Promoter structure and transcription initiation sites of the human death receptor 5/TRAIL-R2 gene[1]

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Abstract The death receptor 5 (DR5) is a receptor for tumor necrosis factor-related apoptosis-inducing ligand and is able to induce apoptosis in various tumor cells. The expression of DR5 is up-regulated at the transcriptional level by p53, genotoxic stress and so on. To investigate the structure of the DR5 gene promoter, we screened and sequenced a genomic clone containing the 5′-flanking region of the DR5 gene. RNase protection assays showed two major transcription start sites around −122 and −137 upstream of the translation initiation codon ATG. Transient transfections with serial 5′-deletion mutants identified the minimal promoter element spanning −198 to −116. Site-directed mutagenesis demonstrated that the DR5 gene promoter has no typical TATA-box, but has two Sp1 sites responsible for the basal transcription activity of the DR5 gene promoter.

Key words: Death receptor 5; Tumor necrosis factor-related apoptosis-inducing ligand; Promoter; Transcription; Sp1 site

1. Introduction

Death receptor 5 (DR5, also called tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-R2, Apo2, TRICK2 or KILLER) [1–5] is a member of the TNF receptor family and is a receptor for TRAIL [1–5]. TRAIL is selectively cytotoxic against tumor cells but not against normal cells in vitro and in vivo [6–10], which therefore make it a good target molecule for cancer therapy. To date, four receptors for TRAIL including DR4, DR5, DcR1 and DcR2 have been identified [1–5,11]. DR4 and DR5 induce apoptosis through an intracellular death domain [1–5]. In contrast, DcRs do not induce apoptosis due to the presence of mutation or deletion in the death domain [1–5,11]. On the other hand, a tumor-suppressor gene p53 has been reported to transactivate DR5 gene expression [12–14]. In addition, genotoxic reagents such as doxorubicin, etoposide and MMS, and γ-radiation are also known to induce the expression of DR5 in a p53-dependent or -independent manner [15–19]. The synthetic retinoid CD437 induces apoptosis together with up-regulation of DR5 in lung and prostate carcinoma cells [20–22]. Moreover, the synthetic glucocorticoid dexamethasone and the cytokine interferon-γ induce apoptosis and DR5 expression in cell lines with mutant p53 [23].

As stated above, DR5 gene is a key target molecule for cancer therapy and the expression of the gene is regulated by a variety of factors. However, even the basic structure of the DR5 promoter has not been reported. To investigate the regulatory mechanisms of DR5 gene expression, it is crucial to determine the basic structure of the DR5 gene promoter. Thus, in this study, we clarified the basic structure, transcription initiation sites, and minimal promoter elements of the human DR5 gene promoter.

2. Materials and methods

2.1. Cloning of the DR5 promoter region

On the basis of human DR5 cDNA sequences [1–5], two oligonucleotides, 5′-CCGCAATCTCTGCGCCCACAAAATACACCG (sense) and 5′-GTTTCAGCCCTTAAAGTAGATCGGGCATCG (antisense), were synthesized. These oligonucleotides were labeled with [γ-^{32}P]ATP for use as probes, and used to screen the human λPS library (Mo Bi Tec, Göttingen, Germany).

2.2. RNase protection assay

PCR products produced from DR5 genomic DNA were subcloned into the pGEM T-easy vector (Promega). RNA probes were synthesized with T7 RNA polymerase (MAXI script, Ambion). The RNase protection assay was carried out using an RPA III kit (Ambion). The sequencing reaction for preparing size markers was performed using a T7 Sequencing kit (Amersham Pharmacia Biotech).

2.3. Plasmid construction

*Sac*I–*Nco*I fragment digested from the DR5 promoter region of genomic DNA was subcloned into the *Sac*I–*Nco*I site of pGVB2 luciferase assay vector (Toyo ink, Tokyo, Japan) to produce pDR5/*Sac*I. pDR5/*Bam*HI was generated by subcloning *Bam*HI–*Nco*I fragment of DR5 genomic DNA into pGVB2. Other deletion mutants were generated with deletion kits (Takara, Tokyo, Japan) after *Sac*I–*Hin*dIII digestion of pDR5/*Sac*I. Mutations in the TATA-like box and two Sp1 sites were generated with a QuickChange Site-Directed Mutagenesis kit (Stratagene).

2.4. Cell culture, transfection and luciferase assays

MCF7 cells were maintained at 37°C in Dulbecco's modified Eagle medium with 10% fetal calf serum. Culture cells (3×10^4/well) were seeded on 12-well plates 24 h before transfection. Plasmids (0.5 μg) were transfected into cells using the DEAE-dextran method (Cell-Phect, Amersham Pharmacia Biotech). After 48 h, the cells were harvested. Then luciferase assays were performed using luciferase assay

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[1] The nucleotide sequence of the DR5 gene promoter is in the DDBJ/EMBL/GenBank databases with the following accession number: AB054004.

Abbreviations: DR5, death receptor 5; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TNF, tumor necrosis factor; β-gal, β-galactosidase

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reagents (Promega) and a luminometer. Each experiment was repeated at least three times. Transfection efficiency was standardized by co-transfection of the pACT-β-galactosidase (β-gal) plasmid (kind gift from Dr. S. Ishii). Data were analyzed using a Student's *t*-test and differences were considered significant from controls when $P < 0.05$.

3. Results and discussion

3.1. Cloning and nucleotide sequence of the 5′-flanking region of human DR5 gene

We carried out molecular cloning, and clarified the sequences of the 5′-flanking region of DR5 gene from a human genomic library. As shown in Fig. 1, there are multiple potential transcription regulatory factor binding sites that might transcriptionally regulate DR5 gene expression, such as c-Ets 2, AML-1a, c-Myb, Sp1 and GATA-1. The p53 transactivates DR5 gene through an intronic p53 binding site [14]. We also found a p53 binding site at the same region (+235 to +254). Interestingly, the nuclear factor κB (NFκB) binding site lies between +385 and +394 in intron 1. Gibson et al. reported that the increase in DR5 expression following etoposide treatment was blocked by inhibition of NFκB activation, and proposed that etoposide-induced expression of DR5 is mediated through an NFκB signaling pathway [15]. In addition, NFκB stimulates TRAIL-induced apoptosis by activation of DR5 [24]. It might be possible that the NFκB activates DR5 ex-

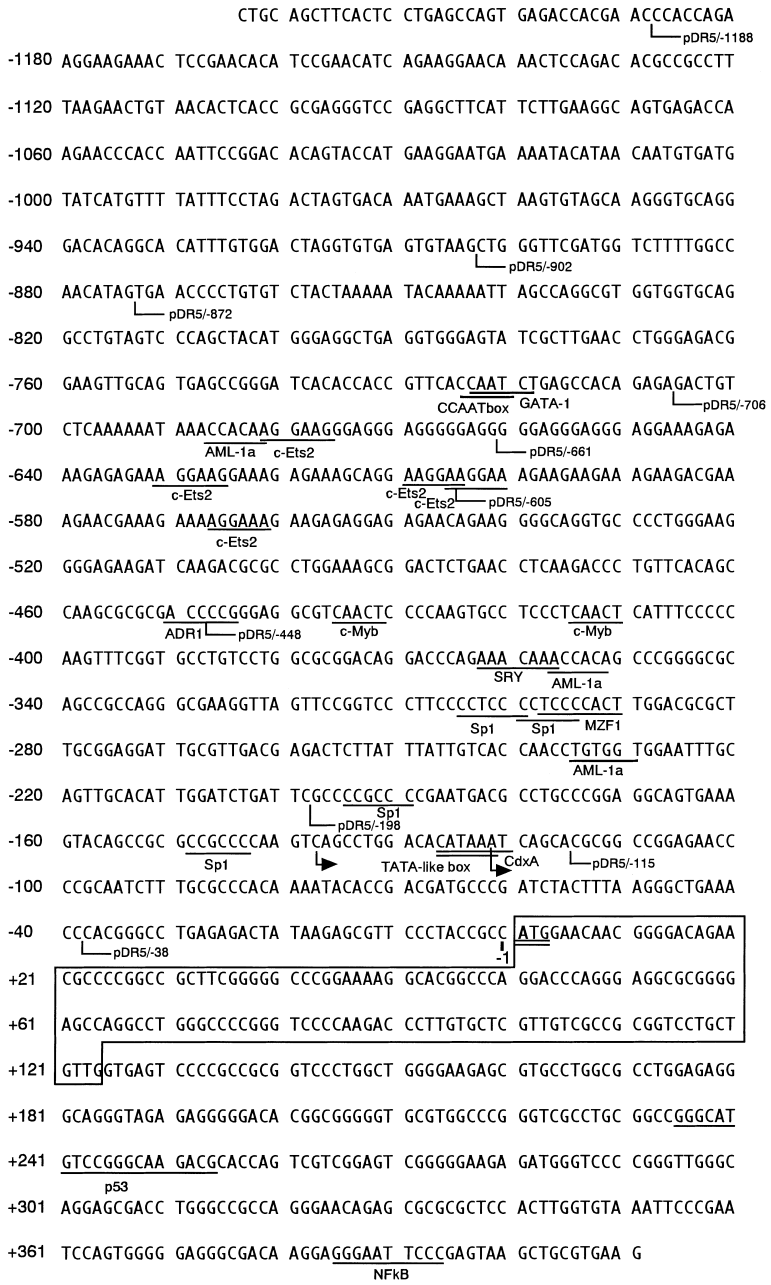

Fig. 1. The 5′-flanking region of the human DR5 gene. The ATG translational initiation codon is shown by the double underline and the adenine residue is designated as +1. The binding sites of potential transcription factors are underlined and indicated below the sequence. The transcription start sites mapped in Fig. 2 are demonstrated with arrowheads. The terminal ends of 5′-deleted constructs are shown at each base. The coding region in exon 1 is boxed.

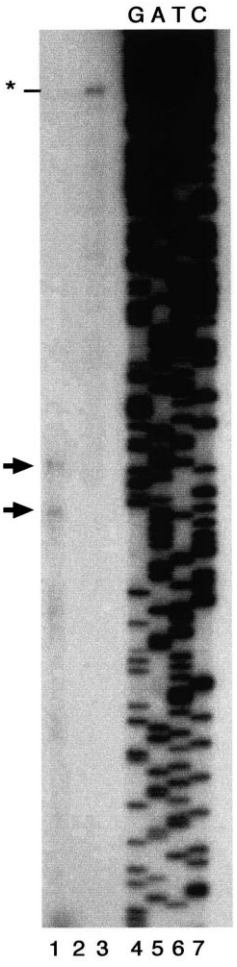

Fig. 2. RNase protection assay to determine the transcription start sites of the human DR5 gene. ^{32}P-labeled antisense riboprobe spanning −347 to +45 was hybridized to total RNA isolated from MCF7 cells and yeast tRNA. The fragments protected by RNase A and T1 digestion were separated on a denaturing polyacrylamide gel. Major transcription start sites at −137 and −122 (arrows) were identified by comparison to the nucleotide sequence of M13 mp18 ssDNA. *: non-digested riboprobe.

pression via the binding site in intron 1. The sequence between −680 and −541 of the 5′-flanking region of the DR5 gene was rich in G and A. TRAIL is a ligand for DR5, and the promoter region of TRAIL also contains a GA rich region [25]. Both DR5 and TRAIL genes may be regulated via this region.

3.2. Identification of transcription initiation sites of DR5 gene

We performed an RNase protection assay to determine the transcription initiation sites of the human DR5. Total RNAs were prepared from MCF7 cells in which DR5 is expressed [18]. RNAs were hybridized with DR5 genomic sequence-derived RNA probes, and the RNA fragments digested by RNase A and T1 were analyzed. The major longer signals were detected around −137 and −122 upstream of the first ATG translation start codon (Fig. 2, lane 1). These protection patterns seemed to be sequence-specific because yeast RNAs did not provide such signals (Fig. 2, lane 2).

3.3. Demonstration of promoter activity of the DR5 gene

*Sac*I–*Nco*I fragment of the 5′-flanking region of the DR5 gene was subcloned into luciferase assay vector and transient luciferase assay was performed (Fig. 3). The full length construct (pDR5/*Sac*I) demonstrated enough luciferase activity compared with that using vacant vector, pGVB2. This finding indicates that the fragment of the 5′-flanking region has authentic promoter activity. Next we generated a series of 5′-deletion mutants. In shorter constructs than pDR5/−605, promoter activities gradually decreased. However, promoter activities of DR5/−115 and DR5/−38 were almost the same as that of vacant vector, pGVB2. These findings indicate that the region spanning −198 to −116 contains the minimal promoter element of the human DR5 gene. Transcription start sites (−137 and −122) illustrated in Fig. 2 are contained within this putative minimal promoter region, which supports this idea.

3.4. Two Sp1 sites are involved in transcription activation of the DR5 gene

The region spanning −198 to −116 contains two Sp1 sites and a TATA-like box site as typical transcription factor binding sites (Fig. 1). Next, we generated constructs harboring mutations in the TATA-like box and two Sp1 sites, and car-

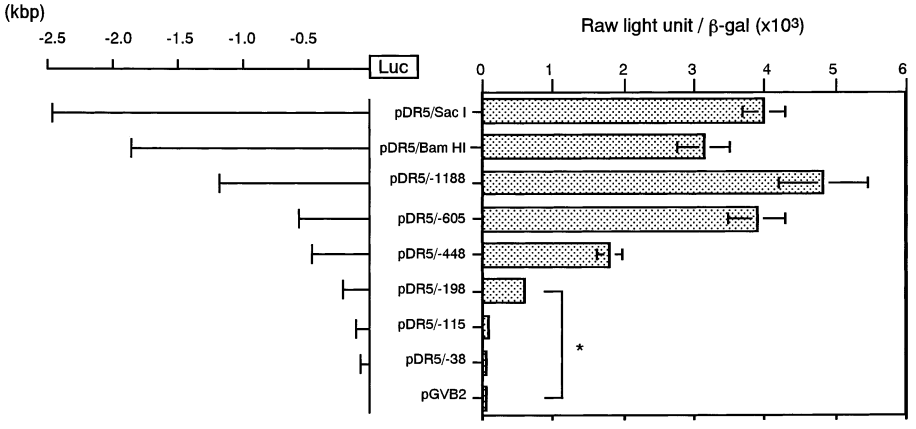

Fig. 3. Deletion analysis of DR5 gene promoter. The reporter plasmids containing various sizes of 5′-deleted human DR5 promoter and luciferase genes were transfected into MCF7 cells. Each activity was normalized by the activity of cotransfected pACT-β-gal (kind gift from Dr. S. Ishii). Data are shown as means ± S.E. ($n = 3$). Structures of relevant plasmids used in this experiment are shown on the left. *$P < 0.01$.

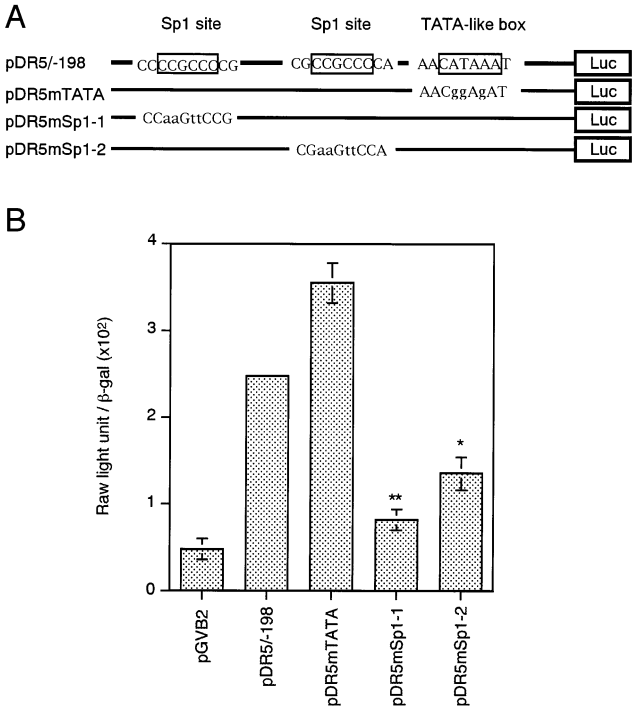

Fig. 4. Mutation analysis of human DR5 gene promoter. A: Structure of reporter plasmids used in this experiment. Two Sp1 sites and TATA-like box were boxed. Substituted nucleotides are shown as small characters. B: The reporter plasmids were transfected into MCF7 cells. Each activity was normalized by the activity of co-transfected pACT-β-gal. Data are shown as means ± S.E. ($n = 3$). $^{**}P < 0.002$, $^{*}P < 0.025$ against the activity by pDR5/−198.

ried out the transient luciferase assay (Fig. 4). Each mutation in the Sp1 sites decreased the promoter activity. In contrast, mutations in the TATA-like box somewhat stimulated the promoter activity. These findings indicate that the DR5 gene promoter is a TATA-less promoter, and that the two Sp1 sites are important for basal transcription activity. Ubiquitously expressed genes often lack a TATA-box but have GC-boxes such as Sp1 binding sites within their promoters [26,27]. The human DR5 gene is expressed ubiquitously in multiple tissues. The findings of this study show that the human DR5 gene promoter belongs to the class of ubiquitously active, TATA-less and GC-box-containing promoters.

The DR5 gene is regulated by p53, which is a tumor-suppressor gene and deficient in more than half of all malignant tumors. Therefore, chemical compounds that can stimulate the DR5 gene should compensate for the absent function of p53. We proposed that methods of up-regulating p53 target genes would be useful for cancer therapy, and termed this method 'gene-regulating chemotherapy' [28,29]. As a model for this, histone deacetylase inhibitors such as butyrate or trichostatin A stimulate the p21/WAF1 gene, being a p53 target gene, through the Sp1 sites of the promoter independent of p53, resulting in cell cycle arrest [30,31]. Thus, our findings and methods in this report may be useful for screening the regulators of human DR5 gene expression, and for analyzing these regulatory mechanisms in the hope that this will lead to novel cancer therapy.

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References

[1] Pan, G., Ni, J., Wei, Y.-F., Yu, G.-L., Gentz, R. and Dixit, V.M. (1997) Science 277, 815–818.
[2] Sheridan, J.P., Marsters, S.A., Pitti, R.M., Gurney, A., Skubatch, M., Baldwin, D., Ramakrishnan, L., Gray, C.L., Baker, K., Wood, W.L., Goddard, A.D., Godowski, P. and Ashkenazi, A. (1997) Science 277, 818–821.
[3] MacFarlance, M., Ahmad, M., Srinivasula, S.M., Fernandes-Alnemri, T., Cohen, G.M. and Alnemri, E. (1997) J. Biol. Chem. 272, 25417–25420.
[4] Walczak, H., Degli-Eaposti, M.A., Johnson, R.S., Smolak, P.J., Waugh, J.Y., Boiani, N., Timour, M.S., Gerhart, M.J., Schooley, K.A., Smith, C.A., Goodwin, R.G. and Rauch, C.T. (1997) EMBO J. 16, 5386–5397.
[5] Screaton, G.R., Mongkolsapaya, J., Xu, X.N., Cowper, A.E., McMichael, A.J. and Bell, J. (1997) Curr. Biol. 7, 693–696.
[6] Pitti, R.M., Marsters, S.A., Ruppert, S., Donahue, C.J., Moore, A. and Ashkenazi, A. (1996) J. Biol. Chem. 271, 12687–12690.
[7] Walczak, H., Miller, R.E., Ariail, K., Gliniak, B., Griffith, T.S., Kubin, M., Chin, W., Jones, J., Woodward, A., Le, T., Smith, C., Smolak, P., Goodwin, R.G., Rauch, C.T., Schuh, J.C. and Lynch, D.H. (1999) Nat. Med. 5, 157–163.
[8] Ashkenazi, A., Pai, R.C., Fong, S., Leung, S., Lawrence, D.A., Marsters, S.A., Blackie, C., Chang, L., McMurtrey, A.E., Hebert, A., DeForge, L., Koumenis, I.L., Lewis, D., Harris, L., Bussiere, J., Koeppen, H., Shahrokh, Z. and Schwall, R.H. (1999) J. Clin. Invest. 104, 155–162.
[9] Keane, M.M., Ettenberg, S.A., Nau, M.M., Russell, E.K. and Lipkowitz, S. (1999) Cancer Res. 59, 734–741.
[10] Lawrence, D., Shhrokh, Z., Marsters, S., Achilles, K., Shih, D., Mounho, B., Hillan, K., Totpal, K., DeForge, L., Schow, P., Hooley, J., Sherwood, S., Pai, R., Leung, S., Khan, L., Glinial, B., Bussiere, J., Smith, C.A., Strom, S.S., Kelley, S., Fox, J.A., Thomas, D. and Ashkenazi, A. (2001) Nat. Med. 7, 383–385.
[11] Ashkenazi, A. and Dixit, V.M. (1999) Curr. Opin. Cell Biol. 11, 255–260.
[12] Wu, G.S., Burns, T.F., McDonald III, E.R., Jiang, W., Meng, R., Krantz, I.D., Kao, G., Gan, D.D., Zhou, J.Y., Muschel, R., Hamilton, S.R., Spinner, N.B., Markowitz, S., Wu, G. and El-Deiry, W.S. (1997) Nat. Genet. 17, 141–143.
[13] Wu, G.S., Burns, T.F., McDonald III, E.R., Meng, R.D., Kao, G., Muschel, R., Yen, T. and El-Deiry, W.S. (1999) Oncogene 18, 6411–6418.
[14] Takimoto, R. and El-Deiry, W.S. (2000) Oncogene 19, 1735–1743.
[15] Gibson, S.B., Oyer, R., Spalding, A.C., Anderson, S.M. and Johnson, G.L. (2000) Mol. Cell. Biol. 20, 205–212.
[16] Nagane, M., Pan, G., Weddle, J.J., Dixit, V.M., Cavenee, W.K. and Huang, H.-S. (2000) Cancer Res. 60, 847–853.
[17] Wen, J., Ramadevi, N., Nguyen, D., Perkins, C., Worthington, E. and Bhalla, K. (2000) Blood 96, 3900–3906.
[18] Sheikh, M.S., Burns, T.F., Huang, Y., Wu, G.S., Amundson, S., Brooks, K.S., Fornace Jr., A.J. and El-Deiry, W.S. (1998) Cancer Res. 58, 1593–1598.
[19] Gong, B. and Almasan, A. (2000) Cancer Res. 60, 5754–5760.
[20] Sun, S.Y., Yue, P., Wu, G.S., El-Deiry, W.S., Shroot, B., Hong, W.K. and Lotan, R. (1999) Oncogene 18, 2357–2365.
[21] Sun, S.Y., Yue, P. and Lotan, R. (2000) Oncogene 19, 4513–4522.
[22] Sun, S.Y., Yue, P., Hong, W.K. and Lotan, R. (2000) Cancer Res. 60, 7149–7155.
[23] Meng, R.D. and El-Deiry, W.S. (2001) Exp. Cell Res. 262, 154–169.
[24] Ravi, R., Bedi, G.C., Engstrom, L.W., Zeng, Q., Mookerjee, B., Gelinas, C., Fuchs, E.J. and Bedi, A. (2001) Nat. Cell Biol. 3, 409–416.
[25] Wang, Q., Ji, Y., Wang, X. and Evers, B. (2000) Biochem. Biophys. Res. Commun. 276, 466–471.

[26] Melton, D.W., McEwan, C., McKie, A.B. and Reid, A.M. (1986) Cell 44, 319–328.
[27] Ohbayashi, T., Schmidt, E.E., Makino, Y., Kishimoto, T., Nabeshima, Y., Muramatsu, M. and Tamura, T. (1996) Biochem. Biophys. Res. Commun. 225, 275–280.
[28] Sowa, Y. and Sakai, T. (2000) BioFactors 12, 283–287.
[29] Sakai, T. (1996) Jpn. J. Hyg. 50, 1036–1046.
[30] Nakano, K., Mizuno, T., Sowa, Y., Orita, T., Yoshino, T., Okuyama, Y., Fujita, T., Fujita, N., Matsukawa, Y., Tokino, T., Yamagishi, H., Oka, T., Nomura, H. and Sakai, T. (1997) J. Biol. Chem. 272, 22199–22206.
[31] Sowa, Y., Orita, T., Minamikawa-Hiranabe, S., Mizuno, T., Nomura, H. and Sakai, T. (1999) Cancer Res. 59, 4266–4270.