A Pleiotropic Response Is Induced in F9 Embryonal Carcinoma Cells and Rhino Mouse Skin by All-*trans*-Retinoic Acid, a RAR Agonist but Not by SR11237, a RXR-Selective Agonist

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We evaluated SR11237, a retinoid X receptor (RXR)–specific compound, for its pharmacologic effects on cell differentiation in F9 embryonal carcinoma cells and rhino mouse epidermis. SR11237 can cause RXR/RXR homodimers to form and transactivate a reporter gene containing a RXR-response element. We confirmed, using nuclear receptor co-transfection assays in COS-1 cells, that SR11237 is effective at transactivating a chloramphenicol acetyltransferase reporter gene through RXRs but not retinoic acid receptors. When SR11237 was tested for its ability to modulate cell differentiation, it was inactive on F9 embryonal carcinoma cells and rhino mouse skin. Because differentiation in these systems is known to be regulated by RAR–specific compounds, such as all-*trans*-retinoic acid and (E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl benzoic acid], our results with SR11237 are compatible with the concept that classical retinoid pleiotropic responses are mediated by RXR/RAR heterodimeric nuclear receptors rather than through RXR/RXR homodimers. *Key words: retinoid receptors/heterodimers/homodimers/TTNPB. J Invest Dermatol 102:676–680, 1994*

Retinoids are a class of compounds that regulate the growth and differentiation of normal and neoplastic cells in embryonic and adult tissues [1,2]. These pleiotropic effects are mediated by two classes of nuclear receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Akin to steroid hormone receptors, RARs and RXRs are ligand-activated transcription factors [3]. RARs and RXRs, in the absence of ligand, form a heterodimeric complex (see [4] for references). This heterodimeric complex binds to specific response elements (REs) in regulatory regions of target genes, and when ligand is present, it can modulate gene transcription. RXRs also form homodimeric complexes, but only when ligand is present [5]. These RXR/RXR homodimers activate target genes through unique REs that differ from RXR/RAR-heterodimer REs [6].

The effects of ligands on RARs and RXRs have been studied using co-transfection assays in cells with low levels of nuclear receptors. In such assays, receptors are overexpressed in the presence of a reporter plasmid containing an RAR RE or RXR RE. All-*trans*-retinoic acid (t-RA) (Fig 1) activates only RXR/RAR heterodimers [3]. 9-*cis*-retinoic acid (9-*cis*-RA), a stereoisomer of t-RA, is proposed to be the endogenous ligand for RXR/RXR homodimers [3,7,8]. However, 9-*cis*-RA binds to RARs with high affinity and also acts as an agonist for RXR/RAR-gene transcription responses [3,8]. Some synthetic compounds are reported to be selective for inducing RXR/RXR homodimer formation and activating gene transcription [9]; these compounds are inactive on RXR/RAR heterodimers [9]. Likewise, synthetic retinoids have been identified that activate RXR/RAR heterodimers and not RXR/RXR homodimers [10].

The classical pleiotropic pharmacologic activities of t-RA on cell differentiation and proliferation can be replicated by synthetic compounds that only activate RXR/RAR heterodimers, exemplified by (E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl] benzoic acid (TTNPB) (Fig 1). *In vitro* and *in vivo* systems that are regulated by such retinoids express retinoid nuclear receptors constitutively. To more fully define the role of constitutively expressed retinoid nuclear receptors in mediating retinoid pharmacologic responses, receptor-specific compounds can be used as tools to investigate such questions. To ascertain the potential role of RXR/RXR homodimers in retinoid pharmacologic responses, we evaluated an RXR-specific compound, SR11237 [9] (Fig 1), for its effects on cell differentiation and proliferation. We confirmed that SR11237 is an RXR-specific compound in co-transfection assays; however, it does not exhibit the types of biologic activities in F9 embryonal carcinoma cells or the hairless rhino mouse that one observes with RXR/RAR agonists.

MATERIALS AND METHODS

Synthesis of SR11237 Friedel-Crafts acylation of 1,2,3,4-tetrahydro-1,1,4,4-tetramethyl-naphthalene with 4-cyanobenzoylchloride yielded 4-[(5,6,7,8-tetrahydro-5,5,8,8,-tetramethyl-2-naphthalenyl) carbonyl]-benzonitrile (melting point 94–97°C). Acetalization of the latter compound with ethylene glycol gave 4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1,3-dioxolan-2-yl]-benzonitrile (melting point 146°–148°C), which upon alkaline hydrolysis resulted in SR11237 (4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1,3-dioxolan-2-yl]-benzoic acid) (melting point 257–259°C).

RAR and RXR Transactivation Assays Compounds were tested as agonists on RARs or RXRs using a CAT reporter transactivation assay in COS-1 cells. Expression plasmids coding for human RARα, RARβ, RARγ

Manuscript received September 30, 1993; accepted for publication January 14, 1994.

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Abbreviations: 9-*cis*-RA, 9-*cis*-retinoic acid; REs, response elements; t-RA, all-*trans*-retinoic acid; TRE, thyroid hormone response element; TTNPB, [(E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid].

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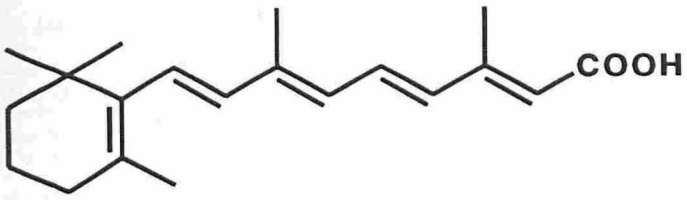
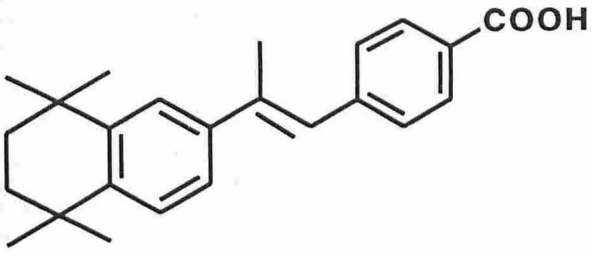
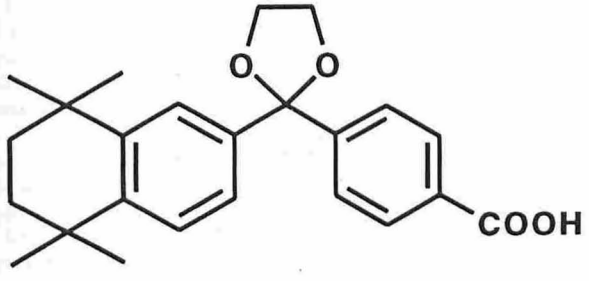


all-*trans*-RETINOIC ACID
(t-RA)



TTNPB



SR11237

Figure 1. Chemical structures for all-*trans*-retinoic acid, TTNPB and SR11237.

or RXRα (pSVL vectors, Pharmacia, Piscataway, NJ) were co-transfected with TRE$_{pal}$-*tk*-CAT or RXRE$_{CRBP-II}$-*tk*-CAT reporter plasmids into COS-1 cells along with a β-galactosidase expression vector (pCH110, Pharmacia). Transfections were performed on cells plated in 162 cm^2 flasks using a synthetic cationic lipid transfection reagent (Transfectam, Promega, Madison WI) according to the manufacturer's instructions. The cells were kept in Dulbecco's Modified Eagle's Medium (DMEM) with 10% charcoal-stripped fetal bovine serum for 24 h. Cells were subcultured into 24-well plates (2×10^5 cells per well). Test compounds were dissolved in ethanol and diluted with DMEM containing 10% charcoal-stripped fetal bovine serum (final ethanol concentration of 0.1%). Cells were incubated with test compounds for 24 h, media were then removed and cells were lysed with 0.25 M Tris-HCl, pH 7.8, by freeze thawing three times. Chloramphenicol acetyltransferase (CAT) activity was assayed using [^{3}H]-acetyl coenzyme A as substrate as described by Chauchereau *et al* [11]. β-galactosidase activity was measured using *o*-nitrophenyl-β-D-galactoside as substrate; the absorbance of the product was measured at 415 nm. CAT activity was divided by β-galactosidase activity to normalize for transfection efficiency.

F9 Embryonal Carcinoma Cells F9 embryonal carcinoma cells (generously provided by Dr. S. Strickland) were grown at 37°C in 5% carbon dioxide/95% air in DMEM with 10% heat-inactivated fetal bovine serum. For treatment with test compounds, cells were plated in 24-well culture plates (200 cells per well) in a total volume of 1.0 ml of media. Test compounds were first dissolved in ethanol at 0.01 M and diluted in culture media (final ethanol concentration of 0.1%). Forty-eight hours after plating, cells were treated with retinoid plus 1.0 mM dibutyryl cyclic adenosine monophosphate and again at 24, 48, and 72 h. Twenty-four hours after the last treatment, media were removed and kept at 5°C.

Laminin in media was measured by non-equilibrium enzyme-linked immunoadsorbent assay as described by Williams *et al* [12]. The primary antibody was rabbit anti-mouse laminin (EY Laboratories, San Mateo, CA) and the second antibody was goat anti-rabbit IgG coupled with horseradish peroxidase (Bio-Rad Laboratories, Melville, NY). Peroxidase activity was measured with *o*-phenylenediamine as substrate. Absorbance of the reaction product was measured at 490 nm in a UV_{max} microplate reader (Molecular Devices, Menlo Park, CA). Mouse laminin (Boehringer Mannheim, Indianapolis, IN) was used to construct standard curves.

Rhino Mouse Evaluations

Utriculus Reduction: Male or female hairless rhino (*hr^{h}hr^h*) mice (Jackson Laboratories, Bar Harbor, ME) were 8–10 weeks old at the beginning of the study. Test compounds were dissolved in an ethanol : propylene glycol vehicle (70 : 30, v/v) and applied topically (0.1 ml) to dorsal trunk skin once daily, five consecutive days per week for 2 weeks. Approximately 72 h following the last treatment, the mice were sacrificed by cervical dislocation. The treated dorsal trunk skin was removed and placed into 0.5% acetic acid for approximately 18 h at 4°C. After this, the epidermis was separated from the underlying dermis. The sheets of epidermis were processed by routine dehydration methods to permanent whole mounts for microscopic examination of utriculi diameters as described previously [13]. For each whole mount (one animal skin/slide), the diameters of ten utriculi in five random fields (n = 50) were measured by image analysis (JAVA, Jandel Scientific, San Rafael, CA). The mean utriculus diameter was calculated for each treatment group. From this, the percent reduction of utriculus diameter compared to the vehicle group was calculated.

Transepidermal Water Loss (TEWL): The rate of evaporation of water from rhino mouse skin was measured using a ServoMed evaporimeter (Kinna, Sweden). Readings were taken on the tenth treatment day, just before the last dosing with test compounds.

Epidermal Keratin and Filaggrin Expression: Rhino mouse epidermal keratin and filaggrin expression was assessed by one-dimensional SDS–gel electrophoresis. The preparation of rhino mouse epidermis and extraction of epidermal proteins was performed exactly as described previously [14]. Urea-soluble material, which contains living layer keratins and filaggrin, was submitted to SDS–gel electrophoresis on 12.5% polyacrylamide precast gels, 10 cm × 10 cm (Daiichi MiniPlus Gel, Integrated Separation Systems, Natick, MA). Gels were stained with Coomasie blue (ISS Pro-Blue, Integrated Separation Systems) to visualize protein bands.

Skin Permeation *In vitro* skin permeation studies were conducted at 32°C in modified Franz diffusion cells (9 mm opening, 10 ml volume, Crown Glass Inc., Somerville, NJ). Female dorsal trunk rhino mouse skin was mounted onto the diffusion cells (n = 3) and 100 μl of a 0.1% SR11237 solution (in ethanol : propylene glycol, 70 : 30, v/v) was applied to the skin surfaces. The diffusion cells remained open to room air throughout the course of the study. Due to the low aqueous solubility of SR11237, 0.5% polysorbate 80 was added to the receptor solution to maintain adequate sink conditions. Based upon previous studies, it was determined that this receptor medium does not alter the skin permeability of this class of compounds. Receptor solutions were sampled (0.3 ml, with replacement) at 2, 4, 8, and 24 h. At 24 h, skin samples were removed from the diffusion cells and washed twice with methanol and once with water. SR11237 was then extracted from the skin samples using a methanol : ethyl acetate (1 : 1) solution. SR11237 in samples was assayed by high-performance liquid chromatography with UV detection. The chromatographic system used was a mobile phase of acetonitrile : water (60 : 40) with 0.1% trifluoroacetic acid (flow rate: 2.0 ml/min), a Spherisorb ODS-2 reverse-phase column, 5 μm (0.4 × 15 cm, Alltech) with UV detection at 240 nm. The detection limit for SR11237 with this method was 10 ng/ml.

RESULTS

RAR and RXR Transactivation Assays We first evaluated SR11237 for its agonist activity on RARs and RXRs in COS-1 cells by co-transfecting receptor expression vectors along with CAT re-

Table I. Effects of SR11237 and t-RA on RAR Transactivation[a]

Compound	Concentration (nM)	Cat Reporter Activity (% of Control)[b] RARα	RARβ	RARγ
SR11237	1	117	91	116
	10	164	125	123
	100	200	164	158
	1000	201	242[c]	149
t-RA	100	1584[c]	1241[c]	572[c]
	1000	2070[c]	1606[c]	713[c]

[a] RAR expression plasmids for RARα, RARβ, or RARγ were co-transfected into COS-1 cells with the TRE_{pal}-*tk*-CAT reporter plasmid.

[b] CAT reporter activity = CAT activity/β-galactosidase activity, where control (no test compound) = 100%.

[c] Significantly different from control (no compound) at p = 0.05 level, Dunnett two-tailed test, based on comparison of normalized CAT activity values. Results shown are from one experiment, n = 3 per concentration.

Table III. Effects of SR11237 and t-RA on F9 Embryonal Carcinoma Cells[a]

Compound	Concentration (nM)	Laminin Released (ng/ml[b])
SR11237	1	−5 ± 10
	10	15 ± 22
	100	−8 ± 4
	1000	185 ± 35
t-RA	0.1	111 ± 39
	1	690 ± 108
	10	2154 ± 56
	100	2724 ± 387
	1000	3330 ± 197

[a] Cells were treated for 96 h with test compounds in the presence of 1.0 mM dibutyryl cyclic adenosine monophosphate as described in *Materials and Methods*.

[b] Calculated by subtracting the mean laminin release value (116.8 ng/ml) of the control group (dibutyryl cAMP treatment alone) from laminin release values of compound plus cAMP groups. Results shown are mean ± SEM from one experiment, n = 3 per concentration.

porter plasmids. TRE_{pal}-*tk*-CAT is activated by RXR/RAR heterodimers or RXR/RXR homodimers, whereas $RXRE_{CRBP\text{-}II}$-*tk*-CAT is specific for RXR/RXR homodimers [5,9]. Table I shows results for RAR transactivation. SR11237 is weakly effective at activating the three RAR subtypes (α, β, γ), whereas t-RA strongly activates all three RARs. The degree of induction of CAT by t-RA through RARγ is lower than RARα and RARβ. The reasons for low transactivation of RARγ by t-RA are unclear, but this pattern of differential transactivation has been observed by others [15]. As shown in Table II, SR11237 dose-dependently activates RXRα. t-RA was also effective at activating RXRα, but TTNPB was only slightly active on RXRα. t-RA is reported to activate RXRα by conversion in cells to 9-*cis*-RA [16,17].

We have thus confirmed that SR11237 is a compound that activates RXR/RXR dimers. We also corroborated the findings of Mangelsdorf *et al* [10] showing that TTNPB is ineffective as a RXR agonist.

F9 Embryonal Carcinoma Cells In the presence of cAMP, F9 embryonal carcinoma cells are induced to terminally differentiate from a neoplastic state to parietal endoderm by retinoids [18]. During differentiation, F9 cells secrete various connective tissue proteins; one of these, laminin, serves as a convenient biochemical marker of cell differentiation [12].

In contrast to t-RA, SR11237 failed to induce differentiation of F9 cells to a parietal endoderm morphology, as assessed by light microscopy. Table III also shows the lack of effect of SR11237 on laminin release at concentrations between 1 and 100 nM; a slight increase in laminin release occurred at 1000 nM SR11237. With t-RA, laminin release was markedly increased in a dose-related manner, which plateaued between 100 and 1000 nM.

Table II. Effects of SR11237, t-RA, and TTNPB on RXRα Transactivation[a]

Compound	Concentration (nM)	CAT Reporter Activity (% of Control)[b] TRE_{pal}-*tk*-CAT	$RXRE_{CRBP\text{-}II}$-*tk*-CAT
SR11237	0.1	76	80
	1	105	108
	10	147	150
	100	524[c]	345[c]
	1000	606[c]	436[c]
t-RA	1000	780[c]	462[c]
TTNPB	1000	262	121

[a] RXRα expression plasmid was co-transfected into COS-1 cells with the TRE_{pal}-*tk*-CAT or $RXRE_{CRBP\text{-}II}$-*tk*-CAT reporter plasmids.

[b] CAT reporter activity = CAT activity/β-galactosidase activity, where control (no test compound) = 100%.

[c] Significantly different from control (no comppound) at p = 0.05 level, Dunnett two-tailed test, based on comparison of normalized CAT activity values. Results shown are from one experiment, n = 3 per concentration.

Rhino Mouse Evaluation The hairless rhino mouse is an allelic mutant that exhibits abnormal hair follicle keratinization. The skin of these mice responds to retinoids with miniaturization of keratin-filled hair follicles (utriculi) and changes in epidermal cytoskeleton protein expression [13,14]. Table IV shows that treatment of rhino mouse skin with t-RA dose-dependently reduces the size of horn-filled utriculi. The rate of TEWL measured by evaporimetry was also enhanced by t-RA in a dose-dependent fashion (Table IV). In contrast, SR11237 did not affect utriculi diameter and caused a small increase in TEWL only at 0.1% (Table IV).

In a second study, we evaluated the effects of SR11237 on keratin and filaggrin expression. In previous work we demonstrated that t-RA and TTNPB profoundly and specifically suppress filaggrin expression in rhino mouse epidermis [14]. Mice were dosed similarly as for utriculus reduction except that during the second week, dosing was performed for four consecutive days and skin was removed 24 h after the last dose. A piece of skin was also taken for hematoxylin and eosin–stained vertical histologic sections.

Unlike 0.05% t-RA, 0.1% SR11237 did not suppress the expression of filaggrin in rhino mouse epidermis, as assessed by SDS–gel electrophoresis (data not shown). We also observed that keratin K17 was induced by t-RA, consistent with our previous findings [14]; however, K17 was not induced in epidermis of SR11237-treated skin. By histology, skin sections from 0.1% SR11237-treated mice were indistinguishable from vehicle-treated skin sections. Skin from these two treatment groups exhibited the characteristic abnormal epidermal architecture of rhino mice, with numerous hyperkeratinized follicular sacs (utriculi) and interfollicular epidermis consisting of 2–4 epidermal cell layers. In contrast, skin treated with 0.05% t-RA had miniaturized follicles completely lacking in keratinized material and a hyperplastic epidermis consisting of 5–8 epidermal cell layers.

To verify that SR11237 penetrates rhino mouse skin, we also assessed its permeation into and through excised skin. SR11237 permeated through rhino mouse skin after an extended lag time. Only the 24-h samples from the receptor medium contained detectable SR11237 (mean ± SD = 4.4 ± 1.1 μg/cm²). Results from the skin extraction indicated that 0.47 ± 0.19 μg of SR11237 was found in the skin after 24 h. These findings for permeation of SR11237 into and through rhino mouse skin are similar to that seen with other pharmacologically-active retinoids (M. Corbo, unpublished observations). Thus, these results exclude the possibility of

Table IV. Effects of SR11237 and t-RA on Rhino Mouse Skin

Treatment[a]	Dose (%)	Utriculus Diameter (μm)[b]	% Utriculus Reduction Versus Vehicle	TEWL (g/m²/h)[b]
Vehicle	—	118.9 ± 0.6	—	12.9 ± 1.2
SR11237	0.001	119.8 ± 1.1	−0.8	17.2 ± 2.1
	0.01	118.3 ± 0.5	0.5	19.7 ± 1.4
	0.1	119.0 ± 1.4	0	22.1 ± 2.4
t-RA	0.0005	77.7 ± 1.4	34.7	23.3 ± 2.2
	0.005	51.1 ± 1.8	57.0	33.9 ± 5.9
	0.05	30.2 ± 1.3	74.6	56.8 ± 5.0

[a] Mice were treated topically with test compounds for 2 weeks (five consecutive days per week) as described in *Materials and Methods.*
[b] Results are mean ± SEM from one experiment, n = 4 mice per treatment group.

pharmacologic inactivity of SR11237 being due to its lack of permeation into rhino mouse skin.

DISCUSSION

We confirmed the specificity of SR11237 as a compound that activates a RXRE, presumably by RXR/RXR homodimers [9]. SR11237, unlike t-RA or TTNPB, was far less effective as an agonist on RXR/RAR heterodimer-activated gene responses. When SR11237 was tested for its ability to modulate cell differentiation, it proved to be inactive on F9 embryonal carcinoma cells and rhino mouse skin. Although we did not evaluate TTNPB (an RAR-selective agonist) in these studies, previous work with the rhino mouse [14,19] and F9 cells [18,20] shows it to be a potent regulator of cell differentiation. Thus, our results give strong support to the concept that classic retinoid pleiotropic responses are mediated by RXR/RAR-heterodimeric nuclear receptors rather than through RXR/RXR homodimers.

We used F9 cells and rhino mouse skin to evaluate SR11237 for pharmacologic activity because they undergo marked changes in their differentiation state when treated with vitamin A analogs (polyenes) as well as non-polyene synthetic retinoids [12–14, 18–20]. Moreover, F9 cells and epidermal skin cells are reported to constitutively express mRNA for RARα and RARγ [21,22]. Epidermal cells also express high levels of RXRα [22]. Although it has not been shown directly, one can infer that F9 cells probably contain RXR for these cells to form RXR/RAR heterodimers and respond to RXR/RAR-specific agonists.

A question that remains unanswered is whether or not RXR agonists like SR11237 do induce RXR homodimers to form in F9 cells or rhino mouse skin, even in the absence of observable pharmacologic effects. Constitutively expressed RXRs are present as heterodimeric complexes with RARs, vitamin D_3, and thyroid hormone receptors [4]. In cases where the content of non-RXR nuclear receptors exceeds RXRs, RXR agonists would have to cause RXR homodimers to form from RXRs that are bound up in cells as heterodimers. Transfection experiments suggest that this may be unlikely because overexpression of RARs in cells transfected with a RXR expression vector inhibits RXR-mediated transactivation of a $RXRE_{CRBP\text{-}II}$-*tk*-CAT reporter plasmid [23].

Even if it is possible for RXR agonists to induce homodimer formation in cells that constitutively express RXRs, a pharmacologic response is dependent on the presence of target genes that contain RXREs. CRBP-II, which is highly expressed in the small intestine, is one such target gene [23]. Apolipoprotein A1 (apoA1), the major protein component of high-density lipoprotein (expressed in the intestine and liver) also contains a RXRE in its 5′-regulatory gene region [24]. Thus, CRBP-II and apoA1 expression could be used as RXR-specific markers to determine if RXR agonists induce homodimer formation in target tissues.

Whether or not RXR homodimers mediate a unique pleiotropic response that differs from RAR-induced pleiotropic effects remains to be determined. As proposed by Mangelsdorf and colleagues [7,10], RXRs may subserve important physiologic roles in visceral tissues such as the liver, intestine and kidney. Pharmacologic studies with RAR and RXR-specific compounds should be useful in elucidating the function of constitutively expressed RARs and RXRs in fetal and adult tissues.

We thank Dr. Lubing Zhou (The R. W. Johnson Pharmaceutical Research Institute, Toronto, Canada) for providing the RAR, TRE_{pal}-tk-CAT and $RXRE_{CRBP\text{-}II}$-tk-CAT plasmids. Dr. John J. Voorhees (University of Michigan) is gratefully acknowledged for the RXRα vector and for valuable comments on the manuscript. John Mallon, Paul Siock, and Scott White provided excellent technical assistance.

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ANNOUNCEMENT

The International Symposium "Mast Cells in the Cytokine Network" will be held in Berlin, Germany on October 21–22, 1994.

The main subjects will be Mast Cell Ontogeny, Mast Cell Responses to Cytokines, Cytokine Secretion by Mast Cells, Pharmacological Modulation, and Clinical Significance.

Prominent researchers in the field will present overview lectures on these topics. Participants submitting abstracts are given preference for attendance. Deadline for abstract submission is May 2, 1994.

For further information please contact the Congress Office, Frau Fuchs, Department of Dermatology, Freie Universität Berlin, Universitätsklinikum Rudolf Virchow, Augustenburger Platz 1, D-13353 Berlin, Germany, Tel., (49-30)-4505-3131; Fax, (49-30)-4505-3131.