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Mechanism of the Regulation of OCTN1 Transporter (*SLC22A4*) by Rheumatoid Arthritis-Associated Transcriptional Factor RUNX1 and Inflammatory Cytokines

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Running title: Regulation of OCTN1 transporter-gene expression

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Abbreviations:

G3PDH, glyceraldehyde-3-phosphate dehydrogenase; ET, ergothioneine; FBS, fetal bovine serum; IL-1ß, interleukin-1ß; OCTN, organic cation/carnitine transporter; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RA, rheumatoid arthritis; TNFα, tumor necrosis factor-α

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Abstract

Recently, it was reported that organic cation/carnitine transporter 1 (OCTN1, SLC22A4) is associated with chronic inflammatory diseases, such as rheumatoid arthritis (RA) and Crohn's disease. OCTN1 in human is expressed in synovial tissues of individuals with rheumatoid arthritis. Furthermore octn1 in mice is expressed in inflamed joints with collagen-induced arthritis, a model of human arthritis, but not in the joints of normal mice. OCTN1 should be involved in the inflammatory disease and in the present study, the regulatory mechanism of OCTN1 expression was characterized using human fibroblast-like synoviocyte cell line MH7A, derived from RA patients. Luciferase-reporter gene assay and gel shift assay demonstrated that RUNX1, which is an essential hematopoietic transcription factor associated with acute myeloid leukemia and is related to RA and Sp1, is involved in the regulation of OCTN1 promoter activity. Inflammatory cytokines such as IL-1ß and TNFα increased the expression of OCTN1 mRNA. Furthermore, over-expression of NFκB activated promoter activity of OCTN1. These results clearly demonstrate that expression of OCTN1 is regulated by various factors, including RUNX1, inflammatory cytokines and NFκB, all of which are also related to the pathogenesis of RA. Further studies on the physiological substrate(s) of OCTN1 should be done to clarify the roles of OCTN1 in these diseases.

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Introduction

The organic cation/carnitine transporter, OCTN1 (*SLC22A4*) shows a relatively broad tissue distribution and was firstly discovered as a pH-dependent organic cation transporter (Tamai et al., 1997; Yabuuchi et al., 1999). The OCTN family consists of three members, that is, OCTN1 and OCTN2 (SLC22A5) in mice, rats, and humans, and octn3 in mice. Among them, OCTN2 and octn3 predominantly exhibit carnitine transport activity, while OCTN1 has relatively low carnitine transport activity, suggesting a distinct physiological role of OCTN1 from those of OCTN2 and octn3 (Tamai et al., 1998; Tamai et al., 2000; Nezu et al., 1999). OCTN1 is located on the apical membrane of renal proximal tubular epithelial cells and has been suggested to be involved in the tubular secretion of cationic compounds (Tamai et al., 1998; Tamai et al 2003), because OCTN1-mediated transport of organic cations shows similarity to the transport mediated by a proton/organic cation exchanger transport system observed in the renal apical membranes (Tamai et al., 2000; Tamai et al 2003). Furthermore, since OCTN1 is expressed in bone marrow and fetal liver, but not in the adult liver, it was suggested that OCTN1 might be involved in the differentiation, growth or function of blood cells (Tamai et al., 1997; Kobayashi et al., 2004). Recently, it was clearly demonstrated that OCTN1 mediates the transport of ergothioneine that is one of antioxidants, suggesting that the physiological role of OCTN1 is related to the redox reaction (Grundermann et al., 2005).

The chromosomal region 5q31 that includes OCTN1 gene is of particular interest, because it contains many genes involved in immune and inflammatory systems and also because it is suggested to be a susceptible locus for several inflammatory or autoimmune diseases, such as rheumatoid arthritis (RA) and Crohn's disease that have been reported recently based on the frequency of specific single nucleotide polymorphisms (SNPs) in the

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intron 1 of the OCTN1 gene in Japanese, Greek and English patients with RA and Crohn's disease (Tokuhiro et al., 2003; Gazouli et al., 2005; Russell et al., 2006), and the frequency of SNPs in the exons of the OCTN1 gene in patients with Crohn's disease (Peltekova et al., 2004; Yamazaki et al., 2004). SNPs in intron 1 of OCTN1 affect the expression of OCTN1 by lowering the affinity for the transcription factor RUNX1 (AML1) (Tokuhiro et al., 2003) that is expressed mainly in hematopoietic cells. In addition, the RUNX1 gene was independently associated with three autoimmune disorders: systemic lupus erythematosus (SLE), psoriasis, and RA (Prokunina et al., 2002; Helms et al., 2003; Tokuhiro et al., 2003). It was also reported that OCTN1 was expressed in the inflamed joints of mice with collagen-induced arthritis, a model of human arthritis, but not in the joints of normal mice (Tokuhiro et al., 2003). RA is a chronic inflammatory disease in which the synovial environment is characterized by intense immunological activity (Dendorfer et al., 1994). In particular, the macrophage-like synoviocytes and fibroblast-like synoviocytes of the hyperplastic lining layer exhibit an activated phenotype in RA (Buchan et al., 1988; Arend et al., 1995; Panayi et al., 1992). These cells are a major source of several inflammatory cytokines, such IL-1, tumor necrosis factor-α (TNFα), and IL-6 proteins, which play crucial roles in the pathophysiology of RA (Dendorfer et al., 1994; Firestein et al., 1990). Both IL-1ß and TNFα stimulate fibroblast-like synoviocytes to secrete mediators, such as matrix metalloproteinases (MMPs) (Pillinger et al., 2003), and vascular endothelial growth factor (Jackson et al., 1997), that regulate inflammation and connective tissue degradation, respectively. Cytokines also stimulate the NFκB signaling pathway (Barchowsky et al., 2000), which in turn regulates the expression of a wide array of proinflammatory molecules. In addition, monoclonal anti-TNFα antibody, IL-1ß receptor antagonist, and NFκB decoy oligonucleotides have been successfully used to block the activity

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of TNFα , IL-1ß and NFκB both in experimental models and human trials (Tomita et al., 2000). Therefore, elucidating the regulatory mechanism of OCTN1 expression is useful for future treatment of RA and Crohn's disease.

Accordingly, in the present study, the regulatory mechanism of OCTN1 expression was characterized, focusing especially on promoter analysis, using human fibroblast-like synoviocyte cell line MH7A derived from RA patients as a model (Miyazawa et al., 1998). We used several methods to identify the basal transcription factor of OCTN1 and factors, including inflammatory cytokines such as IL-1ß and NFκB, that modulate the expression of OCTN1, since they are known to be closely related to RA (Hiramitsu et al., 2006).

Methods

Materials [γ-^{32}P]Adenosine triphosphate (3,000 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) and Wako Pure Chemicals Industries Ltd. (Osaka, Japan). Antibodies for Sp-1, RUNX1 and NFκB (NFκB p50) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Cell culture and cytokine treatment The cell line (MH7A) was purchased from RIKEN BioResource Center (Tsukuba, Japan) and cultured in 50% RPMI-1640 medium (Sigma-Aldrich), containing 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA) in a humidified incubator at 37°C under 5% CO_2. At 24 or 48 h before harvesting of the cells, the culture medium was replaced with fresh RPMI-1640 and supplemented with phosphate-buffered saline (PBS), with or without IL-1ß or TNFα as required.

Identification of the OCTN1 transcription start site Determination of the transcription start site of the OCTN1 gene was performed by the 5'-rapid amplification of cDNA ends (5'-RACE) method in accordance with the manufacturer's protocol (BD Bioscience Clontech, Palo Alto, CA). The first-round polymerase chain reaction (PCR) was performed using the Marathon cDNA adaptor-specific primer AP1 and OCTN1 gene-specific primer GSP1 (see Table 1; synthesized by Hokkaido System Science Co., Ltd. (Sapporo, Japan)). The second-round PCR was performed using the Marathon cDNA adaptor-specific primer AP2 and OCTN1 gene-specific primer GSP2 (see Table 1; synthesized by Hokkaido System Science Co., Ltd.). The reactions were performed using the following conditions: 95 ℃ for 5 min and then

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35 or 25 cycles of 95 °C for 20 sec, 60 °C for 20 sec, and 72 °C for 5 min. A PCR product of about 0.20 kb was obtained, subcloned into pGEM-T easy vector (Promega, Madison, WI), and sequenced.

Cloning of human OCTN1 promoter The 5'-region of the OCTN1 gene was PCR-amplified using human genomic DNA (BD Biosciences, Palo Alto, CA) as a template, using upstream primer p-2544, downstream primer p+92 (see Table 1; both synthesized by Hokkaido System Science), and Ex Taq DNA polymerase (Takara Shuzo Co. Ltd., Shiga, Japan), based on the reported OCTN1 gene sequence (accession number AB007448). All amplified fragments were sequenced and confirmed not to have any mutation. The finally obtained nuclear base sequence obtained for the promoter region of OCTN1 was submitted to GeneBank (accession number AB252052). Since the upstream and downstream primers were designed to include an internal *Hind*III restriction site and an internal *Xho*I site, respectively, the resulting PCR products were digested with *Hind*III and *Xho*I and ligated into the luciferase reporter gene vector pGL3-Basic (Promega).

Nuclear extract preparation and gel mobility shift assay Nuclear extracts were prepared from MH7A cells based on the method of Schreiber et al. (Schreiber et al., 1989). Briefly, MH7A cells (1 x 10^6 cell) were washed with 10 mL PBS and pelleted by centrifugation at 1,500 x g for 5 min. The pellet is resuspended in 1 mL PBS, transferred into a 1.5 mL tube and centrifuged again by 10,000 x g for 15 sec. The resultant cell pellet was resuspended in 400 µL ice-cold buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1mM DTT, 0.5 mM PMSF, 2 µg/mL aprotinin, 2 µg/mL leupeptine) by gentle pipetting. The cells

were allowed to swell on ice for 15 min, after which 25 μL of a 10 % solution of Nonidet P-40 was added, and was vigorously mixed by vortex mixer for 10 sec. The resultant cell homogenate was centrifuged at 10,000 x g for 30 sec. The obtained nuclear pellet was resupended in 50 μL aliquot of ice-cold buffer B (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2 μg/mL aprotinin, 2 μg/mL leupeptine) and the tube was vigorously rocked at 4 ℃ for 15 min on a shaking platform. The obtained cell lysate was centrifuged for 5 min at 10,000 x g for 10 min at 4 ℃ and the resultant supernatant was used as nuclear extract.

Sense- and antisense-oligonucleotides containing putative Sp1, RUNX1 and NFκB binding sites were synthesized (Hokkaido System Science Co., Ltd.). Their sequences are shown in Table 1 and mapped on the OCTN1 promoter sequence in Fig. 1. Gel mobility shift assays were carried out as described previously (Maeda et al., 2005). Briefly, double-stranded synthetic oligonucleotide was used as a probe. T4 polynucleotide kinase (Toyobo Co., Ltd., Osaka, Japan) and [γ-^{32}P]adenosine triphosphate (3,000 Ci/mmol) were used to label the 5' end of the sense strand oligonucleotide. Binding reactions were initiated by incubating nuclear extracts from MH7A cells (10-15 μg of protein) with 750 ng of poly(dI-dC) (GE Healthcare Bio-Science) and 100 μg of bovine serum albumin (Sigma-Aldrich Co.) for 20 min at room temperature. Double-stranded probe was then added, and incubation was continued for another 30 min at room temperature. The specific antibodies for Sp1, RUNX1 and NFκB (NFκB p50) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Transfections and luciferase assay The deletion-mutant luciferase-reporter plasmids were constructed by PCR using specific primers. Since the upstream and downstream primers were

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designed to include an internal *Hind*III restriction site and an internal *Xho*I site, respectively, the resulting PCR products were digested with *Hind*III and *Xho*I and ligated into the luciferase reporter gene vector pGL3-Basic (Promega). Reporter gene constructs were transfected using LipofectAmine 2000 (Invitrogen) according to the manufacturer's protocol. Briefly, cells were plated in 24-well plates at approximately 0.5 x 10^5 cells per well for 24 h before transfection. Before addition of DNA/liposome complexes, the cells were rinsed with serum-free medium. For each transfection, reporter constructs (0.8 μg) were cotransfected with 0.08 μg of pRL-TK vector (Promega) that includes TK promoter and Renilla luciferase as an internal control in 0.5 ml of serum-free medium by incubating at 37 °C for 6 h. After 6 h, the culture medium was changed to medium containing 10% FBS, and the cells were incubated for 48 h at 37 °C, rinsed twice with PBS, and harvested using passive lysis buffer (Promega). For luciferase assays, cell extracts were mixed with luciferase assay reagent (Promega) for detection in a luminometer (Berthold GmbH, Germany). Relative luciferase activities were shown as the ratio of firefly/*Renilla* luciferase activities and the results were presented as the mean ± S.E.M. of 4 to 7 independent transfection experiments. Sp1 expression vector was a kind gift from Dr. Alexandrea Stewart (University of Pittsburgh) which was reported previously (Kadonaga et al., 1987). RUNX1 expression vector was prepared with LA Taq DNA polymerase, based on the reported RUNX1 gene sequence (Lutterbach and Hiebert, 2000), and ligated into expression vector pcDNA3.1 (Invitrogen). NFκB expression vectors were kind gifts from Dr. Seiichi Tanuma (Tokyo University of Science).

Site-directed mutagenesis Site-directed mutagenesis of the binding sites of Sp1 (GC-box), RUNX1 and NFκB were performed using the QuikChange site-directed mutagenesis kit

(Stratagene, La Jolla, CA) and the oligonucleotides shown in Table 1. Briefly, an OCTN1-derived –180/+92 construct or –2544/+89 construct containing a mutation was generated by PCR using two complementary oligonucleotides mutated in the Sp1 binding site or RUNX1 binding site or NFκB binding site (sequence mut Sp1: 5'-GGTCCTTGGGGGCGGGCGGGCG-3', mut RUNX1: 5'-CCGGGGATGGGGGTGTTTTCCCAAGTGT-3', and mut NFκB: 5'-CAACTTTGAAAATAAATTCCCCCAGTGGGC-3' in Table 1) and Pfu DNA polymerase (Stratagene). The product was digested with *Dpn*I to remove the parental DNA template and to identify the DNA containing the mutation. The obtained mutated plasmids were termed mut Sp1, mut RUNX1, and mut NFκB for mutants in binding sites of Sp1, RUNX1, and NFκB, respectively, and all mutants were sequenced.

Reverse transcription-polymerase chain reaction Total RNA was prepared from MH7A cells using ISOGEN (Wako Pure Chemicals Industries Ltd.). The total RNA content was determined by measuring the absorbance at 260 nm. mRNA level was analyzed using semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). Single-strand cDNAs were constructed using an oligo(dT) primer (Invitrogen) and Improm-II™ reverse transcriptase (Promega). These cDNAs provided templates for PCRs using specific primers (Table 1) at a denaturation temperature of 94 °C for 30 sec, an annealing temperature of 58-62 °C for 30 – 60 sec, and an elongation temperature of 72 °C for 30 sec in the presence of deoxynucleotides (dNTPs) and Ex Taq polymerase (Takara Shuzo Co. Ltd.). Annealing time and temperature were changed as required, depending on the genes. The PCR cycle numbers were titrated for each primer pair to confirm that amplification was performed within a linear

range. PCR products were analyzed by 2 % agarose gel (w/v) electrophoresis and the gels were stained with ethidium bromide for visualization. mRNA levels were quantified by densitometry using light capture (Atto Co., Tokyo, Japan). PCR amplification data were normalized with respect to glyceraldehyde-3-phosphate dehydrogenase (G3PDH). The sets of primers specific for the nucleotide sequences of the transporters are shown in Table 1. The quantitation of each gene was repeated at least three times using RNA sources isolated from independently cultured cells.

Statistical analysis The statistical significance of differences was determined by Student's t test or one-way analysis of variance with Dunnett's post hoc test, with $P < 0.05$ as criterion. Data are shown as the mean ± S.E.M.

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Results

Localization of the transcriptional initiation site and promoter region of the OCTN1 gene

To localize the promoter region of the OCTN1 gene, the transcription start site was identified by 5'-RACE methods. By using a downstream oligonucleotide (Table 1) that corresponded to nucleotides +142 to +160 and +271 to +292 of the OCTN1 cDNA sequence as published by Tamai et al. (1997) (accession number AB007448), a PCR product was amplified and sequenced (see supplemental data). The obtained sequences suggested that the transcription initiation site was 20 bp upstream from the start of the published OCTN1 cDNA sequences (accession number AB252052).

Analysis of the 5'-flanking region of the human OCTN1 gene

Based on the above finding, the upstream genomic region of the OCTN1 was identified using the Human Genome Resource of NCBI and NIH. A 2544 bp fragment of the 5'-flanking sequence of the OCTN1 gene was obtained by PCR amplification (Fig. 1). PCR was performed with primers p-2544 and p+92 (Table 1) and human genomic DNA as a template. Figure 1 shows the sequence of the 5'-flanking 2544 nt relative to the transcription start site up to the translation start site. Potential transcription factor recognition sites were identified by using the program TRANSFAC 4.0 (BIOBASE GmbH, Germany) and revealed that this region lacks canonical TATA and CAAT boxes, but contains several putative transcription factor binding sites such as the GC-box (Sp1 binding site) at –59 to –50, RUNX1 at –65 to –60, -391 to – 386, -1119 to –1114, -1383 to –1377, -1563 to –1558, -1958 to –1953, -1996 to –1991, and –2457 to –2452, GATA at –2167 to –2162, and NFκB at –2243 to –2234, as shown in Fig. 1.

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Constitutive activity of the OCTN1 enhancer-promoter region in MH7A cells

To functionally characterize the OCTN1 promoter activity, a series of promoter deletion mutants were constructed in a reporter gene vector pGL3-basic that expresses firefly luciferase. Promoter activities of all deleted constructs between p-2544 Luc and p-61 Luc were compared with that of pGL3-basic (Fig. 2). Transfection of the p-2544 Luc plasmid into MH7A cells, which contained the promoter region from –2544 to +92 nts, stimulated luciferase activity about 35-fold compared with the pGL3-basic (no promoter insert) (Fig. 2). Other constructs, p-2142, p-1682, and p-1263 Luc, stimulated luciferase activity 40-fold compared with the pGL3-basic. The constructs p-699 Luc, p-189 Luc, and p-150 Luc stimulated luciferase activity 25-, 15-, and 20-fold compared with pGL3 basic, respectively. A shorter construct that stimulated luciferase activity, p-150 Luc, which includes the RUNX1 binding site (Fig. 1), was sufficient to confer residual promoter activity in MH7A cells. The shortest construct, p-61 Luc, stimulated luciferase activity 3-fold. Accordingly, first of all, we focused on p-189 Luc, since it included a putative Sp1 binding site (GC-box) and a RUNX1 binding site. Although p-189 Luc showed lower promoter activity than p-150 Luc, it did maintain promoter activity.

Identification of putative Sp1 and RUNX1 binding sites in OCTN1 promoter region

Since the region from –189 to +92 nt contains the consensus binding sites for Sp1 and RUNX1, those transcription factors may bind to those regions and regulate the expression of OCTN1. To identify the composition of nuclear proteins that interact with this cis-acting motif, we used gel mobility shift assays. Gel mobility shift assays were conducted using nuclear extracts derived from MH7A cells and a putative Sp1 and RUNX1 binding motif

oligonucleotide within the human OCTN1 promoter. The OCTN1 promoter region from the –168 to –159 nt included one putative Sp1 binding site and the –80 to –60 nt included one putative RUNX1 binding site (Fig. 1). As shown in Fig. 3, three protein complex bands, L, M, and H, were observed (Fig. 3, lane 1). The protein-DNA complexes shown in Fig. 3 were further analyzed using antibody directed to Sp1 nuclear protein (Fig. 3, lane 3) and excess oligonucleotides as self competitor (Fig. 3, lane 2). The shifted complexes banding to the OCTN1 oligonucleotides were all subject to competition by excess oligonucleotides (Fig. 3, lane 2). Shifted complex band H was super-shifted by Sp1-specific antibody (Fig. 3, lane 3). The oligonucleotide with a mutated Sp1 binding site did not show the bands corresponding to H, M, and L complexes (Fig. 3, lanes 4 and 5). Furthermore, as shown in Fig. 3, one protein complex band was observed using RUNX1 probe (Fig. 3, lane 6). The shifted complex was competed by an excess amount of self oligonucleotide (Fig. 3, lane 7) and super-shifted by RUNX1-specific antibody (Fig. 3, lane 8).

Effect of mutation of Sp1 binding sites on OCTN1 expression.

To assess the importance of Sp1 binding to OCTN1 promoter regions for basal promoter function, mutations of the Sp1 binding sites were introduced into p-189 Luc by site-directed mutagenesis. The resulting reporter gene plasmids p-mut Sp1 Luc contained mutations of nucleotides at –162 and –161 (GG to TT) (Table 1). Introduction of these mutations abrogated Sp1 binding in mobility shift assays (Fig. 3 lanes 4, and 5). GC-box mutation did not change the luciferase activity compared with wild type, while the activity of mutant p-mut Sp1 Luc was no longer stimulated by overexpression of Sp1 (Fig. 4). These results demonstrate that Sp1 is associated with OCTN1 transcriptional regulation, but is not

involved in basal transcriptional regulation of OCTN1.

Stimulation of the OCTN1 promoter in MH7A cells by induced expression of Sp1 and RUNX1.

To investigate the effect of exogenously expressed Sp1 and RUNX1 on OCTN1 promoter function in MH7A cells, an expression plasmids coding for Sp1 and RUNX1 were introduced into MH7A cells together with several OCTN1 promoter constructs. As shown by closed columns in Fig. 4, cotransfection of the Sp1 plasmid enhanced OCTN1 promoter-driven luciferase activity 1.8-fold (p-189 Luc) compared with cotransfection of empty vector lacking Sp1. However, Sp1 did not activate p-150 Luc or p-mut Sp1 that lacks the GC-box or has a mutated Sp1 binding site, respectively (Fig. 4). As shown in Fig. 5, RUNX1 enhanced the promoter activity of p-150 Luc about 2-fold, whereas RUNX1 did not activate p-61Luc that does not include the putative RUNX1 binding site. Furthermore, a mutation of the RUNX binding site did not induce promoter activity. These results demonstrated that RUNX1 is associated with OCTN1 transcriptional regulation. However, proximal RUNX1 was not associated with basal promoter activity of OCTN1, because the mutation of RUNX1 in the OCTN1 promoter did not affect the promoter activity (Fig. 5). Accordingly, transcription factors other than Sp1 or RUNX1 may be important for basal transcriptional regulation of the OCTN1 gene. Moreover, the effect of the upstream RUNX binding site on OCTN1 promoter activity was studied. It was shown that RUNX1 enhanced the promoter activity of p-699 Luc that includes two putative RUNX1 binding sites by about 2.5-fold. In addition, the promoter activities of p-189 Luc, p-1263 Luc, and p-1682 Luc that include one, four, and six putative RUNX1 binding sites, respectively, tended to increase activity, while the differences were not

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statistically significant (Fig. 5B).

Effects of IL-1ß and TNFα on the expression of OCTN1 and OCTN2.

IL-1ß and TNFα are inflammatory cytokines and are associated with RA. Therefore, we examined the effects of IL-1ß and TNFα on transcriptional activation of OCTN1 and OCTN2. As shown in Fig. 6, expression of OCTN1 mRNA was activated 2- to 3-fold relative to the control by treatment with 1 ng/ml IL-1ß or 10 ng/ml TNFα for 24 and 48 h, respectively (n=3, normalized to G3PDH, $p<0.05$). However, expression of OCTN2 mRNA was affected by neither IL-1ß nor TNFα (Fig. 6). Accordingly, OCTN1 and OCTN2 are presumed to be regulated by distinct mechanisms. The mechanism of up-regulation of OCTN1 by IL-1ß was further analyzed using promoter constructs. Only p-2544 Luc that included the NFκB binding site construct was activated by IL-1ß (Fig. 7).

Effects of NFκB on the expression of OCTN1

NFκB is a transcription factor and plays an important role in the regulation of immune response (Barchoesky et al 2000). Therefore, we examined the effect of overexpression of NFκB on OCTN1 promoter activity. NFκB consists of two proteins, p50 and p65. When expression plasmids of p50 and p65 were transfected into MH7A cells, p-2544 Luc luciferase was activated (Fig. 8). Furthermore, p-2544 Luc promoter activity induced by IL-1ß was decreased by mutations of the NFκB binding site (Fig. 9A). In addition, when expression plasmids of p50 and p65 were transfected in MH7A cells, promoter activity of p-2544 Luc with mutations of the NFκB binding site was not activated (Fig. 9B). We also examined whether or not the NFκB binding site was functional by gel mobility shift assay. As

shown in Fig. 3, two protein complex bands (H and L) were observed (Fig. 3, lane 9). The shifted complexes were competed by excess self oligonucleotide (Fig. 3, lane 10) and super-shifted by NFκB specific antibodies (NFκB p50) (Fig. 3, lane 11). The results shown in Fig. 3 demonstrated that the slower-migrating protein complex (H) and fast-migrating protein (L) might contain p50/p65 and p50/p50, respectively, as shown in previous study (Huang et al., 2006). These results demonstrated that IL-1ß and TNFα are associated with OCTN1 transcriptional regulation via the NFκB signaling cascade.

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Discussion

In the present study, the regulatory mechanism of an expression of OCTN1, which might be associated with chronic inflammatory diseases such as RA (Tokuhiro et al., 2003) and Crohn's disease (Peltekova et al., 2004), was characterized by focusing on promoter activity using the human fibroblast-like synoviocyte cell line MH7A derived from RA patients as a model (Miyazawa et al., 1998).

First of all, we determined the transcription start site of OCTN1. The promoter region of OCTN1 obtained in the present study included several consensus recognition sites for both ubiquitously expressed transcription factors, such as Sp1 (Kadonaga et al., 1987), and transcriptional factors related to RA, such as RUNX1 and NFκB (Tokuhiro et al., 2003; Miyazawa et al., 1998). The OCTN1 promoter region had one consensus sequence for Sp1 close to the transcription site (Fig. 1), and an examination of promoter activity in MH7A cells supported the involvement of Sp1 in the expression of OCTN1. Binding of Sp1 to this region of the OCTN1 promoter was confirmed by both gel mobility shift assays and supershift analysis using an anti-Sp1 antibody (Fig. 3). The results shown in Fig. 3 demonstrated that the slower-migrating protein complex (H) contains Sp1, while the two faster migrating (M and L) complexes may contain Sp3, as shown in previous study by McClure et al. (1999). Co-expression of exogenous Sp1 stimulated OCTN1 promoter activity up to 2-fold in MH7A cells (Fig. 4). Targeted deletion of the Sp1 binding site from –165 nt to –160 nt (GGGCGG) abolished inducibility of the OCTN1 promoter by Sp1 (Fig. 4). However, mutation of the GC-box in the OCTN1 promoter did not lead to complete loss of promoter activity. Accordingly, it was suggested that Sp1 is involved in the regulation of OCTN1 expression, but is not associated with basal transcriptional regulation of OCTN1. There may be other

transcription factors for basal transcriptional regulation of OCTN1. Some researchers have suggested that Sp1 is involved in cell differentiation, as it is expressed at high levels in hematopoietic stem cells, fetal cells, and spermatids (Saffer et al., 1991; Suzuki et al., 1998). Accordingly, it is possible that Sp1 plays a role in tissue specific expression of OCTN1 rather than basal transcriptional regulation.

It is known that the expression of serum amyloid A (SAA) is induced by IL-1ß after activation by Sp1 and SAF (Ray et al., 1999). Moreover, the DNA binding affinity and amount of Sp1 were increased by IL-1ß (Ray et al., 1999). However, in the present study, luciferase activity of p-189 Luc that includes the Sp1 binding site was not induced by IL-1ß (Fig. 7). Therefore, it appears that the expression of OCTN1 was not induced via Sp1 after treatment with IL-1ß.

Previously, it was demonstrated that SNPs in intron 1 of OCTN1 affected the expression of OCTN1 by lowering the affinity for RUNX1 (Tokuhiro et al., 2003), which is an essential hematopoietic transcription factor (Roumier et al., 2003). So, we investigated the role of RUNX1 in the OCTN1 promoter region. The present results suggested that RUNX1 binds to the putative proximal RUNX1 binding site on the OCTN1 promoter and activates the promoter (Figs. 3 and 5). However, RUNX1 was not associated with basal promoter activity of OCTN1, because mutation of RUNX1 in the OCTN1 promoter region led to a partial loss of the promoter activity (Fig. 5). Previous findings suggested that the expression of OCTN1 is negatively regulated by RUNX1 via intron 1 (Tokuhiro et al., 2003). However, the present results indicate that RUNX1 activated OCTN1 promoter via the proximal RUNX1 binding site (Fig. 5). RUNX1 activated luciferase activity of p-150 Luc but not p-61 Luc (Fig. 5B). In addition, we confirmed that the region from the –62 to –67 nt is a functional site for RUNX1

binding considering from the results of gel mobility shift assay and site-directed mutagenesis (Figs. 3 and 5). Therefore, OCTN1 expression is likely to be regulated by RUNX1 via the –62 to –67 nt region. At present, the mechanism that RUNX1 is associated with OCTN1 expression via the RUNX1 binding sites at the promoter region and intron 1 of the OCTN1 gene is not clear. It is possible that different co-factors are associated with RUNX1 for the regulation of OCTN1 via those binding sites at the promoter and intron 1. Accordingly, further studies on the role of RUNX1 in regulating of OCTN1 expression are needed.

Human OCTN1 is strongly expressed in hematopoietic tissues, such as bone marrow and fetal liver as well as kidney, but not in adult liver (Tamai et al., 1997). In addition, when the expression of OCTN1 was suppressed, the proliferation and differentiation of K562 cells, a human leukemia cell line, were inhibited (unpublished observation). Moreover, RUNX1 knockout embryos develop normal blood islands and show progress through the yolk sac phase of hematopoiesis, but die between E11 and E12.5 (Okuda et al., 1996). Before death, the liver rudiment contains primitive nucleated erythrocytes, but lacks all definitive erythroid, myeloid, and megakaryocytic cells, indicating a complete block of the development of the definitive hematopoietic program in the absence of RUNX1. Therefore, it is possible that the expression of OCTN1 is regulated by RUNX1 in fetal liver as well as rheumatoid tissue and OCTN1 is associated with proliferation and differentiation of hematopoietic stem cells. Moreover, since p-150 Luc that includes the proximal RUNX1 binding site was not activated by IL-1ß (Fig. 7), the expression of OCTN1 regulated by RUNX1 may be independent of the cascade of inflammatory cytokines. Therefore, it is possible that the role of RUNX1 is the tissue-specific regulation of certain genes.

In the present study, we also examined the effect of inflammatory cytokines on the

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expression of OCTN1, since these cytokines are related to RA. There is a report that the level of OCTN1 mRNA was increased two fold by TNFα, but no such response was observed with OCTN2 mRNA in fibroblast-like synoviocytes from individuals with RA (Tokuhiro et al., 2003). The selective increase of OCTN1 by TNFα is consistent with our results using MH7A cells (Fig. 6). Up-regulation of OCTN1 promoter activity by IL-1ß was shown to involve positions the – 2244 to –2253 nt, by gel mobility shift assay, deletion analysis, and over-expression of NFκB (Figs. 3, 7, and 8). IL-1ß/TNFα stimulated the translocation of p65 and p50 from the cytosol to the nucleus of human RA synovial fibroblasts (Gomez et al., 2005). IL-1ß and TNFα play important roles in sideration and progress of RA, such as proliferation of synoviocyte, production of protease, and induction of adhesion proteins. In fact, RA is a chronic inflammatory disease characterized by the proliferation of the synovial membrane into a highly vascularized tissue known as pannus, and inflammation. The pannus consists of several distinct cell types, which include resident fibroblast-like synoviocytes and infiltrating mononuclear cells capable of producing inflammatory cytokines such as IL-1, TNFα and IL-6 (Feldmann et al., 1996). The present study showed that OCTN1 expression was regulated by inflammatory cytokines related to RA. Therefore, it is possible that OCTN1 is associated with proliferation of the sinovial membrane and fibroblast-like synoviocyte cells. However, there is no direct evidence of a contribution of OCTN1 to sideration and progression of RA at present.

The present study demonstrated that OCTN1 expression is regulated by RUNX1 and inflammatory cytokines, such as IL-1ß and NFκB. Although RUNX1 and these cytokines are known to be associated with RA, the mechanisms through which they are involved remain unclear. Their physiological roles and the mechanism through which OCTN1 acts in RA and Crohn's disease might be explained by considering the roles of ergothionein, that was recently

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found to be a substrate of OCTN1 (Grudermann et al., 2005). Colognato et al. reported that ergothioneine that is a good substrate of OCTN1 should inhibit apoptosis by the induced oxidant stress in PC12 cells (Colognate et al., 2005). In addition, we investigated the expression of OCTN1 and transport of ergothioneine by OCTN1 in PC12 cells. As a result, the transport of ergothioneine was mainly accounted for by OCTN1 in PC12 cells (unpublished observation). Moreover, it was reported that reactive oxygen species play a significant role in the pathogenesis of RA (Ozturk et al., 1999). Therefore, it is possible that the expression of OCTN1 is induced in inflammation to suppress the oxygen species by transporting anti-oxidants, such as ergothioneine. Further analysis will also be required to specify the substrate(s) of OCTN1 in order to establish its physiological role in inflammation and in the pathogenesis of RA other than ergothioneine.

In conclusion, we showed that OCTN1 expression is regulated by several transcription factors, i.e., Sp1, RUNX1, and NFκB, and the regulation by these factors might explain the involvement of OCTN1 in autoimmune diseases, such as RA and Crohn's disease.

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We thank Dr. Alexandrea Stewart and Dr. Seiichi Tamuma for their generous gifts of the expression vectors of Sp1 and NFκB, respectively.

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Footnotes

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Figure Legends

Fig. 1 **Nucleotide sequence and structure of the 5'-flanking region of the OCTN1 gene**

The nucleotide sequence of the 5'-flanking region of the human OCTN1 gene from –2544 to +92 nt is shown. Nucleotide numbers are relative to the transcription start site. Consensus binding sites for putative regulatory elements are underlined, and the respective transcription factors are given above the sequence. Flags denote the size of the deletional constructs used for functional promoter studies.

Fig. 2 **Deletion analysis of the 5'-flanking region of the human OCTN1 gene in MH7A cells**

Deletional fragments in the 5'-upstream region of the human OCTN1 gene are illustrated at the left. Each deleted promoter fragment was ligated into the luciferase reporter plasmid pGL3-Basic. Numbers indicate distance in base pairs from the transcription start site. The MH7A cells were transiently transfected with eight chimeric promoter constructs ranging from nt -2544 to +92 relative the transcription start site. Transfection efficiency was normalized by cotransfection of pRL-TK, and the promoter activity was measured as relative light units of firefly luciferase per unit of *Renilla* luciferase. Promoter activity is shown as the factor of induction of luciferase relative to background activity measured in cells transfected with pGL3-Basic alone (Basic). Results are expressed as the mean ± S.E.M. of 4 to 7 independent transfection experiments. Horizontally striped boxes, vertically striped boxes, and black boxes show putative GC-box, NFκB, and RUNX1 binding sites, respectively.

Fig. 3 **Gel mobility shift competition assay and supershift analysis of Sp1, RUNX1, and NFκB-bound protein complexes**

Gel mobility shift assays were carried out with nuclear extract from MH7A cells. Lanes 1, 4, 6, and 9, radio-labeled probe alone; lane 2, 5, 7, and 10, probe with 5 pmol of unlabeled oligonucleotide (self competition); lanes 3, 8, and 11, probe with Sp1, RUNX1, or NFκB specific antibodies. The two or three shifted protein complexes are labeled from largest to smallest as H and L or H, M, and L for NFκB and Sp1, respectively. The arrows and asterisk indicate the super-shifted complexes and RUNX1 complex, respectively.

Fig. 4 **Effect of Sp1 on OCTN1 expression in MH7A cells**

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(A) Schematic sequences of the –189 and –150 regions are shown. (B) MH7A cells were co-transfected with the p–189 Luc, p–150 Luc, or p-mutSp1 Luc reporter gene construct plus either Sp1 expression plasmid (closed columns) or empty vector as a control (open columns). Luciferase activity is shown as the ratio of firefly/*Renilla* luciferase activities and data represent the mean ± S.E.M. of 4 to 7 independent transfection experiments. An asterisk indicates a statistically significant difference by Student's t test ($p < 0.05$).

Fig. 5 **Effect of RUNX1 on OCTN1 promoter activity in MH7A cells**

(A) Schematic sequences of the –150 and –61 regions are shown. (B) MH7A cells were co-transfected with the p-1682 Luc, p-1263 Luc, p-699 Luc, p-189 Luc, p–150 Luc, p–61 Luc, or p-mutRUNX1 Luc reporter gene constructs plus either RUNX1 expression plasmid (closed columns) or empty vector as a control (open columns). Luciferase activity is shown as the ratio of firefly/*Renilla* luciferase activities and data represent the mean ± S.E.M. of 4 to 7 independent transfection experiments. An asterisk indicates a statistically significant difference by Student's t test ($p < 0.05$).

Fig. 6 **Effects of IL-1ß and TNFα on OCTN1 mRNA expression in MH7A cells**

MH7A cells were cultured in either the absence (open columns) or presence of IL-1ß (1 ng/mL) (closed column) or TNFα (10 ng/mL) (horizontally striped column) for 24 h (A) or 48 h (B) before harvest, respectively. mRNA levels of OCTN1 and OCTN2 were analyzed by semi-quantitative RT-PCR. mRNA expression is shown as the ratio of OCTNs mRNA/G3PDH mRNA and data represent the mean ± S.E.M. of 3 independent experiments. An asterisk indicates a statistically significant difference from the control by one-way analysis of variance with Dunnett's post hoc test ($p < 0.05$).

Fig. 7 **Effect of IL-1ß on promoter activity of OCTN1 in MH7A cells**

(A) Schematic sequences of the –2544 and –2142 regions are shown. (B) MH7A cells were cultured in either the absence (open columns) or presence of IL-1ß (1 ng/mL) (closed columns) for 24 h. Luciferase activity is shown as the ratio of firefly/*Renilla* luciferase activities and data represent the mean ± S.E.M. of 4 to 9 independent transfection experiments. An asterisk indicates a significant difference by Student's t test ($p < 0.05$).

Fig. 8 **Effect of NFκB on promoter activity of OCTN1 in MH7A cells**

MH7A cells were co-transfected with p50 and/or p65 expression vectors together with the OCTN1 reporter gene construct (p-2544 Luc). Luciferase activity is shown as the ratio of

firefly/*Renilla* luciferase activities and data represent the mean ± S.E.M. of 5 independent transfection experiments. An asterisk indicates a statistically significant difference by one-way analysis of variance with Dunnett's post hoc test ($p < 0.05$).

Fig. 9 **Effect of mutagenesis of the NFκB binding site on promoter activity of OCTN1 in MH7A cells**

MH7A cells were transfected with OCTN1 reporter gene construct (p-2544 Luc or p-mutNFκB Luc). (A) MH7A cells were cultured in either the absence (open columns) or presence of IL-1ß (1 ng/mL) (closed columns) for 24 h. Luciferase activity is shown as the ratio of firefly/*Renilla* luciferase activities and data represent the mean ± S.E.M. of 4-9 independent transfection experiments. (B) MH7A cells were co-transfected with p50 and/or p65 expression vectors together with the OCTN1 reporter gene construct (p-mutNFκB Luc). Luciferase activity is shown as the ratio of firefly/*Renilla* luciferase and data represent the mean ± S.E.M. of 5 independent transfection experiments. An asterisk indicates a significant difference by one-way analysis of variance with Dunnett's post hoc test ($p < 0.05$).

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Table 1

Sequences of oligonucleotides used for chimeric plasmid construction, PCR and gel mobility shift assays. The bold type indicates the differences of oligonucleotides of the mutated binding sites from native binding sites in OCTN1 promoter.

Oligonucleotide	sequence (5′ to 3′)
OCTN1	
p-254	CTCGAGAGGAATGGTAAATTCAGGATCAGG
p-2142	CTCGAGAGCAATGGCAAATAGTCGAGTGG
p-1682	CTCGAGTGGCTTTACCTCCCTCTCTGTTC
p-1263	CTCGAGTGCAAAATCTCCAAAGAGGTGAGC
p-699	CTCGAGTCATCCCTTCCAGGCTGTGG
p-189	CTCGAGGGAAGCCCCCGTCAGGTCC
p-150	CTCGAGGAAGCACAGGGCGGAGACAG
p-61	CTCGAGTCCCAAGTGTACAGTGGCATCAAGC
p+92	AAGCTTCGCTCCCCAAGGATGTTAGAACGTTCC
For quantity	
OCTN1	ACCTCAGTGGGTTACTTTGCTC
	GGTAGAGCTCAGCAGTGAAGAC
OCTN2	GGGCAAGTTTGGAGTCACGG
	AGCAAGTCAGACACAGGTCAAGAG
For 5′-Race	
AP1	CCATCCTAATACGACTCATATAGGGC
2RDT	CGAGTCAAGTCGACGAAGTGC
GSP1	CCAGGAACACGACTGACATACC
GSP2	CCACTGCCGCTCCGAAAC
For cloning	
RUNX1 sense	TACCAACCAAGAAGGGGCGGA
RUNX1 antisense	TCTAGATTTCTGATGCATCAGAGCAGAG
Gel Shift Assay	
Sp1	CCTTGGGGGCTTGCGGGCGCGCGAAGC
RUNX1	CCGGGGATGGGGGTGTGGTCCCAAGTGT
NFκB	TTGAAGGGAAATTCCCCCAGTCC
Mutagenesis	

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Sp1	GGTCCTTGGGGGC**TT**GCGGGCG
RUNX1	CCGGGGATGGGGGTGT**TT**TCCCAAGTGT
NFκB	CAACTTTGAA**AAT**AAATTCCCCCAGTGGGC

Fig. 1

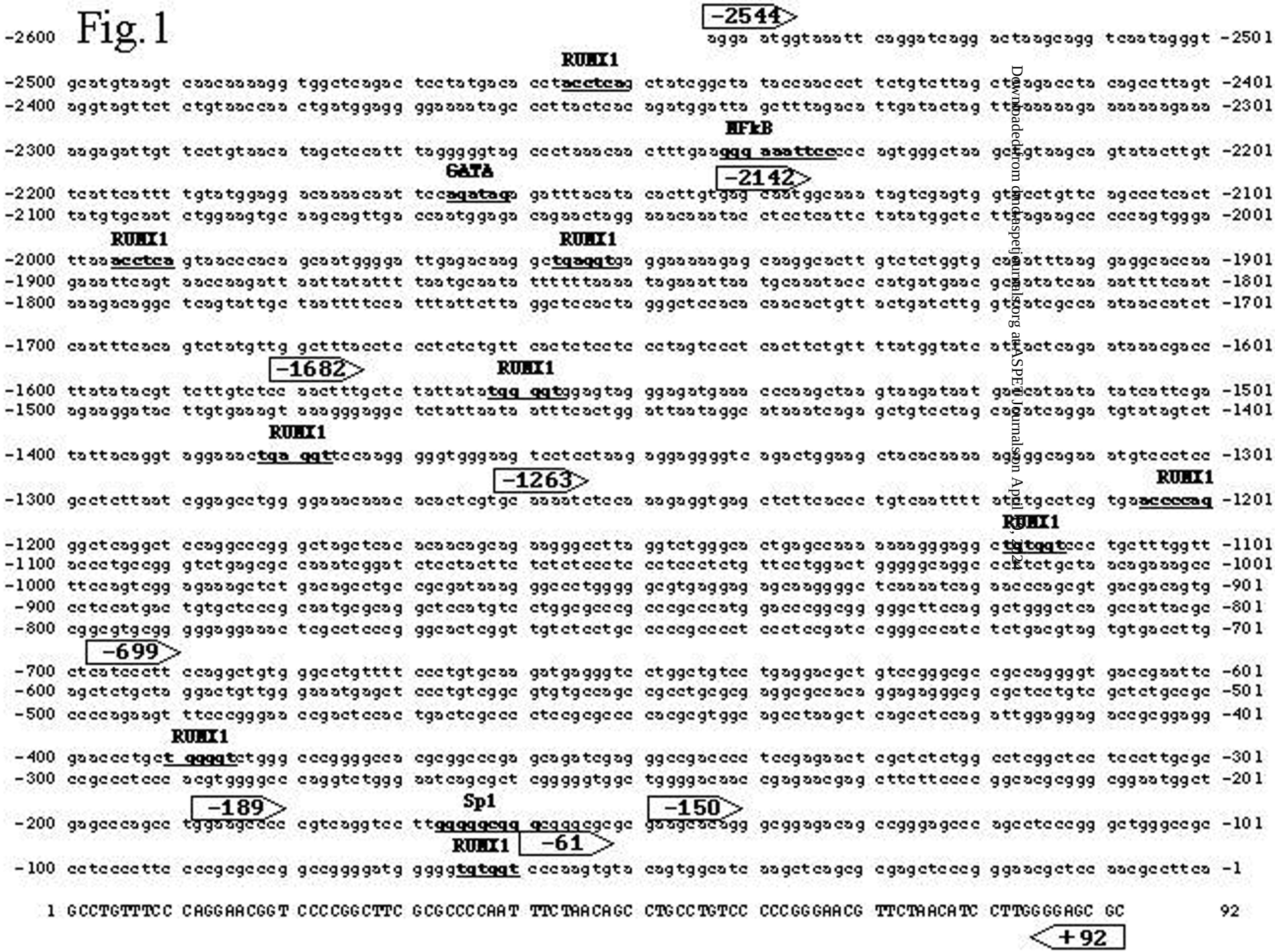

Fig.2

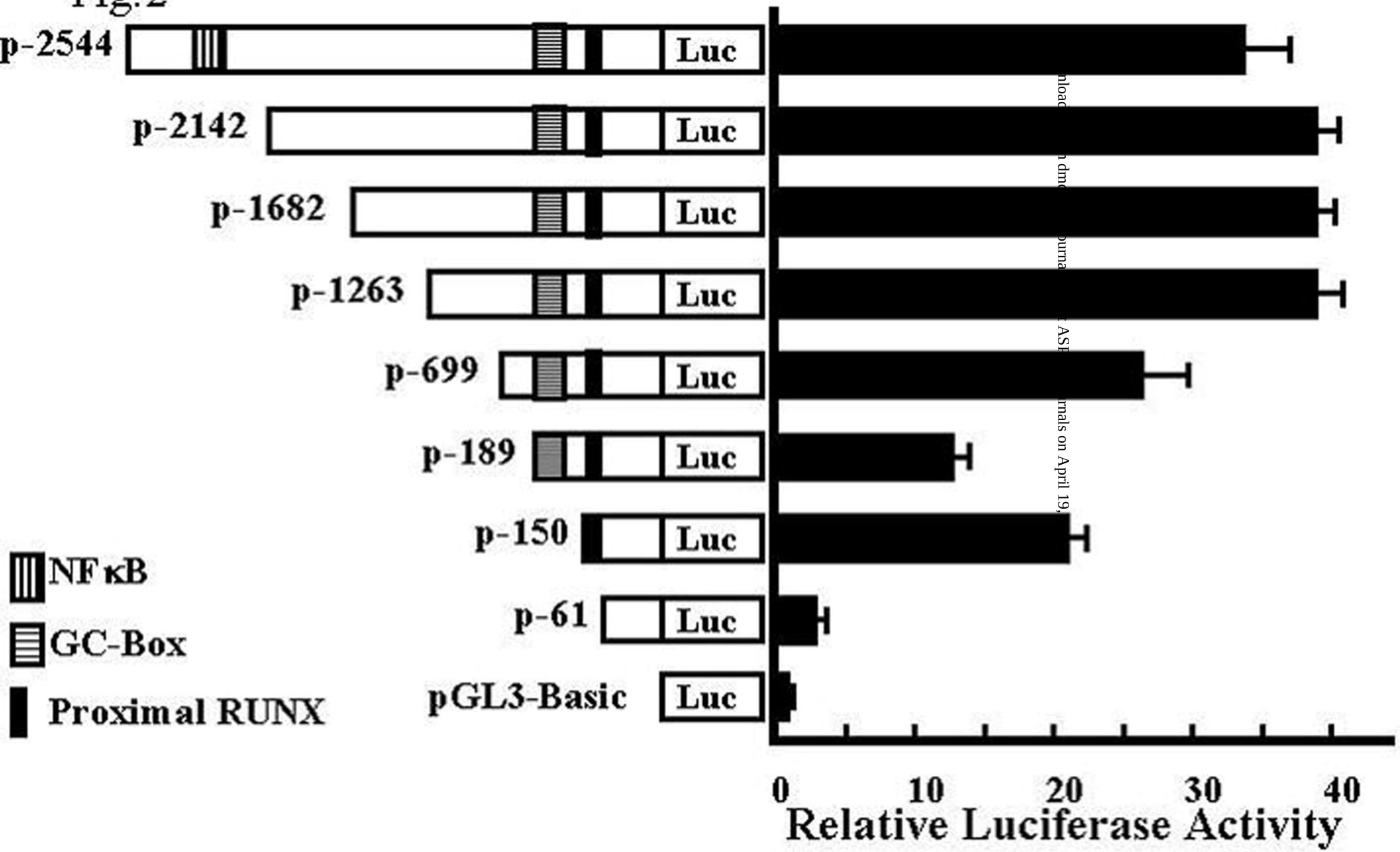

Fig. 3

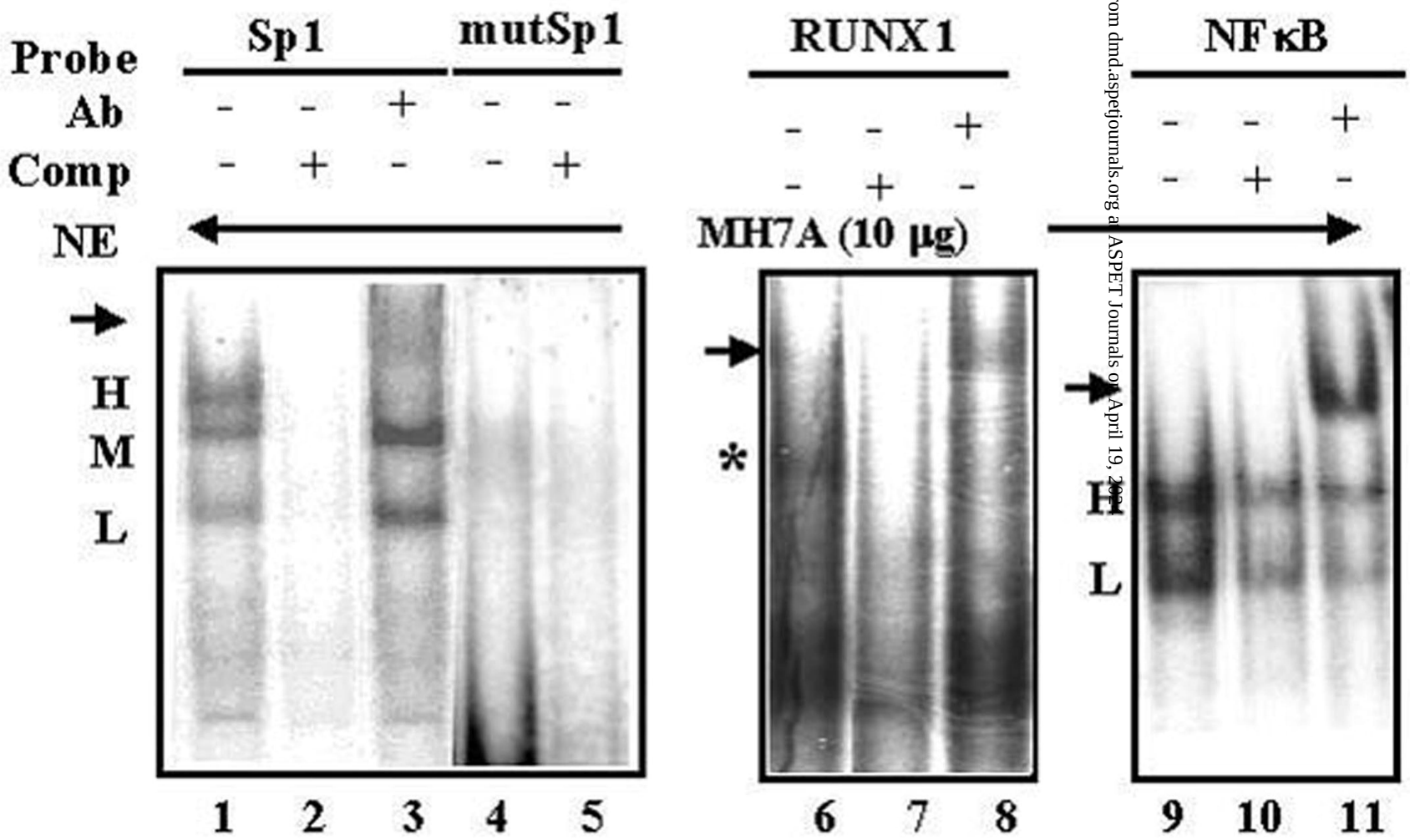

Fig. 4

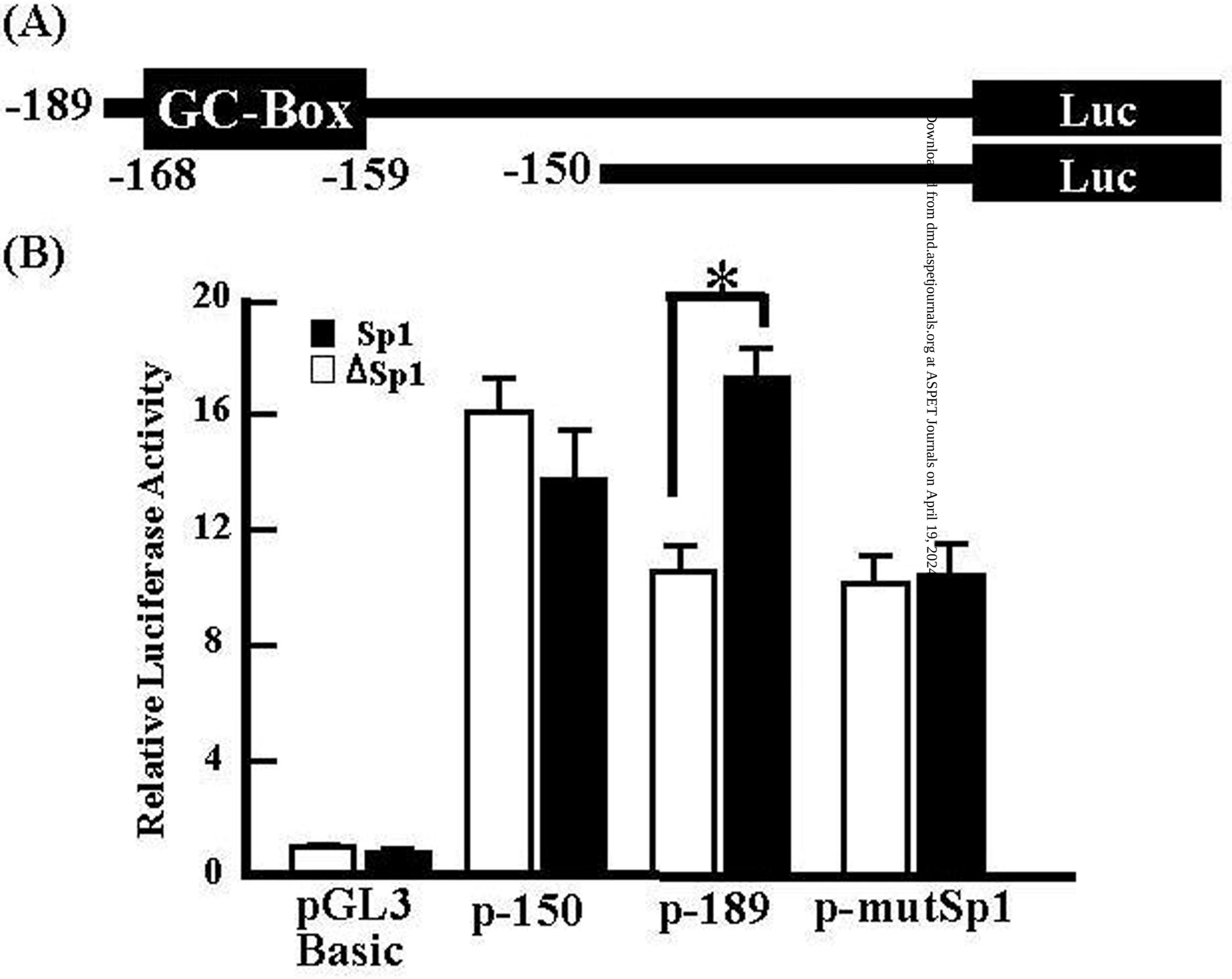

Fig. 5

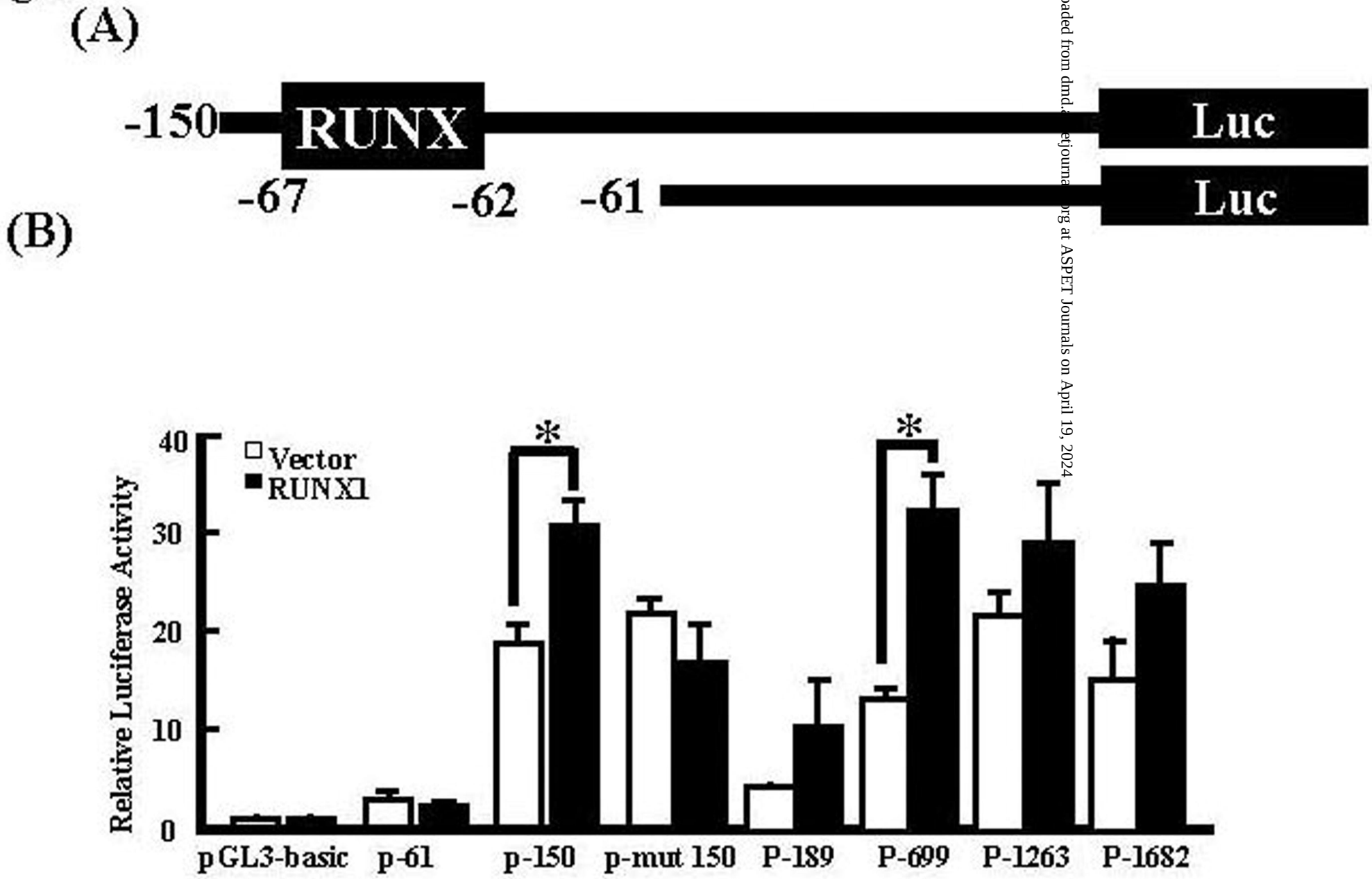

Fig. 6

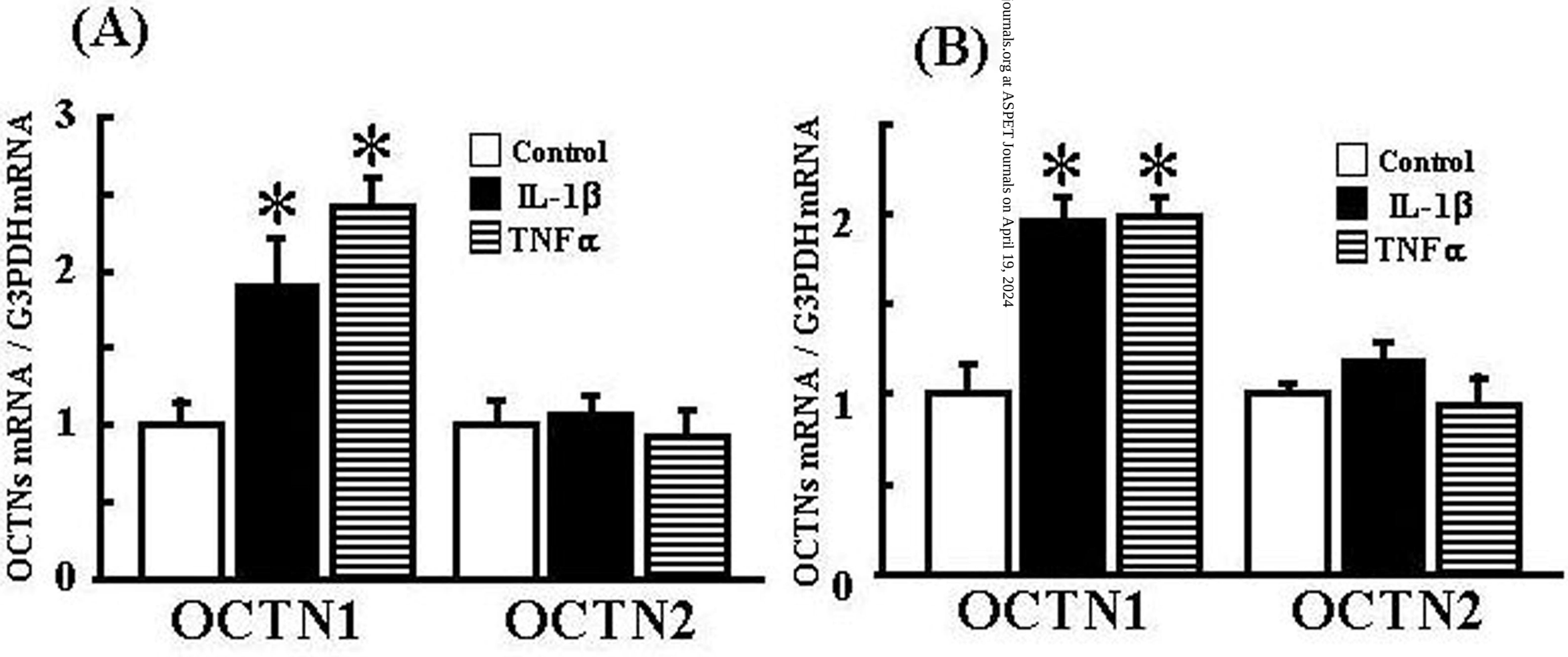

Fig. 7

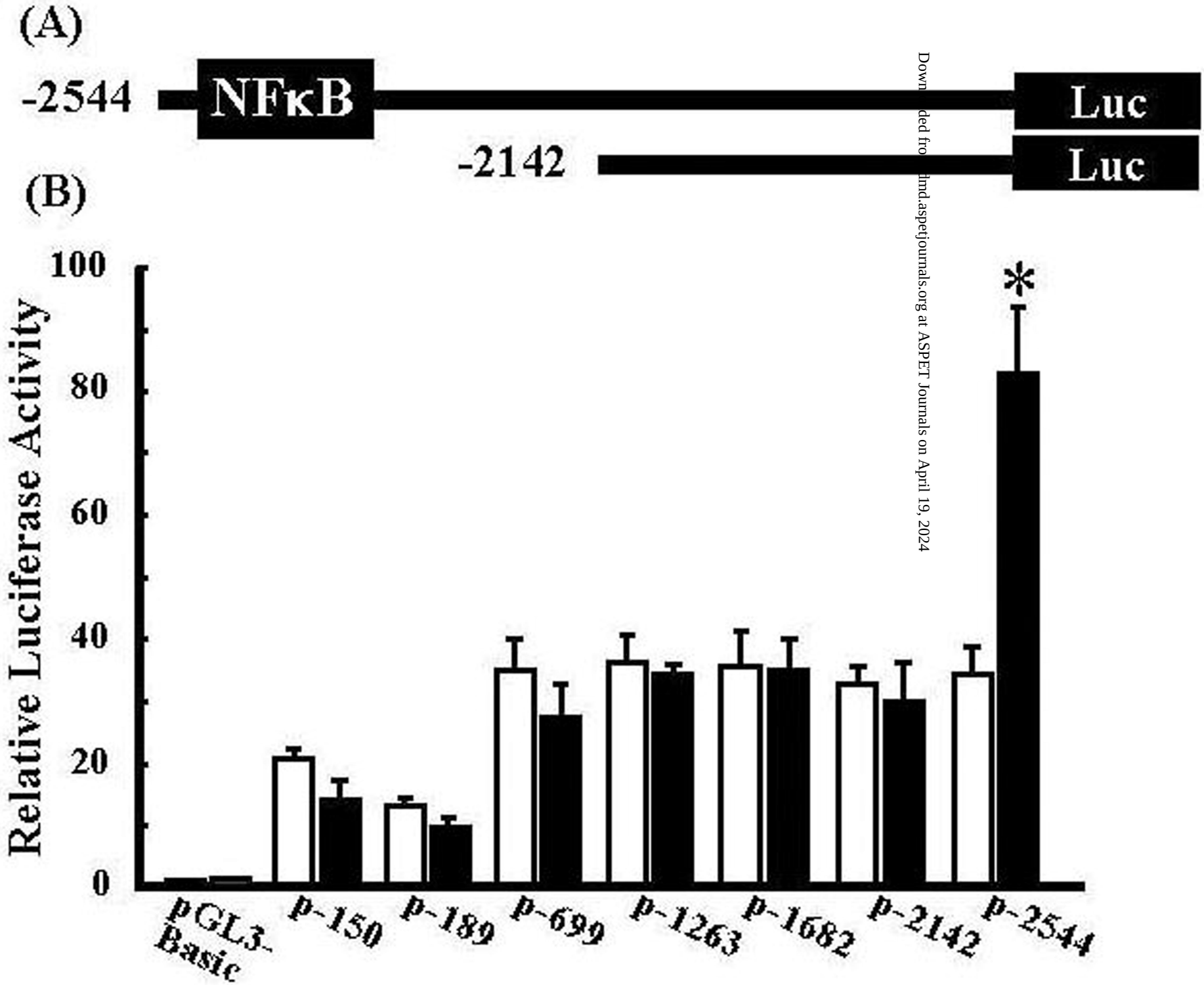

Fig. 8

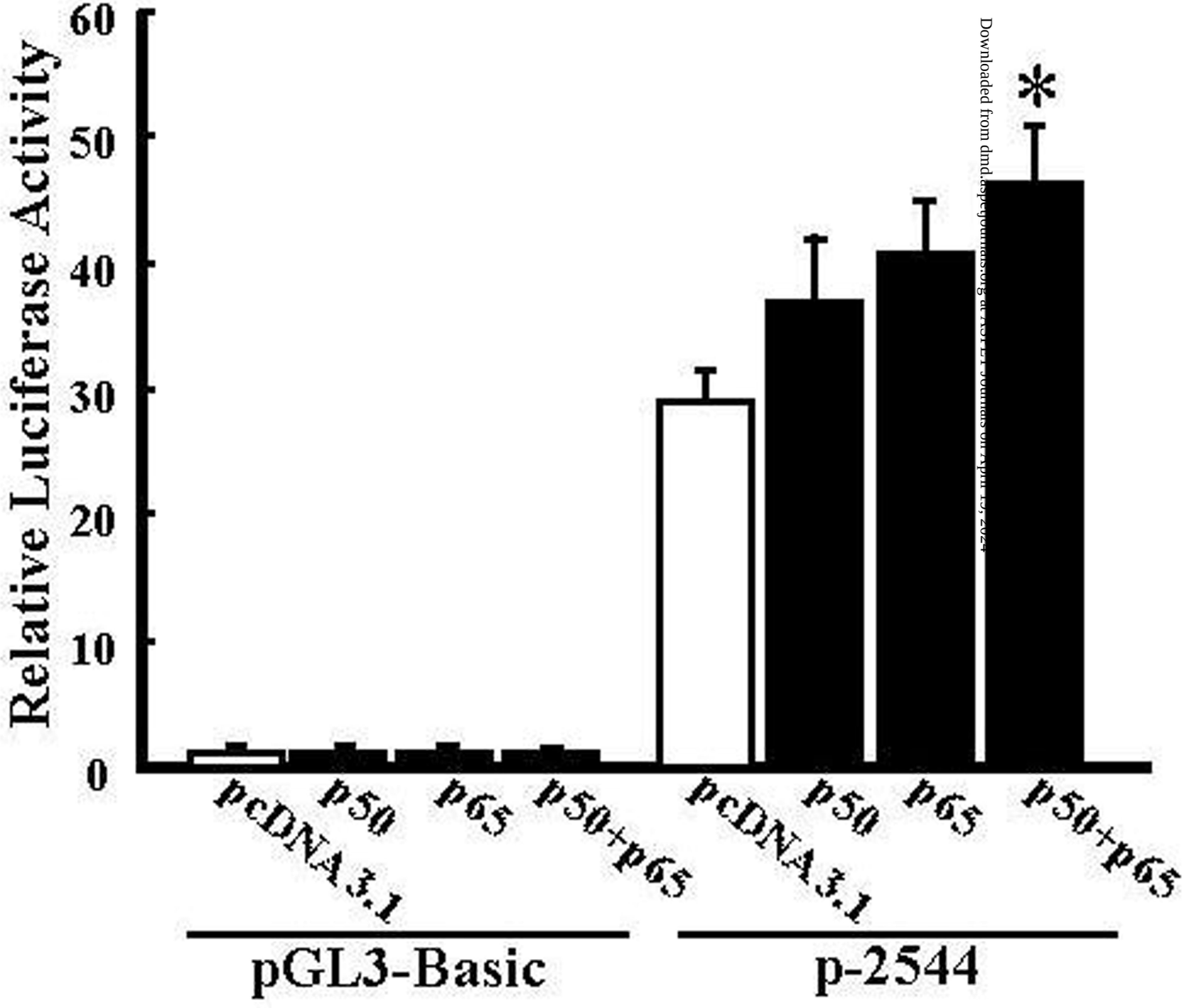

Fig. 9