ACTIVITY AND METABOLISM OF SELECTED HETEROAROTINOIDS IN F9 CELLS

By

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ACTIVITY AND METABOLISM OF SELECTED

HETEROAROTINOIDS IN F9 CELLS

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LIST OF SYMBOLS

9*c*-RA	9-*cis*-retinoic acid
13*c*-RA	13-*cis*-retinoic acid
AP-1	activator protein-1
APL	acute promyelocytic leukemia
β-C	β-carotene
cAMP	cyclic adenosine monophosphate
CBP	CREB binding protein
CRABP	celluar retinoic acid-binding protein
CRBP	cellular retinol-binding protein
CREB	cAMP response element binding protein
dbcAMP	dibutyryl cyclic adenosine monophosphate
DEPC	diethyl pyrocarbonate
DEPC-H_2O	water treated with diethyl pyrocarbonate
DR-1	direct repeat-1 (repeat seperated by 1 nucleotide)
DR-2	direct repeat-2 (repeat seperated by 2 nucleotides)
DR-5	direct repeat-5 (repeat seperated by 5 nucleotides)
HAT	histone acetylase activity
HDAC	histone deacetylase activity
het(s)	heteroarotinoid(s)
HPLC	high performance liquid chromatography
L	hormone ligand

mRNA	messenger RNA
N-CoA	nuclear co-activator
N-CoR	nuclear co-repressor
ODC	ornithine decarboxylase
PPAR	peroxisome proliferator activator receptor
RAc	retinyl acetate
RAL	retinaldehyde
RAR	retinoic acid receptor
RAR$\alpha^{-/-}$	mutant lacking RARα gene on both alelles
RAR$\gamma^{-/-}$	mutant lacking RARγ gene on both alelles
RARE	retinoic acid response element
RE	retinyl ester
ROH	retinol
rRNA	ribosomal RNA
RXR	retinoid "X" receptor
RXRE	retinoid "X" response element
SMRT	silencing mediator for retinoid and thyroid receptors
SPARC	secreted protein, acidic and rich in cysteine
TOC	tracheal organ culture
TPA	12-O-tetra-decanoylphorbol-13-acetate
TR	thyroid receptor
t-RA	all-*trans*-retinoic acid
VDR	vitamin D receptor

CHAPTER 1

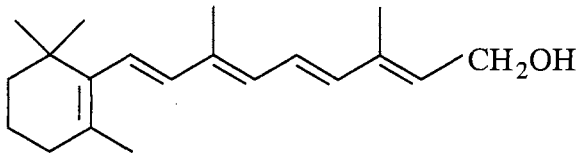
INTRODUCTION TO RETINOIDS

Early Research on Vitamin A

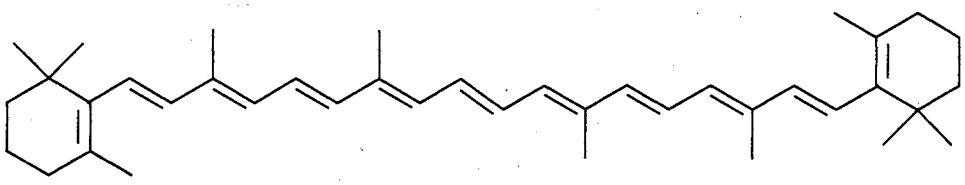
Vitamin A is required for many life processes including reproduction, growth, metabolism, differentiation, hematopoiesis, bone development and embryogenesis (Sporn et al. 1994). Research on vitamin A began in the early 1900s with egg yolk extracts.

In 1913, McCollum and Davis identified a fat-soluble substance in butterfat, egg yolk, and cod liver oil that promoted growth in rats. They called this substance "fat soluble A" to distinguish it from essential water-soluble nutrients they called, "water soluble B". In 1920, Drummond renamed fat-soluble A simply "vitamin A". Karrer determined the structure of β–carotene in 1930 and vitamin A (retinol) in 1931, clarifying the relationship between vitamin A and β-carotene in plants. (See structures in Figure 1.) Thomas Moore described this early vitamin A research in detail (1957).

In the late 1960s, the mechanism of vitamin A activity was partially elucidated with evidence that vitamin A affected RNA synthesis (Zachman et al. 1967). Another clue came in the early 1970s with the discovery that steroid hormones act through a family of DNA-binding receptors to regulate transcription (reviewed by Evans 1988). By the mid 1980s some researchers hypothesized that receptors, similar to those for steroid hormones, may be responsible for retinoid activity (Sporn et al. 1984). The term "retinoid" was coined by Sporn in 1976 to refer to "all natural forms of vitamin A and



retinol (vitamin A)



β -carotene

Figure 1. The Chemical Structures of Vitamin A and its Plant Source, β-Carotene.

synthetic analogs of vitamin A". In 1985, Sporn and Roberts offered a revised definition for retinoids – in terms of receptor activity – even though a receptor was yet to be identified. They suggested a retinoid is "a substance that can elicit specific biological responses by binding to and activation of a specific receptor or set of receptors, with the program for the biologic response of the target cell residing in the retinoid receptor rather than in the retinoid ligand itself". Initially, the cellular retinoic acid-binding protein (CRABP) and cellular retinol-binding protein (CRBP) were suspected to be receptors (Sporn et al. 1984). But CRABP and CRBP were not homologous to the steroid receptors, and a more similar receptor was sought.

Simultaneously, in different labs, Giguère, Petkovitch and their respective labs isolated a human orphan receptor cDNA and showed that it encoded a retinoic acid activated transcription factor belonging to the steroid and thyroid hormone superfamily (Giguère et al. 1987, Petkovitch et al. 1987). This receptor was named RAR for retinoic acid receptor. Surprisingly, a second retinoid receptor was soon found and named RXR for "retinoid X" receptor, since its ligand was initially unknown (Mangelsdorf et al. 1990). The identification of retinoid receptors marked a new era in retinoid research.

Retinoid Receptors

Retinoid receptors are members of the steroid and thyroid hormone receptor superfamily and have been reviewed extensively (Mangelsdorf 1994, Manglesdorf et al. 1994, Chambon 1996). Transactivation (transcription rate enhancement by diffusible factors) was the first described known function of these nuclear receptors. For transactivation, the receptors form dimers that interact with specific sequences (response

elements) in target genes' promoters. When a ligand hormone binds to its receptor, mRNA synthesis is upregulated. (See Figure 2.)

There are six known retinoid receptors: RARα, β and γ and RXRα, β and γ (Petkovich et al. 1987, Giguère et al. 1987, Benbrook et al. 1988, Brand et al. 1988, Krust et al. 1989, Hamada et al. 1989, Giguère et al. 1990, Mangelsdorf et al. 1990, Mangelsdorf et al. 1992, Yu et al. 1991). The naturally occurring ligands are all-*trans*-retinoic acid (*t*-RA) for RARs and 9-*cis*-retinoic acid (9*c*-RA) for both RARs and RXRs (Levin et al. 1992, Heyman et al. 1992). Both RARs and RXRs require dimerization for transactivation activity. RARs heterodimerize with RXRs and require only a RAR ligand. RXRs form heterodimers with RAR and many other receptors including VDR (vitamin D receptor), PPAR (peroxisome proliferator activator receptor) and TR (thyroid receptor) (Mangelsdorf and Evans 1995). In these heterodimers, RXR ligand is usually not required for activity, but exceptions have been observed (Mangelsdorf 1994, Kliewer et al. 1992). RXR/RXR homodimers also form, and these require 9*c*-RA for activity (Mangelsdorf et al. 1994).

Retinoid response elements consist of a halfsite consensus sequence (AGGTCA) configured in several structure patterns, including direct repeats and palindromes (Umesono et al. 1988, Umesono et al. 1991, Näär et al. 1991). The response element for RAR ligands and RXR ligands are called RARE (retinoic acid response element) and RXRE (retinoid "X" response element), respectively. RAREs are direct repeats of the half site separated by 2 or 5 nucleotides (DR-2 and DR-5), and RXREs are direct repeats separated by one nucleotide (DR-1) (Mangelsdorf et al. 1994).

The RAR/RXR heterodimers activate transcription at RAREs in the presence of RAR ligand (Umesono et al. 1991), but these heterodimers can also transrepress genes. Transrepression is the down-regulation of transcription by diffusible factors. In the

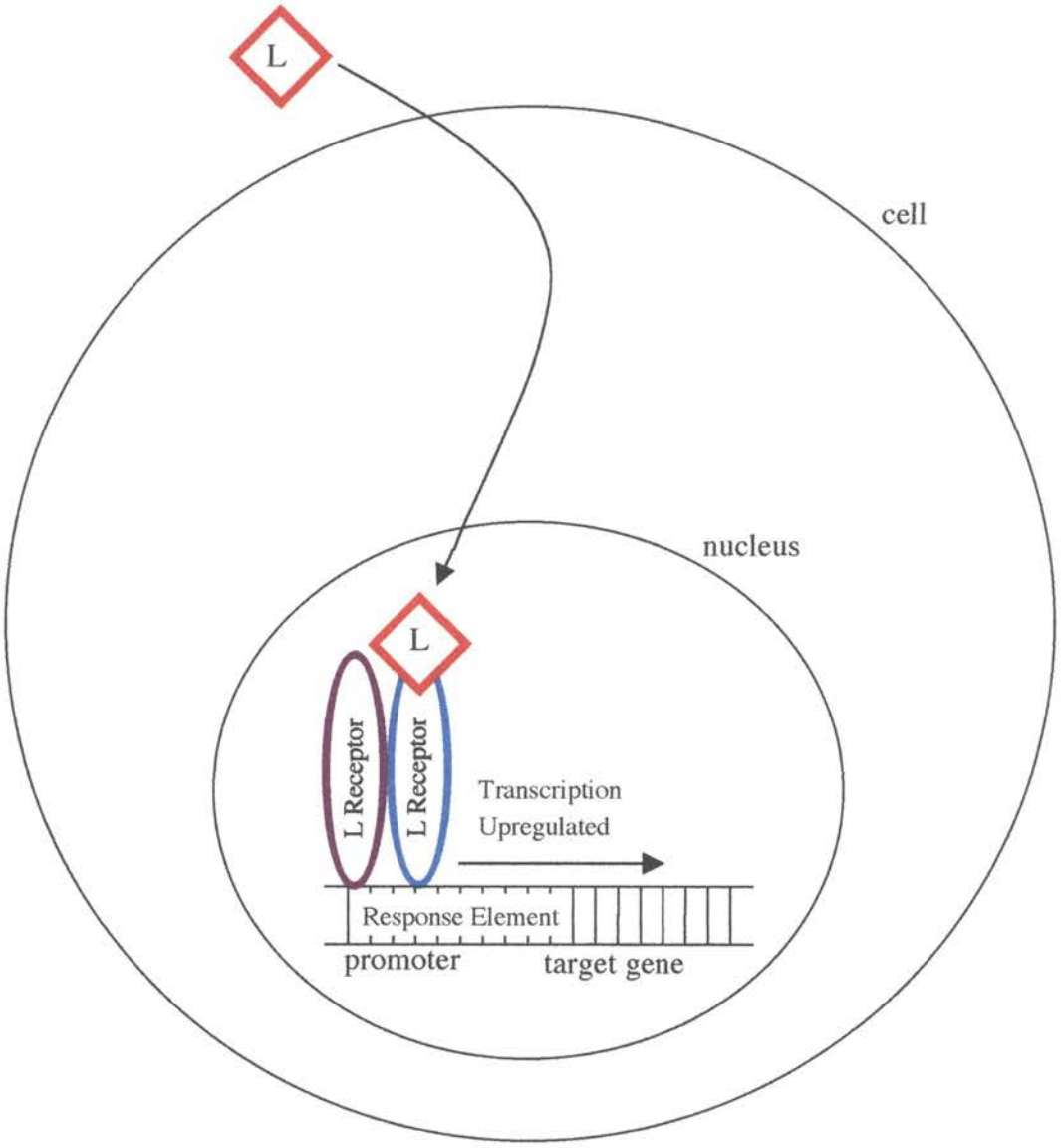


Figure 2. General Model of Transactivation Induced by Hormone Ligands Interacting with Their Receptors. Hormone ligand (L) enters the nucleus and binds its receptor (L receptor). The dimerized receptors, which are bound to their response element in the promoter of a target gene, are activated by hormone binding, and mediate an increase in transcription of the gene.

absence of ligand, RAR/RXRs transrepress at RAREs (Damm et al. 1989, Baniahmad et al. 1990). RXR/RAR heterodimers repress gene transcription at RXREs also, with RAR ligand having little effect (Mangelsdorf et al. 1991). A summary of the ways in which RAR, RXR, retinoid ligands, and response elements interact to affect transcription is shown in Figure 3.

Co-repressors and Co-activators

In recent years, transcriptional regulation by retinoid receptors is becoming clearer. Interactions with other proteins called co-activators and co-repressors are required for receptor function. Many co-activators and co-repressors appear to act by remodeling chromatin at the nucleosome level (Grunstein 1997, Lemon and Freeman 1999). High levels of histone acetylation are associated with transcriptionally active chromatin, and lack of histone acetylation is associated with transcriptional inactivation. Acetylating of core histone amino termini leads to weaker binding of DNA. Deacetylation of core histones is expected to increase their affinity for DNA. Many co-activators have (or recruit factors with) histone acetylation (HAT) activity (Felsenfeld 1996, Brownell and Allis 1996). Co-repressors act in part by recruiting factors with histone deacetylase (HDAC) activity (Wolffe 1997).

There are numerous co-activator families are often shared by several nuclear receptors (Table 1). CBP/p300 are common components in many regulated transcription systems, including many outside the steroid nuclear receptor family (Shikama et al. 1997). (CBP is a CREB-binding protein, and CREB is a cAMP response element binding

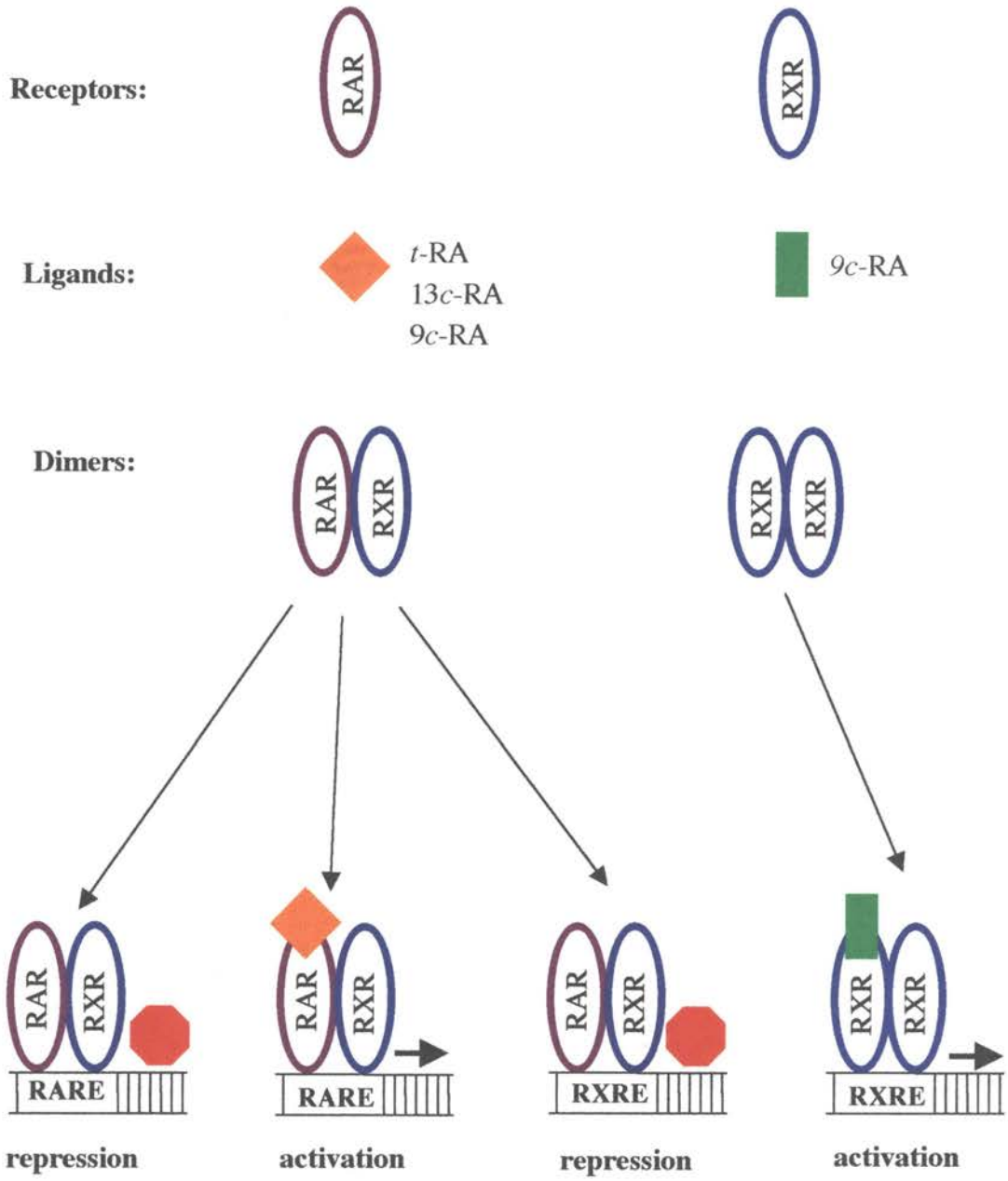


Figure 3. Retinoid Receptor Dimers Act to Repress and Activate Transcription at Different Response Elements.

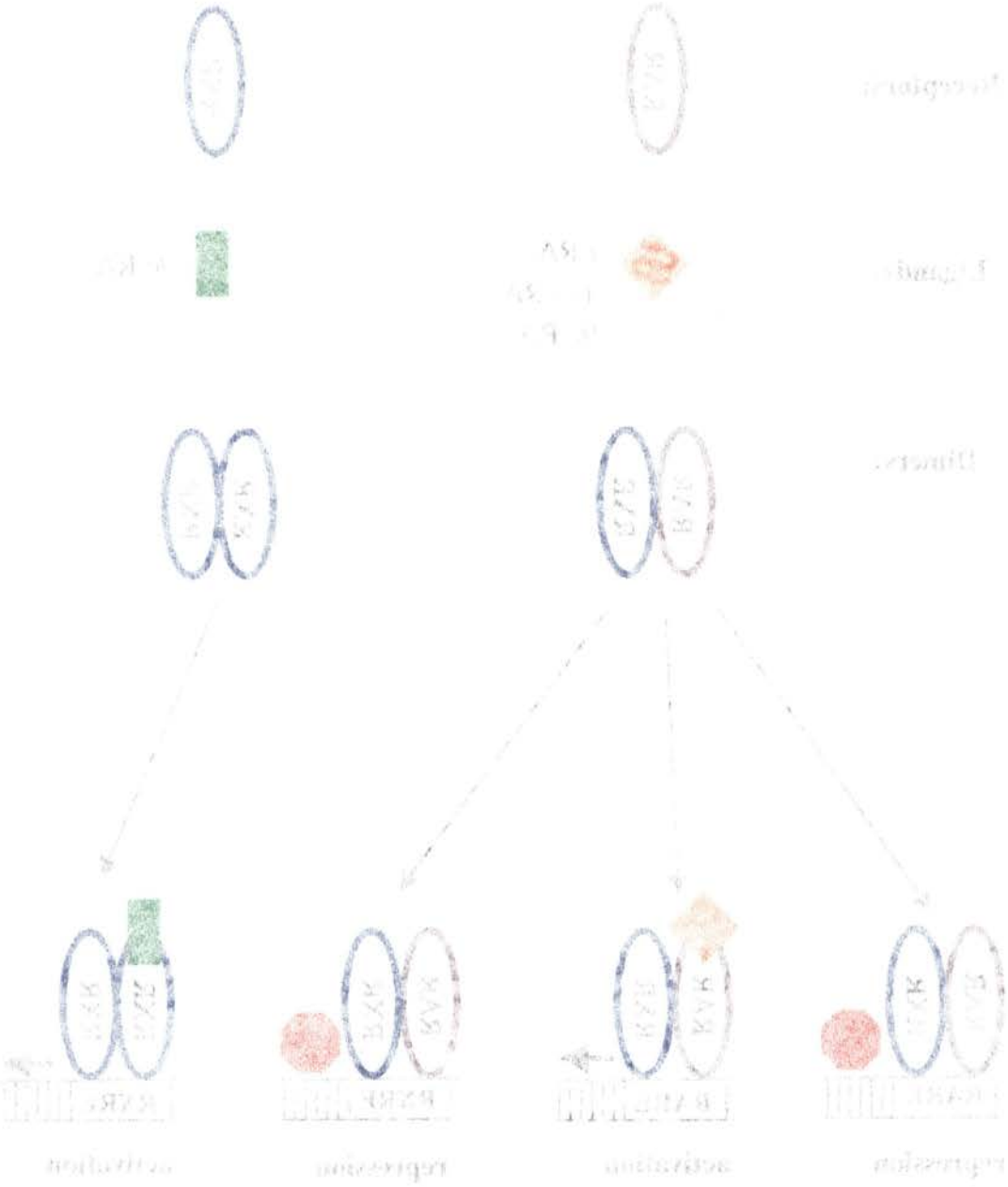


TABLE 1

CO-ACTIVATORS OF RETINOID (AND OTHER STEROID) RECEPTORS

Co-activator	HAT	Comments	References
CBP/p300	yes	ligand binding stimulates interaction with receptor	Bannister et al.1996 Chakravarti et al. 1996 Hanstein et al. 1996 Janknecht et al. 1996 Kamei et al. 1996 Ogryzko et al. 1996 Yao et al. 1996
P/CAF	yes	interacts with CBP/p300 and members of NcoA family	Blanco et al. 1998 Korzus et al. 1998
SRC-1/NCoA-1	yes	interacts with CBP/p300 HAT activity specific for core histones H3 and H4	Onate et al. 1995 Spencer et al. 1997 Yao et al. 1996
TIF2/GRIP1/NCoA-2	no	Mediates transcription through CBP dependent and independent pathways	Hong et al. 1997 Voegel et al. 1998
p/CIP/ACTR/A1B1	yes	binds directly to receptors, recruits CBP and P/CAF	Chen et al. 1997 Torcia et al. 1997
HMG-1	?	interacts with RARs displaces histone H1 from chromatin	Nagpal et al. 1999 Zhao et al. 1993

protein.) Many of the co-activators have HAT activity, and one co-activator can remove histone H1, the histone that seals off each nucleosome.

Fewer co-repressors have been identified. The most prominent are N-CoR (nuclear receptor co-repressor) and SMRT (silencing mediator for retinoid and thyroid receptors). Dissociation of N-CoR is required for ligand-dependent transactivation by RAR/RXR heterodimer on RAREs (Kurokawa et al. 1995). On RXRE elements, N-CoR remains associated with a RAR/RXR heterodimer even in the presence of RAR ligands, presumably leading to repression of the RXRE target gene (Kurokawa et al. 1995). SMRT is another co-repressor that interacts with unliganded RAR mediating transcriptional silencing, with ligand causing a dissociation of SMRT (Chen and Evans 1995). Retinoid structure may directly affect co-repressor release, and in this way, effect transactivation efficiency (Hong et al. 1999). SMRT and N-CoR act at least in part by recruiting the co-repressor mSin3 complex and HDACs (Alland et al. 1997, Heinzel et al. 1997, Nagy et al. 1997).

AP-1 Repression

Another retinoid receptor function, which does not require DNA interaction, is activator protein-1 (AP-1) repression. Retinoid receptors RARα, RARβ, and RXRα can mediate repression of the transcription factor AP-1 (Nicholson et al. 1990, Schule et al. 1991, Salbert et al. 1993). AP-1, a c-Jun/c-Fos heterodimer, usually mediates proliferation signals (Angel and Karin 1991, Kovary et al. 1991). Retinoid receptor dimerization is not necessary for AP-1 repression (Salbert et al. 1993). Recently, Zhou et al. have demonstrated that liganded RARs interfere with c-Jun/c-Fos dimerization,

preventing formation of active AP-1 complexes (1999). Other studies point to competition for CBP, a common co-activator, as the mechanism of retinoid AP-1 repression (Kamei et al. 1996).

Retinoid Metabolism

The central pathway of retinoid metabolism is shown in Figure 4 (Goodman and Blaner 1984, Blaner and Olson 1994). Retinoids are ingested as retinyl esters or as β-carotene, which are converted by ester hydrolysis or oxidative cleavage and reduction to retinol in the intestine. Retinol is absorbed, esterified, and packaged into nascent chylomicrons. The retinyl ester-bearing chylomicrons enter the blood via the lymphatic system. After lipolysis, the liver takes up the chylomicron remnant. The liver can convert small amounts of retinyl ester to *t*-RA to be released into the blood, and it can store the ester form in stellate cells. The liver releases retinol into the blood, which is taken up by cells. Cells in most tissues can convert retinol to retinal and then to retinoic acid (Napoli 1996).

Retinoids and Cancer

In 1925, the connection between retinoids and cancer was first observed by Wolbach and Howe who saw similarities in the epithelium of vitamin A-deficient organs and neoplastic tissue. In 1955, Lasnitzki demonstrated that preneoplastic changes induced by the carcinogen 3-methylcholanthrene in mouse prostate glands were inhibited

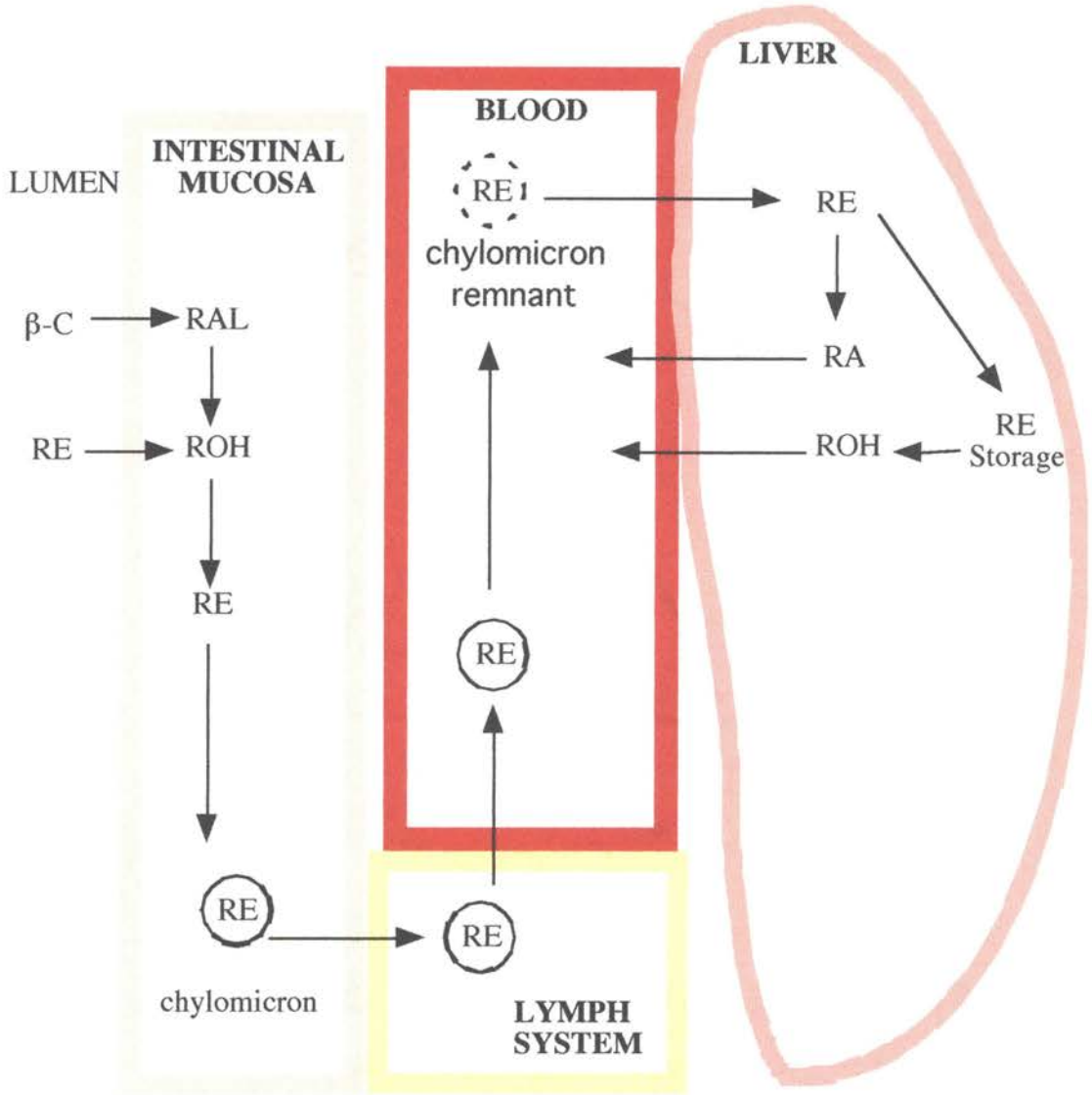


Figure 4. The Central Pathway of Vitamin A Metabolism.
(b-C = b-carotene, RE = retinyl esters, RAL = retinaldehyde ROH = retinol and RA = retinoic acid.) For verbal description, see text.

and reversed by vitamin A *in vitro*. In the early 1980s, epidemiologic studies showed that low serum retinol levels were associated with an increased cancer risk (Wald et al. 1980, Kark et al. 1981). The established anti-cancer activity of retinoids led to their use in cancer treatment.

Retinoids have shown promise in the treatment of a variety of cancerous or precancerous diseases including acute promyelocytic leukemia, head and neck cancer, actinic keratosis, cervical dysplasia, squamous cell carcinoma, basal cell carcinoma, mammary dysplasia and non-small-cell lung carcinoma (Bollag et al. 1992, Hong and Itri 1994). Acute promyelocytic leukemia (APL) is treated with *t*-RA, which induces remission (Huang et al. 1988, Castaigne et al. 1990, Warrell et al. 1991). All-*trans*-retinoic acid is also used to treat cervical dysplasia (Meyskens et al. 1993). 9*c*-RA also induces remission in APL (Miller et al. 1996). Retinoic acid (13-*cis*) has shown promise in the treatment of head and neck cancer (Hong et al. 1990). 13*c*-RA also reverses premalignant oral leukoplakia lesions (Hong et al. 1986). Recently, 13*c*-RA was found effective in the treatment of neuroblastoma (Matthay et al. 1999). Nagpal and Chandraratna have reviewed retinoids in chemotherapy (1996). Retinoid treatment is limited by its undesirable side effects, such as mucocutaneous irritation, increase in plasma triglycerides, headache, bone toxicity and teratogenicity (Armstrong et al. 1994).

Heteroarotinoids

The desire to retain the anti-cancer activities of natural retinoids without their toxicity has led to the development of many novel synthetic retinoids. Heteroarotinoids (hets) are synthetic retinoids with a partially saturated ring containing at least one

heteroatom, fused to an aromatic ring (Figure 5) (Waugh et al. 1985). Hets are often less toxic than *t*-RA (Dawson et al. 1984, Benbrook, et al. 1997b). Hets HS-acid and HO-acid (Figure 5) are three fold less toxic than *t*-RA, based on maximum tolerated dose (MTD) to 10% weight loss (Benbrook et al. 1997b, Lindamood et al. 1990, Lindamood et al. 1987). Hets HS2-acid and HO2-acid are also less toxic than *t*-RA (Dawson et al. 1984). Although hets are less toxic than *t*-RA, they still exhibit anti-cancer retinoid activity.

Past studies have demonstrated that heteroarotinoids exhibit anti-cancer activities. Hets (including HO-acid, HS-acid, and HO2-acid) block induction of ornithine decarboxylase (ODC) by 12-O-tetra-decanoylphorbol-13-acetate (TPA) (Spruce et al. 1987, Dawson et al. 1984, Spruce et al. 1991). The ability of a compound to repress ODC in this assay correlates with its ability to suppress formation of TPA-induced skin papillomas in mice (Verma et al. 1977, 1978 and 1979). Hets reduced the number of papilloma tumors formed when a mouse was treated with a carcinogen with no retinoid toxic side effects observed (Dawson et al. 1984). Heteroarotinoids, including HS-acid and HO2-acid reverse keratinization (induce differentiation) in tracheal organ cultures (TOC) from vitamin A deficient hamsters (Waugh et al. 1985, Dawson et al. 1984). Some hets, including HS-acid and HO2-acid induce differentiation in HL-60 cells (Spruce et al. 1987, 1991), a well-established activity of *t*-RA (Breitman et al. 1980). Results from ODC, TOC, HL-60 and papilloma tumor assays were obtained prior to the work presented in this dissertation and are summarized in Figure 6. More assays have been completed during the past five years, including studies with cervical carcinoma cells, growing rats and receptor-specific transactivation.

The CC-B cervical tumor cell line (Benbrook et al. 1997a) was used for transactivation and growth inhibition studies (Benbrook et al. 1997b). These cells contain integrated copies of RARβ2-RARE-tk-CAT reporter plasmid, which was used to evaluate the ability of heteroarotinoids to induce transcription of the RARβ RARE

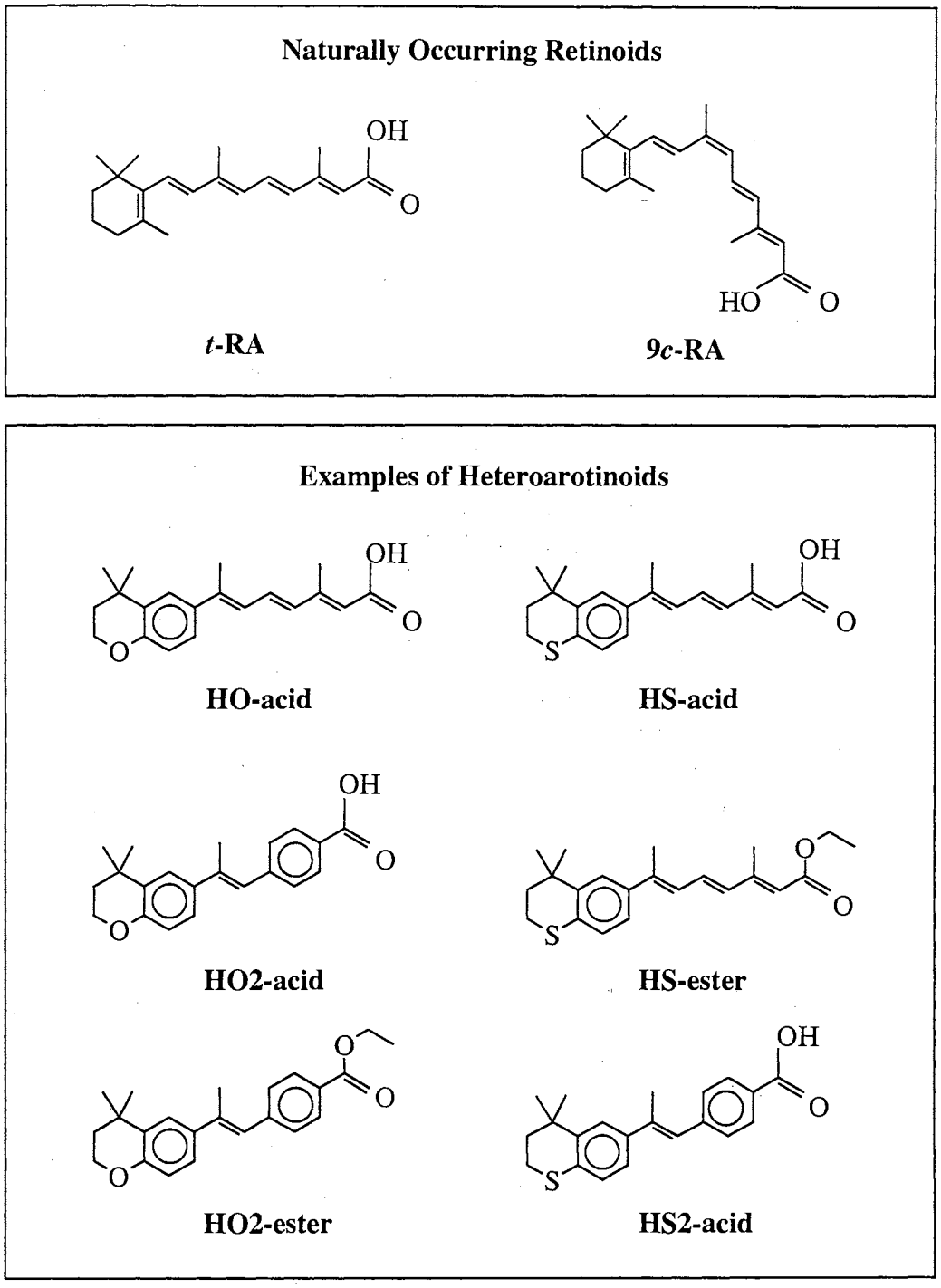


Figure 5. Naturally Occurring Retinoids and Examples of Heteroarotinoids.

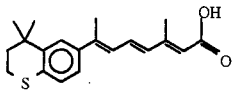
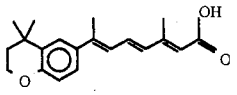
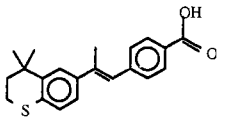
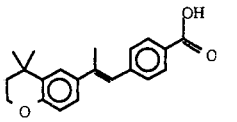
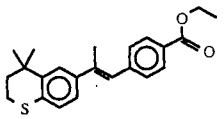
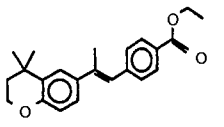
Heteroarotinoid	Assay	Reference(s)
 HS-acid	ODC HL-60 Differentiation HEL Differentiation CC-B Growth Inhibition Rat Growth	Spruce et al. 1987, 1991 Spruce et al. 1987, 1991 Benbrook et al. 1997 Benbrook et al. 1997 Simms-Kelley et al. 1998
 HO-acid	ODC HEL Differentiation CC-B Growth Inhibition	Spruce et al. 1987 Benbrook et al. 1997 Benbrook et al. 1997
 HS2-acid	ODC TOC Tumor Inhibition	Dawson et al. 1984 Dawson et al. 1984 Dawson et al. 1984
 HO2-acid	TOC HEL Differentiation CC-B Growth Inhibition	Waugh et al. 1985 Benbrook et al. 1997 Benbrook et al. 1997
 HS2-ester	ODC TOC HEL differentiation CC-B Growth Inhibition Tumor Inhibition	Dawson et al. 1984 Dawson et al. 1984 Waugh et al. 1985 Benbrook et al. 1997 Benbrook et al. 1997 Dawson et al. 1984
 HO2-ester	ODC TOC HL-60 Differentiation HEL Differentiation CC-B Growth Inhibition Rat Growth	Spruce et al. 1987 Dawson et al. 1984 Waugh, et al. 1985 Spruce et al. 1987 Benbrook et al. 1997 Benbrook et al. 1997 Thorne 1993

Figure 6. Demonstrated Activity of Select Heteroarotinoids.

promoter. HS-acid, HS2-ester, and HO2-acid exhibited 53, 50, and 31 percent of *t*-RA's activity, respectively. Inhibition of CC-B growth rate was determined by counting cells after a seven-day incubation. Heteroarotinoids HS-acid, HS2-ester and HO2-acid inhibited growth to 62, 70, and 83 percent of untreated cultures, respectively.

Supporting growth in rats is a classic vitamin A activity. To test heteroarotinoids for this activity, young vitamin A-deficient rats were fed a diet lacking vitamin A, but supplemented with a heteroarotinoid. Three heteroarotinoids supported growth in rats. HS2-ester had the strongest activity, followed by HO2-ester and HS-acid (Thorne 1993, Simms-Kelley et al. 1998, Simms-Kelley et al. 1999).

Transactivation of the receptors RARα, RARβ, RARγ and RXRα were determined for many hets including HS-acid, HO2-acid and HS2-ester (Benbrook et al. 1998). Transactivation was evaluated by transfecting the appropriate response element with a luciferase reporter. Then an expression plasmid with a specific receptor (RARα, RARβ, etc.) was transfected into the same CV-1 cells. Activity of the hets was compared to that of 9*c*-RA, since it activates both RAR and RXR receptors (see Table 2). HS-acid, HO2-acid, and HS2-ester were active in transactivating at RAREs with all three RARs. Of these hets, only HS-acid had RXRα transactivation activity.

F9 Embryonal Carcinoma Cell Line

F9, a murine carcinoma cell line, serves as a model of retinoid-induced differentiation. This embryonal carcinoma cell line originated from a tumor started by grafting a 6-day old embryo to a mouse host testis (Bernstine et al. 1973). Monolayer F9

TABLE 2

RETINOID TRANSACTIVATION EFFICACY* AND POTENCY**
(Benbrook et al. 1998)

Heteroarotinoid	RARα EC_{50} (n*M*) (Potency (%))	RARβ EC_{50} (n*M*) (Potency (%))	RARγ EC_{50} (n*M*) (Potency (%))	RXRα EC_{50} (n*M*) (Potency (%))
HS-acid	1400 (103)	43 (99)	12 (106)	2700 (121)
HO2-acid	1100 (76)	190 (88)	200 (76)	NA (5)
HS2-ester	620 (62)	110 (45)	84 (65)	NA (2)

* EC_{50} (n*M*) is the concentration required for maximal response.
** Potency (%) is maximal response relative to that of 9*c*-RA.
NA = Not Applicable, no substantial receptor was observed.

cells differentiate to primitive endoderm when treated with *t*-RA, and to parietal endoderm upon treatment with *t*-RA and cAMP agents, such as dibutyryl cAMP (dbcAMP) or methyl xanthines (Hogan and Taylor 1981, Strickland et al. 1980). Parietal endoderm cells are extra-embryonic epithelial cells in the mouse blastocyst (Figure 7).

Morphological changes are associated with F9 differentiation (Darrow et al. 1990). Treatment with *t*-RA only results in primitive endoderm cells that are larger, more flat, and more spread out. Treatment with *t*-RA and dbcAMP leads to parietal endoderm cells that are more rounded. During retinoid-induced differentiation of F9 cells, many genes are activated. Some of these genes are upregulated by retinoic acid directly; their promoters contain RAREs. Hoxa1, RARβ, and laminin B1 all have RAREs in their promoters (Langston and Gudas 1992, de Thé et al. 1990, Vasios et al. 1989). Genes which are upregulated include transcription factors, retinoid receptors, extracellular matrix proteins and cytoskeletal proteins (Gudas et al. 1994).

F9 cells express all RARs and RXRs (Zelent et al. 1989, Martin et al. 1990, Wan et al. 1994), but RARβ mRNA is present in high amounts only after *t*-RA treatment (Hu et al. 1990). Receptor requirements for F9 differentiation are complex. Some promoters are preferentially activated by specific receptors (Nagpal et al. 1993), but some degree of functional redundancy among receptor isoforms does exist (reviewed by Kastner et al.1995).

F9 differentiation with either receptor null mutants or receptor-specific ligands has been evaluated to determine which receptors are involved in differentiation (reviewed by Chiba et al. 1997). For example, RARγ-specific ligands induce differentiation by themselves, but RARα- and RARβ-specific ligands induce differentiation poorly (Taneja et al. 1996). $RAR^{-/-}$ (lacking RAR gene on both alleles) mutants differentiate very poorly, but $RAR\alpha^{-/-}$ mutants differentiate normally (Boylan et al. 1995). See Table 3 for a

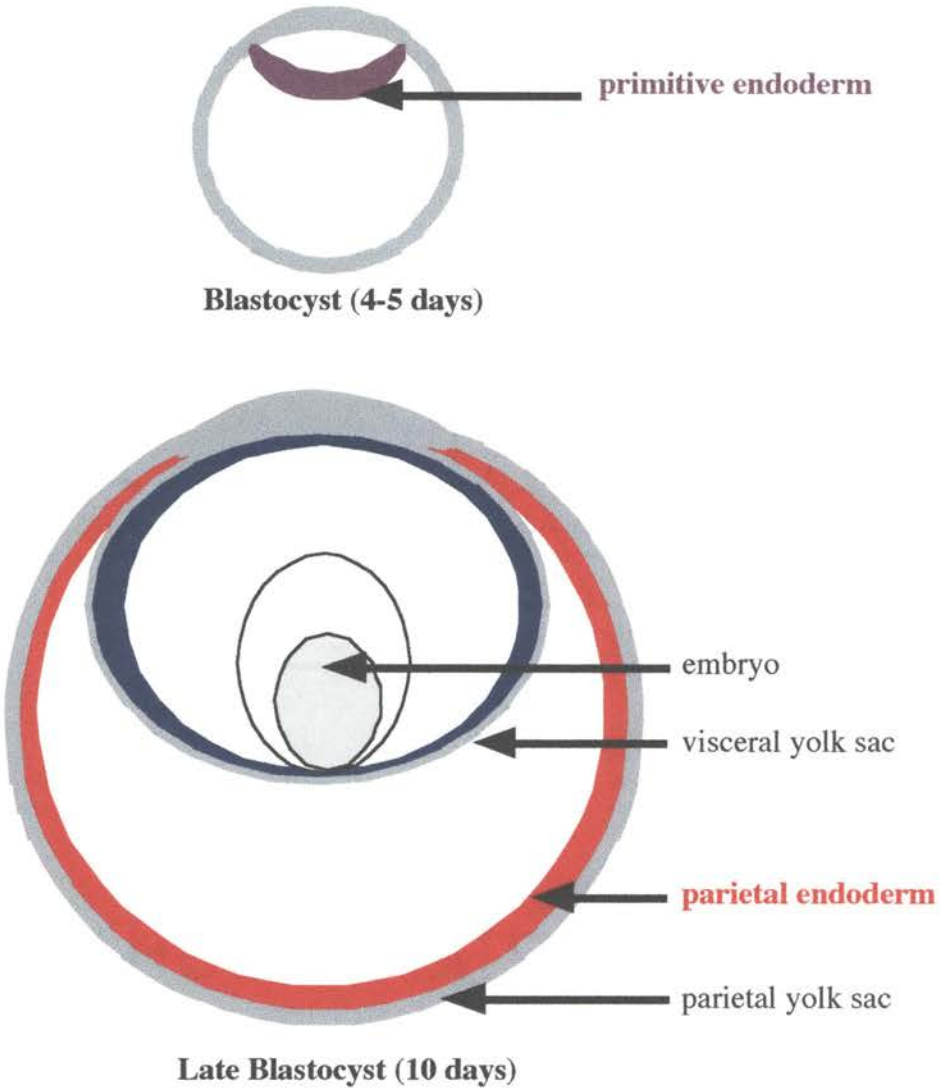


Figure 7. Location of the Primitive and Parietal Endoderm in the Mouse Blastocyst.

TABLE 3

SPECIFIC LIGAND AND MUTANT RECEPTOR STUDIES OF RETINOID RECEPTOR ACTIVITY IN F9 DIFFERENTIATION

RXRs	RARs
RXR ligand inactive by itself (Roy et al. 1995)	RARα ligand inactive by itself (Taneja et al. 1996)
RXRα$^{-/-}$ cells differentiate poorly (Clifford et al. 1996)	RARα$^{-/-}$ cells differentiate normally (Boylan et al. 1995)
RXR ligand is required at suboptimal levels of RARγ ligand (Roy et al. 1995, Taneja et al. 1996)	RARβ ligand is barely active by itself (Taneja et al. 1996)
RXRα can be poorly replaced by RXR(β+γ) provided RARγ is present (Chiba et al. 1997)	RARβ$^{-/-}$ cells do not differentiate completely and do not exhibit growth arrest (Faria et al. 1999)
	RARγ ligand active by itself (Taneja et al. 1996)
	RARγ$^{-/-}$ cells differentiate poorly (Boylan et al. 1993)

summary of all the mutant and specific ligand studies. The heterodimer RARγ/RXRα plays a central role in retinoid-induced F9 differentiation (Chiba et al. 1997). RARβ also plays an important role in the growth arrest and late features of differentiation (Faria et al. 1999).

Retinoid transactivation, as opposed to transrepression of AP-1, appears to be the mechanism of F9 differentiation. Anti-AP-1 specific retinoids (which transrepress AP-1, but lack RARE transactivation ability) have an impaired ability to induce F9 differentiation (Fanjul et al. 1994). RAREs are in the promoters of many differentiation-specific genes, including Hoxa1 (Langston et al. 1992), laminin B1 (Vasios et al. 1989) and Bmp2 (bone morphogenic protein) (Heller et al. 1999). The transcriptional co-activator p300 is required for *t*-RA-induced differentiation of F9 cells, although CBP is not required (Kawasaki et al. 1998).

Cyclic-AMP agents enhance differentiation to the parietal endoderm. The effect of cAMP does not affect the "early" genes, such as Hoxa1, but enhances transcription of genes expressed at late times such as laminin B1 and SPARC (secreted protein, acidic and rich in cysteine) (Gudas et al. 1994). Cyclic-AMP response element binding protein (CREB) may play a role in regulating cAMP inducibility of promoters in F9 cells (Gudas et al. 1994). Phosphorylated CREB is present in the nucleus of F9 cells only after *t*-RA treatment (Kingsley-Kallesen et al. 1999) which may explain why dbcAMP treatment alone does not induce differentiation marker genes.

F9 Retinoid Metabolism

Retinoic acid is quickly taken up, converted to polar metabolites and excreted in F9 cells (Napoli et al. 1981, Napoli et al. 1982, Kalin et al. 1984, Gubler et al. 1985). In F9 cultures, retinoic acid is the most abundant metabolite in the cells (Williams et al. 1985). All metabolites found in the cells are also found in the medium - at higher concentrations (Williams and Napoli 1985). Some metabolites of *t*-RA are active in differentiation, but all are less active than *t*-RA (Roberts et al. 1981). The first step of *t*-RA metabolism is its conversion to 4-hydroxyretinoic acid and 4-oxoretinoic acid, followed by conversion to more polar metabolites (Roberts et al. 1981). A mouse P450 enzyme (P450RAI) catalyzes the formation of 4-hydroxyretinoic acid, 4-oxoretinoic acid and 18-hydroxyretinoic acid (Abu-Abed et al. 1998).

In F9 cells, cellular retinoic acid binding protein (CRABP) is important for retinoid activity. Two isoforms exist, CRABP-I and CRABP-II. CRABP-I moderates cellular response to *t*-RA by facilitating catabolism or sequestering *t*-RA, keeping it from receptors (Boylan et al. 1991, 1992). CRABP-II directly interacts with RAR and facilitates the formation of the RAR·retinoic acid complex (Dong et al. 1999). Retinoids which possess differentiating activity can usually bind CRABPs; however, retinol can induce differentiation at high concentrations, even though it does not bind CRABPs (Ong et al. 1994). The rate of *t*-RA degradation increases with increasing levels of CRABP-I (Boylan et al. 1992). Sensitivity to *t*-RA-induced differentiation is inversely correlated to cellular level of CRABP-I (Boylan et al. 1991).

CHAPTER 2

F9 DIFFERENTIATION

Introduction

F9, a murine embryonal carcinoma cell line, serves as a model of retinoid-induced differentiation. This cell line originated from a 6-day old embryo grafted to a mouse host testis (Bernstine et al. 1973). F9 cells differentiate to primitive endoderm with the addition of all-*trans*-retinoic acid (*t*-RA) and to parietal endoderm upon addition of *t*-RA along with agents which increase intracellular cAMP, such as dibutyryl cAMP or methyl xanthines (Hogan and Taylor 1981, Strickland et al. 1980).

Morphological changes are associated with F9 differentiation (Darrow et al. 1990). Undifferentiated F9 cells grow in crowded groups, with sharp borders; individual cells are difficult to distinguish in these groups. Treatment of F9 cells with *t*-RA alone results in primitive endoderm cells that are larger, polygonal, and spaced out. Treatment with *t*-RA and cAMP agents leads to parietal endoderm cells which are like primitive endoderm cells except they are more rounded and less polygonal.

Although differentiation can be recognized by the above morphological changes, induction of parietal endoderm-specific genes further confirms differentiation. SPARC (Secreted Protein Acidic and Rich in Cysteine) is a parietal endoderm product which appears to be involved in morphological changes (Everitt and Sage 1992) and is specifically induced in parietal endoderm cells (Mason et al. 1986, Wang et al. 1985).

This collagen-binding glycoprotein interacts with the extracellular matrix and is often highly expressed in tissues undergoing morphogenesis (Lane and Sage 1994). SPARC may control cell-cell interactions (Everitt and Sage 1992). In F9 cells differentiated with *t*-RA and cAMP agents, SPARC mRNA is induced 20-31 fold in 3 to 4.5 days (Mason et al. 1986, Wang et al. 1985).

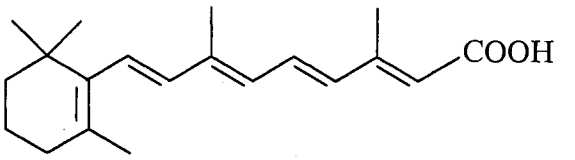
The purpose of the F9 differenitiation experiments was to evaluate selected heteroarotinoids for their ability to differentiate F9 cells to parietal endoderm cells since this is a classical activity of *t*-RA. Differentiation was assessed by observing cell morphology and accumulation of SPARC mRNA.

The heteroarotinoids studied (Figure 8) were selected based on their activity in previous assays (Figure 6 and Table 2) and for structure/activity relationship studies. For example, HS2-acid and HS-acid are structurally alike, except for the presence of a second aryl ring in HS2-acid. Thus, the effect of the second ring on the compound's ability to induce differentiation can be determined.

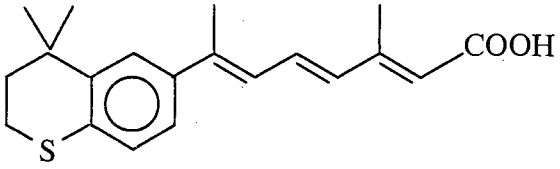
Experimental Procedures

Materials

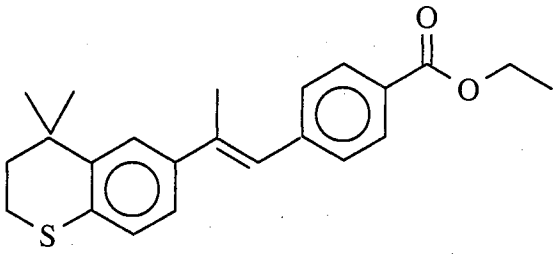
All-*trans*-retinoic acid (*t*-RA) was obtained from Hoffmann-La Roche, Inc. Dr. K. Darrell Berlin and his students, Oklahoma State University, supplied the heteroarotinoids HS-acid, HS2-ester, and HO-acid. Dibutyryl cyclic AMP (dbcAMP) was purchased from Sigma (#D-0627). F9 cell culture was obtained from American Type Culture Collection



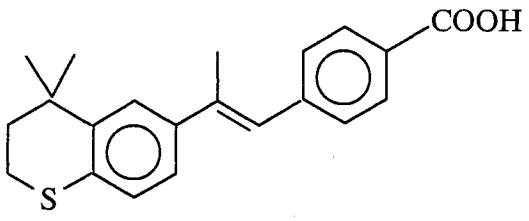
***t*-RA**



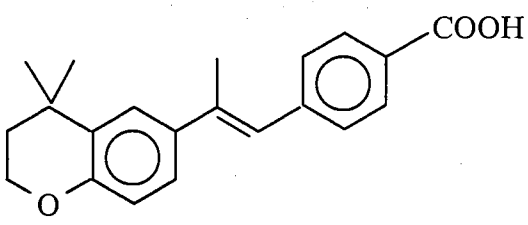
HS-acid



HS2-ester



HS2-acid



HO2-acid

Figure 8. Structures of Retinoids Assayed in F9 Differentiation Assay.
All-*trans*-retinoic acid served as a positive control for F9 differentiation. The four hets (HS-acid, HS2-ester, HS2-acid and HO2-acid) were evaluated for their differentiation activity. In this text, the nomenclature utilized for hets correlates to their structure. For example, HS2-acid is a Heteroarotinoid with a Sulfur heteroatom and 2 aryl rings (diaryl). All esters are ethyl esters. Chemical Abstract information is given in Appendix 5.

(CRL-1720). The plasmid pSPARC was the kind gift of Dr. Ivor Mason, UMDS Guy's Hospital, London. Sources of common materials are given in the protocols in Appendix 2.

HS2-acid Preparation

HS2-acid was prepared from HS2-ester by alkaline hydrolysis. The HS2-ester was dissolved in ethanol (≈1 *M*), one volume 10% KOH was added, and the solution was incubated at 50 °C for 20 minutes. An acidic hexane extraction was performed to recover the HS2-acid product. Then HCl (4 *M*) was added until the pH dropped to 2, and then 2.5 volumes of hexane were added. The tube was shaken until solution was transparent, with no precipitate visible. The hexane layer was removed, dried under nitrogen and the residue was dissolved in ethanol. HPLC analysis was performed to ensure the reaction was complete.

Cell Culture

F9 cells were grown in gelatin-coated cell culture flasks which were prepared as follows: A 0.1% gelatin solution was prepared from porcine gelatin (Sigma #G-2625) and autoclaved twice. Gelatin solution (20-25 mL) was added to each 75 cm^2 flask (Corning #25110-75), and then the flasks were stored at 4 °C for at least two hours. After removing the gelatin solution, the flasks were rinsed three times with sterile nanopure® H_2O, then stored under sterile conditions until use.

F9 cells were maintained in Dulbecco's Modified Eagles Medium (Sigma #D5671) supplemented with 15% fetal bovine serum (Sigma #F4135), 4 m*M* glutamine

(Sigma #G-5763),100 U/mL penicillin G (Sigma #P-4687) and 100 μg/mL streptomycin (Sigma #S-9137). Cells were grown in a humidified 37 °C incubator with 10% CO_2 and subcultured every 3-4 days.

A detailed description of the subculturing protocol is provided in Appendix 2. Briefly, cells were removed by trypsin/EDTA (Sigma #T-4049), pelleted at 400 × g for 15 minutes, and resuspended in 1-10 mL of medium. This cell solution was then gently passed through a 21 gauge needle 6-8 times to separate small aggregates. Cells were counted with a hemacytometer (protocol in Appendix 2) and routinely replated at 3×10^6 cells/75-cm^2 flask.

F9 Retinoid Treatment (Standard)

Twenty-four hours prior to treatment, the F9 cells were subcultured and plated at 1.3×10^4 cells per 75 cm^2 flask with 15 mL of fresh medium. F9 cells attached to the gelatin-coated flasks during this 24 hours, after which, treatment solutions were added.

Treatment solutions were prepared in several steps. Retinoids were first dissolved in a minimal volume of ethanol, then this solution was added to medium that was used to treat cells. Retinoids were dissolved in absolute ethanol to make > 3.13×10^{-3} *M* solutions. Spectrophotometry (Table 4) was used to determine the dilution necessary to obtain 3.13×10^{-3} *M* retinoid-ethanol stocks. Medium-retinoid treatment solutions were prepared by adding 10 μL of retinoid-ethanol stock solution to 12.5 mL of medium to give concentrations of 2.5×10^{-6} *M* retinoid and 0.08% ethanol (see Table 5). Five mL of medium-retinoid treatment solution were added to each appropriate flask 24 hours after subculturing (Table 6). (Final concentrations in culture were calculated to be 0.5 μ*M*

TABLE 4

SPECTROSCOPY OF SELECT HETEROAROTINOIDS

Retinoid	λ_{max} (nm)	ε ($10^4/M$ cm)	Reference
t-RA	350	4.5	Robeson 1955
HO2-acid	307	2.5	Madler 1997
HS-acid	355	3.8	*
HS2-ester	326	2.6	Madler 1997
HS2-acid	319	2.5	Madler 1997

*These values were determined as follows: A 2.00 m*M* HS-acid solution (in ethanol) was prepared based on mass (mg balance was employed). The maximum absorbance was recorded for five dilutions of the stock, with $0.4 \leq$ Absorbance ≥ 1.9. Molar absorptivity (ε) was calculated as the slope of the line: Absorbance = ε (*M* HS) + b.

TABLE 5

COMPOSITION OF MEDIUM TREATMENT SOLUTIONS

Treatment Solutions	*t*-RA (μM)	het (μM)	ethanol (%)	dbcAMP (mM)
ethanol-medium	-	-	0.080	-
t-RA-medium	2.5	-	0.080	-
Het-medium	-	2.5	0.080	-
dbcAMP-medium	-	-	-	5 .0

TABLE 6

VOLUMES OF TREATMENT SOLUTIONS AND PLAIN MEDIUM ADDED TO EACH F9 FLASK

Flask	medium (plain) (mL)	medium-ethanol (mL)	medium-dbcAMP (mL)	medium-*t*-RA (mL)	medium-het (mL)
no treatment	10.0	-	-	-	-
ethanol	5.0	5.0	-	-	-
ethanol/dbcAMP	-	5.0	5.0	-	-
t-RA	-	-	5.0	5.0	-
Het	-	-	5.0		5.0

TABLE 7

FINAL CONCENTRATION OF EACH COMPONENT IN F9 FLASKS

Treatment	ethanol (%)	dbcAMP (mM)	*t*-RA (μM)	Het (μM)
no treatment	-	-	-	-
ethanol	0.016	-	-	-
ethanol/dbcAMP	0.016	1.0	-	-
t-RA	0.016	1.0	5	-
Het	0.016	1.0	-	5

retinoids and 0.016% ethanol.) All manipulations with the retinoids were performed in low light or yellow light.

Dibutyryl cyclic AMP (dbcAMP) is a membrane permeable cAMP analog used along with *t*-RA to induce F9 differentiation to parietal endoderm. dbcAMP was added to medium to make a medium-dbcAMP treatment solution with a concentration of 5 m*M* dbcAMP. At treatment time, 5.0 mL of each medium-dbcAMP treatment solution was added to the appropriate flasks (Table 6). The final concentration of dbcAMP was 1 m*M*.

Each time cells were treated with heteroarotinoids, both negative and positive controls were included. Negative controls included cultures that were untreated, treated with ethanol (vehicle) only, and treated with ethanol and dbcAMP. A culture treated with both dbcAMP and *t*-RA was the positive control and the standard to which the het responses were compared. Het treatments also included the dbcAMP. See Table 7 for a summary of experimental design showing final treatment concentrations.

F9 Retinoid Treatment (12 hour)

One set of cells was treated using a limited retinoid exposure time. The treatment method was essentially the same as the standard method, except: The initial treat ment included retinoid only, no dbcAMP. After 12 hours of retinoid exposure, the media were removed, cells were rinsed with phosphate buffered saline, and medium containing dbcAMP was added to all flasks. All concentrations were the same as the standard treatment.

Morphological Assessment

After 96 hours of treatment, cultures were examined at 100× magnification, and photographs were taken of representative fields (5-10 fields were examined). The cultures were divided into three catagories:

0 ≈< 20% of cells have clearly defined edges

1 ≈ 20-80% have clearly defined edges

2 ≈> 80% have clearly defined edges

With this scale, "0" indicates baseline levels of differentiation and "2" indicates full differentiation. Cells were not measured nor counted; it was easy to judge the striking morphological difference between differentiated cells and stem cells.

Total RNA Isolation

Total RNA was isolated using a modified version of the Chomczynski and Sacchi method (1987). See protocol in Appendix 2. Each 75 cm^2 flask was processed separately. Cells (monolayer) were rinsed with PBS and then lysed while inside the culture flask. A phenol extraction was followed by sodium-isopropanol precipitation. The RNA was electrophoresed in a formaldehyde gel to assure the RNA was not degraded.

mRNA Isolation

Poly-adenylated (poly A) mRNA was isolated from total RNA using Oligotex™ resin (Qiagen). Oligotex™ resin consists of small beads with $dC_{10}T_{30}$ oligonucleotides

covalently linked on the outside to bind poly A tails of mRNAs. The manufacturer's spin-column protocol was used. An extra rinsing step was added to further remove rRNA as suggested by the handbook.

RNA gels

The complete RNA gel protocol is in Appendix 2 and is abbreviated here. Gel box and comb were treated with 3% H_2O_2 for at least two hours prior to use. RNA concentrations were determined by A_{260} measurements, and all samples had $A_{260}/A_{280} >$ 1.7. A 75 mL formaldehyde gel (11×14 cm^2 gel box) was prepared. Gels were 1.2% agarose (Biorad high strength analytical), 2.2 *M* formaldehyde (Sigma #F-8775), and MOPS (running buffer). Formaldehyde was added last, after heating and dissolving the agarose.

Samples were prepared for the gel as follows: First, RNA was diluted with DEPC H_2O (diethyl pyrocarbonate-treated water, Appendix 1) to give 3-5 μg of RNA in the same volume for all samples. Next, the following was added to each sample: 0.182 volumes of 10X MOPS (see protocol in Appendix 2), 1.82 volumes of formamide (Fisher), 0.636 volumes of formaldehyde (Sigma), and 1.0 μL of 100 mg/mL ethidium bromide (Biorad). Next, the samples were placed in a 70 °C bath for 10 minutes, and then cooled on ice. Gels were run in a hood with a peristaltic pump to recirculate buffer. After electrophoresing, a photograph of each gel was made under UV light.

Mapping pSPARC

Several digests with DNA gel electrophoresis were utilized to map the SPARC plasmid. Until the exact map was known, it was impossible to make an RNA probe. These digests also confirmed that a SPARC cDNA sequence was being used to produce the probe. This work is presented in Appendix 3.

SPARC Probe

Since a pure plasmid sample was desired for probe synthesis, a CsCl gradient preparation of the plasmid was performed by the Molecular Pathology Core Laboratory at the University of Oklahoma Health Sciences Center. Quantification was determined by A_{260} (one absorbance unit = 50 ng/μL). The plasmid was linearized using EcoRV (Promega); there was only one restriction site, and it was within the SPARC cDNA sequence (Figure 9). (See restriction digest protocol in Appendix 2.) Digestion completion was confirmed by DNA agarose gel electrophoresis (protocol in Appendix 2).

RNA probe synthesis of antisense SPARC was performed using 1 μg of the linearized plasmid. Two μL of NTP label (digoxigenin) mix (Roche Molecular Biochemicals), 2 μL of 10X transcription buffer (Promega), 1 μL of RNase-OUT (Gibco-BRL), 10.5 μL of DEPC-treated H_20 and 2 μL of SP6 RNA polymerase (Promega) were added to plasmid. After gentle mixing, the sample was incubated at 37 °C for two hours. The RNA synthesis reaction was stopped by adding 2 μL of 200 m*M* EDTA, pH 8 (Biorad #161-0728). The SPARC probe was quantitated using digoxigenin-labeled control RNA (Roche Molecular Biochemicals #1585746) according to the manufacturer's protocol. The probe was stored at -80 °C.

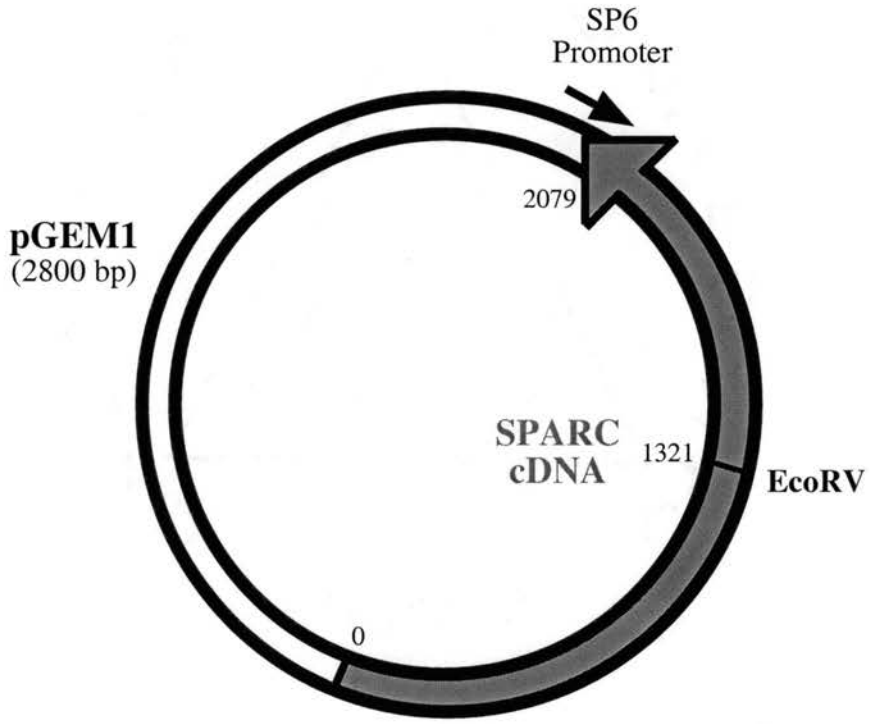


Figure 9. Map of pSPARC Showing EcoRV Restriction Site.
The complete map of pSPARC is shown in Appendix 3. Digestion at the EcoRV site allows for an RNA probe (antisense SPARC) to be syntheized using RNA polymerase SP6. This probe will be complementary to SPARC mRNA.

GAPDH Probe

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a constituitively expressed gene and its mRNA was used as an internal standard to normalize the quantity of RNA on blots. An antisense RNA probe for GAPDH was produced from a linearized template (Ambion #7792) using the same protocol used for SPARC. This probe was similarly quantitated and stored at -80 °C.

Northern Blotting

Hybridization of SPARC (or GAPDH) probe was performed according to Boehringer Mannheim's Genius System User's Guide (Roche Molecular Biochemicals). Hybridization and detection protocols are described in detail in Appendix 2. Briefly, membranes were prehybridized for one hour before the probe was added. Probe concentrations were 50 ng probe per mL of hydridization solution (DIG easy-Hyb from Roche). Probes were used up to three times. Hybridizations were performed overnight at 68 °C unless otherwise specified. Hybridization washes included two washes at room temperature with 2X hybrid wash and two washes at 68 °C with 5X hybrid wash.

Probe detection began with rinsing in detection wash buffer for 5 minutes. The membrane was then washed in blocking solution for 30 minutes at room temperature. Next, the anti-DIG-AP (anti-digoxigenin-alkaline phosphatase, Roche Molecular Biochemicals) was diluted in blocking solution and incubated with the membrane for 30 minutes at room temperature. Excess antibody was removed by two washes with malate buffer. In a hyridization bag, equilibration with detection buffer was followed by the addition of 1 mL of the detection buffer containing 10 μL of CSPD®, (Roche Molecular Biochemicals #1655884), the alkaline phosphatase substrate. (The chemical name of

CSPD is found in Appendix 2 in the detection protocol.) Excess liquid was drained from membrane. The membrane was re-sealed in a hybridization bag and placed at 37 °C for 15 minutes to enhance the light reaction. An X-ray film was exposed by the blot. Films from each blot were scanned with a Biorad densitometer (GS-700), and MultiAnalyst (version 1.0.2)was used to quantitate signal.

Dot Blots

Dot blots were used to quantitate SPARC mRNA relative to GAPDH (internal standard) mRNA. A Hybridot® dot blot manifold (Life Technologies) was used to blot mRNA onto a nylon membrane (MSI magnagraph). First, a dilution series of each total RNA sample was made. Typically, two-fold dilutions were made for a total of 5 solutions per original RNA sample. All samples/dilutions were diluted to 50 µL with TE (tris/EDTA Sigma), pH 8. To each sample 35 µL of 20X SSC (in General Solution Recipes, Appendix 1) and 20 µL of formaldehyde (Sigma) were added. Samples were incubated at 60 °C for 15 minutes to denature the RNA and then cooled on ice.

The dot blot manifold was treated for at least 2 hours in 3% H_2O_2 prior to use. The nylon membrane was supported by 2 pieces of Whatman No. 2 filter paper. A low vacuum was used to empty wells slowly (≈500 µL / 30 minutes). Wells were rinsed with 500 µL of 20X SSC before and after RNA sample application. UV crosslinking (120 mJ) was performed immediately following blotting (UV Stratalinker™ 1800, Stratagene). The method of evaluated dot blot data is described in detail in Appendix 4.

Results and Discussion

HS2-acid Preparation

Alkaline hydrolysis of HS2-ester produced a single peak when analyzed by reverse-phase HPLC (Figure 10). The product eluted earlier and thus was more polar than the parent compound. No HS2-ester peak was observed in the product chromatogram, indicating complete conversion. The product exhibited a λ_{max} of 323 nm; the reported λ_{max} of HS2-acid is 319 nm in ethanol (Madler et al. 1997). This difference in wavelength can be accounted for by solvent differences. The mass spectrum analysis was consistent with HS2-acid being the product (Figure 11). For these reasons, the product was concluded to be HS2-acid.

Probe Synthesis

A RNA probe was used since it hybridizes at higher temperatures, allowing for more specificity. A SPARC RNA probe was synthesized by using the cDNA plasmid, pSPARC. This plasmid was created from pGem1 with a full length SPARC cDNA cloned into the BamH1 (5') and HindIII (3') sites (see Appendix 3). A template for an RNA SPARC probe was obtained by digesting pSPARC with EcoRV. Complete digestion was confirmed by gel electrophoresis (Figure 12). Complete digestion prevents unwanted labeling of vector DNA.

Digoxigenin-labeled RNA probes for SPARC and GAPDH were then synthesized using SP6 polymerase. (The GAPDH template was purchased from Ambion.) The resulting probes were quantitated using standard labeled (digoxigenin) RNA from Roche

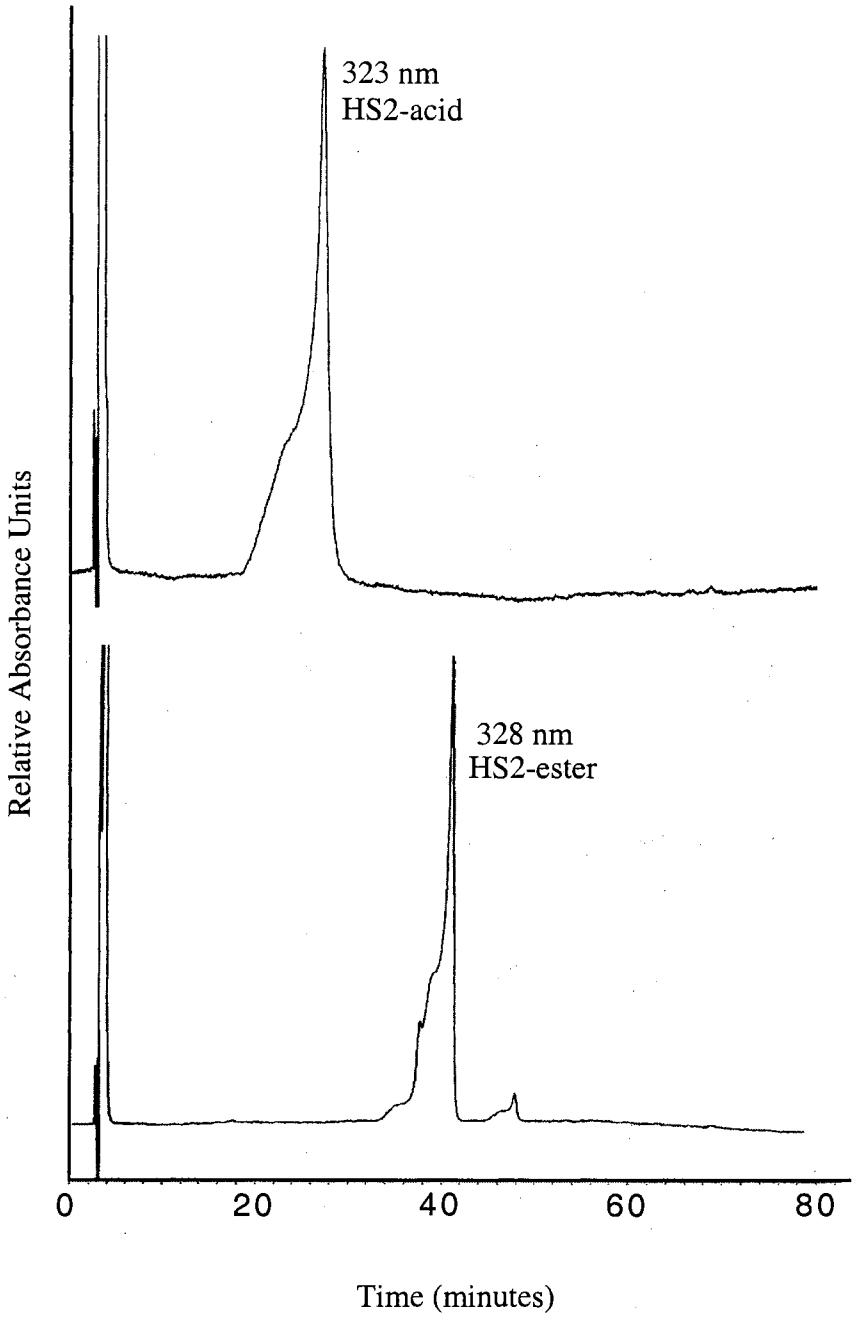


Figure 10. HPLC Analysis of HS2-acid Synthesis from HS2-ester.
HS2-acid was prepared by alkaline hydrolysis of HS2-ester. The reverse-phase HPLC chromatograms of the acid product (top) and the parent compound (bottom) are shown. Solvent was held at 25% 0.01 *M* acetic acid:75% methanol for 5 minutes, then was adjusted linearly, reaching 100% methanol at 60 minutes, which was maintained until 80 minutes. Injected samples were 300 μL with concentrations of 0.5 m*M*. The HS2-acid product shows no peak at the time of the HS2-ester parent.

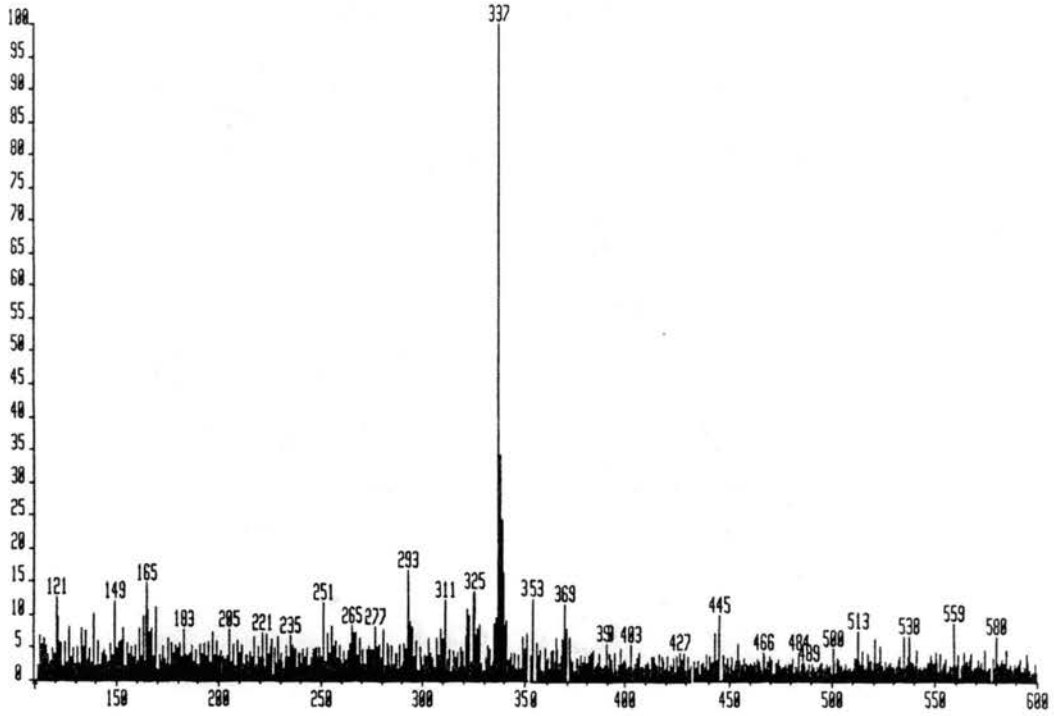


Figure 11. Mass Spectral Analysis of Product from Alkaline Hydrolysis of HS2-ester. This mass spectral analysis was performed by M-Scan, Inc. using Fast Atom Bombardment on a VG Analytical ZAB 2-SE.

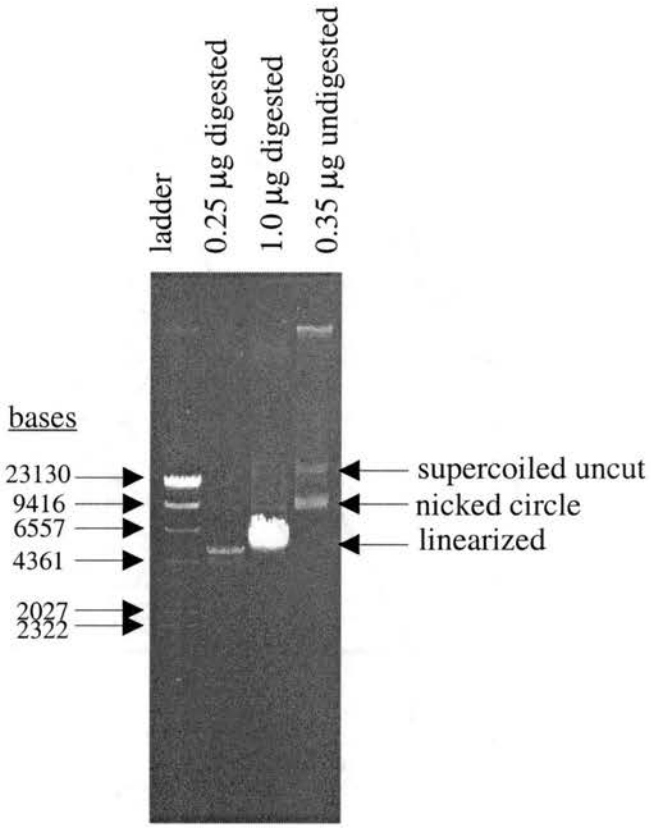


Figure 12. EcoRV Digest of pSPARC.
EcoRV (3 units per µg DNA) was used to digest 10 µg pSPARC. Digested and intact pSPARC were electrophoresed in a 0.7% agarose gel (see protocol for DNA gel in Appendix 1.) The ladder is Lambda/Hin (Promega), and the sizes are shown on the left. The size of the linearized template is shown by the 0.25 µg lane and is consistent with 4.9 kb (kilo bases) expected for linearized pSPARC. The overloaded 1 µg digested lane and the undigested lane demonstrate the extent of the digest.

Molecular Biochemicals. By comparing to the standard dilution series, the SPARC and GAPDH probes were estimated to be 150 ng/μL and 230 ng/μL, respectively (Figure 13). Quantitation is an important step, because it verifies that the probe is active and it approximates how much probe should be used with each hybridization.

Problem with SPARC Detection

Quantitation of SPARC mRNA was desired, but this mRNA presented a special problem. The SPARC transcript (2.1 kb) is close to the same size as the mouse 18S rRNA (1.9 kb). Probes sometimes have a low affinity for rRNA, and this can cause a large, dark band, since there is so much rRNA. Binding of the SPARC probe to 18S rRNA band produced a very large band which obscured the true SPARC band on blots made from gels (Figure 14). Not only was the SPARC probe not specific, its legitimate band could not be seen. Of course, if a probe binds to anything besides its target gene, then it should not be used for quantitation on dot blots. The specificity problem was overcome by purifying the total RNA to obtain mRNA only. The following experiment illustrates the non-specificity of the SPARC probe with total RNA and its specificity with mRNA.

Total RNA from both *t*-RA/dbcAMP-treated and untreated F9 cells was electrophoresed on a gel with mRNA from the same sources. The gel was allowed to run very long - until the bromophenol blue was at the end of the gel. This allowed the SPARC and 18S rRNA bands to separate. A blot was made and hybridized with SPARC probe. On the total RNA lanes, both rRNA bands are apparent, and on the treated lanes, the SPARC message is seen just above the 18S rRNA. (See left blot, Figure 15). On the mRNA lanes (right blot, Figure 15) no rRNA bands are present, and a SPARC band is present in the *t*-RA-treated mRNA lane only. GAPDH was detected on the same blot to

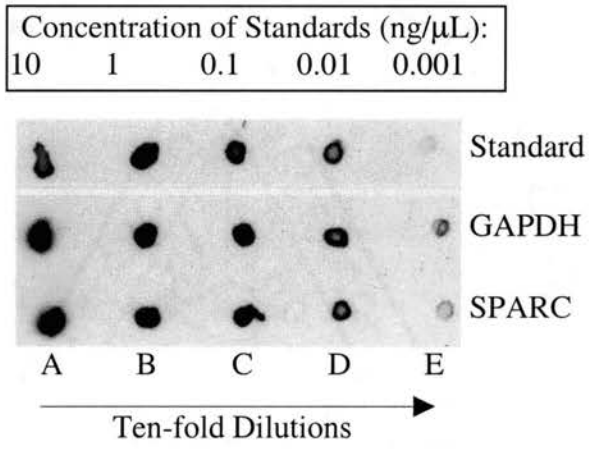


Figure 13. SPARC and GAPDH Probe Quantitation.
The standard is digoxigenin-labeled control RNA, GAPDH RNA probe and SPARC RNA probe were serially diluted ten-fold and spotted on a nylon membrane. After UV crosslinking the RNA to the membrane, the standard detection protocol for the digoxigenin label (protocol in Appendix 1) was performed. The standard has a concentration of 10 ng/μL for dilution A. The probes are diluted 1:10 in dilution A. Standard dilutions B-E are (signal versus concentration) in the linear range. Probe dots C-E were within this linear range and were used to calculate the concentration of the probes.

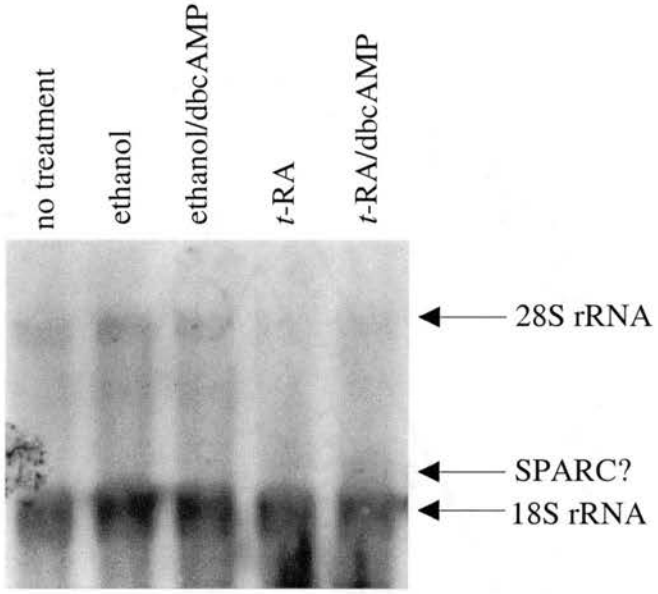


Figure 14. SPARC Probe Detection on Total RNA Gel Blot.
Three micrograms total RNA per lane were electrophoresed in a formaldehyde gel. RNA was blotted onto a a nylon membrane and crosslinked and then hybridized with SPARC probe. (Detailed protocols given in Appendix 2.) The main band detected by the SPARC probe in this total RNA blot is the 18S rRNA, not SPARC. The presence of a SPARC band (which should be present in *t*-RA and *t*-RA/dbcAMP lanes) is questionably distinguishable. The SPARC band may be hidden under the large 18S rRNA band and/or hidden by the signal of degraded 28S rRNA (vertical "stripes" of signal). The SPARC probe is obviously not specific with total RNA; therefore, dot blots of this RNA could not be used to quantitate SPARC RNA.

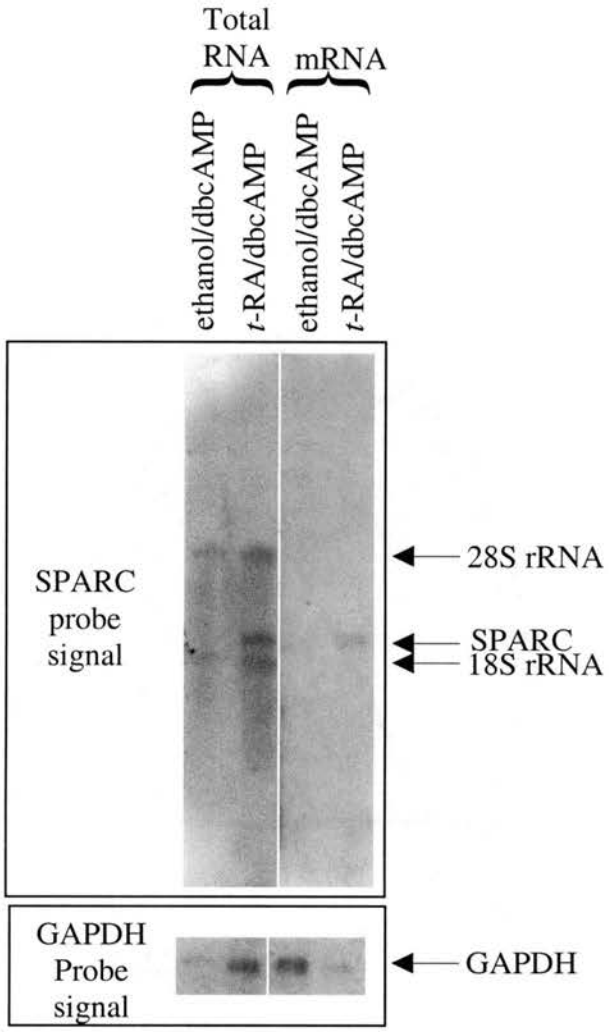


Figure 15. Elimination of rRNA Reveals SPARC mRNA Induction.
Total RNA (left) and mRNA (right) from both negative control (ethanol/dbcAMP) and positive control (*t*-RA/dbcAMP) treated F9 cells was electrophoresed in a formaldehyde gel and blotted onto a nylon membrane. After crosslinking, RNA on the membrane was hybridized with SPARC probe (upper picture) and GAPDH probe (lower picture). (Detailed protocols are given in Appendix 1.) Three μg of total RNA was loaded in the left two lanes, but the first well leaked. The total RNA *t*-RA/dbcAMP lane produced three bands when hybridized with SPARC probe: SPARC, 18S rRNA and 28S rRNA. Poly A RNA was isolated from total RNA (22 μg for *t*-RA/dbcAMP and 27 μg for ethanol/dbcAMP) using Qiagen's Oligotex mRNA kit. (mRNA was not quantitiated after isolation.) Although the mRNA *t*-RA/dbcAMP lane is underloaded compared to the ethanol/dbcAMP lane (see GAPDH difference), this retinoid-treated lane does have a more prominent SPARC band.

illustrate the induction of SPARC in the treated lanes (bottom, Figure 15). As evidenced by the low level of GAPDH in the treated mRNA lane, this lane was under-loaded (contains less RNA) compared to the untreated mRNA lane. Yet, a SPARC band was only present in this under-loaded lane. This indicates SPARC induction with *t*-RA treatment. The probe is specific for SPARC in the absence of rRNA.

Effect of dbcAMP on SPARC

Once a valid SPARC detection method was working, the differentiation assay was optimized by evaluating the effect of dbcAMP. The literature suggested SPARC mRNA was induced some (no numbers given) with *t*-RA alone (Mason et al. 1986, Wang et al. 1985). It was also desired to verify that SPARC was not induced by dbcAMP without a retinoid present.

F9 cultures were treated with either dbcAMP, *t*-RA, dbcAMP/*t*-RA, HO2-acid or dbcAMP/HO2-acid. Dot blots were performed (Figure 16), and SPARC mRNA levels were quantiated as described in Methods. Dibutyryl-cAMP did increase *t*-RA's SPARC mRNA levels, and dbcAMP treatment alone did not affect SPARC mRNA levels (Figure 17). Without dbcAMP, *t*-RA treatment induced SPARC mRNA levels to around 4-fold, compared to approximately 13-fold with dbcAMP. Using dbcAMP produced a similar increase in SPARC levels with HO2-acid treatment. Also, SPARC levels were comparable between untreated, ethanol, and dbcAMP/ethanol treatments showing that dbcAMP by itself does not enhance SPARC mRNA levels.

Dibutyryl cAMP was used in all later treatments since it gave a more easily discernible effect. Heteroarotinoids may not have as much activity as *t*-RA, and less than a

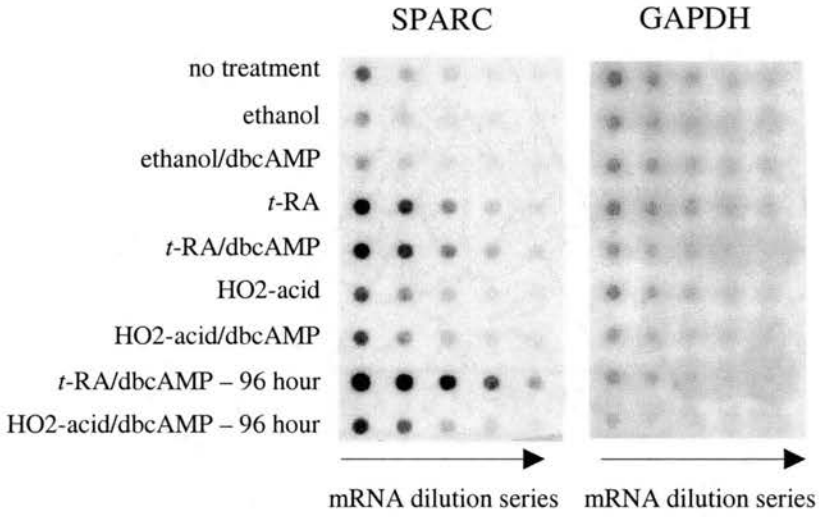


Figure 16. SPARC and GAPDH Dot Blots from Optimization Experiments.
A dot blot was produced from mRNA dilutions in which the most concentrated dot contained the mRNA isolated from ≈ 1 μg of total RNA. Dilutions were two-fold. A previous GAPDH northern dot blot (not shown) was used to determine the amount of GAPDH signal per μL mRNA solution; these concentrations were then used to produce even loading of GAPDH signal (and thus even loading of RNA) for the dot blot above. These blots were hybridized with SPARC probe (left) and then with GAPDH probe (right) at 68 °C overnight. F9 cells were treated with 5×10^{-7} *M* retinoid (*t*-RA or HO2-acid) and 1 m*M* dbcAMP as specified. Incubation time after treatment was 72 hours, except the bottom two rows, which were incubated 96 hours.

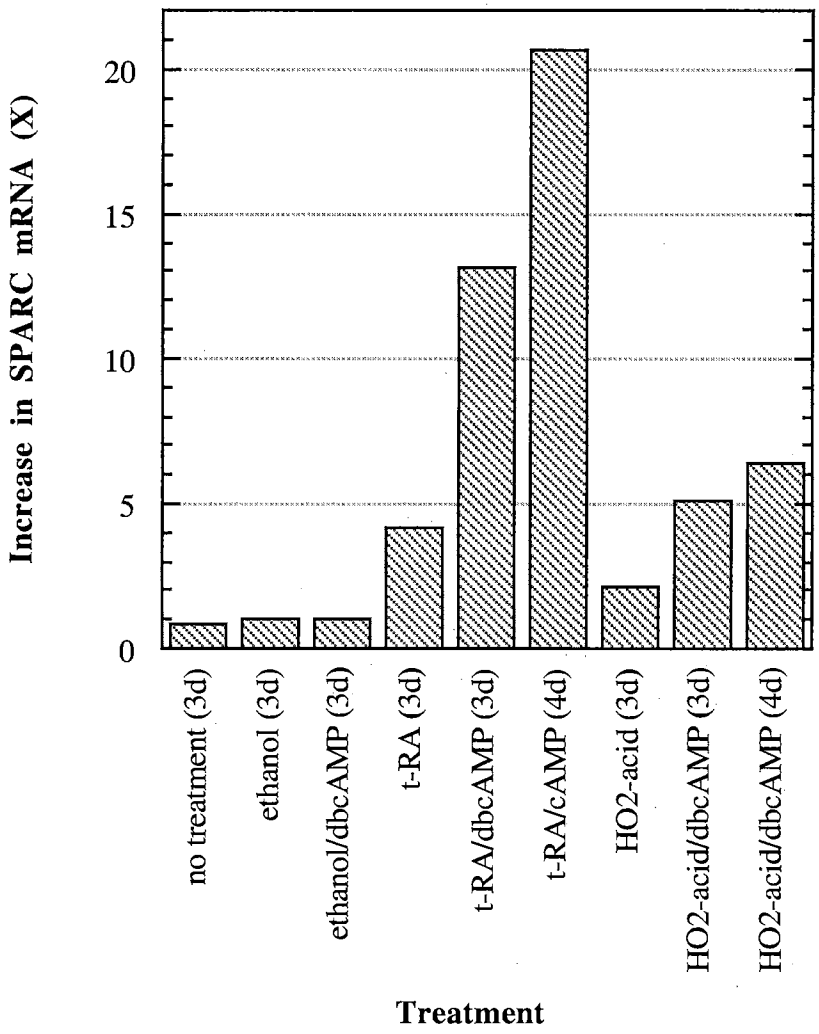


Figure 17. Effect of dbcAMP and Incubation Time on SPARC mRNA Levels with *t*-RA and HO2-acid Treatments.
Data from dot blots (Figure 16) were analyzed as described in Appendix 4. The F9 cultures (3×10^4 cells per 75 cm^2 flask) were treated with 5×10^{-7} *M* retinoid (*t*-RA or HO2-acid) and 1 m*M* dbcAMP as indicated on the x-axis. RNA was isolated at the end of 72 or 96 hours (3d or 4d, respectively). SPARC mRNA was quantitated from mRNA dot blots as described in Appendix 2.

four-fold induction would be hard to interpret. Moreover, dbcAMP does not affect SPARC mRNA levels on its own.

Effect of Treatment Length

Since different treatment lengths (3-4.5 days) were described in the literature (Mason et al. 1986, Wang et al. 1985), the effect of treatment time on SPARC mRNA levels was investigated. Seventy-two and 96-hour *t*-RA/dbcAMP and HO2-acid/dbcAMP treatments were performed with F9 cells that were subcultured and treated at the same time. A treatment period longer than 96 hours was not used, since the cells were near confluency after 96 hours. (It was feared the stress of subculturing might affect the assay adversely.)

Ninety-six hours of *t*-RA/dbcAMP treatment produced a 21-fold induction, whereas 76 hours produced only 13-fold induction (Figures 16 and 17). SPARC levels also increased with the extra 24 hours when treated with HO2-acid, although not as dramatically. The 96-hour treatment protocol was used in all assays since it produced higher levels of SPARC mRNA.

Heteroarotinoid-Induced F9 Differentiation

The ability of heteroarotinoids to induce F9 differentiation was determined by evaluating changes in cell morphology and SPARC mRNA levels when cultures were incubated 96 hours after treating with het/dbcAMP.

Morphology

Photographs of two sets of treated cells are shown in Figures 18-20. Negative control cultures (no treatment, ethanol, and dbcAMP/ethanol) consisted entirely of crowded groups of cells, with individual cells only easily recognized on the outside edges of the groups (Figure18). Cultures treated with *t*-RA/dbcAMP (positive control) featured individual cells that were larger and more spaced-out than the controls (Figure 19). All four het treatments produced a mixture of these two distinct morphology types (Figure 20, Table 8). According to the morphology, hets seem to induce differentiation in a subpopulation of the cells.

Upon close examination, two morphologies are observed in the *t*-RA/dbcAMP-treated cells. Primitive endoderm cells are large and spaced out but retain an angular, geometric shape (labeled in Figure 19). Parietal endoderm cells are like the primitive endoderm cells, except they are rounded (labeled in Figure 19). The het treatments induced primarily the primitive endoderm morphology, with very few cells exhibiting parietal endoderm morphology (labeled in Figure 20).

All cells were plated at the same density before the treatment. There are fewer cells in the differentiated cultures (cells were not counted, but it was visually obvious.) This was expected, because with differentiation comes a decrease in proliferation (Rosenstraus et al. 1982). However, differences in cell number do not account for the crowded versus well-spaced morphology. Control cultures do have plenty of unused surface area, but they remain crowded anyway; they have less contact inhibition. Morphological changes suggest increases in SPARC levels, since this protein is involved in the morphological transition (Everitt and Sage, 1992).

A third type of treatment was performed in which time exposed to retinoids was limited to 12 hours. In this treatment set, the retinoid and dbcAMP treatments were

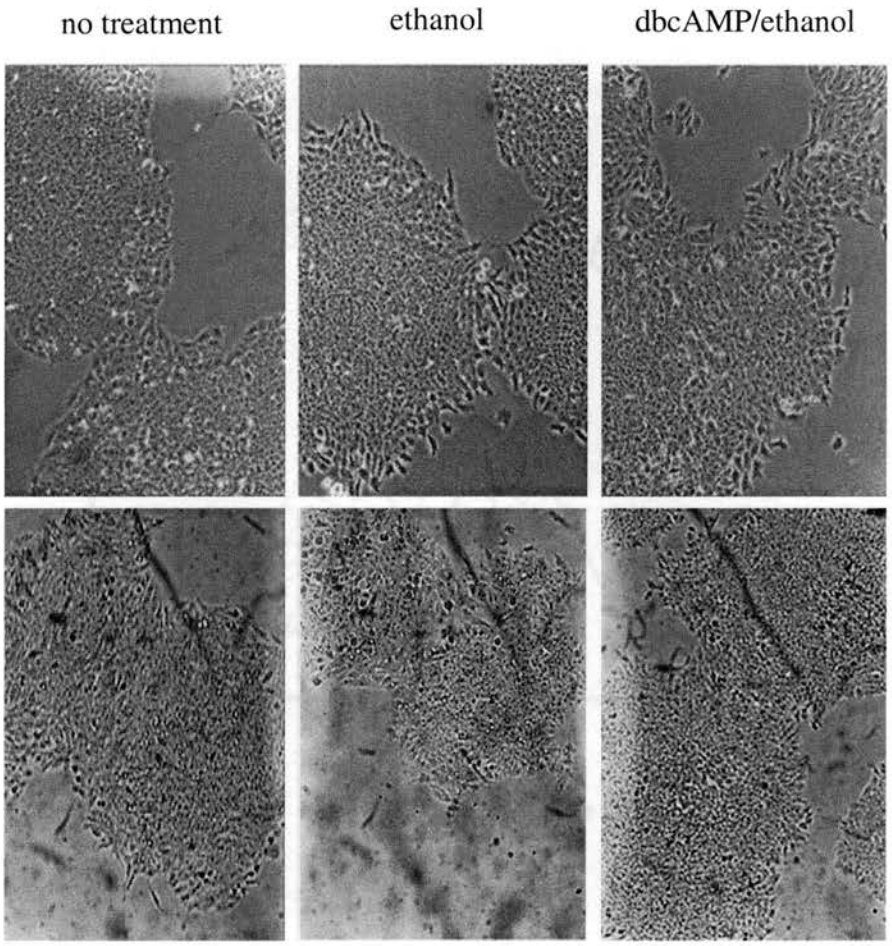


Figure 18. Negative Control F9 Cultures: Stem Cell Morphology.
Two sets of cultures incubated 96 hours after treatment (no treatment, ethanol, or dbcAMP/ethanol) are shown. Concentrations were 0.016% ethanol and 1 m*M* dbcAMP. These photographs were taken at 100× magnification and then reduced to 3/8ths their original size. The crowded morphology is typical of F9 stem cells.

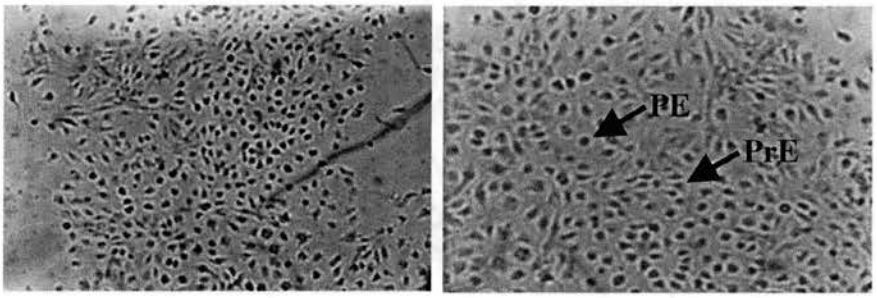


Figure 19. *t*-RA/dbcAMP-treated F9 Cultures: Differentiated Morphology.
Two sets of cultures incubated 96 hours after treatment with 0.5 μ*M* *t*-RA and 1 m*M* dbcAMP are shown. Photographs were taken at 100× magnification and then reduced to 3/8ths their original size. Cells exhibited both primitive endoderm (PrE) and parietal endoderm (PE) morphologies. (The difference in these morphologies is the polygonal shape of PrE versus the rounded shape of PE.)

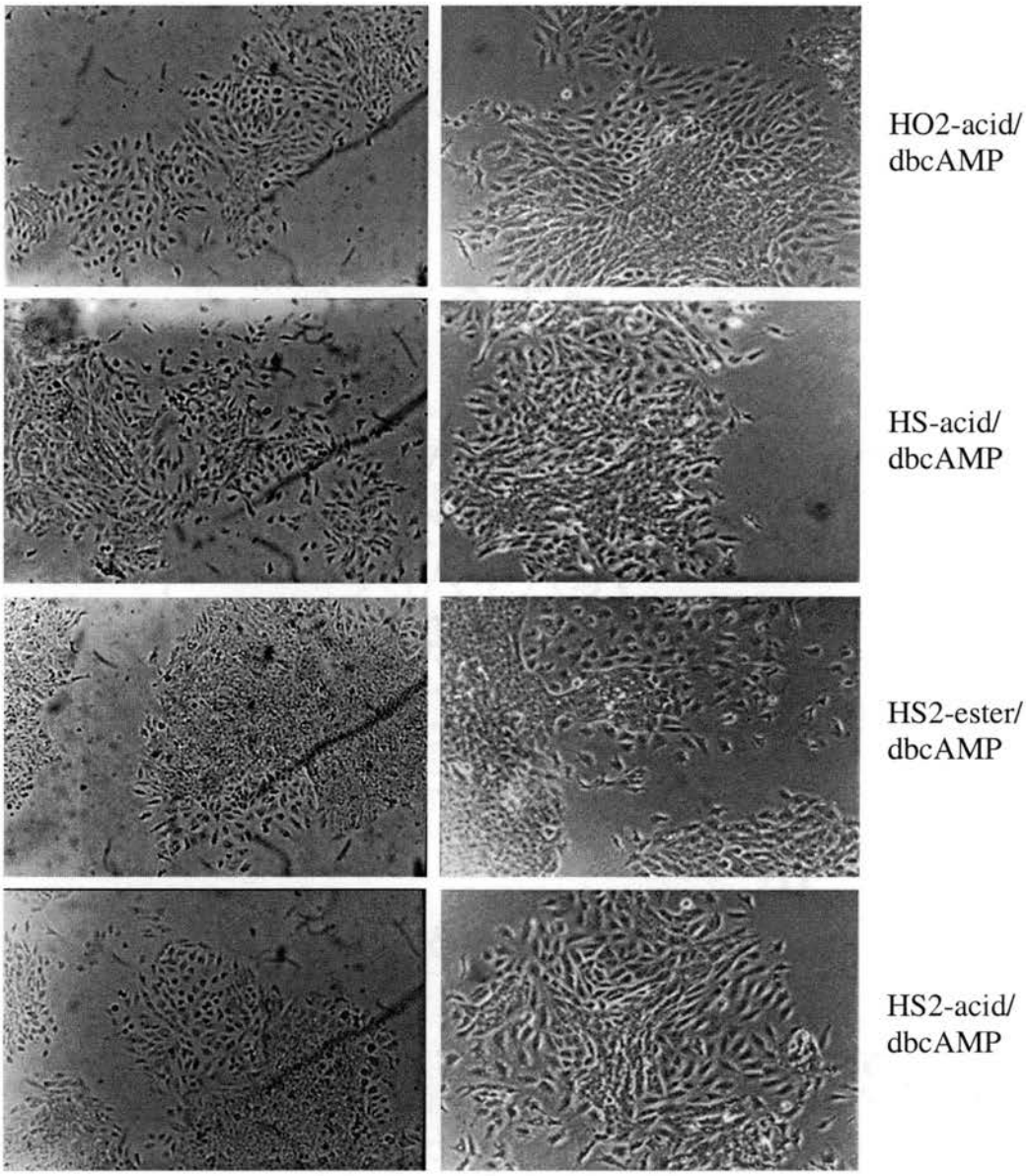


Figure 20. Heteroarotinoid/dbcAMP-treated F9 Cultures: Mixed Morphology. Two sets of cultures incubated 96 hours after treatment with 0.5 μM heteroarotinoid and 1 mM dbcAMP are shown. Photographs were taken at 100× magnification and then reduced to 3/8ths their original size. Stem cell (SC) and primitive endoderm (PrE) were the most common morphologies observed, but a few cells with the large, rounded morphology of parietal endoderm (PE) were noted.

TABLE 8

MORPHOLOGY EXHIBITED BY F9 CULTURES AFTER 96-HOUR TREATMENT

Treatment	Morphology Score
no treatment	0
ethanol	0
ethanol/dbcAMP	0
t-RA	2
HS-acid	1
HS2-acid	1
HS2-ester	1
HO2-acid	1

administered separately. Cells were incubated with retinoids for 12 hours, and then media were removed and medium with dbcAMP was added. Concentrations were the same as the standard protocol. The cells then incubated the remaining 84 hours. Photographs of these cells are shown in Figure 21.

The 12-hour retinoid treatment produced some interesting differences to the 96-hour treatment. The 12-hour *t*-RA/dbcAMP treatment (Figure 21) produced a much more mixed morphology than the 96-hour treatment (Figure 19). In fact, the 12-hour *t*-RA/dbcAMP treatment looks similar to the 96-hour heteroarotinoid treatments (Figure 20). Thus, the "partial" differentiation seen in 96-hour het-treated cultures is similar to a limited treatment with a known inducer, *t*-RA. Moreover, differentiation activity of two hets (HO2-acid and HS2-ester) was noticeable hindered by the shorter treatment. In these treatment cultures, no groups of differentiated cells were found after scanning the flask for several minutes. Two hets, HS-acid and HS2-acid produced similar morphologies with both the 12-hour and 96-hour treatments. The failure of HO2-acid and HS2-ester to induce differentiation with the shorter exposure time suggests that transport may be a limiting step or metabolism may be required.

SPARC mRNA

After the 96-hour treatment, total RNA was isolated, and a total RNA gel was run to confirm that the RNA was intact. As seen in Figure 22, ribosomal RNA bands are easily distinguishable, with the 28S bands brighter than the 18S bands. This shows the RNA was intact.

Poly A RNA was isolated from total RNA and run on a gel to show the loss of rRNA bands and establish the migration distances for known sizes of RNA (Figure 23). A

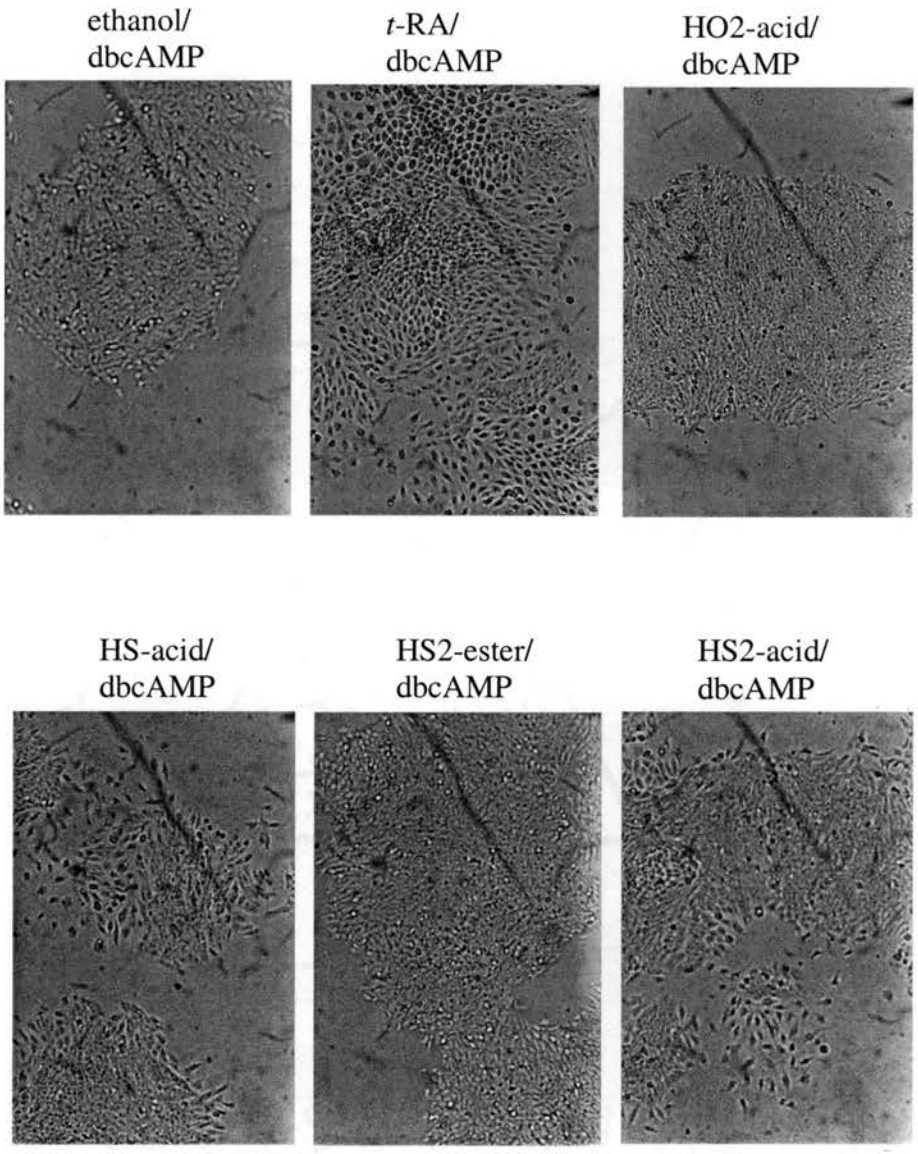


Figure 21. Twelve Hour Incubation with Retinoid/dbcAMP .
These F9 cultures were processed like the other 96-hour treatments, except that the initial retinoid treatment media (lacking dbcAMP) were removed and replaced with fresh medium (containing dbcAMP) after 12 of hours incubation. The cells were then incubated the remaining 84 hours. Concentrations of retinoids and dbcAMP were the same as the 96-hour treatments. Photographs were taken at 100× magnification and reduced to 3/8ths their original size. No groups of differentiated cells were found in HO2-acid and HS2-ester cultures. The *t*-RA-treated culture consisted of a more mixed morphology (PrE and PE) than seen when *t*-RA was not removed after 12 hours.

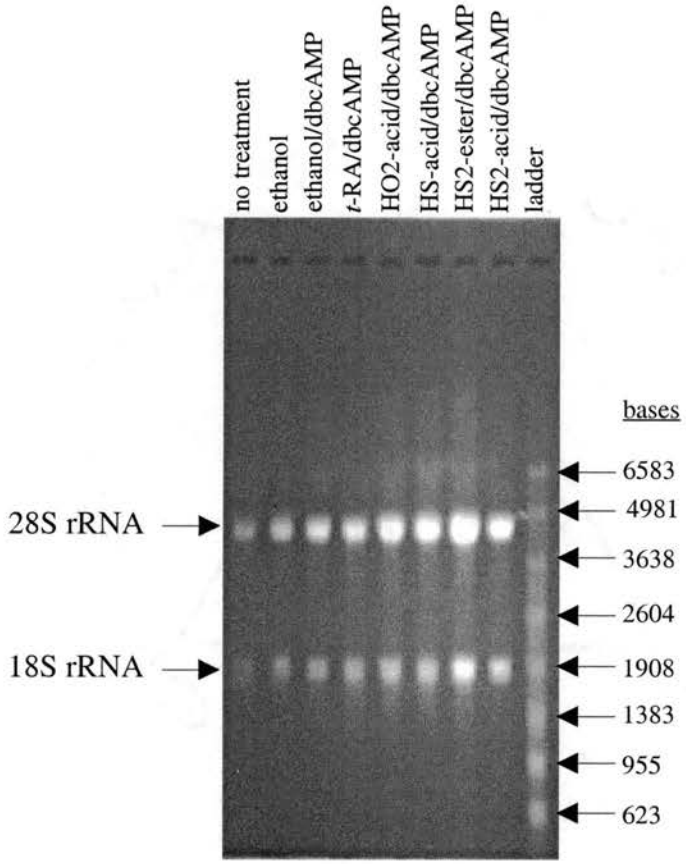


Figure 22. Total RNA Gel Showing Intact RNA.
RNA was isolated after 96 hours incubation with treatments shown at top. RNA was quantitated using absorbance at 360 nm. Three μg total RNA per lane were electrophoresed in a 1.2% agarose formaldehyde gel. The ladder is Promega's RNA marker (PRG 3191); 8 μg were loaded. This blot verifies that the RNA is intact. Since the ribosomal RNA bands are clearly visible and the 28S rRNA bands are brighter than the 18S rRNA bands, this RNA is intact. Although this gel appears to be loaded unevenly, this did not affect SPARC quantitation. SPARC qunatitation was determined from a dot blot, not a gel.

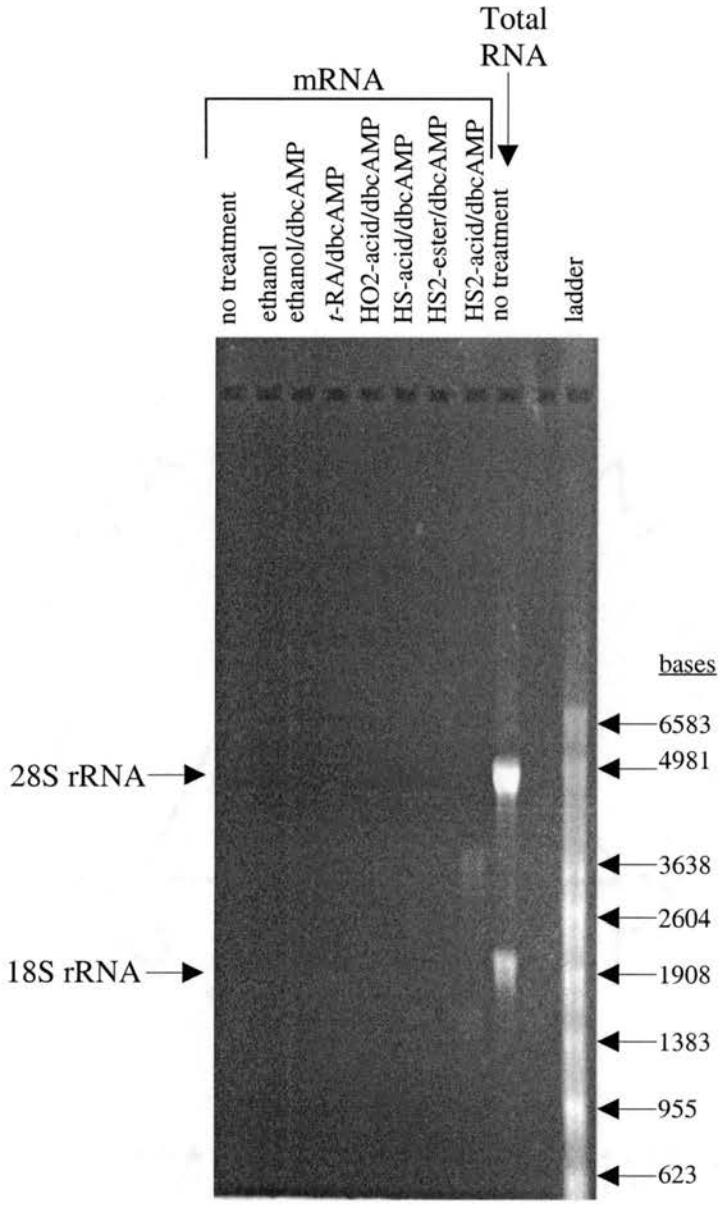


Figure 23. mRNA Gel Showing Lack of rRNA Bands.
RNA was electrophoresed in a 1.2% agarose formaldehyde gel. (Protocol for RNA gel in Appendix 2.) The mRNA loading was normalized according to a GAPDH dot blot (not shown); equal amounts of GAPDH signal were loaded in all mRNA lanes. This was equivalent to the mRNA isolated from ≈1 μg of total RNA per lane. The total RNA lane (no treatment) contains 3 μg of total RNA. The purpose of this lane was to establish the position of the rRNAs, principally for the blot made from this gel. The ladder (Promega, RNA marker) was used to establish sizes on the blot made from this gel. No rRNA was apparent in the mRNA lanes in this gel. However, the lack of rRNA signal on a blot (made from this gel) probed for SPARC (Figure 24) was required to show that rRNA would not interfere with SPARC mRNA quantitation on a dot blot.

blot was made from this gel and probed for SPARC (Figure 24). The SPARC probe proved to be specific, indicating a size of 2.10 to 2.25 kb. (SPARC mRNA's actual size is 2.1 kb.)

A dot blot was made and probed with both GAPDH and SPARC probes (Figure 25). Induction of SPARC was calculated as described in Appendix 4. SPARC mRNA levels were induced in all retinoid-treated cultures (Figure 26). The *t*-RA/dbcAMP-treated cells produced the highest levels of SPARC mRNA, and the negative controls produced the lowest levels. All four het/dbcAMP treatments produced SPARC mRNA levels higher than negative controls, but lower than *t*-RA/dbcAMP.

F9 differentiation depends primarily on RARγ activity (Boylan 1993, Taneja et al. 1996, Chiba et al. 1997); however, RARβ activity is required for later stages of differentiaton and growth arrest (Faria et al. 1999). Heteroarotinoids HS-acid, HO2-acid, and HS2-ester have exhibited RARγ and RARβ activity with transfected receptors and a reporter protein (Benbrook et al. 1997b). Although transactivation activity has not been determined for HS2-acid, the activity seen for HS2-ester may actually be due to its acid form, since esterases are considered ubiquitous. For these reasons, HS2-acid probably has transactivation activity, like the other three hets studied here. Therefore, it is not surprizing that the four hets induce F9 differentiation.

The importance of het structural features to differentiation - such as one versus two aryl rings - was not observed in this experiment. Rather, the four hets produced similar degrees of differentiation. No het lacked activity, and no het had activity equal to *t*-RA. This may be because the necessary transactivation activities (RARβ and RARγ) are similar for the hets. (RXR activities are not similar, but RXR ligand is not required for F9 differentiation.)

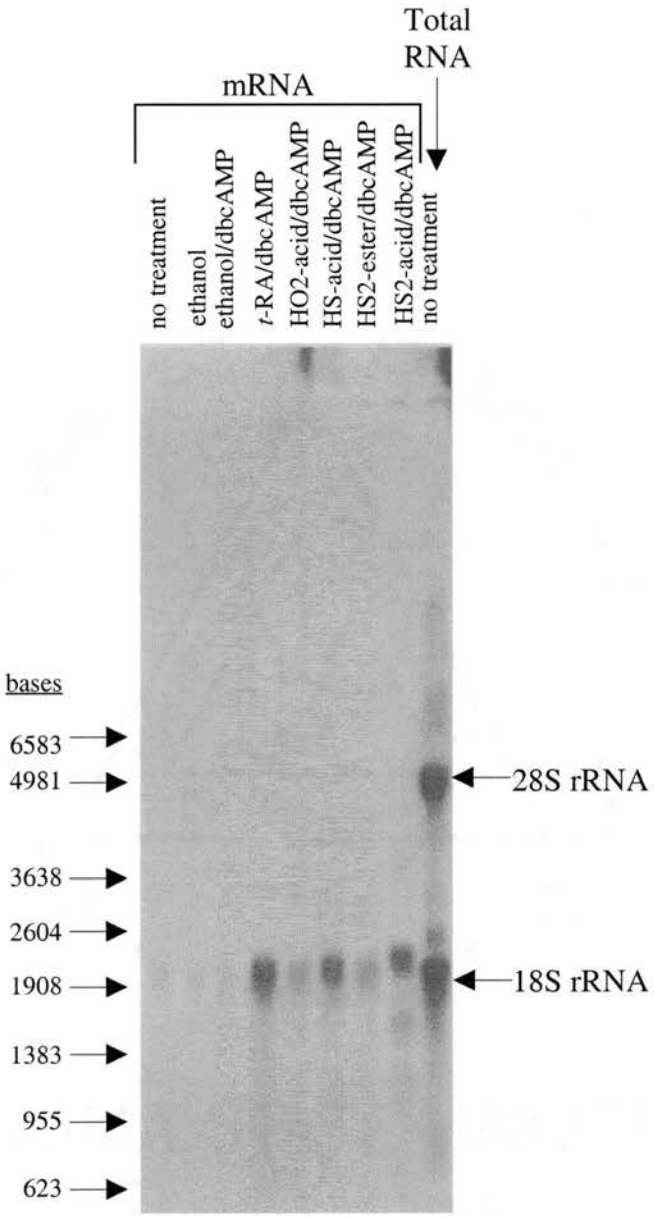


Figure 24. SPARC Northern from mRNA Gel.
This blot was created from the RNA gel shown in Figure 17. RNA was transferred to a nylon membrane, crosslinked to membrane and then hybridized at 68 °C with digoxigenin-labeled SPARC probe. (Protocols are in Appendix 2.) Ribosomal RNA does not appear in mRNA lanes. The SPARC probe is specific for a band that is 2.10-2.25 kb; the actual size of SPARC mRNA is 2.1 kb.

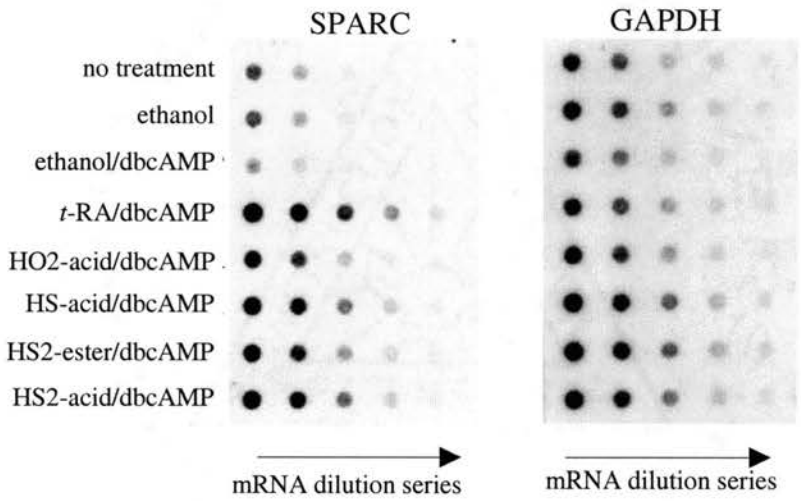


Figure 25. SPARC and GAPDH mRNA Dot Blots Showing SPARC Induction.
A dot blot was produced from mRNA dilutions in which the most concentrated dot contained the mRNA isolated from ≈ 1 μg of total RNA. Dilutions were two-fold. A previous GAPDH northern dot blot (not shown) was used to determine the amount of GAPDH signal per μL of mRNA solution; these concentrations were then used to produce even loading of GAPDH signal (and thus even loading of RNA) for the dot blot above. These blots were hybridized with SPARC probe (left) and then with GAPDH probe (right) at 68 °C overnight. The *t*-RA/dbcAMP treatment produced the highest levels of SPARC, the heteroarotinoid/dbcAMP treatments produced higher levels than the negative controls (no treatment, ethanol, and ethanol dbcAMP).

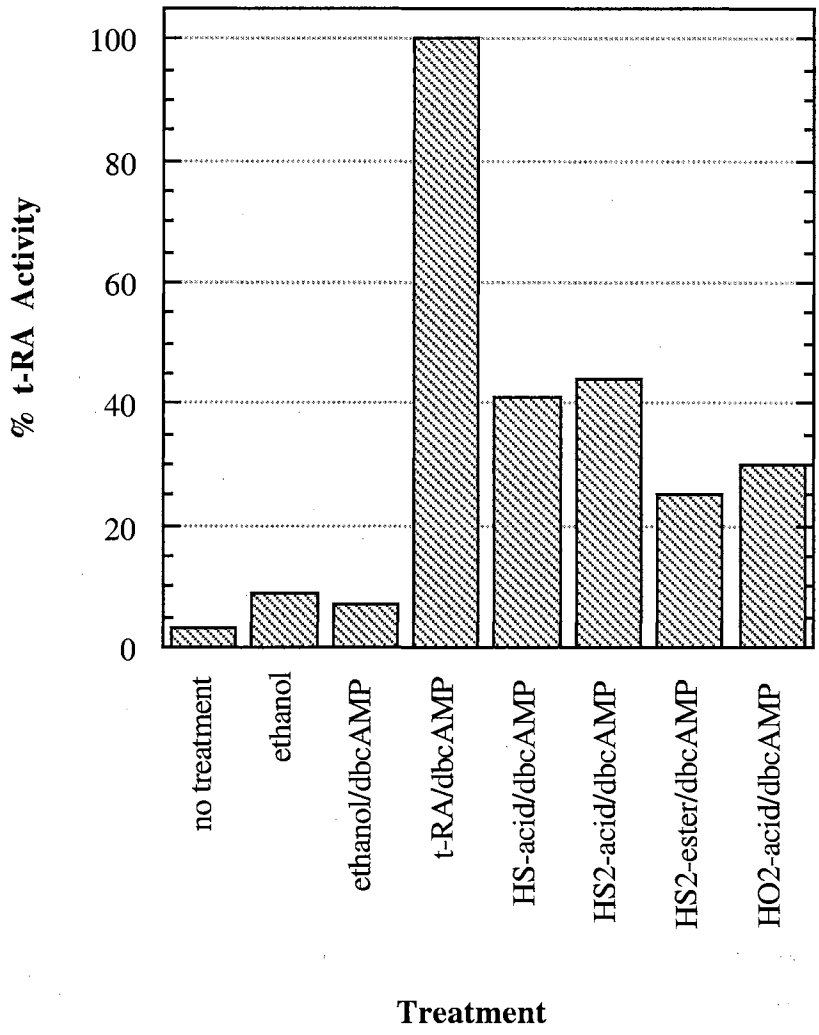


Figure 26. SPARC Levels After Various Treatments Relative to *t*-RA-Induced Levels. The data from dot blots shown in Figure 25 were analyzed as explained in Appendix 4. Briefly, linear plots were made for each dilution series (each treatment) for both SPARC and GAPDH northerns. For each treatment, the slope of its SPARC line was divided by the slope of its GAPDH line, thus normalizing SPARC to the amount of RNA present. This SPARC/GAPDH value was then normalized to that of *t*-RA/dbcAMP (the positive control).

SPARC mRNA is not induced directly by retinoids; a complex set of events precede its up-regulation. The exact receptor roles and sequence of differentiation events are not fully understood, but, due to the delay of SPARC induction after treatment, more than one event must occur first. SPARC is induced late in differentiation (Wang et al. 1985). For this reason, induction of SPARC is not merely another measure of transactivation, but a complex retinoid activity.

In conclusion, the four heteroarotinoids evaluated (HO-acid, HS-acid, HS2-ester, and HS2-acid) produced differentiated cell morphology, mainly primitive endoderm with some parietal endoderm morphology. These heteroarotinoids also induced increased levels of SPARC mRNA, a parietal endoderm-specific product. Therefore, these heteroarotinoids were capable of inducing differentiation in F9 cells.

CHAPTER 3

F9 HETEROAROTINOID METABOLISM

Introduction:

Heteroarotinoid activity in F9 cell culture depends on receptor binding and metabolism. Since metabolism may modify het concentrations during incubation, the metabolism of selected hets in F9 culture was studied.

All-*trans*-retinoic acid is readily taken up, metabolized, and excreted in F9 cells (Napoli et al. 1981, Napoli et al. 1982, Kalin et al. 1984, Gubler et al. 1985). It was desired to compare how quickly the heteroarotinoids were converted compared to *t*-RA. Slower metabolism might increase activity. All four hets (HO2-acid, HS-acid, HS2-ester and HS2-acid) in the differentiation study were quantitated at the end of the 96-hour differentiation incubation.

Some synthetic retinoids may exert their activity by inhibiting the metabolism of *t*-RA. Although retinoid levels in the medium ($\approx 10^{-8}$ *M* retinol) were insufficient to induce differentiation (as seen by negative controls in Chapter 2) active retinoids may accumulate in the cells if retinoid metabolism is inhibited.

The purpose of the metabolism studies was to: 1) determine how much of the treatment retinoid remains at the end of a 96-hour incubation and 2) evaluate *t*-RA metabolism in the presence and absence of hets (HS-acid, HO2-acid).

Experimental Procedures

Materials

Retinyl acetate was purchased from Sigma (#R-4632). Heteroarotinoids were provided by Dr. K. Darrell Berlin, Department of Chemistry, Oklahoma State University. Chromotography solvents used were methanol (OPTIMA, Fisher #A454), water (HPLC grade, Fisher #W5) and acetic acid (glacial, Fisher #A507).

Medium Extraction

The protocol for medium extraction is in Appendix 1 and was adapted from Nelson et al. (1965). Briefly, 25.0 or 50.0 μL of retinyl acetate internal standard (2.1×10^{-4} *M*, in ethanol) was added to a known quantity of medium to be extracted. One volume ethanol (95%) was added, and the pH was adjusted to 2 with 3 *M* HCl. One volume of hexane was added, and the tube was shaken by hand and then placed on ice for 20 minutes. The hexane layer was removed, and the aqueous layer was extracted a second time with one volume of hexane. The two hexane layers were combined and dried under nitrogen. The samples were stored dry at -20 °C.

High Performance Liquid Chromatography (HPLC)

HPLC (reverse-phase) analysis was performed using Waters Model 6000A pumps, a Waters 996 photodiode array detector and a Whatman Partisil 5 ODS-3 (0.47×23.5 cm, with 5 μm particles) Column. Methanol and 0.010 *M* acetic acid (prepared from glacial

acetic acid and HPLC grade water) were used as solvents. Data were analyzed using Waters' Millenium Chromatography Manager Software version 2.1.

Concentration Calculations

Each medium sample was spiked with a known amount of retinyl acetate (RAc) prior to extraction. Absorbance peaks for the compound being quantitated and RAc were determined on HPLC using a photodiode array detector. Peak areas were calculated using Millinium software. Standards for *t*-RA, HO2-acid, HS-acid, HS2-ester, HS2-acid and RAc were chromatographed and standard curves (area versus molarity) were plotted.

Concentrations of retinoids in medium samples were calculated as follows: Using the linear equation from the RAc standard plot, RAc area yields *M* RAc injected into the HPLC. The fraction of RAc injected (compared to amount spiked into medium sample) was then calculated. Using the unknown retinoid's peak area and its standard curve, the *M* of the injected sample was calculated. The concentration of the unknown retinoid in the original medium was calculated:

$$M \text{ of retinoid in medium} = \frac{(\text{volume injected})\,(M \text{ of retinoid in injected sample})}{(\text{volume of medium extracted})\,(\text{fraction of RAc injected})}$$

Example data and calculations are given in Appendix 4.

Disappearance of Retinoids During Differentiation Assay

F9 cell cultures were treated with 5×10^{-7} *M* *t*-RA, HO2-acid, HS-acid, HS2-ester and HS2-acid; see "F9 Retinoid Treatment (Standard)" in Chapter 2 Methods. Flasks containing the same medium, but no F9 cells were also treated ("no cell" flasks). After 96 hours, media and cells were collected. Cells were analyzed for differentiation, and medium was analyzed for remaining heteroarotinoids. Two mL of medium were used for each extraction. For each medium sample analyzed, three extractions were performed. Each replicate extract sample was chromatographed separately, so three concentration determinations were made for each medium sample.

Effect of Heteroarotinoids on *t*-RA Metabolism

The F9 cells were plated at 2.5×10^5 cells per 75 cm^2 with medium containing 1 μM *t*-RA. Twenty-four hours after *t*-RA pretreatment, medium was removed, and cells were rinsed twice with room temperature phosphate buffered saline (PBS) (Sigma #1000-3). A total of 25 mL of treatment media were then added to the cultures (at time = 0 hours). (See Table 9 for volumes of each medium used. Table 10 summarizes experiment design, showing t = 0 concentrations of components.) A sample of each treatment medium (that never had cell contact) was reserved; these treatment media were used to verify initial concentrations of retinoids in the treatments (at 0 hours). At 8 and 16 hours 2.5 mL of medium was removed from each flask and placed at –20 °C. At 24 hours all remaining media was removed and placed at –20 °C. Between 2.0 and 2.5 mL of (exact volume noted) medium was extracted for each concentration determination. Initial treatment media (t = 0) and 24-hour media (t = 24) were extracted in triplicate, and the resulting concentration values were averaged.

TABLE 9

VOLUME MEDIA (mL) ADDED AT TREATMENT TIME

Flask	mL			
	2.5 μ*M* *t*-RA-Medium	2.5 μ*M* HO2-acid-Medium	2.5 μ*M* HS-acid-Medium	0.125% ethanol-medium
t-RA/no cell	5			5
HO2-acid/no cell		5		5
HS-acid/no cell			5	5
ethanol				10
t-RA	5			5
t-RA/HO2-acid	5	5		
t-RA/HS-acid	5		5	
HO2-acid		5		5
HS-acid			5	5

Each flask also had 15 mL of F9 medium (the standard medium described in Chapter 2 Methods, under Cell Culture). Media listed above are made from the standard medium, with the additive indicated.

All retinoid-medium solutions contain 0.125% ethanol.

TABLE 10

INITIAL μ*M* CONCENTRATIONS OF COMPONETS IN F9 CELL CULTURE

		μ*M*		
Flask	Cells*	*t*-RA	HO2-acid	HS-acid
t-RA/no cell	None	0.5		
HO2-acid/no cell	None		0.5	
HS-acid/no cell	None			0.5
ethanol	Yes			
t-RA	Yes	0.5		
t-RA/HO2-acid	Yes	0.5	0.5	
t-RA/HS-acid	Yes	0.5		0.5
HO2-acid	Yes		0.5	
HS-acid	Yes			0.5

*Cells were plated at a density of 2.5×10^5 cells per 75 cm^2 flask 24 hours before the above treatment.

Results and Discussion

Disappearance of Retinoids During Differentiation Assay

The retinoid treatment concentrations were slightly higher than intended. All four retinoids were used at 0.6 μM, instead of the intended 0.5 μM. This does not affect the differentiation assay, since this concentration is below the *t*-RA toxicity limit of 1 μM.

The disappearance of retinoids during the differentiation assay was evaluated for all four hets (HO2-acid, HS-acid, HS2-ester and HS2-acid). In this section, it is important to distinguish between the two ways retinoids may be lost during the differentiation assay:

"Instability" or "stability" will be used to refer to how well a compound remains intact *exclusive* of cell action.

"Metabolism" will be used to refer to cell actions on the compounds.

"Disappearance of retinoid" will refer to the sum of stability and metabolic effects.

Disappearance of Retinoids with No Cells Present *(Instability)*

Retinoids were added to medium and placed in flasks without cells. These flasks were subjected to the same 96-hour incubation as cultures were. After incubation, medium was analyzed for treatment compounds. The purpose of these flasks was to determine retinoid stability during the treatment protocol exclusive of cell metabolism. By comparing retinoid stability in "no cell" flasks to results when cells were present, retinoid metabolism was evaluated.

All diaryl heteroarotinoids (HO2-acid, HS2-ester and HS2-acid) were more stable than HS-acid (Figure 27). HS-acid and HS2-acid are structurally equivalent except for the second ring, and yet their stabilities are different. Only 56% of HS-acid remained after 96 hours, and 90% of HS2-acid remained. Stability appears to be associated with the presence of the second ring in diaryls.

Disappearance of Retinoids with F9 Cells Present *(Instability + Metabolism)*

How much parent retinoid remains at the end of the differentiation assay? This is an important question, because the real "incubation period" may or may not be the same for all retinoids tested, since some may be quickly converted. Medium was collected at the end of the differentiation assay and analyzed for remaining retinoids. Both compound instability and cell metabolism contribute to retinoid loss during the 96-hour incubation.

There was a wide variation in retinoid loss during the incubation (Figure 28). Two retinoids, *t*-RA and HS-acid, were undetectable after the 96-hour incubation. About two thirds of HS2-ester remained, and nearly all of HS2-acid and HO2-acid remained. These differences follow trends in structure summarized in Figure 29.

HS-acid has the closest structure to *t*-RA of all heteroarotinoids studied. It lacks the second ring of the others (diaryls), and also has a terminal carboxyl group. Similar to *t*-RA, HS-acid was not detectable in the medium after 96 hours in F9 culture. (The detection limit was approximately 0.05 μM.)

The diaryl acids (HO2-acid and HS2-acid) were more stable, with nearly all of these compounds remaining at the incubation conclusion. HS2-acid and HO2-acid have a very different size and shape than *t*-RA and HS-acid – due to the second ring – and must not be vulnerable to the same degradation as are the straight chain retinoids.

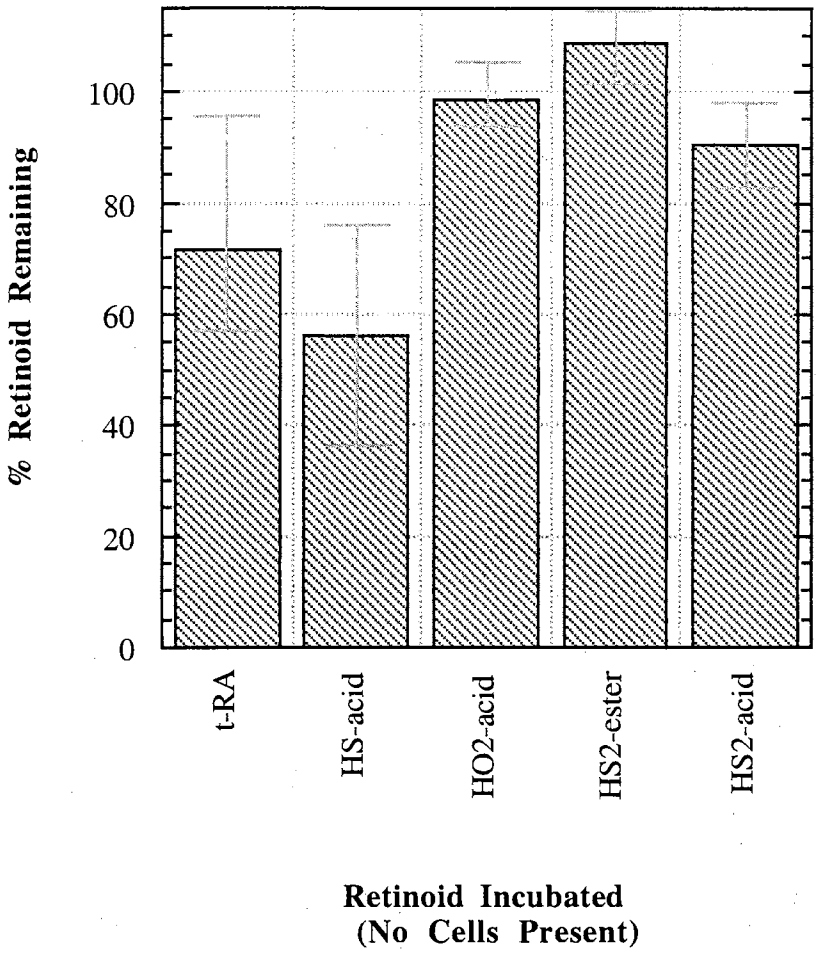


Figure 27. Percent Retinoid Remaining After 96-Hour Differentiation Assay in the Absence of F9 Cells.
F9 cell cultures were treated with 5×10^{-7} *M* retinoid as described in Methods, Chapter 2. After 96 hours of incubation, media were removed from flasks and frozen until they could be extracted. The protocol for the hexane extraction is given in Appendix 2. The percent retinoid remaining was determined using an initial concentration for the retinoid as determined by performing the same extraction protocol on media mixed at treatment time. For each media sample, three separate extractions and concentration determinations were performed.

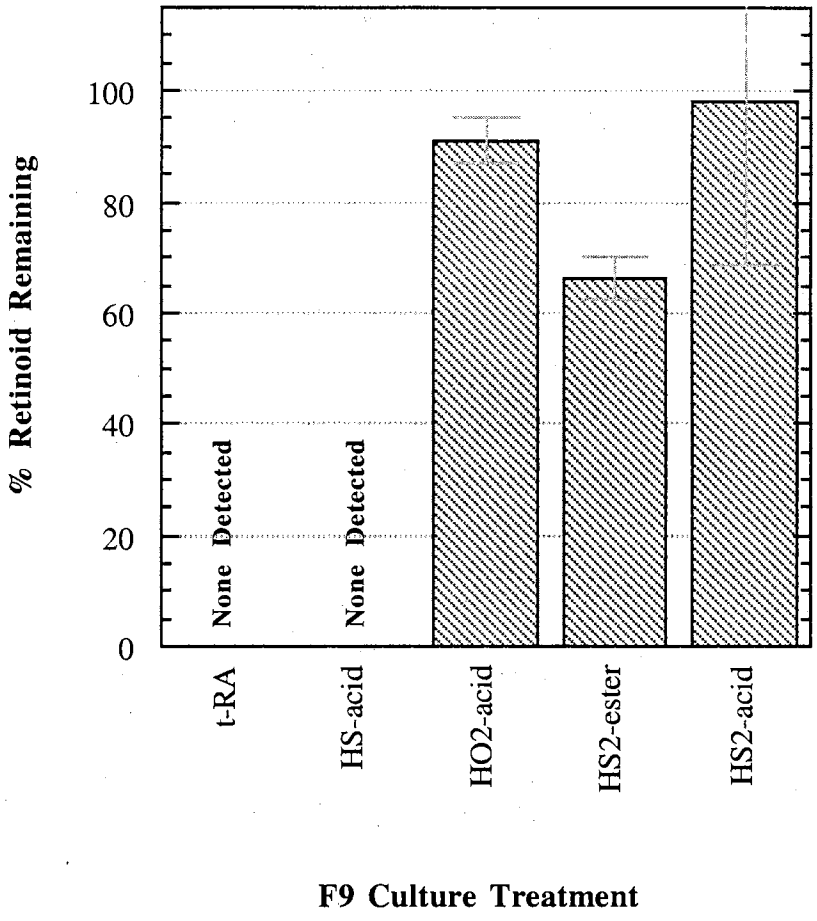


Figure 28. Percent Retinoid Remaining After 96-Hour Differentiation Assay with F9 Cells.
F9 cultures (1.3×10^4 cells per 75 cm^2 flask) were treated with 5×10^{-7} M retinoid 24 hours after subculturing. (See standard retinoid treatment in Methods, Chapter 2.) After incubating 96 hours, media were removed for analysis. Three 2.0 mL samples of each medium was extracted and chromatographed on HPLC to determine retinoid concentration.

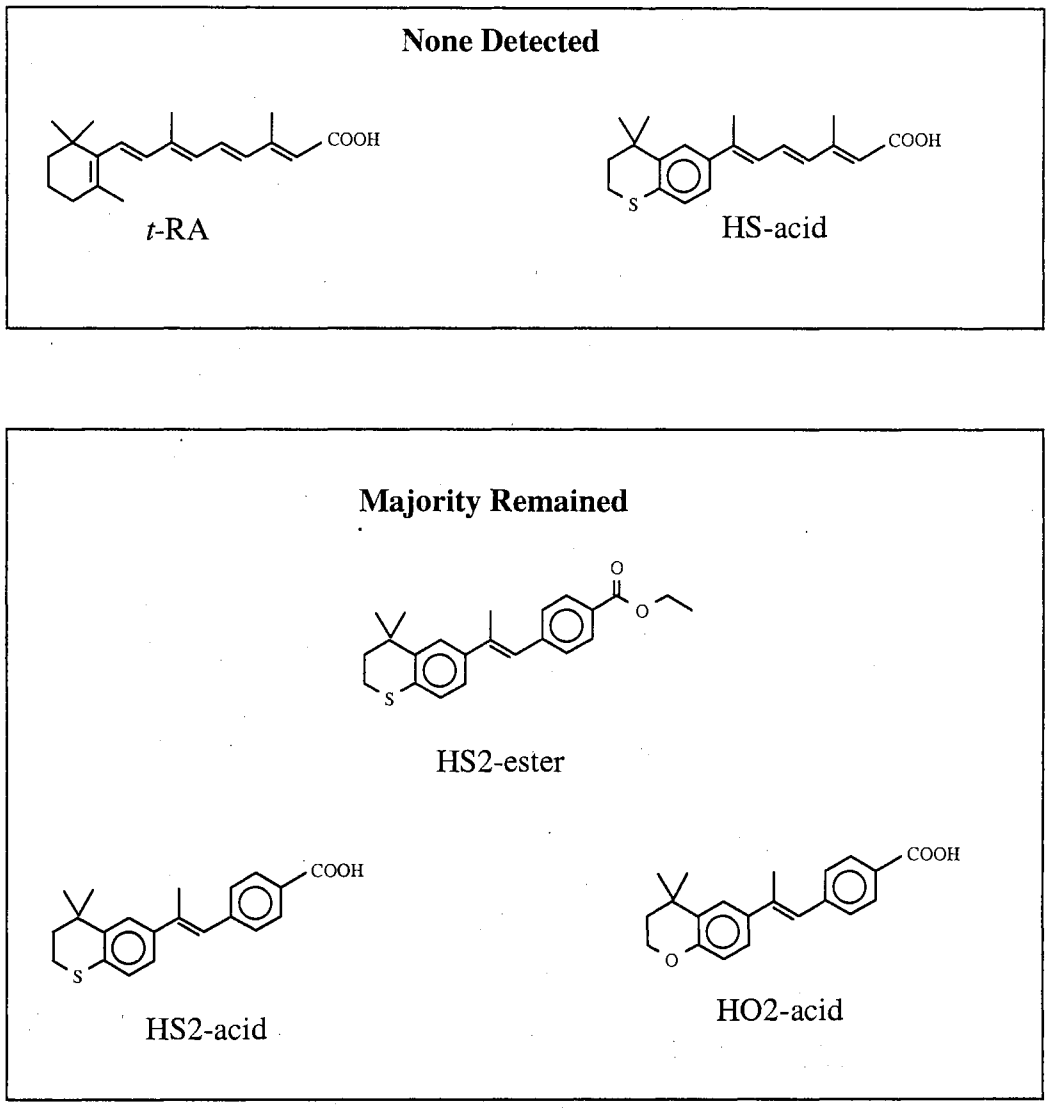


Figure 29. Stability of Retinoid During Treatment Correlates with Structure.
The amount of retinoid present at the end of the 96-hour differentiation incubation was higher for diaryls. The only monoaryl tested, whose structure resembled *t*-RA, was not detectable.

For this differentiation assay, a retinoid's persistence to the end of the incubation does not have a profound effect on its ability to induce differentiation. Retinoic acid, the strongest inducer of differentiation, was among the most quickly degraded retinoids tested. Also, the retinoids with the highest end concentrations, HO2-acid and HS2-acid, were not better inducers. This suggests that a retinoid's presence over several days does not have an overriding effect on its F9 differentiating activity in this assay.

Even though the complete differentiation process takes three to four days, only 3-8 hours exposure to *t*-RA is needed to initiate this process (Alonso et al. 1991, Dong et al. 1990). Therefore, a retinoid does not have to persist the entire 96 hours to be active in the differentiation assay. Yet, the persistence of a retinoid for the 96-hour incubation may contribute some to its activity.

Metabolism of Retinoids (Loss w/ cells – Loss w/o cells)

By comparing the results from flasks with and without F9 cells, one can determine if the cells appear to play a role in the disappearance of the retinoid. (See Figures 27 and 28.) F9 cells metabolize *t*-RA, HS-acid, and HS2-ester. In these cases, the loss of the retinoid is significantly enhanced by the presence of cells. For heteroarotinoids HS2-acid and HO2-acid, the presence of cells does not alter the loss of retinoid significantly, so metabolism must be poor.

If diaryls are not readily metabolized by F9 cells during the differentiation assay, then why is there a substantial (one-third) loss of HS2-ester after 96 hours incubation? Does this contradict the trend of diaryls being more enduring? The one structural feature that places HS2-ester in a different category than the other two diaryls is the presence of the ethyl ester group. In fact, this is the *only* difference between HS2-ester and HS2-acid, yet

the ester is metabolized and the acid is not. Therefore, the ester group must play a role in its metabolism; ester hydrolysis was investigated.

HS2-ester Hydrolysis

It was suspected that HS2-ester would be hydrolyzed by F9 cells (and other cell types) to form HS2-acid, but it had not been confirmed. In looking at Figures 27 and 28, it is clear that HS2-ester is converted by some action of the cells, for it was stable in the cells' absence. On HPLC chromatograms, a peak with the same retention time and λ_{max} as HS2-acid appeared in HS2-ester samples when cells had been present, but was absent in the "no cell" HS2-ester extracts (Figure 30). The area of this peak was consistent with the conversion of HS2-ester to HS2-acid. It was big enough to account for most HS2-ester disappearance, but not so big as to be stoichiometrically impossible. Finally, hydrolysis of the ester satisfies the conclusion above that the ester group must play a role in HS2-ester's metabolism. It was concluded that this peak was indeed HS2-acid, and calculations were performed to determine its concentration (Figure 31).

Almost all HS2-ester was accounted for by HS2-ester (66±4%) and HS2-acid (24±4%), indicating that HS2-acid is the major metabolite of the ester. Further metabolism of HS2-acid is not likely to be significant in this assay, since (as shown in Figures 27 and 28) it has already been established that in the 96-hour period little HS2-acid is metabolized.

Effect of Heteroarotinoids on *t*-RA Metabolism

In these metabolism studies, the rate at which *t*-RA was metabolized was evaluated in the presence and absence of a het (HO2-acid or HS-acid). After *t*-RA pre-treatment,

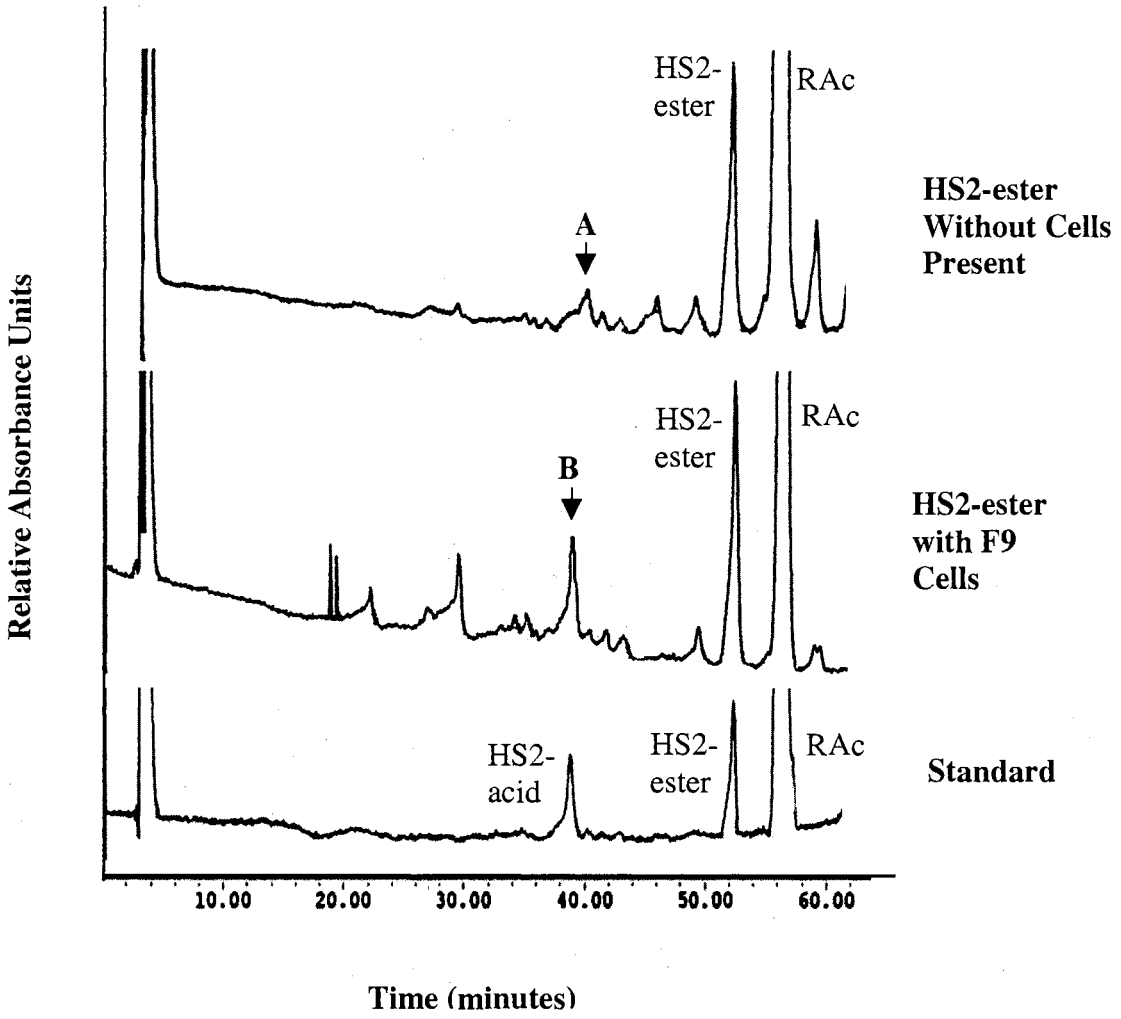


Figure 30. HPLC Analysis of Media After HS2-ester Treatment With and Without F9 Cells.

Media from two 96-hour differentiation flasks were analyzed; both flasks were treated with HS2-ester, but only one contained F9 cells. Media was extracted and HPLC analysis was performed (see Methods) using the following solvent program: 50% 0.1 M acetic acid (HAc):50% methanol was held for 5 minutes. Then concentrations were adjusted linearly to 25% HAc: 75% methanol at 15 minutes; finally, concentrations were adjusted linearly to 100% methanol at 70 minutes (chromatograms above were truncated). The chromatograms shown are at 328 nm. In the standard (bottom), HS2-acid has a λ_{max} of 323 nm. The spectrum of peak A (top) revealed a λ_{max} of 347 nm. This confirms that there is no HS2-acid formed without cells present. Peak B (middle) has a λ_{max} of 323 nm and has a retention time that corresponds to HS2-acid, indicating that F9 cells metabolize HS2-ester to HS2-acid.

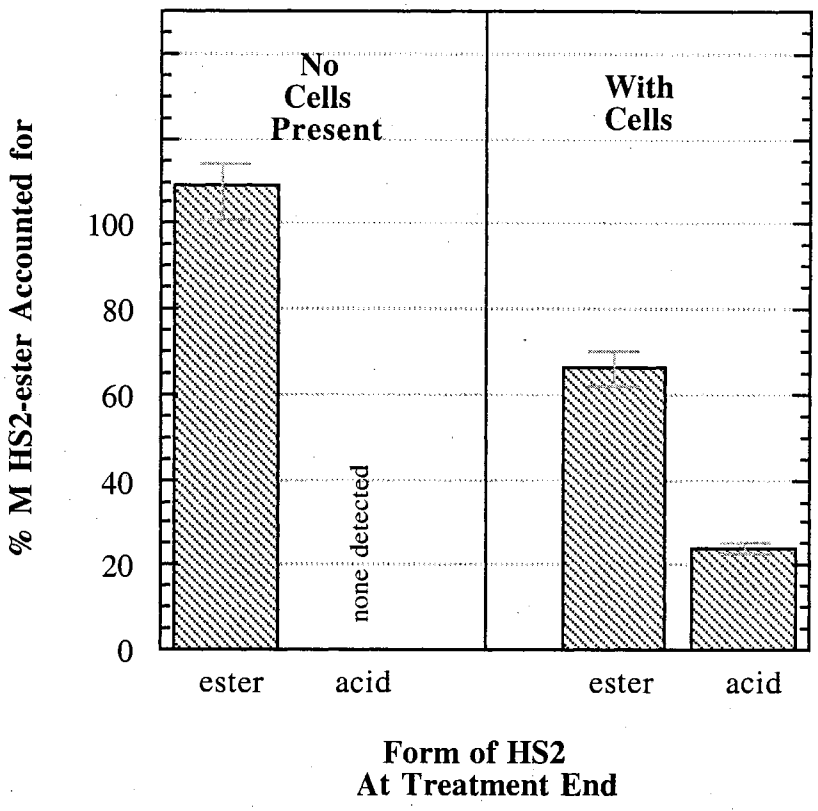


Figure 31. HS2-ester and HS2–acid Present 96 Hours After HS2-ester Treatment. F9 cell cultures were treated with 5×10^{-7} *M* HS2-ester 24 hours after subculturing at a density of 1.3×10^4 cells per 75 cm^2 flask. After incubating 96 hours, medium was removed, extracted and analyzed by HPLC. Chromatograms are shown in Figure 30. HS2-acid was present in the flask containing cells, but not when cells were absent, indicating that the F9 cells were responsible for the ester hydrolysis.

t-RA was added alone, with HO2-acid or with HS-acid. The other two heteroarotinoids, HS2-ester and HS2-acid, were not evaluated in this assay; they were not available at the time this experiment was performed. Another flask was prepared with the same medium and *t*-RA, but without F9 cells. Samples were taken from the flasks over the next 24 hours. The purpose for this experiment was to determine if the presence of either het affected the rate at which *t*-RA was metabolized by the cells.

Two major differences in these metabolism studies and the 96-hour differentiation metabolism studies. First, a greater number of cells per flask (2.5×10^5 instead of 1.3×10^4) was used in these cultures. This greatly increases the rate at which retinoid is converted. Secondly, all cells were pre-treated with *t*-RA for 24 hours before adding the treatment retinoids. This induces *t*-RA metabolism, to prevent a lag time before metabolism began. The pre-treatment with *t*-RA may or may not affect the metabolism of the hets; this was not determined.

The results of the media extraction and HPLC analysis are shown in Figure 32. (As an example, raw data and standard curves for *t*-RA calculations are shown in Appendix 4.) The *t*-RA is not completely stable in the media during incubation even without the presence of cells. Yet, with F9 cells present *t*-RA disappeared more rapidly. Clearly, the F9 cells did metabolize *t*-RA in this short time. The presence of neither HO2-acid nor HS-acid affected *t*-RA metabolism in this study.

The inability of HO2-acid and HS-acid to affect *t*-RA metabolism in this assay, suggests that their F9 differentiation activity is due to their intrinsic retinoid activity, not to an ability to alter *t*-RA metabolism. These compounds probably do not bind CRABP-I more efficiently than *t*-RA; this protein plays a central role in *t*-RA degradation (Boylan and Gudas 1992). If HO2-acid and HS-acid do share some metabolic enzymes with *t*-RA, then they must be less desirable substrates than *t*-RA.

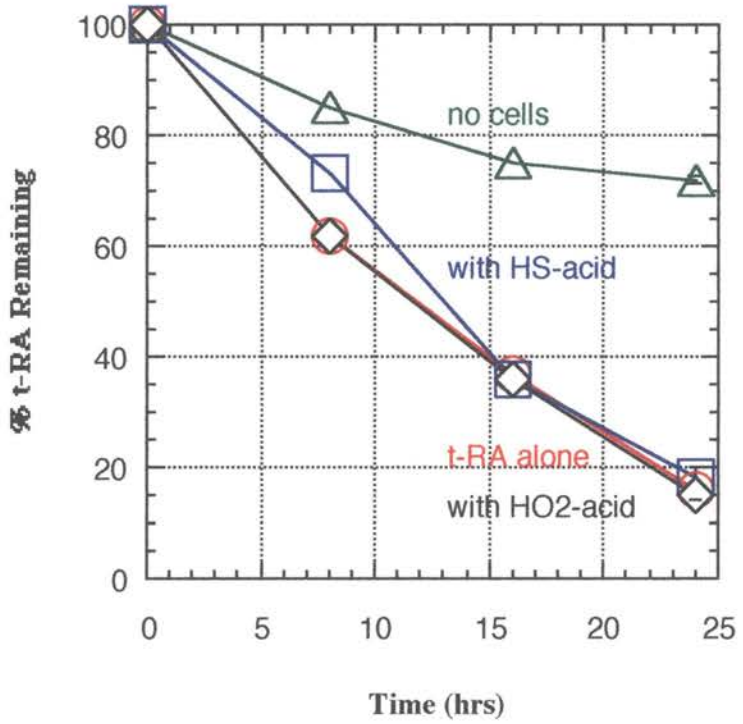


Figure 32. Effect of F9 Cells, HO2-acid and HS-acid on *t*-RA Concentration. Medium containing 0.5 μ*M* *t*-RAwas incubated with F9 cultures at 37 °C with 10% CO_2 by itself *t*-RA alone (○), with HO2-acid (◇) and with HS-acid (□) also present. Additionally, media containing 0.5 μ*M* HS-acid was incubated in the absence of cells (△). F9 cell cultures were plated at 2.5×10^5 cells per 75 cm^2 flask and pretreated with 1.0 μ*M* *t*-RA to induce retinoid metabolism. In this study, the presence of heteroarotinoids HO2-acid and HS-acid did not affect the at which rate *t*-RA was metabolized.

In addition to monitoring *t*-RA in this experiment, the concentrations of the heteroarotinoids were also analyzed. HO2-acid showed evidence of being metabolized by the cells (Figure 33), but was its metabolism was unchanged by *t*-RA presence. HS-acid was quickly metabolized by the cells (Figure 34), as seen in the 96-hour differentiation experiments. Surprisingly, *t*-RA suppressed the metabolism of HS-acid. This suppression was most obvious in the first time period (from t = 0 to t = 8 hours). After the first time period, the rate HS-acid is lost is similar with and without *t*-RA present. If *t*-RA inhibits HS-acid metabolism, then why does it not affect all time periods? Simply because *t*-RA is not present at the same concentration at all times periods (Figure 32). By the end of the second time period (t = 16 hours) only 37% of the *t*-RA remained.

In conclusion, the study of *t*-RA metabolism in the presence of HS-acid or HO2-acid showed these hets do not affect *t*-RA metabolism to any significant extent. This supports the idea that these hets differentiate F9 cells by their intrinsic retinoid activity and not by altering *t*-RA metabolism. Furthermore, HS-acid metabolism is suppressed by the presence of *t*-RA, suggesting a shared metabolic pathway.

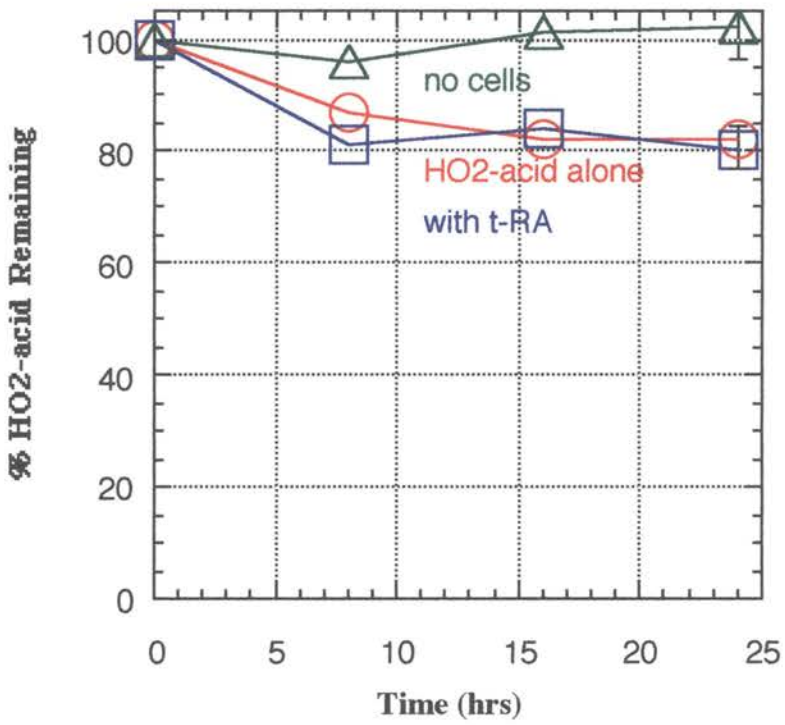


Figure 33. Effect of F9 Cells and *t*-RA on HO2-acid Stability.
Medium Containing 0.5 μ*M* HO2-acid was incubated with F9 cultures at 37 °C with 10% CO_2 by itself (○) and with *t*-RA (□) also present. Additionally, medium containing 0.5 μ*M* HO2-acid was incubated in the absence of cells (△). F9 cell cultures were plated at 2.5×10^5 cells per 75 cm^2 flask and pretreated with 1.0 μ*M* *t*-RA to induce retinoid metabolism. The presence of *t*-RA did not have an effect on HO2-acid metabolism in this study. The F9 cells do metabolize HO2-acid, as seen by the difference observed in the absence of cells.

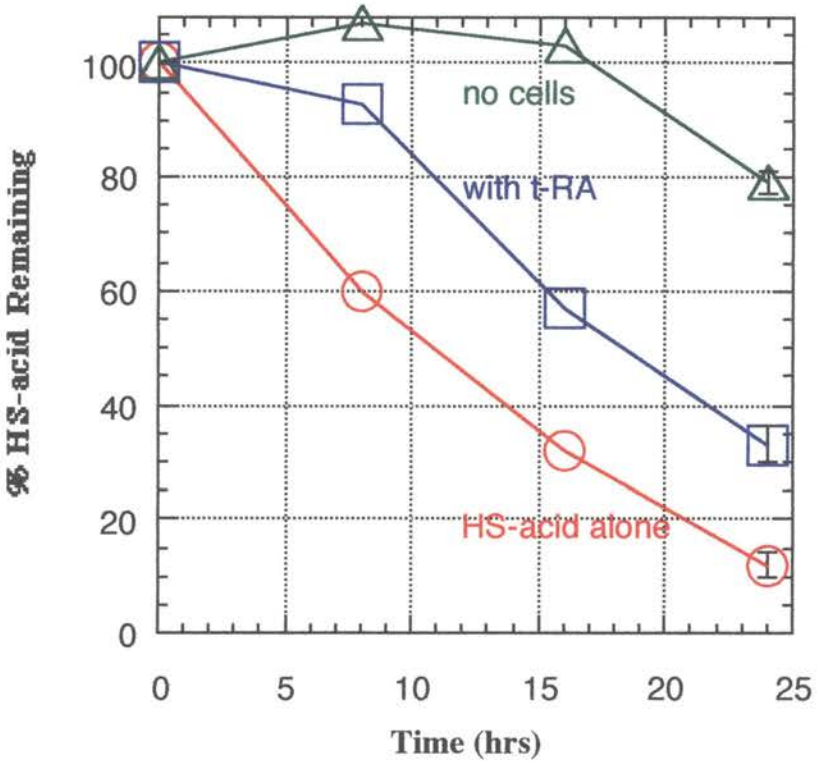


Figure 34. Effect of F9 Cells and *t*-RA on HS-acid Stability.
Medium Containing 0.5 μ*M* HS-acid was incubated with F9 cultures at 37 °C with 10% CO_2 by itself (○) and with *t*-RA (□) also present. Additionally, media containing 0.5 μ*M* HS-acid was incubated in the absence of cells (△). F9 cell cultures were plated at 2.5×10^5 cells per 75 cm^2 flask and pretreated with 1.0 μ*M* *t*-RA to induce retinoid metabolism. Although the original purpose of this study was to see if HS-acid affected *t*-RA metabolism, it was found that the presence of *t*-RA inhibited HS-acid metabolism.

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APPENDIX 1

PROTOCOLS AND SOLUTIONS

General Solutions

DEPC-treated H_2O

Add 1.0 mL of DEPC (diethyl pyrocarbonate, Sigma #D4628) per liter nanopure® H_2O. Stir vigorously for a least one hour. Autoclave 30 min for 250 mL, 1 hour for 1 L.

Note: During the long autoclave cycle, DEPC is degraded into volatile components. The completed solution "DEPC-treated H_2O" does not contain DEPC.

20X SSC
3*M* NaCl
300 m*M* Na Citrate
pH 7.0

Add 43.83 g of NaCl and 22.05 g of Na citrate to about 240 mL of nanopure® H_2O. Adjust pH to 7.0 with 1 *M* HCl. Bring up volume to 250 mL with nanopure® H_2O. DEPC treat. Autoclave.

Maleate buffer
0.1 *M* maleic acid
0.15 *M* NaCl
pH 7.5

Add 11.61 g of maleic acid, 8.77 g of NaCl and 7.09 g of NaOH to ≈ 980 mL of nanopure® H_2O. Adust pH with 5 *M* NaOH. Bring up volume to 1.00 L with nanopure® H_2O. DEPC treat. Autoclave.

Gelatin-Coated Flasks

It is necessary to coat the bottom of culture flasks with gelatin to help F9 cells attach (Darrow et al.1990).

Materials:
gelatin from porcine skin (Sigma #G-2625)
75 cm^2 flasks (Corning #25110-75)

	For ten 75 cm^2 flasks
0.1% gelatin solution	0.250 g of gelatin/250 mL of H_2O
autoclaved nanopure® H_2O	850 mL

Procedure:

1. Prepare gelatin solution using nanopure® H_2O.
2. Autoclave gelatin solution and water.
3. Let gelatin cool to room temperature, then autoclave gelatin again and allow to cool.

Under sterile conditions:

4. Add 20-25 mL of gelatin solution to each 75 cm^2 flask.
5. Place flasks at 4 °C for at least 2 hours. (Make sure flasks are oriented correctly.)
6. Remove gelatin solution.
7. Add 25 mL of sterile water, rock gently, then delete water. Repeat this step two times.
8. Place flasks back in sterile bag or autoclaved tray and store at room temperature.

F9 Medium Preparation

Components:
*FBS, 15% (v/v) fetal bovine serum, heat inactivated (Sigma #F-4135)
+penicillin G 100 Units/mL (1575 U/mg) (Sigma #P-4687)
+streptomycin 100 µg/mL (Sigma #S-9137)
+glutamine 4 m*M* (Sigma #G-5763)
*DMEM (Dulbecco's Modified Eagles Medium with 4.5 g of glucose/L, Sigma #D-5671)
*according to Darrow et al. (1990)
+according to ATCC (American Tissue Culture Collection)

Preparation and Storage of Components:

FBS: Heat inactivate if not already done for you: 55 °C for 20 minutes; store at -20 °C.

Antibiotics: Antibiotics will lose their activity slowly in an aqueous solution. According to Sigma catalog, these antibiotics are active only 3 days at 37 °C, but longer at 4 °C. Freeze stock to prevent loss of activity.

Make stock solution, aliquot, store frozen, -20 °C.
0.1905 g of penicillin G (Sigma # P-4687)
0.3000 g of streptomycin (Sigma # S-9137)
30.0 mL of nanopure® H_2O

Sterile filter stock (0.2 μm filter).
Aliquot into sterile microcentrifuge tubes (1 mL each) in sterile hood.
Store at -20 °C.

Glutamine: Glutamine converts to glutamate slowly in an aqueous solution!
Make stock solution, aliquot, and store frozen at -20 °C.
For 40 mL: 1.17 g of glutamine
40.0 mL of nanopure® H_2O
Sterile filter stock (0.2 μm filter). Aliquot into sterile centrifuge tubes (4 mL each) in sterile hood. Store at -20 °C.

DMEM: Store at 4 °C.

Procedure for Medium Preparation:

1. Autoclave bottle.

2. Thaw: antibiotics, glutamine, and FBS in 37 °C bath. (DMEM may be prewarmed also.)

Medium	100 mL	200 mL
FBS	15 mL	30 mL
antibiotics	1 mL	2 mL
glutamine	2 mL	4 mL
DMEM	82 mL	164 mL

3. Make sure glutamine is dissolved - it tends to precipitate if solution is still cold.

4. In the hood, combine above in 0.2 μm bottle top filter, and apply vacuum to pull through. (All of it may not fit at once.)

5. Store at 4 °C.

Cell Count

This procedure was adapted from the Sigma Cell Culture (1997) catalog.

Materials
hemacytometer
trypan blue solution 0.4% (w/v) in normal saline (Fisher #MT-25-052-LI)

Procedure

1. Prepare cell suspension in medium.

2. Dilute cell suspension with trypan blue: 20 μL of the cell suspension + 20 μL of trypan blue (Dilution Factor = 2)

3. Let solution stand 5-15 min. (Too long will cause all cells to stain.)

4. With cover slip in place, pipet 10 μL of the diluted cell solution into each "V" on the hemacytometer.

5. If greater than 10% of cells appear clustered, you must repeat entire procedure after pipeting or syringing cells to separate clumps.

6. Count viable (white, not blue) cells. Count cells on the top and left touching the middle line of the perimeter of each square. Do not count cells touching the middle line at the bottom and right sides. Start with one chamber and count all cells in center square and three corner squares. Repeat with second chamber.

7. In all squares counted, the cells number should range between 20-50 per square; if not, repeat after adjusting dilution factor.

8. Use the average square count to calculate number of cells:
   Cells/mL = (average count per square) × (10^4) × (Dilution Factor)
   Dilution Factor = v_2/v_1 = trypan blue volume + cells volume / cells volume

Subculturing F9 Cells

F9 cells must be subcultured every 3-4 days (Darrow et al. 1990). This protocol was adapted from American Tissue Culture Collection (source of F9 cells.)

Materials:
0.25% trypsin/EDTA solution, sterile (Sigma #T-4049), 10 mL per 75 cm^2 flask
F9 medium - You need 10 mL for each 75 cm^2 flask you have + 10 mL for each new seeded flask you make.

DMSO for freezing stock (dimethyl sulfoxide Sigma #D-2650)
gelatin-coated flasks (See protocol in Appendix 1.) Expect each flask of cells to produce 5 newflasks.
syringes, sterile (plastic, disposible), 10 mL
needles 21 gauge
50 mL sterile centrifuge tubes
microcentrifuge tubes, a few
pipets, sterile, various sizes

Procedure:

1. Warm medium and trypsin in 37 °C bath.

2. Observe cells: Note morphology, presence of contaminants (discard!), crowding, confluency.

3. Remove medium.

4. Add 10 mL of trypsin to each flask.

5. Take all flasks to microscope. Gently rock flasks, so the solution flows across cells. Observe a flask every few minutes. Determine when cells are completely detached (all cells move when rocked under scope) and when groups of cells are clusters (about 30 cells at largest), not large sheets. When this occurs, return to hood and add 10 mL of medium to each flask to stop trypsinizing action.

6. Place all solutions in two-50 mL centrifuge tubes and balance liquid levels.

7. Centrifuge at 400-500 × g for 15 minutes to pellet cells.

8. Discard supernatant. Using a sterile pipet with bulb, resuspend cells in 1 to 10 mL of medium per tube. (Less medium should be used when planning to freeze.)

9. If cells are not in 50 mL tube, place in one; this will make working with the syringe much easier.

10. Using the 10 mL syringe, gently pass cells through a 21 gauge needle six times (pull in, push out three times.) Minimize shear stress on the cells by doing this slowly. You must have a single-cells suspension to count cells accurately.

11. Count cells (see protocol) and calculate number of flasks or number of tubes to freeze:

Each New Flask:	<u>75 cm^2 flask</u> 3×10^6 cells 25 mL of medium (total)
Each 1 mL Freezing Tube:	5×10^6 to 1×10^7 cells (more is better) 0.45 mL of medium 0.45 mL of FBS 0.1 mL of DMSO

12. For new flasks: Prepare new flasks by just mixing medium and cells in correct amounts, place in flask(s), and return flask(s) to incubator (10% CO_2 and 37 °C.)

For new freezing tubes: Prepare total solution (cells, medium, FBS, and DMSO) for all tubes in one container, then distribute into cryotubes (Corning #25702). Place tubes in either a Nalgene "Mr. Frosty" or a Styrofoam box, and place container in -80 °C.

RNA Isolation with In-Flask Lysis

This protocol is a modified version of the Chomczynski and Sacchi (1987) method.

Materials

Day 1:

RNase-free microcentrifuge tubes
RNase-free pipet tips
disposible cell scrapers
disposible pipets (25mL, 5mL)
PBS, phosphate buffered saline, pH 7.4 (Sigma #1000-3), on ice
G solution:
guanidine thiocyanate (Sigma #G-9277)
sodium acetate pH 7.0 (Sigma #S-2889)
sarcosyl (sodium salt of N-lauryl sarcosine, Sigma #L-9150)
2-mercaptoethanol (Sigma #M-3148)
3.0 *M* sodium acetate, pH 4.0

phenol:chloroform:isoamylalcohol 125:24:1, pH 4.7, water saturated (Sigma #P-1944)
isopropanol, on ice (sigma I-9516)
SDS (sodium dodecyl sulfate, Fisher #BP166)

At a Later Time:

70% ethanol, on ice
sterile water or 0.5% SDS or TE/.5% SDS for dissolving purified RNA (TE is tris EDTA, Sigma #T-9285)

Solution Preparation:

0.75 *M* sodium citrate stock solution (for making G solution):
Dissolve 5.51 g of Na Citrate in 20 mL of nanopure® H_2O. Adjust pH to 7.0 before bringing volume up to 25 mL.

10% sarcosyl stock solution (for making G solution):
Dissolve 1.00 g of sarcosyl in 10.0 mL of nanopure® H_2O.

3 *M* NaAc, pH 4.0
Use 6.15 g of NaAc for 25 mL of solution.

Use concentrated HCl to lower pH to 4.0 before bringing volume up to 25 mL; it will take about 5 mL of 12 *M* HCL. (Do not mix NaAc and HAc to obtain pH, if this is done the [Na^+] will be too low to precipitate RNA.)

G solution

4 *M* guanidine thiocyanate, 25 m*M* sodium acetate, 0.5% sarcosyl and 0.1 *M* 2-mercaptoethanol

For:	100 mL	10 mL
g guanidine thiocyanate	47.28	4.728
mL 0.75 *M* Na citrate stock	3.33	0.333
mL 10% sarcosyl stock	5.00	0.5
µL of 2-mercaptoethanol	70.1	0.701
nanopure® H_2O to volume		

0.5% SDS

Add 0.125 g of SDS to 25 mL of DEPC-treated H_2O.

Procedure

For approximately 5×10^6 cells (one 75 cm^2 flask, ≈80% confluent)

Day 1:

Everything can be done at benchtop - sterile conditions are not necessary. Use fresh pipets, cell scrapers, etc. for each flask to avoid cross-contamination.

1. Remove medium.

2. To rinse cells, add 25 mL of ice-cold PBS to each flask, tilting to wash cells. Pour out. Rinse again.

3. Add 400 µL of G solution to each flask. Use a cell scraper to spread G solution over entire cell surface. Use a sterile disposible pipet to remove the "slime" and carefully place in a labeled microfuge tube.

4. To microfuge tube, add 27 µL of 3 *M* NaAc (pH 4) and vortex.

5. Add 0.5 mL of phenol:chloroform:isoamyl (pH 4.2) and vortex.

6. Centrifuge 15 minutes at 12,000 × g (11,000 rpm on our centrifuge).

7. Collect aqueous phase (upper) and place in new labeled tube.

8. Determine volume, and add one volume of ice-cold isopropanol.

9. Place at -20 °C for minimum of 30 minutes. (RNA can be stored as an isopropanol precipitate for up to one year at –20 °C.)

End of Day 1. Continue only when ready to use RNA.

Later:

10. Centrifuge 20 minutes at 12,000 × g (4 °C).

11. Remove isopropanol.

12. To wash pellet, add one volume 70% ethanol (ice-cold), centrifuge at 12000 × g for five minutes, remove ethanol. Wash again.

13. After removing final ethanol wash, lay tube on counter to dry pellet about 15 min.

14. RNA can be resuspended in sterile H_2O (store at -80 °C) or 0.5% SDS or TE/0.5% SDS.

Formaldehyde Gel Electrophoresis for Northern

Quantities listed are for a 75 mL gel which is 5 mm thick in the BRL model H5, 11 × 14 cm^2 gel box.

Materials:
DEPC-treated H_2O, one liter (See General Solutions in Appendix 1.)
MOPS, morpholinopropane sufonic acid (Fisher #BP-308)
EDTA, ethylenediaminetetraacetic acid (Bio-Rad #161-0728)
bromophenol blue (Sigma #B-5525)
xylene cyanol (Sigma #X-4126)
glycerol (Fisher #BP-229)
ethidium bromide (Bio-Rad #161-0433)
formaldehyde (Sigma # F8775) 37% solution is 12.3 M, pH must be >4.0, if not, deionize
formamide, "Super Pure" (Fisher #BP228-100) If any yellow color present, deionize.
agarose, high strength analytical grade (Bio-Rad #162-0125)
peristaltic pump

Preparation of Solutions:

10X MOPS buffer

0.2 *M* MOPS	41.85 g	10.46 g
80 m*M* sodium acetate	6.56 g	1.64 g
10 m*M* EDTA	3.72 g	0.93 g
	1000 mL	250 mL

Adjust pH to 7.0 with 5 *M* NaOH. DEPC treat. Autoclave (turns yellow). Buffer will turn yellow (straw colored buffer OK, darker NOT OK). Store in dark at 4 °C.

gel-loading buffer	For 5.0 mL gel-loading buffer:
50% glycerol	2.5 mL of glycerol
1 m*M* EDTA	25 µL of 200 m*M* EDTA
0.1% bromophenol blue	0.005 g of bromophenol blue
0.1% xylene cyanol	0.005 g of xylene cyanol
	2.5 mL of DEPC-treated H_2O

ethidium bromide (500 μg/mL)
For 5.0 mL of 500 μg/mL ethidium bromide, add 250.0 μL of ethidium bromide (10 mg/mL) to 5.0 mL of DEPC-treated H_2O.

Procedure:

RNA aliquots:

Aliquot RNA to be run. One lane should have 1 to 20 μg RNA. All lanes should have equal amounts of RNA. Check volume of RNA allowable for well, etc.

RNA concentration (μg/mL) = $40 \times A^{260}$

Gel Preparation:

Pretreat the gel apparatus (box, comb, gel bed) for several hours with 3% hydrogen peroxide.

Gel thickness should not exceed 5 mm for good transfer. (Midi gel box (BRL model H5) is 11×14 cm^2, so 75 mL gel maximum.)

1. Remove hydrogen peroxide from gel apparatus, rinse with 95% ethanol and set up for gel pouring.

2. Prepare running buffer (1X MOPS) by diluting with DEPC-treated H_2O - make 900 mL.

3. Prepare an agarose-formaldehyde gel:

   For a 75 mL, 1.5% gel: 1.12 g of agarose
   7.5 mL of 10X MOPS
   54.1 mL of DEPC-treated H_2O

4. Microwave to dissolve agarose. Let cool to about 60 °C (still hot, but can touch to elbow for a few seconds).

5. In hood, add 13.4 mL of formaldehyde (gel is 2.2 *M*) while swirling.

6. Pour in gel tray and to solidify 30 minutes, then add running buffer.

Sample Preparation:

For 10 prong (2×8 mm^2) comb in $9 \times 11 \times 0.5$ cm^3 midi gel, each well hold a maximum of 64 μL.

1. Use DEPC-treated H_2O to bring all RNA volumes to the same volume:

RNA	5.5 μL	V μL

2. Add (μL):

10X MOPS	1.0	$0.182 \times V$
formamide	10.0	$1.82 \times V$
formaldehyde	3.5	$0.636 \times V$
ethidium bromide (100 μg/mL)	1.0	$1.0 \times V$

5. Heat samples at 70 °C for 10 min; cool quickly on ice.

6.

Add loading buffer:	2 μL	$0.364 \times V$
Total loading volume:	**23 μL**	**4.18 × V (+1)**

Running the Gel:

Run Gel in hood, since formaldehyde will evaporate.

1. Prerun gel 5 minutes at 5 V/cm or 135 V for midi gel.

2. Set up pump; turn off.

3. Load samples and run at about 100 volts (20-55 volts is good for overnight).
4. When dye fronts are in gel, start pump.

5. Run about 3 hours - watch dye, let run about 3/4th the way.

6. Take picture under UV light.

7. Inspection:
   18S and 28S rRNAs show clearly stand out. In mice:
   18 S is 1869 nucleotides
   28 S is 4712 nucleotides
   The ratio 28S:18S signal should be 2:1.

Northern Blot: Transfer RNA from Formaldehyde Gel to a Nylon Membrane

Materials:
20X SSC - 250 mL (See General Solutions in Appendix 1.)
6 pieces of Whatman #2 filter paper
paper towels
nylon membrane (MSI #NJOHY320F5)

Procedure:

1. Equilibrate gel in transfer buffer, 20X SSC for 2X 15 min

2. Cut to gel-size:

6 pieces of filter paper

1 piece of nylon membrane (Touch only with glove or forceps on edge.)
paper towels, to create 3-4" stack

3. Make sandwich:

top	3 filter papers (WET with 20X SSC)
	membrane (WET with 20X SSC), marked-see below*)
	gel (RNA up, open wells down)
	3 filter papers (WET with 20X SSC)
bottom	saran wrap

4. Roll sandwich with test tube to flatten

5. Seal edges with four pieces of saran wrap.

6. Stack paper towels on top.

7. Place large book (like Fisher catalog) on top.

8. Leave overnight.

Next Day:

9. Carefully disassemble stack.

10. Immediately, UV crosslink RNA to membrane.

*Mark membrane with lanes, and an "X" in upper right corner of side facing up (RNA will be on bottom.) From then on, orienting the X in the upper left will orient RNA face up, gel running top to bottom, and lanes going left to right.

Northern Hybridization

For a 100 cm^2 membrane. This protocol was adapted from the DIG System User's Guide from Roche Molecular Biochemicals.

Materials:
SDS (sodium dodecyl sulfate, Fisher #BP-166)
20X SSC (See General Solutions in Appendix 1.)
hybridization oven (Biometra)

Day 1: 20 mL of DIG Easy Hyb (Roche Molecular Biochemicals #1603558) at 68 °C.
10 mL of DIG Easy Hyb *for a new probe* at 68 °C.
probe

Day 2: 120 mL of 2X SSC wash at room temperature
120 mL of 0.5X SSC wash at 68°C

Hybridization Wash Solutions:

0.5X SSC wash	2.0X SSC wash
6.25 mL of 20X SSC	25.0 mL of 20X SSC
0.25 g of SDS	0.25 g of SDS

Dissolve SDS and 20X SSC in 250 mL of nanopure® H_2O. DEPC treat. Autoclave.

Procedure:

1st Day:

1. After crosslinking RNA to membrane, place blot in a bag containing 20 mL of DIG Easy Hyb. Seal bag and incubate at 68 °C at least 1 hour (longer OK).

2. Prepare probe:

   New probe:
   Heat-denature probe in boiling water bath 10 min. Dilute the probe in 10 mL of DIG Easy Hyb (A minimum of 1.25 mL per 50 cm^2 membrane).

   Re-used probe:
   Thaw probe and denature by heating to 68°C for 10 min.

3. Discard DIG Easy Hyb from bag. Add probe/DIG Easy Hyb solution. Allow RNA probe to hybridize overnight at 68 °C.

2nd Day:

4. Recover probe solution. Store at -20 °C.

5. Wash membrane twice in 60 mL of 2X SSC wash at room temperature.

6. Wash membrane twice in 60 mL of 0.5X SSC wash at 68 °C.

Proceed with detection protocol.

Detection of Digoxigenin Probe

For a 100 cm^2 membrane. Adapted from Genius Users' Handbook (Roche Molecular Biochemicals).

Materials:
11 mL of detection buffer
14 mL of 10X blocking buffer
206 mL of maleate buffer (See General Solutions, Appendix 1.)
450 µL of Tween-20 (Fisher #BP337-100)
anti-DIG-AP (anti-digoxigenin-alkaline phosphatase, Roche Molecular Biochemicals #1093274)
*CSPD® (Roche Molecular Biochemicals #1655884)
plastic heat-seal bags

Solution Preparation:

10X blocking buffer
10% blocking reagent (Roche Molecular Biochemicals #1096176) in maleate buffer (See General Solutions, Appendix 1). Dissolve blocking reagent by storing with low heat (do not boil). Too much heat causes coagulation. DEPC treat. Autoclave. Aliquot and store at -20 °C.

blocking solution
14 mL of 10X blocking buffer + 56 mL of maleate buffer

maleate wash
150 mL of maleate buffer + 450 µL of Tween-20

detection buffer
0.1 *M* trisHCl, 0.1 *M* NaCl and 50 m*M* $MgCl_2$ with pH 9.5
No DEPC can be used in this solution - only DEPC-treated H_2O - since DEPC reacts with tris. For 100 mL:
1.58 g of tris HCl
0.58 g of NaCl
5.0 mL of 1 *M* $MgCl_2$
80 mL of DEPC-treated H_2O
Adjust pH to 9.5 with 5*M* NaOH. Bring volume up to 100 mL with DEPC-treated H_2O. Sterile filter. Aliquot 11 mL/tube and store at -20 °C.

Procedure:

In Rubbermaid® container:

1. Rinse 1-5 minutes in 50 mL of maleate wash with 0.3% Tween-20.

2. Incubate 30 minutes in 50 mL of blocking solution.

3. In sterile tube, dilute 2 µL of anti-DIG-AP to 20 mL with blocking solution.

4. Incubate 30 minutes in 20 mL of anti-DIG-AP/blocking solution.

5. Wash twice for 15 minutes each with 50 mL of maleate wash with 0.3% Tween-20 each time.

In sealed bag:

6. Equilibrate 2-5 minutes in 10 mL of detection buffer.

7. Dilute10 µL of CSPD® (vial 5) in 1 mL of detection buffer.

8. Remove detection buffer, and incubate membrane in sealed bag for 5 minutes in the 1 mL of CSPD® solution.

9. Remove from bag, let excess liquid drip off and blot membrane (DNA side up) briefly on Whatman #2 paper.

10. Seal damp membrane in a bag and incubate 5-15 minutes to enhance the reaction at 37 °C in H_2O bath.

11. Expose film for 20-30 minutes initially. It continues to produce light for at least 24 hours.

*CSPD® is a light-generating substrate for akaline phosphatase. It is: disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate.

Northern Blot Strip

For a 100 cm^2 membrane.

Materials:
DEPC-treated H_2O
Stipping solution

Solution Preparation:

Stripping solution
50 m*M* tris HCl (Fisher #BP-153)
1% SDS (Fisher #BP-166)
60% formamide (Fisher #BP-228)
pH 8.0

Formamide must be added fresh. Prepare the rest of the solution ahead:

Dissolve 1.58 g of trisHCl in 75 mL of DEPC-treated H_2O. Adjust pH to 8.0 with 5 *M* NaOH. Bring up volume to 80.0 mL. Add 2.00 g of SDS. Autoclave. Aliquot 8 mL per tube. Store at –20 °C.
Before use: Add 12 mL of formamide to each tube.

Procedure:

1. Add formamide to complete the stripping solution. (See above.)

2. Preheat stripping solution to 68 °C.

3. Rinse the membrane twice, 10 minutes each, in 50 mL of DEPC-treated H_2O at room temperature in a plastic tray.

4. Shake the membrane in a hybridization bag for one hour with the stripping solution at 75 °C.

5. Rinse the membrane twice, 10 minutes each, in 50 mL of DEPC-treated H_2O at room temperature in a plastic tray.

6. Dry the membrane on a paper towel with a filter paper lightly placed on top. Store blot dry at room temperature.

Digestion of DNA with Restriction Endonucleases

This procedure is adapted from Current Protocols (1992). In mapping pSPARC, EcoRI and PvuII were used, and in making a probe template EcoRV was used.

Materials
PvuII (Promega #R6331)
EcoRI (Promega #R6011)
EcoRV (Promega #R6351)
6X DNA gel loading buffer (See preparation in next section)
10X buffer for each nuclease (comes with enzyme)

Procedure

1. Pipet into a microfuge tube:

x µL	DNA sample
2 µL	10X buffer for enzyme
(18-**x**-**y**) µL	sterile nanopure® water
y µL	enzyme (use 1-5 U/µg DNA*)
20 µL	Total Volume

*The volume of enzyme should not be > 2 µL if total volume of mixture is 20 µL.

2. Incubate one hour at 37 °C.

3. Stop the reaction by adding: 5 µL 6X DNA gel loading buffer

   Sample is ready for agarose gel electrophoreses.

DNA Agarose Gel Electrophoresis

A Horizon™ 58 Gel Electrophoresis Unit was used in all DNA gels. The accompanying manual provides detail instructions on how to prepare apparatus, pour gel, etc.

Materials
agarose (Bio-Rad #162-0133)
tris base (Fisher #BP-152)
boric acid (Sigma #B-0252)
EDTA (Bio-Rad #161-0728)
xylene cyanole FF (Sigma #X-4126)
bromophenol blue (Sigma #B-5525)
glycerol (Fisher #BP-229)
ethidium bromide (Bio-Rad#161-0433)

TBE Buffer Preparation:

For 100 mL of 10X TBE, combine 10.8 g of tris base, 2.75 g of boric acid, 2.0 mL of 0.50 *M* EDTA, and water to volume. Sterile filter to maximize shelf life. This is plenty buffer for 2 gels.

6X loading buffer:

For 5.0 mL, add 0.0125 g of xylene cyanole FF, 0.0125 g of bromophenol blue, 1.5 mL of glycerol and sterile water to 5.0 mL.

Gel Preparation:

Combine agarose (mass based on desired percent) and 25.0 mL of 1X TBE in a 250 mL flask. Microwave and swirl (careful for superheated liquid). Allow flask to cool until you can touch it comfortably, but its still very hot. Add 25.0 µL of 0.1 mg/mL ethidium bromide.

Sample Preparation:

Add calculated amount of 6X loading buffer.

Hexane Extraction of F9 Medium

This protocol was adapted from Nelson et al. 1965.

1. Add 2.0 mL of medium to a clean dry vial with cap.

2. Add 25-50 µL of (10^{-4} *M*) retinyl acetate to medium sample.

2. Add 2.0 mL volume of 95% ethanol.

3. Add 7 drops of 3 *M* HCl with a disposable pipette to the tube. Carefully mix the vial and check the pH. The pH should be 2.0.

4. Add 3 mL of hexane and cap. Shake well, by hand, one minute.

5. Cool vial in an ice bucket for 20-30 minutes in the dark.

6. Carefully, without shaking the vial, extract the upper hexane layer and avoid the aqueous (bottom) layer and interface.

7. Transfer the hexane layer to a clean labeled vial.

8. Repeat steps 4-7 to obtain a second hexane extract.

9. Evaporate hexane under N_2 until dryness with no heat applied.

Store dry sample under nitrogen at -20 °C.

APPENDIX 2

ANALYSIS OF DOT BLOTS

As an example of how data were evaluated, the complete analysis of one dot blot is shown here (Figure 25; the blot from the four heteroarotinoids studied in the differentiation assay). First, the film was scanned and the signal ($OD \times mm^2$) was determined using Multianalyst software. The signal data are shown in Table 11. Next, data for each sample was plotted, Signal vs. Dilution Factor. Points that were beyond the linear range were eliminated. When target RNA is too concentrated, the detection method gives a lower (often-erratic) apparent signal. All linear data were plotted and a linear fit was performed (Figure 35).

The above analysis was completed for both SPARC and GAPDH probes using the same RNA blot. Slopes of these lines represent relative quantities of SPARC or GAPDH mRNAs. SPARC signals were normalized by dividing the slope of the SPARC line by the slope of the GAPDH line for each sample (Table 12). SPARC was quantitated using dot blots with signals in the linear range and normalized by GAPDH as described above. Fraction (or percent) SPARC, relative to *t*-RA induced levels, was used to compare treatments (Table 12).

TABLE 11

EXAMPLE SIGNAL DATA: DENSITOMETRIC SIGNAL FOR DILUTION SERIES OF SAMPLES

Relative Concentration	Signal (OD × mm2)							
	no treatment	ethanol	ethanol/ dbcAMP	*t*-RA/ dbcAMP	HO2-acid/ dbcAMP	HS-acid/ dbcAMP	HS2-ester/ dbcAMP	HS2-acid/ dbcAMP
50	5.3934	5.3080	3.6696	17.845	12.029	16.747	13.735	16.543
25	3.3649	3.4226	2.9718	15.041	6.2541	9.6537	7.7767	10.096
10	2.6482	2.7047	2.6220	6.6412	3.1742	4.1840	3.5585	4.3563
5	2.5797	2.5406	2.4754	3.6746	2.5918	2.9100	2.7839	2.9007
2.5	*	*	*	2.7581	2.4988	2.6081	2.5522	2.5066

*These "dots" were indistinguishable from the surrounding background area.

All digits reported by MultiAnalyst are shown here. Not all of the digits are significant. The number of significant figures is not limited until the final calculation.

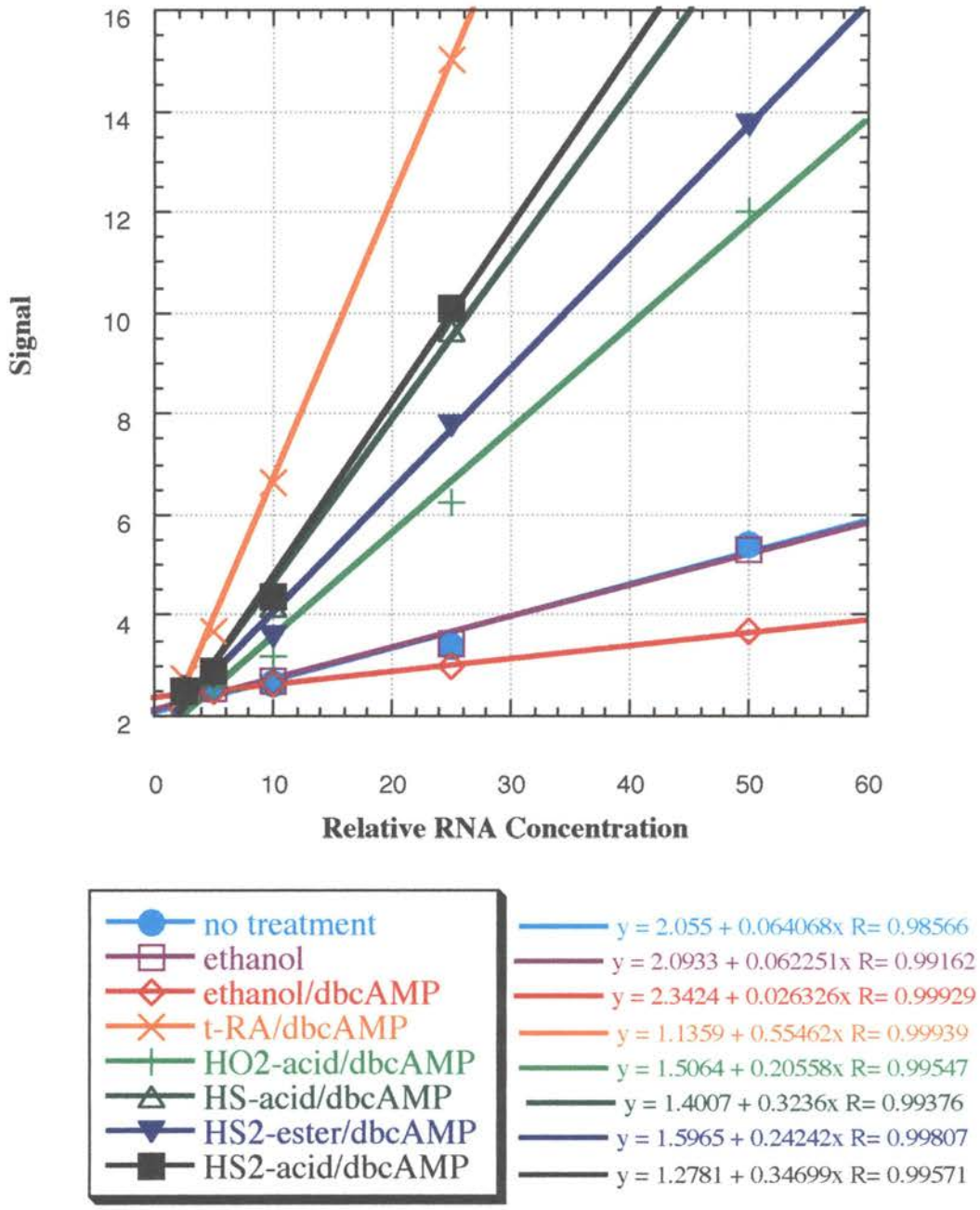

Figure 35. Plotted Linear Dot Blot Signal Data.
The linear data from the SPARC northern of a mRNA dot blot. A dot blot was made and hybridized with SPARC probe (Figure 25). The signal data is shown in Table 11. Here the signal data is plotted against the relative mRNA concentration for each dot. Each dilution series (from one original RNA sample) forms a line, with the slope being directly proportional to the amount of SPARC signal in the original mRNA sample. This method assures signals are within the linear range. See note on the number of significant figures under Table 12.

TABLE 12

EXAMPLE DATA: INDUCTION OF SPARC NORMALIZED TO GAPDH AND AS A FRACTION OF *t*-RA ACTIVITY

Treatment	SPARC*	GAPDH*	$\frac{\text{SPARC}^*}{\text{GAPDH}}$	Fraction *t*-RA Activity
no treatment	0.0641	0.1463	0.4380	0.12
ethanol	0.0623	0.1727	0.3604	0.10
ethanol/ dbcAMP	0.0263	0.1011	0.2604	0.070
t-RA/ dbcAMP	0.5546	0.1492	3.7185	1.0
HO2-acid/ dbcAMP	0.2056	0.1864	1.1031	0.30
HS-acid/ dbcAMP	0.3236	0.2708	1.1948	0.32
HS2-ester/ dbcAMP	0.2424	0.2795	0.8672	0.23
HS2-acid/ dbcAMP	0.3470	0.2349	1.4771	0.40

*Although four digits were shown for the slopes above, this number of significant digits is unreasonable. The signal data had five digits, which is how Multianalyst (the software used to analyze densitometer data) reported it. But the densiometric varies according to how the areas quantitated are selected and how much background varies. However, a large number of significant digits were maintained until the end of the calculation, when the number of significant digits was reduced to two.

APPENDIX 3

DETERMINATION OF SPARC PLASMID MAP

A SPARC cDNA clone was a gift from Dr. Ivor Mason, UMDS Guy's Hospital, some time ago, and the map of this plasmid was unknown. Dr. Mason was contacted and he described three possible plasmids, which he had shared with other labs. One plasmid he described was named pSPARC and contained the full length cDNA cloned into the BamH1 (5') and HindIII (3') sites in the multiple cloning site of pGEM1. The map of pGEM1 was found in the EMBL (European Molecular Biology Laboratory) Data Library. The entire plasmid is 2868 base pairs. The sequence for the 2079 base pair SPARC cDNA was also found in the EMBL Data Library, and a map of the pSPARC plasmid could be deduced (Figure 36).

Digests were performed using PvuII and PvuII/EcoRI to determine the identity of our cDNA clone. (Protocols for restriction digestion and DNA gel electrophoresis are found in Appendix 1.) A PvuII digest of pSPARC would produce fragments of 412, 449 and 4059 base pairs. The PvuII digest of our cDNA clone produced three fragments that were consistent with pSPARC base pairs according to a 100 base pair ladder (Roche Molecular Biochemicals #1721933). (See Figure 37.)

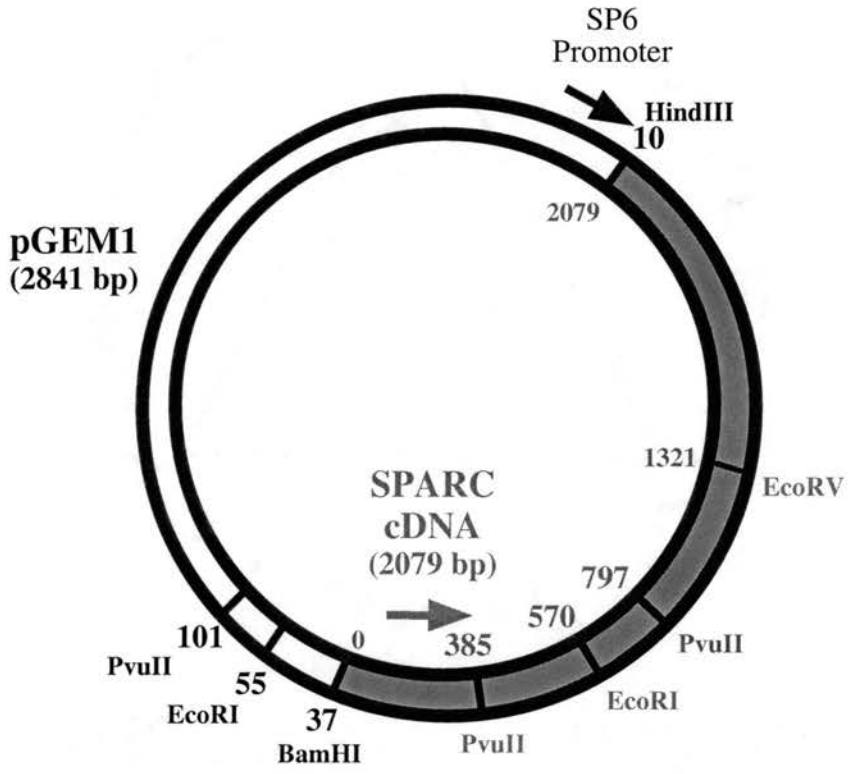

Figure 36. Map of pSPARC. A full length cDNA cloned into the BamHI (5') and HindIII (3') sites in the multiple cloning site of pGem. Sequences for both pGem1and SPARC cDNA are available on the EMBL Data Library. Restriction sites for SPARC cDNA were determined by MacVector.

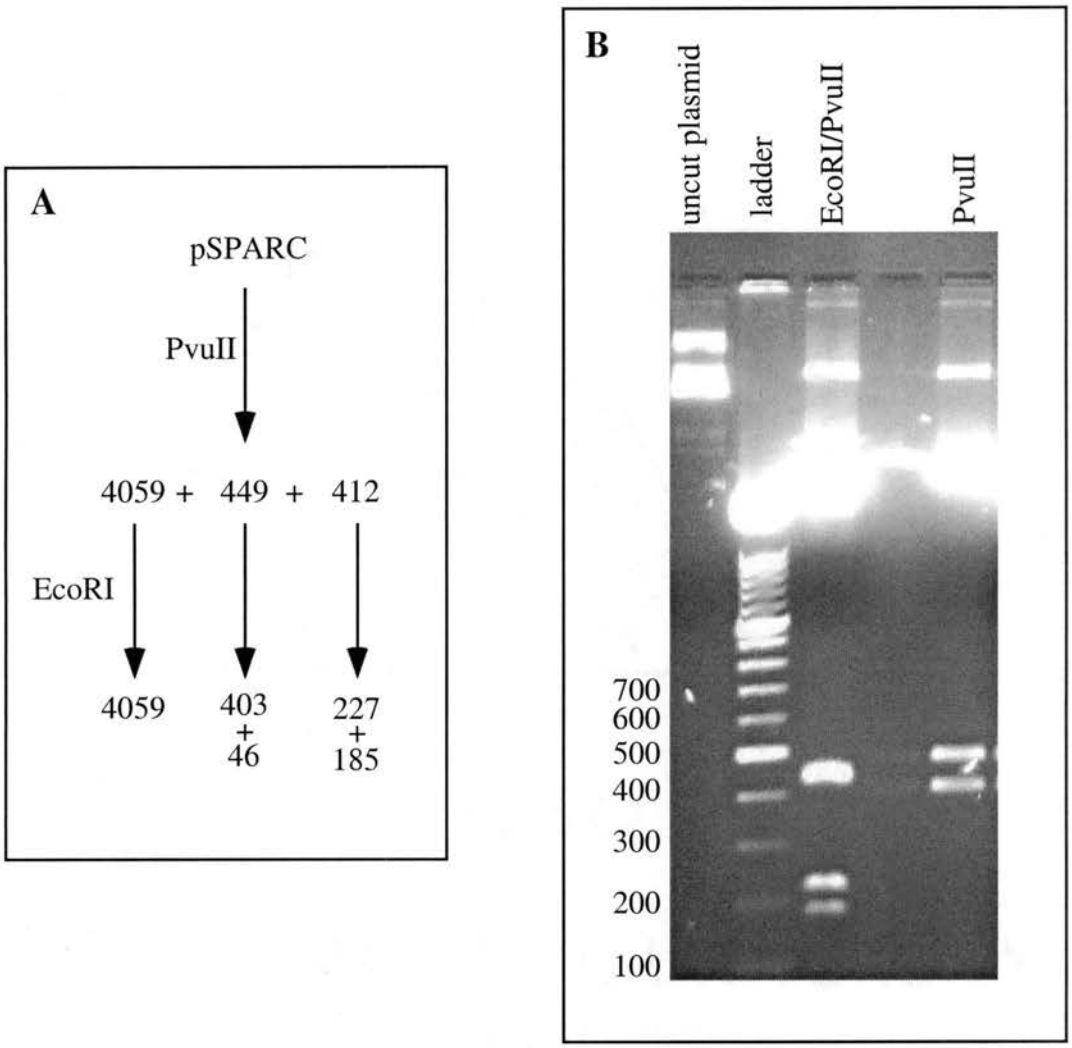

Figure 37. Digest of SPARC cDNA Clone and Predicted Digest Fragments for pSPARCR. Panel A shows the predicted fragments (as number of base pairs) formed by digests with PvuIII and PvuII/EcoRI. Panel B shows a DNA gel run on digests of our SPARC cDNA clone. One μg of uncut plasmid was run; 4 μg of each digest was run. Each of the smaller two fragments created by PvuII are cut by PvuII, as is predicted for pSPARC.

The 412 base pair fragment is the SPARC sequence from nucleotide 385 to 797; it contains the sole SPARC EcoR1 restriction site at nucleotide 570. Further digestion of this fragment with EcoRI would produce 185 and 227 base pair fragments. The 449 base pair fragment contains the EcoRI site from the vector and additional digestion with EcoRI would produce 46 and 403 base pair fragments. The 46 base pair fragment would be very difficult to detect, due to its small size, and hence limited ability to bind ethidium bromide. The PvuII/EcoRI digest of our cDNA clone produced fragments consistent with pSPARC (Figure 37). The 185 and 227 base pair fragments were not observed until the amount of DNA and ethidium bromide were increased, and so it is acceptable that the 46 base pair fragment is not apparent. Since the combined fragment patterns agreed with that of pSPARC, it was concluded that our cDNA clone is pSPARC.

APPENDIX 4

EXAMPLE HPLC DATA AND ANALYSIS

One set of data is shown here to illustrate the analysis of HPLC peak areas. This data is from the study of the effect of hets (HO2-acid and HS-acid) on *t*-RA metabolism. Table 13 shows the areas of *t*-RA and the internal standard, RAc for each chromatogram. These areas were determined from a chromatogram, which plotted the maximum absorbance between 280 nm and 500 nm. For each chromatograph, 300 μL of an extract sample were injected. For the samples in Table 13, 50.0 μL of RAc were used in each extraction; RAc was 2.1×10^{-4} *M* for samples *t*-RA-t0-1, -2, and -3 and RAc was 2.1×10^{-3} *M* for all other samples. The final percent was rounded to two significant figures since this was supported by the standard deviations.

Calculations began with determining the fraction of the internal standard that was injected into the HPLC (versus how much was added to the medium sample). The molarity of RAc injected was determined from the area using the equation on the standard curve (Figure 38). Next, the fraction injected was determined:

$$\text{Fraction injected} = \frac{M \text{ RAc injected} \times 300\ \mu\text{L}}{M \text{ RAc added to medium} \times \mu\text{L added to medium}}$$

The molarity of *t*-RA was calculated using its equation from the standard curve (Figure 38). Lastly, the concentration of *t*-RA was calculated as:

$$M \text{ of retinoid in medium} = \frac{(\text{volume injected})\,(M \text{ of retinoid in injected sample})}{(\text{volume of medium extracted})\,(\text{fraction of RAc injected})}$$

TABLE 13

EFFECT OF HETS ON *t*-RA METABOLISM:
DETERMINATION OF *t* -RA CONCENTRATIONS FROM HPLC DATA

Sample	RAc Area (A × min)	*t*-RA Area (A × min)	mL Medium Extracted	n*M* *t*-RA in Medium	% *t*-RA Remaining
t-RA t0-1	6780009	662543	2.50	557	
t-RA t0-2	6626642	677097	2.50	581	
t-RA t0-3	6503351	677310	2.50	592	
t-RA t8	473586	351488	2.40	360	0.62
t-RA t16	549713	208989	2.35	212	0.37
t-RA t24-i	599276	61744	2.50	80.0	0.14
t-RA t24-1		91626	2.50	110	0.19
t-RA t24-2		60505	2.50	85.5	0.15
t-RA t24-3		82859	2.50	103	0.18
t-RA/hs t8	484657	429521	2.42	419	0.73
t-RA/hs t16	565321	211269	2.35	209	0.36
t-RA/hs t24-i	614471	104833	2.50	109	0.19
t-RA/hs t24-1		86628	2.50	106	0.18
t-RA/hs t24-2		68175	2.50	91.4	0.16
t-RA/hs t24-3		102584	2.50	118	0.20
t-RA/HO2-acid t8	593424	405237	2.32	355	0.62
t-RA/HO2-acid t16	422097	166338	2.42	210	0.36
t-RA/HO2-acid t24-1	533211	58835	2.50	85.4	0.15
t-RA/HO2-acid t24-2	560359	59559	2.50	82.7	0.14
t-RA/HO2-acid t24-3	543979	68884	2.50	91.9	0.16
no cell *t*-RA t8	565712	545966	2.29	489	0.85
no cell *t*-RA t16	593023	525589	2.42	431	0.75
no cell *t*-RA t24-1	544796	478476	2.50	409	0.71
no cell *t*-RA t24-2	593023	525589	2.50	417	0.72
no cell *t*-RA t24-3	508191	468050	2.50	423	0.73

Samples are labeled according to the retinoids present, the time the sample was taken and the repetition number. For example, "*t*-RA/HO2-acid t3-1" is a sample with both retinoids (*t*-RA and HO2-acid); the sample was taken at 24 hours, and it was repetition number one from that medium sample. Sample names with "no cell" in them represent the flask prepared with medium and retinoids and no F9 cells. The internal standard was left out in some samples (no value for RAc area). In these samples, the average fraction recovered was used in place of fraction of RAc recovered. Additionally, one more extraction was performed (samples labeled "-i") to confirm the others values.

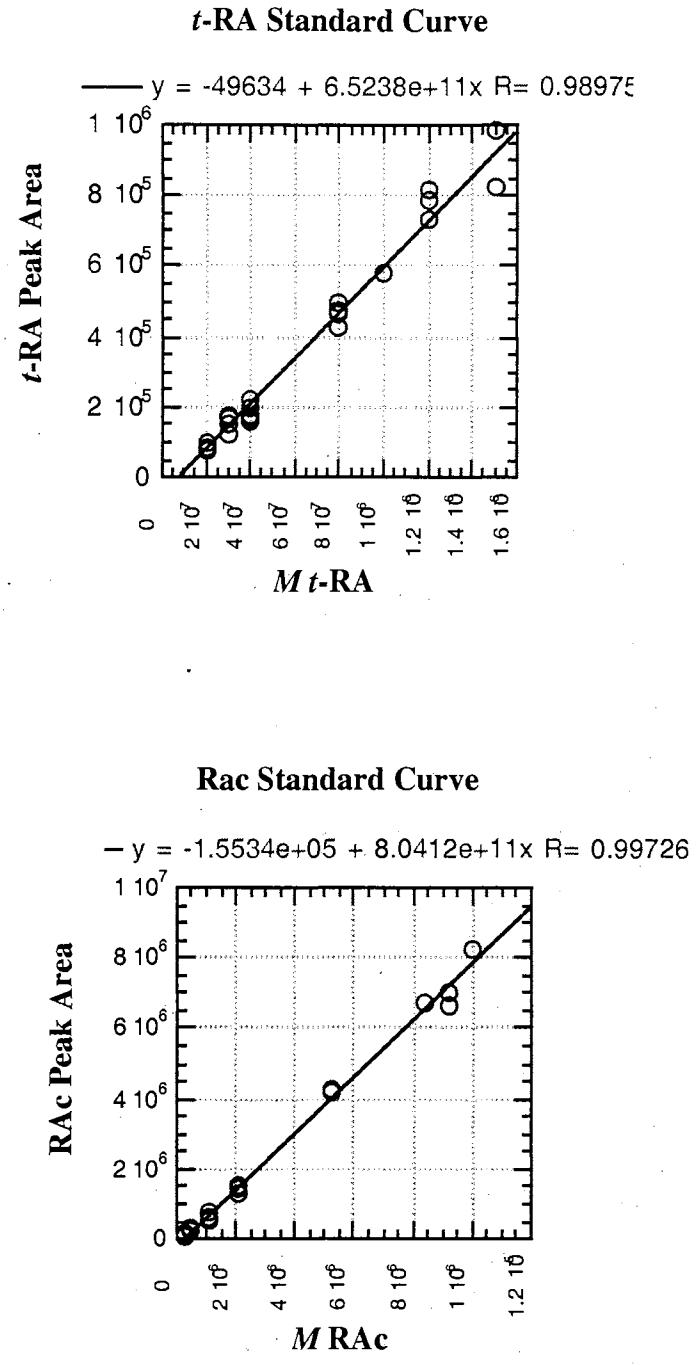

Figure 38. Standard Curves for *t*-RA and RAc .
These standard curves were created by analyzing *t*-RA and RAc peaks on a maxplot chromatogram. (Maxplot shows the absorbance max between 280 and 500 nm for each time in the chromatogram.) Standards were chromatographed at different times interspersed between experimental samples.

APPENDIX 5

CHEMICAL ABSTRACT INFORMATION FOR SELECTED HETEROAROTINOIDS

Table 14 gives chemical abstract registry numbers and names for the four heteroarotinoids used in this study.

TABLE 14

CHEMICAL ABSTRACT INFORMATION FOR SELECTED HETEROAROTINOIDS

Heteroarotinoid	Chemical Abstract Registry Number	Chemical Abstract Index Name
HO2-acid	88579-29-7	benzoic acid, 4-[(1E)-2-(3,4-dihydro-4,4-dimethyl-2H-1-benzopyran-6-yl)-1-propenyl]-
HS-acid	108695-20-1	2,4,6-octatrienoic acid, 7-(3,4 -dihydro-4,4-dimethyl-2H-1-benzothiopyran-6-yl)-3-methyl (2E,4E,6E)-
HS2-ester	88579-35-5	benzoic acid, 4-[2-(3,4-dihydro-4,4-dimethyl-2H-1-benzothiopyran-6-yl)-1-propenyl]-, ethyl ester (E)-
HS2-acid	91587-07-4	benzoic acid, 4-[(1E)-2-(3,4-dihydro-4,4-dimethyl-2H-1-benzothiopyran-6-yl)-1-propenyl]-, (E)-

VITA

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