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ORIGINAL PAPER

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Targeting gene expression of the mouse uroplakin II promoter to human bladder cells

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Abstract Differential expression of the desired gene product in the target tissue is central to the concept of gene therapy. One approach is to use a tissue-specific promoter to drive therapeutic genes. To investigate the feasibility of tissue-specific gene therapy for bladder cancer using the mouse uroplakin II (UPII) promoter and its transcriptional control, the efficacy of this promoter as well as fragments in regulating gene expression were qualitatively and quantitatively analyzed in bladder and non-bladder tissue cell lines using DNA transfection. Our results demonstrate that the mouse UPII promoter actively drives gene expression in BIU-87, a bladder cancer cell line. Little promoter activity was detected in the non-bladder tissue cell lines. Furthermore, deleting the 5′ end 1.5 kb of the UPII promoter by PCR, the activity was significantly decreased but was bladder-specific. However, deleting the 3′ end 143-bp of the UPII promoter, the activity was hardly detected in any tissue cell lines. The activity of the 3′ end 143-bp of the UPII promoter was detected in both bladder cancer and stomach cancer cell lines. These data demonstrate that the mouse UPII promoter has a high activity in human bladder cells and a low basal activity in human non-bladder cells. This suggests that targeting the gene expression of the mouse UPII promoter could be used to treat human bladder cancer. The enhancer was contained in the region of the 1.5 kb of the 5′ end of the mouse UPII promoter. The core promoter was located in the region of the 143 bp of the 3′ end.

Keywords UroplakinII promoter · Bladder neoplasm · Gene expression regulation

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Introduction

Of all urinary cancers, transitional cell carcinoma (TCC) of the bladder is the most common malignancy [15]. Each year, approximately 50,000 new patients are diagnosed with this disease, resulting in about 11,000 deaths annually [5]. Over the past few years, the administration of intravesical chemotherapy or immunotherapy has been used to prolong the progression-free interval after initial transurethral resection [3, 22]. However, the rate of recurrence is about 20%, with as many as 30% of these recurrent tumors presenting with a higher grade and with invasive properties [2]. Today, gene therapy for cancer has become a more realistic approach because of rapid advances in molecular genetic techniques [4, 16, 11]. Differential expression of the desired gene product in the target tissue is essential to the concept of this type of therapy [12, 13, 14, 19]. One such approach is to use a tissue-specific promoter to drive therapeutic genes, because tissue-specific promoters can only be expressed differentially in these cells, minimizing systemic toxicity [1].

A group of bladder tissue-specific proteins, uroplakins (uroplakinIa UPIa, uroplakinIb UPIb, uroplakinII UPII, uroplakinIII UPIII), have been identified and characterized [20]. The expression of uroplakins was shown to be limited to bladder-derived cells by RT-PCR and immunohistochemistry [9, 23]. Therefore, the promoters that direct the expression of the uroplakin genes may be useful in constructing tissue-specific vectors for bladder cancer gene therapy.

In 1995, a mouse UPII promoter was found by Lin and co-workers [8]. This promoter can cause a bacterial lacZ gene to be expressed in the suprabasal cell layers of mouse urothelium, but not in other tissues. To date, however, the mouse UPII promoter cloned by Lin has not been demonstrated to have human bladder tissue specificity or transcriptional control of the promoter.

In this report, we study the promoter activity of the mouse UPII promoter in different human cell lines and analyze the region for promoter activity and regulation. We subcloned four fragments of mouse UPII promoter by PCR or restriction endonuclease. The five promoters were inserted into the pEGFP-1 plasmid with green fluorescence protein (GFP) and the pGL3-basic plasmid with the luciferase gene as the reporter gene. Because the GFP and luciferase assay were very sensitive and quantitative, we were able to measure the promoter specificity using DNA transfection in both human bladder and non-bladder cell lines.

Materials and methods

Subcloning of the UPII promoter

A 3.6 kb fragment of mouse UPII promoter was introduced into the placF vector at the SalI site (a gift from T.T. Sun). Using the placF template by pfx, two oligonucleotide primers, tcctcgcccttgctcaccat and ccggaattcaggtaacgcagagacacag, were designed for PCR amplification. A DNA fragment of approximately 2.0 kb was created for designating UPII-1 (from about 2.0 kb to 1 bp). This UPII-1 DNA fragment was digested with *SacI* to create the 1,270 bp UPII-2 DNA (from about 1,270 bp to 1 bp). The latter DNA fragment was digested with *ApaI* and *BamHI* to create the 143 bp UPII-3 DNA (from about 143 bp to 1 bp). UPII DNA fragment was digested with *EcoRI* and *ApaI* to create the approximately 3.4 kb UPII-4 DNA (from about 3.6 kb–143 bp) (Fig. 1).

These five DNA fragments were introduced into the pEGFP-1 vector with green fluorescence protein (GFP, Clontech) and pGL3-basic vector with luciferase (Promega) to construct recombinant plasmid DNA. The construct with the cytomegalovirus (CMV) promoter was used as a positive control to indicate transfection and expression efficiency. The construct with no promoter was used as a negative control to determine the levels of non-specific transcription. The construct with the CMV-driven β-galactosidase was used as an internal control to normalize levels of luciferase. The recombinant plasmid was purified and the plasmid concentration was determined by spectrophotometer.

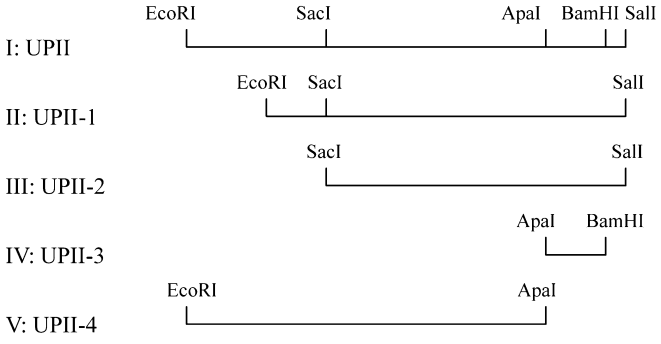

Fig. 1 Subcloning of the uroplakinII promoter and fragments. *I*: (*UPII*) the entire length of the uroplakinII promoter is 3,572 bp. *II*: (*UPII-1*) 5′ end 1.5 bp of UPII was deleted by PCR. Length of UPII-1 is 2 kb. *III*: (*UPIII-2*) 5′ end 750 bp of UPII-1 was deleted by restriction endonuclease *SacI*. Length of UPII-2 is 1,250 bp. *IV*: (*UPII-3*) 3′ end 143 bp was deleted of UPII by restriction endonucleases *ApaI* and *BamHI*. Length of UPII-3 is 143 bp. *V*: (*UPII-4*) 3′ end 143 bp was deleted of UPII by restriction endonuclease *ApaI*. Length of UPII-4 is 3,429 bp

Cell line culture and maintenance

Bladder cancer cell line (BIU-87), kidney cancer cell line (GRC-1), prostate cancer cell line (DU145), vascular endothelium cell line (EC), stomach cancer cell line (AGS-1) and skin fibroblast cell line (CRL-7014) were derived in our laboratory. They were maintained in RPMI 1640 with 10% fetal bovine serum (FBS, Hyclone).

GFP vector transfection and GFP assay

Five GFP recombinant plasmid DNAs were transfected into BIU-87, GRC-1, DU145 and EC cell lines by Lipofectamine 2000 (GIBCO). Cells were trypsinized from plates, resuspended in RPMI 1640 with 10% FBS to 4×10^5 cells/ml and plated into a 24 well plate at a concentration of 2×10^5 cells and grown overnight until $\approx$70% confluent. Cells were washed twice with 500 ul RPMI 1640 medium. A total of 1 ug DNA or 2 ul Lipofectamine 2000 was mixed into 50 ul RPMI 1640 medium. This mixture was added into plates. Cells were incubated at 37°C for 6 h, then added to 600 ul RPMI 1640 with 20% FBS into plates and incubated at 37°C for 24 h. The GFP activity of cells was detected using a confocal microscope. Cells were trypsinized from plates and washed twice with phosphate buffer saline (PBS). The washed cells were detected by flow cytometry.

Luciferase vector transfection and luciferase assay

A total of 0.8 ug luciferase recombinant plasmid DNA and 0.2 ug β-galactosidase plasmid were cotransfected into BIU-87, AGS-1, CRL-7014 and EC cell lines by Lipofectamine 2000. The steps were the same as for the transfection of GFP recombinant plasmid DNA. They were repeated twice in every cell lines and constructs. After 24 h of cotransfection, 50 ul 1×Reporter Lyses Buffer (Promega) was added to cover the cells and a single freeze-thaw was performed to ensure complete lysis. β-galactosidase levels were measured using 20 ul lysate by ONPG methods. Luciferase values were determined by the adding 100 ul luciferase assay reagent to the residual 20 ul cell lysate and then measuring of light emission for 1.2 s in a luminometer (microplate). L/G values were acquired for transfection efficiency in all cells by dividing the luciferase values from the test construct by the β-galactosidase expression from the transfected pCMV-β-galactosidase plasmid.

Results

Construction of GFP and luciferase expression vectors

In order to analyze the activity and specificity of the UPII promoter and fragments, we constructed 5 GFP and 5 luciferase vectors. The expression vectors were characterized by digestion with restriction endonuclease and electrophoresis on 1% agarose gel. The recombinant plasmids were correct (data not showed).

5 mouse UPII promoters and fragment-activated GFP expression in the human cell lines

Seven plasmids, the first containing the CMV promoter, the second the mouse UPII promoter, the third the UPII-1 fragment, the fourth the UPII-2 fragment, the fifth the UPII-3 fragment, the sixth the UPII-4 fragment and the seventh with no promoter, were transfected into BIU-87, EC, GRC-1 and DU145 cells by Lipofectamine. The plasmid containing the CMV promoter showed a significant increase in GFP activity; about 50% by flow cytometry in all cell lines, indicating that the DNA transfection for each cell line was efficient. Compared to the negative control plasmid, the GFP expression from the mouse UPII promoter plasmid exhibited a higher activity in human bladder cancer cells than in other cell lines (5-10/HP vs 0-2/HP). The positive rate in BIU-87 was 4.34%, but in EC it was 0 by flow cytometry. In all cell lines, the GFP expression from the UPII-3 plasimd was high in both bladder cancer cells and vascular endothelium cells. In contrast, the expression of the UPII-4 plasimd was very low. The positive rates in all cells were 0 by flow cytometry (Fig. 2, Table 1) were:

1. BIU87 (positive control); BIU87-UPII, BIU87-UPII-1. BIU87-UPII-2
2. EC (positive control); EC-UPII, EC-UPII-1, EC-UPII-2
3. BIU87 (negative control); BIU87-UPII-3, BIU87-UPII-4
4. EC (negative control); EC-UPII-3, EC-UPII-4

5 mouse UPII promoter and fragment-activated luciferase expression in the human cell lines

Five plasmids and the β-galactosidase plasmid were cotransfected into BIU-87, AGS-1, CRL-7014 and EC cell lines by Lipofectamine, and parallel control transfections were carried out with pCMV-GL3, no promoter

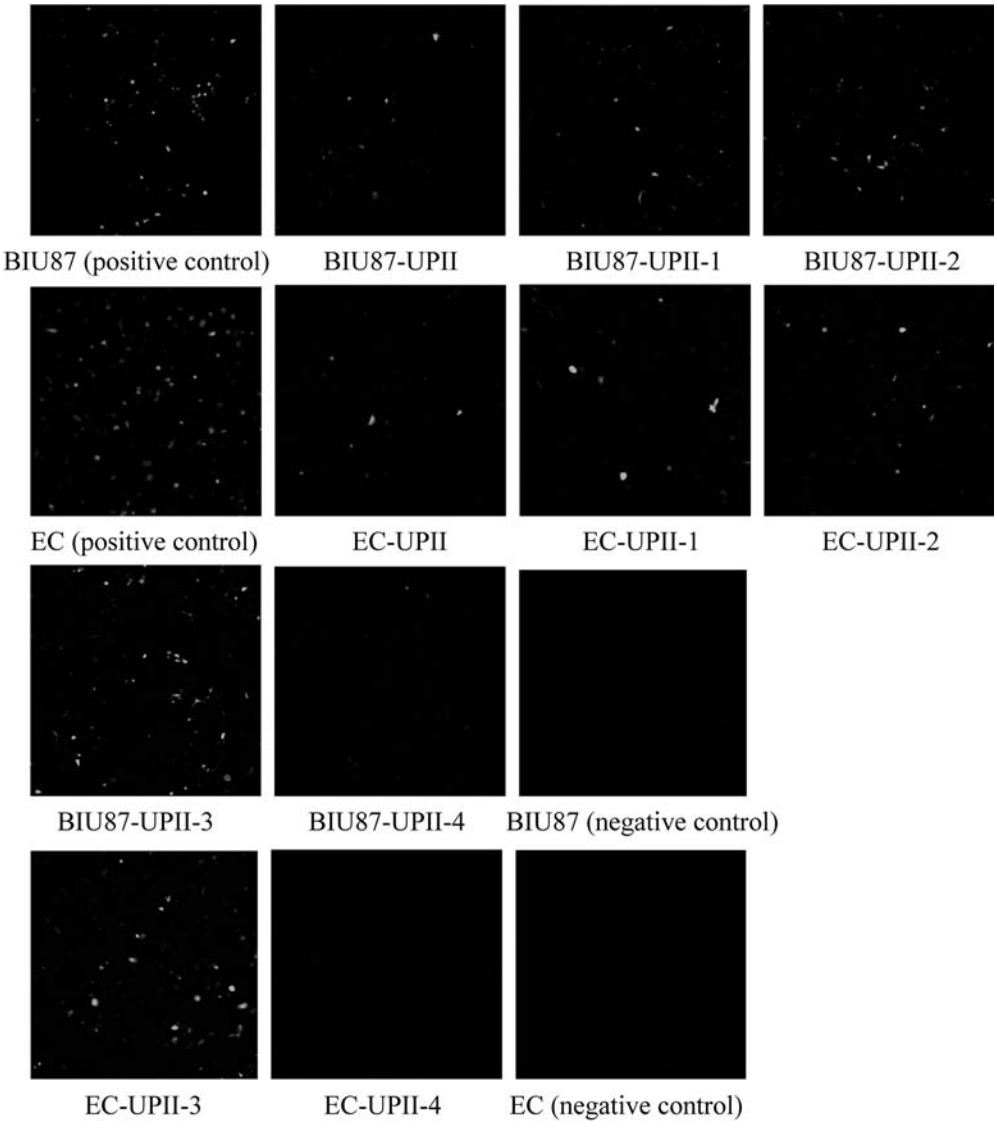

Fig. 2 The activity of the mouse uroplakinII promoter in BIU-87 and EC. Positive green signals from UPII, UPII-1, UPII-2 were more and stronger in the BIU-87 than in the EC cell line. Positive green signals from UPII-3 were strong in both BIU-87 and EC cell lines. Positive green signals from UPII-4 were hardly detected in either the BIU-87 or the EC cell lines

Table 1 The activity of GFP in BIU-87 and EC by flow cytometry. The GFPexpression of UPII, UPII-1, UPII-2, UPII-3 was more in the BIU-87 than in theEC cell line, but the GFP expression from UPII-4 was 0 in both the BIU-87 and EC cell lines

	Positive control	pEGFP-UPII	pEGFP-UPII-1	pEGFP-UPII-2	pEGFP-UPII-3	pEGFP-UPII-4
BIU-87	51.7%	4.34%	5.55%	6.14%	9.65%	0
EC	51.7%	0	1.63%	0.94%	4.47%	0

plasmid and pCMV-β-galactosidase. The level of expression from mouse UPII promoter and fragment plasmids normalized against cotransfected pCMV-β-galactosidase was carried out as described above. For each cell line and constructs, the ratio of luciferase activity to β-galactosidase activity (L/G) was calculated. Compared to the negative control plasmid, L/G values showed a luciferase expression from the mouse UPII promoter in human bladder cancer cells of 1.8–8.2-fold higher than in non-bladder cell lines. The luciferase expression from UPII-1 and UPII-2 was still tissue-specific, but the luciferase expression in each cell line was significantly lower than for UPII. UPII-3 could drive luciferase expression in both bladder cancer and stomach cancer cell lines but the luciferase expression in each cell line was also significantly lower than UPII. The luciferase expression from UPII-4 was detectable but very low in BIU-87 and hardly detectable in other cell lines (Fig. 3).

Discussion

Surgical resection, chemotherapy and radiotherapy are currently used for the treatment of bladder cancer. None of these have led a decrease in recurrence rate [6]. In the past few years, several new approaches for treating bladder cancer have been proposed, including gene therapy. Successful gene therapy requires not only the identification of an appropriate therapeutic gene, but also a delivery system by which the gene can be delivered to the desired target cell types both efficiently and accurately [10]. One such approach is to use a tissue-specific promoter to drive the expression of the therapeutic genes so that they are expressed only in the specific target cell types and systemic toxicity is minimized [18].

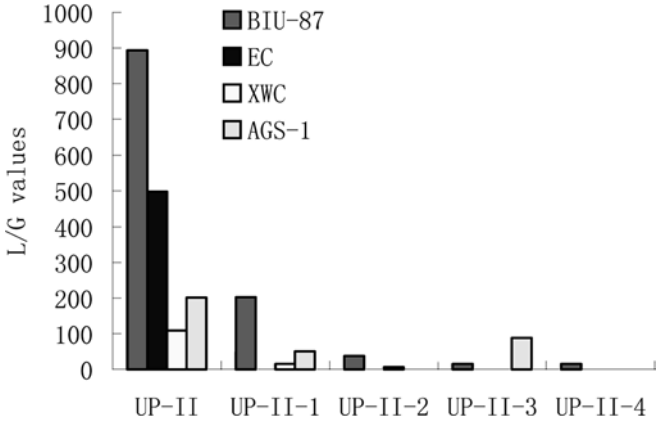

Fig. 3 L/G values of the uroplakinII promoter and fragments in human cell lines. The luciferase expression from UPII, UPII-1, UPII-2 was higher in BIU-87 than in other cell lines. The luciferase expression from UPII in BIU-87 was higher from UPII-1 and UPII-2. The luciferase expression from UPII-3 was expressed in both BIU-87 and AGS-1 cell lines, but the luciferase expression from UPII-4 was hardly detected in either BIU-87 or other cell lines

To set up such a tissue-specific gene expression vector, we used the UPII promoter. UPII, a bladder tissue-specific gene which has been studied extensively [7, 17, 21], has been identified in humans, mice and cows. A mouse UPII promoter was found in 1995 [8]. An understanding its unique transcriptional control mechanism may prove to be beneficial in developing target-specific expression vectors for bladder cancer gene therapy. In this study, we combined the GFP and luciferase assays to characterize the role of the mouse UPII promoter in human bladder cancer cells as well as the transcriptional control of the promoter. Our results demonstrated that: (1) the mouse UPII promoter has a high activity in human bladder cells and a low basal activity in human non-bladder cells. This suggests that targeting gene expression of the mouse UPII promoter could be used to treat human bladder cancer. (2) Enhancer was contained in the region of 1.5 kb of 5′ end of mouse UPII promoter. (3) The core promoter was contained in the region of 143 bp of 3′ end. These features of the UPII promoter are fundamental to the development of a target-specific vector for treating bladder cancer via gene therapy.

The mouse UPII promoter characterized by Lin and associates has demonstrated tissue specificity in the mouse [8]. Our results indicate that the promoter is also bladder tissue specific in humans. High tissue specificity of the UPII promoter and the CMV promoter was demonstrated using plasmid DNA transfections in BIU-87 and negative control cells including the GRC-1, DU145, EC, AGS-1 and CRL-7014 cell lines. The plasmid containing the UPII promoter has a nearly identical structure to the plasmid containing the CMV promoter. Both plasmids were used to transfect all six cell lines. The fact that the high expression of the GFP and luciferase gene was demonstrated in all six cell lines transfected with the CMV containing plasmid suggests that the CMV plasmid could transfect the cell lines with high efficiency. Low luciferase expression was demonstrated in all five negative control cell lines. Because the structures of all seven plasmids are very similar, we believe that gene expression in these cell lines reflects

promoter activity. It might be plausible that the low expression in the UPII promoter plasmid-transfected negative control cells was a result of poor transfection efficiency. However, because these plasmids have nearly identical structures to that of the CMV containing plasmid, it seems highly unlikely that the UPII promoter plasmids would fail to transfect all five negative control cell lines while the CMV containing plasmid did. Furthermore, it also seems unlikely that the UPII promoter plasmids would fail to transfect all five negative control cell lines while simultaneously demonstrating high transfection efficiency in the BIU-87 cells. The most reasonable conclusion is that the difference in gene expression between the BIU-87 and the negative control cell lines is the result of promoter activity.

It is remarkable that the UPII promoter can be decreased by the deletion of some fragments and loses its tissue specificity. We have proposed that an enhancer function was contained in the region of 1.5 kb of the 5′ end of UPII promoter. By deleting this region the activity of the promoter decreased significantly. When deleting the region of 143 bp of the 3′ end of the UPII promoter, the activity of the fragment was hardly detected, but when the fragment only contained the region of 142 bp of the 3′ end of UPII promoter, the activity resumed and lost tissue specificity. This result shows that the region of 143 bp of the 3′ end is the key fragment of the promoter. It may be a core promoter.

In conclusion, we demonstrated that the bladder tissue specificity of the mouse UPII promoter could be used to develop a target specific gene expression vector for human bladder cancer gene therapy. Further, the transcriptional control of UPII promoter was undertaken to define the enhancer regulatory and core regulatory elements.

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