응용약물학회지, The Journal of Applied Pharmacology 6, 276-282(1998)

# A Retinoid Antagonist Inhibits the Retinoic Acid Response Element that Located in the Promoter Region of the Cytomegalovirus

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(Received May 13, 1998; accepted June 30, 1998)

**Abstract** – Retinoids regulate a wide variety of biological processes such as cellular proliferation and differentiation in many cell types. They have also shown to stimulate replication of several viruses including human cytomegalovirus (CMV). Retinoid signalling pathway involves two distinct subfamilies of nuclear receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs) that bind to specific retinoic acid response elements (RAREs) in the promoter regions of retinoid-target genes. Here, we characterized RAREs in the regulatory regions of the CMV and of the hepatitis B virus (HBV). The viral RAREs, *i.e.*, CMV-RARE and HBV-RARE, are composed of two consensus RARE half-sites (A/GGGTCA) arranged as a direct repeat separated by 5-bp and 1-bp, respectively. The RAREs were activated by both RAR/RXR heterodimers and RXR homodimers in transient transfection experiments. We also found that COUP-TFα (chicken ovalbumin upstream promoter-transcription factor α) and COUP-TFβ repressed the retinoid response of the viral elements. Further we demonstrated that previously known retinoid antagonist, SR11330, repressed retinoid-induced transactivation of the CMV-RARE. These results implicate Vitamin A, it's nuclear receptors and COUP-TFs as important regulators of the CMV and HBV pathogenesis and the SR11330 as potential negative modulator of such retinoid-dependent processes.

**Keywords** □ RAR, RXR, retinoic acid response element, HBV, CMV, and COUP-TF, and antagonist

Retinoic acid and its natural and synthetic derivatives of Vitamin A (retinoids) regulate a wide variety of essential biological functions such as vertebrate development, maintenance of homeostasis and differentiation (Lotan, 1981 and Gudas *et al.*, 1994). Recently it has been reported that the expression of human immunodeficiency virus Type I (HIV-I) in macrophages is enhanced by retinoic acid (Zeichner *et al.*, 1992). Retinoic acid has been also reported to promote cellular differentiation and replication of cytomegalovirus (CMV) in embryonal cells suggesting viruses may take advantage of the retinoids for their own replication (Turpin *et al.*, 1992, Poli *et al.*, 1992, Huan and Siddiqui, 1992, Ghazal *et al.*, 1992, Maio and Brown, 1988, Zeichner *et al.*, 1992, and Angulo *et al.*, 1994).

The effects of retinoids are mainly mediated by specific nuclear receptors that belong to the steroid/thyroid hormone receptor superfamily. Two classes of nuclear receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) have been cloned (Pfahl *et al.*, 1994). These receptors function as ligand activated transcriptional factors that bind to specific response sequences on the regulatory region of target genes and thereby regulate the transcriptional expression of these genes (Pfahl *et al.*, 1994, Zhang and Pfahl, 1993, Green and Chambon, 1988, Zhang *et al.*, 1992a. Kastner *et al.*, 1995, and Mangelsdorf and Evans, 1995). All-*trans* retinoic acid (all-*trans* RA) and 9-cis retinoic acid (9-*cis* RA), the two known active derivatives of vitamin A, essentially function as hormones by interacting with specific retinoid receptors (Heyman *et al.*, 1992, Zelent *et al.*, 1989 and Petkovich *et al.*, 1987). RARs require RXRs for efficient transactivation, resulting RAR/RXR heterodimers that recognize a retinoic acid response element (RARE), usually consisting of A/GGGTCA or similar core motifs arranged as a direct repeat with either 2- or 5-bp spacing. In addition, RXR can function as homodimers that recognize DR-1 (direct repeat with 1-bp spacing)-type RAREs or an inverted repeat element with no spacing or 9-bp spacing in the presence of its ligand, 9-*cis* RA (Zhang *et al.*, 1992b). The magnitude of the retinoid response can be modulated by the expression of

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other intracellular protein factors. For instance, chicken ovalbumin upstream promoter-transcription factors (COUP-TFs) can repress retinoid response, probably through their strong binding to the RAREs or through dimerization with RXR (Cooney *et al.*, 1992, Tran *et al.*, 1992, and Orchard. *et al.*, 1993). Synthetic retinoid antagonists could be an another tool to restrict the retinoid-induced biological effects. Recently, several groups of retinoid antagonists have been developed with a potential that could repress the retinoid-dependent transcription and replication of viruses (Lee *et al.*, 1994 and Lee *et al.*, 1996).

In the present investigation, we characterized the retinoic acid response elements located in the regulatory region of the CMV and human hepatitis B virus (HBV). Further, the COUP-TFs and a retinoid antagonist, SR 11330, were demonstrated to inhibit retinoid-dependent transactivation of the viral retinoic acid response elements.

## MATERIALS AND METHODS

### Retinoids

All-*trans* RA and 9-*cis* RA were purchased from Sigma (St. Luis, MO, USA). SR11330 was kindly provided by Dr. M. I. Dawson (Stanford Research Institute, CA, USA). Retinoids stock solutions (10 mM) were made in dimethylsulfoxide/ethanol (1 : 1) and were maintained at -20℃. Further dilutions were made in cell culture medium prior to use.

### Plasmids

The receptor expression plasmids pECE-RARα, pECE-RARβ, pECE-RARγ, and pECE-RXRα, as well as pECE-COUP-TFα (ear-3) and pECE-COUP-TFβ (ARP-1) expression plasmids, have been described earlier (Zhang *et al.*, 1992a, Tran *et al.*, 1992 and Lehmann *et al.*, 1994). The HBV-RARE and CMV-RARE reporter constructs, *i. e.*, HBV-*tk*-CAT and CMV-*tk*-CAT, were obtained by inserting either one or two copies of the corresponding oligonucleotide sequences shown in Fig. 1 with additional 5'-GATC overhangs into the *Bgl*II site of pBLCAT$_2$ that carries a *tk* promoter (Luckow and Schutz, 1987) as described (Pfahl *et al.*, 1990). The constructs were sequenced to verify the copy number and orientation. The reporters containing two copies of HBV-RARE (⟶⟶) and CMV-RARE (⟶⟵) were used in transient transfection experiments otherwise indicated.

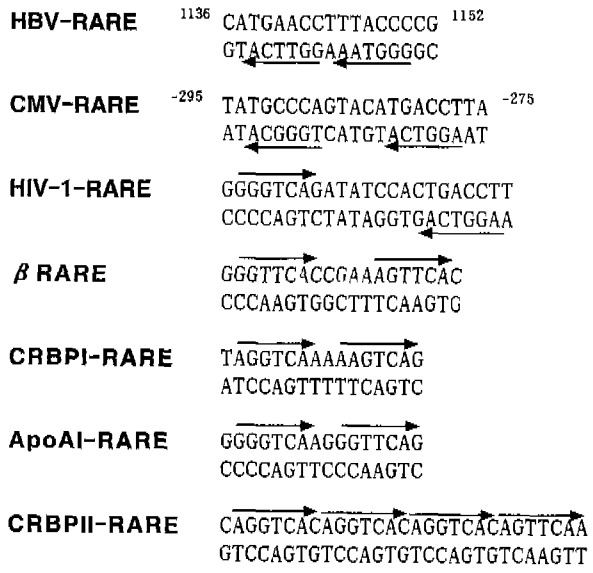


**Fig. 1.** Putative RARE sequences found in the regulatory regions of the HBV and CMV. HBV-RARE and CMV-RARE sequences were compared with other known RAREs (Pfahl *et al.*, 1994). Sequences that are closely related to the A/GGGTCA motif are indicated by arrows.

### Tissue culture, transient transfection and CAT assays

CV-1 cells were maintained in Dulbecco's modified eagle's (DME) medium supplemented with 10% fetal calf serum in a $CO_2$ incubator. A modified calcium phosphate precipitation procedure was used for transient transfection (Lee *et al.*, 1994 and Pfahl *et al.*, 1990). Briefly, CV-1 cells (50,000 to 75,000 cells/well) were seeded in a 24-well culture plate. Reporter plasmid (100 ng) and β-galactosidase (β-gal) expression vector (150 ng) were mixed with carrier DNA (pBluescript) to 1 μg of total DNA per well. After 24 hours of retinoid or vehicle treatment, Chloramphenicol acetyltransferase (CAT) activity was determined (Pfahl *et al.*, 1990). Counts per minute, normalized for transfection efficiency by the corresponding β-gal activity, were expressed as relative CAT activities.

## RESULTS AND DISCUSSION

### The RAREs in the promoter/enhancer regions of the cytomegalovirus (CMV) and the hepatitis B virus (HBV).

The transcriptional activation activity of retinoids and their receptors is mainly mediated by response elements composed of A/GGGTCA or similar sequences arranged as 1-, 2-, or 5-bp spacing. Several synthetic or natural RARE sequences are shown in Fig. 1. To investigate pos-

sible involvement of retinoids and their receptors in the regulation of CMV and HBV gene expression, we searched possible RARE sequences in the regulatory region of the HBV and CMV that control the expression of the viral genome.

Inspection of the sequences in the promoter/enhancer region revealed the putative RARE sequences in the regulatory region of CMV and HBV. The RARE-like sequences in the enhancer I that stimulates the core and X region promoters of HBV (1138 to 1150) is arranged as a direct repeat with 1-bp spacing similar to the one identified in the cellular retinol binding protein type II (CRBPII) gene. The RARE in the major immediate-early gene of human CMV promoter (-293 to -277) is arranged as a direct repeat with 5-bp spacing similar to the one identified in the promoter of RARβ gene, βRARE (Fig. 1). These types of sequences, *i.e.*, DR-1 and DR-5 have been shown to confer RXR homodimer and RAR/RXR heterodimer activity, respectively (Pfahl *et al.*, 1994).

The RARE in the long termianl region of HIV-1 has been reported previously (Orchard *et al.*, 1993 and Lee *et al.*, 1994). The HIV-1-RARE was composed of two consensus half-sites arranged as a palindrome separated by 9 nucleotide. The fact that such pathogenic viruses are containing tools for efficient retinoid response, suggests that the viruses take advantage of retinoids for their own replication although pathogenesis and consequences of the viral infection are largely different.

**Transient transfection experiments showed that the putative RAREs are activated by RAR and RXR.**

RAREs can be activated by RAR/RXR heterodimers or RXR homodimers (Pfahl *et al.*, 1994, Zhang *et al.*, 1992a and Zhang *et al.*, 1992b). To examine whether the DR-1 and DR-5 elements found in the CMV and HBV genome function as RARE, oligonucleotides corresponding to the RARE sequences were synthesized and cloned into the reporter gene vector pBLCAT$_2$, which contains the thymidine kinase (*tk*) promoter and CAT gene. The resulting reporter was tested for its retinoid responsiveness by transient transfection in CV-1 cells.

The putative viral RARE sequences allowed a significant induction of CAT activity in response to retinoids in the presence of retinoid receptor expression vectors. Cotransfection of RXRα expression vector alone with CMV-RARE-*tk*-CAT resulted in a strong induction of the reporter gene expression in response to 9-*cis* RA

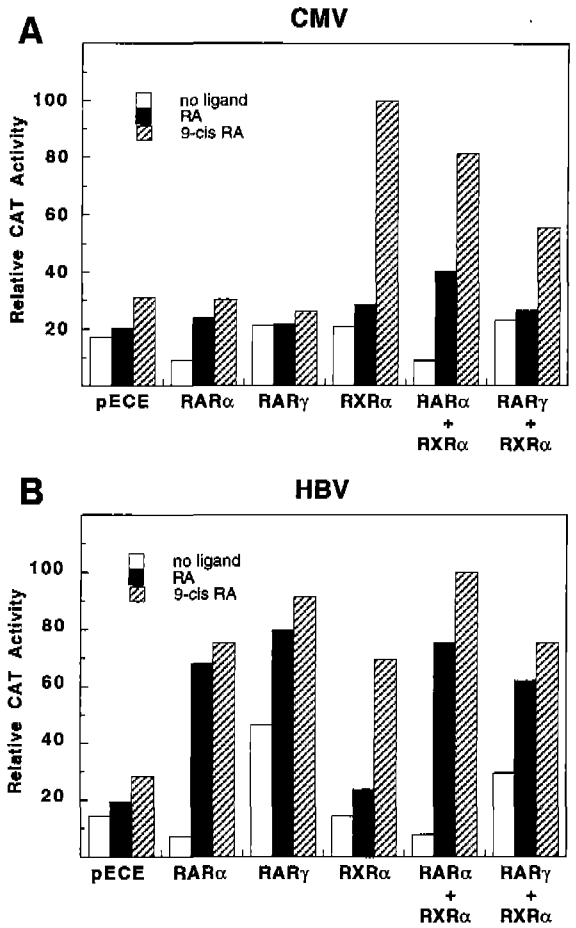


**Fig. 2.** Transcriptinal activity of the CMV-RARE (—×—) (A) and HBV-RARE (—→—) (B) in CV-1 cells. To determine the transcriptional activity of HBV-RARE and CMV-RARE, CV-1 cells were transfected with CMV-RARE-*tk*-CAT and HBV-RARE-*tk*-CAT and the indicated combinations of pECE-RARα (10 ng) and pECE-RXRα (10 ng) expression vectors, treated with $10^{-7}$ M all-trans RA for 24 hours and assayed for CAT activity. The results shown represent the means of three independent experiments.

(Fig. 2A), suggesting RXR homodimers are good activators of the response element. Compared to the RXR homodimers, RARs/RXRs heterodimers could be activated by either all-*trans* RA or 9-*cis* RA since both retinoids bind and transcriptionally activate RARs and RXRs (Heyman *et al.*, 1992, Zelent *et al.*, 1989 and Petkovich *et al.*, 1987). When both RARα and RXRα expression vectors were cotransfected the reporter gene activity was induced by either all-*trans* RA or 9-*cis* RA, indicating RAR/RXR heterodimer also activate the viral response elements. Comparing RARα/RXRα heterodimers, we noted weak induction with RARγ/RXRα heterodimers by all-*trans* RA. Similarly, cotransfection

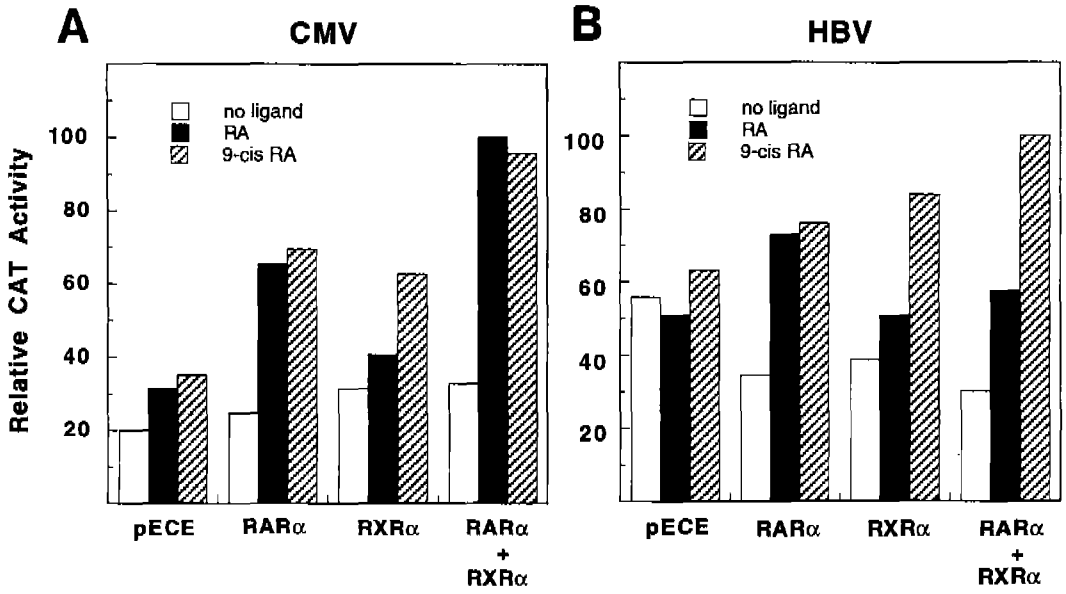


**Fig. 3.** Transcriptinal activity of the CMV-RARE (⟶) (A) and HBV-RARE (⟶) (B) in CV-1 cells. To determine the transcriptional activity of CMV-RARE and HBV-RARE reporter genes that containing sigle copy of the RARE sequences, CV-1 cells were transfected with CMV-RARE-*tk*-CAT, HBV-RARE-*tk*-CAT and the indicated combinations of pECE-RARα (10 ng) and pECE-RXRα (10 ng) expression vectors, treated with $10^{-7}$ M all-trans RA for 24 hours and assayed for CAT activity. The results shown represent the means of three independent experiments.

of RARα or RARγ alone with HBV-RARE-*tk*-CAT induced significant CAT activity in response to either 9-*cis* RA or all-*trans* RA. Cotransfection of RXRα expression vector alone also resulted in a significant induction of the reporter gene activity in response to 9-*cis* RA (Fig. 2B).

The induction of the reporter gene activity by retinoids

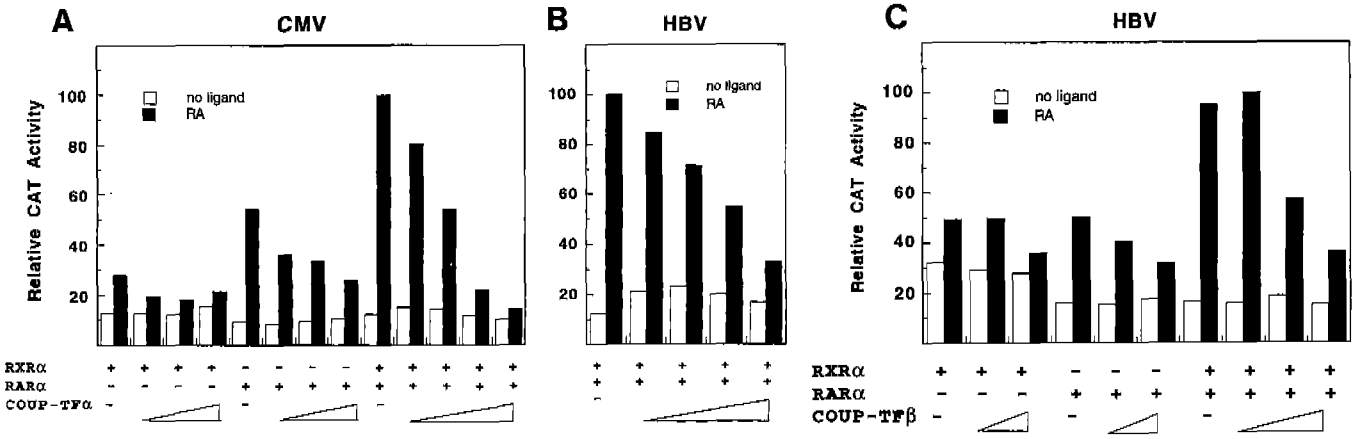


**Fig. 4.** Repression of retinoid-induced activation of the CMV-RARE (⟶) (A) and HBV-RARE (⟶) (B) by COUP-TFα. The CMV-RARE-tk-CAT and HBV-RARE-*tk*-CAT reporter plasmids were cotransfected together with pECE-RARα and pECE-RXRα expression vectors into CV-1 cells in the presence of the increased amounts of COUP-TFα expression vectors (1.25, 2.5, and 5 ng for RXRα alone and RARα alone; 1.25, 2.5, 25 and 50 ng for RXRα and RARα). Transfected cells were treated with no retinoid (open bars) and $10^{-7}$ M all-trans RA (closed bars), and assayed 24 hours later for CAT activity. Data shown represent means of duplicate experiments. (C) Repression of retinoid-induced activation of the HBV-RARE (⟶) by COUP-TFβ. The HBV-RARE-*tk*-CAT reporter plasmids were cotransfected together with pECE-RARα and pECE-RXRα expression vectors into CV-1 cells in the presence of the increased amounts of COUP-TFβ expression vectors (1.25 and 25 ng for RXRα alone and RARα alone; 1.25, 25 and 50 ng for RXRα and RARα). Transfected cells were treated with no retinoid (open bars) and $10^{-7}$ M all-trans RA (closed bars), and assayed 24 hours later for CAT activity. Data shown represent means of duplicate experiments.

may be due to an artificial RARE created by inserting multiple copies of responsive sequences into pBLCAT$_2$. Therefore we analyzed reporter gene activity with a reporter containing single copy of the viral RARE sequences. As shown in Fig. 3, the viral reporter genes of single copy were activated by retinoid receptors in a similar manner with that of the multi-copy reporter genes. Taken together the data show that RAR/RXR heterodimers as well as RXR homodimers are potent activators of the both CMV- and HBV-RAREs.

**COUP-TFs repressed retinoid-induced transcriptional activation of the viral RAREs.**

The expression of other transcriptional factors such as COUP-TFs can modulate the magnitude of the retinoid response. COUP-TFs are orphan members of nuclear receptor superfamily that are encoded by two distinct genes, COUP-TFα and COUP-TFβ (Cooney *et al.*, 1992 and Tran *et al.*, 1992). COUP-TF could repress transcriptional activity of a number of nuclear receptors including thyroid hormone receptors, vitamin D receptor and RARs. Several different mechanisms may be involved to the repression including direct competition of COUP-TFs for DNA binding sequences, heterodimerization with RXRs and suppression of transcription by COUP-TF homodimers (Miyajima *et al.*, 1988, Ladias *et al.*, 1991 and wang *et al.*, 1989). When COUP-TFα expression vector was cotransfected, a dramatic repression of the transcriptional activity of retinoid receptors on the viral RAREs was observed (Fig. 4A and 4B) COUP-TFα effectively repressed RAR/RXR heterodimer activity (Fig. 4) as well as RXR homodimer activity (data not shown). Similar results were obtained with COUP-TFβ (Fig. 4C). Therefore, COUP-TFs orphan receptors may function as negative regulators of the retinoid-induced replication of the HBV and CMV by interfering transcriptional activity of the viral RAREs.

**Retinoid antagonists inhibited all-trans retinoic acid-induced transcriptional activation of the viral RAREs.**

Recently several groups of retinoid antagonists have been developed with a potential to modulate retinoid activity *in vitro* and *in vivo* (Lee *et al.*, 1994, Lee *et al.*, 1996, Umemiya *et al.*, 1996, Kasechika 1994, Eyrolles *et al.*, 1994, Apfel *et al.*, 1992 and Kaneko *et al.*, 1991). Umemiya and his colleague reported retinoid antagonist (LE135) that bind to but not activate RARs (Umemiya *et al.*, 1996). Some benzimidazole and benzodiazepine derivatives exhibited antagonistic effects against differentiation inducing activity on human promyelocytic leukemia cell line HL-60 (Kagechika, 1994). We have previously reported that a novel class of retinoid antagonists, *i.e.*, SR11330, SR11334, and SR11335, that inhibited transcriptional activation of RAR/RXR heterodimer and RXR homodimer (Lee et al., 1994 and Lee et al., 1996). An antagonist, SR11335, has been shown to repress retinoic acid-induced transcriptional activity of HIV-1-RARE that probably by competing with all-*trans* retinoic acid for binding to RARs, thereby preventing the induction of conformational changes of the receptors necessary for transcriptional activation (Lee et al., 1996). Similarly, retinoid-induced transcriptional activation of CMV-RARE was repressed by one of the previously reported

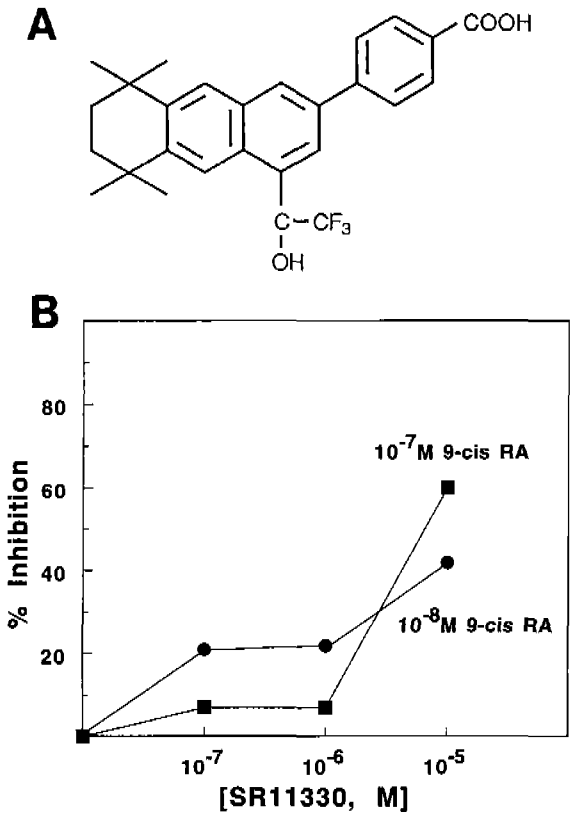


**Fig. 5.** Repression of retinoid-induced activation of the CMV-RARE (—×—) by the retinoid antagonist, SR11330. (A) Structure of the retinoid antagonist, SR11330. (B) CV-1 cells were cotransfected with the CMV-RARE-*tk*-CAT reporter plasmids and 10 ng pECE-RARα and 10 ng pECE-RXRα expression vectors. Transfected cells were treated with $10^{-7}$ or $10^{-8}$ M 9-*cis* RA in the presence or absence of the indicated concentrations of the retinoid antagonists. Inhibition obtained with the retinoid antagonist treatment that completely abolished 9-*cis* RA-induced transcriptional activity was considered as 100% inhibition. Data shown represent means of three independent experiments.

retinoid antagonist, SR11330 (Fig. 5A). The inhibition obtained was most significant at $10^{-8}$ M all-trans RA but relatively weak at $10^{-7}$ M of all-*trans* RA (Fig. 5B).

Our data demonstrated that the presence of a functional RAREs in the regulatory region of HBV and CMV that are shaped as DR-1 and DR-5, respectively. Recently, others have described retinoic acid response elements in the regulatory region of the viruses elsewhere (Huan and Siddiqui, 1992, Ghazal *et al.*, 1992 and Angulo *et al.*, 1996). Ghazal and his colleagues characterized three retinoid-response elements in the CMV promoter (Angulo *et al.*, 1996). Among these, REc located in -291 to -175, is the same as the CMV-RARE that we identified in the present investigation. Similarly Huan and Siddiqui reported an enhancer element which binds RXR efficiently (Huan and Siddiqui, 1992 and Garcia *et al.*, 1993). We demonstrated that the element, HBV-RARE, is activated by the RAR/RXR heterodimer as well as RXR homodimer. Because the elements are located in the main regulatory region that determines viral transcription and replication, the RAREs described here and others allow retinoids to exert a major effect on the transcription of the viral genome. We found that COUP-TFs may be important cellular factors that modulate transcriptional activity of the viral RAREs. Since COUP-TFs have a complex expression pattern that are probably due to delicate regulatory mechanisms (Soo saar *et al.*, 1996), further studies on the expression of the COUP-TFs in tissues that are susceptible for viral infection are guaranteed. Importantly, retinoid antagonists could repress retinoid- induced transactivation of the viral RAREs, suggesting a potential clinical use of such synthetic retinoids to repress retinoid-dependent viral replication and the resultant pathogenesis of the viruses.

## ACKNOWLEDGEMENT

This study was supported by a Yonsei university faculty research grant for 1996.

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