FEBS Letters 354 (1994) 311–314

FEBS 14765

The existence of a growth-specific DNA binding factor for the promoter region of mouse ST2 gene

Shin-ichi Tominaga[a,*], Masako Kato-Yamazaki[a], Ken Yanagisawa[a], Kiyoshi Kawakami[b], Tsunao Tetsuka[a]

[a]*Department of Biochemistry II, 3311-1 Yakushiji, Minamikawachi-machi, Kawachi-gun, Tochigi 329-04, Japan*
[b]*Department of Biology, Jichi Medical School, Tochigi, Japan*

Received 4 October 1994

Abstract A comparison of the 5'-flanking regions of human and mouse ST2 genes revealed the presence of two highly conserved DNA sequences. The promoter activity assay with a luciferase gene as a reporter showed that the deletion of the upstream conserved region diminished the transcriptional activity in growing BALB/c-3T3 cells. By electrophoretic mobility-shift analysis, the presence of a factor that binds to the positive regulatory region of the mouse ST2 gene was found in growing but not in quiescent BALB/c-3T3 cells. These results suggest the functional importance of this conserved region and the requirement of a binding factor for the expression of the ST2 gene.

Key words: Cell cycle; Cell growth; Interleukin 1 receptor; Immunoglobulin superfamily; Transcription factor

1. Introduction

The ST2 gene was originally identified as one of the genes induced by growth factor stimulation in BALB/c-3T3 cells [1]. Nucleotide sequence analysis revealed that the ST2 protein is a member of the immunoglobulin superfamily and is very similar to the extracellular portion of interleukin 1 receptors [1,2]. Furthermore, we found ST2L, which has a transmembrane domain and a cytoplasmic domain, probably expressed by alternative splicing as reported by Bergers et al. for the rat ST2 homologue, the Fit-1 gene [2,3]. Thus, the viewpoint that the ST2/ST2L gene may be a member of the cytokine receptor family is very intriguing, and therefore, the regulatory mechanisms of the ST2 gene expression may be important for elucidating the regulation of cell proliferation. The serum responsiveness of ST2 gene expression was reported to be dependent on the sequence located at 3.6 kb upstream of the transcription initiation site [4]. However, as described, the function of this region may not be enough to explain the whole mechanism regulating ST2 gene expression [4,5].

Here we report that the proximal 5'-flanking region is important for the transcription of the ST2 gene, and the existence of a DNA binding factor that is detected in growing but not in quiescent BALB/c-3T3 cells.

2. Materials and methods

2.1. Cell culture

The BALB/c-3T3 cells (clone A31) were kindly provided by Dr. C. Stiles (Harvard Medical School), and they were cultured as described [6].

2.2. Harr plot analysis

The nucleotide sequence was determined by the dideoxynucleotide chain termination procedure as described [1]. A dot matrix analysis of the mouse and human sequences was performed using the GENETYX program with a window size of 20, a minimal percent score of 80.

2.3. Transfection and reporter gene assays

Four fragments of different lengths, from nucleotide numbers 597 to 972 (A), 677 to 972 (B), 768 to 972 (C), and 846 to 972 (D), of the mouse ST2 gene, were generated by the polymerase chain reaction (PCR) (see Fig. 3A and [7]). The *Sac*I site and *Hin*dIII site were included at the ends of the forward and reverse primers, respectively. The amplified fragment was inserted into the *Sac*I and *Hin*dIII sites of the pUC19 plasmid to confirm that the nucleotide sequence of the PCR product was correct, and then the fragment was inserted into the *Sac*I and *Hin*dIII sites of the luciferase expression vector, Pica Gene (TOYO INK, Co.).

For the construct possessing an internal deletion, the construct A in pUC19 was digested at nucleotide number 738 (according to [7]) with *Ssp*I, and thereafter, it was treated with nuclease *BAL*31 (Takara Shuzo, Co.) and ligated (Fig. 3B). The deletion mutant lacking 19 bp (from nucleotide number 720 to 738) (E) was used in this study.

Growing BALB/c-3T3 cells were transfected in 10-cm dishes (3×10^5 cells/dish) using a modified calcium phosphate transfection protocol [8,9]. The total amount of transfected DNA (20 μg) was kept constant in each experiment. A vector (2 μg) expressing β-galactosidase from the EF-1α promoter [10] was included as an internal control. Levels of luciferase were determined 48 h later [11] and normalized for β-galactosidase expression.

2.4. Preparations of nuclear extracts

BALB/c-3T3 cells were grown in 15-cm dishes, and cells were harvested at the Go state; 2, 6, 10, 12, and 16 h after serum stimulation; or at continuously growing states (50% and 90% confluency). Then the nuclear extracts were prepared as described [12]. The protein concentration of the nuclear extracts was determined by the method of Bradford.

2.5. Electrophoretic mobility-shift analysis

Electrophoretic mobility-shift analysis was performed as described previously [13]. The probe was prepared by annealing chemically synthesized oligonucleotides, and then it was inserted into the *Xba*I–*Kpn*I site of pUC119. On the other hand, the mouse and human competitors were synthesized by PCR, and they were directly cloned into the pCR^{II} vector (Invitrogen). After confirmation that the nucleotide sequences were correct, the plasmids were propagated and purified by centrifugation through a CsCl gradient. For the probe, the *Xba*I–*Kpn*I fragment was excised. The mouse competitor fragment was excised by *Eco*RI, and the human competitor fragment was excised by *Bst*XI. Then, they were purified by preparative polyacrylamide gel electrophoresis. The nucleotide sequence corresponding to the probe as well as those of the competitor fragments are shown in Fig. 2B. The probe was labelled with [γ-^{32}P]ATP.

*Corresponding author. Fax: (81) (285) 44 2158.

SSDI 0014-5793(94)01152-4

3. Results and discussion

3.1. Conserved regions in the human and mouse ST2 promoters

The mouse ST2 gene, containing the 5'-flanking region of 882 bp upstream of the transcription initiation site, was previously cloned and the whole nucleotide sequence was determined [7]. We also cloned the human genomic DNA for the ST2 gene [14]. Fig. 1 shows the partial nucleotide sequence of the 5'-flanking region of the human ST2 gene. The arrow in Fig. 1 indicates the 5'-edge of the longest ST2 cDNA obtained so far. The transcription initiation site has not been determined yet due to the lack of an appropriate human cell culture system to carry out a primer extension experiment. As shown in Fig. 2A, a dot matrix analysis of the murine and human sequences revealed the presence of two close regions of high homology starting approximately 200 bp upstream and ending 50 bp upstream of the transcription initiation site of the mouse ST2 gene. A direct alignment of this region is depicted in Fig. 2B. To avoid confusion, the nucleotide numbering is according to the previous report [7]. The upstream (X) and the downstream (Y) conserved regions are boxed.

3.2. The upstream conserved region is significant for the transcription of ST2 gene in the growing state

The comparison between mouse and human genes led us to focus on the two conserved regions. Conservation between the species suggests a functional importance of the sequences. To study the role of the putative promoter sequence, luciferase fusion constructs were prepared that contained 286 (A), 206 (B), 115 (C), and 37 (D) bp of the 5'-flanking region of the mouse ST2 gene (Fig. 3A). These plasmid constructs were transiently transfected into growing BALB/c-3T3 fibroblasts, and their luciferase activities were determined and normalized for β-galactosidase expression as described in section 2. As shown in Fig. 3A, the deletion of the upstream region (from 677 to 767) containing the upstream conserved region (X) diminished the luciferase activities by 23-fold. Furthermore, as shown in Fig. 3B, internal deletion of this region diminished the transcriptional activity by 2.9-fold. These results suggest that this region (X) is important for the transcription of the ST2 gene in the growing state.

```
AAGCTTCTTAGTCACACTCTCCTTCTCTTTCTCTGCACTCTATGCACTCTAGAAAAGCTC   60
CTTTTTTTTTTTCTTCATCCAGGCAGAGAGGCCTACTGGGACTTAAATCCAAGGAGCTGA  120
AATCTGTTTTGGGATGGGGTGGAGTCACATTCTGGAACCTAGACAGAGAATTTCTAAGTT  180
CCAGAAAGTGCTGCTTACTTCGCATTTCCTCTCCCCCCACCTTTGCTTTTGAAACTCCTG  240
GCACCAATGCTGCCAAGGCTGGCGGAGCTTTCCTGAGTGGTGTCTGCCAAATGAGGAGTC  300
AAGGAATATCTGGAAAGGCAGCCTCCAGGTCCCCGATGTCAAGACCATTTAGAACTGAAA  360
GTGTCCCAATATCGGGGTACAGGCAATAAGCATTAGTTATTAATCAGCCTGAGAAGTTGA  420
TTCTAAAATAGGAGGAAATGATTCAATTATTTCCTCTCAAGGGATTACTCAATGTTGTTT  480
TTATGTTTAAATATTTATTTGTCAACATCAAGAATTCTTAGTACATGATGCACCAGCATT  540
TTTGAACAAGTCATAGATTTGGCCACAAATCAAATTTCAGGATGGGAGGAGTGTCTCCCC  600
TTTAAAATAGAAGAGAGTGAGTAGTCTATGAGGAGGGACCTACAAAGACTGGAAACTATT  660
CTTAGCTCCGTCACTGACTCCAAGTTCATCCCCTCTGTCTTTCAGTTTGGGTAAGCATCA  720
ATTACTTATCTAAAATTTGTAATAAGAAAAGTCTTCATAATTCATGATTGTGTTTATCTT  780
TATGTTAGTAAATTTCTATGTTGGTTCTAT                                 810
```

Fig. 1. Partial nucleotide sequence of the 5'-flanking region of the human ST2 gene. The human ST2 genomic DNA was isolated as described [14]. The nucleotide sequence was determined as described in section 2. Exon 1 is boxed. The arrow indicates the 5'-edge of the longest ST2 cDNA obtained so far.

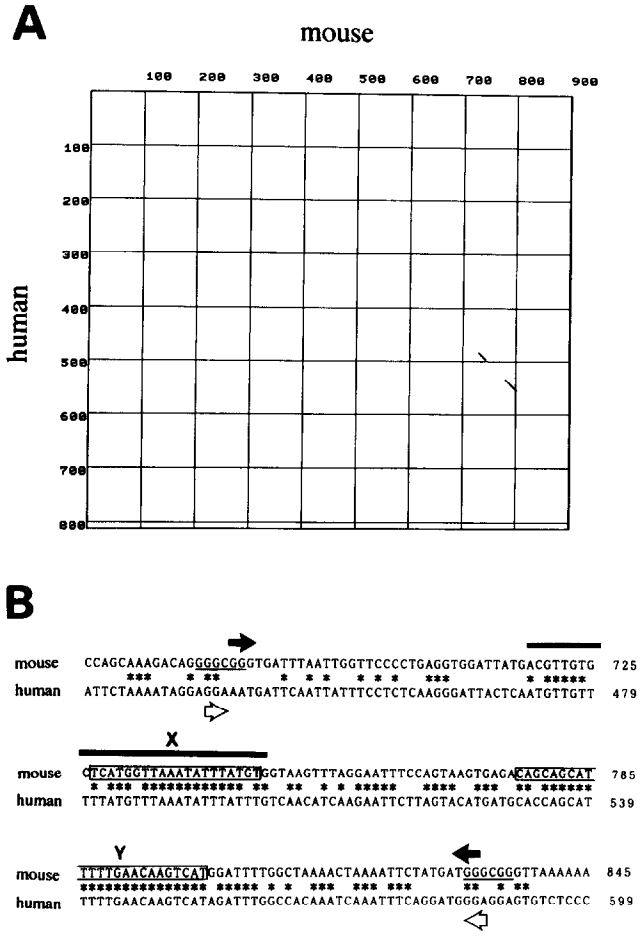
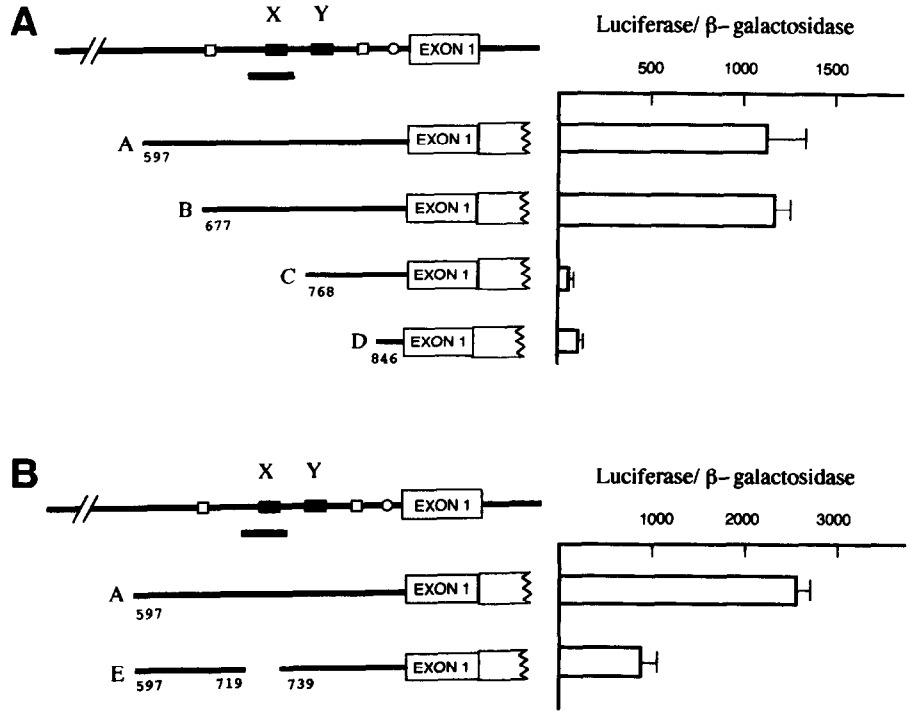
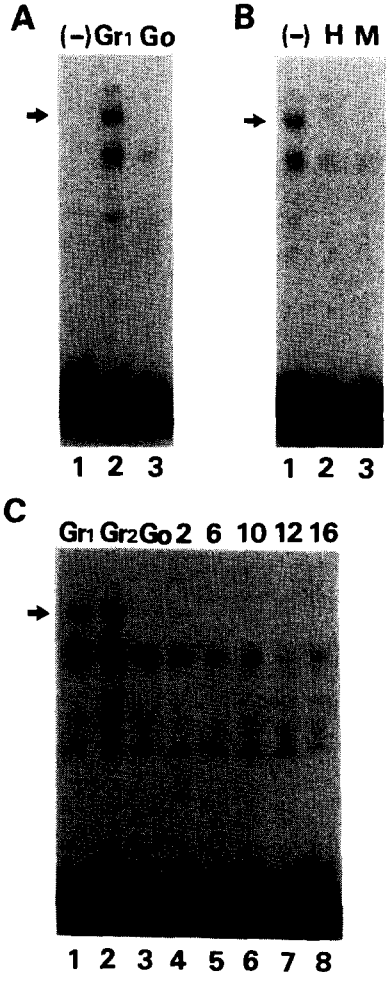


Fig. 2. Harr plot analysis of the mouse and human ST2 genomic DNA. Panel A: the 5'-flanking regions of mouse ST2 genomic DNA [7] and human ST2 genomic DNA (Fig. 1) were compared using the Harr plot method as described in section 2. Panel B: a direct alignment of the homologous regions in the 5'-flanking regions of mouse and human ST2 genomic DNAs. Identical nucleotides are shown by asterisks. Two boxed sequences (X and Y) indicate the conserved regions clarified by Harr plot analysis. The thick bar, the region between closed arrows, and the region between open arrows indicate the nucleotide sequences of the probe, the mouse competitor, and the human competitor for the electrophoretic mobility-shift analysis, respectively. The positions of two GC boxes in the mouse 5'-flanking region are underlined.

3.3. Growth-specific factor-binding to the 5'-regulatory region of ST2

For a biochemical characterization of potential *trans*-acting factors that might be involved in the ST2 transcriptional regulation, nuclear extracts were prepared from growing, quiescent, and serum stimulated BALB/c-3T3 cells as described in section 2. The fragment probe of 30 bp (see the thick bar in Fig. 2B) including the entire upstream conserved region (X) was prepared, purified, and radiolabelled. Electrophoretic mobility-shift analysis with this probe and the growth- or quiescence-specific nuclear extracts from BALB/c-3T3 cells revealed the presence of a complex that is detected in the nuclear extract of the growing cells, but not in that of the quiescent cells (Fig. 4A).





↑

Fig. 3. Expression of luciferase reporter gene constructs in growing BALB/c-3T3 cells. Panel A: fragments of different lengths corresponding to the mouse promoter region, starting at nucleotide number 597(A), 677(B), 768(C), and 846(D), and each containing 90 bp of the first non-coding exon, were fused to the Pica Gene vector as described in section 2. The open squares and the open circle indicate the positions of GC boxes and a TATA-like sequence, respectively. The black boxes (X and Y) indicate the positions of highly conserved regions (see text). The thick bar represents the position of the probe used for the electrophoretic mobility-shift assay (see Fig. 4). Fusion constructs A, B, C, and D were transfected into exponentially growing BALB/c-3T3 cells. Luciferase activities were determined and normalized as described in section 2. Each lysate was assayed twice. Data are presented by arbitrary units as the mean of 3 independent experiments from which the background using the Pica Gene basic vector was subtracted. The thin bar indicates a standard error. Panel B: the reporter plasmid (E) possessing an internal deletion in the upstream conserved region (X) was constructed as described in section 2. Luciferase activities were determined as in Panel A.

←

Fig. 4. Electrophoretic mobility-shift analysis of the nuclear extracts from BALB/c-3T3 cells. Panel A: nuclear extracts from quiescent BALB/c-3T3 cells(Go) and growing cells at 50% confluency (Gr1) were analyzed with the labelled probe as described in section 2. In lane 1, the analysis was performed in the absence of nuclear extracts (−). Panel B: the analysis was carried out with nuclear extract from growing cells (Gr1) in the absence (−), or in the presence of a 50-fold molar excess of the human competitor (H), or in the presence of a 50-fold molar excess of the mouse competitor (M). Panel C: nuclear extracts from cells in various stages: growing cells at 50% confluency (Gr1) (lane 1); growing cells at 90% confluency (Gr2) (lane 2); quiescent cells (Go) (lane 3); cells at 2 (lane 4), 6 (lane 5), 10 (lane 6), 12 (lane 7), and 16 h (lane 8) after serum stimulation were analyzed by the probe. The amounts of the nuclear extract protein used are 2.1 μg for Gr1; 2.9 μg for Gr2; 2.6 μg for Go; 2.4, 2.3, 2.8, 2.3 and 2.8 μg for 2, 6, 10, 12 and 16 h after stimulation, respectively.

Addition of a 50-fold molar excess of the human and mouse competitors into the reaction mixture prevented the formation of the retarded complex, suggesting that the binding of the factor to the probe is specific, and the factor is common for the mouse and human ST2 gene (Fig. 4B). Furthermore, as shown in Fig. 4C, the factor is detected only in continuously growing cells and not detected in the growth-stimulated cells from the Go state. The trials to transfect quiescent BALB/c-3T3 cells with the fusion constructs to see the serum responsiveness failed, due to poor transfection efficiency in our system.

Trüb et al. reported that a TRE located 3.6 kb upstream of the transcription initiation site is important, but not sufficient for the transcription of the ST2 gene [4,5]. Our data that there are highly conserved sequences in the 5'-flanking region between two species and that deletion of the upstream conserved region diminished the transcriptional activity of the ST2 gene in growing cells, together with the presence of a growth-specific binding factor for this region, suggest the significance of the proximal promoter region in addition to the 3.6-kb distal region. There might be an interaction between some *trans*-acting factors binding to the proximal promoter region and those binding to the 3.6-kb distal region. The factor that we describe here could be a candidate for a part of the interacting factors.

Purification and identification of this possible *trans*-acting factor will help us to elucidate how the expression of the ST2 gene is regulated.

Acknowledgements: We thank Dr. Shigekazu Nagata for helpful discussions and Dr. Stephanie M. Jung for reading the manuscript. We also thank Ms. Mayumi Masubuchi, Reiko Izawa and Yuko Watanabe for their technical assistance. This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas, Ministry of Education, Science and Culture, Japan.

References

[1] Tominaga, S. (1989) FEBS Lett. 258, 301–304.
[2] Yanagisawa, K., Tsukamoto, T., Takagi, T., Tetsuka, T. and Tominaga, S. (1993) FEBS Lett. 318, 83–87.
[3] Bergers, G., Reikerstorfer, A., Braselmann, S., Graninger, P. and Busslinger, M. (1994) EMBO J. 13, 1176–1188.
[4] Trüb, T., Kalousek, M.B., Fröhli, E. and Klemenz, R. (1994) Proc. Natl. Acad. Sci. USA 91, 3896–3900.
[5] Kalousek, M.B., Trüb, T., Schuermann, M. and Klemenz, R. (1994) J. Biol. Chem. 269, 6866–6873.
[6] Tominaga, S. and Lengyel, P. (1985) J. Biol. Chem. 260, 1975–1978.
[7] Tominaga, S., Jenkins, N.A., Gilbert, D.J., Copeland, N.G. and Tetsuka, T. (1991) Biochim. Biophys. Acta 1090, 1–8.
[8] Wigler, M., Pellicer, A., Silverstein, S. and Axel, R. (1978) Cell 14, 725–731.
[9] Copeland, N.G. and Cooper, G.M. (1979) Cell 16, 347–356.
[10] Mizushima, S. and Nagata, S. (1990) Nucleic Acids Res. 18, 5322.
[11] de Wet, J.R., Wood, K.V., Deluca, M., Helinski, D.R. and Subtamani, S. (1987) Mol. Cell. Biol. 7, 725–737.
[12] Kawakami, K., Yanagisawa, K., Watanabe, Y., Tominaga, S. and Nagano, K. (1993) FEBS Lett. 335, 251–254.
[13] Kawakami, K., Scheidereit, C. and Roeder, R.G. (1988) Proc. Natl. Acad. Sci. USA 85, 4700–4704.
[14] Tominaga, S., Yokota, T., Yanagisawa, K., Tsukamoto, T., Takagi, T. and Tetsuka, T. (1992) Biochim. Biophys. Acta 1171, 215–218.