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# Regulation of the Expression of the Prostate-specific Antigen by Claudin-7

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**Abstract.** Claudins are a family of proteins involved in forming tight junctions between cells. Here we describe two forms of claudin-7 (CLDN-7), a full-length form of CLDN-7 with 211 amino-acid residues and a C-terminal truncated form with 158 amino-acid residues. These two forms of CLDN-7 are able to regulate the expression of a tissue-specific protein, the prostate-specific antigen (PSA), in the LNCaP prostate cancer cell line. We also found that the expression of CLDN-7 is responsive to androgen stimulation in the LNCaP cell line, suggesting that this protein is involved in the regulatory mechanism of androgen. Both forms of claudin-7 are expressed in human prostate, kidney and lung samples, and in most samples, the full-length form of claudin-7 was predominant. However, in some prostate samples from healthy individuals, the truncated form of claudin-7 is predominantly expressed. Our results demonstrated that unlike other claudins, CLDN-7 has both structural and regulatory functions, and the two forms of CLDN-7 may be related to cell differentiation in organ development.

**Key words:** Claudin — PSA — Gene regulation — Membrane protein — Androgen

## Introduction

Claudins are components of tight junctions (TJs) between epithelial cells (Furuse et al., 1998a, b; Sonoda et al., 1999). The TJ structure is important for restricting lateral diffusion of lipids and membrane proteins, thereby physically defining the border between the apical and basolateral compartments (Anderson & van Itallie, 1995, Tsukita, Itoh & Furuse 1999; Tsabita, Furuse & Itoh, 2001). Moreover, TJs form a regulated barrier for diffusion of solutes through the paracellular pathway to control the microenvironment on each side of the epithelium (Simon et al., 1999). Thus, such a structure is important to various organs for maintaining their growth environments and performing their functions.

The structural functions of claudins have already been described. It is expected that by regulating the expression of TJ proteins, the growth of organs can be modulated, because TJ proteins are critical to the epithelial cells covering organs and their uptake of nutrients. To date, more than 20 claudins have been identified (Furuse et al., 1998a; Morita et al., 1999a, b, c; Simon et al., 1999; Tsukita & Furuse, 2000), and some show tissue specificity (Peacock, Keen & Inglehearn 1997; Paperna et al., 1998; Morita et al., 1999b; Simon et al., 1999). For example, rat claudin-3 (RVP.1) has been identified in rat ventral prostate cells after androgen starvation (Peacock et al., 1997), mouse claudins-4, -7 and -8 are expressed only in the lung and kidney, and mouse claudin-6 is expressed in embryos but not in adults (Morita et al., 1999b). In human tissue, the expression profiles may not be the same. For example, high-level expression of claudin-4 has been detected in colon, and intermediate levels of claudin-4 expression have been detected in prostate, placenta, lung, and pancreas and low levels in small bowel, kidney, and uterus (Long et al., 2001). The tissue specificity of claudins strongly suggests that many claudins may have functions in addition to being structural components of TJs.

In this manuscript, we study the regulatory mechanism of the prostate-specific antigen (PSA). PSA is an important protein in prostate cancer diagnosis, used to define disease stages and cancer cell malignancy (Muschenheim et al., 1991, DeVere

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White et al., 1992; Zietman et al., 1994; Oefelein et al., 1995; Di Silverio et al., 1996; Vicini et al., 1997, Nash & Melezinek, 2000), and is specifically expressed in prostate cells. Although this property is very important in both disease diagnosis and cell differentiation studies, the regulatory mechanism of PSA expression has not yet been fully elucidated. We expect that PSA expression is regulated by proteins that are specifically expressed in prostate cells. To identify these regulatory genes, we used subtractive hybridization to isolate genes expressed in the PSA-expressing prostate cell line, LNCaP, but not in the non-PSA-expressing prostate cell line, DU145. We identified a PSA regulator, the claudin 7 (CLDN-7).

## Materials and Methods

### Cell Culture

The LNCaP and DU145 cell lines were purchased from American Type Culture Collection (ATCC), and the 293T cell line was obtained from I.S.Y. Chen of the UCLA School of Medicine. The other cell lines used have been described previously (Pang et al., 2000, 2001). All cell lines were maintained in RPMI medium supplemented with 10% fetal bovine serum.

### RNA Isolation and Dot Hybridization

RNA was isolated from $10^7$ cells from each cell line, using a guanidinium thiocyanate method. An aliquot of isolated RNA was checked for the quality of RNA by gel electrophoresis. Total RNA was then purified using the PolyATract mRNA isolation kit from Promega (Madison, WI) to remove polyA(−) RNA. The polyA(+) RNA samples were resuspended in 100 μl DEPC-treated water and stored at −70°C. Five μ1 of polyA(+) RNA from each sample was spotted to a nylon filter (Roche Molecular Biochemicals, Indianapolis, IN), followed by hybridization with $^{32}$P-labeled cDNA fragments used as probes.

### Use of RT-PCR to Assess the Expression of CLDN-7 mRNA, β-Actin, PSA and other Claudins

Five μl of the purified polyA(+) RNA samples isolated using the PolyATract mRNA isolation kit (described above) was used for reverse transcription. Oligo-dT nucleotides were used as the primer. The resulting cDNA samples were used for PCR amplification, with oligonucleotide primers to amplify mRNA fragments of CLDN-7, β-actin and PSA, as described previously (Pang et al., 1995). The samples were amplified for 25 thermal cycles at 94°C for 1 min, followed by 65°C for 1 min during each cycle. The primers for amplification of CLDN-7 were AGGCATAATTTTCATCGTGG (5′ primer) and GAGTTGGACT TAGGGTAAGA GCG (3′ primer). Primers for β-actin and PSA were as described previously (Pang et al., 1995). Primers for CLDN-1 are GTGATAGCAA TCTTTGTGGC (5′ primer) and CCTCTGTGTC ACACGTAGTC (3′ primer); CLDN-2, CTACGGGACT TCTACTCACC and CTCACTCTTG ACTTTGGGAG; CLDN-3, TGCTGTTCCT TCTCGCCGCC and CTTAGACGAA GTCCATGCGG; CLDN-4, CTTCTACAAT CCGCTGGTGG and TTACACGTAG TTGCTGGCAG; CLDN-5, GACTCGGTGC TGGCTCTGAG and CGTAGTTCTT CTTGTCGTAG; CLND-6, TGAGGCCCAA AAGCGGGAGC and CGTAATTCTT GGTAGGGTAC; CLND-8 TCATCCCTGT GAGCTGGGTT and TGGAGTAGAC GCTCGGTGAC; CLDN-9, AGGCCCGTAT CGTGCTCACC and ACGTAGTCCC TCTTGTCCAG.

### Suppression Subtractive Hybridization (SSH)

The SSH kit purchased from Clontech (Palo Alto, CA) was used according to the manufacturer's protocol to perform SSH (Diatchenko et al., 1996, 1999).

### Preparation of the cDNA Library

We prepared cDNA from LNCaP cells and prostate cancer samples from two patients. PolyA + RNA was isolated using a cesium gradient centrifugation method (Perry et al., 1972; Chirgwin et al., 1979), followed by removal of polyA(−) RNA with the PolyATract mRNA isolation kit from Promega. The isolated polyA+ RNA was reverse-transcribed to cDNA, using an oligo-dT primer with a Not I sequence tag. The single-stranded cDNA was processed to double-stranded cDNA, using *E. coli* DNA polymerase I, RNase H, and *E. coli* ligase. A Sal I adaptor was then added to the end of the resulting cDNA. After digestion with the Not I restriction endonuclease, the cDNA was inserted into the pCMV-SPORT6 plasmid, which had been precut with Not I and Sal I (purchased from Invitrogen, Carlsbad, CA).

### Isolation of Full-length cDNA of CLDN-7

The cDNA clones isolated from dot hybridization were used as probes to screen a prostate cancer cDNA library with a colony hybridization method. The positive clones were sequenced to confirm that they contained the sequence homologous to the probe cDNA sequences.

### DNA Transfection

Liposome FUGENE-6 (Roche Molecular Biochemicals) was used to introduce DNA into the prostate cell line, LNCaP. Cells ($2 \times 10^4$) were plated into each well of 24-well plates 16 h prior to lipofection. Plasmid DNA (2.0 μg) was mixed with FUGENE-6 liposome in 50 μl of RPMI for 10 min at room temperature before addition into cells. At 8 h, the cell culture was washed once, followed by addition of fresh medium into the cell cultures.

### Luciferase Assays and Determination of EGFP Expression

The expression levels of the luciferase gene in transfected cells were quantified with the assay kit purchased from Promega, and the expression levels of the EGFP were determined by counting EGFP-positive cells by fluorescent microscopy.

### Construction of a CLDN-7 Lentiviral Vector

Plasmid pCDNA3.1-CLND-7 was digested with restriction endonucleases Nde I and Asp 718. A fragment of 1.5 kb containing a part of the CMV promoter and a part of CLDN-7 cDNA from nt +1 to 1016 (Asp 718) was recovered by gel electrophoresis. This fragment was then inserted into the pHR' lentiviral transduction plasmid (Naldini et al., 1996; Pang et al., 2001) that had been

digested with the same restriction endonucleases. The resulting plasmid was used to generate a corresponding lentiviral vector by a cotransfection method. The CLDN-7 insert encodes a truncated CLDN-7 with 16 amino-acid residues removed from the C-terminal. The prepared lentiviral vector stocks were titrated by measuring the contents of Gag protein p24 in the prepared viral stocks.

### Infection of LNCaP and 293T Cells by Lentiviral Vectors

Cells ($2 \times 10^4$/well) were plated into 24-well tissue culture plates 24 h prior to infection. Various amounts of viral vector (multiplicity of infection (MOI) ranging from 0.1 to 10) were added to each well. At three hours post-infection, the cells were washed and fresh medium was added.

## Results

### Isolation of cDNA Fragments Derived from Genes Specifically Expressed in LNCaP Prostate Cells

We isolated genes differentially expressed in a PSA-producing prostate cancer cell line LNCaP, but not in the non-PSA-producing prostate cell line, DU145, by suppressive subtractive hybridization (SSH) (Diatchenko et al., 1996, 1999). Over 100 cDNA clones were obtained. Of the first 20 sequenced cDNA fragments, 11 showed no homology to any reported gene. These 11 clones were further characterized, using dot hybridization to confirm their tissue specificity, and three showed strong prostate-tissue specificity. The mRNA of the sixth clone (LN6) has shown significantly higher expression in the LNCaP cancer cell line (Fig. 1).

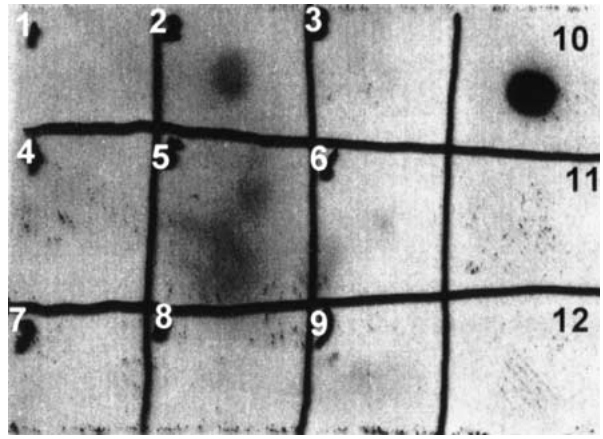


**Fig. 1.** Isolation of tissue-specific cDNA fragments using subtractive hybridization. Tissue-specific expression of a cDNA fragment, LN6, shown by dot-blot hybridization. Dot 2 is the mRNA isolated from LNCaP cells, and other dots are mRNAs from the following cell lines: 1) LNCaP-D, a derivative of LNCaP with no expression of PSA; 2) LNCaP; 3) DU145, a prostate cell line with no PSA expression; 4) AT84, a mouse oral cell line; 5) SCC4, an oral cell line; 6) oral cell line Tu139; 7) oral cell line Tu177; 8) fibroblast cell line HT1080; and 9) HeLa cells. Dots 6 (Tu139) and 9 (HeLa) show low expression of LN6. Dot 10 is the positive control of 0.1 μg of plasmid LN6, and Dots 11 and 12 are blank.

### Isolation of Full-length cDNA Clones Homologous to the Sixth Clone of LNCaP cDNA

The cDNA fragment of this clone (LN6) was used as a probe to screen cDNA libraries prepared from the LNCaP prostate cell line and two prostate tumor samples prepared in our laboratory. Three cDNA clones of approximately 1.6 kb were obtained, and DNA sequencing confirmed that all three were homologous to LN6. These three full-length cDNA clones were subsequently inserted into our prostate-specific vector, pPSAR-PCPSA-P, downstream of the PSA (PSAR-PCPSA) promoter (Pang et al., 1995, 1997), or into pCDNA3.1 vectors (Invitrogen, Carlsbad, CA) under the control of the cytomegalovirus (CMV) promoter. DNA sequencing revealed that cDNA clone 1 contains a major open reading frame (ORF) encoding a peptide of 158 amino-acid residues that is homologous to the human claudin-7 (CLDN-7) peptide sequence, but with a truncation of 53 amino-acid residues from the C-terminal. The other two cDNA clones are identical, and encode a peptide of 211 amino-acid residues, which is the same as the full-length human claudin-7 amino-acid sequence published in GenBank (NCBI Accession #AJ011497). Although these two cDNA clones contain an open reading frame encoding a peptide identical to the CLDN-7 sequence published in GenBank, they use different initiation site. Thus the generated mRNA is different.

### Comparison of Our cDNA Sequences to GenBank Claudin 7 Sequence

Although the amino-acid sequences of the isolated cDNA clones are similar or identical to the claudin-7 sequence published in GenBank, our cDNA clones have different transcriptional initiation and 3′ untranslated sequences, suggesting that they are regulated by different mechanisms (Fig. 2). In comparison to claudin-7 reported in GenBank, cDNA clone 1 has an extra sequence in the major ORF. Because there is a stop codon contained within this extra sequence, the major ORF ended at amino-acid position 158 rather than at 211, as in human claudin-7 (Fig. 2). Using computer analysis (program from Baylor College of Medicine; the internet page is http://searchlauncher.bcm.tmc.edu/), several structural domains have been postulated. When compared with normal claudin-7, the structure of the truncated

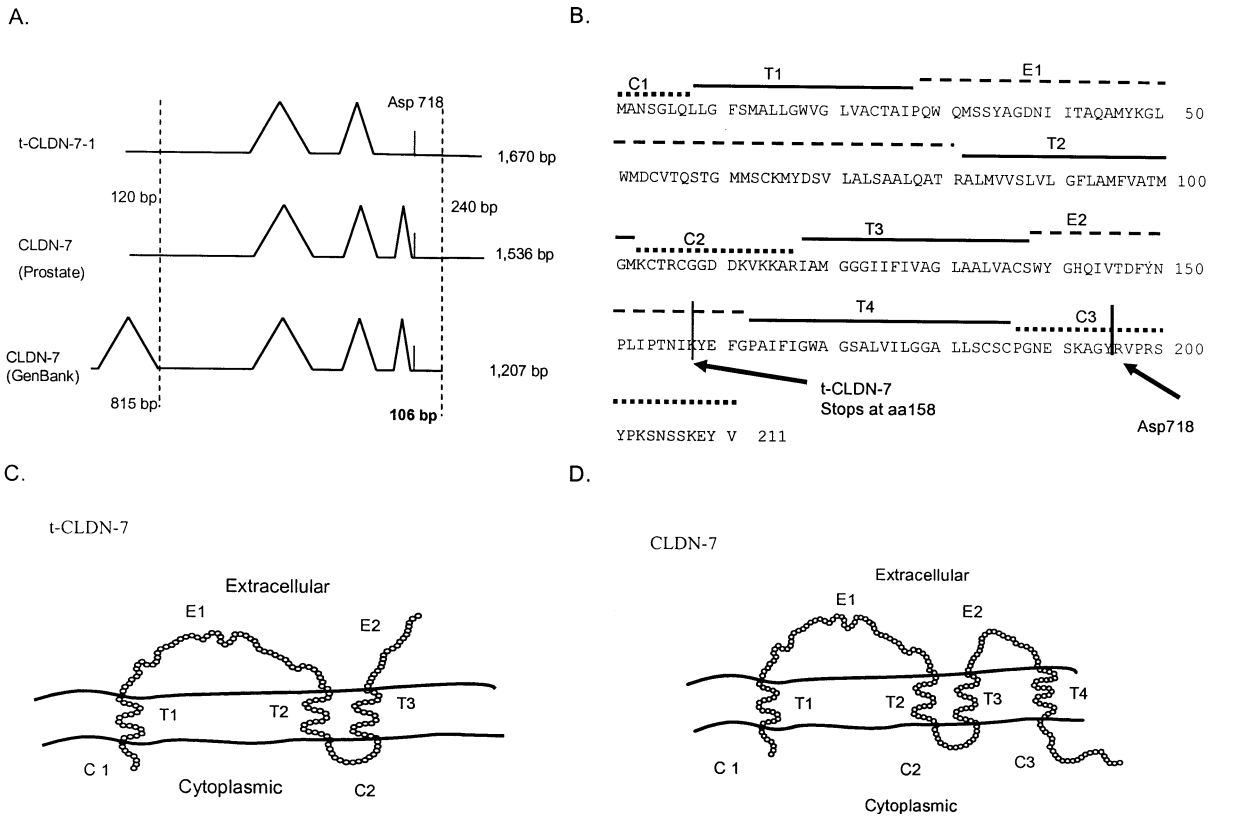


**Fig. 2.** Messenger RNA and amino-acid sequences of the truncated and full-length forms of CLDN-7. (*A*) Comparison of our CLDN-7 cDNAs with the mRNA of the human claudin-7 sequence reported from GenBank (Acc# AJ011497). Our truncated CLDN-7 cDNA is approximately 1,650 bp in length, and full-length CLDN-7 is approximately 1,550 bp, whereas the mRNA of claudin-7 from GenBank is 1,207 nucleotides. Upward deflections indicate the positions of introns. (*B*) Amino-acid sequences and the structural domains according to computer analysis of the coding regions of CLDN-7. C1, C2, and C3 are three cytoplasmic sequencing domains, T1–4 are transmembrane domains and E1 and E2 are the two extracellular domains. Sequencing domains were obtained by using a program from Baylor College of Medicine; the internet page is http://searchlauncher.bcm.tmc.edu/. (*C*, *D*) Two-dimensional structures of the two CLDN-7 forms shown by computer-aided programs.

claudin-7 (t-CLDN-7) contains only three transmembrane domains, and the third cytoplasmic domain (C3) is also truncated (Fig. 2).

## Tissue-specific Regulation of PSA Expression by Both Normal and Truncated CLDN-7

We used the plasmids containing the two isolated cDNA clones controlled by the PSA (PSAR-PCPSA) promoter (Pang et al., 1997) to transfect the LNCaP cell line. A low dose (0.1 μg) of the truncated or normal CLDN-7 cDNA vector significantly increased the activity of the PSA promoter, suggesting that both forms of CLDN-7 positively regulate PSA expression at low levels of claudin-7 (Fig. 3). When higher doses (>0.5 μg) of these gene vectors were used, dramatic inhibition of the PSA promoter was observed (Fig. 3). We have also quantified the full-length gene of the truncated CLDN-7 in the pCDNA3.1 vector driven by the CMV promoter. Because the CMV promoter is stronger than the PSA promoter, significant increases in Lux reporter gene expression were demonstrated when 0.01 μg of pCDNA3.1-t-CLDN-7 was used (approximately 10-fold increase, from $5.7 \times 10^5$ RLU/μg to $4.7 \times 10^6$ RLU/μg). Similar to the results obtained with PSA vectors, at concentrations >0.5 μg/well in 24-well plates, the truncated CLDN-7 gene completely inhibited expression of the PSA promoter (Fig. 3*C*). We also used the EGFP gene as a reporter gene to assess the regulatory function of CLDN-7, and a similar regulatory pattern was demonstrated (Fig. 3*D*). However, because EGFP is not a very sensitive protein reporter and this protein has much longer half-life, the regulatory effects by CLDN-7 are not as obvious as that shown in panels *A* and *B* of Fig. 3, using luciferase.

We have cotransfected the CLDN-7 plasmids with the plasmid that carries the luciferase gene driven by the PSA promoter into a non-PSA-producing prostate cell line, DU145. We found that

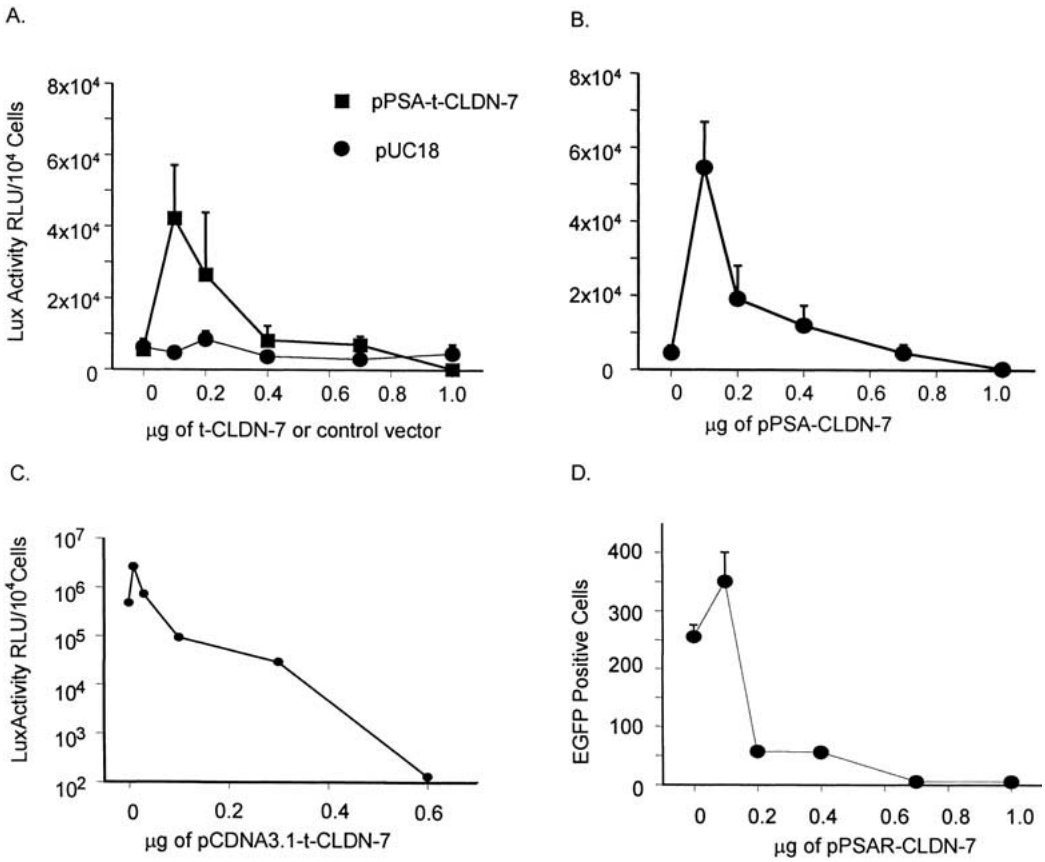


**Fig. 3.** Regulatory function of the two forms of CLDN-7 in mediating the expression of the PSA promoter. (*A*, *B*) Regulation of the PSA promoter (PSAR-PCPSA promoter) by transfection of plasmids carrying either CLDN-7 forms. PSA promoter activity was quantified by measuring luciferase expression levels. (*C*) Regulation of the PSA promoter by t-CLDN-7 in a plasmid vector controlled by the CMV promoter. (*D*) Regulation by CLDN-7 of the PSA promoter driven by the EGFP gene in a plasmid vector controlled by the PSA promoter. PSA promoter activity was quantified by measuring expression levels of EGFP. LNCaP cells ($2 \times 10^4$) were plated into each well of 24-well plates one day prior to transfection. The plasmid, carrying either the EGFP or the Lux gene driven by the PSA promoter (1.0 μg), was mixed with the CLDN-7 plasmids before transfection. Carrier DNA containing the PSA promoter was used to adjust the total volume of DNA to 2 μg for each transfection. Transfections were performed using FUGENE-6 (Roche Biochemicals). The activity of the PSA promoter was assessed two days post-transfection.

the expression of CLDN-7 in this cell line did not stimulate the PSA promoter (*data not shown*), suggesting that other factors that are only expressed in LNCaP cells are involved in the stimulation process.

## The C-terminal of CLDN-7 Is not Involved in PSA Gene Regulation

Although previous reports demonstrated that the C-termini of claudins are important in binding to ZO-1 protein in TJs (Furose, Sasaki & Tsukita, 1999; Itoh et al., 1999), it seems not essential for claudin-7 to regulate PSA gene expression. The C-terminal-truncated form of CLDN-7, t-CLDN-7, shows no significant difference in regulating the expression of PSA gene when compared to the full-length CLDN-7, suggesting that the C-terminal of CLDN-7 is not essential for PSA regulation. We also constructed a truncated form of CLDN-7 by removing the sequence downstream of the Asp 718 site, which removed 16 amino-acid residues from the C-terminal. This form of CLDN-7 showed no significant differences when compared to its parental protein, indicating that the C-terminal amino-acid residues are not required for regulating the PSA gene (Fig. 4).

The effect of the C-terminal-truncated CLDN-7 in regulating the expression of the endogenous PSA gene was also assessed. It was inserted into a lentiviral vector and driven by the CMV promoter. When cultured LNCaP prostate cells were infected by the lentiviral vector that expresses the CLDN-7 protein, significant increases of PSA mRNA were detected by RT-PCR, suggesting that the endogenous PSA promoter that drives PSA gene expression is also stimulated by expression of CLDN-7 (Fig. 5).

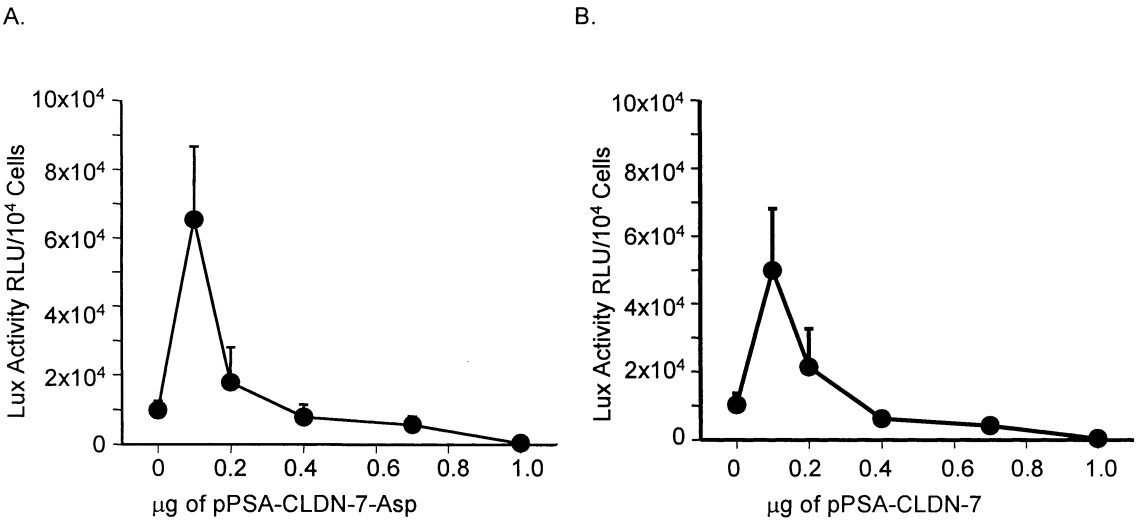


**Fig. 4.** The C-terminal sequence of CLDN-7 is not involved in PSA regulation. Plasmids containing either full-length CLDN-7 (*A*) or CLDN-7 with C-terminal truncation of 16 amino-acid residues (*B*) were used to cotransfect LNCaP cells with the plasmid containing the PSA-luciferase gene-expression cassette. The cell culture and the transfection methods were as described in the legend of Fig. 3. Two days post-transfection, the cells were collected for luciferase assay.

### Both t-CLADN-7 and CLDN-7 are Membrane Proteins

We also studied the location of these two forms of CLDN-7 in cells. To determine the location of the two forms of CLDN-7 in epithelial cells, we fused the EGFP to the N-terminal of the coding regions of these two forms of CLDN-7 in a plasmid carrying the CMV promoter (Fig. 6). DNA transfection of both 293T and LNCaP cell lines with plasmids containing either EGFP-*t*-CLDN-7 or EGFP-CLDN-7 demonstrated that both forms of CLDN-7 localize at the cell membrane, confirming that both are membrane proteins.

### Responsiveness of CLDN-7 Expression to Androgen Stimulation

Because CLDN-7 regulates the PSA gene, which is highly androgen-dependent, we speculated that CLDN-7 is also involved in androgen regulation. LNCaP cell cultures were stimulated with various concentrations of the dihydrotestosterone (DHT) androgen, and the expression of endogenous CLDN-7 quantified by quantitative RT-PCR. LNCaP cells were cultured in RPMI medium supplemented with 10% charcoal-stripped fetal bovine serum, which contains a minimal amount of androgen (Pang et al., 1995). Such androgen starvation provides a low-background cell culture for assessing the effects of androgen in regulating the expression of the endogenous CLDN-7 gene in LNCaP cells. Four days post-androgen starvation, 0, 10, 30, 100 or 300 nM of DHT was added into the culture medium for two days. At that time, all the LNCaP cultures were lysed for RNA isolation. RT-PCR results demonstrated that the expression of the full-length form of CLDN-7 is highly dependent upon androgen stimulation, whereas at low concentrations of DHT, expression of CLDN-7 was low, similar to the expression profile of the PSA gene. At physiological concentrations (30–100 nM) of DHT, maximal expression of CLDN-7 was demonstrated. At a DHT concentration of 300 nM, inhibition of CLDN-7 expression was demonstrated, similar to the results observed with PSA in this experiment (Fig. 7) and our previous report (Pang et al., 1995).

### Characterization of Expression of Two CLDN-7 Forms in Various Tissue Samples

We characterized the expression of two CLDN-7 forms in various tissue samples, using semi-quantitative RT-PCR (Pang et al., 1997). The full-length form of CLDN-7 was found to be actively expressed in all organ samples from patients but not in most of the cancer cell lines tested (Fig. 8*A*). In most tissue samples, the full-length form of CLDN-7 is the predominant form, except in some prostate samples from healthy individuals. These results suggest that t-CLDN-7 plays a tissue-specific role in the prostate. It was interesting to note that neither form of CLDN-7 was expressed in the majority of the cancer cell lines tested (Fig. 8*B*). As a comparison, the expression of other claudins was characterized in three cultured cancer cell lines, including the PSA-producing prostate cell line, LNCaP, the non-PSA-producing prostate cell line, DU145, and the renal cell carcinoma cell line, R11. Significant expression levels of CLDN-6 and

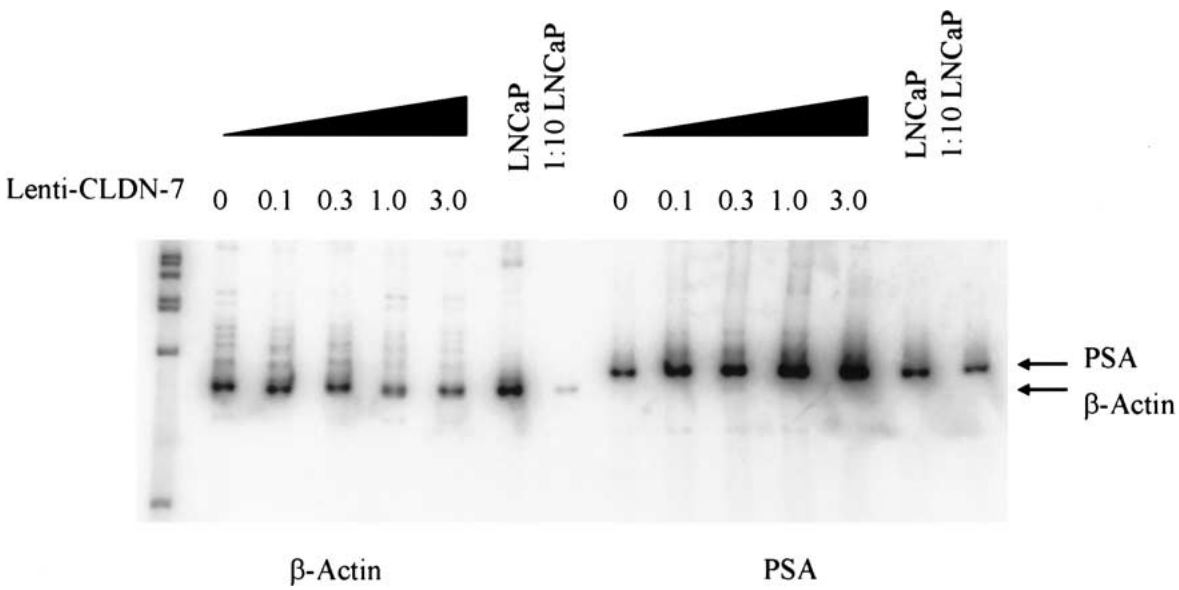


**Fig. 5.** CLDN-7 regulating the expression of endogenous PSA in the LNCaP prostate cell line. LNCaP cells ($2 \times 10^4$) were plated in 24-well tissue culture plates 24 h prior to infection. Various doses (MOI 0–3.0) of lentiviral vector carrying the C-terminal-truncated CLDN-7 were added to each well of LNCaP cell cultures. Two days post-infection, the infected cells were collected for RNA isolation, followed by RT-PCR to quantify expression of the PSA gene. The expression of β-actin was used as a control to normalize the amount of cDNA used to quantify the expression of PSA. LNCaP mRNA collected from other experiments was also quantified as a control.

CLDN-8 were detected in all three of these cell lines. Expression of CLDN-2 was also detected in these three cell lines, with higher levels of expression found in DU145 (Fig. 9). These results suggest that when compared to other claudins, CLDN-7 may be more strongly related to cell differentiation and/or malignancy.

## Discussion

Claudins have been characterized as components of TJs. Some claudins in TJs selectively allow certain materials to pass the tight junction barrier, thereby regulating the supply of nutrients to organs (Morita et al., 1999b; Chen et al., 2000). Our results have shown that in prostate cells, both the truncated and full-length forms of the claudin-7 protein regulate the expression of a tissue-specific protein, PSA, strongly suggesting that some claudins may be able to regulate gene expression. Previous reports have demonstrated that the C-terminal of claudins is highly conserved, and it may be indispensable for a claudin to associate with other claudin peptides and other components of TJs. Here we demonstrate that deletion of the C-terminal of CLDN-7 does not affect the ability of CLDN-7 to regulate PSA expression, suggesting that the gene regulatory function of CLDN-7 is independent of its role in forming TJs. It is intriguing that there are two forms of CLDN-7 in many tissues. Our results demonstrate that the full-length CLDN-7 is the predominant form in the kidney, lung and most prostate samples. However, in some prostate samples, the truncated form of CLDN-7 showed higher levels of expression, indicating that there is a mechanism that regulates the splicing of CLDN-7 mRNA. It is also interesting that only some prostate tissue samples express the truncated form of CLDN-7 more highly than the full-length form. We also noted that in most cell lines, the expression levels of CLDN-7 were very low compared to samples from patients, suggesting that this gene may be involved in cell malignancy or dedifferentiation.

Our results demonstrate that both the truncated and the full-length forms of CLDN-7 are membrane proteins. However, the biological significance of the existence of two forms of CLDN-7 is not clear. Different splicing of claudins has been found in claudin 18 (Niimi et al., 2001). In this paper, four additional spliced mRNAs are demonstrated in stomach and lung, opening the door to a possible role in organ differentiation for some claudins. Because CLDN-7 is highly homologous to well-characterized TJ claudins (e.g., claudin-1), it is likely that CLDN-7 functions similarly to claudin-1 in forming TJs. Previous reports demonstrated that the C-terminal of claudins is critical to the ability of claudins to form TJs. It is probable that full-length CLDN-7 has both structural and regulatory functions, while truncated CLDN-7 might have only a regulatory function. By regulating the splicing of CLDN-7, cells would be able to reg-

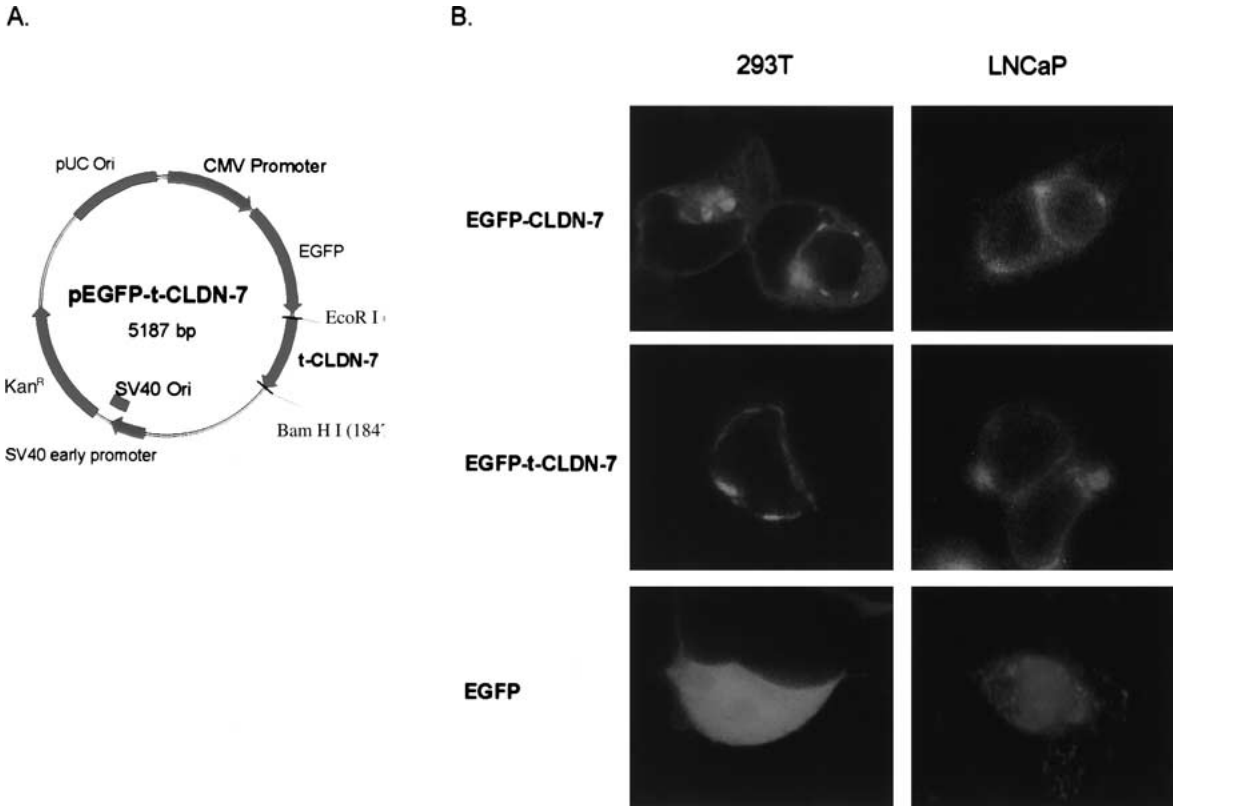


**Fig. 6.** Locations of the two forms of CLDN-7 in epithelial cells. (*A*) The EGFP gene inserted into the N-terminal of t-CLDN-7. The strategy of construction of a plasmid expressing the EGFP-CLDN-7 fusion protein was similar. (*B*) Expression of the fusion proteins created above in 293T and LNCaP cells. Cells ($2 \times 10^4$) were plated in 24-well tissue culture plates 24 h prior to transfection. Plasmids containing the fused genes were transfected into cell cultures, using the FUGENE-6 liposome (Roche Molecular Biochemicals). Expression of the EGFP gene alone transfected by plasmid pEGFP-N1 (purchased from Clontech) in these two cell lines is used as control. Confocal microscopy was used to visualize the expressions of EGFP in the 293T cell line (left three photos) and regular fluorescent microscopy was used to visualize the expressions of EFFP in the LNCaP cell line. Photographs were taken 24 h post-transfection.

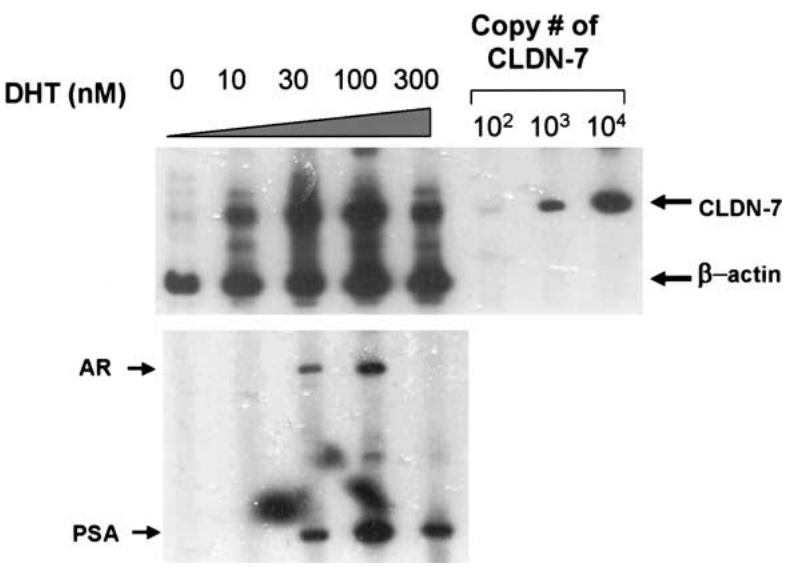


**Fig. 7.** Androgen responsiveness of CLDN-7. Expression of CLDN-7 and PSA in the LNCaP culture stimulated with DHT at various concentrations was quantified, using RT-PCR. LNCaP cells were maintained in RPMI 1640 medium supplemented with 10% charcoal-stripped fetal bovine serum for four days before plating. The cells were trypsinized from T-162 cell culture flasks and plated into 24-well culture plates ($2 \times 10^4$/well) in RPMI medium with 10% charcoal-stripped fetal serum. Various concentrations of DHT were added into individual wells. At 48 h, the cells were lysed for RNA isolation. RT-PCR was performed using a previously described method (Pang et al., 1995).

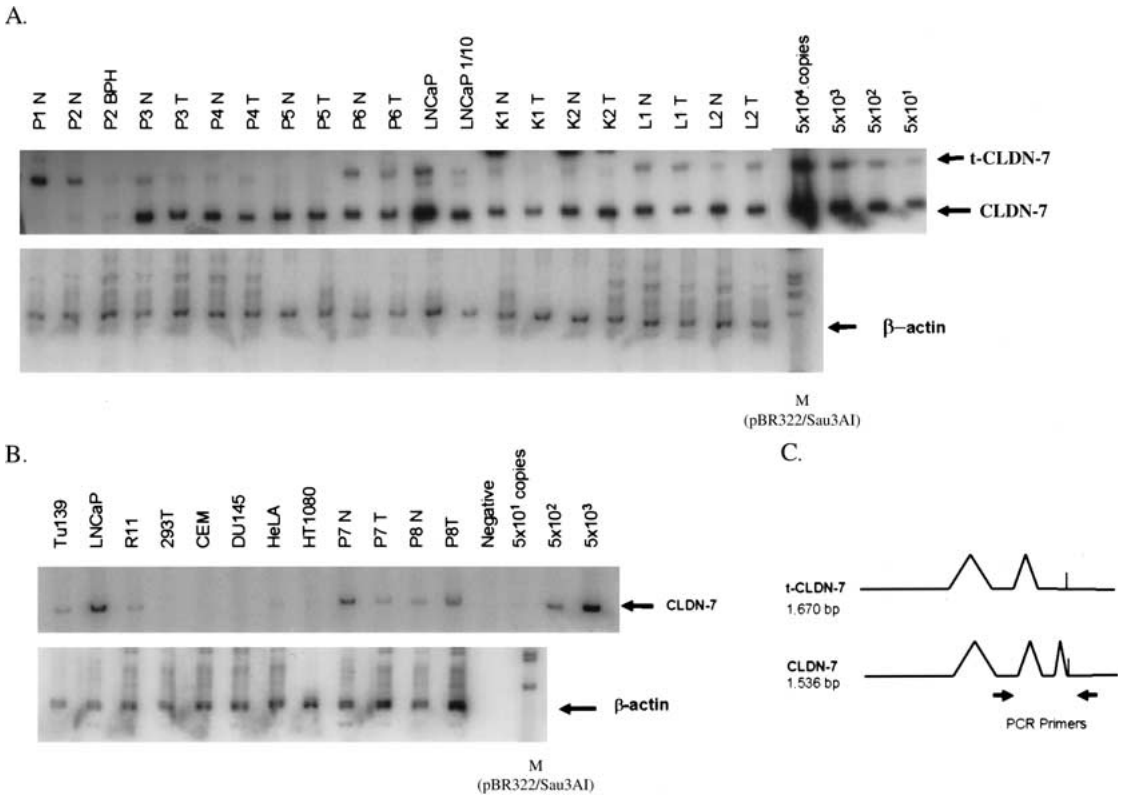


**Fig. 8.** CLDN-7 expression in patient samples and cell lines derived from various organs. (*A*) RT-PCR semi-quantitation of CLDN-7 in human kidney, lung and prostate tissue samples. Tissue biopsies from patients were sliced into small pieces before lysing for RNA isolation. The isolated RNA samples were reverse-transcribed, using AMV reverse transcriptase (Roche Molecular Biochemicals). The resulting cDNA samples were normalized, using β-actin as the indicator. LNCaP RNA samples, either with no dilution or at a 1:10 dilution, were used as positive controls. Equal amounts of the t-CLDN-7 and CLDN-7 plasmids were mixed together to serve as standards to quantify the copy numbers of the two forms of CLDN-7 in the patient samples. P1 to P7 are prostate samples. "N" represents normal, and "T" indicates that the sample is from tumor tissue. P3 BPH is a prostate sample from a patient with benign prostatic hyperplasia, the K1 and K2 samples are from kidney tissue, and L1 and L2 are from lung tissue. (*B*) RT-PCR semi-quantitation of CLDN-7 in human cell lines. Various cell lines were cultured in 24-well plates for two days before collection. RNA samples were reverse-transcribed, followed by PCR, using the same procedure as described above. Plasmids containing either t-CLDN-7 or CLDN-7 genes were used as standards for quantitation. The cell lines were derived from the following organs: Tu139, oral; LNCaP, prostate; R11, renal; 293T, embryonic kidney; CEM, CD4+ T-lymphocytes; DU145, prostate; HeLa, cervix; and HT-1080, fibroblasts. P8 and P9 are prostate samples from patients. (*C*) The positions of the PCR primers for RT-PCR.

ulate the expression of other genes without disturbing the structure of TJs.

It is also notable that while both forms of CLDN-7 are membrane proteins, they regulate PSA gene transcription, which occurs in the nucleus. Thus, a signal transduction pathway to relay the signals from CLDN-7 in the cell membrane to the PSA gene promoter in the nucleus would be required. Our results demonstrate that expression of CLDN-7 in prostate cells is responsive to androgen stimulation; thus, it is possible that CLDN-7 is a component of a signal transduction pathway through which androgen regulates the PSA gene. At least in prostate cells, androgen stimulation can upregulate CLDN-7, and the increase of CLDN-7 can subsequently upregulate the expression of the PSA gene, as well as of other genes. Such a role may be important for cell growth and differentiation. We have cotransfected the plasmid carrying either the truncated or full-length form of CLDN-7 into DU145 cells with the plasmid that contains the PSA promoter-luciferase gene expression cassette. In this cell line neither the truncated nor the full-length CLDN-7 demonstrated any stimulation effect. These results suggest that other proteins that are specifically expressed in LNCaP cells are involved in the stimulation pathway. Furthermore, our results demonstrate that the CLDN-7 protein does not merely stimulate or inhibit the expression of the PSA gene (i.e., at low concentrations, the CLDN-7 proteins stimulate PSA gene expression); at high concentrations, they dramatically inhibit PSA gene expression. Such a bi-phase control profile suggests that there is a complex mechanism, including a feedback control, at the signal transduction pathway.

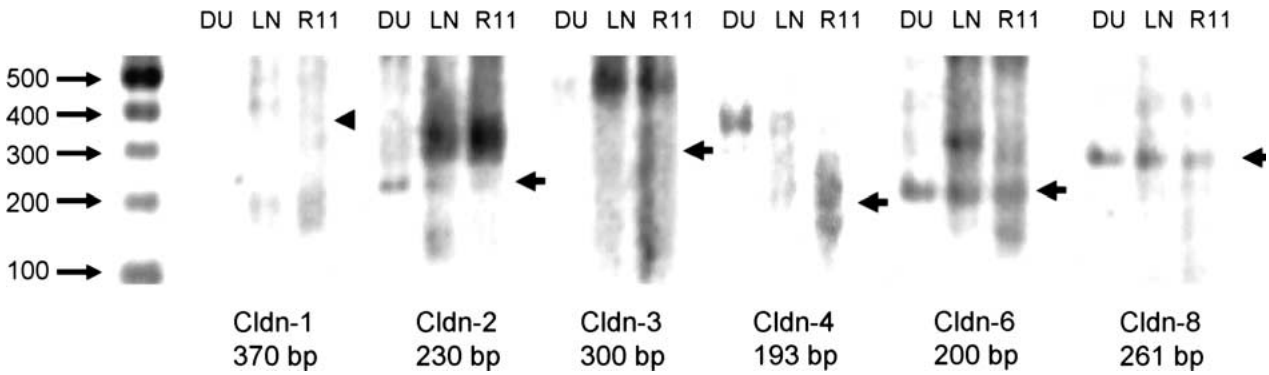


**Fig. 9.** Expression of claudins-2, -6, and -8 in the LNCaP, DU145 and R11 cell lines. mRNA from the PSA-producing prostate cell line, LNCaP, the non-PSA-producing prostate cell line, DU145, and the renal carcinoma cell line, R11, was normalized using β-actin. Significant CLDN-7 expression was demonstrated in LNCaP but not the other two cell lines, as described above. Significant expression levels of claudins-2, -6 and -8 were detected in all three cell lines. No significant levels of expression of claudins-1, -3 and -4 were detected. Arrows indicate the expected locations of PCR products derived from the mRNA of these genes. The expression of CLDN-7 and t-CLDN-7 in LNCaP, DU145 and R11 cells is shown in Fig. 8*B*. In a separate experiment, claudin-5 expression was examined in these three cell lines, but no positive band was detected in any of them (*data not shown*). The sequences of the primers used for RT-PCR were described in Materials and Methods, and the expected sizes of these PCR products are indicated.

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