Analysis of the Promoter Region of the *Xenopus* General Type *TFIIS* (*TCEA1*) Gene

Fumihiko Kugawa* and Masatada Aoki

Department of Biological Pharmaceutical Sciences, College of Pharmacy, Nihon-University, 7–7–1 Narashino-dai, Funabashi, Chiba 274–8555, Japan

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TFIIS is a general transcription elongation factor that interacts with Elongin (SIII), a product of the tumor suppressor gene for the human hereditary disorder von Hippel-Lindau (VHL) disease. Here, we have studied the promoter region of the *Xenopus* general type *TFIIS* (*XGTFIIS*) gene using a luciferase assay. The previously cloned region 1600-bp upstream of the *XGTFIIS* gene was divided into 12 sequential deletion fragments, and each fragment was inserted into the pGL3-Basic vector, which was used as a reporter for a luciferase assay in the *Xenopus* cultured cell-line, XTC. Except for the proximal promoter region close to the transcription start sites, no positively or negatively regulating element was found for *TFIIS* gene expression within the 1600-bp upstream region of the *XGTFIIS* gene. Of the two previously identified transcription start sites, the one that is distal to the ATG codon was preferred over the proximal one.

Key words —— von Hippel-Lindau disease, *Xenopus laevis*, general type TFIIS, genomic cloning, transcription start site, luciferase assay

INTRODUCTION

Cooperative progress in molecular biology and human genetics recently revealed the genes and products responsible for some human hereditary disorders. Among them, Xeroderma pigmentosum (XP),[1] Cockayne syndrome (CS),[2] and von Hippel-Lindau (VHL) disease[3] are unique, because their responsible genes are closely related to general transcription factors. RNA polymerase II is indispensable for the transcription of protein-encoding genes, a process that also requires general transcription factors.[4–6] The general transcription factors TFIIH (initiation factor) and Elongin/SIII (elongation factor) are the gene products responsible for XP and VHL disease, respectively.

VHL disease is a rare hereditary disorder (1/36000) that predisposes individuals to a variety of cancers, such as clear-cell renal carcinoma.[7] Recently, one VHL gene product, Elongin (SIII) A, was shown to interact with another general transcriptional-elongation factor, TFIIS.[8,9] There are two classes of general elongation factors that function in eukaryotic transcription. One includes TFIIF,[10] ELL,[11] and Elongin (SIII),[12] and the other, TFIIS, promotes elongation by reactivating RNA polymerase II that has been arrested by any of a variety of impediments, including a specific DNA sequence referred to as the "intrinsic arrest site."[13–15] Thus, a detailed study of *TFIIS* gene regulation should reveal its connection to human genetic disorders resulting from transcriptional malfunctions.

We have studied TFIIS in *Xenopus laevis*,[16,17] because this animal is very useful for studying development, and because we observed an intriguing oscillation of the *Xenopus TFIIS* mRNA expression during oogenesis.[16] After we first cloned the *Xenopus* TFIIS cDNA, other researchers identified two additional types of *TFIIS* genes in *Xenopus laevis*, and the one we cloned turned out to be the general type *TFIIS* (*TCEA1*) of *Xenopus laevis* (which we refer to here as *XGTFIIS*).[18] In our previous genomic cloning of the *XGTFIIS* gene,[17] we found that *XGTFIIS* has two unique transcription start sites. Here, to reveal the contribution of these two start sites to *XGTFIIS* gene expression, we analyzed the promoter activity of the *XGTFIIS* gene using sequentially deleted reporter plasmids.

MATERIALS AND METHODS

Plasmid Construction —— The *XGTFIIS* genomic clone (–1730 to +214) had already been obtained by us (DNA Data Bank of Japan; DDBJ accession No. AB047824).[17] Twelve sequentially deleted fragments of the *Xenopus* genomic sequence, listed in Fig. 1, were made by PCR amplification. For the antisense primer, 5′-ATA CCC GGG CCC TCG CTC G-3′, was used; it contained a *Sma* I site

*To whom correspondence should be addressed: Department of Biological Pharmaceutical Sciences, College of Pharmacy, Nihon University, 7–7–1 Narashino-dai, Funabashi, Chiba 274–8555, Japan. Tel.: +81-47-465-6779; Fax: +81-47-465-7182; E-mail: jfkugawa@pha.nihon-u.ac.jp

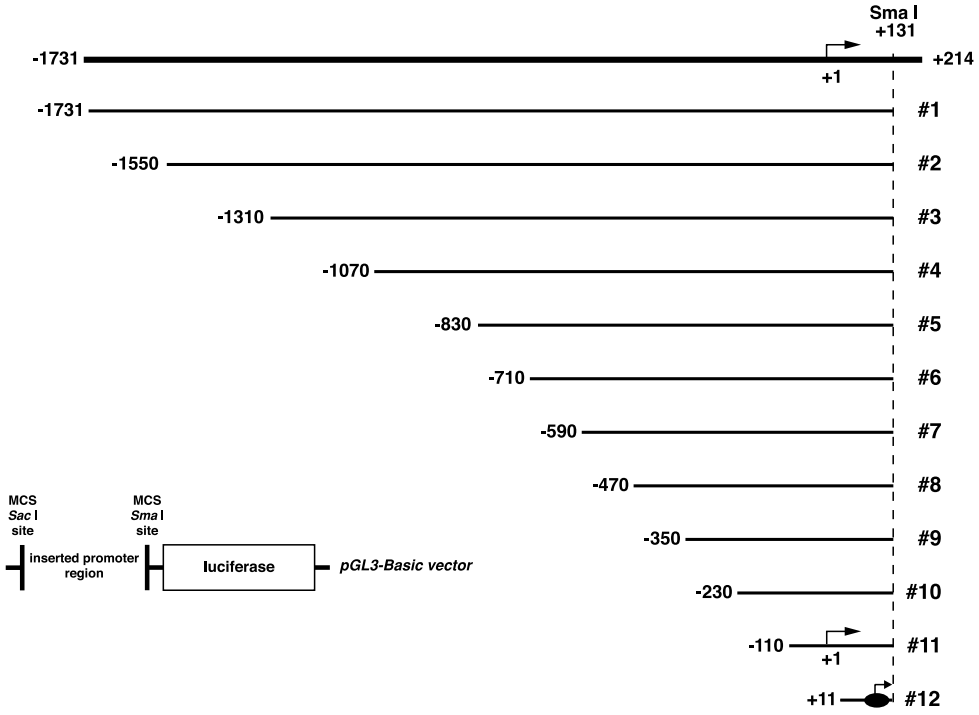

Fig. 1. Deletion Mutants of the Upstream Genomic DNA Structure of *Xenopus laevis* General Type *TFIIS*

Sequential deletions of 240 or 120 bp from the 5′ side of the genomic clone of the *XGTFIIS* gene were made. The upper, thick line indicates the genomic DNA sequence of *XGTFIIS*. The +1 indicates the more distal of the two transcription start sites (details in the text). The closed oval with the arrow indicates the other, leaky transcription start site. The *Sac* I restriction enzyme site and the *Sma* I site were inserted at the 5′- and 3′-ends of each fragment, respectively. The numbers listed to the right of each fragment are the names of the constructs. The inset shows the structure of the pGL3-Basic vector; details are available at the Promega home page (www.promega.co.jp/lit/pgl3b.html).

at +126. For the sense primers, the ATA-GAG CTC (*Sac* I site) sequence was attached to 15-mers at the 5′-end of each fragment (#1–#12, Fig. 1), and PCR amplification was conducted with a TaKaRa Premix Taq kit (TaKaRa, Japan). The PCR protocol was performed according to the manufacturer's recommendations. The amplified fragments were then double-digested with *Sac* I and *Sma* I, and the purified fragments were subcloned into the multi-cloning site of the pGL3-Basic vector (Promega, U.S.A.), using a standard protocol.[19] The accuracy of the sequentially deleted constructs was confirmed by sequencing both strands using a Thermo Sequenase Cycle Sequencing kit (USB Corporation, U.S.A.) and Li-Cor's DNA Analyzer Gene Reader 4200 (Li-Cor, U.S.A.).

Cell Culture, DNA Transfection, Luciferase Assay, and β-Galactosidase Assay —— The *Xenopus laevis* XTC cell line (a gift from Emeritus Professor Koichiro Shiokawa, University of Tokyo, Japan) was maintained at 22°C in Leibovitz's L-15 medium diluted to 60% and supplemented with fetal calf serum to 10%. XTC cells were transfected using the lipid-based FuGENE 6 Transfection Reagent (Roche Diagnostics Corporation, U.S.A.). All transfection experiments were performed in triplicate using 60-mm-diameter tissue culture dishes (IWAKI GLASS, Japan). XTC cells were co-transfected with the pGL3-Basic vectors and the pSV-β-GAL vector (Promega), which harbors the β-galactosidase gene and was used as an internal control of transformation efficiency. The luciferase assay was conducted using Promega's Luciferase Assay System (Promega). The luminometer was an ATTO AB-2200 Luminescencer. The β-galactosidase assay was conducted using Promega's β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer (Promega). The luciferase and β-galactosidase activities were measured using 20 μl and 150 μl of the XTC cell lysate, respectively. The detailed protocols for the luciferase assay and for β-galactosidase measurement were according to the manufacturer's recommendations.

RESULTS AND DISCUSSION

The transcription elongation factor TFIIS consists of three functional domains: the N-terminal (I),

central (II), and C-terminal (III). Domains II and III have been recognized as stimulators of the arrested RNA polymerase II elongation complex, which enable transcription to resume.[20,21] The key to understanding the function of the N-terminal domain I[22] was its homology to two other transcription elongation factors, Elongin (SIII) A[12] and CRSP70.[23] In addition, a recent biochemical study conducted by Elmendorf *et al*. indicated that Elongin negatively regulates TFIIS-induced nascent transcript cleavage.[9] These observations led us to hypothesize that TFIIS might play a role in the occurrence of VHL disease. Moreover, the unique function of TFIIS in proofreading arrested RNA polymerase II suggests to us that a malfunction of the *TFIIS* gene expression early in development could give rise to serious hereditary disorders.

In our previous studies, we discovered, using the *Xenopus* oocyte development system, that *XGTFIIS* mRNA expression oscillated during *Xenopus* oogenesis,[16] and by genomic cloning, we found that like other basic transcription factors, *XGTFIIS* has a unique TATA-less transcriptional start site; this is a characteristic feature of house-keeping genes.[17] These findings suggested that a unique transcription regulation mechanism could be involved in *XGTFIIS* gene expression. That study also revealed that there are two unique transcription start sites in the *Xenopus TFIIS* gene: one is –142 bp from the methionine (Met) codon, and the other is a leaky region *ca*. 100 bp upstream from the Met site.[17] Although the existence of multiple transcription start sites for a particular gene is not unusual, determining which start site is used predominantly is essential for understanding this unique transcription elongation gene. Indeed, there are few reports about the gene regulation of *TFIIS*,[24,25] even though there are several reviews on the function of TFIIS as a transcriptional protein.[5,8,14]

In Fig. 1, we show the 12 sequentially deleted genomic DNA fragments that were prepared for the construction of luciferase assay vectors. The plus 1 (+1) transcription start site was in the #11 fragment and the other leaky transcription start site, which is located about 100 bp downstream of the +1 site, was in the #12 fragment (shown as a closed oval).[17] The PCR-amplified sequential deletion fragments (#1–#12, Fig. 1) were subcloned into the pGL3-Basic vector between the *Sac* I and *Sma* I sites of the multiple-cloning region (Fig. 1 inset). XTC cells were co-transfected with these constructs and the pSV-β-GAL vector, and the luciferase assay was conducted to identify the regions that could affect the promoter activity of the *XGTFIIS* gene. The transfection efficiency was evaluated according to the β-galactosidase activity of the co-transfected pSV-β-GAL vector. The value of relative luciferase activity, RLA, was calculated as follows: (luciferase counts/μg per ml protein concentration in XTC cell lysate)/(β-galactosidase concentration/μg per ml protein concentration in XTC cell lysate). Both evaluations were done in triplicate.

The results of the luciferase assay of the transfected *Xenopus* XTC cells are shown in Fig. 2. In the *Xenopus* TFIIS 5′-genomic sequence (from construct #1 through construct #12), the RLA value ranged from its highest value at 3.7×10^7 (#5) to the lowest value at 5.8×10^6 (#12). However, we could not identify the particular region that promotes or suppresses *TFIIS* gene expression in the region between –1731 and –110 bp (#1–#11). In the regions containing the transcription start site (+1) and the second, leaky transcription start site, the deletion of a 120-bp fragment (from –110 in #11 to +11 in #12) caused a drastic decrease in the RLA value (from 2.3×10^7 to 5.8×10^6). This result strongly suggested that the +1 transcriptional start site, which is the most distal of the two transcription start sites, plays the dominant role in the transcription of this gene.

The cDNA of *TFIIS* genes has been cloned from many creatures that are available for experimental use, and the functions of these genes have been studied extensively. However, there are few reports on the genomic cloning of these genes, especially the general-type *TFIIS* gene, or on their gene regulation. Oh *et al*. conducted a promoter activity assay for the *Drosophila melanogaster TFIIS* gene and showed that a region just proximal to the transcription start site (–113 to +1) is required for its efficient expression.[24] They also reported that there was no other positively or negatively regulating element for *Drosophila TFIIS* gene expression in the region between the +1 site and –3100 bp. Our current study (indicating no specific regulatory elements in the 5′-upstream region of the *XGTFIIS* gene) roughly coincides with their report, and our results also emphasize the importance of the proximal region to the transcription start site for *Xenopus TFIIS* gene expression.

In a preliminary experiment, we transfected XTC cells with three different vectors: pGL3-Basic, pGL3-Control (Promega), and construct #11 (pGL3-Basic vector harboring the #11 fragment). We then conducted a luciferase assay for each transfection.

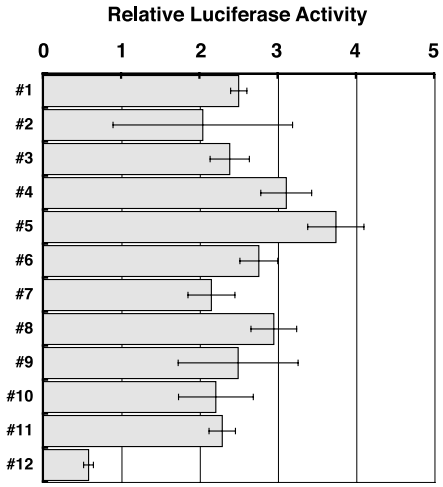

Fig. 2. The RLA (Relative Luciferase Activity) Value of XTC Cells Transfected with the Promoter-Luciferase Fusion Plasmids

The luciferase activity of cells transfected with each deletion fragment in a pGL3-Basic vector was calculated as explained in the text. The numbers on the X-axis times 10^7 give the RLA value. The bars indicate ± S.D., $n = 3$.

The results showed that the RLA value of the pGL3-Control vector and construct #11 transfection were almost same; however, that of the pGL3-Basic vector was *ca*. 1/100 of their RLA values (data not shown). Since the pGL3-Control vector has an SV40 promoter and enhancer 5′ and 3′ of the luciferase gene, this result suggested that the promoter activity, which is in construct #11 (–110 to +11) was as strong as SV40. Thus, further close investigation of construct #11 might lead to finding a strong promoter function for *Xenopus TFIIS* gene expression.

Our present study of *TFIIS* gene regulation was conducted using *Xenopus laevis* because of its advantages for early development studies. The relatively easy handling of *Xenopus* oocytes will enable us to microinject *TFIIS* siRNA or TFIIS antibodies into them in future experiments. The accumulation of data from basic gene-regulation studies using this experimental animal should provide a more detailed understanding of the gene regulation of *TFIIS* and its possible participation in human genetic disorders like VHL disease.

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