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Transcription initiation sites and promoter structure of the human TRAIL-R3 gene[1]

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Abstract TRAIL-R3 is a decoy receptor for TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), a member of the tumor necrosis factor ligand family. In several cell types decoy receptors inhibit TRAIL-induced apoptosis by binding TRAIL and preventing its binding to TRAIL pro-apoptotic receptors. Here we report the cloning of the promoter region of human TRAIL-R3 and the mapping of the transcriptional start sites. This gene contains a consensus TATA box and the minimal promoter lies within the first 33 nucleotides upstream of the transcription start site. Transient transfection assays of luciferase reporter plasmids demonstrate that human TRAIL-R3 promoter can be induced in doxorubicin-treated MCF-7 cells in a p53-independent manner.

Key words: TRAIL-R3; Decoy receptor; Promoter structure; Transcription; Apoptosis; Doxorubicin

1. Introduction

TRAIL (tumor necrosis factor (TNF)-related apoptosis-inducing Ligand) is a member of the TNF ligand family [1,2]. As with other members, TRAIL is a type II transmembrane protein which is able to induce apoptosis after binding to its pro-apoptotic membrane receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5/TRICK2/KILLER) [3,4]. Unlike Fas ligand and TNF-α, TRAIL selectively induces apoptosis in tumor cells without being toxic in non-transformed cells [5]. This observation raises expectations regarding the clinical use of TRAIL as a new anti-tumor agent. Great efforts are being made to elucidate TRAIL biology and the mechanisms of TRAIL-induced apoptosis. TRAIL binds to four membrane receptors [6]. The pro-apoptotic receptors TRAIL-R1 [7] and TRAIL-R2 [8] contain a cytoplasmic death domain which is involved in the apoptotic signal. In contrast, the decoy receptors TRAIL-R3 (DcR1/TRID/LIT) [7,9–11] and TRAIL-R4 (DcR2/TRUNDD) [12] do not signal apoptosis since they either lack the intracellular death domain (TRAIL-R3) or have it truncated (TRAIL-R4). In several cell types the decoy receptors have been reported to inhibit TRAIL-induced apoptosis by competing the binding of TRAIL to the pro-apoptotic receptors [13]. However, other mechanisms can also be responsible for the inhibition of TRAIL-mediated apoptosis as in certain cells lacking decoy receptors resistance to TRAIL has been reported [14,15].

TRAIL-R3 is a glycosylphosphatidylinositol (GPI)-anchored cell surface protein that may prevent TRAIL from binding to pro-apoptotic receptors. In this respect, cleavage of the GPI anchor by phospholipase C treatment of TRAIL-R3-expressing cells could result in marked sensitization to TRAIL-induced apoptosis [16]. In addition, forced expression of TRAIL-R3 in TRAIL-sensitive cells reduces ligand-induced apoptosis [7,9]. Interestingly, TRAIL-R3 is highly expressed in a majority of normal human tissues while very low levels of mRNA were found when different cell lines representing various tumor types were analyzed [7,9,10]. These observations have made TRAIL-R3 a possible marker for tumor resistance.

Regulation of TRAIL receptors has been commonly studied at mRNA and protein levels, but little is known regarding the basic structure of TRAIL receptor promoters and the transcription factors that control their expression. The involvement of p53 in the regulation of the TRAIL-R2 promoter has been described [17], and hypermethylation of the human TRAIL-R3 and TRAIL-R4 promoters has been suggested to be important for the down-regulation of these receptors in neuroblastoma and other tumor types [18]. It has been recently reported that activator protein 1 (AP-1) regulates TRAIL-R1 expression via the AP-1 binding site located at −350/−344 within the gene promoter region [19]. Earlier findings demonstrated that TRAIL-R3 mRNA levels are enhanced by genotoxic agents and ionizing radiation in a p53 wild-type cell line [20]. Later, it was shown that induction of expression of the TRAIL-R3 gene in response to DNA damage is restricted to p53 wild-type cell lines [21]. In addition, transcription factors of the NF-κB family may also be involved in the regulation of TRAIL-R3 gene expression [22].

Despite the publication of most of the human genome sequence [23], there are still some gaps and so far the promoter structure and the genomic organization of human TRAIL-R3 are not available. In this paper we describe the TRAIL-R3 promoter sequence and we identify the transcription initiation

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[1] The nucleotide sequence of the human TRAIL-R3 promoter has been submitted to GenBank (accession number AF524869).

Abbreviations: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TNF, tumor necrosis factor; GPI, glycosylphosphatidylinositol; AP-1, activator protein 1

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sites, as well as minimal functional promoter elements. The induction of promoter activity by the anti-tumor drug doxorubicin is also addressed.

2. Materials and methods

2.1. Cell culture

MCF-7 human breast cancer cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 40 mg/l gentamicin at 37°C in a humidified 5% CO_2/95% air incubator. MCF-7 cells stably expressing the human papillomavirus type 16 E6 protein (MCF-7^{E6}) were maintained in culture as described [24]. Doxorubicin (Sigma) was employed at 500 ng/ml.

2.2. Cloning of the human TRAIL-R3 promoter

The genomic sequence lying upstream of the human TRAIL-R3 cDNA was isolated employing the Human Promoter Finder DNA Walking Kit (Clontech) according to the manufacturer's instructions. Polymerase chain reactions (PCRs) were carried out using human TRAIL-R3-specific primers corresponding to the 5′ region of the published cDNA sequence (GenBank accession number AF012536) and the supplied adapter primers. The sequences of the gene-specific primers were TR3#3rp (5′-CTATCAGAAATCGTTGGCTGAGTT-CCCTAACGGTAG-3) and TR3#4rp (5′-CTGAGTTCGTGCGCG-TGGAGAGGTTCC-3′). A single 2.7 kb PCR product from the secondary reactions was cloned directly into the pGEM-T vector (Promega).

2.3. Plasmid constructs

TRAIL-R3 promoter constructs were created by cloning a 1683 bp *Bam*HI–*Sal*I DNA fragment from the pGEM-T 2.7 kb construct described above into the *Bam*HI–*Sal*I sites of the pXP2-Luc reporter plasmid [25]. The 506 bp, 126 bp and 33 bp pXP2 reporter constructs were made from the 1683 bp pXP2 plasmid by using unique restriction sites within the TRAIL-R3-specific DNA fragment. This construct was digested with *Bam*HI combined with either *Hin*dIII, *Stu*I or *Sac*II enzymes, the 5′ ends were refilled and the blunt-ended plasmid religated. As an internal negative control, a 2.7 kb antisense construct was obtained by cloning the 2.7 kb *Sal*I fragment from the pGEMT vector into the *Sal*I-digested pXP2 vector. The pC53-SN3 (wt p53) plasmid was generously provided by Dr. M. Oren (Weizmann Institute of Science, Rehovot, Israel). The pI-391LUC reporter plasmid has been recently described [26]. All restriction enzymes were obtained from Roche Biochemicals. T4 DNA ligase and T4 DNA polymerase were purchased from New England Biolabs.

2.4. S1 nuclease analysis

For nuclease S1 protection assay, an antisense oligonucleotide (5′-TGAGTTCGTGCGCGTGGAGAGGTTCCCACAGCTGCACTGC-CAGAATCGGAG-3′) spanning the putative initiation site (based on the consensus TATA box detected within the promoter sequence) was designed. This oligonucleotide was kinased using the polynucleotide kinase enzyme (Roche Biochemicals) and [γ-^{32}P]ATP; the labelled oligonucleotide was purified in a G25 Spin Column (Roche Biochemicals).

Total RNA from MCF-7 or mouse liver cells was extracted using Trizol Reagent (Invitrogen) according to the manufacturer's protocol. Fifty micrograms total RNA was precipitated and resuspended into 15 μl of 2×hybridization buffer. The RNA solution was then mixed with 10^6 cpm of ^{32}P-labelled oligonucleotide and 15 μl of formamide, heated at 68°C for 10 min and incubated overnight at 37°C. After this incubation, samples were treated with S1 nuclease (Gibco-BRL) for 30 min at 37°C according to the manufacturer's instructions. The S1-digested products were analyzed on a denaturing 8% polyacrylamide gel. Four microliters of the non-digested and diluted (1/800) labelled oligonucleotide (i.e. probe) was run at the same time. For a detailed protocol visit our website: http://www2.cbm.uam.es/bc-015.

2.5. Primer extension assays

Primer extension assays were performed employing the M-MLV reverse transcriptase (Gibco-BRL). The antisense oligonucleotides TR3#1rp (5′-CGCTCTGTCCCCAGAGTTCCCTAACGGTAG-3′) and PE#2 (5′-GCTCCTTCACCCCTTGCATCTCTGG-3′) were end-labelled with [γ-^{32}P]ATP to a specific activity of 4–6×10^{10} cpm/μg. 1.5×10^6 cpm of labelled oligonucleotide was mixed with 20 μg of total RNA from either control or 24 h doxorubicin-treated MCF-7 (20 μg of tRNA was employed as a negative control), dNTPs at a final concentration of 2 mM, dithiothreitol (final concentration 0.01 M) and First Strand Buffer. The samples were heated at 68°C for 5 min and then allowed to cool down to 42°C. The annealed primers were extended with M-MLV reverse transcriptase for 1 h at 42°C; the extended products were analyzed on denaturing 8% polyacrylamide gels. Sequencing reactions performed on a promoter-containing plasmid with the same oligonucleotides (Sequenase Quick-Denature plasmid sequencing kit, USB) were run at the same time for accurate determination of the 5′-end termini.

2.6. Transient transfection and reporter activity assay

Transient transfection experiments were performed in MCF-7 cells by using a modified version of the calcium phosphate precipitation procedure [27]. Approximately 90% confluent cell cultures were split 1/12 onto 35 mm diameter tissue culture dishes. The following day the medium was replaced with 1.4 ml of fresh supplemented Dulbecco's modified Eagle's medium; 4 h later DNA precipitates were added dropwise to cells. The cells were left in contact with the precipitates for 12 h, and then the medium was replaced. Precipitates were generated by mixing 0.5 ml of 1×HEPES-buffered saline (0.5% HEPES, 0.8% NaCl, 0.1% dextrose, 0.01% anhydrous Na_2HPO_4, 0.37% KCl, pH adjusted to 7.05) with 5.0 μg of plasmid DNA (typically, 0.5 μg luciferase reporter plasmid, 0.25 ng of β-galactosidase (β-gal) plasmid plus carrier DNA (pGL3Basic) up to 5.0 μg) followed by the addition of 5 μl of 2.5 M $CaCl_2$. Precipitates were allowed to form at room temperature for 20 min before addition to the cells. For doxorubicin treatment, cells were allowed to grow for 10 h after transfection and then overnight treated with 500 ng/ml doxorubicin For a detailed protocol visit our website: http://www2.cbm.uam.es/bc-015.

Luciferase activity was measured according to the instructions of the Luciferase system kit (Promega) in a luminometer Monolight 2010 (Analytical Luminescence Laboratory, San Diego, CA, USA). Transfection efficiency was normalized by cotransfection with 0.25 ng of the pCMV-β-gal (Promega). Transfection experiments were performed in triplicate.

2.7. Reverse transcriptase (RT) and PCR assays

Total RNA from MCF-7 cells was isolated with the Trizol RNA isolation system (Invitrogen) and the M-MLV reverse transcriptase (Gibco-BRL) was used for the RT reaction, following the manufacturer's recommendations. An aliquot of the RT reaction was analyzed by PCR for both TRAIL-R3 and β-actin expression using Taq polymerase (Promega) and the following pairs of primers: TRAIL-R3 forward: 5′-GAAGAATTTGGTGCCAATGCCACTG-3′; TRAIL-R3 reverse: 5′-CTCTTGGACTTGGCTGGGAGATGTG-3′; β-actin forward: 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′; β-actin reverse: 5′-CTAGAAGCATTTGCGGTGGACGATGGA-GGG-3′. PCR cycle conditions were 30 s at 95°C, 45 s at 60°C and 1 min at 72°C. After running PCR samples for 27 cycles, the products were resolved on a 1% agarose gel and visualized with ethidium bromide.

2.8. Computer analysis

Search for regulatory elements was performed using the TFSEARCH ver.1.3, Threshold: 76.0 (http://www.cbrc.jp/research/db/TFSEARCH.html).

3. Results

3.1. Cloning the 5′ upstream region of the human TRAIL-R3 gene

The upstream regulatory region of the human TRAIL-R3 gene was amplified by nested PCR using two gene-specific primers, TR3#3rp and TR3#4rp, spanning regions near the translation start site, and two adapter primers, AP-1 and AP-2, from the adapter-ligated human genomic DNA libraries. A 2.7 kb PCR product was amplified, directly cloned into pGEMT vector and sequenced (GenBank accession number AF524869). The 3′ end of the sequence matched the gene-

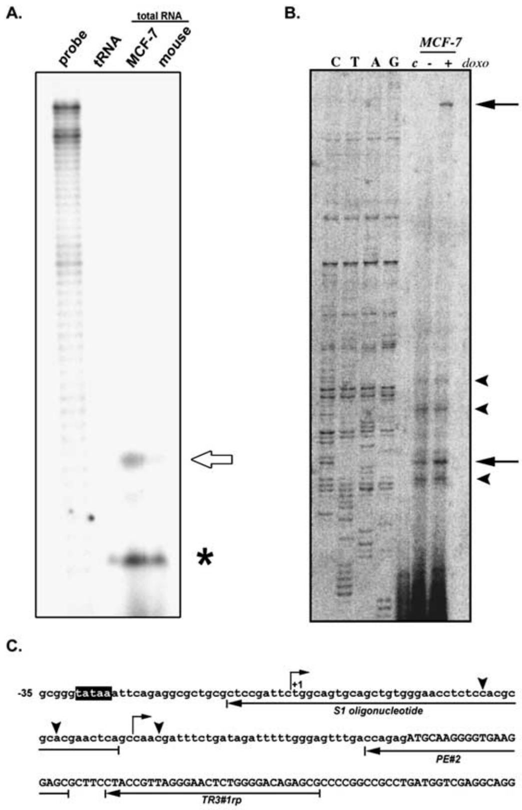

Fig. 1. Characterization of the 5′ end of human TRAIL-R3 transcripts. A: The S1 nuclease protection assay was performed by employing the antisense S1 oligonucleotide detailed in the figure. The samples were loaded onto an 8% denaturing polyacrylamide gel. Open arrow indicates the specifically protected DNA fragment. Asterisk denotes a non-specific signal. B: Primer extension mapping of the 5′ ends in the human TRAIL-R3 mRNA. A 30-mer oligonucleotide (TR3#1rp) was hybridized to total RNA from MFC-7 cells either untreated (−) or treated with doxorubicin (doxo, 500 ng/ml) for 24 h (+). tRNA was employed as a negative control (c). Samples were loaded onto an 8% denaturing polyacrylamide gel together with a sequencing reaction performed with the same oligonucleotide employed for the primer extension assay. C: Sequence of the human TRAIL-R3 promoter showing the mRNA transcription start sites. Arrowheads point to non-induced transcription start sites. Doxorubicin-induced transcription start sites are indicated by bent arrows. Black box denotes the TATA element. The location and name of the oligonucleotides employed are detailed. The coding region is shown in upper case. The major transcriptional start site is indicated (+1).

specific primer and the upstream sequence of TRAIL-R3 cDNA [11].

The TRAIL-R3 upstream sequence was analyzed for regulatory regions using the TFSEARCH ver.1.3 program. The presence of a consensus TATA box sequence as well as potential binding sites for known transcription factors strongly suggested that this genomic region probably contained the human TRAIL-R3 promoter.

3.2. *Identification of the transcription start site of the human TRAIL-R3 gene*

The transcription initiation start site was mapped by S1 nuclease protection and primer extension assays (Fig. 1). Because TRAIL-R3 is expressed in MCF-7 cells [28,29], total RNA from this cell line was employed. As negative controls, tRNA and total RNA from mouse liver cells were used. We initially carried out S1 protection assays to test whether the transcriptional start site mapped close to the consensus TATA box identified within the cloned sequence (Fig. 1). As shown in Fig. 1A, a fragment of the S1 oligonucleotide was specifically protected when the labelled oligonucleotide was hybridized to MCF-7 RNA sample. The band indicated with an asterisk in Fig. 1A represents a non-specific signal as it is present not only in the human and mouse lanes but also in the lane corresponding to tRNA. To precisely locate the initiation start site, primer extension assays were performed. As human TRAIL-R3 expression is up-regulated in MCF-7 cells upon doxorubicin treatment (C. Ruiz de Almodóvar and A. López-Rivas, unpublished results), total RNA isolated from MCF-7 cells, either treated with doxorubicin for 24 h or left untreated, was employed. The extended products were specifically detected only when RNA from MCF-7 cells was used (Fig. 1B). Two major bands were selectively induced in samples from doxorubicin-treated cells. The upper site mapped within the S1 oligonucleotide sequence whereas the second site was located 45 nucleotides downstream. These start sites were also mapped when a second oligonucleotide (PE#2) was employed (data not shown). These results confirmed that the upper transcriptional start site lies within the region identified by the S1 experiment and is located 25 nucleotides downstream of the TATA box (Fig. 1C). This residue was given the position +1. Recently, a TRAIL-R3-specific cDNA from a human library displaying the longest 5′ end has been reported (GenBank accession number BC017852). The fact that the first nucleotide of this sequence fits to the transcriptional start site identified above reinforces our results.

3.3. *Activity of the TRAIL-R3 gene promoter*

To determine whether the 5′ upstream region of the human TRAIL-R3 gene contains the basic elements for basal promoter activity, a 1683 bp *Bam*HI–*Sal*I fragment (−1641, +42) generated by digestion of the 2.7 kb genomic was cloned into the pXP2 vector [25], upstream of the luciferase reporter gene. Three additional pXP2-based constructs were obtained spanning 506, 126 and 33 bp upstream of the initiation start site. MCF-7 cells were transiently transfected with these reporter plasmids and 24 h later the relative luciferase activity

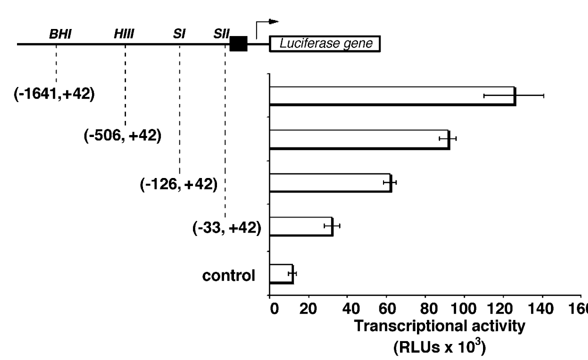

Fig. 2. Deletion analysis of the human TRAIL-R3 gene promoter. The reporter plasmids were transfected into MCF-7 cells. Restriction sites employed to generate these constructs are detailed in the schematic diagram (*BHI* = *Bam*HI; *HIII* = *Hin*dIII; *SI* = *Stu*I; *SII* = *Sac*II). Black box denotes the TATA box. One out of four independent experiments performed is shown. Control indicates the activity of the pXP2 parental luciferase vector. Transfections were normalized to the activity of a cotransfected pCMV-β-gal plasmid. The results are shown as means ± S.D.

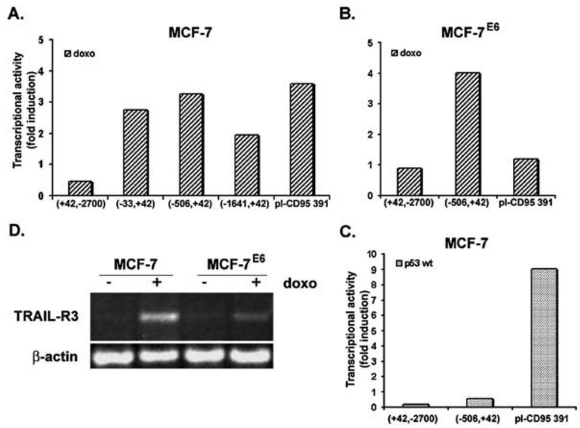

Fig. 3. Doxorubicin (doxo) can induce TRAIL-R3 promoter in a p53-independent manner. MCF-7 (A) and MCF-7^{E6} cells (B) were transfected with the reporter plasmids indicated in the figure, containing either the human TRAIL-R3 promoter or the CD95 intronic p53-responsive enhancer element. Ten hours after transfection, cells were overnight treated with doxorubicin at 500 ng/ml. C: MCF-7 cells were co-transfected with the constructs indicated in the figure and a p53 expression vector. Luciferase activity is expressed as fold induction over the baseline levels of transfected untreated cells (A, B) or wt p53/empty vector-transfected cells (C). One out of three experiments performed is shown. D: RT-PCR products from cells either treated (+) or not (−) with doxorubicin (500 ng/ml for 24 h) were analyzed in the p53-positive (MCF-7) or p53-negative (MCF-7^{E6}) cell lines.

was measured. The basal transcriptional activity progressively increased with the length of the 5′ upstream region of the human TRAIL-R3 promoter (Fig. 2). These results indicate that all these regions appear to contain elements that contribute to the overall promoter activity and that the region spanning from nucleotides −33 to +42 contains the minimal promoter elements of the human TRAIL-R3 gene.

3.4. Doxorubicin can induce the human TRAIL-R3 promoter activity in a p53-independent manner

The primer extension results indicate that transcriptional activity of the human TRAIL-R3 gene increased when MCF-7 cells were treated with doxorubicin. Transient transfection experiments were performed to test whether the basal transcriptional activity of these human TRAIL-R3 promoter constructs was affected upon doxorubicin treatment. To this end, MCF-7 cells were transiently transfected and luciferase activity was measured upon overnight treatment with doxorubicin. The results indicated that the transcriptional activity of the human TRAIL-R3 promoter-containing constructs is stimulated by doxorubicin (Fig. 3A). As a negative control, the 2.7 kb PCR-amplified genomic fragment was cloned in antisense orientation into pXP2 plasmid (+42, −2700). In these experiments the highest fold induction was displayed by the −506,+42 promoter fragment, thus suggesting that the key elements responsive to doxorubicin must be located within the −506 and +42 bp region of the human TRAIL-R3 promoter. As a control for doxorubicin-induced promoter activity, the construct pI-CD95 391LUC corresponding to the CD95 gene is included. This construct contains a region of intron I of the CD95 gene that confers regulation by p53 [26]. In order to investigate whether the induction of TRAIL-R3 promoter by doxorubicin is dependent on p53 we performed reporter assays in MCF-7 cells expressing human papilloma virus type 16 E6 protein, which induces p53 degradation and thus prevents p53 accumulation in response to genotoxic damage [24]. Results shown in Fig. 3B indicate that p53 is not required for doxorubicin-induced TRAIL-R3 promoter activity. In MCF-7^{E6} cells, the control doxorubicin-inducible, p53-dependent pI-CD95 391LUC construct is not activated by the DNA-damaging drug (Fig. 3B). Further evidence for the lack of involvement of p53 in doxorubicin-induced TRAIL-R3 promoter activity in MCF-7 cells is shown in Fig. 3C. In these co-transfection experiments, the control p53-inducible pI-CD95 391LUC plasmid is clearly activated by wt p53 (nine-fold). In contrast, co-transfection of the TRAIL-R3 promoter construct (−506, +42) and wt p53 vector does not result in transcriptional activation over the baseline activity of empty vector-transfected cells.

To determine whether or not endogenous expression of TRAIL-R3 could be increased by doxorubicin in a p53-dependent manner, RT-PCR experiments were carried out. As shown in Fig. 3D, doxorubicin efficiently induces the endogenous TRAIL-R3 mRNA level in MCF-7 cells. Likewise, doxorubicin was able to increase the TRAIL-R3 mRNA levels in the absence of wt p53 (MCF-7^{E6}). In these experiments, the doxorubicin-mediated induction of TRAIL-R3 mRNA was stronger in MCF-7 cells, therefore suggesting that p53 mediates additional signals that contribute to TRAIL-R3 gene expression.

4. Discussion

In spite of the advance in the human genome sequence and the mapping of the TRAIL receptors to the same chromosomal locus, 8p22–21 [8,30,31], the sequence of the human TRAIL-R3 gene is still unknown. In this work we have cloned and defined the minimal promoter region of this decoy receptor. Our results indicate that the smallest promoter construct analyzed containing the TATA box is enough to drive the expression of the TRAIL-R3 human gene promoter. The transcriptional activity progressively increases with the size of the 5′ fragment of the promoter. This suggests that functional binding sites for transcription factors that cooperate with the basal transcription machinery to control human TRAIL-R3 mRNA expression must be located upstream of this minimal promoter fragment.

In this study, several transcription start sites were found in non-treated MCF-7 breast carcinoma cells. It is particularly relevant that upon doxorubicin treatment, there is an increase in the rate of transcription from two major start sites. As a result, the amount of mRNA and protein expressed would probably increase and the final impact could result in the resistance of these cells to TRAIL-induced apoptosis. Doxorubicin is a drug currently used for breast cancer treatment and the results from our work suggest that treatment of breast carcinoma cells could raise the level of this decoy receptor in these cells. This should be considered in potential clinical trials employing TRAIL as an anti-cancer agent.

The transcriptional regulation of the TRAIL receptors has been approached in a few studies. Rel/NF-κB transcription factors have been shown to protect TRAIL-induced apoptosis by up-regulating the human TRAIL-R3 expression in HeLa cervical carcinoma cells [22]. As a new level of transcriptional regulation, it has been suggested that hypermethylation of the promoter of the TRAIL decoy receptors, human TRAIL-R3 and TRAIL-R4, is involved in the down-regulation of the expression of these genes in neuroblastoma and other tumor types, such as brain, colon and skin cancers [18]. Moreover, it has been shown that TRAIL-R3 is a p53-regulated gene highly expressed in primary tumors of gastrointestinal tract [21]. Although we have not observed p53-mediated regulation of the TRAIL-R3 promoter, we cannot exclude that p53-responsive sites may be located in other regions of the gene, as occurs in TRAIL-R2 and CD95 genes [32,33]. These sites could account for the higher induction of TRAIL-R3 gene expression observed in p53-positive MCF-7 cells versus MCF-7^{E6} cells. In addition, treatment with doxorubicin may also activate other transcription factors, like NF-κB and AP-1 [34,35]. In this context, this work could be the starting point for the analysis of the human TRAIL-R3 gene at the transcriptional level. Based on computer analysis, different transcription factors could bind to the human TRAIL-R3 promoter region and regulate its expression. Further studies dissecting the *cis*- and *trans*-acting elements involved in the transcriptional regulation of TRAIL-R3 promoter will facilitate the study of the mechanisms that control the expression of this anti-apoptotic decoy receptor, a protein with tumor resistance properties.

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