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**Retinoic Acid-Independent Regulation of RAR β 2 Gene
Expression During Cardiac Development**

**A Thesis Submitted to the School of Graduate Studies
University of Ottawa**

**In Partial Fulfilment of the Requirements for the Degree of
Master of Science
Department of Pharmacology
Faculty of Medicine**

By

Craig A. Crippen[®]



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Abstract

RAR β has been shown to be expressed in the developing heart at the 8 somite stage. Differentiation of EC cells with DMSO produces a mixture of embryonic cardiac, skeletal, endodermal and other mesodermal derivatives. The RAR β transcript reaches a maximum at day 7 of DMSO differentiation, concurrent with beginning of beating of cardiac muscle cells. Four different isoforms of RAR β have previously been identified. These include RAR β 1,2,3 and just recently RAR β -4. The RAR β -2 isoform is predominantly expressed in the differentiated cardiac muscle cells. RT-PCR with isoform specific primers has been used to identify RAR β -2 as the predominant isoform. The RAR β -2 and RAR β -4 isoforms are transcribed from the promoter P2 which is associated with the RARE β enhancer element, whereas the RAR β -1 and RAR β -3 isoforms are transcribed from the upstream P1 promoter which has no RARE. These results show, that like RA-treated EC cells, expression of the RAR β -2 isoform in DMSO differentiated P19 cells predominates.

This increase in RAR β -2 mRNA is not associated with an increase in transcriptional activation of the RARE β -2/4 promoter. Stable transformants of the RARE β -2-CAT reporter gene construct were pooled and differentiated with either RA or DMSO. The results obtained demonstrate that RA induced CAT activities increased to a maximum of 188 fold by day 3. Alternatively,

differentiation with DMSO showed no increase in CAT activity, demonstrating that this the RARE β -2 enhancer was not activated during mesoderm formation. The increase in RAR β -2 mRNA levels was not due to another enhancer element as evidenced by nuclear run-on analysis.

To determine whether the expression of RAR β -2 is dependent on muscle, a mutant P19 cell line (D3), incapable of differentiating into cardiac or skeletal muscle following aggregation and exposure to DMSO was exposed to DMSO. The results show that there is no induction of RAR β upon differentiation of the defective P19 cell line. Since treatment of P19 cells with DMSO also forms skeletal muscle as well as cardiac muscle, myo D expressing P19 cells were aggregated in the absence of DMSO. The results show that while many of these cells differentiate into skeletal muscle, no induction of RAR β mRNA is observed.

RAR β 2 mRNA levels seem to be regulated by a post-transcriptional mechanism. The stability of the DMSO induced RAR β 2 message was determined using the transcriptional inhibitor actinomycin-D on day 4 and 7 of differentiation. The half life of the message was found to be approximately equal on both days. We postulate that stabilization of the message occurs at an earlier time point, and that accumulation of the message occurs up until Day 7 of differentiation.

Calcium chelating experiments were performed to determine if a lack of calcium would have an effect on the level of expression of the RAR β 2 isoform. No

change in RAR β 2 expression was seen following pretreatment for 48 hours with the calcium chelator EGTA.

Simultaneous administration of RA and DMSO to aggregating P19 cells blocked the up-regulation of Brachyury expression in these cells. No beating cardiac muscle was formed, however neuroectoderm was found to be the predominant cell type, indicating that the DMSO differentiating effect was usurped by the presence of RA. Addition of RA on day 2 of DMSO-differentiating P19 cells resulted in a variety of cell types not including neuroectoderm or beating cardiac muscle. Brachyury expression was not noticeably altered in this case, indicating that its transient induction expression pattern was already determined.

The effect of adding both DMSO(inducer of mesoderm formation) and RA(inducer of neuroectoderm formation) to P19 cells simultaneously resulted in the appearance of neuroectoderm. The results show a high level of Id expression as compared to those of normally DMSO differentiated P19 cells, indicating that neuroectoderm formation is accompanied by Id expression. It was also observed that addition of RA on day two of differentiation resulted in the formation of a variety of cell types, not including neuroectoderm or beating cardiac muscle.

The data presented here clearly demonstrate RA-independent regulation of RAR β -2 gene expression during mesoderm formation. The mechanism for

RAR β 2 up-regulation during mesoderm formation is believed to be at the posttranscriptional level as evidenced by nuclear run-on analysis. Elucidating this mechanism of up-regulation would be of great importance in understanding the role of retinoid receptors throughout development and in the adult.

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LIST OF ABBREVIATIONS

ARE	AU-rich element
ATP	adenosine triphosphate
AUBF	AU-binding factor
BES	N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid
bHLH	basic helix-loop-helix
°C	degrees Celsius
CAT	chloramphenicol acetyltransferase
Ci	curie
CTP	cytidine triphosphate
dCTP	deoxycytidine triphosphate
dUTP	deoxyuridine triphosphate
DBS	donor bovine serum
DEPC	diethyl pyrocarbonate
DMSO	dimethylsulfoxide
cDNA	complementary deoxyribonucleic acid
DTT	dithiothreitol
EC	embryonal carcinoma
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β -aminoethyl ether) N,N,N',N'-tetraacetic acid
EtBr	ethidium bromide

FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GTF	general transcription factor
GTP	guanidine triphosphate
kb	kilobase
ml	millilitre
mM	millimolar
mRNA	messenger ribonucleic acid
ng	nanogram
NP-40	nonidet P-40
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethyl sulfonyl fluoride
RA	all-trans retinoic acid
RAR	retinoic acid receptor
RARE	retinoic acid response element
RT-PCR	reverse-transcriptase polymerase chain reaction
RXR	retinoid-X receptor
SDS	sodium dodecyl sulfate
SSC	sodium chloride/sodium citrate
SSPE	saline-sodium phosphate-EDTA
UV	ultraviolet
μ Ci	microcurie

μg	microgram
μM	micromolar
UTR	untranslated region

Chapter I: Introduction

Part A - Mediating the Retinoid Signal

Vitamin-A is known to play an important role in normal growth, vision, and maintenance of tissue (Sporn et al., 1994) (Wolbach and Howe., 1925). The first most striking finding of the action of Vitamin-A is its ability to act in the positional identity of certain embryonic tissues such as the vertebrate limb, CNS, retina, and body axis (Hofmann and Eichele., 1994). Animals on vitamin-A deficient (VAD) diets develop a variety of symptoms including widespread squamous metaplasia and/or keratinizing squamous metaplasia of epithelia (Underwood., 1994) (Wolbach and Howe., 1925). Additionally, offspring of VAD females display a characteristic spectrum of congenital malformations (Wilson and Warkany., 1948) (Wilson and Warkany. 1949) (Warkany et al., 1948) including the heart and aortic arches, respiratory tract, diaphragm, eye, and urogenital tract. Administration of retinol to the diet of these VAD animals reverses all or nearly all of these congenital malformations. Interestingly, the gestational stage of administration of retinol determines which VAD-induced malformation is prevented, suggesting that Vitamin-A is required at several different stages of development.

The finding that excess retinoid administration also caused dysmorphogenesis lends further credence to the proposal that retinoids are key developmental factors.

The carboxylic acid derivative of retinol, retinoic acid (RA) is known to be a more potent teratogen than retinol, indicating that RA is the biologically active form of the vitamin. This theory is supported by the observation that post-partum VAD-induced defects may be either prevented or reversed by exogenous RA (except for vision) (Thompson et al., 1964).

The discovery of nuclear receptors for retinoids expanded our understanding of how retinoids could potentially influence levels of gene expression (figure 1-1). The incidence of developmental defects has also correlated with alterations in the level and/or pattern of expression of specific genes, some of which possess retinoic acid response elements (RAREs) in their promoter regions (Chambon., 1994). This observation suggests that retinoic acid acts through its receptors and directly regulates the expression of certain genes essential for normal development.

There are two classes of retinoid-binding proteins that are implicated in this signaling pathway. The first class includes the cellular retinol-binding proteins I and II (CRBPI and CRBP II) and the cellular retinoic acid binding proteins I and II (CRABPI and CRABPII). The two isoforms share amino acid sequence similarity, but are immunologically distinct and exhibit different affinities for retinoids (Bailey and Siu., 1988). It is also clear that these cellular binding proteins exhibit distinct patterns of expression in the developing embryo and the adult

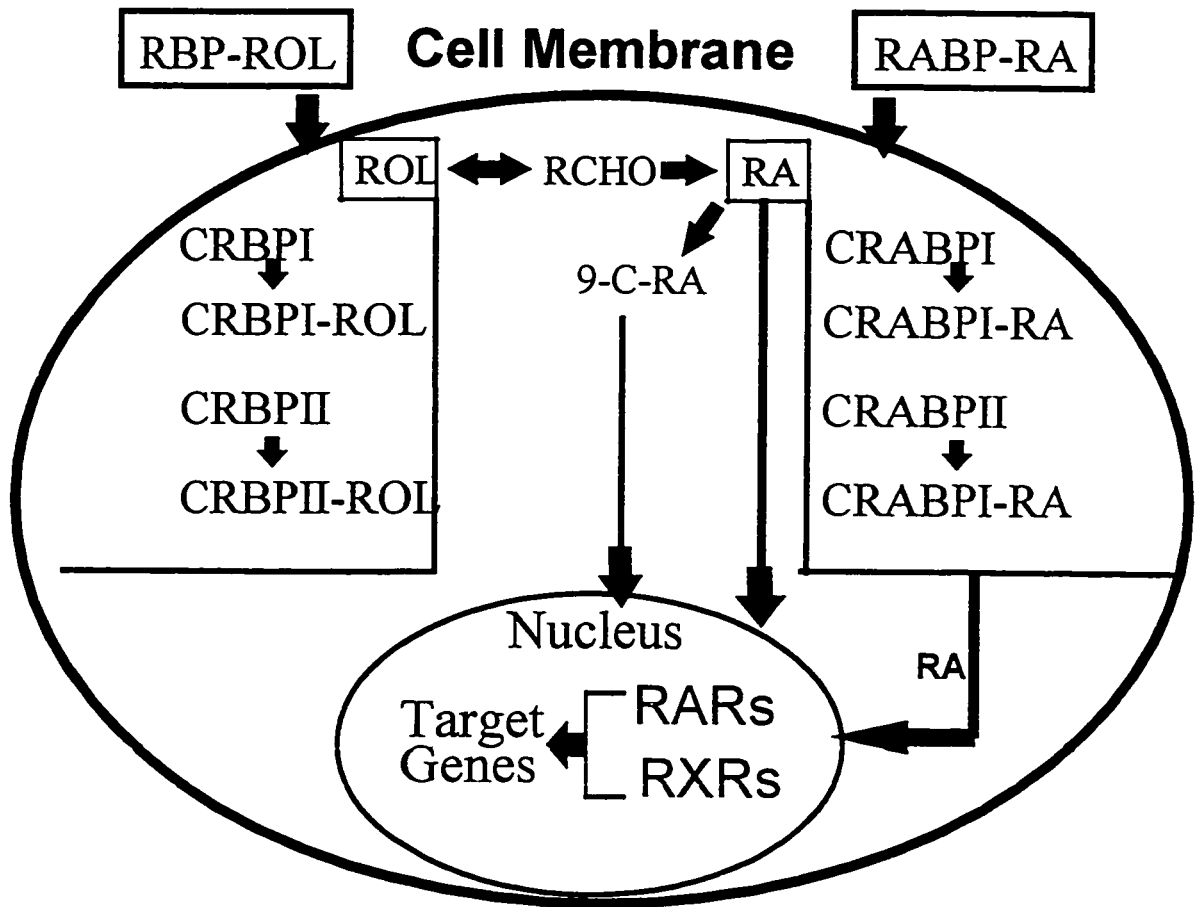


Figure 1-1: Action of retinoids with their cognate receptors. Vitamin-A is metabolized to retinol with the body, which can then undergo isomerization to retinaldehyde and then *all-trans*-retinoic acid, the carboxylic acid derivative of retinol. Retinoic acid can then be isomerized into *9-cis* retinoic acid. Retinol and retinoic acid have cytoplasmic binding proteins termed cytosolic retinol binding protein I and II, and cytosolic retinoic acid binding protein I and II. *9-cis* retinoic acid and *all-trans* retinoic acid are believed to act directly on gene expression by entering the nucleus and binding to retinoid receptors. (ROL) retinol, (RCHO) retinaldehyde, (RA) all-trans retinoic acid, (RBP) retinol binding protein, (RABP) retinoic acid binding protein, (CRBPI and CRBP II) cellular retinol binding protein I and II, (CRABPI and II) cellular retinoic acid binding protein I and II, (9-C-RA) 9-cis retinoic acid, (RARs) retinoic acid receptors, (RXRs) retinoid X receptors.

organism suggesting that they do indeed play a specific role in retinoid signaling. The exact role of these cytosolic proteins is still unknown but it has been suggested that they may be involved in retinoid storage, metabolism, and the regulation of the free level of biologically active retinoids within a given cell (Livrea and Packer., 1993) (Boylan and Gudas., 1992) (Fiorella and Napoli., 1991).

The second class of retinoid binding proteins involved in transducing the RA signal are the retinoic acid receptor family (RAR), and the retinoid-X receptor family (RXR). These receptors belong to a large superfamily of ligand-inducible transcription factors that include the steroid, Vitamin-D and thyroid hormone receptors, the insect ecdysteroid receptor and a number of orphan receptors whose ligands are still as yet unknown (Mangelsdorf et al., 1995).

The nuclear receptor superfamily can be broadly divided into four classes based on their dimerization and DNA binding properties. Class I receptors include the known steroid hormone receptors, which function as ligand induced homodimers and bind to DNA half-sites organized as inverted repeats. Class II receptors heterodimerize with RXR and characteristically bind to direct repeats. Class III receptors bind primarily to direct repeats as homodimers. Class IV receptors typically bind to extended core sites as monomers. Most of the orphan receptors fall into class II and IV categories.

A characteristic feature of these nuclear receptors is that they bind to regulatory regions in DNA called hormone response elements (HREs) and mediate gene expression. The unique ability of nuclear receptors, which sets them apart from other DNA binding proteins, is that they can act on the DNA through a highly conserved DNA binding domain (C) that contains two “zinc finger” motifs which allows the receptor to bind tightly to a particular DNA response element (Umenoso and Evans., 1989) (Leid et al., 1992) (Freedman et al., 1988) (figure 1-2). The DNA binding domain of each of these receptors spans a “core” of 66 residues that forms a highly conserved domain encompassing the two zinc finger modules followed by a carboxy-terminal extension. The core domain is conserved across all members of the receptor family and constitutes α -helices, one of which (the recognition helix) engages the major groove to make specific contacts with the bases of the half-site (Schwabe et al., 1993) (Luisi et al., 1991).

The ligands of these nuclear receptors bind within the ligand binding domain (E) at the carboxy terminus of the receptor. Also, within the ligand binding domain are sequences involved in nuclear translocation (Picard and Yamamoto., 1987) (Guiochon-Mantel et al., 1989), dimerization (Fawell et al., 1990), transactivation (Webster et al., 1988) (Webster et al., 1989) (Tora et al., 1989) (Lees et al., 1989) (Kumar et al., 1988) (Danielian et al., 1992) and interaction with heat shock protein (Dalman et al., 1991). It has been revealed that the ligand binding

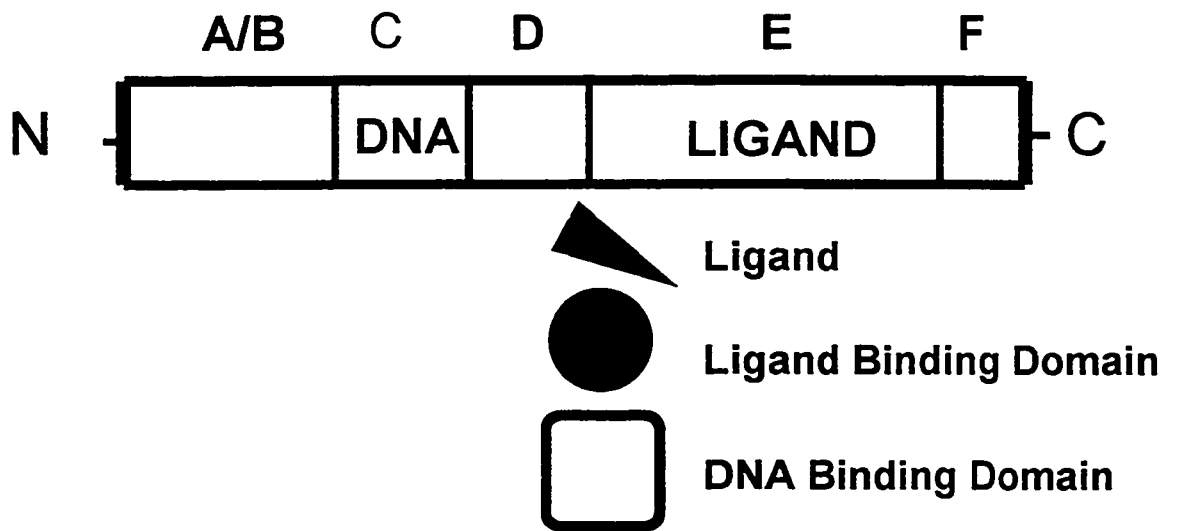
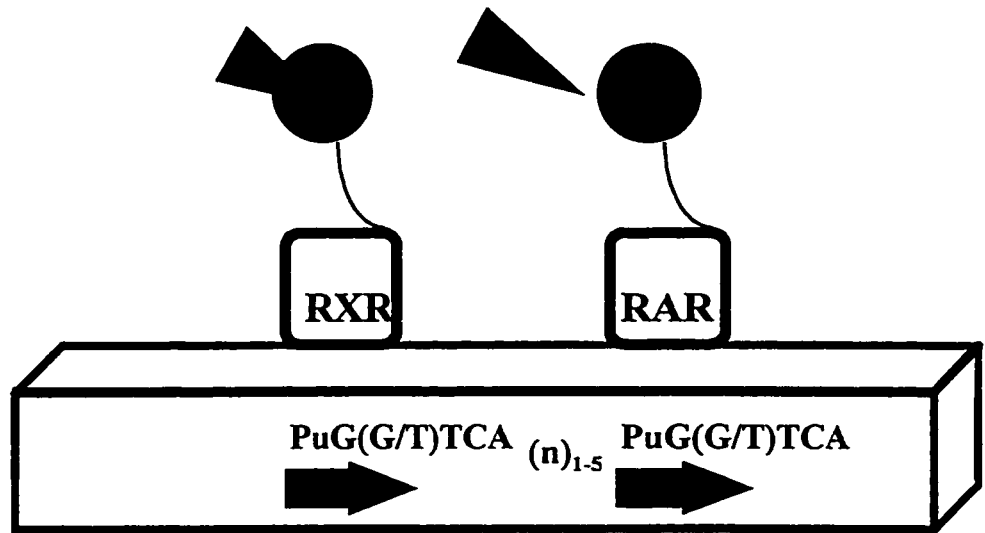


Figure 1-2: Nuclear receptor domains and their binding sites on DNA. Retinoic acid receptors (RARs) and retinoid X receptors (RXRs) may interact together in a heterodimeric combination on a hormone response element (HRE) consisting of a core hexad consensus sequence by binding to a direct repeats of this sequence spaced by five nucleotides (DR5). The receptors have multiple domains from A-F in which there are conserved DNA binding regions (region C), ligand binding domains (region E), AF-2 transactivation domains (region E), dimerization domains (region E) and nuclear translocation (region E) (Pu) purine, (RAR) retinoic acid receptor, (RXR) retinoid X receptor.

domain comprises a carboxy-terminal region of approximately 225 amino acids that is capable of autonomous ligand binding.

The intrinsic transcriptional activation region (AF-2) located within the ligand-binding domain controls ligand-dependent transcriptional events. Inactivation of the AF-2 domain in the mouse estrogen receptor by deletion completely blocks transcriptional activation while not affecting DNA or steroid binding (Danielian et al., 1992). The ligand-binding domain of any of the retinoid receptors, when fused to the DNA-binding domain of GAL-4 is capable of mediating retinoid-induced transcriptional activation from a GAL-4 reporter gene (Allenby et al., 1993). The deletion of the AF-2 region is responsible for the lack of transcriptional activation by v-erb-A, a mutant version of the thyroid hormone α receptor with oncogenic activity (Zenke et al., 1990) (Damm et al., 1989). COUP and ARP-1 which belong to the orphan receptor group because their ligand is unknown also lack an AF-2 region (Wang et al., 1989) (Ladiaz and Karathanasis., 1991). Since deletion of the AF-2 region does not influence DNA binding, it is not surprising that v-erb-A, COUP, and ARP-1 are involved in the repression of transcriptional activation mediated by other members of the nuclear receptor superfamily.

RAR α , RAR β , and RAR γ mutants with deleted AF-2 regions are unable to activate transcription and act as potent negative transcriptional regulators. The retinoic acid receptor differs from the estrogen receptor in that deletion of the

AF-2 region produces a receptor unable to bind 9-cis RA and only capable of binding all-trans RA. These observations indicate that the ligand-binding domain and the transactivation domain in the intact receptor function interdependently.

Additionally, in all nuclear receptors, there is a non-conserved transactivation domain (AF-1) which functions in a ligand-independent manner (Tora et al., 1989) (Nagpal et al., 1993), found in the N-terminal region of all nuclear receptors. This region can be shown to function in a ligand-independent autonomous manner. Also, both the AF-1 and AF-2 transactivation domains can act synergistically with one-another to activate transcription depending on the promoter (Tora et al., 1989) (Nagpal et al., 1992) (Nagpal et al., 1993).

The action of retinoids as potent effectors of cellular proliferation, differentiation, and developmental pattern formation is thought to occur solely through the binding of retinoic receptors to RAREs which are found in the promoter regions of retinoic-acid responsive genes. It has been found that RAR dependent gene activity requires the presence of RXR. However RXR-dependent gene activity is thought to be mediated by RXR homodimers and can be inhibited by the presence of RAR. RXR heterodimers typically bind to two half-sites consisting of a minimal core hexad consensus sequence PuG(G/T)TCA that may be arranged as two direct repeats, inverted repeats, or everted repeats depending on which response element is being bound (figure 1-2).

The uniqueness of each retinoid receptor is what gives this signalling pathway its diversity. The presence of a direct repeat (DR) response element was initially suggested by the studies on thyroid hormone (Koenig et al., 1987) and retinoic acid response elements (RAREs) (Leid et al., 1992). Later, a model was developed in which HREs for the Vitamin-D receptor (VDR), TR, and RAR are composed of DRs spaced by 3,4, or 5 nucleotides (ie., DR3, DR4, DR5 respectively) (Umenoso et al., 1991). The subsequent demonstration that a DR1 serves as an RXR and peroxisome proliferator response element and a DR2 is a second RARE has expanded this model to the so-called 1-5 rule. By nature, all receptors that bind to either DRs or palindromic HREs do so as either heterodimers or homodimers. There are also a number of orphan receptors that can bind to only a single core element as a monomer. Because DRs are asymmetric, heterodimer complexes should bind these elements in an asymmetric fashion. It has now been established that on DR3, DR4, DR5, RXR occupies the 5' half-site and the partner (VDR, TR, RAR respectively) occupies the 3' half-site (Zechel et al., 1994) (Perlman et al., 1993) (Kurokawa et al., 1993). On DR1, RXR can bind as both a homodimer and as a heterodimer with RAR. Interestingly, the polarity of the RXR/RAR heterodimer may be reversed. In many cell types, the consequence of this reverse polarity binding is that the RAR/RXR heterodimer is a potent repressor of the ligand-activated RXR homodimer (Kurokawa et al., 1995). Surprisingly, ligand-induced transcription activities for RXR are suppressed when complexed with VDR, TR, and RAR (Kurokawa et al., 1994). The restriction of RXR ligand binding within several of

these heterodimers indicates that 9-cis retinoic acid responsiveness of RXR is not a necessary consequence of heterodimerization. Thus, RXR is a critical component of heterodimer formation, which in turn is critical to generating diversity of hormone responses.

All three RAR isoforms bind all-trans RA and 9-cis RA with high affinity whereas the three RXR isoforms are only activated by 9-cis RA. Interestingly the amino acid sequence conservation of a particular RAR type is greater across species than observed for the three RAR types in a given species. It seems as though these receptors have been conserved through evolution to serve an important role involving retinoid signaling.

The RXR class of nuclear receptors contains three subtypes, RXR α , RXR β , and RXR γ , that exhibit high homology among themselves and in their ligand and DNA binding domains. The F domain is absent. The above observations suggest that if RA-dependent transcriptional regulation in vivo is dependent on RAR/RXR heterodimers, then the pleiotropic effects of retinoids may be due, at least in part, in the regulated expression of different heterodimeric combinations, as well as the availability of free ligand within the cell.

A model for the ligand activation of RXR/RAR heterodimers, states that in absence of ligand, the RXR/RAR heterodimer complexed with corepressor(s) has a low affinity for DNA (Minucci et al., 1997). Ligand binding to RAR

stimulates displacement of the co-repressor(s) after which the heterodimer gains access to the RARE. Subsequent association with a co-activator(s) may stimulate interaction with the general transcription factors (GTFs) leading to transcriptional activation. However, binding of the ligand to the RXR partner does not by itself allow displacement of the co-repressor and does not allow heterodimer access to DNA. Binding of the ligand to both of the RXR/RAR heterodimer partners results in displacement of the co-repressor and association of multiple co-activators that provides greater stability in heterodimer DNA binding and interaction with GTFs leading to a higher transcriptional response.

The promoter region of the RAR β -2 gene contains three copies of this DR5. Induction of the RAR β 2 gene by RA has been well-documented in many cell lines including F9 (Sucov et al., 1990) (De The et al., 1989) and P19 cells at the level of transcription via a RARE present in the P2 RAR β 2 promoter (Mendelsohn et al., 1994a).

Each of the RAR subtypes themselves have multiple isoforms generated as a result of the existence of two promoters. Additionally, the appearance of splice variants increases the number of subtypes. The isoforms share a common motif with each isoform for a given RAR type diverging only in the 5' untranslated region and the A region (figure 1-3). Furthermore, the expression of the second of each RAR isoform (ie., RAR β 2, α 2, γ 2) is modulated by RA through

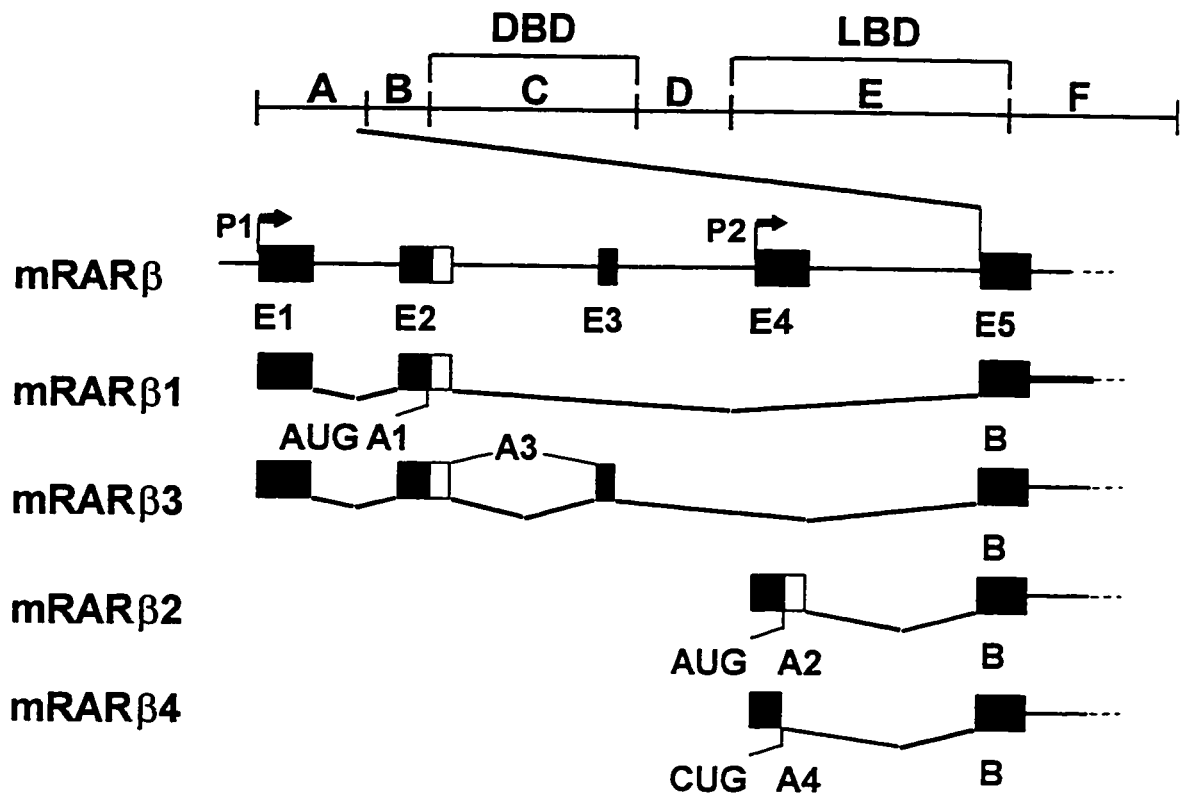


Figure 1-3: Structure of the RAR β gene. The mouse retinoic acid receptor beta gene (RAR β) has four isoforms generated by alternative splicing which differ only in the A region. The RAR β 1 and RAR β 3 isoforms are driven from the P1 promoter whereas the RAR β 2 and RAR β 4 isoforms are driven from the P2 promoter which contains a RARE. (DBD) DNA binding domain, (LBD) ligand binding domain, (RARE) retinoic acid response element), (E1, E2...) exon1, exon2..., (mRAR β) mouse retinoic acid receptor beta.

RAREs present in the promoter regions of each of these genes, suggesting a further level of interactive signalling in this transduction pathway. Expression of RAR β in the mouse embryonic heart at the 8-somite stage (Livrea and Packer., 1993) has led us to investigate the expression of RAR β in these mixed cell populations.

Another mechanism, explaining the appearance of RAR β 1/ β 3 transcripts which are driven by the P1 promoter, demonstrates that accumulation of β 1/ β 3 transcripts by RA is not due to increased transcript initiation, but due to an RA-dependent release of a block in RNA chain elongation (Mendelsohn et al., 1994a). Clearly, transcriptional induction is not the sole mechanism by which retinoic acid exerts its developmental effects.

In a previous study, transgenic mice were generated in order to determine the expression of an RA-induced reporter construct in the developing mouse embryo (Rossant et al., 1991). Three copies of the oligonucleotide encoding the RARE were inserted upstream of the hsp68 promoter containing the lacZ gene. The RAREhsplacZ construct was then injected into the fertilized eggs of mice. Reproducible patterns of activation of this construct were established and suggest that at least one of the effects of RA are mediated by the direct interaction of the RARs and their cognate response elements. This study indicates that the RARE present in the RAR β 2 promoter region can direct specific spatial and temporal expression of an hsplacZ transgene during mouse

embryogenesis. Interestingly, the pattern of expression of the transgene does not entirely mimic the expression of the endogenous RAR β gene during development. Early during embryogenesis, the transgene shows a more spatially restricted pattern of expression and a more precise time of onset of expression than reported for any of the RARs. However, later in development, the pattern of expression of the transgene became more complex and tissue restricted. These contradictory observations in levels of expression of the transgene and the endogenous RAR β gene suggest a possible post-transcriptional mechanism in the regulation of RAR β gene expression.

Part B - Control of mRNA stability

Messenger RNA(mRNA) degradation in eukaryotic cells is important in determining the levels and regulation of gene expression. Recently, many different, yet somewhat related mechanisms by which eukaryotic mRNAs are degraded have been elucidated. One mRNA decay pathway is initiated by shortening of the poly(A) tail followed by decapping and 5' to 3' exonucleolytic degradation of the transcript (Peltz et al., 1991) (Decker and Parker., 1994) (Bernstein and Ross., 1989). The length to which a poly(A) tail must be shortened for subsequent decay may differ between mammals and yeast. It is possible that deadenylation might lead to the loss of the poly(A)-binding protein (PABP) which might trigger further decay events (Shyu et al., 1991) (Chen et al., 1994).

In yeast, experiments suggest that deadenylation of the poly(A) tail to an oligo(A) tail exposes the RNA allowing it to become a substrate for a decapping reaction, thus exposing the transcript to 5' to 3' exonucleolytic decay. An important observation is that 5' to 3' decay is blocked by either deletion of an exonuclease gene (XRN1) (Larimer and Stevens., 1990) or insertion of secondary structure mRNA fragments that lack the cap structure and accumulate after deadenylation. The observation that deadenylation can result in decapping of the mRNA (Muhlrad et al., 1994) (Muhlrad et al., 1995) suggests some sort of interaction between the 5' and 3' ends. Even though there is no direct evidence

for this phenomenon, the abundance of circular polysomes seen in electron micrographs suggests that the 5' and 3' ends may be closely associated.

mRNA decay may also be initiated by decapping and 5' to 3' decay of the transcript independent of poly(A) tail shortening. An example is the degradation of the yeast PGK1 mRNA containing an early nonsense codon (Hagan et al., 1995). The generation of the destabilizing signal requires both the failure to translate a significant part of the coding region and the presence of specific sequences downstream of the nonsense codon (Zhang et al., 1995).

Eukaryotic mRNAs can be degraded via endonucleolytic cleavage prior to deadenylation. This is found to occur from experimental observations of transcripts such as the transferrin receptor (TfR), and *Xenopus* Xlhbox2B mRNA where mRNA fragments are detected in-vivo that correspond to the 5' and 3' portion of the transcript and are consistent with internal cleavage within the 3' UTR (Brown et al., 1993) (Binder et al., 1994).

Another closely related pathway to degradation entails 3' to 5' decay after shortening of the poly(A) tail. However, it should not be expected that deadenylation necessarily exposes transcripts to 3' to 5' degradation since several mRNAs can be quite stable with essentially no poly(A) (Decker and Parker., 1993) (Chen et al., 1994). Importantly, an individual transcript can simultaneously be a substrate for more than one mechanism of decay (Muhlrad

et al., 1995) (Higgs and Colbert., 1994). This observation implies that mutations that inactivate one pathway may not always have significant effects on mRNA stability. Several sequence elements that promote rapid poly(A) shortening have been identified. These include sequences within the c-fos coding region and 3'-UTR which contains a prototypical AU-rich element (ARE) (Chen et al., 1994) and in the yeast MFA2 3'-UTR (Muhlrad and Parker., 1992). Several proteins have been identified which interact with the ARE (Zhang et al., 1993) (Katz et al., 1994). However, only one of these proteins, ARE/poly(U) binding factor has been shown to stimulate decay in vitro (Brewer., 1991).

The conservation of an adenylate/uridylate (AU)-rich region was first noted for genes coding for cytokines. (Caput., 1986) The first direct experimental evidence that the ARE can function to promote mRNA destabilization came from inserting a well-conserved region of 5' nucleotides containing AUUUA motifs from the 3' UTR of human granulocyte-macrophage-colony-stimulating factor (GM-CSF) (Shaw and Kamen., 1986). mRNA was inserted into the 3' UTR of β -globin mRNA. The otherwise stable β -globin mRNA was destabilized.

AREs vary in size from 50 to 150 nucleotides and generally contain multiple copies of pentanucleotide AUUUA. Some important observations concerning the necessity of AREs have recently been made. First of all, the presence of AUUUA motifs even in an AU-rich region does not guarantee a destabilization function of an ARE (Chen and Shyu., 1994). Secondly, AUUUA motifs play a

critical role in determining the destabilizing ability of c-fos, and GM-CSF AREs. For example the AUUUA motifs in the c-fos ARE together have an additive effect on destabilization as successive mutations of each motif leads to gradual loss of destabilizing function (Chen et al., 1994). Analysis of the c-fos ARE has demonstrated the importance of a large number of U residues. Also, several non-functional AU-rich sequences can be converted to potent destabilizing elements by placing the c-fos U-rich domain II immediately downstream (Chen and Shyu., 1994).

It is thus now clear that AUUUA need not be an integral part of a functional ARE, rather each ARE represents a combination of functionally and structurally distinct sequence motifs or domains such as AUUUA motifs, UUAUUUA(U/A)(U/A) nanomers, U stretches, and/or U-rich domains. The combination of these sequence features within an ARE determines the level of destabilizing function.

In order to understand how various AREs function, the role of various trans-acting factors (binding proteins) that interact with the AREs will have to be assessed. Numbers of, apparently distinct proteins (Zhang et al., 1993) (Katz et al., 1994) (Brewer., 1991) (Bohjanen et al., 1991), have been described that bind with high affinity to RNAs containing AUUUA repeats and/or U-rich regions. As of yet there is no direct evidence that any of the ARE-binding proteins play a role in mRNA degradation or stabilization in vivo, however, the binding activity of

ARE-binding proteins can correlate inversely or proportionately with the stability of mRNA that have an ARE.

The complexity of the pathways in which mRNA degradation occurs is attributed to both the deadenylation-dependent and deadenylation-independent pathways that exist. An integrated model of mRNA turnover may be proposed in the following way: all polyadenylated mRNAs would be degraded by the deadenylation-dependent pathway by default in association with deadenylation-independent mechanisms specific to individual mRNAs or classes of mRNAs. The overall rate of mRNA decay would be a function of its susceptibility to these pathways (Decker and Parker., 1994).

Part C - P19 Embryonal Carcinoma (EC) Cell line

P19 cells have been used for these experiments because the EC system is now assumed to be a reasonable substitute for the embryo for developmental studies. The questions that now arise concern the ability of a cell to commit to a specific cell lineage such as neuron or muscle. An approach to solving this problem is proposed by observations that allow various drugs to direct the differentiation of an EC cell line into different cell types.

The P19 cell line is an EC cell line derived from a primary teratocarcinoma formed by grafting a 7.5 day embryo into an adult testis (McBurney and Rogers., 1982). These cells are pluripotent, grow rapidly in culture, and can form a variety of embryonic tissues when injected into blastocysts.

Little or no differentiation results from aggregation of P19 cells, followed by four or five days in suspension, and finally by plating onto tissue culture grade surfaces. The majority of cells remain as EC cells (figure 1-4) and a few differentiate to form cells that resemble extraembryonic endoderm.

P19 cells can however be induced to differentiate extensively in culture by exposing the cells to certain drugs (McBurney et al., 1982) (Jones-Villeneuve et al., 1982) (Edwards et al., 1983), including dimethylsulfoxide (DMSO) and

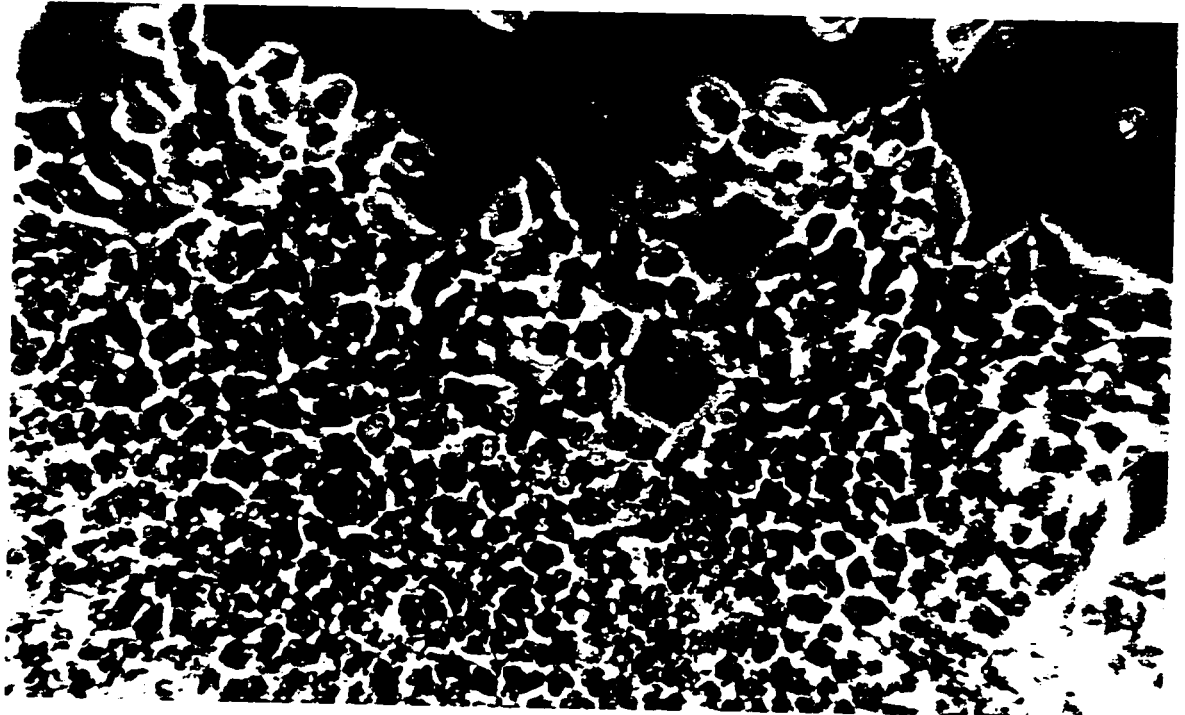


Figure 1-4: Proliferating P19 cells. Cells are maintained in 3:1 DBS/FBS α -MEM medium for a period of 2 days to attain confluence. This figure is representative of cells maintained in the exponential growth phase for a period of 1.5 days

retinoic acid (RA). For both DMSO and RA, the drugs need only be present for the first forty-eight hours of culture aggregation to be effective.

In the presence of 1% DMSO, aggregates of P19 cells develop large amounts of muscle tissue. (McBurney et al., 1982). Six to seven days after the experiment is initiated by cell aggregation and exposure to DMSO, cardiac muscle is present and contracting rhythmically (Figure 1-5). By day 9, skeletal muscle becomes evident. The number of myosin-containing cells reaches 10-15% by 8-9 days. The other cells in DMSO-treated cultures have not been well-characterized. Some have cytokeratin-containing intermediate filaments, indicating that they are epithelial in origin. The majority of the other cells are probably mesodermal derivatives due to indications of abundant and probably elastic fibers. A few EC cells remain (1%) in these cultures, but no neurons or glia are present.

The second group of drugs effective in influencing P19 differentiation are the retinoids. Exposure of P19 cells to RA at 1 μ M concentrations results in the formation of neurons, astrocyte glial cells, and fibroblast-like cells (McBurney et al., 1982) (figure 1-6).

The DMSO effect seems to be unique to P19 cells, where the effect of RA is more widespread, effecting differentiation in a variety of cell lines. DMSO is of no physiological significance, however retinoids are naturally occurring and are

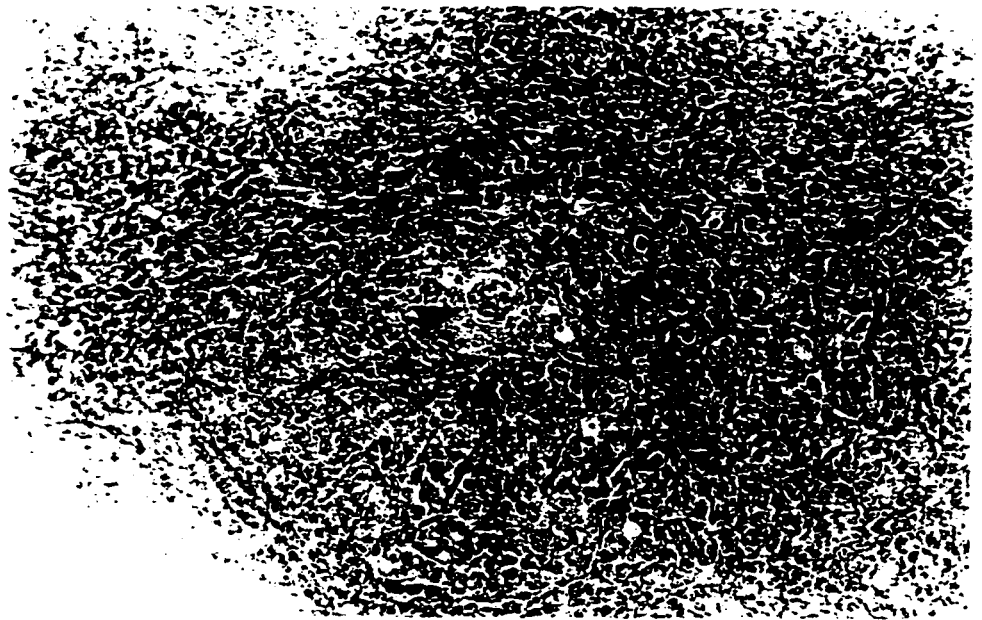


Figure 1-5: Beating cardiac muscle in DMSO-differentiated P19 cells: P19 cells were aggregated in bacterial plates for a period of four days in 1% DMSO. The cells were then plated and maintained on tissue culture plates without DMSO for 3 additional days. These cells are representative of day 7 differentiated P19 cells within which there are mesodermal and endodermal cell types including beating cardiac muscle(▲).

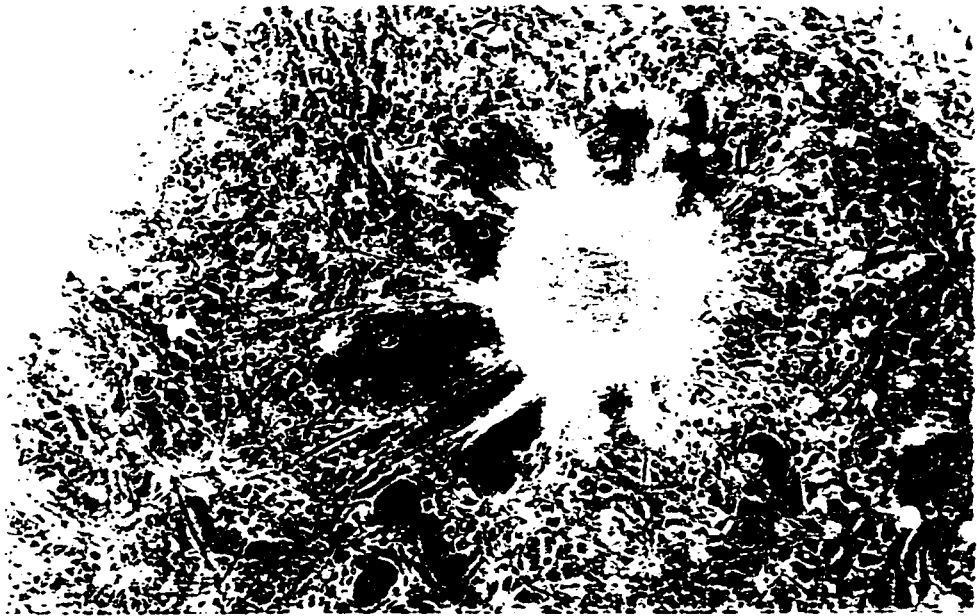


Figure 1-6: Neuron formation in RA-differentiated P19 cells: P19 cells were aggregated in bacterial plates and exposed to 1 μ M RA for a period of four days. The cells were then plated and maintained without RA for an additional four days. This figure is representative of RA-differentiated P19 cells on day 8 of the differentiation process in which neuroectoderm is predominantly formed and neuronal projections are clearly present from the differentiated cell aggregates.

believed to play determinant roles in development. Both of these drugs are believed to act by "inducing" uncommitted EC cells to differentiate along a limited number of developmental avenues.

It has been previously shown that a mutant of the P19 cell line does not differentiate in response to RA (Pratt et al., 1990). This mutant cell line (RAC65) expresses an RAR α gene (RAR α') that is truncated in the C-terminus region and behaves as a dominant negative repressor of RA-induced gene expression. The DNA binding region of the RAR α' gene is intact, however, the RA-binding domain has been partially deleted and thus acts as a dominant negative repressor of RAREs containing promoters. In this RAC65 cell line, RA-induced expression of Hox 1.6, Hox 1.4 and Hox 1.3, is markedly decreased while RAR β induction is equivalent to untreated cells (Pratt et al., 1993). The RAR β transcript is not apparent in northern blots for untreated cells, yet is present in RA treated RAC65 cells, suggesting regulation of the transcript by posttranscriptional mechanism(s).

Is RAR β expressed exclusively in differentiating cardiac myocytes? As mentioned previously, differentiation of P19 cells with DMSO results in a variety of cell types including cardiac and skeletal muscle. We wish to determine whether expression is ubiquitous or restricted to one cell type. In order to accomplish this, we will determine messenger RNA levels of RAR β in the mutant

P19 cell line D3 which is unable to differentiate in DMSO as well as in myoD expressing P19 cells which form skeletal muscle upon aggregation.

Part D - Brachyury and Id in Muscle Development

In the vertebrate embryo, cardiac muscle derives from the splanchnic mesoderm, part of the lateral mesoderm. The Brachyury gene is essential for the development of posterior mesoderm in the mouse. Embryos, lacking a functional Brachyury gene die at 10 days of gestation due to insufficient mesoderm formation (Beddington et al., 1992). Since no Brachyury transcripts are found in adult tissues, those areas affected by its mutation require the protein for mesoderm formation or maturation.

In *Xenopus* embryos, mesoderm-inducing factors such as activin A and basic FGF have been shown to induce Brachyury expression rapidly and in the presence of cycloheximide (Smith et al., 1991). These results indicate that this is an immediate-early response to mesoderm induction. Additionally, injection of Brachyury mRNA results in ectopic mesoderm development (Cunliffe and Smith., 1992).

The P19 line of EC cells (McBurney and Rogers., 1982) when aggregated and differentiated with DMSO shows transient bursts of Brachyury expression which precede the appearance of markers of cardiac muscle (Vidricaire et al., 1994). The presence of Brachyury is aggregation, but not DMSO dependent. Mesoderm only forms with exposure to DMSO following aggregation, thus it is postulated that DMSO enhances the cells response to Brachyury protein,

possibly through the release of calcium from intracellular stores (Morley and Whitfield., 1993). In this same study, Brachyury mRNA expression in RA differentiated cells is restricted to those cells exposed to intermediate RA concentrations that induce mesodermal differentiation.

The basic helix-loop-helix (bHLH) proteins play an important role during embryonic development and cellular differentiation. The basic domain is required but not sufficient for the DNA binding activity of these proteins, because dimer formation is also required. Most bHLH proteins bind to a DNA sequence (CANNTG). It has been shown that, depending on the type of dimer formed (homodimer versus heterodimer), these proteins bind to DNA with altered affinities and sequence specificities (Blackwell and Weintraub., 1990) (Davis et al., 1990) (Murre et al., 1989). Generally, the binding affinity of the heterodimers is much higher than that of the homodimers for a given DNA site.

Several members of the bHLH family of transcription factors appear to play a pivotal role in mesodermal development including MyoD (Weintraub et al., 1991), and Twist (Wolf et al., 1991). The function of mammalian HLH proteins has been most clearly established in muscle cells. Understanding the mechanisms that regulate muscle differentiation in tissue culture led to the discovery of 4 myogenic skeletal muscle-specific bHLH proteins: MyoD (Davis et al., 1987), myogenin (Wright et al., 1989) (Edmondson and Olson., 1989), Myf-5 (Braun et al., 1989), and MRF4 (Braun et al., 1990) (Miner and Wold., 1990)

(Rhodes and Konieczny., 1989). The DNA binding activity of tissue-restricted HLH proteins such as MyoD may be modulated by their interaction via the HLH domain with more widely expressed HLH members such as E12 and E47 (Murre et al., 1989a) which form heterodimers with tissue-specific bHLH proteins (Lassar and Weintraub., 1992).

A subset of HLH proteins, the Id family, lack the basic DNA-binding domains characteristic of bHLH proteins. The Id proteins are thought to inhibit the transcriptional effect of bHLH proteins through dimerization. Such Id/bHLH heterodimers are incapable of binding to DNA. Four Id genes have been isolated: Id1 (Benezra et al., 1990), Id2 (Sun et al., 1991), Id3 (Christy et al., 1997), Id4 (Reichmann et al., 1994).

Id genes are highly expressed in undifferentiated growing cells as well as in some tumor cells, but down-regulated in quiescent cells and during differentiation (Barone et al., 1994)(Kreider et al., 1992) (Jen et al., 1992) (Biggs et al., 1992) (Christy et al., 1997) (Benezra et al., 1990). It has been postulated that Id proteins may serve as general inhibitors of differentiated states by blocking the activity of bHLH proteins that are required for specific differentiation programs.

The fact that all known Id proteins can inhibit the binding of MyoD/E2A protein complexes to the E-box of the MCK enhancer (Reichmann et al., 1994) (Christy

et al., 1997) (Sun et al., 1991) (Benezra et al., 1990) supports the hypothesis that the HLH proteins without basic domains may be involved in inhibitory myogenesis. Consistent with a role in myogenesis, Jen et al., 1992 demonstrated that overexpression of the Id1 gene in the C2C12 murine muscle cell line leads to a delay in the onset of muscle cell differentiation.

Although skeletal and cardiac muscle express many of the same muscle-specific genes, skeletal muscle bHLH proteins are not expressed in the heart. However, indirect evidence suggests that bHLH proteins may participate in the control of cardiac muscle gene expression (Litvin et al., 1993). Heart formation during vertebrate embryogenesis involves the commitment of mesodermal precursor cells to the cardiac lineage and the subsequent formation of a primitive heart tube, which undergoes looping, formation of the outflow tract, and atrial and ventricular cavities and septation to form the mature four-chambered heart. Very little is actually known of the genetic pathways controlling cardiac morphogenesis or differentiation.

What are the effects of retinoic acid on P19 cells that have entered or will enter the cardiac myogenic program? Retinoic acid has been shown to have inhibitory effects on the expression of the skeletal muscle determination factor *myf5* while it enhances the expression of *myoD* and *myogenin* in C2.7 mouse myogenic cells (Carnac et al., 1993). Excess RA has many teratogenic effects on cardiac development *in vivo*. Depending on the animal model system and timing of the

addition of RA these effects vary. RA-induced cardiac truncation is seen in the chick. Increasing doses of RA result in deletion of the ventricles, the atrium and the sinus venosus. Excess RA is also associated with aorticopulmonary septation failure (Zelent et al., 1991). Since P19 cells constitutively express the RAR α and RAR γ receptors (Pratt et al., 1990), while expressing the RAR β during cardiac muscle differentiation, these cells should provide an *in vitro* model of the molecular effects of RA on cardiac myogenesis and function.

Since the Brachyury gene is known to be necessary for mesoderm formation in both *Xenopus* and the mouse and that *Id* is an inhibitor of muscle cell differentiation, we will investigate the ability of high concentrations of retinoic acid to alter their expression levels in P19 cells destined to differentiate into mesodermal and endodermal cell types, including beating cardiac muscle. These results should indicate whether the Brachyury and *Id* genes are sufficient for cardiac muscle formation in the face of a potent teratogen such as retinoic acid. We will also observe whether beating cardiac muscle or neuroectoderm is formed when RA is added at various times during the differentiation process.

Chapter II - General Materials and Methods

A. Cell Culture

P19 and RAC65 cells were maintained in α -MEM (Gibco/BRL) supplemented with 2.5% fetal bovine serum (FBS)(Cansera) and 7.5% donor bovine serum(DBS)(Cansera). Cells were plated at density sufficient to attain confluence in two days. At this point, the media was removed by aspiration, and the cells washed twice with 1X phosphate buffered saline (PBS) solution. 2ml of trypsinizing solution (0.1% trypsin, 1mM EDTA in 1X PBS) was then added to the cells, and incubated for 1 minute at 37°C. The cells were subsequently resuspended in the trypsinizing solution with a pasteur pipette and suction bulb. The trypsinizing solution was neutralized by adding the solution of single cells to new cell culture plates containing fresh media.

B. Cell Experiments

RA-Differentiation

Retinoic acid can be present for as little as 4 hours and still be effective in inducing the irreversible differentiation of virtually all cells in a culture (Berg and McBurney. 1990). Both P19 and RAC65 cells were exposed to a final concentration of 1 μ M *all-trans* retinoic acid (*t*-RA) dissolved in ethanol for a period of 24 hours before total cellular RNA isolation.

DMSO-Differentiation

At concentrations of 0.5%-1% (v/v) DMSO efficiently induces P19 cell aggregates to develop into a wide variety of mesodermal and endodermal cell types (McBurney and Jones-Villeneuve et al., 1982); (Edwards and Haris et al., 1983). P19 and RAC65 cells were aggregated in the presence of 1% DMSO in 10ml bacterial plates for a period of four days with a media change after 48 hours. Following this, the cells were plated on tissue culture dishes with standard α -MEM (2.5% FBS, 7.5% DBS) supplementation for the remaining period of differentiation.

C. Transfection Experiments

DNA uptake by cells in culture is markedly enhanced when the nucleic acid is presented as a calcium phosphate-DNA co-precipitate (Graham and van der Eb, 1973). A more highly efficient protocol for introducing DNA into these cells involves allowing the calcium phosphate-DNA coprecipitate to form slowly in the tissue culture medium during prolonged incubation (6 hours) and under controlled conditions of pH and CO₂ tension (Chen and Okayama, 1987). 9 μ g of RARE β -CAT DNA plus 6 μ g of pgk-puromycin plasmid was mixed with 0.5ml of 0.25 M CaCl₂. 0.5 ml of 2X BES-buffered saline (50mM BES (N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid), and 280mM NaCl, 1.5mM Na₂HPO₄(2H₂O)) was added dropwise to this mixture and left to stand for 20 minutes at room temperature. The CaCl₂/DNA/BES-buffered saline solution was added to the media dropwise while gently swirling. The cultures were then

incubated for 6 hours at 37°C in a humidified incubator with a 5% CO₂ atmosphere. After 6 hours, the media was removed by aspiration and rinsed once with fresh media. Fresh media was then added and the culture incubated for 24 hours at 37°C in a humidified incubator in an atmosphere of 5% CO₂. Following 18-24 hours of incubation in nonselective medium to allow expression of the transferred gene(s) to occur, the cells are trypsinized and replated in a selective medium containing 2µg/ml of puromycin.

D. Assay for chloramphenicol acetyltransferase activity

The chloramphenicol acetyltransferase (CAT) enzyme is a widely used reporter gene for indirect assay of promoter activity in transfected mammalian cells. To assay putative promoters in mammalian cells, a derivative of pSV2CAT has been constructed (pSVOCAT (Gorman and Moffat et al., 1982)) in which the promoter region of SV40 is replaced by the promoter being tested. Acetylated forms of chloramphenicol produced by the activity of CAT can be measured at the endpoint of an enzymatic reaction by liquid scintillation.

On different days of differentiation, following transfection of RAREβ2-CAT, either with 1µM RA or 1% DMSO, the medium was removed and the cells washed 3 times with 10ml 1X PBS. The dishes were let to stand at an angle for 2-3 minutes to allow the last traces of PBS to drain to one side. The last traces of PBS were removed by aspiration. 200µl of 0.25M Tris(pH 7.8) was added to each plate and the cells were scraped into a 1.5ml microfuge tube and stored at

-20°C. Analysis of the samples involves freeze/thawing the cell suspension 3 times with gentle vortexing during the thawing phase. Equivalent amounts of protein from each sample (50-100µg) was made up to 30µl and mixed with 20µl of 100mM Tris-Cl(pH 7.8) and heated for 10 minutes at 65°C to inactivate deacetylases in the extract. The samples were then centrifuged at 12,000 rpm for 10 minutes at 4°C and the supernatant was removed and transferred to a fresh tube. The sample was then transferred to a water bath set at 37°C. The reaction involved adding 27µl of 1.0M Tris pH 7.8, 64µl of 5mM chloramphenicol, 67.4 µl H₂O, 1.5µl ³H-acetyl CoA, 40µl cell extract and heating for 1 hour at 37°C. Following this incubation, the samples were put on ice and extracted twice with 200µl ice-cold ethyl acetate, vortexing for 1 minute each time. The upper phase was added to a scintillation tube containing 3ml of scintillation fluid. This extraction procedure was then repeated and the upper phase added to the scintillation tube. The amount of radioactivity was then determined by liquid scintillation counting.

E. Plasmid Constructs

All expression plasmids were based on the promoter sequences from the mouse pgk gene (Adra and Boer et al., 1987). The RAREβ-CAT reported construct was the gift of Dr. Henry Sucov (Salk Institute) and consists of a 3.5 kb Xho1-Sal1 fragment containing 3kb of promoter sequence driving the CAT gene. Thanks to Dr. Magnus Pfahl (LaJolla) for mouse RARβ cDNA, and Dr. Michael McBurney

(Ottawa) for the pgk-myo-D expression vector, the P19 mutant D3 cell line and the cardiac actin cDNA.

F. RNA Isolation

Total cellular RNA was isolated by the lithium chloride-urea method. Media was removed from the cells by aspiration and the cells were then washed twice with 1X PBS. 2ml of 3M LiCl/8M Urea was then added to the cells and stored at -20°C. The cell culture plates were scraped with a rubber policeman into a 15ml polypropylene falcon tube. The cell suspension was then polytroned at 21,000 rpm for two minutes at 4°C and then stored at 4°C overnight. The following day, the suspension was centrifuged at 7000 rpm for 30 minutes at 4°C. The supernatant was then removed by aspiration and 0.5ml 3M LiCl was added to the remaining pellet. Using 26 gauge syringes, the pellet was resuspended in 3M LiCl and transferred to a 1.5ml eppendorf tube. The 15ml falcon tube was then rinsed with 0.5ml 3M LiCl, that was then added to the eppendorf tube. This eppendorf tube was then centrifuged at 12,000 rpm for 15 minutes at 4°C to pellet the selectively precipitated RNA. The supernatant was removed by aspiration and washed with 70% EtOH before resuspending in 200µl diethylpyrocarbonate (DEPC)-treated H₂O (RNase free). 20µl of this solution was mixed in 980µl DEPC H₂O and added to a quartz cuvette for the spectrophotometric quantitation of mRNA at 260 and 280 nm (Sambrook and Fritsch et al., 1989).

G. Northern Blot Analysis

20 μ g of total cellular RNA was separated on 1% agarose-formaldehyde gels (15ml 10X RNA gel buffer (200mM MOPS, 50mM NaOAc-3H₂O, and 10mM EDTA), 7.6ml formaldehyde, and 1.5g agarose in 127.4ml H₂O). RNA samples were precipitated with ethanol and sodium acetate, and resuspended in 30 μ l RNA sample buffer (50% deionized formamide, 10% formaldehyde, and 1X RNA gel buffer). 4 μ l RNA loading buffer (containing 5% ethidium bromide) was then added to the samples which were then electrophoresed for 2-3 hours at 120V in a running buffer containing 5% formaldehyde and 1x RNA gel buffer. The separated RNA was then transferred overnight with a 20X SSC solution to a nylon membrane (MSI). Following transfer, the blot was then UV crosslinked and stored in a plastic bag at 4°C.

The UV crosslinked blots were incubated in hybridization solution (50% deionized formamide, 25% 20X SSPE, 1% SDS, 10% 50X Denhardt's solution, and 5% salmon sperm DNA (5ml/ml)) for at least two hours. Probe labeling was carried out using the Prime-It Random Primer Labeling Kit (Stratagene). 50-100ng of template DNA in a final volume of 45 μ l of H₂O was denatured at 100°C for five minutes. The denatured DNA was then quick cooled on ice and added to the pre-made reaction tube containing enzyme, buffer, and nucleotides. 50 μ Ci of [α -³²P]dCTP was then added to the reaction mixture and incubated for 10 minutes at 37°C. The radiolabelled probes were then denatured again at 100°C for five minutes and quick cooled on ice before adding

to the hybridization solution. The hybridization was carried out overnight at 42°C in a water bath. Following hybridization, the membranes were washed twice for 20 minutes in 2X SSPE and 0.1% SDS at room temperature and then twice for 5 minutes in 0.1X SSPE and 0.1% SDS at 65°C. The membranes were then exposed to DuPont film at -80°C.

Re-probing of the blots was accomplished by stripping the blots at 65°C for 1 hour with a mixture of 50% DF and 2X SSPE in H₂O. The blots were then washed for 1 minute in 0.1X SSPE at room temperature. Pre-hybridization was then carried out as previously outlined.

H. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The reverse transcriptase reaction was performed by adding 1 µg of mRNA to 4 µl random hexamers(1:100 dilution), 6.5 µl DEPC H₂O, and 0.5 µl RNase inhibitor. This mixture was then heated to 70°C for 10 minutes and quenched on ice. 4 µl first strand buffer (Gibco/BRL), 1 µl 10mM dNTPs, 2 µl DTT (0.1M), and 1 µl Reverse Transcriptase (Gibco/BRL) was then added to the reaction mixture and incubated at 42°C for 1 hour. At the end of the reaction, the enzyme was heat inactivated at 95°C for 5 minutes and quenched on ice.

An 5 µl aliquot of this 20 µl reaction was then used in a 100 µl PCR reaction. The reaction consisted of 10 µl of 10X PCR buffer, 2 µl 10mM dNTPs, 3 µl 50mM

MgCl, 5 μ l primers (10 μ M each), 0.5 μ l Taq polymerase and 69.5 μ l ddH₂O. The mixture was overlaid with 70 μ l mineral oil. Tubes were then pulse centrifuged to collect the contents at the bottom of the tube. The tubes were incubated initially for five minutes at 94°C to completely denature the DNA template. Following this step, 30 cycles of PCR amplification were completed as follows: denaturing at 94°C for 45 seconds, anneal at 55°C for 30 seconds, extend at 72°C for 90 seconds. Extension of the template was accomplished by incubating for an additional 10 minutes at 72°C and maintained at 4°C. Samples were stored (if necessary) at -20°C.

The common 3' primer that was used to amplify the RAR β 2 and RAR β 1/ β 3 isoforms was derived from the B region of the mRAR β cDNA, amino acids 161-168 (5'-GGCGGACGTCTTCACGAAACTT-3'). Amplification of the RAR β 2 and RAR β 1/ β 3 isoforms require 5' sequence-specific primers. (5'-CGTGGACTTTTCTGTGCGGCTCG-3') was used for amplification of the RAR β 2 isoform while (5'GTGATTCTGGACCTGTAGTAA-3') is the other 5' primer used for amplification of the RAR β 1/ β 3 isoforms. Following amplification, 20 μ l aliquots were electrophoresed on a 1.2% agarose gel and stained with ethidium bromide(EtBr). The RAR β 2 specific probe was fabricated using RAR β 2 specific primers: (5' primer 5'-CGTGGACTTTTCTGTGCGGCTCG-3, 3' primer 5'-GTATGCCGGTGCTGCCATT-3') and a RAR β DNA template. The PCR protocol

used for amplification of the RAR β 2 specific probe was the same as previously mentioned.

I. Southern Blot Analysis

20 μ l aliquots from the 100 μ l RT-PCR reactions were loaded into an agarose DNA gels (1% agarose, 1X TAE (0.04M Tris-acetate, 0.001M EDTA), 2 μ l ethidium bromide) and electrophoretically separated. The gel was then denatured in a 1.5 M NaCl and 0.5M NaOH solution for 45 minutes and then neutralized in a solution of 1.5M NaCl and 1M Tris (pH 7.4) for an additional 45 minutes. The DNA was then transferred to a nylon membrane (MSI), UV cross-linked, blocked with prehybridizing solution, and probed with multi-prime labelled cDNA fragments.

J. Slot-Blotting DNA

The RAR β exon 3 DNA probes consist of a RT-PCR product derived from primers 5'-GCACAGAAAACCTCCACG-3' (nucleotides 389-406) and 5'-AGTATGCCGGTGCTGCCATT-3' (nucleotides 593-611). A Pst1 digest of α -Tubulin, and linearized pTZ18R (Pharmacia) were used as positive and negative controls respectively. Exon 7 from RAR β was fabricated using primers (5'-GGTGGCATGAAATGGCTGAT-3') and (5'-TATACCCCAGAGCAAGACAC-3'). DNA was prepared for slot-blotting by diluting the DNA in TE(pH 8.0) up to 400 μ l and adding 40 μ l 3M NaOH. The samples were then incubated for 45 minutes at 60-70°C (to destroy RNA and denature DNA), cooled to room temperature and

neutralized by adding 440 μ l 2M NH₄OAc(pH 7.0). Following this procedure, the DNA was immobilized by UV-crosslinking onto the nylon membrane. The membranes were then pre-hybridized for at least 2 hours at 42°C prior to addition of RNA probe from nuclear runoff procedure.

K. Nuclear Run-On Analysis

Isolation of nuclei from P19, DMSO and RA-treated cells was performed by removing the medium from the cells and rinsing twice with 5ml ice-cold PBS. The cells were scraped, resuspended in 5ml ice-cold 1X PBS, and collected in 15-ml polypropylene tubes. The cells were then pelleted by centrifugation for 2 minutes at 500xg, and the supernatant removed by aspiration. The pellet was loosened with gentle vortexing for 5 seconds, and 4ml of NP-40 lysis buffer (10mM Tris-Cl, pH 7.4, 10mM NaCl, 3mM MgCl₂, 0.5% (v/v) NP-40) was added while vortexing as the buffer is added. The cells were then incubated in the lysis buffer for 5 minutes on ice and then centrifuged at 1400 rpm for 5 minutes at 4°C to pellet the nuclei. The nuclear pellet was resuspended in 4ml of NP-40 lysis buffer by gently vortexing and centrifuged again at 1400 rpm for 5 minutes at 4°C. The supernatant was then removed by aspiration and nuclei were stored at -100°C in 200 μ l glycerol storage buffer (50mM Tris-Cl, pH 8.3, 40% (v/v) glycerol, 5mM MgCl₂, 0.1mM EDTA).

To perform nuclear runoff transcription, the nuclei were thawed at room temperature and centrifuged at 8000 rpm for three minutes at 4°C. Excess

supernatant was removed by aspiration prior to adding 63 μ l of initial reaction solution (20 μ l 5X reaction buffer(1.5M (NH₄)₂SO₄, 0.5M Tris pH 7.9, 0.02M MgCl₂, 0.02M MnCl₂, 1M NaCl, 2mM EDTA, 0.5mM PMSF), 1.5 μ l 0.1M DTT, 10 μ l 100mM Creatine Phosphate, 1 μ l 100mM GTP, ATP, CTP, 2.5 10U/ μ l RNase inhibitor, 1 μ l 100mM PMSF, 10 μ l 50% glycerol, 15 μ l DEPC H₂O.) and 15 μ l of 10mCi/ml [α -P³²]UTP to pelleted nuclei. The mixture was then incubated for 30 minutes at 28°C with periodic mixing.

Following the transcription reaction, 10 μ l of 9mg/ml DNase(Gibco) and 10 μ l of 11mg/ml tRNA was added to the reaction solution and incubated at 37°C for 15 minutes. Following this, 1ml of TRIzol (Gibco/BRL), and 200 μ l of chloroform was added to the mixture. The mixture was then shaken by hand for 15 seconds and incubated at room temperature for 3 minutes prior to centrifuging at 12,000 rpm for 15 minutes at 4°C. The top layer (aqueous phase) was then transferred to a fresh tube and precipitated by adding 500 μ l isopropanol, and incubating the samples at -20°C for 30 minutes. The samples were then centrifuged again at 12,000 rpm for 10 minutes at 4°C. The supernatant was then aspirated and the pellet resuspended in 250 μ l of 20mM Hepes (pH 7.5), 5mM EDTA, 62.5 μ l 1M NaOH, and left on ice for 10 minutes. The reaction was then quenched to adding 125 μ l 1M Hepes (free acid, pH is around 5.5). RNA was precipitated by adding 50 μ l of 3M NaOAc, 1000 μ l EtOH and leaving at -80°C for 15 minutes. The tubes were then centrifuged at 12,000 rpm for 15 minutes at 4°C to pellet

the RNA. The pellet was washed with 70% EtOH. The RNA pellet was resuspended by heating at 65°C for 15 minutes in 500µl of hybridization solution. A comparative analysis between samples was conducted by measuring the number of counts in a 5µl aliquot. Each sample was then normalized to the sample with the lowest counts. This ensured that equivalent amounts of signal were allowed to hybridize to each membrane. The RNA was then denatured at 85-90°C for 10 minutes and then added to the DNA fragments immobilized onto a nylon membrane and hybridized for 48 hours at 42°C. The membrane was washed with 2X SSC and 0.1% SDS at room temperature, and if necessary, washed with 0.1X SSC, and 0.1% SDS at 65°C for five minutes to remove non-specific binding of RNA probe. The blot was exposed to DuPont film at -80°C for at least one week.

L. Actinomycin-D Experiments

Actinomycin-D is proposed to act by binding to a premelted DNA conformation found within the transcriptional complex. This acts to immobilize (or pin) the complex, preventing the elongation of growing RNA chains (Sobell., 1985). Actinomycin-D is hence useful in blocking transcription within mammalian cells and measuring mRNA half-life. Cells were cultured as previously described. 20µg of actinomycin-D was added to the cells after day 4 and day 7 of differentiation. Total cellular RNA was collected as previously described ranging from 0 to 5 hours following administration of actinomycin-D. 20µg of RNA from each sample was run on a denaturing RNA gel, transferred to a nylon

membrane and probed with a RAR β 2 specific probe. The extent of RAR β 2 hybridization was quantified by photo densitometry (MCID Imaging Research Inc.) of the autoradiogram and results were plotted after normalization for loading on the basis of hybridization to GAPDH. Half-lives were calculated by regression analysis.

M. Calcium Chelating Experiments:

The calcium chelator, EGTA, has been shown to block the induction of Brachyury expression in aggregated P19 cells if present at concentrations higher than that of calcium in normal medium (Vidricaire and Jardine et al., 1994). Cells were differentiated with 1% DMSO as previously described and 2mM EGTA added to the cells 48 hours prior to total cellular RNA isolation. RNA was collected on day 4, 5, 6, and 7 of differentiation.

Chapter III - Results

RAR β mRNA is induced in EC cells differentiated with DMSO

It has been shown that both P19 and the mutant P19 cell line RAC65 can be successfully differentiated in the presence of 1% DMSO. The variable cell types formed include embryonic skeletal and cardiac muscle as well as other endodermal and mesodermal derivatives (Umenoso and Evans., 1989) (Webster et al., 1989). Northern blot analysis (figure 3-1) clearly indicates that RAR β is not produced in any detectable amounts in undifferentiated P19 or RAC65 cells. RAR β is induced in both P19 and RAC65 cells following RA administration within a few hours, although induction is much less pronounced in the latter. Exposure of both P19 and RAC65 cells to DMSO does not induce RAR β expression within 2 days, however, by Day 4 of differentiation, the RAR β transcript is clearly visible. The level of RAR β mRNA reached a maximum at approximately day 7 of differentiation which coincides with the onset of beating muscle.

RAR β 2 is predominantly expressed in differentiated muscle

Reverse transcriptase polymerase chain reaction (RT-PCR) with isoform specific primers that would either indicate the presence of RAR β 1/ β 3 or RAR β 2 isoforms determined which of the four known RAR β isoforms were expressed in these DMSO differentiated cells. These blots were probed with full-length cDNA from

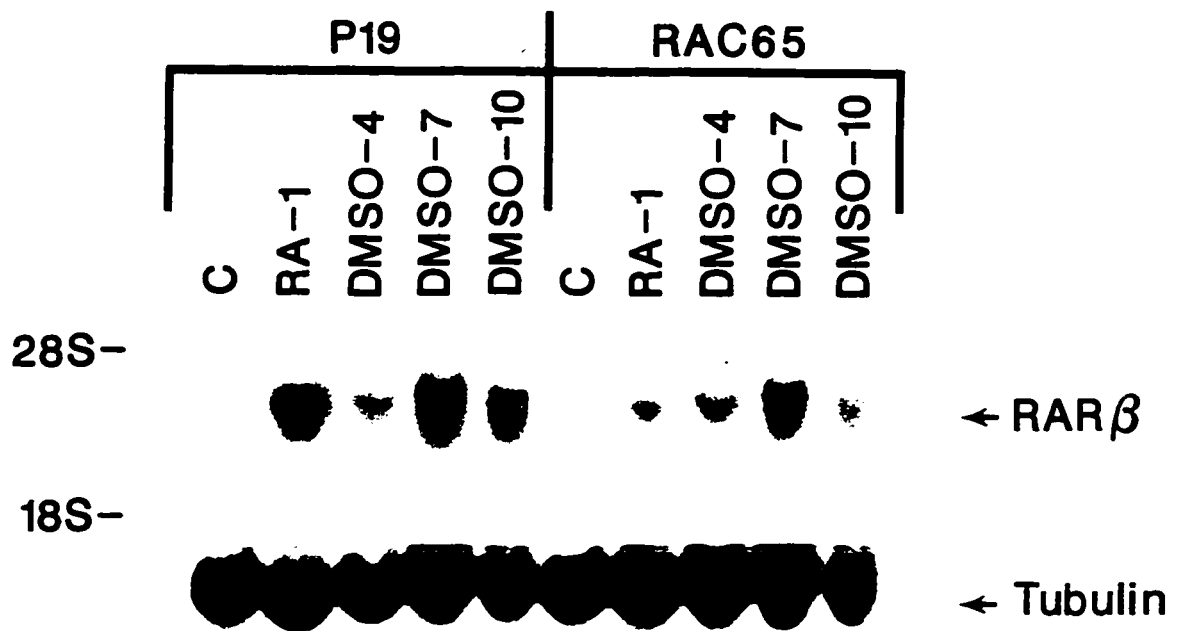
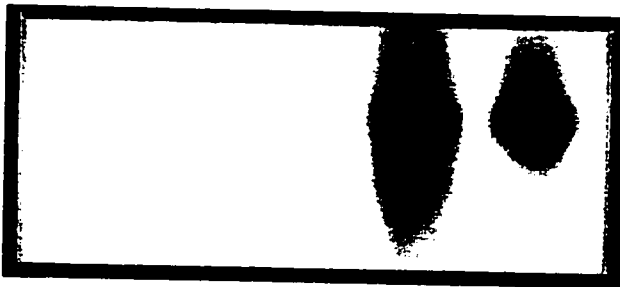
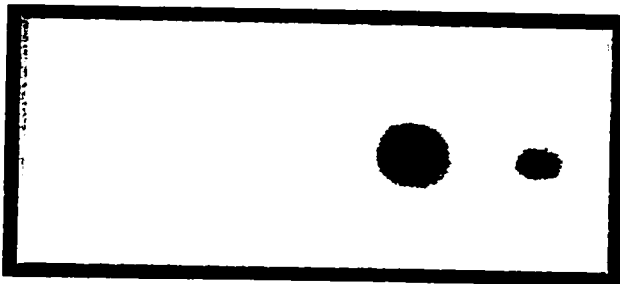


Figure 3-1: RAR β expression in DMSO and RA differentiated EC cells P19 and RAC65 cells(RA nonresponsive) were exposed to either 1 μ M RA or 1% DMSO for neuronal or mesodermal differentiation respectively. Proliferating undifferentiated P19 cells (C) contain undetectable levels of RAR β by northern blot analysis. Alternatively RA differentiation of P19 cells results in a dramatic upregulation of RAR β mRNA. Administration of 1 μ M RA to RAC65 cells results in a slight increase of RAR β indicative of an RA-independent mechanism for RAR β upregulation. Differentiation of both P19 and RAC65 cells with DMSO results in increased RAR β expression in an RA-independent fashion. RAR β expression peaks by day 7, concurrent with the onset of rhythmic contraction in the cardiac muscle cells. An exon 7 probe common to all isoforms was used to identify RAR β expression levels. (C) control, (RA) all-trans retinoic acid, (DMSO) dimethylsulfoxide

W C D R



RAR β 2



RAR β 1/ β 3

Figure 3-2: RAR β 2 and RAR β 1/ β 3 expression in DMSO and RA differentiated P19 cells Southern blot analysis with a probe common to all isoforms in combination with reverse transcriptase polymerase chain reaction (RT-PCR) was conducted with isoform-specific primers for either RAR β 2 or RAR β 1/ β 3 with mRNA isolated from undifferentiated P19 cells (C), DMSO-differentiated P19 cells on day 7 of differentiation (D), or RA-differentiated P19 cells 24 hours after addition of 1 μ M RA (R). From these results it is apparent that the predominant isoform expressed in DMSO-differentiated P19 cells is the RAR β 2 isoform. In order to obtain a sufficient signal to observe RAR β 1/ β 3 expression, the southern blot for these isoforms was exposed approximately 10 times longer than that for the RAR β 2 isoform. (W) water control with no RNA, (C) control RNA from proliferating P19 cells, (D) DMSO-differentiated P19 cells, (R) RA-treated P19 cells.

mRAR β . The results in Figure 3-2 demonstrate that the RAR β 2 isoform is predominantly expressed during DMSO-induced differentiation.

Induction of RAR β in DMSO-differentiated cultures of P19 cells occurs in the absence of an increase in transcriptional activation of the RARE β -2 promoter

In order to assess the involvement of the RARE β promoter in DMSO-induced differentiation, we isolated stable transformants of the RARE β 2-CAT reporter gene construct and differentiated pooled clones of these cells with either 1 μ M RA or 1% DMSO. Figure 3-3 illustrates that RA progressively induced CAT activities starting from day one of differentiation up to a maximal of 188-fold by day 3. RA-induced CAT activity persisted several days after its withdrawal. Transcription from the RARE β 2-CAT reporter plasmid compared with a promoter-less vector transfected control lysate (data not shown) showed that there was no increase in CAT activity in DMSO treated P19 cells at any point during the differentiation process.

DMSO-induced differentiation has no effect on RAR β transcription

Since it was possible that the RARE β 2-CAT construct did not contain a sufficient amount of the promoter region to demonstrate the influence of DMSO on transcription, we performed nuclear run-on analysis of RAR β gene transcription in DMSO differentiated cells. The results in Figure 3-4 show that while there is

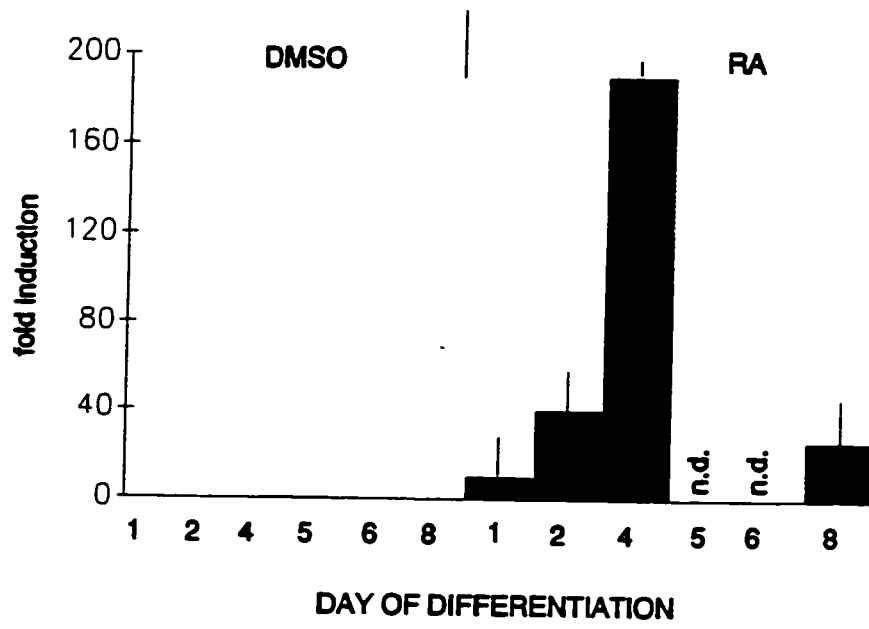
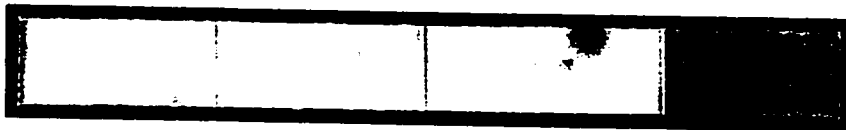


Figure 3-3: Induction of the RARE β 2 promoter in DMSO and RA differentiated cells: P19 cells were stably transfected with the RARE β 2-CAT reporter construct and differentiated with either 1 μ M RA or 1% DMSO. CAT activity was measured from day 1 through day 8 of differentiation for a total of 3 independent experiments performed from one transfection. Results are shown as an average of the three experiments \pm S.E.M.. No induction was seen in DMSO-differentiated RARE β 2-CAT containing P19 cells, however a dramatic induction of CAT activity was seen for those transfected cells differentiated with retinoic acid (maximum of 188 fold on day 4).

D0 D1 D4 RA



RAR β



TUBULIN

Figure 3-4: RAR β transcription in DMSO and RA differentiated P19 cells: Nuclear run-on analysis was performed on P19 cells differentiated with either 1% DMSO or 1 μ M RA. Day 0 and Day 1 of DMSO differentiation showed no significant increase in RAR β transcription. A slight increase was only apparent on Day 4 of DMSO differentiation. Retinoic acid-treated P19 cells were used as a positive control for RAR β transcription 48 hours following administration of 1 μ M RA. A common exon 7 probe was used to identify levels of RAR β expression. α Tubulin was digested with Pst1 and slot-blotted to provide an internal control for the experiment. (D0, D1, D4,) day 1, day 2, day 4 of DMSO differentiation, (RA) 48 hours following 1 μ M RA administration

a very low constitutive level of transcription of the RAR β gene, there is only a very slight increase in RAR β transcription from day 0 to day 4 of DMSO differentiation. Compared to the increase observed for RA-treated P19 cells, this slight increase is not expected to account solely for the dramatic upregulation of RAR β 2 mRNA. Since the level of RAR β expression dramatically increases by day 4 of differentiation (Figure 3-1), the lack of transcriptional induction at this time suggests some other mechanism for the increase in expression.

RAR β is not expressed in D3 mutant P19 cells or in MyoD expressing P19 cells differentiated into skeletal muscle by aggregation

Aggregation and treatment of P19 cells with DMSO results in the differentiation of these cells into a variety of tissue types. While cardiac muscle and skeletal muscle primarily form during differentiation, endoderm and mesoderm are also formed (Edwards et al., 1993) (Rudnicki and McBurney., 1987). While the RAR β 2 isoform appears to be expressed prior to skeletal muscle formation, it is still possible that skeletal cells might express this receptor. In order to determine this, we have differentiated P19 cells which stably express myoD (P19(MyoD)). P19(MyoD) cells form cultures of skeletal muscle without the evolution of other cell types following aggregation in the absence of DMSO (Skerjanc et al., 1994). We have therefore looked at the levels of RAR β mRNA in pooled populations of P19 cells stably transfected with myoD following aggregation to address this question. Figure 3-5 shows that while a large

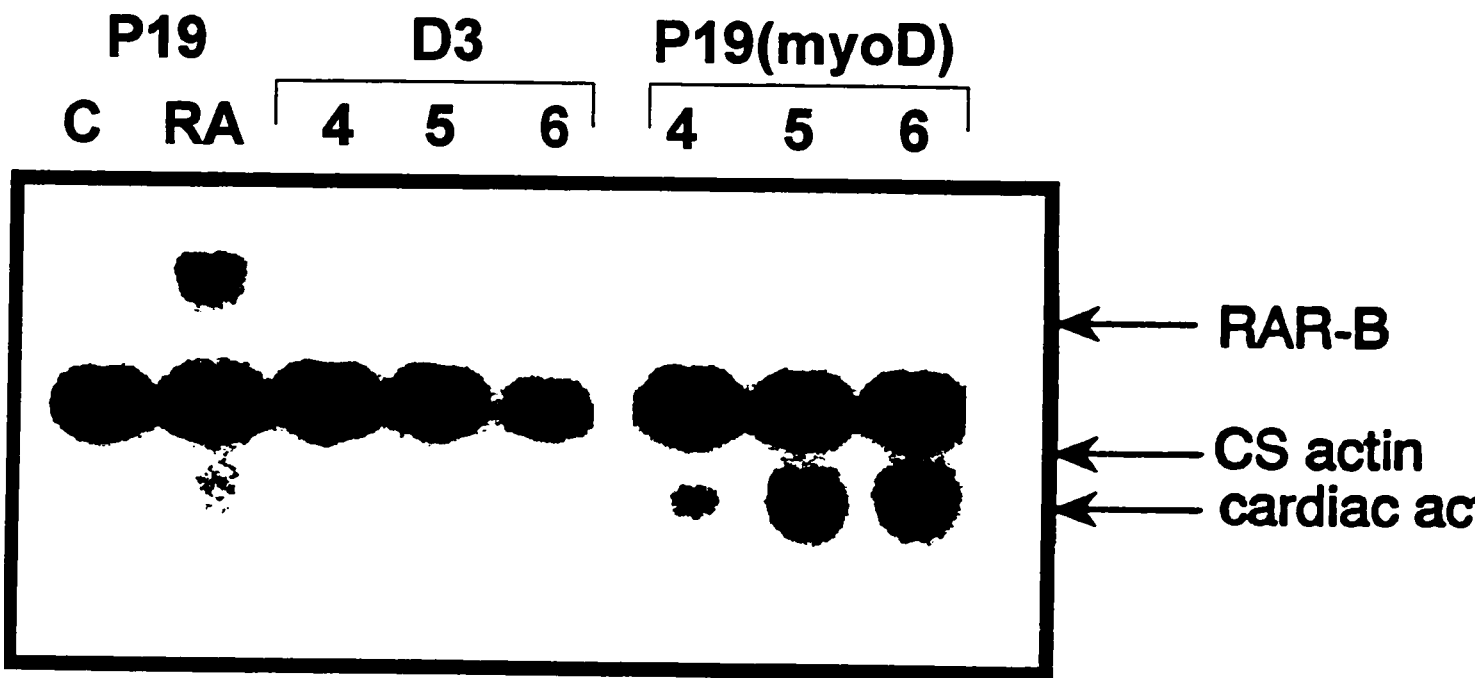


Figure 3-5: RAR β expression in MyoD expressing P19 cells and in D3 aggregated cells: Northern blot analysis was conducted in order to assess which cell type(s) may be responsible for the increased expression of RAR β . P19(myoD) transfected P19 cells were aggregated in the absence of DMSO. Since these cells form cultures of skeletal muscle, as evidenced by the increased expression of cardiac actin, without the evolution of other cell types we were able to conclude that RAR β is not expressed in skeletal muscle that might be formed during the DMSO-differentiation process. The cells of the mutant P19 cell line, D3, which is unable to differentiate into cardiac or skeletal muscle were aggregated and exposed to DMSO. The results showed that no muscle was formed by the apparent lack of cardiac actin demonstrating that RAR β expression is indeed dependant on the presence of muscle. Positive and negative controls were provided by RA-differentiated P19 cells (RA) (48 hours following administration) and undifferentiated P19 cells (C).

proportion of these cells differentiate into skeletal muscle between day 4 and day 6 as evidenced by their expression of increasing levels of cardiac actin, no RAR β mRNA can be seen. Moreover, since this differentiation is achieved by aggregation in the absence of DMSO, it is possible to conclude that the process of aggregation, is on its own, insufficient to induce RAR β expression. As a positive control for RAR β expression, RA-treated P19 cells have been used. A mutant P19 cell line, D3, that is unable to differentiate into cardiac or skeletal muscle following aggregation and exposure to DMSO was used to determine whether the expression of RAR β 2 is dependent on the presence of muscle. Figure 3-5 also shows that when D3 cells were aggregated and exposed to DMSO, no induction of RAR β could be seen on days 4,5, and 6.

RAR β 2 mRNA is not stabilized between day 4 and day 7 of DMSO differentiation

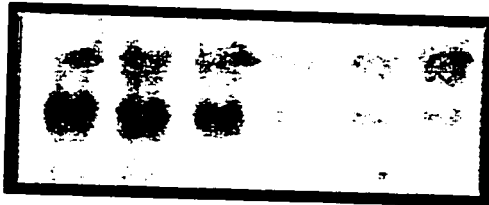
P19 cells were differentiated with 1% DMSO for 4 and 7 days. Actinomycin-D, an inhibitor of transcription, was added at the end of day 4 or day 7 DMSO differentiated P19 cells, and total cellular RNA was collected from zero through five hours. Figure 3-6 is a Northern blot analysis of this experiment probed with a RAR β 2 specific probe. Densitometric readings of these blots has determined that there is no stabilization of the RAR β 2 message between days 4 and 7 of differentiation. Figures 3-7 and 3-8 are logarithmic plots of normalized densitometric readings used for the calculation of mRNA half-life. It has been determined that the half-life of the RAR β 2 message on Day 4 is approximately

DAY 4

DAY 7

0 1 2 3 4 5

0 1 2 3 4 5



RAR β 2



GAPDH

Figure 3-6: Half-life of RAR β 2 on day 4 and day 7 of DMSO differentiation: Northern blot analysis was conducted on Actinomycin-D treated cells isolated from 0 through 5 hours on either day 4 or day 7 of DMSO differentiation in order to assess the half-life of the RAR β 2 message on different days of differentiation. The blots were probed with an isoform-specific RAR β 2 probe as well as the GAPDH control probe.

Day 4 of differentiation

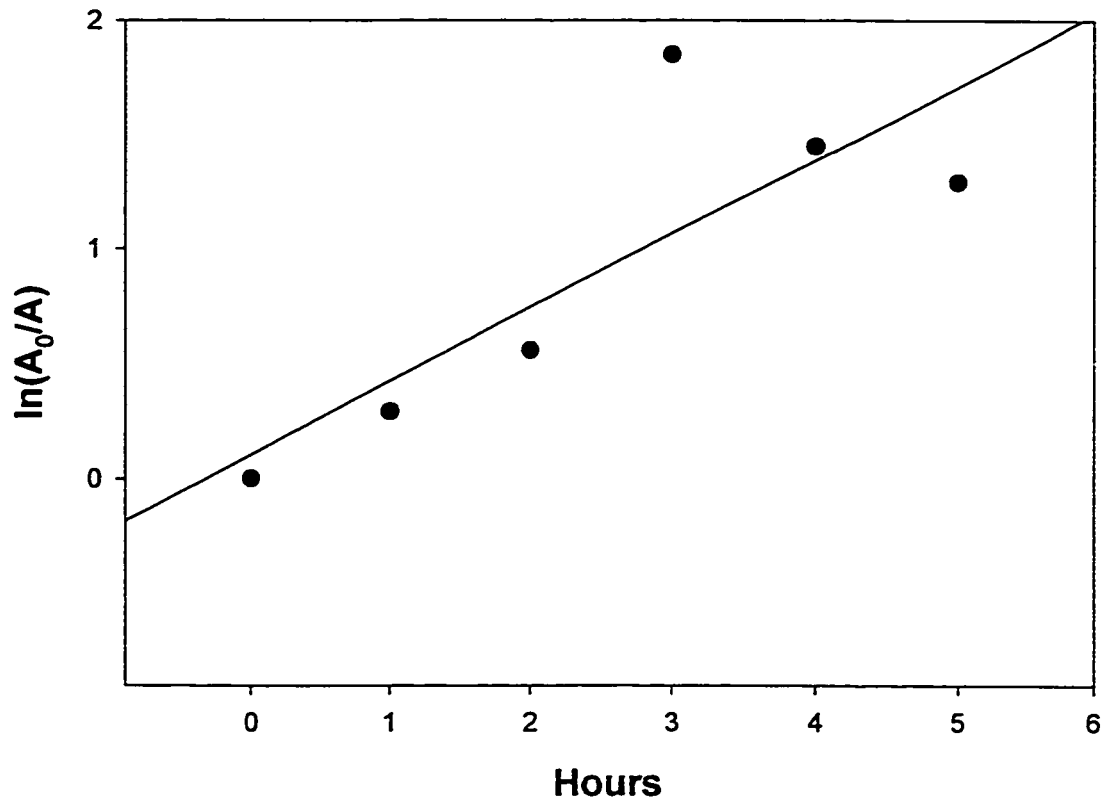


Figure 3-7: Logarithmic plot of RAR β half-life on day 4 of DMSO differentiation: Linear regression analysis of densitometric readings from figure 3-6 demonstrates that the half-life of the RAR β 2 message on Day 4 of DMSO differentiation is 2.2 hours

Day 7 of differentiation

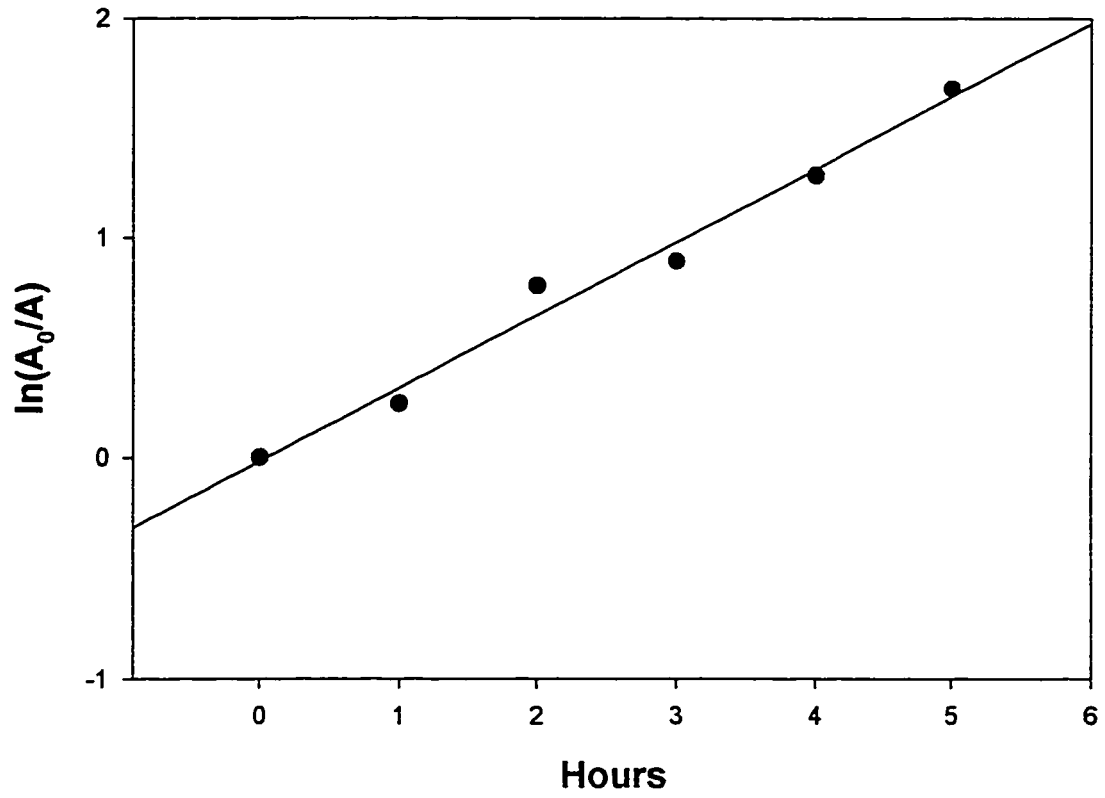


Figure 3-9: Logarithmic plot of RAR β half-life on day 7 of DMSO differentiation: Linear regression analysis of densitometric readings from figure 3-6 demonstrates that the half-life of the RAR β 2 message on day 7 of differentiation is 2.1 hours.

2.2 hours while the half-life of the Day 7 RAR β 2 transcript is approximately 2.1 hours. We propose that because the half-life of the RAR β 2 message on day 4 and day 7 is approximately equal, RAR β 2 mRNA is stabilized before day 4 of the differentiation process. Thus, after this time the observed increase in RAR β message is likely due to accumulation of the already stabilized transcript.

EGTA has no modulating effect on the expression of RAR β 2 mRNA during DMSO differentiation from Day 4 through Day 7

Since DMSO is known to have an effect on intracellular levels of calcium, we investigated the effect of 2mM EGTA on RAR β 2 expression on days 4 through 7 of DMSO-induced differentiation. DMSO differentiated P19 cells were pretreated for 48 hours with 2mM EGTA and collected on days 4,5,6,7. Northern blot analysis with an RAR β 2 isoform specific probe (figure 3-9) demonstrates that chelation of calcium with EGTA does not affect the RAR β 2 mRNA levels to any significant extent.

RA prevents Brachyury expression in P19 cells differentiated with DMSO

P19 cells when aggregated and exposed to DMSO differentiate into a variety of mesodermal and endodermal cell types (McBurney et al., 1982). We investigated the expression of the Brachyury gene when P19 cells were differentiated with DMSO and treated with 1 μ M RA on day 0 and day 2 of the

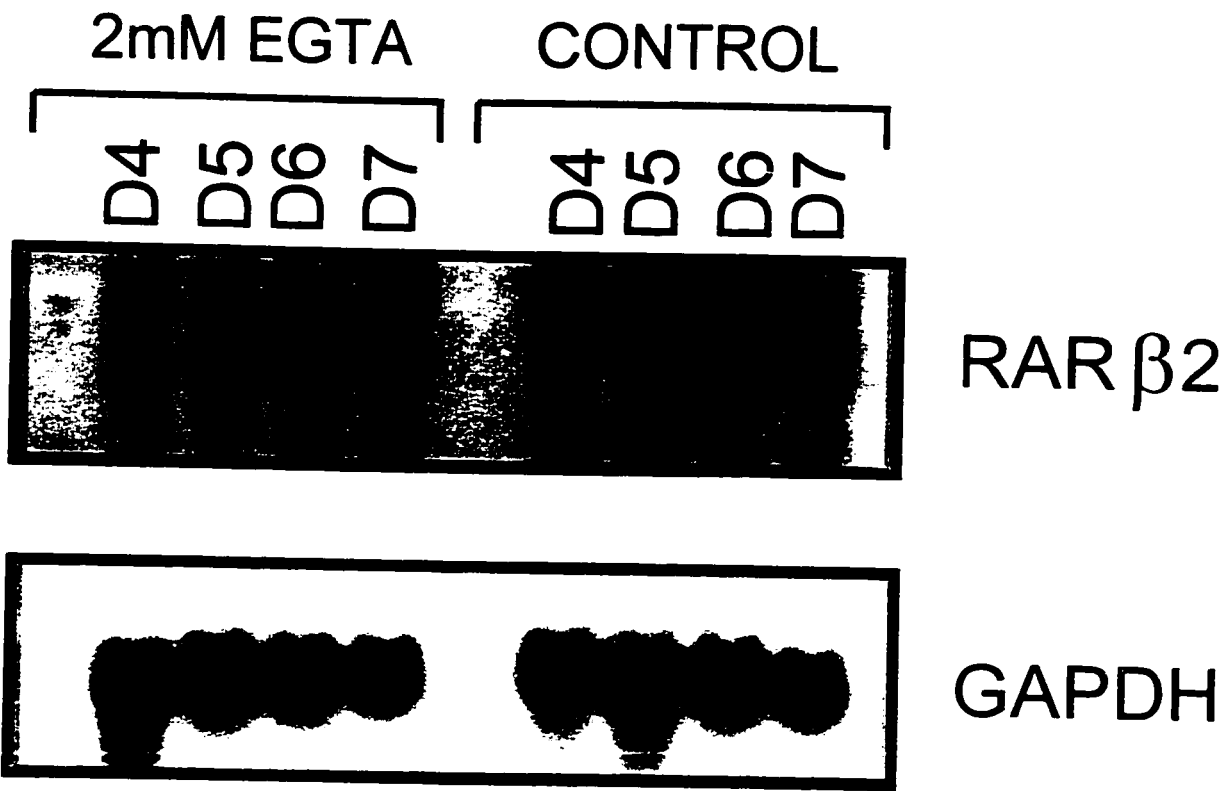


Figure 3-9: Expression of RAR β 2 in EGTA-treated DMSO-differentiated P19 cells: Northern blot analysis was conducted on DMSO-differentiated P19 cells isolated on day 4,5,6, and 7 of differentiation and DMSO-differentiated P19 cells exposed to 2mM EGTA 48 hours prior to total RNA isolation. The blot was probed with the RAR β 2 isoform-specific probe as well as the internal control GAPDH. The results indicate that calcium chelation by EGTA has no effect on the level of RAR β 2 expression in DMSO-differentiated cells. (D4, D5, D6, D7) Day 4, Day 5, etc....,

differentiation process. Brachyury expression has been shown to be expressed transiently in these cells before the appearance of markers of the differentiated cell types (Vidricaire et al., 1994). We differentiated the cells with DMSO and added RA on either day 0 or day 2 and observed Brachyury mRNA expression. Brachyury mRNA was present at very low to undetectable levels in undifferentiated P19 cells and dramatically increased to peak at day 2 before declining in DMSO treated P19 aggregates (Figure 3-10). Aggregation and treatment with both 1 μ M RA and 1% DMSO added on day 0 resulted in neuroectoderm formation by day 7, not including beating cardiac muscle. Brachyury was not transiently expressed in these cells, indicating that mesoderm formation did not take place. When RA was added 48 hours into the differentiation process, there was no alteration in Brachyury expression on day 4 through 10, however a mixture of cell types were not including beating cardiac muscle or neuroectoderm. Thus, even though Brachyury expression was not altered, the formation of beating cardiac muscle could not take place. This supports the finding that Brachyury expression is not sufficient for cardiac muscle formation.

Id expression is transiently downregulated in DMSO-differentiating P19 cells

Figure 3-10 illustrates that the expression of Id mRNA is downregulated on day 1 and 2 of DMSO differentiation as compared to proliferating P19 cells. The

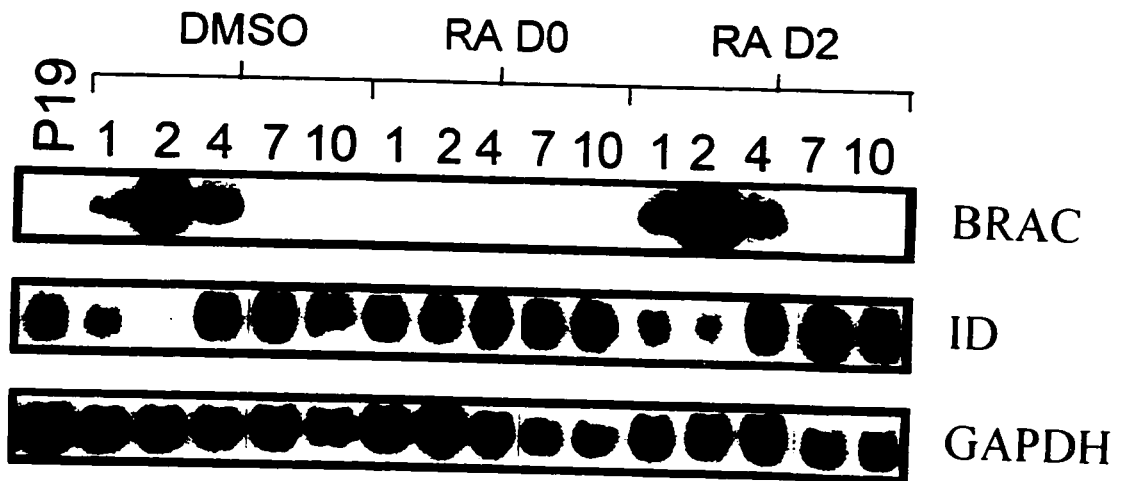


Figure 3-10: Brachyury and Id expression in differentiated P19 cells: In order to assess the ability of RA to influence the formation of cardiac muscle, both Brachyury and Id mRNA levels were examined in P19 cells differentiated with DMSO, with DMSO and 1 μ M RA(added on day 0 of differentiation), and with DMSO and 1 μ M RA(added on day 2 of differentiation). The results indicate that Brachyury expression is transiently upregulated in DMSO-differentiated cells, but is not apparent in both DMSO and RA treated cells when RA is added simultaneously with DMSO on day 0. Id expression levels are downregulated on the first 2 days of P19 differentiation with DMSO and appear to increase back to normal levels by day 4. When RA is added 2 days into the DMSO differentiation process, Id levels mimic that seen in DMSO-differentiated P19 cells, but when RA is added simultaneously with DMSO, Id levels remain constant. This finding is consistent with the observation that when RA is added simultaneously with DMSO, neuron formation is prominent on day 8 of the differentiation process. (P19) DMSO-differentiated P19 cells, (RA D0) 1 μ M RA added on day 0 of differentiation, (RA D2) 1 μ M RA added on day 2 of differentiation, (BRAC) Brachyury

levels of Id however, have increased by day 4 of differentiation back up normal levels found in undifferentiated P19 cells. The simultaneous administration of both RA and DMSO results in the evolution of neuroectoderm, consistent with RA-induced differentiation. This finding is paralleled by the lack of downregulation of Id mRNA on day 1 and 2 of differentiation. Administration of 1 μ M RA for 48 hours on day 2 of differentiation does not appear to have an effect on Id mRNA levels following exposure. Nevertheless, no beating muscle could be found by day 7 to 10 of differentiation. It is not clear what cell types were formed from the simultaneous administration of 1 μ M RA on day 2 of the differentiation process, but it was quite apparent that beating muscle nor neuroectoderm was not present. These results show that the transient downregulation of Id on day 1 and 2 of the muscle differentiation process is not sufficient in itself for cardiac muscle formation.

Chapter IV - General Discussion

Presently, the primary mechanism for the mediation of the RA signal is through the RAR and RXR classes of nuclear receptors. Retinoic acid is believed to upregulate the expression of its own receptor RAR β through two distinct mechanisms. As reported by Mendelsohn et al., 1994a, the modulation of the RAR β 1/ β 3 transcripts occur via a RA-dependent release of a block in RNA chain elongation. In the case of the RAR β 2/ β 4 isoforms, RA-inducibility is mediated by a response element present in the P2 promoter region (Hoffman et al., 1990) (De The et al., 1990). The RARE present in the RAR β promoter region is comprised of two direct repeats of the core sequence 5'-GTTCAC-3' which can be activated in several different cell types which express RARs (Sucov et al., 1990) (De The et al., 1990). It has previously been shown that the RAR β isoform is also induced by RA in P19 EC cells (Pratt et al., 1990). The transcriptional enhancer activity of the RARE β has to the point been the sole mechanism by which the increase in RAR β 2 expression has been demonstrated. This study clearly demonstrates that RAR β gene expression is strongly induced in a RA-independent manner for P19 cells aggregated and differentiated with (DMSO).

At the presomitic stage of mouse development (day 7.5 to day 8), p.c., RAR β expression in transverse sections at the level of the primitive streak, is lower in the midline than in the lateral regions, thought not being tissue related.

Expression of RAR β in the newly formed mesenchyme lateral to the streak at early stages was reflected at later stages as expression in the lateral plate mesoderm, indicating that association with the heart (Rossant et al., 1991). At the 4-7 somite stage of development (day 8 p.c.), the two heart tubes come together and fuse in the ventral midline as the foregut forms, and the heart begins to beat. In embryos of the 8-10 somite stage (day 9 p.c.), RAR β transcripts are detected in the lateral and intermediate mesoderm and as in the previous stages, absent from the somites.

The tissue specific and temporally restricted expression of RAR β during mouse embryogenesis suggests a specific role for this receptor during development. Since the presence of RAR β transcripts is thought to be due to activation of the RARE in its promoter region, it is surprising that in transgenic mice, harbouring a RARE β -LacZ reporter gene construct, there is no cardiac expression at the 8-10 somite stage of development. This observation implies that posttranscriptional mechanisms may play a large role in the level of RAR β gene expression. Taken together, our observations that RAR β expression peaks in DMSO differentiated cells shortly after the onset of cardiac muscle contraction, and that RAR β transcripts appear in the heart at specific times during development strongly suggests that the RAR β plays a pivotal role in normal cardiac development as evidenced by Osmond et al., 1991, who observed that excess levels of retinoic acid in the early chick embryo prevents fusion of the heart tubes. They believe that one of the teratogenic effects of retinoic-induced malformations seen in

mammalian embryos and humans (Lammer et al., 1985) may be related to a disruption of precardiac cell-matrix interactions and inhibited cell migration during cardiac formation.

Recent genetic evidence supporting the concept that RXR/RAR heterodimers act as transcriptional transducers of the RA signal during development has demonstrated a role for retinoid receptors in heart development. Mice embryos null for RXR α display a decrease in thickness of the ventricular myocardium (Kastner et al., 1994). This thin myocardium is believed to be attributed to a decrease in the number of "primary" myocytes in the primitive myocardial wall. Interestingly, RAR α 1 null mutants do not demonstrate any disruption of embryonic development and thus suggests combinatorial or overlapping functions for the various RAR isoforms. RAR α null mutants do however display a spectrum of abnormalities related to Vitamin-A deficiency. Thus RAR α 1 and RAR α 2 are largely functionally redundant.

RAR β 2 null mutants have little or no effect on development (Mendelsohn et al., 1994b), whereas combination mutants result in many developmental abnormalities including the heart (Luo et al., 1996). These studies have indicated that multiple RAR β isoforms are implicated in RA signaling in vivo. Comparisons of the phenotypes from this compound null mutant with other previously reported phenotypes of RAR compound mutants give much insight into the functional roles of the RAR β and RAR α isoforms in development.

The observation that RAR β 2 mRNA is upregulated in DMSO-differentiated P19 cells which contain primary mesodermal derivatives including cardiac muscle supports the hypothesis that this upregulation may be cardiac-cell specific. Our experiments with myoD expressing p19 cells rule out the possibility that the receptor is expressed in mesoderm derived skeletal muscle. Additionally, by using the D3 mutant P19 cell line, we have established that the induction of RAR β is dependent on the presence of muscle as no increase in RAR β mRNA was evidenced throughout the differentiation process. From this data, we can thus conclude that the gene is expressed in either the embryonic cardiac muscle or the germ layer or both.

A study which generated a null mutation for the RAR β gene (Luo et al., 1995) (all isoforms disrupted) in the mouse found that mice homozygous for the RAR β mutation are viable and fertile, and exhibit no obvious external or internal abnormalities including the heart. This result indicates that the RAR β gene is not essential for normal cardiac development. This result is unexpected because the specific expression pattern of the RAR β gene suggests that it plays an important and distinct role in mouse development (Mendelsohn et al., 1992) (Ruberte et al., 1993) (Ruberte et al., 1991) (Dolle et al., 1990) (Dolle et al., 1989). Also, the specific spatial and temporal expression of an RARE-hsp- β galactosidase transgene during mouse embryogenesis was shown to mimic closely the expression of the RAR β gene. This observation significantly complicates assigning a specific function(s) to each RAR β gene. This in

combination with other studies have demonstrated that none of the individual RAR isoforms play a pivotal role in the process of development.

As previously reported by Zelent et al., 1991, small amounts of RAR β 1 and RAR β 3 transcripts were present in RA-treated P19 cells, in accordance with the hypothesis that RA-induced RAR β 1/ β 3 transcript accumulation results from a release of a block to elongation. We have also found the presence of small amounts of the RAR β 1 and RAR β 3 transcripts by RT-PCR and Southern Blot analysis in DMSO differentiated cells. Clearly however, the major transcript as shown by isoform specific RT-PCR is the RAR β 2 isoform.

Compound mutants with the RAR β 2 isoform ($\alpha\beta$ 2) have demonstrated abnormalities of the heart and great vessels, all of which have been described in the offspring from VAD rats (Wilson et al., 1953) (Wilson., 1949). These data demonstrate that RARs are transducing the retinoid signal necessary for proper myocardial growth, aorticopulmonary and ventricular septation, and patterning of aortic arches. Even though none of these studies has specifically implicated the RAR β 2 isoform as necessary for cardiac differentiation, due to significant functional redundancy among receptors, the data presented here strongly suggest a very specific role for the RAR β 2 isoform in cardiac development.

The lack of a large transcriptional induction of either a RARE β 2 reporter gene or the endogenous RAR β in DMSO differentiated P19 cells suggests a

posttranscriptional mechanism for the regulation of RAR β 2 mRNA in developing cardiac muscle/mesoderm. The nuclear run-on experiment indicates a constitutive basal activity from the RAR β gene promoter. Mendelsohn et al., 1994a, has shown a small amount of hybridization to the RAR β 2 first exon in nuclear run-on experiments that increases in the presence of RA. Taken together these results suggest that regulation of RAR β 2 mRNA occurs through a primarily posttranscriptional mechanism in developing cardiac muscle/mesoderm.

A possible mechanism for this may be differentiation dependent stabilization of the RAR β 2 transcript. Our actinomycin-D experiments on Day 4 and Day 7 of differentiation show no increase in stability of the RAR β 2 transcript. Due to very low constitutive RAR β 2 levels in undifferentiated P19 cells, it is difficult to assess the half-life of this transcript in vitro. We have proposed that the RAR β 2 message is stabilized early-on in the differentiation process, (before day 4) and that between days 4 and 7 of differentiation, we are observing accumulation of the transcript. In addition to stabilization of the transcript, we have observed a small, yet observable increase in the level of transcription of the message. Taken together, this data suggests that a combination of transcriptional and posttranscriptional events are involved in the increased expression of the RAR β 2 message.

The 3'-UTR region of the RAR β gene contains four copies of the AUUUA motif. As previously mentioned, the presence of this motif in the A-U rich element (ARE) does not guarantee any kind of destabilizing function since it could be inactivated by neighbouring sequences. The existence of trans factors which interact with labile mRNAs at or near the AUUUA destabilizing elements might play a role in the half-life of RAR β mRNA (Wodnar-Filipowicz and Moroni., 1990) (Schuler and Cole., 1988). A-U binding factor -AUUUA (AUBF-AUUUA) RNA complexes have been previously shown to protect mRNA from rapid degradation in certain instances, leading to regulation of gene expression (Malter et al., 1990).

The mechanism of action of DMSO on biological systems is unknown, but the similarity of the responses of various cells to DMSO suggests that a common mechanism may be involved in inducing the differentiation of these cells. Based on studies of $^{45}\text{Ca}^{2+}$ uptake over 24 hours, it has been suggested that in MEL cells, DMSO triggers a Ca^{2+} surge which is necessary but not by itself sufficient to induce differentiation (Tsiftsoglu et al., 1981) (Bridges et al., 1981). Therefore DMSO action may be mediated at least partly through Ca^{2+} and Ca^{2+} mediated processes. DMSO has been shown to act on an inositol-trisphosphate-independent Ca^{2+} storage site (Morley and Whitfield., 1993). Whether the presence of calcium during the process of differentiation was sufficient to stabilize the RAR β 2 transcript, we performed a calcium chelation experiment with EGTA and measured RAR β 2 mRNA levels. This experiment demonstrates

that the presence of a specific calcium chelator(EGTA) had no effect on the presence of the RAR β 2 mRNA message, indicating that calcium does not seem to play a significant role in the regulation of RAR β 2 mRNA during cardiac muscle/mesoderm. Whether this finding rules out involvement of binding proteins which may stabilize the transcript remains to be seen, but Ca²⁺ does not appear to play a role in mediating RAR β 2 stabilization during DMSO differentiation.

The observation that Brachyury expression is transiently upregulated in differentiating P19 cells supports the hypothesis that this gene is required for mesoderm formation. Brachyury is known to be necessary but not sufficient for mesoderm formation in the mouse (Willison., 1990) (Wilkinson et al., 1990) (Hermann et al., 1990). The differentiation of P19 cells with DMSO forms mesodermal cell types including cardiac as well as skeletal muscle. Exposure of P19 cells to 1 μ M RA at either the beginning of differentiation or shortly after(day 2) has established if the differentiation program initiated by aggregation and exposure of DMSO could be interrupted or altered. The results obtained suggest that RA has a stronger differentiating effect on the cells than DMSO as evidenced from the formation of neurons in day 0 RA-treated cells. The expression of Brachyury following administration of RA on day 2 did not change indicating that the course of Brachyury expression had been previously determined. However, no cardiac muscle or skeletal muscle types were observed to have formed. This again demonstrates the more potent ability of RA

to affect the differentiation process. The lack of Brachyury up-regulation in P19 cells treated with RA as well as DMSO suggests that mesoderm is not formed with this type of exposure. Consistent with this hypothesis is the lack of cardiac beating muscle in these cell cultures. That RA has apparently no effect on Brachyury mRNA levels when administered on day 2 of differentiation implies that this molecular pathway has already been initiated. However, no cardiac muscle is present from days 7 to 10 of differentiation indicating that other important steps within the process of differentiation were altered by the presence of retinoic acid.

Id expression has been linked to cellular proliferation as well as differentiation. In addition to Brachyury expression, we also wanted to investigate the expression levels of Id in DMSO-differentiated cells with or without RA being added on either day 0 or day 2 of differentiation. P19 cells aggregated and exposed to DMSO show a dramatic down-regulation of Id levels on day 1 and 2 of differentiation. However, the levels return to that previously found in proliferating P19 cells by day 4 of differentiation. Addition of RA on day 0 of DMSO differentiation resulted in the maintenance of Id levels through to day 10. From this and the observation that only neurons were formed by day 10 of differentiation, it appears that the initiation of muscle formation did not take place in this experiment. Addition of RA on day 2 had no effect on Id levels even though no beating cardiac muscle was formed. Since Id levels remain high, it is possible that Id remains bound to various bHLH proteins, influencing levels of

gene expression, possibly not allowing the cells to differentiate as they normally would outside RA's influence.

RA appears from these results to have a more dominant effect over DMSO in the process of differentiation. This is supported by the finding that RA acts through nuclear receptors to alter levels of gene expression while DMSO does not appear to be physiologically significant and appears to act more indirectly in the differentiation process, possibly through regulation of intracellular calcium levels.

These observations of both Brachyury and Id expression suggest that RA has a more profound effect on cell fate during differentiation, perhaps because it acts more directly through nuclear receptors to alter gene expression. Though the mechanism of DMSO-induced differentiation has not yet been elucidated, it seems likely that DMSO acts more indirectly in the differentiation process, possibly through regulation of intracellular calcium levels.

The expression levels of both Brachyury and Id appear to play an important role in cardiac muscle formation. High levels of expression of each of these genes within cells could give us insight into the genes necessary for muscle development. Thus, transfection studies with these genes in P19 cells will prove useful in determining their role as molecular mediators of the differentiation process.

It still remains to be determined by what mechanism RAR β 2 is upregulated in DMSO-differentiated cells. Additional stability experiments performed on earlier days of differentiation could give insight into whether the increase in RAR β 2 expression is due to mRNA stabilizing mechanisms. Transfection of full-length RAR β under control of a weak promoter into P19 cells followed by DMSO-treatment should show whether stabilization of the transcript is the primary mechanism by which its upregulation is accomplished. This novel finding that RAR β 2 is upregulated in an RA-independent fashion has added to the level of complexity in which retinoids participate in the process of development.

The results of this study have concluded that expression of the RAR β 2 isoform is strongly induced in differentiating cardiac muscle derived from P19 embryonal carcinoma cells in a RA-independent manner and that the RAR β 1/3 isoforms are expressed at lower levels in differentiating cardiac muscle. The mechanism by which this upregulation occurs is believed to be post-transcriptional and may involve mRNA stabilization. Also, it has been shown that the transient increase and transient decrease in Brachyury and Id expression respectively, are not sufficient for cardiac muscle formation in DMSO differentiating P19 cells.

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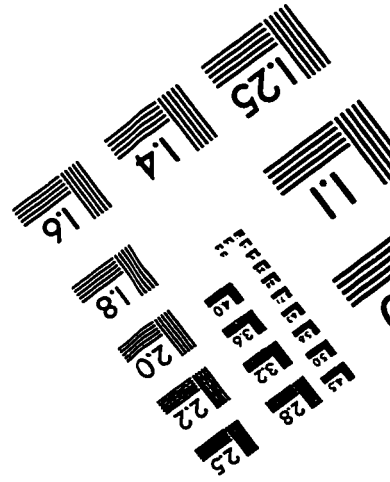
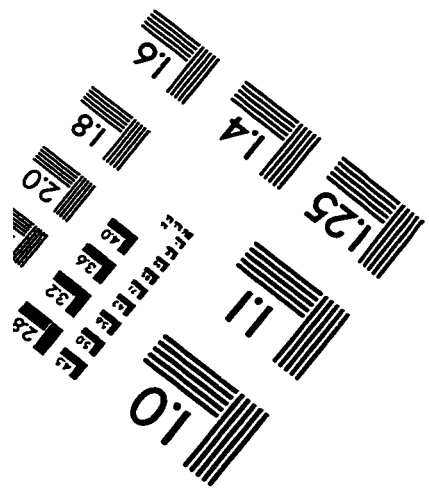
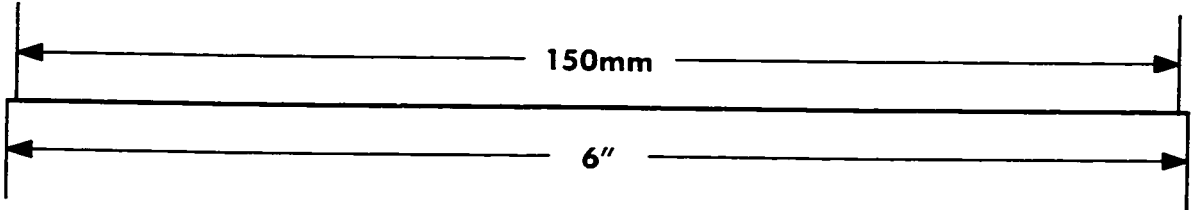
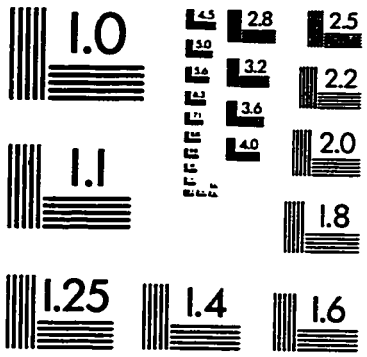
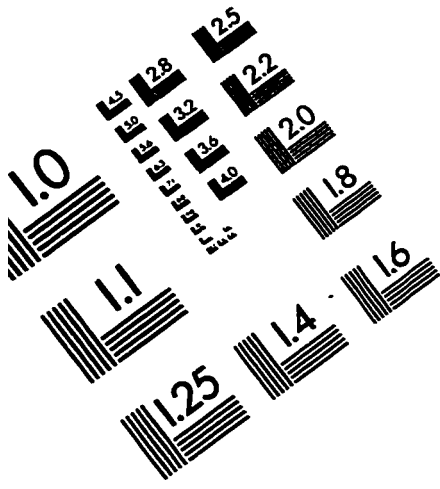
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