, K., Marsh, J.D., 2002. A 27 bp cis-acting sequence is essential for L-type calcium channel α_{1c} subunit expression in vascular smooth muscle cells. Biochim. Biophys. Acta 1577, 401–411.

Flesch, M., 2001. On the tail of cardiac specific transcription factors. Cardiovasc. Res. 50, 3–6.

Hadri, L., Ozog, A., Soncin, F., Lompré, A.-M., 2002. Basal transcription of the mouse sarco(endo) plasmic reticulum Ca^{2+}-ATPase type 3 gene in endothelial cells is controlled by Ets-1 and Sp1. J. Biol. Chem. 277, 36471–36478.

Heinemeyer, T., et al., 1999. Expanding the TRANSFAC database towards an expert system of regulatory molecular mechanisms. Nucleic Acids Res. 27, 318–322.

Lam, E., et al., 1995. A novel FK506 binding protein can mediate the immunosuppressive effects of FK506 and is associated with the cardiac ryanodine receptor. J. Biol. Chem. 270, 26511–26522.

Lee, H.C., 2002. Cyclic ADP-ribose and NAADP. A story of two calcium messengers. In: Lee, H.C. (Ed.), Cyclic ADP-ribose and NAADP. Structure, Metabolism and Functions. Kluwer, Dordrecht, pp. 1–21.

Maki, N., et al., 1990. Complementary DNA encoding the human T-cell FK506-binding protein, a peptidylprolyl cis-trans isomerase distinct from cyclophilin. Proc. Natl. Acad. Sci. U. S. A. 87, 5440–5443.

Marks, A.R., 1996. Cellular functions of immunophilins. Physiol. Rev. 76, 631–649.

Marks, A.R., 2001. Ryanodine receptors/calcium release channels in heart failure and sudden cardiac death. J. Mol. Cell. Cardiol. 33, 615–624.

Nakagawara, N., et al., 1995. Assignment of CD38, the gene encoding human leukocyte antigen CD38 (ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase), to chromosome 4p15. Cytogenet. Cell Genet. 69, 38–39.

Nata, K., et al., 1997. Human gene encoding CD38 (ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase): organization, nucleotide sequence and alternative splicing. Gene 186, 285–292.

Nata, K., et al., 2004. Molecular cloning, expression and chromosomal localization of a novel human *REG* family gene, *REG III*. Gene 340, 161–170.

Nishida, K., Otsu, K., Hori, M., Kuzuya, T., Tada, M., 1996. Cloning and characterization of 5′-upstream regulatory region of Ca^{2+}-release channel gene of cardiac sarcoplasmic reticulum. Eur. J. Biochem. 240, 408–415.

Noguchi, N., et al., 1997. Cyclic ADP-ribose binds to FK506-binding protein to release Ca^{2+} from islet microsomes. J. Biol. Chem. 272, 3133–3136.

Oikawa, T., Yamada, T., 2003. Molecular biology of Ets family transcription factors. Gene 303, 11–34.

Okamoto, H., Takasawa, S., 2002. Recent advances in the Okamoto model. The CD38-cyclic ADP-ribose signal system and the regenerating gene protein (Reg)-Reg receptor system in β-cells. Diabetes 51 (Suppl. 3), S462–S473.

Remy, P., Baltzinger, M., 2000. The Ets-transcription factor family in embryonic development: lessons from the amphibian and bird. Oncogene 19, 6417–6431.

Rossi, D., Sorrentino, V., 2002. Molecular genetics of ryanodine receptors Ca^{2+}-release channels. Cell Calcium 32, 307–319.

Sasamori, K., et al., 2004. Cyclic ADP-ribose, a putative Ca^{2+}-mobilizing second messenger, operates in submucosal gland acinar cells. Am. J. Physiol. 287, L69–L78.

Suske, G., 1999. The Sp-family of transcription factors. Gene 238, 291–300.

Takasawa, S., Nata, K., Yonekura, H., Okamoto, H., 1993. Cyclic ADP-ribose in insulin secretion from pancreatic β cells. Science 259, 370–373.

Takasawa, S., et al., 1998. Cyclic ADP-ribose and inositol 1,4,5-trisphosphate as alternate second messengers for intracellular Ca^{2+} mobilization in normal and diadetic β-cells. J. Biol. Chem. 273, 2497–2500.

Tang, W.X., Chen, Y.F., Zou, A.P., Campbell, W.B., Li, P.L., 2002. Role of FKBP12.6 in cADPR-induced activation of reconstituted ryanodine receptors from arterial smooth muscle. Am. J. Physiol. Heart Circ. Physiol. 282, H1304–H1310.

Wang, Y.X., et al., 2004. FFKBP12.6 and cADPR regulation of Ca^{2+} release in smooth muscle cells. Am. J. Physiol. Cell Physiol. 286, C538–C546.

Valdivia, H.H., 1998. Modulation of intracellular Ca^{2+} levels in the heart by sorcin and FKBP12, two accessory proteins of ryanodine receptors. Trends Phamacol. Sci. 19, 479–482.

Verger, A., Duterque-Coquillaud, M., 2002. When Ets transcription factors meet their partners. BioEssays 24, 362–370.

Yazawa, S., et al., 2003. Gene expression of FK506-binding protein 12.6 and 12 during chicken development. Comp. Biochem. Physiol., A Mol. Integr. Physiol. 136, 391–399.