Molecular Cloning and Characterization of Bovine CYP26A1 Promoter

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The retinoic acid (RA) plays an important role in the growth and development of many cells, and bioactive RA concentration is regulated by several enzymes, including CYP26A1. The expression of the CYP26A1 gene is regulated by RA, and the CYP26A1 gene is one of the candidates for RA-responsive genes. Although CYP26A1 genes are cloned from several animals, cloning of the CYP26A1 gene from cows has not been reported yet. The promoter region of CYP26A1 from cows was cloned by PCR and analyzed by sequence alignment with human and mouse CYP26A1. The RA-responsive element (RARE), DR-5 (TGAACTgggTGAACT), was located in this region and was perfectly conserved. The promoter region of bovine CYP26A1, which contains DR-5, was ligated to the luciferase reporter gene on transient transfection assays. The expression of CYP26A1-Luc promoter was activated by ATRA treatment in lung-derived mtCC cells. Co-transfection with RAR-α or -β with ATRA significantly activates the expression of CYP26A1-Luc promoter; however, it was less effective with either RAR-γ or RXR-γ. In addition, the endogenous gene expressions measured by Q-RT-PCR in mtCC cells were not significantly affected by ATRA treatment for 2 days; however, the expression of the endogenous CYP26A1 gene was diminished sharply at day 3 with ATRA treatment. In conclusion, the promoter region of bovine CYP26A1 contains conserved DR-5 RARE, which functions as a binding site for RAR-α or -β, and it is involved in the regulation of CYP26A1 gene expression and the control of RA signaling in mtCC cells.

Key words: ATRA, CYP26A1, RAR, RARE (responsive element), RXR

Introduction

Retinoic acid (RA) is a member of family of retinoid, which is derived from retinol or vitamin A. RA is also a member of steroid hormone, a small lipophilic molecule, and it cannot be synthesized in any animal and is derived from food [32]. RA is an important biological regulator of gene expression and is involved in the growth and development of the cells as well as cell death [21, 25]. RA is also a critical regulator of embryo development [2, 17, 30] and RA generated anterior and posterior axis by acting as an intercellular signal during early embryonic stages [2, 9]. Some RAs are now in uses in cosmetic ingredient as antioxidant in anti-aging products [27], and RAs are used in treating dermatological problems such as acne and wrinkles [20]. RAs also can be used in cancer therapy and chemoprevention as therapeutic drug [10]. One of the retinoic acid, called all-trans-retinoic acid (ATRA) can be used in a wide range of tumors including lung cancer [1, 4, 10]. The other form of retinoic acid, 9-cis retinoid is also used in the treatment of Kaposi’s sarcoma and breast cancers [1, 18].

RA is the bioactive metabolite of vitamin A (retinol) and RA is synthesized from retinol via enzymatic reactions. First oxidation of retinol to retinaldehyde is mediated by alcohol dehydrogenases (ADHs) or retinol dehydrogenases (RDHs) and then a second oxidation of retinaldehyde to retinoic acid is mediated by retinaldehyde dehydrogenases (RALHDs) [9, 26, 34] (Fig. 1A). Bioactive RA in excess amounts could be harmful to cells and the oxidation of RA is needed to degrade as biologically inactive form [32, 34]. Enzymes that metabolize excess retinol to prevent toxicity include three cytochrome P450 (CYP) enzymes known as CYP26A1, CYP26B1 and CYP26C1 [2, 34]. The oxidative metabolism of RA by CYP enzymes helps to maintain a proper concentration of RA for the critical RA signaling within the cells [13, 24].

The CYP26A1 plays an important role in the clearance of bioactive RA by catalyzing the oxidation of ATRA to 4-oxo-RA, inactive forms of metabolite of RA [29, 32] (Fig. 1A).
The expression of CYP26A1 gene was regulated by RA including ATRA and thus CYP26A1 is belong to a member of RA regulated genes and in this way RA signaling is tightly regulated within cells and tissues [24, 29]. The action of RA is determined after binding to their receptor, retinoic acid receptor (RAR) and retinoid X receptor (RXR) [3, 25]. RAR and RXR, as transcription factors, are belong to families of nuclear receptors that bind a specific sequences of DNA and regulate transcription of target genes [8, 11] (Fig. 1B). This superfamily has three major functional domains for the ligand binding, the activation domain and the DNA binding domain (DBD) [3, 8]. The DBD of RAR and/or RXR binds to the upstream sequences of RA responsive genes in the nucleus [5, 7]. RA acts as a ligand to activate transcription of target genes by the binding of RAR/ RXR as a dimer to the specific sequences of DNA called retinoic acid response elements (RARE) [3, 7, 12]. One of this RARE sequences contains a direct repeat (DR) of the consensus hexamer motif of AGGTCA separated by 5 base-pairs (bp) spacer as the DR-5 or it contains a direct repeat of TGACCT (complementary sequence of AGGTCA) with a spacer of 5 bp as in the reverse orientation [3, 5, 7, 12] (Fig. 1B). Many genes which are regulated by RA are carrying a RARE in the promoter region of DNA and the ligand bound RARs or RXRs could activate the transcription of target genes by binding to this RARE motif [7, 12]. The CYP26A1 gene is induced by RA treatment and it is known that the RARE motif is located within the promoter region as a DR-5 in this gene [13, 15, 16] (Fig. 1A).

The RAR is a nuclear receptor and transcription factor which is activated by all-trans retinoic acid or 9-cis retinoic acid [8, 14]. There are three types of RAR (RAR-alpha, -beta, and -gamma). RAR-β gene is known as a RA responding gene and a DR-5 RARE motif in the promoter region of the RAR-β gene is responsible for the binding of RAR [7, 12]. The promoter regions of CYP26A1 from cow was cloned and sequences were compared with other animals to find a conserved RARE as a binding site for RAR. The promoter region of CYP26A1 from cow which contain RARE DR-5 motif was ligated to the luciferase reporter gene to study the functional role of ATRA in the expression of CYP26A1 gene. The functional role of DR-5 RARE motif as a binding site for RAR or RXR for the expression of bovine CYP26A1 gene was examined by the expression of reporter gene in mtCC cells after ATRA treatment. The mtCC cells, which were originated from Clara cell specific lung cancer [23], were used for the transient transfection assays. Although there are many studies for the roles of RA in lung and lung cancer, there is very limited information for the expression of CYP26A1 in lung and lung origin cell lines. This CYP26A1 promoter construct with DR-5 will be helpful for the understanding of functional role of CYP26A1 gene in the lung. The expression of this reporter gene was further analyzed by the co-transfection of RAR/RXR with ATRA treatment to understand the molecular mechanism of RA signal system in the lung. In addition, the endogenous CYP26A1 gene expressions by ATRA treatment was analyzed by Q-RT-PCR in order to investigate the role of ATRA for the expression of CYP26A1.
gene in lung cell lines.

Materials and Methods

Molecular cloning of the bovine CYP26A1 promoter region

Bovine genomic DNA fragments were isolated from tissue samples from local market in order to clone and characterized the chromosome region of CYP26A1 promoter. Genomic DNA isolations were prepared by using QIAprep Dneasy Blood and Tissue Kit (QIAGEN, Valencia, CA, USA) using 25 mg of bovine meat samples as recommended protocol by the manufacturer. Using isolated genomic DNA from above samples, PCR (45 s at 95°C, 45 s at 60°C, and 1 min at 72°C; 35 cycles) was performed for the cloning of bovine CYP26A1 promoter. The amplification products from each PCR reaction were analyzed by electrophoresis in the 1% agarose gel. The sequences of oligonucleotide primers forward primer, 5’-GGACCTTCCAAGACTTCAGGA-3′ and reverse primer, 5’- GACTTC CCGCGCCACC -3 which yield sequences of oligonucleotide primers forward primer, 5’-GGACCTTCCAAGACTTCAGGA-3′ and reverse primer, 5’- GACTTC CCGCGCCACC -3 which yield 629 bp region of promoter of bovine CYP26A1. The sequences of 629 bp region of promoter of bovine CYP26A1 were obtained (Macrogen, Seoul, Korea) after several rounds of PCR using different samples of genomic DNA. Positive clones were obtained after ligation of these amplified promoter region of CYP26A1 from bovine samples into pGEM-T Easy Vector (Promega, Madison, WI, USA). Sequence data from upstream of the coding region of CYP26A1 genes from human and mouse were retrieved from NCBI (www.ncbi.nlm.nih.gov) using GenBank index number as GI: 192807295 for human and GI: 52851568 for mouse gene and sequences were aligned using ClustalW2 program (www.ebi.ac.uk/Tools/clustalw2/index.html).

Plasmid construct for the transient transfection assays

The promoter region of bovine CYP26A1 obtained above was ligated to the firefly luciferase reporter gene, the pGL3-Basic Luc (Promega, Madison, WI, USA). After selection of a positive clone containing both bovine CYP6A1 gene and the pGL3-Basic Luc, a plasmid construct was isolated by using Plasmid mini extraction kit (BIONEER, Daejeon, Korea). This construct was used to observe the effect of ATRA and its retinoid receptors for the expression of bovine CYP26A1 in vitro transient transfection assays by using mtCC cells [23].

Cell culture and transient transfection assays

ATRA and antibiotics were obtained from Sigma Chemical Co. (St. Louis, MO, USA). RPMI-1640 (phenol red-free), Hanks’ balanced salt solution (HBSS), Minimal essential medium (MEM), McCoy’s 5A Medium, Dulbecco’s modified Eagle medium (DMEM), were purchased from Gibco BRL (Gaithersburg, MD, USA). Fetal bovine serum (FBS), trypsin, and antibiotic-antimycotic (ABAM) were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Mouse transformed Clara Cells (mtCC) cells [23] used for transient transfection assays were provided by Dr. Francesco J. DeMayo at Baylor College of Medicine, Houston, TX, USA. MtCC cells were cultured at 37°C in a humidified atmosphere with 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS), penicillin (100 IU/ml), and streptomycin (0.1 mg/ml). MtCC cells were grown to 60-70% confluency on 24 well culture dishes and transfected with a mixture of 0.5 μg reporter plasmids and 5 ul of the Superfect transfection reagent (QIAGEN, Valencia, CA, USA) as recommended by the manufacturer. For co-transfection assays with RARs or RXR, the cells were transfected with a 50 ng of expression vector and empty eukaryotic expression vector was used as a control in co-transfection studies. Transfected cells were incubated for 3 hr and then washed with DMEM to remove the transfecting agent. Cells were then fed with DMEM with 10% FCS and incubated for 24 hr at 37°C. The cells were harvested, centrifuged for 5 minutes, and re-suspended in 10 ul of passive cell-lysis buffer (Promega, Madison, WI, USA). The cell debris was cleared by centrifugation and protein concentration was measured using Bradford reagent (Bio-Rad, Hercules, CA, USA). Luciferase (Luc) activities were measured by luminescent signals using a commercial kit (Promega, Madison, WI, USA) according to the manufacturer’s protocol and normalized per μg of the protein. All transfection experiments were carried out in replicates of three and repeated at least three times.

RNA isolation and quantitative real-time RT-PCR

Total RNA was extracted from mtCC cells using the Qiagen (Valencia, CA) RNAeasy total RNA isolation kit. The quantitative real-time RT-PCR analysis was conducted on RNA isolated from mtCC cells and expression levels of CYP26A1 were measured by real-time RT-PCR according to
The DNA sequence of the 629 bp of proximal promoter region of bovine CYP26A1 gene. RARE as DR-5, which is underlined and bold, indicates the sequence for the binding site RAR or RXR and RARE is the retinoic acid response element.

TATAAA, a binding site for TATA binding protein (TBP), is also underlined, (B) The DNA sequence alignment of the promoter region of bovine (Bos), human (Homo) and mouse (Mus) CYP26A1 genes. DR5 represents the direct repeat of a hexamer sequence of TGAACT separated by 5 base pairs and DR-5 and TATA box sequence are highlighted and underlined. DR-5 is located just upstream of the putative TATA box sequence, (C) The DNA sequence alignment of the DR-5 motif in cow, human and mouse CYP26A1 genes. Mismatched sequences are indicated as underlined.

Fig. 2. (A) The DNA sequence of the 629 bp of proximal promoter region of bovine CYP26A1 gene. RARE as DR-5, which is underlined and bold, indicates the sequence for the binding site RAR or RXR and RARE is the retinoic acid response element. TATAAA, a binding site for TATA binding protein (TBP), is also underlined, (B) The DNA sequence alignment of the promoter region of bovine (Bos), human (Homo) and mouse (Mus) CYP26A1 genes. DR5 represents the direct repeat of a hexamer sequence of TGAACT separated by 5 base pairs and DR-5 and TATA box sequence are highlighted and underlined. DR-5 is located just upstream of the putative TATA box sequence, (C) The DNA sequence alignment of the DR-5 motif in cow, human and mouse CYP26A1 genes. Mismatched sequences are indicated as underlined.
Results and Discussion

Cloning and characterization of the bovine CYP26A1 promoter region

It is known that CYP26A1 gene is responsible for the enzymatic breakdown of RA and the expression of CYP26A1 gene is regulated by its own substrate RA and thus CYP26A1 gene is one of candidates for RA regulated genes [24, 29, 30]. RA, as a member of steroid hormone, binds to its receptor and this binding of RA receptor to the DNA of RA responding genes occurs at specific sequences of RA response elements known as RARE [8, 11(Fig. 1B)]. The cloning of CYP26A1 gene from several animals are reported and RARE was identified in the promoter region of human and mouse CYP26A1 gene [15, 29]. However, cloning of the promoter region of CYP26A1 from cow was not reported yet. The promoter regions of CYP26A1 from cow were amplified by genomic PCR and cloned to study the role of RA and its retinoid receptors. The amplified PCR product of the promoter region of bovine CYP26A1 contains 629 bp with a conserved TATA box in their 3' end (Fig. 2A). Sequence data from 629 bp of bovine CYP26A1 promoter were used for the sequence alignment with human and mouse sequence of CYP26A1 promoter and revealed 79% (454/572) similarity with human gene and 90% (198/220) similarity with mouse gene (Fig. 2B). However, sequence alignment with Bubalus bubalis (water buffalo) CYP26A1 gene (GI: 594101314) revealed 99% (620/629) similarity (data not shown). Sequence alignment of bovine CYP26A1 with human and mouse genes exhibited only 198 bp region of the promoter among three animals showed highly significant homology (>79%) and 72 bp sequence within 198 bp region exhibited a 97% sequence homology between cow, mouse and human sequences (Fig. 2B). Further analysis of this 72 bp sequence of CYP26A1 promoter region revealed that there is a RARE motif with DR-5 \texttt{[TGAACTTGGG-TGAACT]} which consist of a direct repeat of a hexamer sequence of TGAACT separated by 5 base pairs (DR-5) in the proximal promoter region (Fig. 2C). It is located just upstream of the putative TATA box sequence and this RARE DR-5 motif is perfectly identical among cow, human and mouse CYP26A1 genes (Fig. 2C), suggesting that the DR-5 is a functionally important motif for the regulation of CYP26A1 gene expression. The DR-5 might serve as RARE for the binding site of RA receptors and several genes that are regulated by RA are known to carry DR-5 RARE motif within the promoter region of the target genes [15, 16].

Regulation of CYP26A1 gene expression by ATRA in the mtCC cells

A 629 bp region of bovine CYP26A1 promoter obtained above was ligated to a luciferase reporter gene in order to understand molecular mechanism of the RARE DR-5 motif of this gene (Fig. 3). This CYP26A1-reporter construct was transiently transfected into mtCC cells which are Clara cell specific cell line originated from mouse Clara cell specific lung cancer [23]. Treatment of ATRA (10 μM) in mtCC cells resulted in a 3 fold increase in Luc activity derived from the transcription of the bovine CYP26A1 gene (Fig. 3). Similar results were obtained when cells were exposed to ATRA at concentration of 1 μM (data not shown). It suggests that ATRA has a significant effect on the transcription of bovine CYP26A1 gene in transient transfection system when the lung mtCC cells were used and the up-regulation of bovine CYP26A1 gene is dependent on the ATRA treatment. Co-transfection of RAR and RXR were performed to analyze the function of the RARE DR-5 motif of the bovine CYP26A1 gene in mtCC cells and the effects of ATRA treatment were examined in the presence of RARs (RAR-α, -β, or -γ) or RXR-γ expression vectors. The co-transfection of RAR-α with ATRA treatment (10μM) resulted in a significant increase (7

![Fig. 3. Effect of ATRA on the transcriptional activity of bovine CYP26A1. Mouse transformed Clara cells (mtCC) were used for transient transfection assays for the expression of the bovine CYP26A1. CYP26A1 gene expression by ATRA treatment (10 μM) was measured by the Luc activities of CYP26A1-Luc reporter plasmid.](image-url)
fold increase) and co-transfection of RAR-β with ATRA treatment (10 μM) also exhibited 5 fold increase in bovine CYP26A1 transcriptional activity (Fig. 4). However, the co-transfection of RAR-γ or RXR-γ with ATRA treatment (10 μM) resulted in less significant increase (2-3 fold increase) in the same lung cells (Fig. 4). These results suggest the differential regulation of bovine CYP26A1 occurs by different types of RAR isoforms. Especially up-regulation of this gene by RAR-α or RAR-β might be a result of specific and preferential binding to DR-5 RARE motif. It could be also explained by differential specificity of ligand in the binding of different types of retinoic receptor isoforms [7, 12, 15, 16]. The 692 bp of the bovine CYP26A1 promoter might be responsible for the up-regulation of CYP26A1 gene by the binding of RAR-α and RAR-β to the conserved DR-5 motif in the presence of ATRA. Results with mutations of this DR-5 motif and different types of ligands are needed for the clear answer for the specific role of DR-5 in retinoid signal in lung cells.

In order to confirm the relationship between ATRA and the expression of CYP26A1 gene in lung originated mtCC cells, quantitative RT-PCR analysis was performed for endogenous CYP26A1 gene expression by replacing the medium with ATRA at every day. Endogenous CYP26A1 gene expressions were measured with ATRA treatment in a time dependent manner without co-transfection of any types of retinoid receptor. Results of quantitative RT-PCR assay exhibited that expression of the endogenous CYP26A1 gene was slightly increased at first day and slightly decreased at second day by the ATRA treatment (10 μM) in mtCC cells (Fig. 5). However, the expression of endogenous CYP26A1 gene was diminished sharply at three days by the continuous exposure to ATRA (10μM) in lung mtCC cells (Fig. 5). The molecular mechanism for the repression of endogenous CYP26A1 expression by 3 days ATRA treatment could not be explained clearly in this point. Retinoid treatment might suppress cancers of many tissues including lung tumor and ATRA, as the most active form of RA, is currently used as a chemotherapy drug [19, 28]. It is also known that ATRA is involved in the process of programmed cell death (also known as apoptosis) in certain types of tumor cells [21, 33]. The levels of gene expression of the CYP26A1 may play a role in apoptosis by controlling RA metabolism [33]. In addition, higher levels of CYP26A1 gene expression were observed in various tumors [6, 31]. However, the molecular mechanism responsible for the CYP26A1 expression in different types of tumors remains unclear [6, 14, 22]. As mentioned, very limited information is available for the expression of CYP26A1 in lung and lung cancer. MtCC cells could be a good model system to study the function of CYP26A1 gene in RA signal in vitro. The expression of CYP26A1 reporter construct was activated and exhibited a high responsiveness to RA treatment in these cells (Fig. 3 and 4) and also the endogenous expression of CYP26A1 gene was observed when the cells are treated with ATRA (Fig. 5).

In summary, CYP26A1 gene is belong to a family of RA

![Figure 4](image1.png)

**Fig. 4.** Transcriptional activation of bovine CYP26A1 by different isoforms of retinoid receptors. The effects of ATRA treatment (10 μM) mtCC lung cell line on the expression of bovine CYP26A1 were examined in the presence of RARs (RAR-α, -β, or -γ) or RXR-γ expression vectors.

![Figure 5](image2.png)

**Fig. 5.** The effects of ATRA on the endogenous CYP26A1 gene expressions in mtCC cells. Endogenous CYP26A1 gene expressions were measured with ATRA treatment in a time dependent manner by quantitative RT-PCR. The medium containing ATRA (10 μM) was replaced with fresh ATRA at every day for three days in mtCC cells.
responding genes which their expression is directly controlled by ATRA. The 629 bp of the promoter region of bovine CYP26A1 was cloned and sequence analysis revealed that the RARE as DR-5 is located in this region. This functional DR-5 might serve as a biding site for RAR-α or RAR-δ for the expression of CYP26A as ATRA dependent way in mtCC cell. Continuance treatment of ATRA for three days decreased endogenous CYP26A1 gene expression in mtCC cells and it suggests that RA signal is a critical regulator of CYP26A1 gene in lung mtCC cells. In conclusion, DR-5 might serve as a biding site for RAR-α and RAR-δ to control RA metabolism in mtCC cells.

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초록: 소 CYP26A1 유전자 프로모터의 molecular cloning 및 특성

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레티노산(NA)는 많은 유형의 세포에서 성장 및 발달에 중요한 역할을 수행하며 생체 활성화에 적합한 NA의농도는 CYP26A1 등 여러 가지 효소에 의해 조절된다. CYP26A1의발현은 NA에 의해서 조절되며 CYP26A1은NA에 반응하는 유전자 중 하나이다. CYP26A1 유전자의 클로닝은 여러 동물에서 보고되어 있지만, 소에서CYP26A1 유전자의 클로닝은 아직 보고되지 않았다. 소로부터 CYP26A1의 프로모터 부위를 중합효소 연쇄반응을 이용하여 클로닝한 후 다른 동물과 염기서열 비교분석 결과 RARE DR-5 (TGAACTtgggTGAACT)의존시를 확인하였고, DR-5의 염기서열은 분석한 종에서 완전히 일치하였다. DR-5 motif를 함유한 소의 CYP26A1 프로모터 부위를 luciferase리포터 유전자에 결합한 후 transient transfection에 의해 promoter 발현을 분석하였다. 폐 레세포주인 MTCC세포에서 CYP26A1 promoter의 발현은 ATRA의 처리에 의하여 촉진되었다. CYP26A1 유전자의 발현은 ATRA의존으로 RAR-α 및 RAR-β에 의하여 현저하게 촉진되었다. 그러나 RAR-γ나 RXR-γ는 CYP26A1 발현에 발려된 영향을 미치지 않는다고 하였다. 또한 MTCC 세포주가 생성하는 내인성 CYP26A1 유전자 발현을 Q-RT-PCR로 분석한 결과 1-2일간의 ATRA 처리에 의해서는 현저한 영향을 받지 않았으나, 3일 동안 ATRA를 처리한 셰에서 CYP26A1의 발현이 현저하게 감소하였다. 결론적으로, 소의 CYP26A1 유전자 프로모터 부위에 존재하는 DR-5 RARE는 RAR-α 및 RAR-β의 결합부위로 작용하여 MTCC 세포에서 CYP26A1 유전자 발현 조절의 RA signal의 조절에 관여하는 것을 확인하였다.