

Retinoic Acid Upregulates Cone Arrestin Expression in Retinoblastoma Cells through a *Cis* Element in the Distal Promoter Region

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PURPOSE. This study was initiated to investigate the molecular mechanisms of activation of expression of the human cone arrestin (hCAR) gene by retinoic acid (RA), in an in vitro model of retinoblastoma cells.

METHODS. Human retinoblastoma Weri-Rb-1 or Y79 cell lines were cultured in the absence or presence of RA analogues with transcription or translation inhibitors for various periods. The mRNAs encoding hCAR, retinoic acid receptor (RAR), and retinoid X receptor (RXR) subtypes were analyzed by Northern blot. Immunoblot analysis of hCAR protein was also performed. The hCAR promoter's activity and its responsiveness to RA treatment was evaluated by transient transfection of the hCAR promoter-luciferase reporter constructs, followed by promoter deletion analysis to map the specific regions responsible for the RA response.

RESULTS. In our in vitro model, both all-*trans* RA and 9-*cis* RA induced hCAR mRNA in a time- and dose-dependent manner. RA's effect was blocked by either RNA or protein synthesis inhibitors; however, hCAR mRNA's stability was not affected by RA, as determined by RNA decay experiments. Although all RAR and RXR subtypes were detected, only RXR γ and RAR α increased dramatically after treatment with RA. An RXR-specific agonist, but not an RAR-specific agonist, also increased hCAR mRNA and protein expression in both Weri-Rb-1 and Y79 cells. RA stimulated hCAR promoter-luciferase activity in transient transfection studies. Subsequently, a region between -852 and -702 of the hCAR promoter, with RA-responsive elements (RAREs), was discovered to be responsible for the RA response.

CONCLUSIONS. The hCAR gene is transcriptionally upregulated by RA acting through *cis* elements within -852 to -702 of the hCAR 5' flanking region. Based on the cumulative data, RXR γ is most likely the RA receptor subtype involved in hCAR regulation by RA. (*Invest Ophthalmol Vis Sci.* 2002;43:1375-1383)

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Arrestins constitute a superfamily of regulatory proteins that downregulate activated and phosphorylated G protein-coupled receptors (GPCRs). Cone arrestin (CAR, also known as X-arrestin or arrestin4) is a novel arrestin originally identified by our laboratory and independently identified by another group.^{1,2} Although the molecular structure and limited tissue distribution of cone arrestin are known, its physiological role in GPCR regulation and its regulation of gene expression are poorly understood.

Similar to rod arrestin, cone arrestin has a highly restricted, cell-specific expression pattern. The hCAR mRNA and protein are localized to all classes of retinal cone photoreceptor cells and a subpopulation of pinealocytes but are below detection in other cell types.^{1,3} The tissue-specific expression of cone arrestin indicates that specific factors must control the expression pattern of this gene. In developing and adult rod photoreceptors, neural retina leucine zipper (NRL) and c-Fos (a member of the bZIP transcription factors) play a fundamental role in the initiation and regulation of rod-specific gene expression, and thyroid hormone receptor is essential for the development of green cone photoreceptors in mice.^{4,5} However, the genetic zip code information that drives expression of cone arrestin in all classes of cone photoreceptors is not known.

Vitamin A plays a critical role in normal vision. Its acid form, RA, is a potent modulator of hormonal transcription control and is critical in early eye and photoreceptor development and differentiation.⁶⁻¹⁰ RA controls transcription and translation of the *Drosophila* opsin gene,^{11,12} and RA supplementation increases rod arrestin mRNA in mouse.¹³ In addition, studies on rat retina have shown that RA triggers the differentiation of embryonic retinal cells into photoreceptors.¹⁴

Retinoid actions are mediated mainly by two classes of nuclear receptor: retinoic acid receptor (RAR), and retinoid X receptor (RXR).¹⁵⁻¹⁷ RARs and RXRs each comprise three subtypes: α , β , and γ , and are members of the steroid-thyroid hormone receptor superfamily, whose members function as ligand-activated transcription factors.¹⁵⁻¹⁷ RARs and RXRs recognize specific DNA sequences, designated RAREs.¹⁸ Of the known natural retinoids, 9-*cis* RA is a high-affinity ligand for both RARs and RXRs, whereas all-*trans* RA is a ligand only for RARs. In addition to retinoid receptors, a number of orphan receptors, such as chicken ovalbumin upstream promoter-transcription factors (COUP-TFI and COUP-TFII), whose ligands are unknown, have been implicated in the regulation of the retinoid response.^{19,20}

To explore the fundamental regulatory mechanisms controlling the expression of the cone arrestin (hCAR) gene, we examined both Weri-Rb-1 and Y79 human retinoblastoma cell lines. Both of these cell lines are immortalized and are derived from two independent retinoblastomas, which are intraocular childhood cancers of photoreceptor origin.²¹ Previously, we have demonstrated that hCAR is induced by RA at both the mRNA and protein levels in both cell lines.²² In this study, we sought to elucidate the dynamics of RA upregulation of expression of hCAR mRNA and its underlying molecular mechanisms in these cell lines.

MATERIALS AND METHODS

Cell Culture and Treatment

Weri-Rb-1 and Y79 retinoblastoma cells (American Type Culture Collection, Manassas, VA) were maintained and treated as previously described.²² A 2% B27 serum substitute (GibcoBRL, Grand Island, NY) replaced the 10% fetal bovine serum in the medium. The following ligands or chemicals were used: all-*trans* RA (1 or 10 μ M), 9-*cis* RA (1 or 10 μ M), 4-(E-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl)benzoic acid (TTNPB, 1 μ M), LG100268 (LG268, 1 μ M), cycloheximide (CHX, 20 μ g/mL), actinomycin D (ActD, 10 μ g/mL), and the drug vehicle dimethyl sulfoxide (DMSO, control). All tissue culture media and supplements were obtained from Irvine Scientific (Santa Ana, CA). All reagents and chemicals used to treat the cells were purchased from Sigma (St. Louis, MO), except LG268, which was generously provided by Mark D. Leibowitz of Ligand Pharmaceuticals, Inc. (San Diego, CA).

Northern Blot Analysis

Northern blot analysis was performed with 20 μ g total RNA isolated from either Weri-Rb-1 or Y79 cells, as previously described.²² Specifically bound radiolabeled cDNA probe was visualized by exposure to a phosphorescence imager screen (PhosphorImager; Molecular Dynamics, Sunnyvale, CA), and relative mRNA levels were normalized for loading variability by subsequently hybridizing with an [α -³²P] dCTP-labeled β -actin or 18S rRNA cDNA probe on the same membrane.²³

Immunoblot Analysis

Cells were treated as described, and whole cell homogenates were prepared according to previously published procedures.²² Equal amounts of proteins were resolved by 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were electrophoretically transferred to nitrocellulose membranes (Immobilon-P; Millipore, Bedford, MA), according to previously published protocols.²⁴ The immobilized proteins were detected with anti-hCAR peptide polyclonal antibody (Luminaire Founders, LUMIF, 1:200,000; Research Genetics, Huntsville, AL) and anti-rabbit (1:10,000; Bio-Rad Laboratories, Richmond, CA) secondary antibody, with an enhanced chemiluminescence kit (Amersham, Arlington Heights, IL).

Plasmid Constructs

Gene fragments of 3350, 2027, 1084, 941, and 429 bp from the 5' flanking region of the hCAR gene, all starting from the 5'-noncoding region of the hCAR cDNA (41 bp downstream of the transcription start site) and going upstream, were isolated with a kit (Human Genome-Walker; Clontech, Palo Alto, CA), according to the manufacturer's instructions. All fragments in a cloning vector (pGlow-TOPO TA; Invitrogen, San Diego, CA) were subcloned into a luciferase reporter vector (pRL-null; Promega, Madison, WI) in the sense orientation and were named phCAR3309, phCAR1986, phCAR1043, phCAR900, and phCAR388, respectively. The nomenclature used for reporter constructs includes an abbreviation for the plasmid (p), species (h), and gene (CAR) followed by a number that refers to the position of the 5' end of the construct relative to the transcription start site.

Five progressive deletion constructs were derived from the phCAR900 construct. Standard PCR reactions were performed with a shared hCAR antisense primer in exon 1 with an *Eco*RI restriction endonuclease site (italic), 5'-GC/GAA/TTC/CTG/GGG/AGA/GAG/ATG/AAG-3', paired individually with a unique sense primer linked to a *Hind*III restriction endonuclease site located at the following positions (restriction site is italic): -852/-834: 5'-CGAAGCTTAGAGAAGCCGCTTAGAGC-3'; -702/-684: 5'-CGAAGCTTGGGCTGGGAATCTTATAT-3'; -646/-628: 5'-CGAAGCTTCAGACATGTGCATACACA-3'; -301/-283: 5'-CGAAGCTTCTCAGGTTCAAGCCCTC-3'; -282/-264: 5'-CGAAGCTTCAAGGCCAAAACTCTGAA-3'.

The PCR products were cloned into the pRL-null vector through its *Eco*RI and *Hind*III sites and named DR4-DEL1, DR12-DEL2, DR0-DEL3, IR14-DEL4, and DR0-DEL5. Identities of the cloned inserts were confirmed by sequencing.

Transient Transfection and Luciferase Assay

Transient transfection of Weri-Rb-1 and Y79 cells was performed (Superfect transfection reagent; Qiagen, Chatsworth, CA) as previously described.²⁵ Briefly, cells from suspension culture were seeded at a concentration of 5×10^5 cells/mL into 24-well plates. Each transfection contained 0.8 μ g of promoter-reporter construct, 0.2 μ g of pGL3-P plasmid (Promega), which contains a firefly luciferase reporter gene under the control of the simian virus (SV)40 basic promoter (internal control for transfection efficiency), and 4 μ L of the transfection reagent. After a 3-hour incubation, all-*trans* RA (10 μ M), 9-*cis* RA (10 μ M), or dimethyl sulfoxide (DMSO; 0.1%) was added, and the transfected cells were further incubated for 45 hours before being harvested for the luciferase reporter assay. Both firefly and Renilla luciferase activities were assayed with 5 to 20 μ L of crude cell lysate using a commercial luciferase reporter assay system (Dual-Luciferase Reporter Assay; Promega) and a TD-20/20 luminometer.²⁵

Quantitative and Statistical Analysis

The intensity of hybridization signals was quantified on a computer with image-analysis software (ImageQuant, ver. 5.1; Molecular Dynamics). The density of the bands for hCAR and RXR γ transcripts was digitalized and normalized to the corresponding β -actin or 18S value. Statistical analysis was performed using paired Student's two-tailed *t*-tests, and results are expressed as the mean \pm SEM.

RESULTS

RA Induction of hCAR mRNA in Weri-Rb-1 Cells

In previous work in which various stimulatory reagents were used, the expression pattern of hCAR, at both the mRNA and the protein levels, was induced dramatically with all-*trans* RA treatment of either Weri-Rb-1 or Y79 retinoblastoma cells.²² To analyze the dynamics of this induction, we performed a dose-response and time-course analysis of hCAR mRNA expression in Weri-Rb-1 cells after all-*trans* RA treatment. Northern blot analysis showed that all-*trans* RA enhanced a 1.5-kb hCAR mRNA message in a dose-dependent manner, and the maximal effect (20-fold increase) was achieved with 10 μ M all-*trans* RA (Fig. 1). We thus chose 10 μ M all-*trans* RA for the time-course and other experiments with Weri-Rb-1 cells. hCAR mRNA expression was elevated above basal levels with only 1 hour of exposure to all-*trans* RA, reached its maximum induction of 16-fold over the control by 48 hours and remained constant until day 5 (Figs. 2A, 2B). When all-*trans* RA was withdrawn after a 5-day treatment, hCAR mRNA decreased gradually; however, the mRNA level stayed constant when all-*trans* RA treatment was continued (Fig. 2C).

Two active isomers of RA, all-*trans* RA and 9-*cis* RA, influence gene transcription through distinct pathways. To investigate whether 9-*cis* RA also has an effect on hCAR mRNA expression, we performed both dose-response and time-course analyses of hCAR mRNA expression after 9-*cis* RA treatment of Weri-Rb-1 cells. 9-*cis* RA produced hCAR induction in a dose-response and time-course manner similar to that of all-*trans* RA (Fig. 3).

RA Stimulation of hCAR Transcription

The increase in hCAR mRNA level detected by Northern blot analysis may be due to an increase in the rate of transcription, the stabilization of previously transcribed mRNA, or a combination of both mechanisms. To distinguish among the three

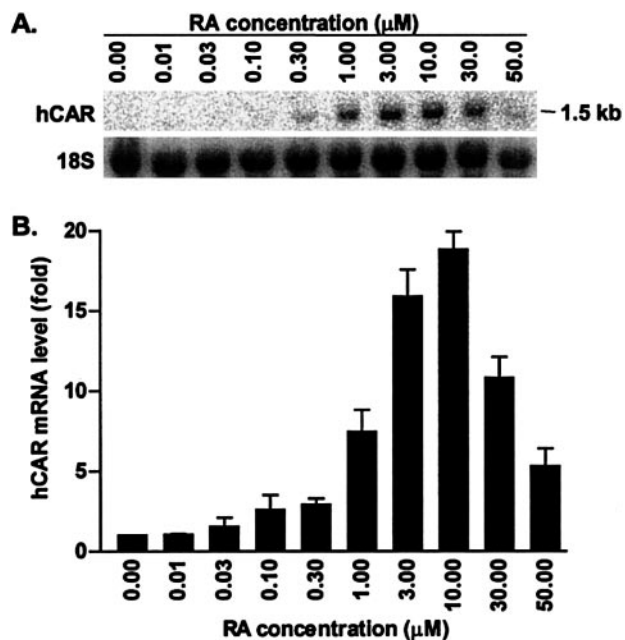


FIGURE 1. Detection of hCAR mRNA expression by Northern blot analysis in treated Weri-Rb-1 cells at increasing concentrations of all-trans RA. Total RNA was extracted from untreated Weri-Rb-1 cells or cells treated with all-trans RA for 6 days at the indicated concentrations. Twenty micrograms of each sample was applied and electrophoresed in a denaturing agarose gel, transferred to a membrane, processed, and hybridized with the appropriate radiolabeled probe. To quantitate gel loading and transfer efficiency, the membranes were striped and re-probed with a radiolabeled 18S probe. (A) Representative blot for hCAR and 18S mRNA expression (20 μg total RNA per lane). (B) Comparison of hCAR signals in treated (all-trans RA) and untreated (DMSO) cells. Data are the mean of duplicate experiments expressed as multiple of control (DMSO) level.

possible mechanisms, ActD, a known inhibitor of transcription was used to block gene transcription. When all-trans RA and ActD were added to Weri-Rb-1 cells simultaneously, the increase in hCAR mRNA in response to RA was blocked, indicating that ongoing mRNA synthesis is required for enhanced hCAR mRNA expression (Figs. 4A, 4B).

To investigate whether RA treatment can also modify post-transcriptional mechanisms to stabilize hCAR mRNA, we examined the effect of all-trans RA on hCAR mRNA's stability. Transcription in DMSO- (control) or all-trans RA-pretreated Weri-Rb-1 cells was blocked with ActD, and the degradation of hCAR mRNA was estimated by Northern blot analysis. The results showed that RA did not alter hCAR mRNA's stability (Figs. 4C, 4D), suggesting that no posttranscriptional regulation was involved.

Note that the size of the hCAR mRNA with ActD treatment for 24 hours was smaller than its normal size of 1.5 kb. The mechanism responsible for this is unclear, although it is well known that ActD binds to either double- or single-stranded DNA with high affinity to block the progression of RNA polymerase along the template DNA, halting the transcriptional machinery.²⁶

Involvement of New Protein Synthesis in Induction of hCAR Gene Expression

The gradual response of the hCAR gene to RA exposure raises the possibility that the stimulation of hCAR mRNA expression may involve the induction of another factor, or factors, that would, in turn, activate the hCAR gene. To clarify this, Weri-

Rb-1 and Y79 cells were cultured for 48 hours in the presence of all-trans RA, with or without 20 μg/mL CHX to inhibit protein synthesis.²⁷⁻²⁹ The upregulation of hCAR mRNA by all-trans RA was blocked by CHX in both cell lines, indicating that the response of hCAR to RA was dependent on the synthesis of intermediate factors (Fig. 5A). Further analysis showed that CHX also blocked the effects of all-trans RA on hCAR mRNA expression with either 3 hours or 24 hours of exposure (Fig. 5B). When CHX was added to the cells that were pretreated for either 3 or 24 hours with all-trans RA, the induction of hCAR mRNA was not prevented (Fig. 5C). These data suggest that the synthesis of the intermediate factor(s) occurred within the first 3 hours of RA treatment.

Expression of RARs and RXRs in Retinoblastoma Cells

RT-PCR analysis using published primers³⁰ revealed that both Weri-Rb-1 and Y79 cells express all subtypes of RAR and RXR transcripts (data not shown), consistent with the human retinal data.³⁰ The RT-PCR results also show that RXRγ is upregulated by RA in both cell lines (data not shown). Amplified cDNA sequences were cloned and their sequence verified, and they were used as probes for Northern blot analysis. Using the same blots as in the all-trans RA time-course experiments, RXRγ gene expression was detected (Figs. 6A, 6B). The expression of RXRγ mRNA was elevated above basal levels after 1 hour of

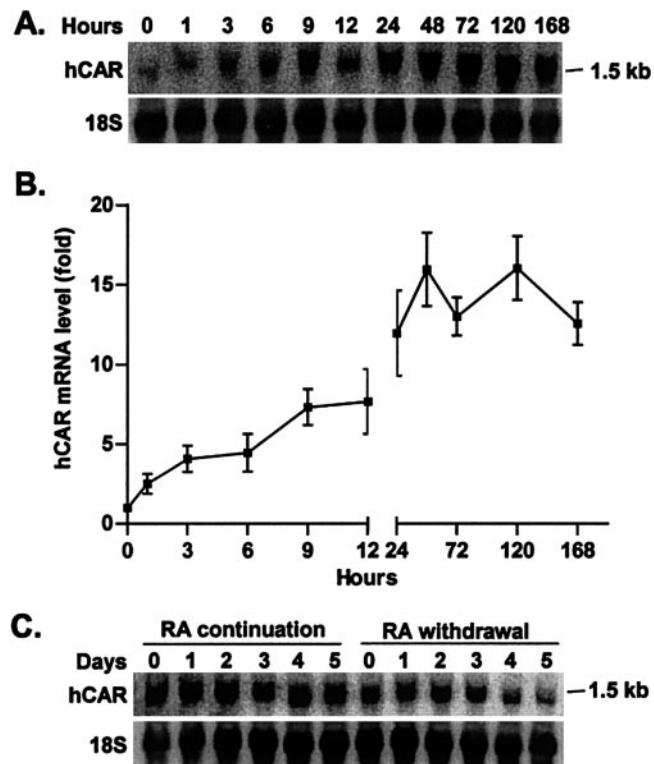


FIGURE 2. Detection of hCAR mRNA by Northern blot analysis in Weri-Rb-1 cells with different duration of RA treatment. Total RNA was extracted from untreated Weri-Rb-1 cells or cells treated with 10 μM all-trans RA for indicated time points and hybridized as in Figure 1. (A) Representative blot for hCAR and 18S mRNA expression (20 μg total RNA per lane). (B) Comparison of hCAR mRNA levels in treated and untreated cells from three independent experiments. Expressed as the mean ± SEM and multiple of the control. (C) Cells were cultured with all-trans RA for 5 days. Half of the cultures were then left in the presence of all-trans RA, and half were removed from all-trans RA treatment for the indicated time. Expression of hCAR mRNA and 18S mRNA is shown.

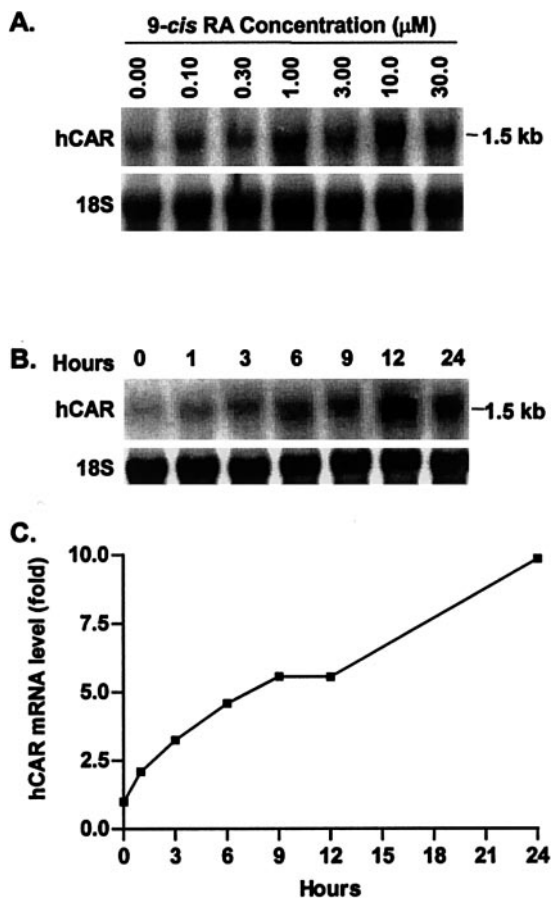


FIGURE 3. Detection of hCAR mRNA by Northern blot analysis in Weri-Rb-1 cells with different concentrations and duration of 9-cis RA treatment. (A) Weri-Rb-1 cells were incubated for 6 days with indicated concentrations of 9-cis RA and data from a representative blot for hCAR and 18S mRNA signals are shown. (B) Weri-Rb-1 cells were incubated with 10 μM 9-cis RA for indicated times in a 24-hour period. Representative blots for hCAR and 18S mRNA expression. (C) Densitometric quantitation of hCAR mRNA expression.

exposure to RA, reached its maximum of 4.5-fold over the control by only 6 hours of exposure and remained constant until day 2. Furthermore, total RNA from Weri-Rb-1 and Y79 cells treated with all-trans RA were analyzed by Northern blot analysis using probes specific for RARα, -β, and -γ and RXRα and -β. Both transcripts of RARα (3.3 and 3.5 kb) were dramatically upregulated by RA in both Weri-Rb-1 and Y79 cells, whereas the mRNA levels of RARβ (3.3 kb) and RXRα (5.5 kb) were not affected (Fig. 6C). RARγ and RXRβ mRNAs were below detectable levels in both cell lines by Northern blot analysis. These results indicate that RA may modulate expression of the hCAR gene through interactions of the RARα/RXRγ heterodimer or RXRγ/RXRγ homodimer with the RAREs in the hCAR promoter region.

Induction of hCAR Expression by RXR-Selective Agonists

To examine the specific pathway involved in RA-induced hCAR expression, a selective agonist for either RAR (TTNPB) or RXR (LG268) was used in our investigation.³¹ The treatment with all-trans RA, 9-cis RA, or LG268 increased hCAR at both mRNA and protein levels (Fig. 7). In contrast, TTNPB had no effect on hCAR expression, indicating that activation of an RXR receptor subtype is necessary for hCAR upregulation. The hCAR mRNA

and protein levels in Figure 7 also demonstrate that all-trans RA had the strongest effect in Weri-Rb-1 cells, whereas 9-cis RA treatment had a more robust effect in Y79 cells. It has been reported that in Y79 cells, 9-cis RA is twice as potent as all-trans RA in increasing another pineal- and retina-specific gene, hydroxyindole-O-methyltransferase (HIOMT).³² Although any induction by all-trans RA may be accounted for by its isomerization to 9-cis RA, the possibility of a different meta-

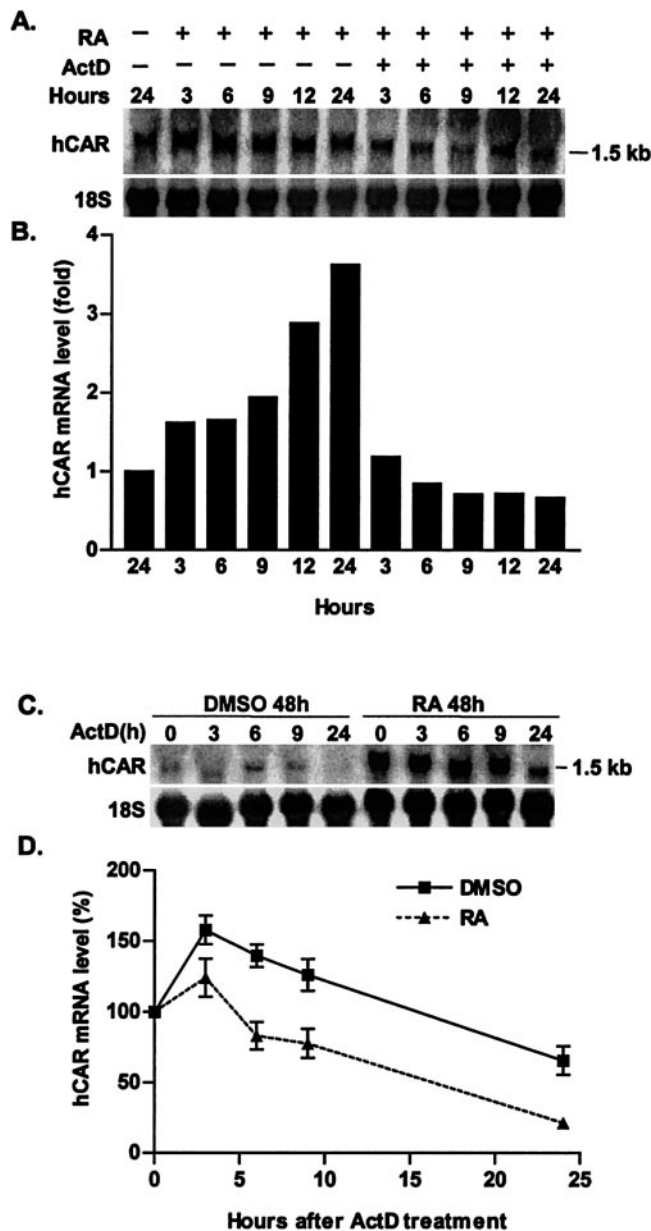


FIGURE 4. Effects of RNA synthesis inhibitor on the RA-mediated hCAR mRNA increase. (A) Weri-Rb-1 cells were untreated or treated with either all-trans RA or all-trans RA and ActD for the indicated times, total RNA was extracted. Representative Northern blots for hCAR and 18S mRNA expression are shown. (B) Densitometric quantitation of hCAR mRNA expression. (C) Weri-Rb-1 cells were pretreated with DMSO or 10 μM all-trans RA for 48 h, then washed and transcription was blocked with ActD (20 μg/ml) for the indicated times, total RNA was extracted. Representative Northern blots for hCAR and 18S mRNA expression are shown. (D) Densitometric quantitation of hCAR mRNA expression. Amount of mRNAs remaining at each time point is expressed as the percentage of that at time 0, which is assigned 100%. Results are expressed as the mean ± SEM (n = 4).

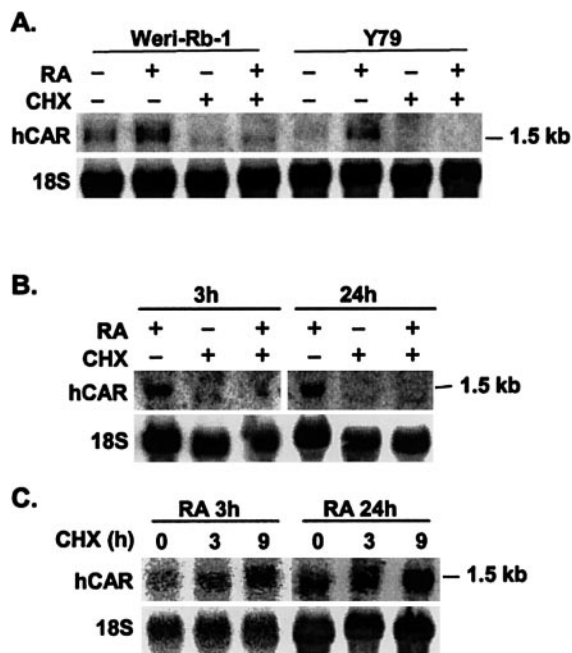


FIGURE 5. Effects of protein synthesis inhibitor on the induction of hCAR mRNA by RA. (A) Weri-Rb-1 and Y79 cells were untreated (–) or treated (+) with all-*trans* RA and/or CHX (20 μg/mL) for 48 hours. Representative Northern blot for hCAR and 18S mRNA signals is shown. (B) Weri-Rb-1 cells were untreated or treated with all-*trans* RA and/or CHX for 3 or 24 hours. Representative Northern blot for hCAR and 18S mRNA signals is shown. (C) Weri-Rb-1 cells were treated with 10 μM all-*trans* RA for 3 hours and 24 hours before addition of CHX for the indicated time. Representative Northern blot for hCAR and 18S signals is shown.

bolic pathway existing in the two cell lines cannot be excluded, despite the similarity in these two cell lines.

RA Stimulation of hCAR Promoter Activity

To test the possibility that RA stimulates hCAR mRNA expression through a direct action on its promoter, luciferase fusion constructs containing hCAR 5' flanking fragments were transiently transfected into Weri-Rb-1 and Y79 cells. Each reporter gene construct directed expression of luciferase that was 2- to 15-fold in Weri-Rb-1 cells and up to 32-fold in Y79 cells over background values (Figs. 8B, 8C). The phCAR1986 had the strongest activity in both cell lines. The longest construct, phCAR3309, had the lowest promoter activity, suggesting the existence of negative regulatory elements between –1986 and –3309.

All the constructs responded to RA treatment, except the shortest (phCAR388) and the longest (phCAR3309), with phCAR900 displaying the maximal RA response in both cell lines (Figs. 8B, 8C). These results strongly imply that RA has regulatory effects on the hCAR promoter, and the *cis*-element, or elements, responsible for RA induction are located between –900 and –388 of the hCAR 5' flanking region. Indeed, analysis of the phCAR900 construct identified several imperfect direct repeats (DRs) or inverted sequences (IRs) of the (A/G)G(G/T)TCA motif, which is the half-site of natural RAREs. These DRs and IRs are spaced by 4 (DR-4), 12 (DR-12), 0 (DR-0), and 14 bp (IR-14) (Fig. 8A). The proximal 392 bp of the hCAR 5' upstream sequence is identical with the published data³⁴ and is highly conserved between human and mouse.³⁵

A Distal Promoter Region of the hCAR Gene Responsible for RA Induction

To ascertain whether one or more of the DR-4, DR-12, DR-0, and IR-14 *cis* elements contribute to RA stimulation of the hCAR gene promoter and therefore pinpoint the potential RARE in the hCAR promoter, 5' progressive deletions were constructed and tested for RA inducibility. Deletion of DR-4 (DR4-DEL1) reduced the baseline level promoter luciferase activity to 64% in Weri-Rb-1 and 62% in Y79 cells, whereas no significant effect was observed on RA stimulation of the promoter activity (Fig. 9). However, the deletion of an additional 132 bp containing the DR-12 element (DR12-DEL2) almost eliminated RA inducibility and greatly reduced the baseline level promoter luciferase activity. This is indicative of the importance of the –852/–702 region, in that the region not only mediates the transcriptional response to RA but also drives the basal expression of the hCAR gene. Further deletions of the rest of the potential RAREs had no significant effect on

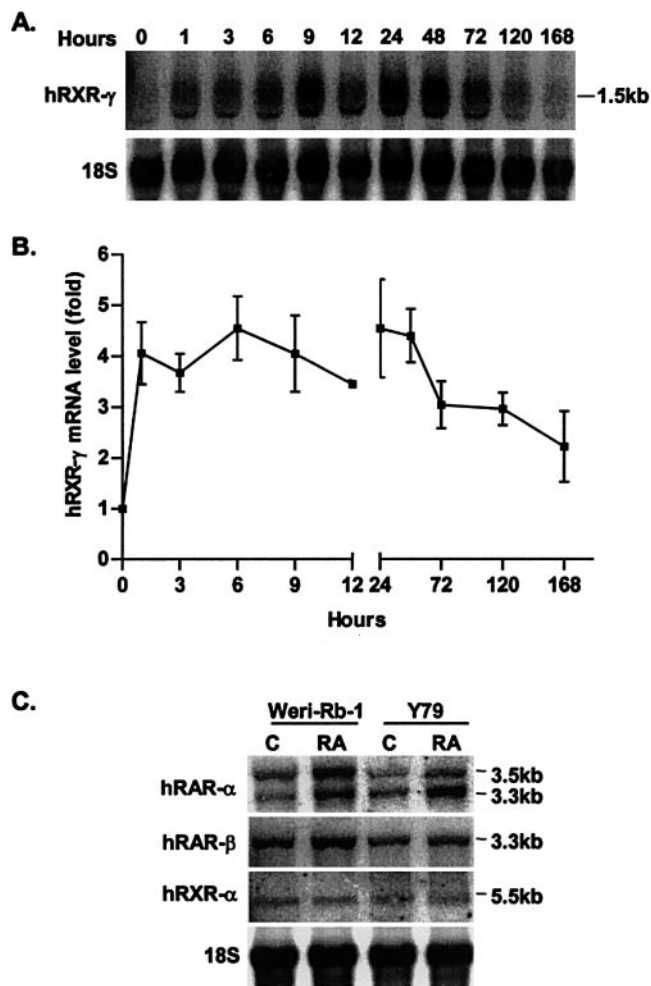


FIGURE 6. Detection of RARs and RXRs mRNA by Northern blot analysis in the absence or presence of RA. (A) Three time-course blots, as described in Figure 1, were striped and rehybridized to a 357-bp human RXRγ cDNA radioactive labeled probe. A representative blot for RXRγ and 18S is shown. (B) Comparison of hCAR mRNA in untreated and treated cells from three independent experiments. Data are the mean ± SEM in multiples of the control level. (C) Weri-Rb-1 and Y79 cells were untreated (C) or treated with all-*trans* RA for 48 hours. Total RNA was extracted as described in Figure 1 and hybridized to an RARα (518 bp), RARβ (435 bp), or RXRα (534 bp) cDNA radioactive probe and rehybridized to an 18S probe. Representative blots are shown.

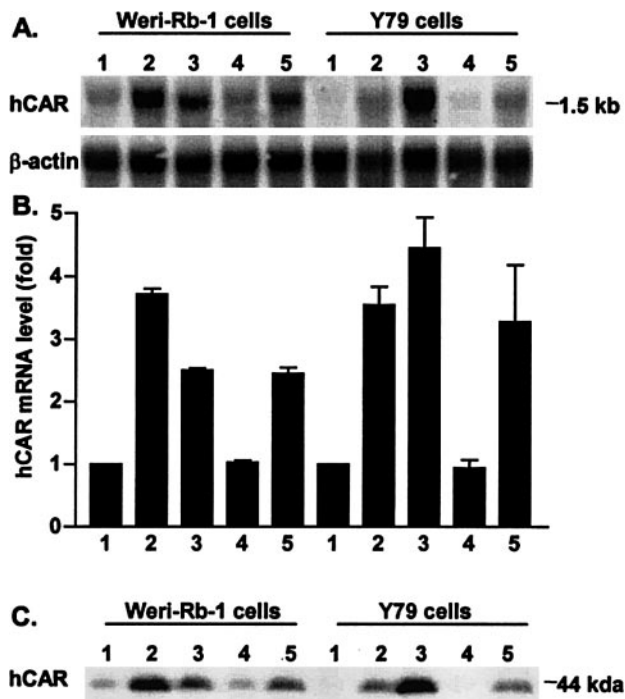


FIGURE 7. Effects of RAR and RXR agonists on hCAR mRNA and protein levels in Weri-Rb-1 and Y79 cells. The cells were incubated for 6 days with DMSO (lane 1, control) and 1 μ M all-trans RA, 9-cis RA, TTNPB, and LG268 (lanes 2, 3, 4, and 5, respectively). (A) Representative Northern blots for hCAR and β -actin mRNA signals are shown. (B) Densitometric quantitation of hCAR mRNA expression. Data are the mean \pm SEM from two independent experiments. (C) A representative immunoblot showing expression of hCAR protein.

either basal promoter activity or RA inducibility in either of the cell lines (Fig. 9).

DISCUSSION

RA induces hCAR expression at both the mRNA and protein levels in human retinoblastoma Weri-Rb-1 and Y79 cells.²² The present study extended these initial observations and explored the molecular mechanisms underlying this regulation.

RA Induction of hCAR Gene Transcription

It has been suggested that transcriptional regulation is a common feature of most retina-specific genes.⁵ This is also true in retinoblastoma cells. A previous study has demonstrated that RA induction of expression of HIOMT in Y79 cells reflects transcriptional regulation and not posttranscriptional modification³² and that laminin regulates expression of the interphotoreceptor retinoid-binding protein (IRBP) at the transcription level in both Weri-Rb-1 and Y79 cells.³⁶

According to our experiments, ongoing mRNA synthesis was necessary for enhanced expression of hCAR mRNA after RA treatment of retinoblastoma cells (Figs. 4A, 4B), but RA did not prolong the half-life of the hCAR mRNA, based on the RNA decay experiments (Figs. 4C, 4D). These results suggest that hCAR activation by RA takes place at the transcriptional level. Transfection studies with the hCAR gene promoter-luciferase constructs further confirm the transcriptional control, because the addition of RA increases the promoter-luciferase activity in both Weri-Rb-1 and Y79 cells (Figs. 8B, 8C).

Effect of RA Receptors on hCAR Gene Expression

Despite a rapid hCAR mRNA induction by RA, which occurred after only 1 hour of treatment, its induction apparently requires de novo protein synthesis (Fig. 5), suggesting that the hCAR gene is a secondary target gene of RA modulation. Previous studies have suggested that the stimulation of *N*-acetyltransferase (NAT) and melatonin by cyclic adenosine monophosphate (AMP) also requires protein synthesis,³⁷ whereas the induction of the IRBP gene by laminin does not require new protein production in human retinoblastoma cells.³⁶

The identity of the protein factor(s) required to induce hCAR gene transcription remains unclear. Our first hypothesis is that the photoreceptor-specific transcription factor, cone-rod homeobox (CRX),^{25,38} may play a fundamental role in the initiation and regulation of the cone-specific gene hCAR transcription, as NRL and *c-fos* do in rods. We have demonstrated that the presence of CRX protein is critical for the activation of the mouse CAR (mCAR) promoter, which has high sequence homology with the hCAR 5' flanking region in the proximal promoter region, including the conserved CRX-binding sites.³⁵ In the present study, the hCAR promoter has much higher basal promoter activity in Y79 cells than in Weri-Rb-1 cells when RA is not added (Figs. 8B, 8C, 9), probably because of the higher CRX expression levels in Y79 cells, as demonstrated by Northern and immunoblot analysis (data not shown). However, although CRX was upregulated by RA treatment in both cell lines, the induction occurred only after a 3-day RA treatment (data not shown). Therefore, CRX is not an appropriate candidate responsible for mediating the RA induction of the hCAR gene transcription. A second possibility is that RA stimulated its own receptor(s) that would, in turn, stimulate hCAR gene transcription. The gene expression profile of RARs and RXRs after RA treatment (Fig. 6) support this hypothesis and suggest that RAR α and RXR γ are the potential candidates as they are dramatically induced by RA treatment of the cells. Recent reports showing that RAR α and RXR γ are found predominantly in cone photoreceptors^{30,39} strongly support our hypothesis that RAR α and RXR γ are the intermediate factors that are upregulated by RA and then stimulate the hCAR gene transcription in retinoblastoma cells, because hCAR is a cone-specific gene. Studies with RAR and RXR agonists demonstrate that the addition of the RXR agonist, but not the RAR agonist, induces expression of hCAR mRNA and protein, suggesting that either an RXR γ homodimer or a heterodimer formed by RXR γ and another unidentified factor or nuclear receptor is responsible for the hCAR upregulation by RA.

To our knowledge, this study is the first direct demonstration of nuclear RA receptors in Weri-Rb-1 and Y79 retinoblastoma cells. The identification of retinoid receptors and the rapid induction of RAR α and RXR γ by RA in these cells provide feasible research tools to study the role of RA and its receptors in regulating gene transcription in these cell lines.

RA Stimulation of hCAR Gene Expression through Distal Promoter Elements

Transient transfection studies showed that RA increased the hCAR promoter-luciferase activity, with the phCAR900 construct exhibiting the highest stimulation in both Weri-Rb-1 and Y79 cells (Figs. 8B, 8C). Sequence analysis of the phCAR900 construct identified several potential RAREs (Fig. 8A). Although natural RAREs have been reported to consist of direct repeats of A/GG (G)/T (T) CA half sites separated by 1-, 2- or 5-bp spacers, everted repeats, or palindromes, growing evidence has shown that RAREs could be more flexible.^{18,40,41} Therefore, any of the *cis* elements that are similar to RAREs in the hCAR 5' flanking region (DR-4, DR12, DR-0, and IR-14) within the phCAR900 construct could confer responsiveness to RA.

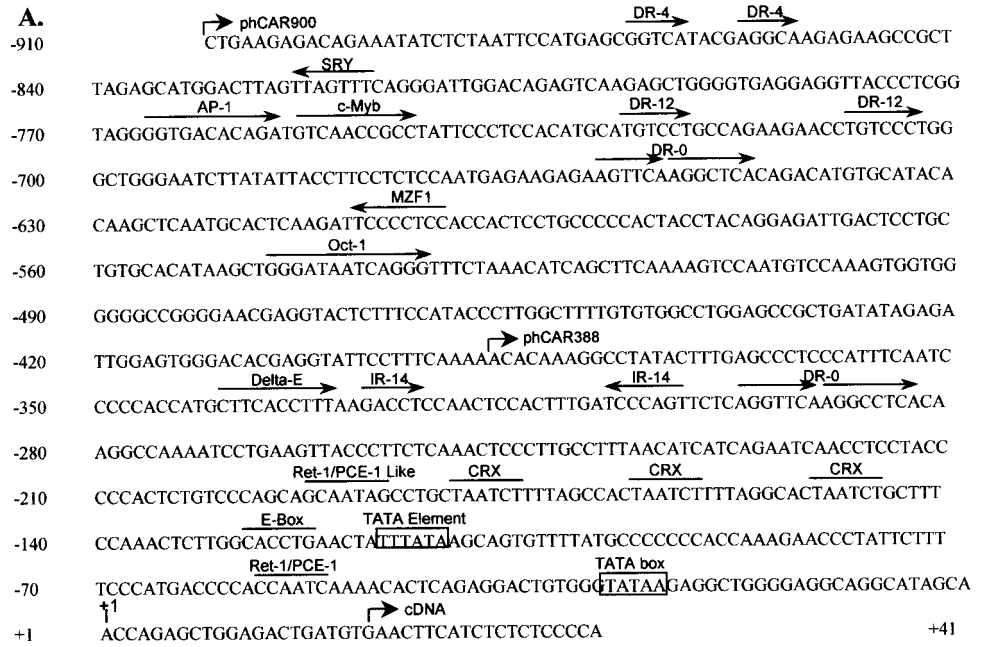
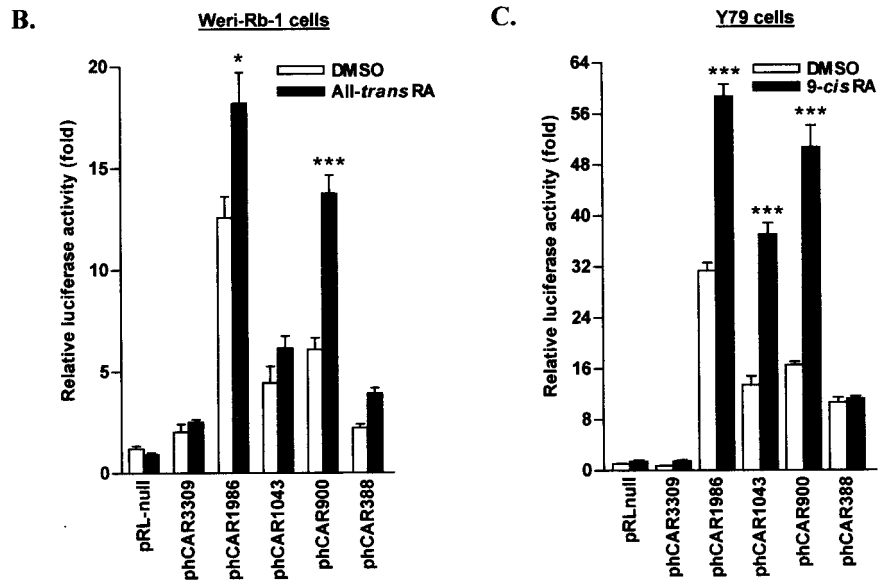


FIGURE 8. The nucleotide sequence of the hCAR 5' flanking fragments and RA effects on hCAR promoter activity. The nucleotide sequence of the 941-bp hCAR 5' flanking fragment (phCAR900, -900/+41) is shown with the potential transcriptional regulatory elements identified by sequence comparisons. The transcription start site is indicated as +1, and the first base of the hCAR cDNA is marked cDNA. The TATA box and the TATA element are boxed. Potential nuclear receptor DNA recognition sites, such as DR-4, DR-12, DR-0, and IR-14 are shown. The *cis* elements that have been described in photoreceptor-expressed genes such as Ret-1/PCE-1, CRX and E-box are shown. Other promoter elements, which were identified by an online promoter analysis program,³³ are also indicated. The five different 5' flanking fragments (phCAR388, phCAR900, phCAR1043, phCAR1986, and phCAR3309) were inserted into the pRL-null luciferase reporter vector and transfected into Weri-Rb-1 (B) and Y79 (C) cells with either vehicle (DMSO) or 10 μ M *all-trans* RA or 10 μ M 9-*cis* RA treatment. The cells were processed and assayed for luciferase activity 48 hours after transfection. The promoterless pRL-null vector was used as a control for basal luciferase activity of the cells, and its luciferase activity was set at 1. Results are expressed as the mean \pm SEM ($n = 4$). * $P < 0.05$, *** $P < 0.001$.



Using progressive deletion experiments, we found that a region between -852 and -702 is responsible for RA's effect on hCAR induction. Notably, this region contains a DR-12 element that comprises two TGTCC(C)T direct repeats separated by 12 nucleotides, similar to the sequence of the mouse rod arrestin in the promoter region (-205 to -185), containing two direct repeats of TGACCT interspersed by a 7-bp insertion (DR-7).^{42,45} This DR-7 element serves as an RARE that is bound and activated specifically by either the RXR α -RAR α heterodimer or the COUP-TF homodimer in the mouse rod arrestin promoter.^{42,43} It is thus reasonable to suggest that either the RXR γ homodimer or a heterodimer of RXR γ and another factor or nuclear receptor binds to the DR-12 element in the hCAR upstream region to activate its gene expression. However, we cannot exclude the possibility that another unidentified element within the -852/-702 region is responsible for the RA induction of the hCAR gene.

Taken together, our experiments clearly demonstrate the molecular and biochemical pathway responsible for the RA

upregulation of hCAR. Because the present studies were performed using retinoblastoma cell lines, the physiological relevance of RA regulation of expression of the hCAR gene in the retina remains to be determined. Because RA induced retinoblastoma cells to express hCAR through the upregulation of RXR γ , which is mainly localized to the cone-photoreceptors in the human retina, it will be interesting to know whether hCAR induction by RA treatment is accompanied by transcriptional alterations of other cone-specific genes and whether RA virtually induces and drives the fate of retinoblastoma cells to differentiate toward a cone photoreceptor phenotype. Global expression analysis of the RA-treated retinoblastoma cells with a microarray system (e.g., Genechip; Affymetrix, Santa Clara, CA) may resolve these issues.

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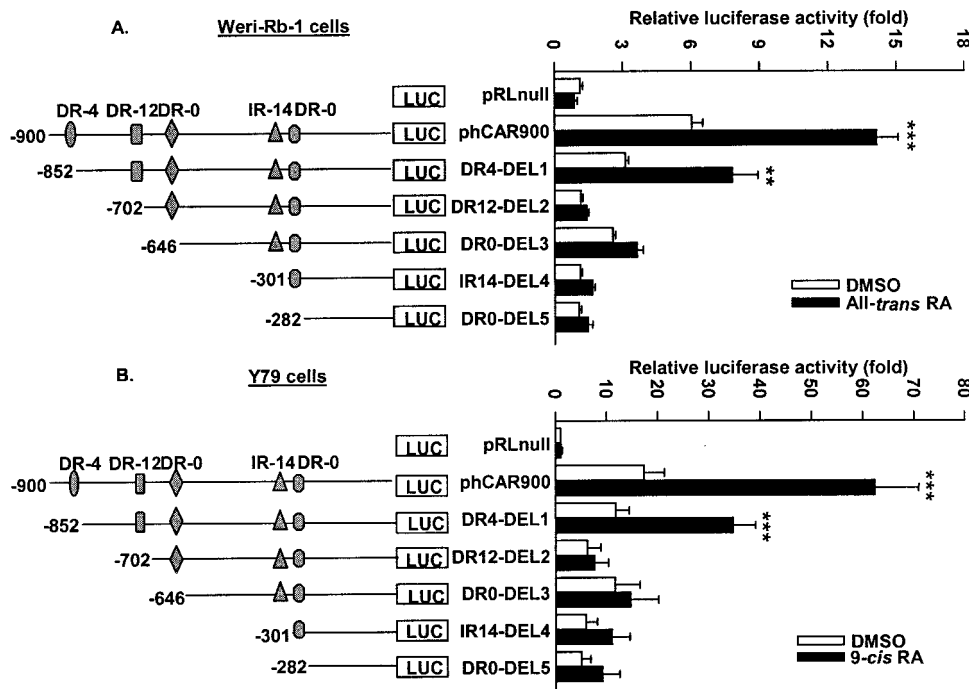


FIGURE 9. Progressive deletion analysis of the phCAR900 construct in cultured Weri-Rb-1 and Y79 cells. Cells were transfected with 5' progressive deletes from the phCAR900 construct and treated with vehicle (DMSO) or 10 μ M all-trans RA in Weri-Rb-1 (A) or 10 μ M 9-cis RA in Y79 (B) cells. The cells were then processed and assayed as described in Figure 8. Results represent means \pm SEM ($n = 3$). ** $P < 0.01$, *** $P < 0.001$.

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