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Presence of a novel exon 2E encoding a putative transmembrane protein in human IL-33 gene

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ABSTRACT

Interleukin-33 (IL-33) is a dual-function molecule that regulates gene expression in nuclei and, as a cytokine, conveys proinflammatory signals from outside of cells via its specific receptor ST2L. There are still a lot of questions about localization and processing of IL-33 gene products. In the course of re-evaluating human IL-33 gene, we found distinct promoter usage depending on the cell type, similar to the case in the ST2 gene. Furthermore, we found a novel exon 2E in the conventional intron 2 whose open reading frame corresponded to a transmembrane protein of 131 amino acids. Dependence of exon 2E expression on differentiation of HUVEC cells is of great interest in relation to human IL-33 function.

1. Introduction

Accumulating evidence suggests the importance of IL-33 in promoting proinflammatory reactions and Th2 immune responses [1,2]. The widely accepted roles of the molecule include dual functions: it both regulates gene expression as a nuclear factor and exerts biological activities from outside of the cell by binding to ST2L-expressing target cells. However, a number of questions remain, such as localization of IL-33 and processing mechanism of IL-33 before acting as a ligand outside of the cell. Therefore, we thought it important to re-evaluate the IL-33 gene itself. We examined the exon-intron organization of the IL-33 gene, and were interested in the large intron 2, which could contain some unknown regulators on IL-33's biological activity. Actually, such an extra exon was found in the case of the ST2 gene [3].

After basal screening by RT-PCR using many primers in intron 2, we focused on a possible novel exon, which we tentatively named exon 2E (extra). Exon 2E is included in the alternatively spliced messenger RNAs. Most interestingly, it contains an open reading frame of 131 amino acids coding for a possible transmembrane protein.

2. Materials and methods

2.1. Cell culture

NHEKs (normal human epidermal keratinocytes) from neonatal foreskin were purchased from Kurabo (Osaka, Japan) and cultured in keratinocyte-serum-free media (K-SFM; Life Technologies, Carlsbad, CA, USA) supplemented with 40 μg/ml of bovine pituitary extract (BPE; Kyokuto Seiyaku, Tokyo, Japan) and 5 ng/ml of epidermal growth factor (EGF; R&D Systems, Minneapolis, MN, USA). At approximately 80–90% confluence, cells were trypsinized, diluted at a 1:4 ratio, and passaged. Cells passaged 3–6 times were used for this study.

HUVECs (human umbilical vein endothelial cells) were purchased from Canbrex BioScience (Walkersville, MD, USA) and were cultured on polystyrene dishes in essential growth medium (EGM) -2 (Canbrex BioScience).

WI38 fibroblastic cells were obtained from JCRB (Japanese Collection of Research Bioresources) and were cultured in DMEM (Sigma Aldrich, St. Louis, MI, USA) supplemented with 10% FBS (Thermo Electron, Melbourne, Australia).

2.2. Tubelike formation of HUVECs

HUVECs were spread at 1.5×10^6 cells per 60 mm cell culture dish, which was pre-coated with Matrigel Matrix (BD Biosciences, San Jose, CA, USA) as described earlier [4]. The cells were harvested at 12, 24, and 48 h after spreading using BD Cell Recovery Solution (BD Biosciences). The layers of cells and the gel were scraped with

Abbreviations: cDNA, complementary DNA; DMEM, Dulbecco's Modified Eagle's Medium; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NP-40, Nonidet P-40; RT-PCR, reverse transcription-polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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4 ml of solution per dish, and then the samples were transferred to a 50 ml conical tube. The conical tube was left on ice for 1 h until the Matrigel was completely dissolved. The cells were then recovered by centrifugation at 300*g* for 5 min at 4 °C.

2.3. Reverse transcription and PCR (RT-PCR)

The total RNA was extracted from the cells, and cDNA was synthesized using 2.0 μg of total RNA with SuperScript III Reverse Transcription Kit (Life Technologies) according to the manufacturer's instructions. PCR was carried out with synthesized cDNA derived from 0.1 μg of total RNA in a final volume of 20 μl using the Advantage 2 PCR Kit (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions. The standard PCR conditions were 95 °C for 10 min, 40 cycles of 94 °C for 1 min, 68 °C for 1 min, and 72 °C for 3 min, followed by treatment at 72 °C for 10 min. The nucleotide sequences of the forward primers were as follows:

1a, 5′-GGGTGAGTAGGAGCAAAATTTCTCATG-3′;
1b, 5′-GAGCTGCAGCTCTTCAGGGAAGAAATC-3′;
1c, 5′-GGAAACCTCATCATCTGAGACCAGCAC-3′;
1312F, 5′-GAGGAGAGGAGGGACCAACCT-3′;
GAPDH, 5′-GATTTGGTCGTATTGGGCGCCT-3′.

The nucleotide sequences of the reverse primers were as follows:

1331R, 5′-GGTTGGTCCCTCCTCTCCTCT-3′;
2ER, 5′-GGCCTTCTGTTGGGATTCTTCTGTCTT-3′;
Ex7-8R, 5′-TTTTCACACTTATGGAGCTCCACAGAG-3′;
GAPDH, 5′-ACTGTGGTCATGAGTCCTTCCACGATA-3′.

Five microliters of PCR products were developed by electrophoresis on 1.0% agarose gels, and the gels were stained with ethidium bromide.

2.4. Construction of plasmids

The primers used for amplification of the 2E131, unspliced, 774-spliced, 789-spliced fragments flanked by the *Kpn* I and *Xba* I sites were as follows: forward, 5′-GGTACCATGTTAACATCACATGTAGG-3′ or 5′-GGTACCAATACTGAAAAATGAAGCCT-3′; reverse, 5′-TCTAGA TGTGACCTCTGCACATACTG-3′. The PCR products were subcloned into pMD20 (TaKaRa Bio, Otsu, Japan) to confirm the nucleotide sequence. Thereafter, the proper fragment was digested with *Kpn* I and *Xba* I, and was then inserted into *Kpn* I/*Xba* I digested pEF6-V5-His (Life Technologies) to construct pEF-2E131, pEF-unspliced, pEF-774-spliced, pEF-789-spliced. pEF6/hST2-V5-His, coding for human soluble ST2 flanked with V5-His, was constructed earlier as described [5].

2.5. Transfection

HEK293T (human embryonic kidney 293T) cells were grown in DMEM supplemented with 10%(v/v) FBS, and transfection was performed with pEF6-V5-His, pEF-2E131, pEF-unspliced,

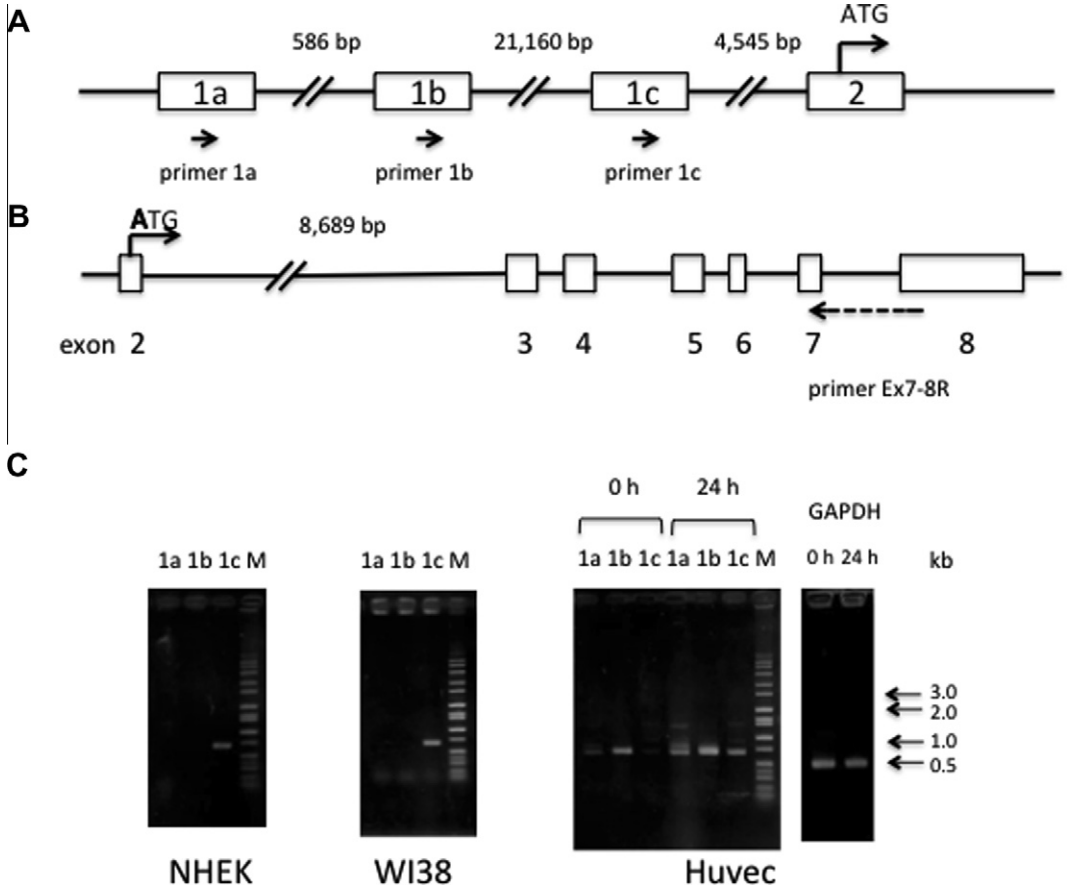

Fig. 1. Cell-type-specific promoter usage of human IL-33 gene. (A) Three reported noncoding exon 1s were named tentatively 1a, 1b, and 1c as described in the text. The distance between each exon is shown above.(B) Exon-intron organization for conventional IL-33 transcript is shown schematically.(C) RT-PCR was carried out with forward primer 1a, 1b, or 1c and reverse primer Ex7-8R. The resultant products were developed by 1% agarose gel electrophoresis as described in Section 2. Cycle number was reduced to 30 in this experiment instead of 40 of the standard condition to detect exon 2E. 0 h and 24 h correspond to proliferating and tubelike formed HUVECs, respectively. The same cDNA samples were analyzed to detect GAPDH by PCR in a condition of 95 °C for 10 min, 30 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min followed by treatment at 72 °C for 10 min, using primers described in Section 2. M represents DNA size markers.

pEF-774-spliced, pEF-789-spliced, or pEF6/hST2-V5-His by a conventional calcium phosphate method [6].

2.6. Western blotting

The culture supernatant was collected and centrifuged to exclude cell debris. The cells attached to 10 cm dishes were washed with PBS, and the cell lysate was prepared by adding 0.5 ml of solubilizing buffer containing 10 mM Tris–HCl (pH 7.8 at 20 °C), 1% (w/v) NP-40, 0.15 M NaCl, 1 mM EDTA, and 1× protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) directly to the cells on the dish. Another 0.5 ml of the solubilizing buffer was added and the remnant cell lysate was recovered. Then the combined solution was sonicated and centrifuged to obtain cleared lysate.

The immunoblotting was performed as described previously [7], with mouse anti-V5 (Life Technologies) and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as the primary antibody and horseradish peroxidase-conjugated anti-mouse IgG (Bio-Rad, Hercules, CA, USA) as the secondary antibody. The bound secondary antibodies were detected using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA).

2.7. Immunohistochemistry

pEF-2E131 was transfected to HEK293T cells cultured on chamber slide glass, and cells were fixed by cold methanol and acetone after 24 h transfection. Empty vector was used as negative control. The cells were treated with primary antibody (anti-V5, 1:200, R960-25, Life Technologies) for overnight at 4 °C, and subsequently with secondary antibody (Alexa Fluor® 546 goat anti-mouse IgG, 1:500, A11003, Life Technologies) for 1 h at room temperature in 20% Blockin One Histo (Nakalai tesque, Kyoto, Japan). Nuclei were stained with Hoechst33342 (1:1000, Wako Chemicals, Osaka, Japan), and slides were mounted in FLUOROSHIELD™ Mounting Medium (ImmunoBioScience, USA). Photographs of cell images were acquired with Biozero BZ-8000 (KEYENCE, Osaka, Japan).

3. Results

We first analyzed the promoter usage of the human IL-33 gene, since three noncoding exon 1s have been deposited in databases so far. Three exon 1s were aligned as shown in Fig. 1A and tentatively named exon 1a <DA713547>, exon 1b <NM_033439.3>, and exon 1c <DA439244> in order of distance. The PCR product between

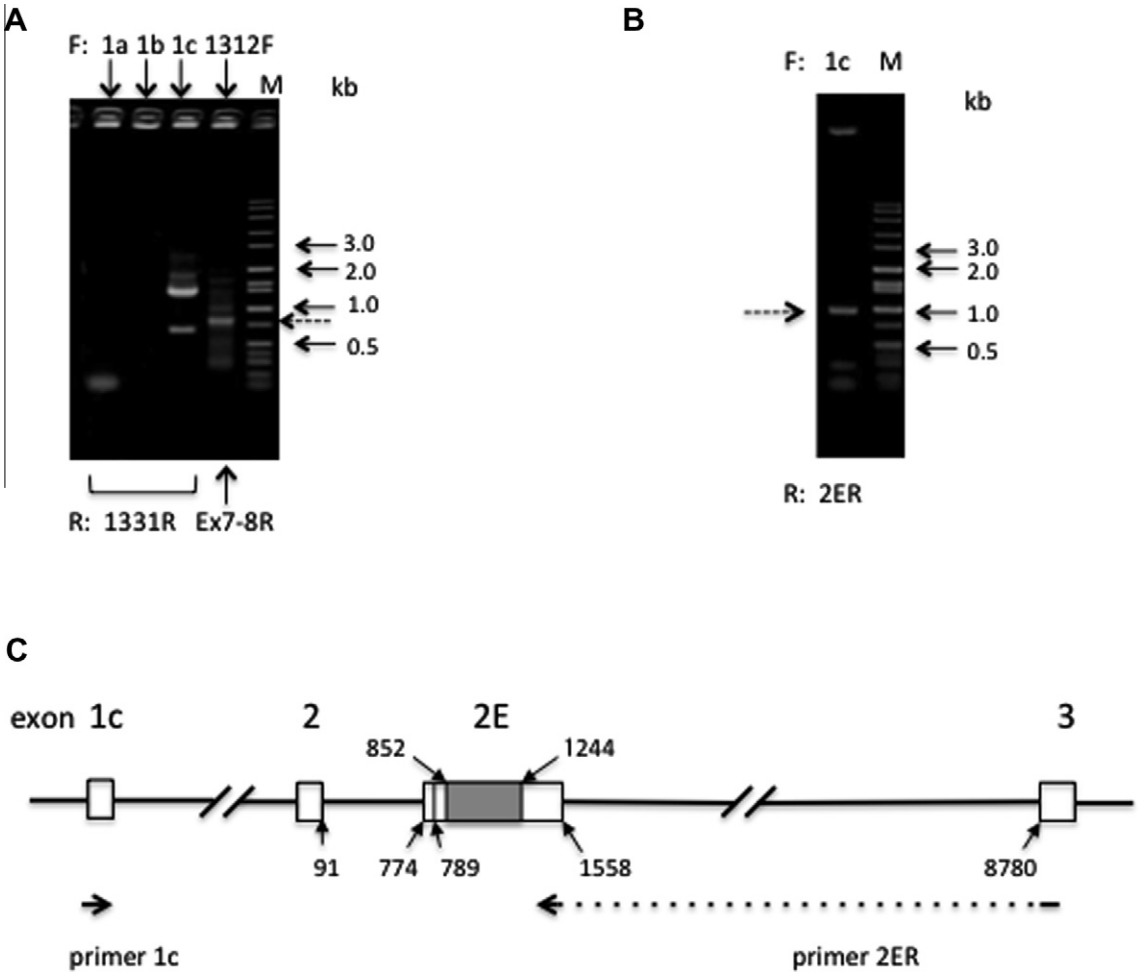

Fig. 2. Promoter flanking exon 1c was used to produce mRNAs containing exon 2E in NHEKs. (A) RT-PCR was carried out as described in Section 2, using forward primers shown above the gel (F) and reverse primers shown below the gel (R). (B) Direct confirmation of the presence of exon 2E with forward primer 1c and bridging reverse primer 2ER (broken arrow). (C) Schematic representation of exon intron organization between 1c and 3 based on the results of the nucleotide sequence determination of (B). Numbers with arrows are nucleotide numbers starting from A in the initiation codon for the conventional IL-33 molecule residing in exon 2. The shaded area corresponds to ORF of 2E131.

the forward primer 1a, 1b, or 1c and the reverse primer Ex7-8R (Fig. 1B) is shown in Fig. 1C. NHEK and WI38 cells used promoter region flanking exon 1c. On the other hand, HUVECs used mainly exon 1b in a continuous growing state (Fig. 1C, 0 h). When HUVECs were cultured on Matrigel and formed tubelike structures (Fig. 1C, 24 h) [4], all three promoters were used. This observation suggests that the promoter usage of IL-33 depended on the cell type, as in the case of ST2 gene promoters reported earlier [8].

Next, we started the search for a possible extra exon in intron 2, which is 8689 nucleotides long. Using more than a dozen forward primers in intron 2 and reverse primers in exon 3, we found several candidate PCR products derived from HUVECs total RNAs (data not shown). Based on this initial screening, the forward primer 1312F, starting from nucleotide number (ntd. No.) 1312, and the reverse primer 1331R, starting from ntd. No. 1331, were synthesized and used for further study. For convenience, we designated ntd. No. 1 corresponding to A of the conventional initiation codon for IL-33 in exon 2 (Fig. 1B, bold letter A).

RT-PCR analysis on total RNAs derived from NHEKs revealed that mRNAs containing a novel exon also used the most proximal promoter, flanking exon 1c (Fig. 2A). Furthermore, nucleotide sequence analysis of the PCR product corresponding to the 3' side of the mRNA (Fig. 2A, broken arrow) showed splicing from ntd. No. 1559 to ntd. No. 8780 of exon 3, which is consistent with the GT-AG rule of splicing (Fig. 2C). Based on this information, we made another reverse primer, 2ER, bridging exons 3 and 2E (named according to extra exon 2).

The PCR products, which was amplified by primers 1c and 2ER (Fig. 2B, broken arrow), were analyzed by nucleotide sequencing, and the final results are shown schematically in Fig. 2C. Interestingly, two very close acceptor sites of the splicing, namely ntd. No. 774 and 789, both matching the GT-AG rule, were found even in the PCR products derived from the same total RNA preparations. Probably, the splicing machinery cannot discriminate between these candidate acceptor sites, and consequently produce two kinds of mRNAs at the same time.

Further studies were performed using HUVECs, since it is well known that proliferating HUVECs can develop into tubelike forms after culturing on Matrigel, and thus would be a nice model for differentiation [4]. Although HUVECs were using promoter 1b rather than 1c, which is the case for NHEKs, the same PCR products containing exon 2E were detected at 12, 24, and 48 h after starting differentiation, but were not detected in proliferating cells (Fig. 3A). The absence of the PCR products at 0 h was not due to RNA degradation (Fig. 3A, GAPDH). Nucleotide sequence determination confirmed the presence of two splicing acceptor sites, as in the case of NHEKs. The upper band in Fig. 3A corresponds to the unspliced product connecting exons 2 and 2E (Fig. 3B). The experiment was repeated three times for 0 h and 24 h, and the results were reproducible.

There was a large open reading frame (ORF) from ntd. No. 852 to ntd. No. 1244 in exon 2E, which was tentatively named the 2E131 protein (Figs. 2C and 3B, shaded area). The nucleotide sequence around the initiation codon of IL-33 and 2E131 were shown in Fig. 3C. The upstream sequence matched Kozak sequence, but the nucleotide immediately after ATG were not G in both cases. Since there are two possible N-glycosylation sites in the molecule (Fig. 4A, N in the thick circle), we constructed an expression plasmid producing the 2E131 protein tailed with the V5-His tag based on the pEF6 vector (see Section 2). Even after digestion with N-glycosidase F for 48 h, the mobility of 2E131 was unchanged in contrast to that of the ST2 protein, which has 9 possible N-glycosylation sites; hence it was hard to obtain complete digestion (Fig. 4B) [9]. Considering the expected molecular weight of recombinant 2E131 and its mobility in SDS–PAGE, the 2E131 protein was devoid of glycosylation, at least in HEK293T cells.

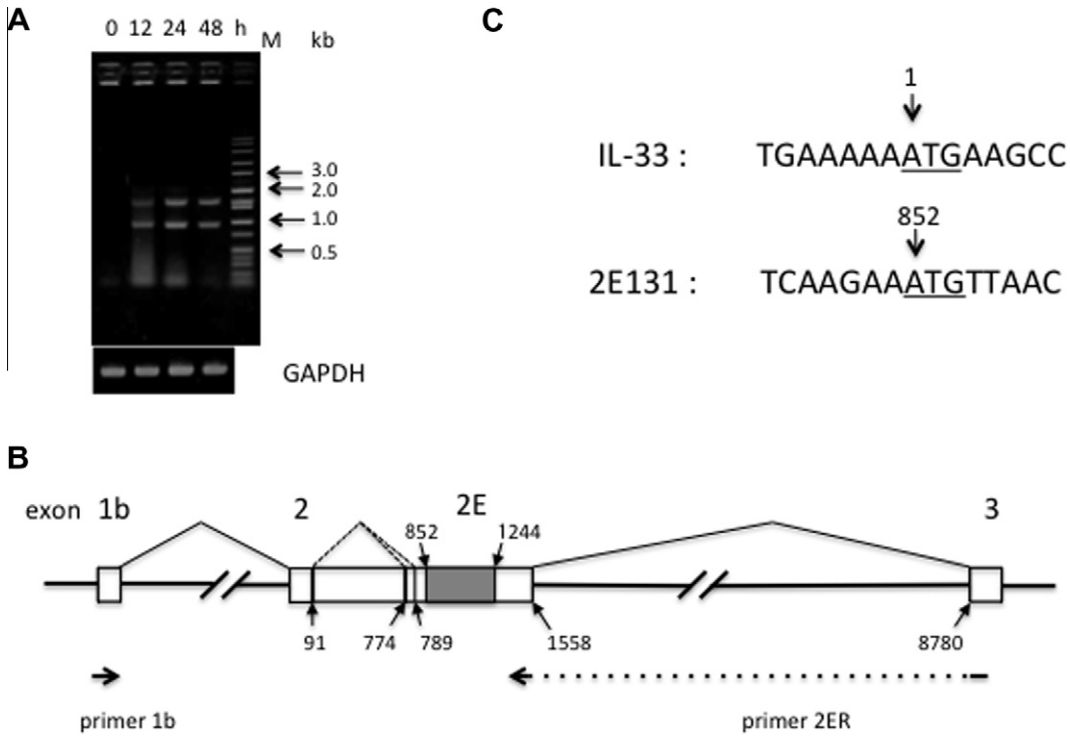

Fig. 3. Tubelike formation-dependent appearance of exon 2E in HUVECs. (A) Total RNAs were extracted before or after 12, 24, or 48 h of spreading on Matrigel, and RT-PCR was carried out as described in Section 2, using primers of 1b and 2ER. The same cDNA samples were analyzed to detect GAPDH by PCR in the condition described in Fig. 1C. (B) Schematic representation of the results of nucleotide sequence determination of the upper and lower bands in (A). Splicing shown by broken lines was carried out in the case of the lower band in (A) but was absent in the case of the upper band in (A). (C) Nucleotide sequence around the initiation codon of IL-33 and that of 2E131 are shown.

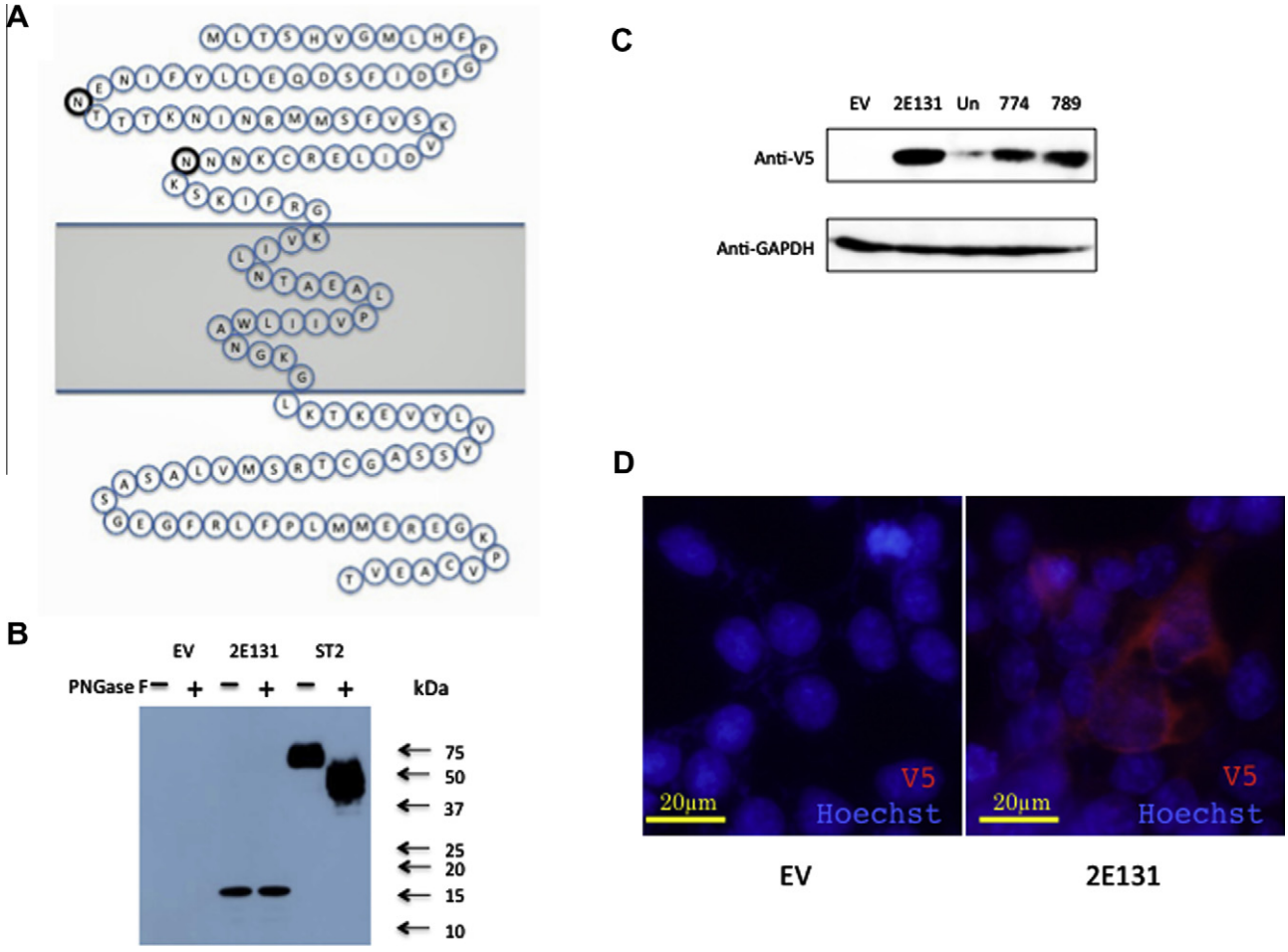

Fig. 4. Deduced amino acid sequence of 2E131. (A) Secondary structure prediction was according to [11]. Amino acids in the shaded region represent the putative transmembrane portion. N in thick circles indicates possible N-glycosylation sites. (B) pEF-2E131 and pEF6/hST2-V5-His were constructed and transfected to HEK293T cells as described in Section 2. pEF6-V5-His (empty vector, EV) was transfected to HEK293T cells as a control. The cell lysate (EV and 2E131) or culture supernatant (ST2) was analyzed by Western blotting with anti-V5 antibody. Each sample was treated (+) or untreated (−) with N-glycosidase F (Roche Applied Science, Mannheim, Germany) according to [12]. (C) pEF6-V5-His, pEF-2E131, pEF-unspliced, pEF-774-spliced, pEF-789-spliced were transfected to HEK293T cells. The cell lysate was analyzed by Western blotting with anti-V5 and anti-GAPDH antibodies. Un, 774, and 789 indicate the cell lysate derived from the transfected cells with pEF-unspliced, pEF-774-spliced, and pEF-789-spliced, respectively. (D) HEK293T cells transfected with pEF-2E131 were observed by fluorescent microscopy. Anti-V5 (red) and Hoechst33342 (blue) images were merged by BZ-8000 analysis software. Left; pEF6-V5-His (EV), Right; pEF-2E131 (2E131).

Besides the unspliced product, we also detected and cloned two spliced products from lower bands (Fig. 3A). These spliced products were deleted the region from 92 to 773 and the region from 92 to 788 as introns, namely 774-spliced and 789-spliced, respectively (Fig. 3B). To confirm whether ATG (No. 852) in exon 2E is a functional initiation codon in the unspliced and two spliced products, we constructed three 2E131-expressing plasmids with different lengths in the upstream regions of ATG, pEF-unspliced, pEF-774-spliced, and pEF-789-spliced. The 2E131 protein was expressed from all constructs in HEK293T cells (Fig. 4C). As the length of upstream region was getting shorter, the level of 2E131expression increased. This result indicates that ATG (No. 852) is a functional initiation codon for the 2E131 protein.

For subcellular localization, we performed immunohistochemistry. As shown in Fig. 4D, the 2E131 protein was expressed mainly in cytoplasmic region.

4. Discussion

There were three splicing patterns between exon 2 and exon 2E: without splicing, or with landing at ntd. No. 774 or 789. In all of the cases of splicing described above, conventional IL-33 protein starting from ATG codon at ntd. No. 1 was prematurely terminated at either ntd. No. 112 or ntd. No. 809 due to the appearance of a termination codon. The longest ORF corresponded to N-terminal truncated IL-33 starting from Met 48 coded in exon 3 in the conventional IL-33 molecule (M48 IL-33). The second-longest ORF was found just in exon 2E, which coded for a 131 amino acid putative transmembrane protein (2E131), shown as the shaded areas in Figs. 2C and 3B. According to sequence analysis, the upper band in the case of NHEKs (Fig. 2A) is the unspliced product connecting exons 2 and 2E and contains the whole 2E131 coding region, but repeated trial of 3'-RACE (rapid amplification of cDNA ends) to identify prematurely terminated RNAs has been unsuccessful thus far. Therefore, we have to leave the band as uncharacterized at present.

Confirmation of ORF expression requires the detection of the protein product directly. Therefore, we tried to make polyclonal antibodies against two candidate peptides synthesized according to epitope prediction. Unfortunately, purified antisera did not work even for the recombinant 2E131. Production of good antibodies is indispensable for this question.

The experiment using immunohistochemistry suggested the subcellular localization of 2E131 in cytoplasm (Fig. 4D). Since

2E131 protein was not N-glycosylated as shown in Fig. 4B, and possesses no signal peptide, 2E131 may be classified to type II membrane protein. Actually, N-terminal sequence adjacent to the putative transmembrane portion is rich in positively charged amino acids (Fig. 4A) [10]. If 2E131 protein resides in inner membranes, it is tempting to speculate that 2E131 has some role in IL-33 translocation from nucleus to cytoplasm and/or processing of IL-33 before secretion, since both molecules are produced from the human IL-33 gene.

Although it is too early to suggest that the appearance of exon 2E is directly related to differentiation, as shown in the experiment using HUVECs (Fig. 3A), the possible expression of a specific product derived from mRNA containing exon 2E in relation to differentiation is intriguing. Of course, the confirmation of 2E131 protein expression in human cells and the elucidation of its biological functions, if any, await further research. The presence of the same mRNAs containing exon 2E in both endothelial cells and keratinocytes motivates us to continue our study in relation to the IL-33-ST2 axis.

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