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Epigenetic Regulation of *Coxsackie and Adenovirus Receptor* (*CAR*) Gene Promoter in Urogenital Cancer Cells

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ABSTRACT

Coxsackie and adenovirus receptor (CAR) is not only a high-affinity receptor for adenovirus, but also a tumor inhibitor in both prostate and bladder cancer lines. Decreased *CAR* gene expression is often detected in cancer specimens; however, the mechanism(s) is still unknown. In this study, we cloned the entire *CAR* gene and characterized the core promoter sequence of the *CAR* gene. The *CAR* gene promoter activity correlated with the differential expression of *CAR* mRNA levels from several urogenital cancer cell lines, indicating that the down-regulation of *CAR* gene expression is mediated by transcriptional regulation. It is known that epigenetic control, such as DNA methylation and histone acetylation of chromatin structure, dictates gene expression. Data from this study indicate that the activation of the *CAR* gene promoter is modulated by histone acetylation, but not by DNA methylation, in urogenital cancer cells. Also, a potent cancer chemotherapeutic agent (FR901228), a histone deacetylase inhibitor, was able to induce endogenous *CAR* gene expression in several urogenital cancer cells. Taken together, epigenetic control of *CAR* gene underlies the down-regulation of this gene in urogenital cancers.

INTRODUCTION

Coxsackie and adenovirus receptor (CAR) is a high-affinity receptor for adenovirus type 5 (1, 2). Many studies have demonstrated that heterogeneous expression of CAR in various tissues (3–5). In urogential cancers, several groups, including us, have reported decreased CAR expression in cell lines and tissue specimens (6–10). These data imply that CAR has physiological functions in urogenital cancers other than as an adenovirus receptor. Structurally, CAR is a typical cell-adhesion molecule with homophilic interaction (7, 8). Our recent studies further demonstrate that the cell-adhesion function of CAR is critical for its growth-inhibitory effect on human bladder and prostate cancer cells (7, 8). Increased CAR expression in CAR-negative cells leads to growth suppression of urogenital cancer cells both *in vitro* and *in vivo* (7, 8). Taken together, the levels of CAR expression in urogenital cancers will have a significant impact on the outcome of adenovirus gene therapy and the cancer progression.

With respect to the differential expression of CAR in urogential cancers, the mechanism(s) of down-regulation of the *CAR* gene is largely unknown. Recent studies by Kitazono *et al.* (11) and Hemminki *et al.* (12) demonstrate that a histone deacetylase (HDAC) inhibitor, FR901228 (13), can increase *CAR* gene expression in several different cancer cell lines, indicating that the critical role of epigenetic control in *CAR* gene expression.

In this study, we cloned and characterized the promoter sequence of the human *CAR* gene. We have identified the core promoter sequence of *CAR* gene and further demonstrated that reduced CAR expression paralleled with the decreased *CAR* promoter activity in urogenital cancer cells. Thus, we believe that agent(s) capable of enhancing *CAR* promoter activity can be potential chemotherapeutic agents for urogenital cancer therapy and can also enhance the efficacy of gene therapy.

MATERIALS AND METHODS

All of the human bladder cancer cell lines used in this study were obtained from American Type Culture Collection (Manassas, VA), and all of the cell lines were cultured in T medium containing 5% fetal bovine serum (6–8). pCMV-β-galactosidase (β-gal) was provided by Dr. Ching-Hai Kao (Indiana University). FR901228 was obtained from Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan).

Determination of the Transcriptional Starting Site of the *CAR* Gene Using 5′ Rapid Amplification of cDNA Ends (RACE) Assay. To determine the transcriptional starting site of the *CAR* gene, a modified 5′ RACE assay, RNA ligase-mediated RACE (obtained from Ambion), was performed using three different primers, CAR6, CAR7, and CAR272 (Fig. 1*A* and Table 1). Total cellular RNA (10 μg) from RT-4 (a CAR-positive cell line) was subjected to this protocol according to the manufacturer's instructions. PCR products were then run in a 2% NuSieve 3:1 agarose gel (Biowhittaker) to determine the length of the cDNAs.

Screening and Subcloning of the *CAR* Gene Promoter From a Human Genomic Library. Human *CAR* gene was obtained by screening a bacterial artificial chromosomal library (CITB from Research Genetics) using a full-length *CAR* cDNA (2160 bp) as a probe. With 17 positive clones, we ruled out pseudogenes by PCR amplification of seven pairs of continuous regions of the entire cDNA sequence as described by Bowles *et al.* (14). Bacterial artificial chromosomal clone 626C16 was selected and further confirmed by Southern blot using a full-length *CAR* cDNA probe, as well as a 5′ upstream region probe (CAR217). CAR217 (248 bp) containing 40 bp of the first exon and a 208-bp untranslated region was generated by PCR with primers CAR G21D (5′-GCTCTATCCCTACCAGAGATG-3′) and CAR7 (Table 1).

Clone 626 C16 was digested with *Hind*III to select fragments containing the 5′ upstream of the untranslated region. On the basis of the predicated *Hind*III fragments from the gene sequence, two fragments were subcloned into pBluescript II SK vector and confirmed by DNA sequencing; one fragment was HF-740 (740 bp) and the other Clone HF4–8 (5309 bp; Fig. 2*A*). To generate a variety of deletion mutants, Clone HF4–8 was further shortened using Erase-A-Base system (Promega) and several deletion fragments: 1196 (−1213 to −18), 1087 (−1213 to −127), 776 (−1213 to −438), 722 (−1213 to −492) and 382 (−1213 to −832) were generated (Fig. 2*A*).

Measurement of *CAR* Gene Promoter Activity Using the Reporter Gene Assay. To test promoter activity of the *CAR* gene, all of the deletion mutants from HF4–8 and HF740 were further subcloned into pGL-3 basic vector (Promega) at *Hind*III and *Nco*I sites. In addition, pGL3–56 was generated by ligating a double-strand synthetic nucleotide containing *CAR* gene sequences (−491 to −436) flanked with *Nhe*I and *Xho*I sites at both ends. Furthermore, pGL3–186 was generated by amplifying a 186-bp fragment using a primer set (5′-ACAGGTCGCATCCCGTGAGC-3′ and 5′-GCAGCCCGTCTCCCACATAC-3′) and further subcloned into pGL3 vector at *Xho*I and *Hind*III sites.

In each transfection experiment, 0.8 μg of DNA and 0.2 μg of pCMV-β-gal DNA were added into each cell line (3×10^5 cells/well) using LipofectAMINE Plus (Invitrogen). Forty-eight hours after transfection, cells were washed twice with cold PBS and lysed with 200 μl of Reporter Lysis buffer (Promega). Cell lysate was collected after twice freeze-thawing and centrifugation; 20 μl of the supernatant were subjected to luciferase assay. All experiments were repeated at least three times in triplicate. The relative reporter gene activity was

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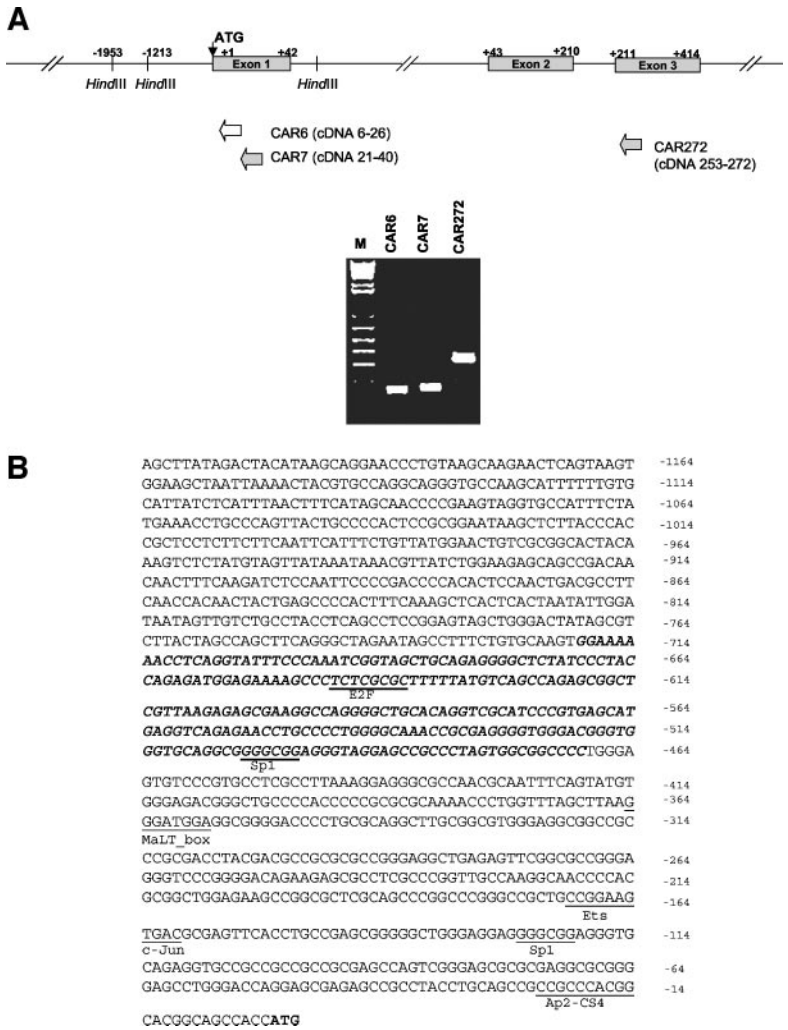

Fig. 1. Determination of 5′-upstream regulatory region of the human the *coxsackie and adenovirus receptor* (*CAR*) gene. *A*, analysis of transcription starting site of the *CAR* gene. The transcriptional starting site was determined using an RNA ligase-mediated rapid amplification of cDNA ends kit with three different primers (CAR6, CAR7, and CAR272), with the corresponding nucleotide position listed in *parentheses*. The *number* depicted *above each exon* represents the nucleotide position of *CAR* cDNA (NM_001338). *M*, 1 kb plus DNA ladder marker (Invitrogen). *B*, DNA sequence of 5′-upstream regulatory region of the *CAR* gene. A total of 1213 bases are depicted as the 5′-untranslated region: the predicted promoter region (*bold italic*), translation initiation site (*bold*), and several potential transcription factors binding sites (*underlined*).

determined by normalizing the luciferase activity with the β-gal activity. For FR9011228 treatment, the agent was added into cells 24 h after transfection and incubated for 24 h before harvesting for luciferase activity assay.

Determination of *CAR* mRNA Expression Using Real-Time Reverse-Transcription PCR Assay. Cells (5×10^5) were plated in a p-100 dish 24 h before the treatment of FR901228. Cells were replaced with fresh medium containing various concentrations of FR901228. After 72 h of drug treatment, total cellular RNA was extracted with RNA-Bee reagent (Tel-Test, Houston, TX). Total cellular RNA (3.2 μg) was subjected to reverse transcription with Superscript II (Invitrogen) with 100 ng random hexamer (Invitrogen). The first strand of cDNA (2.5 μl) was further amplified by a real-time PCR using primerd CAR3 and CAR4 (Table 1). A 40-μl PCR reaction was carried out in a iCycler thermal cycler (Bio-Rad) using 1:100,000 of SyBr-Green (FMC Bioproducts) and Platinum Quantitative PCR SuperMix-UDG mix (Invitrogen), with a denaturing step at 95°C for 2 min, followed by 35 cycles of amplification with 92°C for 15 s, 55°C for 30 s, and 72°C for 2 min, and then an extension step at 72°C for 7 min. The *glyceraldehyde-3-phosphate dehydrogenase* cDNA [G3P4 (5′-AGTGAGCTTCCCGTTCAAC-3′) and G3P7 (5′-GAAGGTGAAGGTCGGAGTCAACG-3′)] was used as the internal control. All experiments were repeated twice in duplicate. Fold of induction of *CAR* mRNA was determined by normalizing the copy number of *CAR* cDNA with the copy number of *glyceraldehyde-3-phosphate dehydrogenase* cDNA of each sample.

Table 1 *PCR primers used in 5′ rapid amplification of cDNA ends (RACE), real-time reverse-transcription PCR, chromatin immunoprecipitation (ChIP) assay and bisulfite genomic sequencing*

Primer name	Application	5′-3′ sequence
CAR3	Real-time PCR	GCCTTCAGGTGCGAGATGTTAC
CAR4	Real-time PCR	CTTGTGCCTCTCGTGTCTACTCTG
CAR6	5′-RACE	AGGAGCACGAAGCACAGCAG
CAR7	5′-RACE	CCACTACTCCGCACAGGAGC
CAR272	5′-RACE	ACTCGGCCTTTCAGATCTGG
CAR5024F	ChIP	ACAGGTCGCATCCCGTGAG
CAR5208R	ChIP	CAGCCCGTCTCCCACATACTG
MethCAR2	Bisulfite genomic sequencing	GGTTAGGGGTTGTATAGGT*YGTAT[a]
MethCAR4	Bisulfite genomic sequencing	CCTCCATCCCTTAAACTAAACCAA

[a] *Y, C or T.

Determination of the Association of Acetylated Histone with *CAR* Gene Promoter Using Chromatin Immunoprecipitation (ChIP) Assay. A ChIP assay was performed to detect the effect of FR901228 on the association of acetylated histone with the promoter region of the *CAR* gene using an anti-acetyl-histone H4 antibody (Upstate Biotechnology). Cells were plated in a p-100 dish 24 h before FR901228 treatment. Seventy-two hours after treatment, an equal number of cells from either with or without FR901228 treatment were subjected to the ChIP assay as described previously (15). DNA fragments were further purified with phenol-chloroform and subjected to PCR reaction [98°C (3 min), 29 cycles of 98°C (30 s), 62°C (30 s), and 72°C (1 min), then 72°C (7 min)] using 3 μl of purified DNA and 1× of ThermalAce DNA polymerase (Invitrogen) with a primer set, CAR5024F and CAR5208R (Table 1), in a 25-μl reaction volume.

Bisulfite Genomic Sequencing. High molecular-weight genomic DNA was obtained from the indicated cell lines and subjected to bisulfite modification (15). Briefly, 1–2 μg (5–10 μl) of genomic DNA were denatured by NaOH (final concentration, 0.2 M), 30 μl of 10 mM hydroquinone (Sigma), and 520 μl of 3 M sodium bisulfite (Sigma; pH 5) and incubated at 50°C for 16 h. Modified samples were purified using Wizard DNA Clean-Up System desalt-

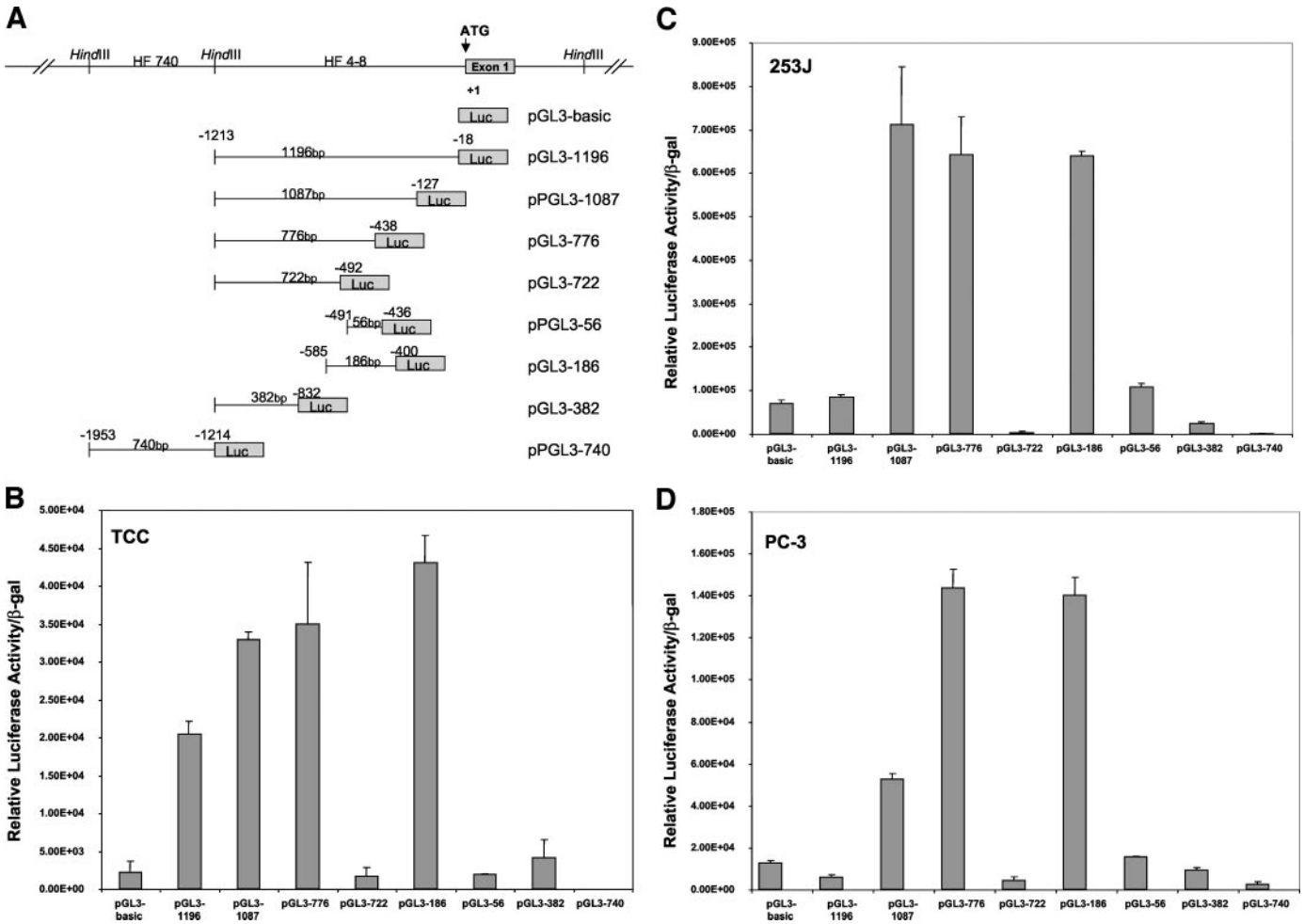

Fig. 2. Mapping the core promoter region of the human *coxsackie and adenovirus receptor* (*CAR*) gene. *A*, schematic representation of the *CAR* gene promoter region and its deletion mutants. A series of reporter gene constructs containing different *CAR* promoter regions was cloned in pGL-3 vector as described in "MATERIALS AND METHODS." *B–D*, luciferase activity of the *CAR* promoter in several urogenital cell lines. Relative luciferase activity was normalized with β-galactosidase activity. Each *number* represents data from triplicate experiments. *Bars*, SD.

ing columns (Promega), followed by ethanol precipitation. Bisulfite-modified DNA (100 ng) was PCR amplified with MethCAR2 and MethCAR4 primers (Table 1) in a 25-μl reaction mixture. A hot start was performed (5 min, 95°C) by adding 0.5 units of HotStar *Taq*DNA polymerase (Qiagen). The PCR products were cloned into TA cloning vector pCR2.1-TOPO (Invitrogen). Five individual clones were sequenced using reverse and forward M13 primers.

RESULTS

Characterization of the Promoter Sequence of *CAR* Gene. To study the regulation of *CAR* gene, we decided to clone the *CAR* gene. Therefore, a human bacterial artificial chromosomal library was screened with *CAR* cDNA probe. With 17 positive clones identified from this library, we found that 10 of 17 clones were pseudogenes based on genomic PCR analyses (data not shown). Nevertheless, Clone 626C16 (~169 kb) containing the entire *CAR* gene was confirmed by Southern blot and DNA sequencing. We further determined the transcriptional starting site of the *CAR* gene by performing a 5′-RACE experiment. As shown in Fig. 1*A*, three primers taken from three different locations of *CAR* cDNA were used, and all of the PCR products depicted the same starting point, that is, 150 bp upstream from the ATG site (Fig. 1*B*). In Clone 626C16, we estimated 30 kb of DNA sequence upstream from the ATG site. Also, the first intron in this gene is quite large and is estimated to be ~34 kb in length. Using the NIH promoter search Web site,[3] we were able to predict the potential promoter sequence of the *CAR* gene located at −470 to −719 (Fig. 1*B*) upstream from the ATG site.

Determination of *CAR* Gene Promoter in Urogenital Cancer Cell Lines. The promoter activity of the *CAR* gene was determined from the deletion mutants generated from Clone HF4−8 and HF740 (Fig. 2*A*). As shown in Fig. 2, *B–D*, the reporter gene activity increased in all three cell lines when the sequence from −18 to −127 was deleted, suggesting that a putative silencer may be present. We also noticed that the reporter gene activity diminished dramatically when the deletion sequence extended beyond −492. Therefore, we believed that the promoter region of the *CAR* gene should be within this region. To further confirm this data, we generated two clones (pGL3–56 and pGL3–186) and demonstrated that only pGL3–186 expressed the same reporter gene activity as pGL3–776 in TCC cells (Fig. 2*B*). Noticeably, the similar pattern of reporter gene activity from each deletion mutant was observed in two other cell lines tested in this study (Fig. 2, *C* and *D*). We, therefore, concluded that the promoter region of the *CAR* gene was located between −585 and −400.

In this experiment, three cancer cell lines expressing different CAR

[3] bimas.dcrt.nih.gov:80/molbio/proscan.

Table 2 *Correlation between coxsackie and adenovirus receptor (CAR) protein expression and CAR promoter activity in urogenital cancer cell lines*

Cell line	CAR protein level (fluorescence-activated cell sorting, %)[a]	CAR promoter activity (luciferase reporter gene)[b]
TCC	8.6	4.4×10^4
WH	24.5	8.8×10^4
PC-3	35.0	1.4×10^5
UMUC3	46.8	2.0×10^5
253 J	93.5	6.5×10^5

[a] Cells were subjected to immunofluorescent staining using RmcB (CAR monoclonal antibody, Ref. 1) and presented as the percentage of cells gated positive by cytometrical analysis (6).

[b] Cells were transfected with pGL-186 plasmid and luciferase activity from each sample was determined as described in "MATERIALS AND METHODS."

levels were used. From our previous studies (7, 8), the levels of CAR expression are as follows: 253J > PC3 > TCC. Data from the reporter gene assay clearly demonstrated that the *CAR* promoter activity in 253J cells was higher than in the other two cell lines tested (Fig. 2, *B–D*). We also noticed a good correlation between the percentage of CAR-positive cells (Table 2) and the luciferase activity of pGL3–186 from each cell line (Fig. 2). Taken together, our data indicated that differential expression of CAR levels among urogenital cancer cell lines correlated with the promoter activity of *CAR* gene.

Epigenetic Control of *CAR* Gene in Urogenital Cancer Cells. To understand the mechanism of the down-regulation of the *CAR* gene in urogenital cancer cell lines, we explored two common known regulatory pathways: DNA methylation and histone acetylation. To detect the methylation status of the *CAR* promoter, we used a bisulfite DNA sequencing assay to determine the density of methylated CpG islands. In general, the methylated CpG islands within the *CAR* promoter region are very scattered (Fig. 3); the total number of methylated CpG islands is very similar between CAR-positive (RT-4, 253J, SWBC, PC-3) and CAR-negative (T24 and TCC) cells. Also, we failed to detect any induction of *CAR* gene expression in cells treated with various concentrations of DNA hypomethylation agent (*i.e.*, 5′-aza-2′-deoxycitidine) using real-time reverse-transcription PCR (Table 3). In contrast, under the same treatment condition, 5′-aza-2′-deoxycitidine was able to induce a positive control gene (*i.e.*, hDAP1/2) expression, recently reported by us (15), in these cells. These data indicated that DNA methylation did not play a role in regulating the *CAR* gene promoter in urogenital cancer.

In addition, we also determined the potential impact of histone acetylation on *CAR* gene expression in urogenital cancer cell lines. An HDAC inhibitor (such as FR901228) has been shown to increase CAR expression and adenovirus sensitivity in several malignant cells (11, 12). However, the role of HDAC inhibitors in urogenital cancer cells is still unknown. To examine the effect of FR901228 on the endogenous CAR expression in urogenital cancer cells, we performed real-time reverse-transcription PCR to detect the steady-state levels of *CAR* mRNA in each cell line. To ensure the quality control of each real-time PCR reaction, we also performed the standard curve of both CAR ($R = 0.997$) and glyceraldehyde-3-phosphate dehydrogenase ($R = 0.990$) as shown previously (16) in every experiment. In general, the linear range of *CAR* cDNA is between 200 copies and 200 million copies (*i.e.*, 6 logs), and the linear range of *glyceraldehyde-3-phosphate dehydrogenase* cDNA is between 700 copies and 700 million copies (*i.e.*, 6 logs). All samples performed in this study were under the linear range of both cDNA. Our data (Fig. 4, *A–C*) demonstrated that FR901228 could induce CAR expression in PC-3, TCC, and T24 cells in a dose-dependent manner. The fold of induction ranged from 15- to 40-fold; it appeared that the higher induction seen in both T24 and PC-3 cells might be attributable to a lower basal CAR level in both cell lines. Noticeably, TCC cells were the most sensitive to FR901228 treatment among three cell lines; the peak of induction was detected at a lower concentration because of the apparent cytotoxicity induced by high concentrations of FR901228 (data not shown).

To evaluate whether the pGL3–186 is indeed a core promoter of human *CAR* gene, we further evaluated the effect of HDAC inhibitor on the activity of this reporter construct. As shown in Fig. 5, the highest luciferase reporter gene activity of pGL3–186 was observed in cells treated with FR901228, indicating that histone acetylation has a direct impact on the *CAR* gene promoter in urogenital cancer cells. To establish the correlation between the status of histone acetylation and CAR expression in cells under the routine culture condition, nuclei from the cell lines with different CAR expression were isolated and subjected to the ChIP assay. Our data (Fig. 6*A*) demonstrated that the association between acetylated histone H4 and core promoter region of the *CAR* gene was detectable in the cell line with higher CAR expression, such as 253J (6, 7), rather than in other cell lines expressing lower levels of CAR, indicating that the chromatin structure surrounding the *CAR* gene promoter maintains an "open" form in 253J cells. Thus, the association of acetylated histone H4 with the *CAR* promoter in 253J cells confirmed the constitutive expression of the *CAR* gene in 253J cell line. Nevertheless, the presence of FR901228 enhanced the association of acetylated histone H4 with the *CAR* gene promoter in TCC, T24, and PC-3 cells (Fig. 6*B*), indicating that the underlying mechanism of *CAR* gene regulation is via epigenetic control. Taken together, we believe that histone acetylation plays a critical role in modulating *CAR* gene expression in urogenital cancer cells.

To test the effect of FR901228 on the adenoviral infectivity, cells were treated with both FR901228 and AdCMVβ-gal, and our results showed a 5–10-fold increase in the β-gal activity in drug treated PC-3 and TCC cells (16). Taken together, our results indicated that epige-

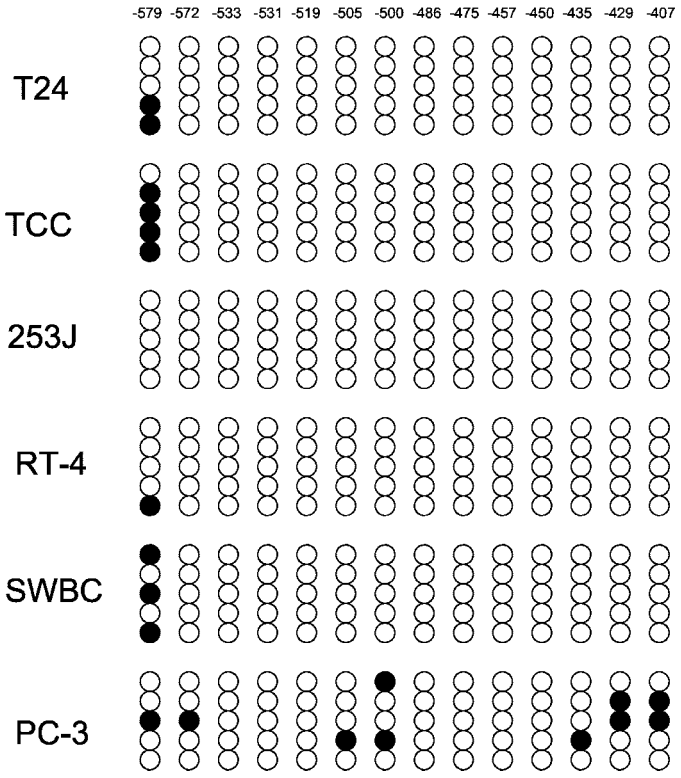

Fig. 3. Characterization of the methylation status in the human *coxsackie and adenovirus receptor* gene promoter from urogenital cancer cells. High molecular-weight DNA isolated from each cell line was subjected to bisulfite genomic sequencing. The *horizontal row* represents each individual PCR clone from each cell line. The *number* indicates each position of CpG island. ○, unmethylated CpG; ●, methylated CpG.

Table 3 *No effect of hypomethylation agent on coxsackie and adenovirus receptor (CAR) mRNA induction in urogenital cancer cell lines*[a]

5′-Aza (μM)	Fold of induction							
	CAR mRNA				hDAB2IP			
	T24	TCC	253J	PC-3	T24	TCC	253J	PC-3
0	1	1	1	1	1	1	1	1
1	0.94 ± 0.03	0.59 ± 0.02	1.01 ± 0.03	1.03 ± 0.05	0.93 ± 0.03	0.82 ± 0.03	3.34 ± 0.02	1.33 ± 0.01
5	1.10 ± 0.02	0.54 ± 0.04	0.84 ± 0.04	0.90 ± 0.05	1.32 ± 0.05	1.68 ± 0.02	3.50 ± 0.05	2.55 ± 0.05
10	0.95 ± 0.02	0.60 ± 0.03	0.30 ± 0.05	1.03 ± 0.09	2.42 ± 0.01	4.59 ± 0.03	4.21 ± 0.02	2.98 ± 0.04

[a] Cells were treated with different concentrations of 5′-aza-2′-deoxycitidine for 96 h.

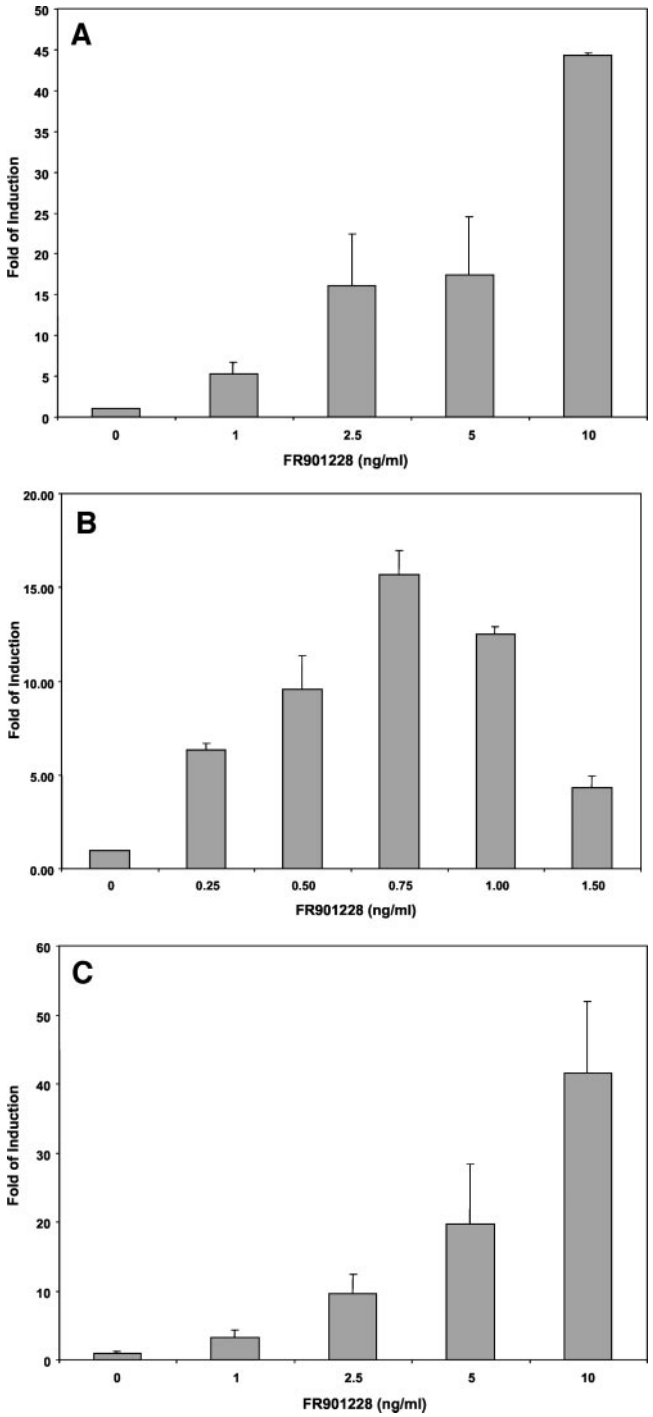

Fig. 4. Effect of histone deacetylase inhibitor on *coxsackie and adenovirus receptor* mRNA expression. An equal amount of total cellular RNA (3.2 μg) prepared from T24 cell (*A*), TCC cell (*B*), and PC-3 cell (*C*) treated with various concentrations of FR901228 was subjected to real-time reverse-transcription PCR. *Bars*, SD.

netic regulation of the *CAR* gene by histone acetylation led to an increased virus uptake in urogenital cancer cells.

DISCUSSION

Recombinant adenoviruses 5 have been widely used in gene-transfer experiments and clinical gene therapy. The entry of adenovirus 5 depends on the binding with its own specific receptor (*i.e.*, CAR) and the subsequent internalization into cell via interaction with integrins, $\alpha_v\beta_3$ or $\alpha_v\beta_5$ (17). From many recent studies, CAR appears to be a high-affinity receptor for adenovirus 2 and 5 (1, 2). Nevertheless, CAR also has other physiological function than as a virus receptor. For example, CAR is a typical cell-adhesion molecule with a homophilic interaction (1, 2, 7, 8), and it colocalizes with ZO-1 in the tight junction of polarized cell (18), suggesting that CAR may be involved in the process of cell differentiation. In addition, recent data reported by Walter *et al.* (19) indicate that the basolateral localization and cell-adhesive property of CAR protein in airway epithelium act as a barrier for preventing the spreading of newly synthesized virus into local environment. Taken together, the presence and the unique localization of CAR in each cell type could affect virus susceptibility of host cell. CAR also plays a critical physiological role in differentiated cells.

From phenotypic changes in cancer cells, it is believed that cancer may undergo cell dedifferentiation. Data from our studies (6–8) further indicate that down-regulation of *CAR* gene expression is often seen in both prostate and bladder cancer cell lines. Thus, increased CAR levels in these cell lines can inhibit their growth both *in vitro* and *in vivo*. Using immunostaining technique, two recent studies (9, 10) also reported that decreased CAR expression in clinical specimens of either prostate or bladder cancer. Taken together, these data support the notion that loss of CAR expression is associated with dedifferentiated phenotypes of urogenital cancers.

Obviously, the decreased CAR expression in urogenital cancers may also impose an obstacle for adenovirus based gene therapy. To circumvent this obstacle, one could change virus tropism by altering the fiber protein of virus (20) or increase endogenous CAR expression by gene transfection. Several recent findings (11, 12, 21, 22) also indicated that some HDAC inhibitors could potentially turn on endogenous *CAR* gene expression in cells. Our studies show that increased *CAR* mRNA expression leads to an elevated CAR protein accumulation on the cell membrane and results in higher virus sensitivity (6–8). These data prompted us to study the regulation of the human *CAR* gene in urogenital cancer cells.

Of the 17 bacterial artificial chromosomal clones, 59% are *CAR* pseudogenes, suggesting that *CAR* exists as a multigene family with many pseudogenes scattered around the human chromosome. Clone 626 C16 (~169 kb) contains all of the introns, exons, and 5′-upstream regulatory sequences confirmed by PCR and 5′-RACE assay. This clone also matched GenBank sequences derived from chromosome 21 where the *CAR* gene has been mapped in previous study (23). This gene does not have a typical TATA box (Fig. 1*B*); however, we were

still able to define the promoter region (186 bp) of the human *CAR* gene located at −400 to −585 bp from the translational initiation site. The promoter activity of this region correlated with the CAR levels in each cell line tested (Table 2). Moreover, there are unique *trans*-factor binding sites (such as E2F, Sp1) located upstream from the promoter region (Fig. 1*B*), which may be critical in modulating *CAR* gene expression in different cell types. For example, two groups report that mitogen-activated protein kinase kinase inhibitor (U0126) can up-regulate CAR expression in colon and pancreatic cancers (24); however, transforming growth factor β and dexamethasone suppress CAR expression in HeLa and glioblastoma cell line (U87MG; Ref. 25).

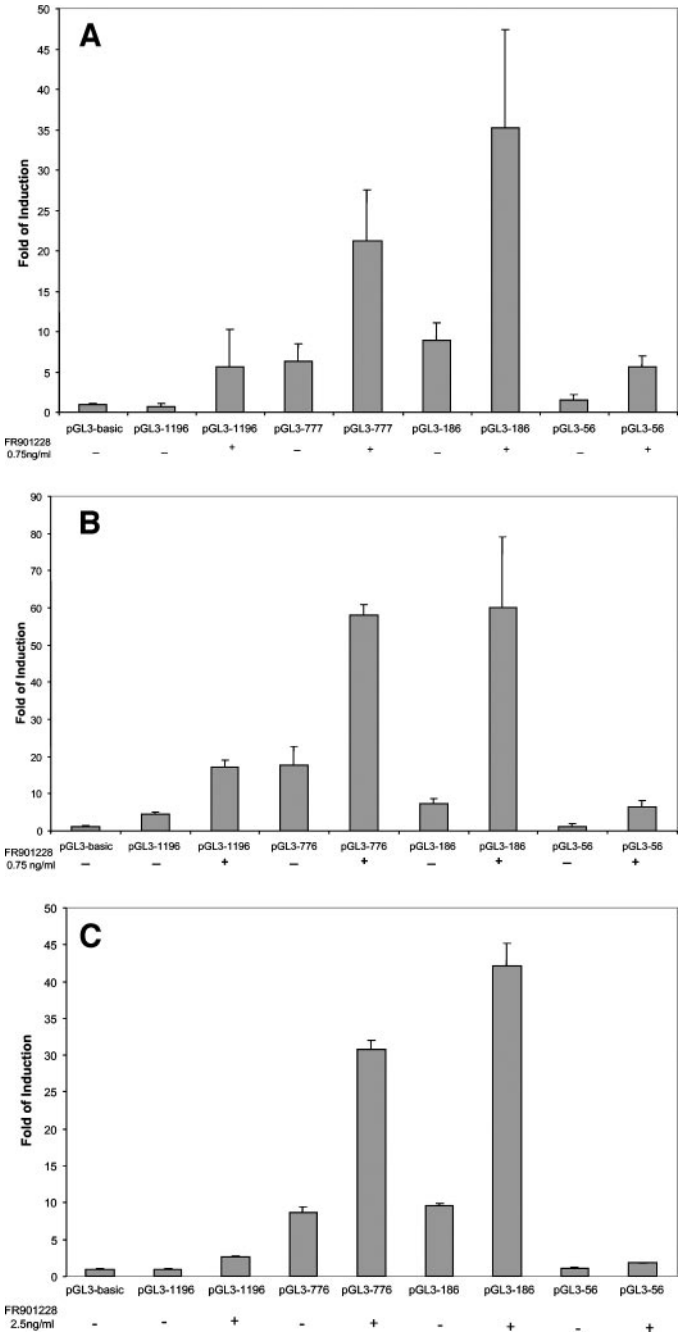

Fig. 5. Inductive effect of histone deacetylase inhibitor on the activity of *coxsackie and adenovirus receptor* promoter. An equal amount of various *coxsackie and adenovirus receptor* reporter gene vectors (0.8 μg) and pCMV-β-galactosidase (0.2 μg) was transfected into 253J (*A*), TCC (*B*), and PC-3 (*C*) cells, and cells were treated with various concentrations of FR901228 and then were subjected to reporter gene assay. Fold of induction was calculated by normalizing with pGL3-basic (=1) in each experiment. *Bars*, SD.

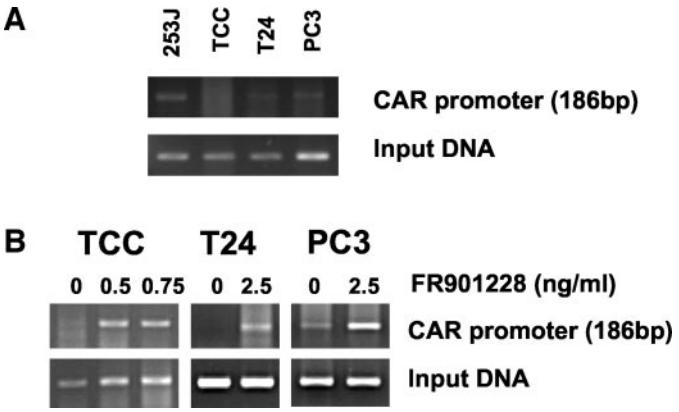

Fig. 6. Analysis of acetylated histone H4 associated with the human *coxsackie and adenovirus receptor* (*CAR*) gene promoter in urogenital cancer cells treated with histone deacetylase inhibitor. *A*, the status of acetylated histone H4 associated with the *CAR* promoter in cells with different CAR expression. *B*, increased levels of histone H4 acetylation associated with the *CAR* gene promoter in cells treated with histone deacetylase inhibitor. Chromatin immunoprecipitation assay was performed using anti-acetyl-histone H4 antibody, and then PCR was carried out with primers set to generate a 186-bp PCR product. The input DNA (*bottom*) derived from total DNA before immunoprecipitation was used a positive control.

Decreased CAR is often observed in urogenital cancer cell lines and specimens (7–10), indicating that CAR could be a potential tumor suppressor. These observations also raise a major concern for the patients who undergo adenovirus-based gene therapy. The purpose of this study was to evaluate whether or not down-regulation of CAR in urogenital cancers is attributable to epigenetic control. We showed that the association of acetylated histone protein with *CAR* promoter DNA correlated with the basal levels of CAR expression (Fig. 6). Up-regulation of the *CAR* gene can be observed in cells treated HDAC inhibitor (Figs. 4 and 5) that blocks the deacetylation process of histone proteins. Although, the promoter region of *CAR* contains many CpG islands, DNA methylation does not seem to play any role in controlling CAR gene expression (Fig. 3 and Table 3). Thus, these data clearly indicate that changes in chromatin structure of CAR promoter enhance the expression of CAR in urogenital cancer cells.

It is known that histone acetylation transferase unfolds nucleosome structure and leads to gene activation (26); histone acetylation transferase has also been identified as a transcriptional activator (27). In contrast, HADC plays a negative feedback role in gene activation by deacetylating histone. Our recent data (16) show that HDAC inhibitors such as FR901228 and trichostatin A (TSA) can enhance not only *CAR* mRNA levels but also virus-mediated gene delivery in PC-3 and TCC cells. FR901228 appears to be more potent in inducing *CAR* gene expression than TSA. FR901228, a bicyclical depsipeptide isolated from *Chromobacterium violaceum*, can induce morphological reversion of H-*ras*-transformed NIH3T3 (28). Currently, this agent has been evaluated in lung cancer patients in a Phase II trial.[4] Using this agent, we were able to demonstrate that the status of the histone acetylation associated with the *CAR* gene significantly impacts on its gene transcription in cells expressing low levels of CAR. These data indicate that epigenetic control of the *CAR* gene modulates CAR expression in human urogenital cancer cells.

In summary, we identified the core promoter sequence from the human *CAR* gene and showed that this promoter activity could be enhanced by HDAC inhibitors but not by DNA hypomethylation agents in urogenital cancer cells. Thus, the human *CAR* gene is highly inducible in cancer cells and the promoter sequence identified in this study can be used as a screening system for searching many other

[4] www.clinicaltrials.gov.

potential inducers. It is likely that the increased CAR expression in target cells could reduce virus dosage and further enhance the therapeutic efficacy of gene therapy.

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