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TECHNICAL NOTE

Transcriptional activation of a hybrid promoter composed of cytomegalovirus enhancer and β-actin/β-globin gene in glomerular epithelial cells *in vivo*

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Transcriptional activation of a hybrid promoter composed of cytomegalovirus enhancer and β-actin/β-globin gene in glomerular epithelial cells *in vivo*. The aim of this study was to seek a promoter, transactivated selectively in renal cells *in vivo* by using transgenic (tg) mouse technology. We generated two kinds of tg mouse lines carrying a green fluorescence protein (GFP) cDNA driven either by cytomegalovirus enhancer and β-actin/β-globin promoter (CX-GFP) or by elongation factor 1α promoter (EF-GFP), and investigated the expression of GFP in the kidney. Microscopic examination of the renal tissues in CX-GFP-tg mice revealed that GFP was expressed only in glomeruli, mainly epithelial cells, but not in tubules, arteries and interstitium. Moreover, *in situ* hybridization demonstrated that GFP mRNA expression was localized in the glomerular cells. In contrast, GFP was not detectable in the kidney in any of the lines of EF-GFP-tg mouse. To exclude the possible involvement of the GFP cDNA as an enhancer, we constructed tg mice carrying the CX promoter driving a human CD4 cDNA. It was confirmed that the expression patterns of human CD4 in the kidney were quite similar to those of GFP in the kidney of CX-GFP-tg mice. These results strongly suggest that CX promoter could be transactivated in glomerular epithelial cells *in vivo*.

Intentional expression of a specific molecule in a living animal by means of gene transduction would provide insight into the physiological and pathophysiological role of the molecule. In this context, numerous transgenic (tg) mice and knockout mice have been developed. However, the systemic effect of the molecule is inevitable in tg mice carrying an uninducible promoter, since the molecule is overexpressed in an uncontrolled fashion from the fetal stage. Therefore, the creation of a cell-specific and inducible promoter, the "molecular switch" in a living animal, would be expected to be a feasible tool for studying the molecular function *in vivo*. However, an ideal promoter has never been found to allow specific expression in a particular cell population in the kidney to date.

Green fluorescence protein (GFP) in Aequorea victoria absorbs blue light and emits green fluorescence. This protein has been used as a reporter gene in molecular biology since it can be detected immediately without any preliminary treatment [1–3]. GFP, in particular, possesses the merit that we can observe its green fluorescence macroscopically as well as microscopically only by exposure to ultraviolet (UV) light. Though the detection of green fluorescence emitted by GFP under UV light is not so sensitive, the visible illumination indicates that GFP is highly transcribed in green fluorescence-positive cells.

We already reported that tg mice, which harbored a GFP cDNA driven by a hybrid promoter composed of cytomegalovirus (CMV) enhancer and β-actin/β-globin promoter (CX promoter), expressed GFP in skeletal muscle, heart, pancreas and kidney [2]. In the present study, we constructed two kinds of tg mouse lines carrying a GFP cDNA driven by either CX promoter or elongation factor 1α promoter (EF-1α promoter), and investigated the expression of GFP in the kidney. In order to identify the cells in which GFP is specifically transactivated by the CX promoter, immunohistochemistry and *in situ* hybridization were performed. We report here that CX promoter was able to induce a selective and strong expression in glomerular epithelial cells in tg mice.

Methods

GFP transgenic mice

Construction of the transgene and production of the tg mice was described in our previous paper [2]. Briefly, a GFP cDNA (gift of Drs. Prasher and Chalfie) [1] was ligated to β-actin/β-globin promoter and CMV enhancer (pCX-GFP) [4]. Thirty lines of tg mice were generated by injecting the *Sal*I/*Bam*HI DNA fragment of pCX-GFP (referred to as CX-GFP-tg mice). Also, a GFP cDNA was ligated to EF-1α promoter (pEF-GFP) [5]. Three lines of tg mice were generated by injecting the *Hin*dIII/*Eco*RI DNA fragment of pEF-GFP (referred to as EF-GFP-tg mice). Human CD4 cDNA was excised from pSP65. T4.8 (gift of Dr. Littman) [6] by *Eco*RI digestion and inserted into the pCAGGS (pCX-hCD4). Five lines of tg mice were also generated by injecting the *Sal*I/*Hin*dIII DNA fragment of pCX-hCD4 (referred to as CX-hCD4-tg mice).

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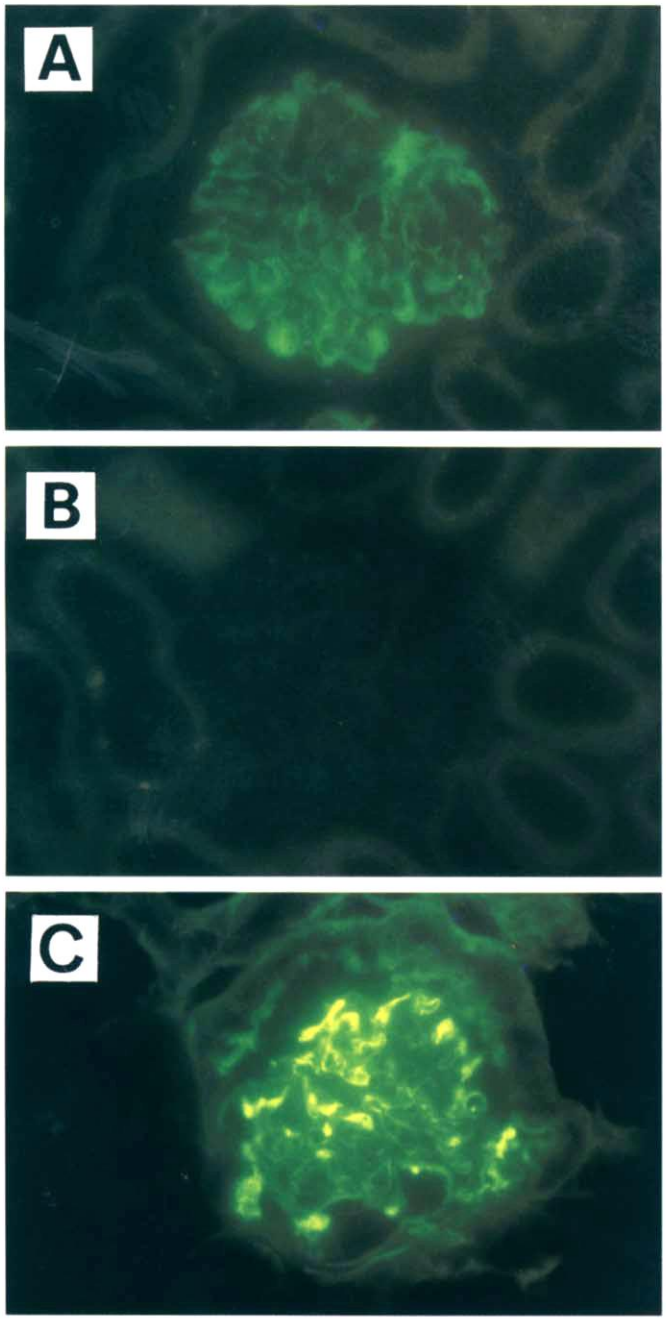

Fig. 1. *Intrarenal expression of GFP and hCD4 in the transgenic mice (×500).* Representative fluorescence photomicrographs of the kidney in the transgenic mice carrying GFP driven by either CX promoter (**A**; CX-GFP-tg mice) or EF-1α promoter (**B**; EF-GFP-tg mice). In the kidney of tg-CX-GFP mice, only glomeruli were illuminated in green, but tubules, arteries and interstitium were not. In contrast, GFP illumination was not observed in the kidney of EF-GFP-tg mice. In the transgenic mice carrying the CX promoter driving human CD4 instead of a GFP cDNA, human CD4 was also localized in glomeruli, mainly epithelial cells with quite similar patterns of GFP expression in tg-CX-GFP mice (**C**).

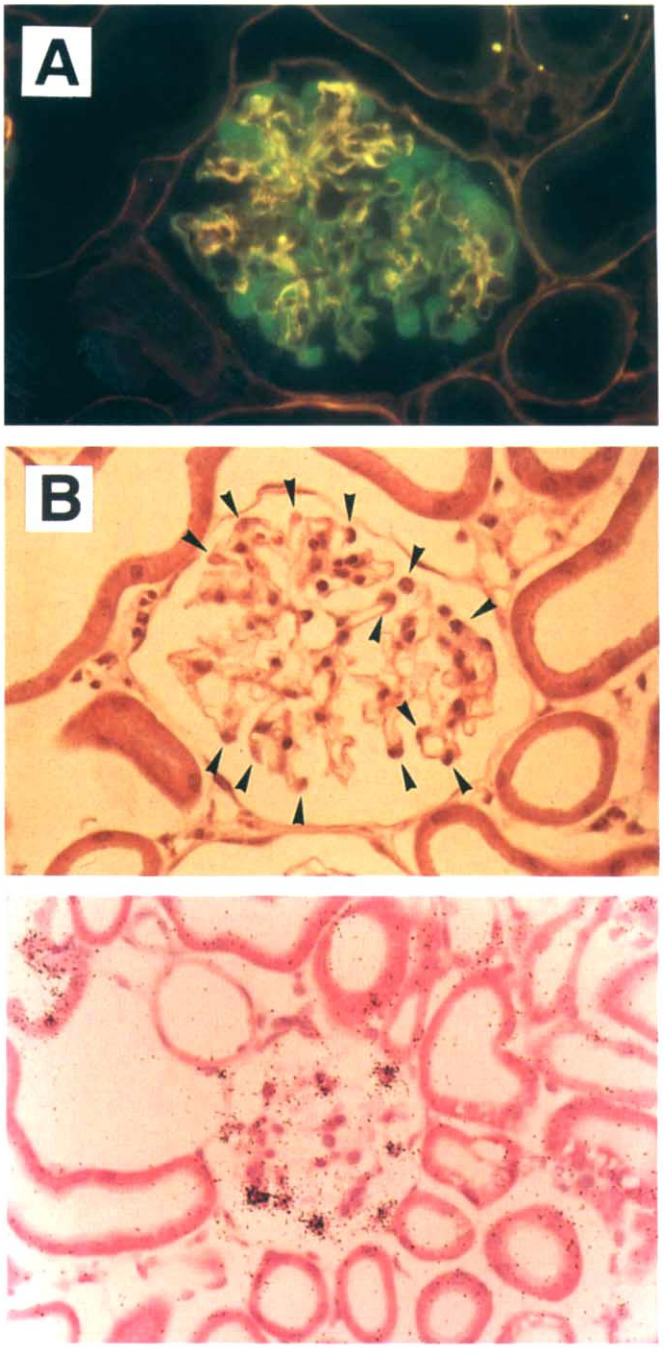

Fig. 2. *(top panels) Localization of GFP expression in the glomerulus assessed by laminin labeling and HE staining (×500).* **A.** Immunofluorescence staining of laminin, a specific marker of GBM, demonstrated that most of the GFP-positive cells (green) were present at the sites just outside of GBM (yellow), indicating that they were glomerular epithelial cells. **B.** This HE staining shows the cells expressing GFP in the same section (arrows). It is evident that most of the GFP-positive cells were glomerular epithelial cells.
Fig. 3. *(bottom panel) The expression of GFP mRNA in the kidney of CX-GFP-tg mice (×300). In situ* hybridization was performed to determine the cells synthesizing GFP *de novo*. In the kidney of CX-GFP-tg mice, the hybridization signal was predominantly present in glomerular cells, presumably epithelial cells and at a much lower level in tubules and interstitium.

Histological detection of GFP

For simple and quick detection of GFP, the mice were deeply anesthetized and various organs were exposed under UV light using a common transilluminator (Spectroline; TC-312A). When transgene was present, organs expressing GFP could be easily visualized macroscopically by the emission of green fluorescence.

Mouse kidneys, after perfused fixation with 4% paraformaldehyde (PFA) solution, were placed in 4% PFA solution for two hours and subsequently in 30% sucrose solution overnight.

Cryosections (4 μm) mounted on glass slides were observed under a Nikon fluorescent microscope equipped with filters DM510 (EX420 to 490 and BT520), which were the same filters used for the detection of fluoresceinisothiocyanate (FITC).

To examine the cellular localization of GFP expression, the sequential sections were stained with an antibody to laminin, a specific marker of glomerular basement membrane (GBM). The sections were incubated with a rabbit polyclonal IgG anti-mouse laminin antibody diluted 1:50 (Becton Dickinson Labware Inc., MA, USA), followed by rhodamine-conjugated anti-rabbit IgG (Chemicon Int., Inc., CA, USA). Thus, GFP positive cells and GBM were revealed by green and red fluorescence, respectively. The preparations were examined under a fluorescent microscope equipped with filters for GFP or rhodamine. The green fluorescence of GFP and the red fluorescence of rhodamine were taken by photomicrograph on the same film by double exposure.

The same sections were then stained with hematoxylin and eosin (HE). We took light photomicrographs of the same glomeruli again in order to allow morphological identification of the GFP-positive cells.

Histological detection of human CD4

Mouse kidneys after perfusion with 0.01 M phosphate buffered saline (PBS) were placed in 30% sucrose solution overnight. Cryosections (6 μm) were incubated with FITC-conjugated anti-human CD4 monoclonal antibody H61 (gift of Dr. Yoshie) [2] for one hour and washed with 0.01 M PBS.

RNA probes and in situ hybridization

To identify the cells synthesizing GFP *de novo*, *in situ* hybridization was performed. A GFP cDNA was inserted into the *Eco*RI site of the pBluescript II SK$^+$. To make ^{35}S-labeled sense and antisense cRNA probes for GFP, *in vitro* transcription was performed using T3 and T7 RNA polymerase, respectively, after linearizing by cutting with *Acc*I. The procedures for *in situ* hybridization were described in our previous paper [7].

Results

Expression of GFP in transgenic mice

To identify the tissue- or cell-specific activity of a particular promoter in tg mice, we employed GFP as a reporter protein because of its quick and simple detection procedure. GFP expression was examined macroscopically in 30 lines of CX-GFP-tg mice and 3 lines of EF-GFP-tg mice. All lines of CX-GFP-tg showed strong expression of GFP in skeletal muscle, heart and pancreas, although the expression could not be detected macroscopically in liver, aorta and intestine. The illumination of GFP was not detected on the surface of the kidney, however, illuminated spots could be seen on the section. In contrast, no organs were illuminated in any of the lines of EF-GFP-tg mouse (data not shown).

Cellular localization of GFP expression in the glomerulus

Next, we examined the intrarenal microscopic expression of GFP in 5 out of 30 lines of CX-GFP-tg mice and 3 lines of EF-GFP-tg mice. Green fluorescence was observed in the glomeruli, but not in the tubules, arteries and interstitium on the kidney section of CX-GFP-tg mice (Fig. 1A). On the sections of other organs including liver, intestine and aorta, illuminated cells were not detected as far as we carefully observed. In EF-GFP-tg mice, green fluorescence was not detected microscopically in any of the organ including kidney (Fig. 1B). Since a GFP cDNA may include an element to allow expression limited to glomerular epithelial cells, we constructed 5 lines of tg mice carrying the CX promoter driving human CD4 instead of a GFP cDNA. It was confirmed that the expression patterns of human CD4 among organs were quite similar to those of GFP in CX-GFP-tg mice. In the kidney, the expression of human CD4 was also localized in glomeruli, mainly epithelial cells (Fig. 1C).

Then, we identified the GFP-positive cells in the glomerulus by means of immunofluorescence staining of laminin, a specific marker of GBM. It was revealed that most of the GFP-positive cells (green) were present at the sites just outside of GBM (yellow), indicating that GFP was highly expressed in glomerular epithelial cells (Fig. 2A). Most of the GFP-positive cells could be identified as epithelial cells on the same section stained with HE afterwards (Fig. 2B). Similarly, the expression of human CD4 in CX-hCD4-tg mice seemed to be mainly limited to epithelial cells.

In situ hybridization of the GFP probe

GFP seemed to exist in glomerular epithelial cells, however, it still remained unclear whether GFP was originally transcribed in glomerular epithelial cells. Therefore, we sought to determine the cells synthesizing GFP *de novo* by using an *in situ* hybridization technique. It was revealed that GFP mRNA in CX-GFP-tg mice was highly expressed in the glomerular cells and was at a much lower level in the tubules and interstitium (Fig. 3). Hybridization signals with the control sense probe were not detected in the kidney. These results strongly suggested that GFP was highly transcribed in glomerular cells, presumably epithelial cells. The expression of GFP mRNA was not detected in the kidney of EF-GFP-tg mice (data not shown).

Discussion

In the present study, we demonstrated that CX promoter activated the transcription of GFP selectively in glomerular epithelial cells as well as in skeletal muscle, pancreas and heart. This is the first report that a promoter was selectively activated in the glomerular epithelial cells *in vivo*. The CX promoter was originally developed as an ubiquitous and strong promoter, pCAGGS [4] by utilizing the character of cytoplasmic β-actin promoter that is highly active in a wide range of cell types as a house keeping gene. Indeed, pCAGGS, was used for a transient transfection in a variety of cells including the kidney fibroblast cell line, mesangial cells and hematopoetic cells. We found that the transcriptional activity of the CX promoter was limited specifically to the tissues that included the glomerular epithelial cells in the tg mice, while the expression of the CX promoter was generally considered to be strong in transient transfection in cultured cells. It is still unclear why the difference in transcriptional activation was observed between *in vivo* glomeruli of tg mice and *in vitro* cultured renal cells; however, it was reported that extracellular matrix (ECM)-cell and cell-cell interactions are essential for the regulation of many genes in differentiated cells and that several expression vectors which work well in tissue culture cells are sometimes inactive *in vivo* and *vice versa* [8]. Furthermore, the CMV enhancer was reported to be more active in the epithelial cells in contact with ECM [8]. Another possibility is that CX promoter may lose the essential *cis*-elements for the transactivation of native β-actin genes in other cells. These possibilities may,

in part, show that CX promoter provided the selective expression in the glomerular epithelial cells in tg mice.

The expression of transgene has often been reported to be different among tg animals [9]. This may be partly due to the copy number and the site of integration into the host genome. However, this glomerular cell-specific activation by CX promoter is not due to the copy number or integration site, since no significant difference was observed in the expression of the transgene among 30 lines of tg mice whose genomes had different copy numbers and integration sites of the transgene.

Another possibility is that a GFP cDNA may include an element to allow the selective expression in glomerular epithelial cells. However, in the kidneys of tg mice carrying the CX promoter driving human CD4 instead of a GFP cDNA, human CD4 expression was also localized in glomeruli with quite similar patterns of GFP expression in tg-CX-GFP mice. Hence, this is not the case where an element of the reporter gene acts as a tissue-specific enhancer for the glomerular epithelial cells.

The CX promoter used in this study is composed of the CMV enhancer, a fragment of chicken β-actin gene (−276 to +969 bp), including a 5′-flanking region, the first exon, the first intron and a part of the second exon, and a fragment of rabbit β-globin gene, including a part of the second and the third exon. This CX promoter contains five putative sp1 binding elements within 220 bp upstream of the CAP site, five putative basic helix-loop-helix (bHLH) transcription factor binding sites (CANNTG), three NF-1 elements (CCAAT) at positions −491, −222 and −91, three CArG boxes (CCWWWWWWGG) at −599, −60 and +685, a NFκB binding site at −482 and a TATA box at −29. The CArG box, in particular, is reported to bind the serum responsive factor [10], and this element is considered to play an important role in the transactivation of gene components of the cytoskeleton, including actins. In cytoplasmic β-actin genes, the CArG box in the first intron is reported to act as a principal enhancer [11].

There are several reports using tg mice harboring a β-actin promoter. Sands et al reported that 7 out of 10 lines tg mice harboring a β-galactosidase cDNA driven by a β-actin promoter (−433 to +61) expressed β-galactosidase in testis, but the rest of the 3 lines expressed it in skeletal muscle and heart [12]. The tg mice harboring a chicken β-actin promoter (−330 to +10) and a 3899 bp of fragment of homeobox gene, Hox-1.1, showed scattered expression in brain, stomach, heart, lung, bladder, muscle and vertebra columun [13]. Merlino et al also generated the tg mice harboring an epidermal growth factor (EGF) cDNA driven by a β-actin promoter (−339 to +1) [14]. The transgene was sporadically expressed in various tissues including spleen, testis, liver, brain and lung. The expression patterns of the transgene in these reports are distinct from our results. The major cause of the different expression among these tg animals is considered to be the presence of the first intron and CMV enhancer. Very recently, Marber et al also showed that the same CX promoter selectively activated transgene 70-kDa heat stress protein (HSP-70) in heart and skeletal muscle in tg mice, although they did not examine the expression of HSP-70 in the kidney [15]. However, further studies are required to examine whether glomerular epithelial cell-specific enhancer localizes in this hybrid promoter.

EF-1α promoter is considered to show a strong activity in various murine tissues and has been utilized for a transient transfection *in vitro*. Therefore, it was expected that EF-GFP-tg mice would ubiquitously express the transgene. From our results, the expression of GFP was not detected under UV light in any of the lines of EF-GFP-tg mouse, although a slight expression of GFP mRNA or its protein might be detectable by highly sensitive analysis, such as reverse transcription-PCR or immunohistochemistry. It has been reported that EF-1α promoter was utilized for the generation of the salt sensitive hypertensive tg mouse strain overexpressing Na^+-H^+ exchanger [16]. In this model, exogenous Na^+-H^+ exchanger was detected in renal tubule cells as well as glomerular epithelial cells by sensitive immunohistochemistry. These results may conflict with our results, however, a weak expression of protein was not our goal. Our aim was to identify the promoter which enables the expression of abundant protein selectively in the kidney, as in the results obtained from the CX promoter.

In conclusion, we developed new lines of tg mice carrying a CX promoter by which selective expression of cDNA occurred in glomerular epithelial cells. This CX promoter may be applicable to the production of a new model mouse with selective glomerular epithelial lesions. We further speculate that a glomerular epithelial cell-specific enhancer element may be identified in the region of this hybrid promoter.

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