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MUSCLE-SPECIFIC GENE EXPRESSION
IN DIFFERENTIATED
EMBRYONAL CARCINOMA CELLS

By

Giovanna Pari

A thesis submitted to
the School of Graduate Studies and Research
in partial fulfillment of the requirements for the degree
of
Master of Science

Department of Biology
University of Ottawa



Giovanna Pari, Ottawa, Canada, 1990



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Abstract

Transcription of the cardiac actin gene is restricted to cardiac and skeletal muscle tissue. The transcriptional regulation of this gene in cardiac muscle was investigated using P19 embryonal carcinoma (EC) cells which can be induced to differentiate into various cell types including cardiac muscle. A chimeric gene consisting of the human cardiac actin promoter linked to the lac Z reporter gene encoding β -galactosidase (β -gal), was permanently transfected into P19 cells. Low levels of expression of the chimeric gene were present in pooled populations of transfected EC cells. Differentiation of these cultures into cardiac muscle resulted in high levels of β -gal activity, while differentiation along the neuroectodermal lineage did not lead to any increase in the expression of the actin-lacZ chimeric gene. These results suggest that the cardiac actin chimeric gene is expressed in the appropriate developmental and tissue-specific manner in differentiated P19 cells.

Unidirectional 5' deletion analysis of the cardiac actin promoter showed that a DNA segment of 237 bp upstream of the transcription initiation site was sufficient to direct muscle-specific expression in differentiated P19 cells. Within this segment, regions between -237 and -158 bp play a role in the transcriptional regulation since their deletion resulted in the loss of expression. However, the cardiac actin 5'-flanking region from -650 to -86 bp was unable to confer tissue-specific expression when placed upstream of the heat-shock protein 68 (hsp 68) promoter. These results therefore indicate that transcription of the cardiac actin gene requires multiple *cis*-acting DNA elements which are located in the proximity of the transcription initiation region of the gene, and more upstream, between -237 and -158 bp.

The transcription of various muscle-specific genes can be activated in different

nonmuscle cell lines by the expression of MyoD, a regulatory factor of skeletal myogenesis. The MyoD1 cDNA placed under the transcriptional control of the phosphoglycerate kinase (pgk)-1 promoter, was transiently transfected in undifferentiated P19 cells with a cardiac actin-lacZ chimeric gene which included the cardiac actin promoter linked to the lac Z reporter gene. Expression from the chimeric actin gene increased approximately ten-fold in the presence of MyoD. However, MyoD did not stimulate transcription from nonmuscle-specific chimeric genes controlled by the pgk or the hsp 68 promoter. A Northern analysis of RNA isolated from P19 cells transiently transfected with the MyoD expression vector showed that MyoD was unable to activate endogenous sarcomeric actins and muscle myosin heavy chain (MHC) genes in P19 cells, thus indicating that additional regulatory factors participate in the activation of muscle-specific genes. Sequential deletions in the cardiac actin promoter linked to the lac Z gene showed that a DNA segment containing 312 bp from the transcription initiation site was sufficient to mediate the MyoD-induced transcriptional activation. MyoD-induced expression involves several regions which are located between -312 bp and -47 bp, and which differ from the sequences required for expression in skeletal muscle cell lines. These results therefore suggest that MyoD activates muscle-specific genes in cooperation with other factors which interact directly or indirectly at the various *cis*-acting regulatory sequences of the target gene.

The role of MyoD in muscle differentiation was investigated in permanent transfections of P19 cells with the MyoD expression vector. The stable transformants expressing MyoD consisted of undifferentiated P19 cells and of various differentiated cell types such as skeletal muscle and spontaneously beating cardiac muscle, which were both identified by immunocytochemistry with a muscle MHC antibody. Northern analysis of RNA isolated from pooled populations of stable transformants containing the MyoD expression vector showed that the transcription of sarcomeric actin genes was associated with the expression of MyoD. In contrast, MyoD transcripts were absent during the *de novo* differentiation of P19 cells into cardiac muscle. Therefore, MyoD is not involved in cardiac myogenesis, but its forced

expression at high levels in P19 embryonal carcinoma cells can lead to the inappropriate expression of differentiated functions. The myogenic differentiation program therefore seems to be controlled by complex interactions among multiple upstream regulatory elements and myogenic factors that are functional only in the appropriate cellular context.

Résumé

La transcription du gène de l'actine cardiaque est limitée aux tissus cardiaque et squelettique. Le contrôle de la transcription de ce gène dans les cellules cardiaques a été examiné avec les cellules embryonnaires carcinomateuses (EC) P19 qui peuvent se différencier en plusieurs types de cellules, incluant les myocytes cardiaques. Un gène chimérique consistant du promoteur de l'actine humaine cardiaque lié à la phase codante du gène lac Z qui dirige la synthèse de la β -galactosidase (β -gal) a été introduit de façon permanente dans les cellules P19. De faibles niveaux d'expression du gène chimérique étaient présents dans les populations groupées de transformants. Suite à la différenciation de ces cellules pour former du muscle cardiaque, des niveaux élevés d'activité β -gal ont été mesurés, tandis qu'avec la différenciation suivant la lignée neuroectodermique, aucune augmentation de l'expression a été notée. Ces résultats suggèrent que l'activité du gène chimérique d'actine cardiaque est régie par les règles du développement lors de la différenciation des cellules P19.

Une analyse par l'exclusion unidirectionnelle de séquences du côté 5' du promoteur de l'actine cardiaque a démontré qu'un segment d'ADN avec 237 bp en amont du site d'initiation de la transcription, était suffisant pour diriger la transcription dans les cellules P19 différenciées. A l'intérieur de ce segment, plusieurs régions entre -237 et -158 bp sont impliquées dans le réglage de la transcription puisque leur retranchement résulte en la perte de l'expression dans les cellules musculaires. Toutefois, la région entre -650 et -86 bp du promoteur de l'actine ne peut conférer le réglage spécifique au muscle lorsqu'elle est placée en amont du promoteur du gène codant pour la protéine 68 Kd de l'effet hyperthermique (hsp 68). Ces résultats indiquent donc que la transcription du gène de l'actine cardiaque nécessite plusieurs

composantes qui se trouvent à proximité du site d'initiation de la transcription et plus en amont, entre -237 et -158 bp.

La transcription de plusieurs gènes spécifiques au muscle peut être activée dans différentes lignées cellulaires non-musculaires à l'aide de l'expression de MyoD, un facteur impliqué dans le contrôle de la myogénèse squelettique. Le cADN de MyoD1 placé sous le contrôle du promoteur du gène de la phosphoglycérate kinase (pgk)-1 a été introduit de façon transitoire dans des cellules P19 non-différenciées avec un gène chimérique consistant du promoteur de l'actine cardiaque lié au gène marqueur lac Z. Le niveau d'expression de ce gène a augmenté approximativement de 10 fois en présence de MyoD. Toutefois, MyoD n'a pas stimulé la transcription de gènes chimériques avec les promoteurs pgk ou hsp 68 dont l'expression n'est pas restreinte aux cellules musculaires. Une analyse "Northern" de l'ARN isolé de cellules transfectées transitoirement avec le véhicule d'expression de MyoD a démontré que les gènes d'actines sarcomériques et de myosines à chaîne lourde musculaires (MHC) endogènes ne sont pas activés par MyoD, ce qui indique que d'autres facteurs participent à l'activation des gènes spécifiques au muscle. L'exclusion de séquences du promoteur de l'actine cardiaque a montré que la région en aval de -312 bp était suffisante pour permettre l'activation transcriptionnelle induite par MyoD. Cet effet implique plusieurs régions situées entre -312 et -47 bp qui diffèrent des séquences requises pour l'expression dans les lignées de muscle squelettique. Ces résultats indiquent donc que MyoD active la transcription de gènes spécifiques au muscle en coopérant avec d'autres facteurs qui agissent directement ou indirectement au niveau des séquences régulatrices du gène indiqué.

Le rôle de MyoD dans la différenciation musculaire a été investigué en transformant de façon permanente des cellules P19 avec le véhicule d'expression MyoD. Les transformants sélectionnés consistaient de cellules P19 non-différenciées et de différents types de cellules différenciées. Parmi ceux-ci, le muscle squelettique et le muscle cardiaque avec une activité contractile spontanée ont été identifiés par immunocytochimie à l'aide d'un anticorps réagissant avec les myosines à chaîne lourde du muscle. Une analyse "Northern" de l'ARN isolé de la population groupée de

transformants contenant le véhicule d'expression de MyoD a montré que la transcription des gènes d'actine sarcomérique est associée avec l'expression de MyoD. La transcription de MyoD n'a toutefois pas lieu durant la différenciation de cellules P19 en muscle cardiaque. MyoD n'est donc pas impliqué dans la myogénèse cardiaque, mais son expression forcée dans les cellules embryonnaires carcinomateuses aboutit à l'expression inappropriée de fonctions différenciées. Le programme de différenciation du muscle semble donc être régi par de complexes interactions entre les multiples éléments régulateurs et les facteurs spécifiques au muscle, qui sont fonctionnels uniquement dans le contexte cellulaire approprié.

Abbreviations

β -gal	β -galactosidase
bp	base pair(s)
BRL	Bethesda Research Laboratories
CAT	chloramphenicol acetyltransferase
CBF	CarG box binding factor
cDNA	complementary DNA
CDTA	trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid
cpm	counts per minute
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EC	embryonal carcinoma
EDTA	ethylene diamine tetraacetic acid
hsp	gene encoding the mouse heat-shock protein 68
Kb	kilobase pair(s)
lac Z	gene encoding <i>Escherichia coli</i> β -galactosidase
M	molar
MCK	muscle creatine kinase
mM	millimolar
μ M	micromolar
min	minute(s)
MHC	myosin heavy chain

MOPS	3-(N-morpholino) propane-sulfonic acid
mRNA	messenger RNA
MyoD	myogenic determination factor
neo	gene encoding neomycin phosphotransferase
ONPG	o-nitrophenyl- β -D-galactopyranoside
PBS	phosphate buffered saline
pgk	gene encoding the somatic cell isoform of phosphoglycerate kinase
RA	retinoic acid
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SRF	serum responsive factor
TE	Tris-EDTA
u	unit
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

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Chapter 1

General Introduction

1.1 Introduction

Cellular differentiation is established and maintained by the expression of specific sets of genes. An important mechanism which permits the regulated expression of genes is the alteration of the transcriptional activity. Transcription of certain genes required for specialized functions is activated while expression of genes associated with the undifferentiated state is down-regulated during differentiation. The biochemical basis for such diversity includes multiple *cis*-acting DNA elements and tissue-specific factors that selectively interact with the regulatory sequences. The presence of *cis*-acting regulatory sequences has mainly been documented by creating mutations in cloned genes and by analyzing their effect in cultured cells where the gene is reintroduced. More recently, proteins that interact with the regulatory sequences of tissue-specific genes have been analyzed, mostly by *in vitro* binding experiments with labelled DNA. The mechanism by which the DNA-protein interactions control the expression of specific genes, and modulate the overall pattern of expression during cellular differentiation remains however a central problem in the study of cell growth and development.

In this study, we investigated different aspects of muscle-specific transcription as a model to study the molecular mechanisms involved in gene regulation during differentiation. Myogenesis is accompanied by the activation of a series of genes

which encode the proteins forming the contractile apparatus (reviewed in Buckingham, 1985). Sequences that govern tissue-specific expression have been identified in some of these genes using permanent skeletal myoblast cell lines. However, little is known about the sequences that control the expression of muscle-specific genes in cardiac muscle. Here, we have studied the transcriptional regulation of the cardiac actin gene in cardiac muscle using P19 cells which can be induced to differentiate *in vitro* into cultures containing cardiac myocytes and other cell types (Section 1.2.2). In addition, we have investigated the activation of the cardiac actin gene in the presence of the muscle-specific regulatory factor MyoD in transient and permanent transfections of P19 cells.

1.2 P19 Embryonal Carcinoma Cells

1.2.1 The Cell Culture System

P19 embryonal carcinoma cells (EC) were isolated from a teratocarcinoma experimentally produced by transplanting a 7.5 day old mouse embryo into the testis of an adult mouse. The teratomas derived in this way consisted of a wide variety of differentiated cells, as well as of undifferentiated cells. Stem cells of the transplanted embryo were isolated from the primary tumour to form the P19 EC cell line (McBurney and Rogers, 1982b). These cells are maintained in an undifferentiated state with routine subculturing.

When placed in the appropriate environment, P19 cells resemble the undifferentiated pluripotent stem cells of the early embryo in their capacity to differentiate. Injection of these cells in a blastocyst, followed by implantation of the embryo in a pregnant mouse results in the formation of an apparently normal mid-gestation fetus which contain some tissues derived from the injected cells (Rossant and McBurney, 1982). EC cells therefore have the capacity to behave as embryonic stem cells, and thus to differentiate into a wide variety of cell types in the appropriate conditions.

1.2.2 The Differentiation of P19 Cells

In vitro, P19 cells exposed to certain drugs can be induced to differentiate into various cell types. In particular, cells treated with a high dose of retinoic acid (RA) develop into neurons, astroglia, and fibroblast-like cells (Jones-Villeneuve et al., 1982). However, striated muscle cells were absent in those differentiated cultures as indicated by the lack of muscle-specific markers in the cells. In the characterization of RA-treated cultures derived from the P19 cell line, neuronal cells were initially identified on the basis of the presence of neurofilament protein, and on their distinctive morphology which includes long branching processes. Cells in RA-treated cultures of P19 cells also contained elevated levels of acetylcholinesterase and choline acetyltransferase activities (Jones-Villeneuve et al., 1982, 1983). The neurons in 7 day old cultures synthesized catecholamines and possessed high affinity uptake sites for the neurotransmitter GABA. In older cultures, the neuronal processes become differentiated into axons and dendrites which form synapses (McBurney et al., 1988). Differentiation of P19 cells with RA also produces other cell types, such as fibroblast-like cells which expressed smooth muscle actin isoforms, and glial astrocytes which were identified by their staining with an antibody to glial fibrillary protein (Rudnicki et al., accepted in Dev. Biol., Jones-Villeneuve et al., 1982).

Following treatment with dimethyl sulfoxide (DMSO), aggregates of P19 cells differentiate along the mesodermal lineage to form cultures containing cardiac and skeletal muscle but no neurons and glia (McBurney et al., 1982a). About 15% of all cells in the differentiated cultures consisted of cardiac and skeletal myocytes which were mostly mononucleate and contained muscle-specific isoforms of myosin and actin proteins. Following 6 days of differentiation, cardiac muscle cells often exhibited spontaneous rhythmic contractions (McBurney et al., 1982a). Skeletal muscle cells represented a negligible proportion of the muscle cells since troponin T, myosin light chain 1/3, and myosin light chain 2 mRNAs which are characteristic of skeletal muscle could not be detected in the RNA isolated from DMSO-treated cultures (Rudnicki et al., accepted in Dev. Biol.). The aggregates also contained other cell types, such as fibroblast-like cells expressing smooth muscle α -actin and cytokeratin, which might be indicative of a myoepithelial or myofibroblastic origin.

Additionally, previous studies reported the formation of an epithelial cell layer at the surface of the DMSO-treated aggregates, prior to the development of cardiac myocytes, thus indicating the complex nature of the DMSO response that leads to the development of muscle cells (Smith et al., 1987).

Electron microscopic studies of the myocytes derived from the DMSO-treated P19 cells showed the presence of many immature myofibrils with the appropriate arrangement of thick and thin filaments and occasional Z-bands. Junctional complexes resembling the intercalated disk of cardiac muscle formed between adjacent cardiac muscle cells (Smith et al., 1987). Further characterization of these cells revealed the presence of four cardiac-specific myosin isoforms: atrial- and ventricular-myosin heavy chain proteins, ventricular-myosin light chain 1, and atrial-myosin light chain 2 proteins (Rudnicki et al., accepted in Dev. Biol.). The cardiac muscle in DMSO-treated P19 cells also develops with the same kinetics as cardiac muscle develops in the embryo. On the basis of these results, it was suggested that myocytes derived from DMSO-treated P19 cells are analogous to early embryonic myocardium cells (Rudnicki et al., accepted in Dev. Biol.). Since there is no continuous line of cardiac myocytes, P19 cells are therefore a unique system to study the *de novo* differentiation of cardiac muscle. In this work, we made use of this system to investigate the activation and regulation of the transcription of a muscle-specific gene, the cardiac actin gene.

1.3 Regulation of Gene Expression

1.3.1 Promoters and Enhancers

Expression of eukaryotic genes transcribed by RNA polymerase II is controlled by multiple *cis*-acting genetic elements which are of two general types: promoter elements and enhancers. Promoter elements operate in the immediate vicinity of the transcription start site, while enhancers regulate transcription from a distance, and in an orientation-independent fashion. Both types of regulatory sequences are recognized by specific DNA-binding proteins that stimulate or inhibit transcription.

Although the interplay between the various DNA-binding factors is not yet understood, it is believed that the distinct pattern of expression of individual genes is determined primarily by the unique combination of DNA-protein interactions which occur at the *cis*-acting regulatory sequences.

Molecular analyses of various genes revealed the presence of several conserved promoter elements located within 100 bp of the transcription initiation site. The most proximal element is an AT-rich region which is designated the TATA box and is located 25 to 30 bp upstream of the cap site. The TATA box functions to position the start site of RNA synthesis, and constitutes a binding site for the transcription factor IID (Nakajima et al., 1988). Specific proteins have also been shown to bind other promoter elements such as the CCAAT and GC boxes (Briggs et al., 1986, Gil et al., 1988). Transcription factors produced by differential splicing of the CTF/NF1 gene bind to the CCAAT box, and activate transcription via a proline-rich domain. The GC box is recognized by the Sp1 factor which includes a glutamine-rich transcription activation domain, and a DNA-binding domain with three regions homologous to the zinc finger motif. Distinct structural motifs in the regulatory proteins therefore play a role in the formation of active transcription complexes.

Like promoters, enhancers contain discrete elements which interact with specific regulatory proteins (reviewed in Serfling et al., 1985). The characterization of the SV40 enhancer has shown that it is composed of many individual elements which are the binding site of one or more transcriptional activator proteins, many of which show cell-specific patterns of distribution (Zenke et al., 1986, Fromental et al., 1988). The different elements termed "enhansons" are distinguished by the proteins that they bind and by their functional properties; some enhansons act as a single copy while others are functional only when combined with an identical or nonidentical enhanson. In the appropriate background, many sites in the enhancer therefore contribute to the transcriptional activation.

1.3.2 Inducible and Tissue-specific Elements

The specialization of cells that make up a differentiated tissue depends on their ability to switch genes on and off in response to extracellular and environmental signals. In addition to the common transcription elements such as the TATA and CAT boxes of basal promoters, these genes contain separate *cis*-acting DNA sequences which confer inducible, tissue-specific or developmental patterns of expression.

Inducible elements are present in genes that respond to heat-shock, exposure to heavy metals, growth factors, or steroids. Mutagenesis studies have shown that short sequences responsible for the transcriptional activation are often reiterated several times, and are either located at a distance from the promoter, or are closely associated to it (reviewed in Maniatis et al., 1987). For example, temperature induction of the *Drosophila* hsp 70 gene involves several heat-shock elements where cooperative binding of the heat-shock transcription factors occurs (Topol et al., 1985). In other genes such as the β -interferon one, regulation of transcription was found to involve negative *cis*-acting DNA elements that bind repressor proteins when transcription is not activated (Goodbourn et al., 1986). Therefore, the transcriptional activation of inducible genes seems to involve the differential interaction of proteins at positive and/or negative regulatory sequences.

Similarly, many *cis*-acting regulatory sequences have been discovered in genes which are specifically expressed in differentiated tissues such as liver, pancreas, lens, lymphocytes and muscle (reviewed in Maniatis et al., 1987, Mitchell and Tjian, 1989). In the phosphoenolpyruvate carboxykinase gene, a distal element regulates expression in hepatocytes, while a proximal sequence enhances expression in adipocytes (Benvenisty et al., 1989). Similarly, the expression of the *Drosophila* yolk protein genes *yp1* and *yp2* in the fat body and ovaries is controlled by two distinct *cis*-acting elements (Garabedian et al., 1985). In the $\beta 3$ -tubulin gene, the major intron is responsible for expression in visceral muscles, while upstream sequences direct the expression in other tissues (Gasch et al., 1989). These results demonstrate that there are different DNA sequence requirements for the expression of a gene in different tissues.

More complex mechanisms of gene regulation seem to be required for certain

genes expressed in many cell types. Experiments with the α -fetoprotein gene in transgenic mice have shown the presence of three separate enhancer sequences located upstream of the tissue-specific promoter. Each enhancer exerts a different influence on the expression in the visceral endoderm, the fetal liver, and the gastrointestinal tract (Hammer et al., 1987). It was suggested that differences in enhancer recognition by tissue-specific factors may explain the different levels of expression that occur in the various tissues.

DNA-binding studies of the immunoglobulin heavy and light chain genes have shown that the enhancer with many copies of the "E" motif, the NF- κ B site and the conserved octamer sequences, is recognized by various proteins, one of which, the Oct-2 activator protein, is produced in B lymphocytes (Ko et al., 1988). Several studies with muscle-specific genes have also shown the binding of both nonmuscle- and muscle-specific factors to the tissue-specific regulatory elements (Section 1.4.1). Interactions of regulatory proteins with DNA elements thus seem to play a role in gene activation, although the specific mechanism by which this occurs has not been elucidated.

1.3.3 Transcription Activators

A model has been proposed to explain how transcription is controlled by multiple activator proteins that bind to various DNA regulatory elements which are located either nearby or at a distance from each other (Ptashne, 1986, 1988). In this model, activators are considered as modular proteins with a highly specific DNA-binding region and a less precisely defined activating region. An activator could therefore bind to DNA and interact with another protein near the transcription start, via looping-out of the intervening DNA. The bound proteins would then participate in the formation of an active transcription complex. In this scheme, multiple weak activators may cooperate when bound to DNA to permit a more efficient interaction with the target protein(s) near the transcription start site. The effect of activators may also be modified according to their relative concentration in the cell, to the degree of phosphorylation, or to the binding with other regulatory proteins. Therefore, changes in the activity of activator proteins by these different

mechanisms would allow the modulation of gene expression in the cell.

The functional analysis of various activator proteins has indicated that they contain distinct domains. Three structural motifs have been proposed for the DNA-binding domains of transcriptional activators: the helix-turn-helix, the zinc finger, and the leucine zipper (reviewed in Struhl, 1989, Landschulz et al., 1988). These motifs are found in various transcription factors, as well as in hormone receptors, oncoproteins, and homeodomain proteins, which are all involved in regulating the expression of specific sets of genes. Similar mechanisms of DNA-protein interactions are therefore used by a wide variety of proteins which modulate the transcription of other genes.

The modular organization of activator proteins has been analyzed in members of the steroid hormone receptor family. In the presence of a specific hormone, these proteins stimulate transcription of steroid hormone responsive genes. "Finger swaps" in which a particular domain of one receptor is replaced by the corresponding sequence from another receptor has led to the identification of discrete domains involved in DNA binding, steroid binding and in the stimulation of transcription (reviewed in Evans, 1988). The replacement of the DNA-binding region of the progesterone receptor by the homologous region in the glucocorticoid receptor, therefore leads to the formation of a hybrid protein which responds to progesterone by activating glucocorticoid-responsive promoters (Giguere et al., 1986, Green and Chambon, 1987). Thus domains responsible for DNA binding and transcriptional activation can be physically separated from the hormone-binding domain in the activator protein.

Modulation of the activity of DNA-binding proteins can be achieved by different mechanisms (reviewed in Mitchell and Tjian, 1989). In yeast, the increase in transcription of heat-shock genes is correlated with an increase in the phosphorylation of heat-shock transcription factors which are bound to the heat-shock DNA elements. Other activator proteins are regulated by protein-protein interactions. In the absence of inductive signal, inactive steroid receptors were found to associate with the heat-shock protein 90, an abundant protein in the cytoplasm. Upon hormone binding to the receptor molecule, dissociation of the complex occurred

to permit nuclear binding of the receptor and thus activation of steroid-dependent genes. Similarly the NF- κ B factor which is involved in regulating the expression of the immunoglobulin light and heavy chain genes, is held inactive by its association with the cytoplasmic inhibitor factor I κ B. Therefore, regulation of the tissue- and signal-dependent transcription of genes can be mediated by changes in the activity of the *trans*-acting proteins which recognize the *cis*-acting DNA elements.

1.4 Gene Expression during Myogenesis

1.4.1 Muscle-specific Transcription

The formation of striated muscle requires the synthesis of muscle-specific proteins that are organized into sarcomeres, which in turn are arranged in myofibril bundles (reviewed in Bagshaw et al., 1982). Studies with cloned satellite cells from human skeletal muscle have shown that the transcriptional activation of muscle-specific genes occurs asynchronously during the differentiation of myoblasts to myotubes (Gunning et al., 1987). In general, the transcription was activated after the fusion of the cells to form myotubes. Cardiac actin was the only muscle-specific transcript that was expressed in myoblasts. Skeletal actin was transiently induced in fusing cultures, while total myosin heavy chain mRNA accumulation lagged behind that of the sarcomeric actins. The other muscle-specific genes tested also possessed distinct kinetics of mRNA accumulation. The asynchronous activation of muscle-specific genes therefore indicated that distinct regulatory mechanisms must be present to specify the specific transcription pattern of each gene.

Members of the actin gene family show different developmental and tissue-specific patterns of expression. Six isoforms of actin have been detected in adult tissues of mammals: two in striated muscle (α cardiac and α skeletal), two in smooth muscle (α and γ smooth muscle actins), and two in nonmuscle cells (β and γ cytoskeletal actins) (Vandekerckhove and Weber, 1979). The sarcomeric actins which are restricted to striated muscle, are regulated during myogenesis. In embryonic skeletal muscle, cardiac actin predominates, while in the adult, cardiac actin

mRNA represents 5% of the sarcomeric actin transcripts (Gunning et al., 1983). Similarly in fetal heart, both isoforms of striated actins are coexpressed at approximately similar levels. Subsequently, cardiac actin is the only isoform expressed in adult cardiac muscle (Mayer et al., 1984).

The promotion of cardiac actin transcription has been investigated to determine the *cis*-acting DNA sequences which confer the distinctive pattern of expression of this gene. Several DNA sequences that permit expression of the human cardiac actin gene in the C2C12 skeletal muscle cell line have been identified: a distal region between -443 and -395 bp and a proximal region between -177 and -118 bp are implicated in the high-level transcription in the skeletal myocytes (Minty and Kedes, 1986b). A detailed mutagenesis analysis of the proximal region showed that a conserved region with the consensus CC(A/T)₆GG referred to as the CarG box, plays the major role in the transcriptional activation of the cardiac actin gene. In particular, the most proximal CarG boxes, CarG boxes 1 and 2, are necessary for transcription, while the more distal ones, CarG boxes 3 and 4, were not required for expression in the C2C12 cells (Miwa and Kedes, 1987). A proximal CarG box sequence was also found to be necessary for muscle-specific transcription of the *Xenopus* cardiac actin gene in *Xenopus* embryos, and of the chicken skeletal actin gene in chicken primary myoblast (Mohun et al., 1989, Grichnik et al., 1988). Additionally, the regulatory region of the human skeletal actin promoter contains a CarG box sequence (Muscat and Kedes, 1987). The CarG boxes would therefore seem to play an important role in the muscle-specific transcription of the sarcomeric actin genes.

The CarG box is found in a number of genes which are not necessarily muscle-specific (reviewed in Taylor et al., 1988). The serum responsive element (SRE) of the protooncogene *c-fos* contains a CarG box sequence which is recognized by a nuclear factor termed serum responsive factor (SRF) (Greenberg et al., 1987, Prywes and Roeder, 1986, 1987). Studies of the proteins that bind to the CarG box sequence in the *c-fos* and in the striated actin genes showed that the SRF is indistinguishable from the CarG binding factor (CBF) (Boxer et al., 1989). In addition, CarG boxes in the *c-fos* SRE, in the interleukin-2 gene, and in the cardiac

actin gene compete for the binding of a similar factor (Phan-Dinh-Tuy et al., 1988). These results suggested that tissue-specific expression of the sarcomeric actin gene results from complex interactions of the CBF with other factors which are found in muscle cells. In support of this, recent studies with gel shifts and footprinting assays revealed that at least seven distinct nuclear proteins including the Sp1 factor, the SRF, the CCAAT box-binding factor CTF/NF-1, interact with known and putative regulatory elements of the human cardiac actin promoter (Gustafson and Kedes, 1989).

Studies with other muscle-specific genes such as troponin I, myosin light chain 1/3, myosin heavy chain and muscle creatin kinase, have permitted the mapping of various *cis*-acting regulatory sequences, but no common conserved sequence for the muscle-specific expression has been identified in these genes (Billeter et al., 1988, Bouvagnet et al., 1987, Konieczny et al., 1987, Jaynes et al., 1988). In the chicken cardiac myosin light chain 2 and in the mouse muscle creatine kinase genes, nuclear factors have been shown to interact with the muscle-specific regulatory elements in the 5'-flanking regions of the genes (Braun et al., 1989a, Buskin and Hauschka, 1989, Horlick and Benfield, 1989). In both cases, muscle and nonmuscle factors interact at various sites within the promoter. Thus the transcriptional regulation of muscle-specific genes appears to involve the cooperation of diverse muscle and nonmuscle *trans*-acting factors at specific sites in the promoter region.

1.4.2 Myogenic Factors

Several factors which are involved in the activation of muscle-specific genes during skeletal myogenesis have recently been identified. The myogenic determination factor 1 (MyoD1) complementary DNA (cDNA) has first been shown to convert fibroblasts cells and other cell types to myoblasts, when expressed at high levels in those cells (Davis et al., 1987, Weintraub et al., 1989). MyoD is a phosphoprotein which is normally present in the nuclei of proliferating myoblasts and differentiated myotubes. Deletion mutagenesis showed that the MyoD protein contains various domains with specific functions: a highly basic region is involved in nuclear localization while a short region which is homologous to the transforming region of the

c-myc oncogene, confers the ability to initiate myogenesis (Tapscott et al., 1988). The region of MyoD that provides the myogenic function has been found to be homologous with a section of the *myc* protein and with regions in the *Drosophila daughterless* protein, in proteins of the *Drosophila achaete-scute* and *twist* gene family, and in proteins that bind to the immunoglobulin kappa chain enhancer (Murre et al., 1989). These proteins play a role in differentiation or in the transcriptional regulation of different genes.

In transient assays, constitutive expression of MyoD leads to the *trans*-activation of various muscle-specific genes thus indicating that MyoD may be a tissue-specific transcriptional activator (Davis et al., 1989, Weintraub et al., 1989). More recently, MyoD has been shown to be a sequence-specific DNA-binding protein, which can interact with enhancer sequences in the MCK gene (Lassar et al., 1989). The expression of sarcomeric genes may therefore be regulated by the MyoD protein during skeletal myogenesis.

Other factors with similar properties as MyoD have been discovered. These include myogenin, Myf-5 and CMD1, which are all expressed specifically in skeletal muscle, and which can induce the myogenic conversion of fibroblast cells (Lin et al., 1989, Braun et al., 1989b, Wright et al., 1989). The *trans*-activation of muscle-specific genes in skeletal myocytes may therefore involve several activator proteins in addition to MyoD. Indeed, other muscle-specific transcription activators are required for expression in cardiac muscle since MyoD and MyoD-related proteins are not expressed in these cells. Additionally the DNA-binding studies of muscle-specific genes (described in Section 1.4.1), indicated that transcription of muscle-specific genes involves the cooperation of various proteins that bind to the different *cis* elements in the genes.

1.5 Thesis Project

In this thesis, we have investigated some aspects of the regulation of gene expression during myogenesis. Previous studies have demonstrated that the expression of the transfected human cardiac actin gene was appropriately regulated during the

differentiation of P19 cells into cardiac muscle-containing cultures (Rudnicki et al., 1988). Since the *cis* regulatory elements of the cardiac actin gene have only been studied in skeletal muscle cell lines, we have focused on the identification of the regulatory sequences that control the expression of this gene in cardiac muscle derived from P19 cell cultures. We first showed that a chimeric cardiac actin gene with 440 bp of the 5'-flanking region was appropriately regulated during the differentiation of P19 cells into cardiac muscle. Using the approach of mutational analysis with the chimeric actin gene, we found that sequences between -237 and -158 bp upstream of the cap site are involved in the cardiac-specific expression of the gene (Chapter 3). In Chapter 4, we examined the effect of a skeletal muscle transcriptional activator, MyoD, on the expression of the mutated cardiac actin promoter in transiently transfected P19 cells. We concluded that multiple elements located between -312 and -47 bp upstream of the cap site are involved in the MyoD-induced *trans*-activation of the transfected cardiac actin chimeric gene. Endogenous sarcomeric actin genes were not subject to regulation by MyoD. To further analyze the effect of MyoD in EC cells, we isolated stable transformants containing a constitutively expressed MyoD chimeric gene. We found that MyoD induced differentiation of P19 cells into various cell types, including cardiac muscle where MyoD normally does not play a role (Chapter 5). These results and the previous ones suggested that cooperation of MyoD with other factors is required for the induction of muscle-specific genes during skeletal myogenesis.

Chapter 2

Material and Methods

2.1 Chemicals and Equipment

Chemicals were obtained from the following companies: BDH Chemicals Limited (Toronto, Ont., Canada), Fisher Scientific Company (Fair Lawn, NJ, USA), Sigma Chemical Company (St. Louis, Mo, USA). Molecular biology reagents including restriction endonucleases and other modifying enzymes, were obtained from either BRL (Gaithersburg, MD, USA), New England Biolabs (Beverly, MA, USA), Boehringer Mannheim Canada (Dorval, Que., Canada), or Pharmacia (Baie d'Urte, Que., Canada). Radiochemicals were received from Amersham Canada Limited (Oakville, Ont., Canada).

The instruments used in the molecular biology experiments include a Beckman J2-21M for centrifugation, a Beckman LS7800 Scintillation Counter for measurements of radioactivity, a Beckman DU-7 Spectrophotometer for measurements of absorbances, and a LKB Ultrosan XL for densitometry. Finally, luminescence was measured with a LKB luminometer.

2.2 Plasmid DNA preparations

2.2.1 Small scale plasmid preparation

Minipreparations of plasmid DNA were made using the modified protocol of Birnboim and Doly (1979). Bacteria were inoculated into 5 ml of "Terrific Broth" (Tartof et al., 1987) containing 100 $\mu\text{g}/\text{ml}$ of ampicillin, and were grown overnight at 37°C with vigorous shaking. Bacteria from 1.5 ml of culture were pelleted by centrifugation for 15 sec at 12,000 g in an Eppendorf tube. The pellet was resuspended in 150 μl of lysis solution (50 mM glucose, 10 mM CDTA, 25 mM Tris-Cl pH 8.0, 4 mg/ml lysozyme). Following a 5 min incubation at room temperature, 200 μl of a denaturation solution (0.2N NaOH, 1% SDS) was added and mixed gently with the lysed bacteria. After storing on ice for 5 min, 150 μl of an ice-cold high salt solution (3M potassium acetate, 1.8M formic acid) was added and mixed by vortexing for 10 seconds. The solution was stored 5 min on ice, and was centrifuged for 5 min in a microcentrifuge at 4°C. The supernatant was extracted once with 450 μl of phenol and once with 450 μl of chloroform/isoamyl alcohol (24:1 V/V). Plasmid DNA was precipitated by adding 900 μl of 95% ethanol to the aqueous phase. After vortexing briefly and incubating 5 min at 4°C, the tube was centrifuged 5 min at room temperature. The pellet containing the plasmid DNA was resuspended in 50 μl of TE (10 mM Tris-Cl pH 8.0, 1 mM EDTA) containing DNase-free pancreatic RNase.

2.2.2 Large scale plasmid preparation

Highly purified plasmid DNA for transfection experiments was isolated using the modified protocol of Marko et al. (1982). Bacteria were grown overnight in 250 ml of "Terrific Broth" (Tartof et al., 1987) containing 100 $\mu\text{g}/\text{ml}$ of ampicillin. The culture was centrifuged at 6,000 rpm for 10 min at 4°C. The pellet was washed with 100 ml of demineralized water and centrifuged again at 6,000 rpm for 5 min at 4°C. The pellet was resuspended in 20 ml of lysis solution (see section 2.2.1), and was left on ice for 30 min. Denaturation solution (40 ml, see section 2.2.1) was

added, and mixed with the bacterial lysate by swirling gently. After storing on ice for 15 min, 30 ml of high salt solution (see section 2.2.1) were added and mixed by shaking. Following a 30 min incubation on ice, the solution was centrifuged at 4°C for 20 min at 10,000 rpm. (All subsequent centrifugations were done at 10,000 rpm and at a temperature of 4°C .) DNA was precipitated by adding two volumes of ice cold ethanol, and by centrifuging for 15 min. The pellet was resuspended in 8 ml of acetate-MOPS (0.1 M sodium acetate, 0.05 M MOPS pH 8.0), and was precipitated again in ethanol. The centrifuged DNA was dissolved in 3 ml of water and mixed with an equal volume of LiCl solution (5M LiCl, 0.05 M MOPS pH 8.0). After a 15 min incubation on ice and a 20 min centrifugation, the supernatant was incubated at 65°C for 15 min. The solution was centrifuged for another 15 min, and the DNA was precipitated from the supernatant as described above. The pellet was dissolved in 2 ml of acetate-MOPS solution, and the DNA was collected by ethanol precipitation and centrifugation. The pellet was next dissolved in 2 ml of loading buffer (6M $NaClO_4$, 50 mM Tris-Cl, 10 mM CDTA pH 8.0). Glass powder suspension (20 ml of loading buffer containing 2 g of ground Whatman GF/A filters) was added to the DNA solution, which was gently mixed for 15 min. The suspension was filtered under vacuum using a Nalgene 0.2 μm filter prewetted with loading buffer. Following a filtration with 100ml of loading buffer, 24ml of elution buffer (0.2M $NaClO_4$, 60 mM Tris-Cl, 10 mM CDTA pH 8.0) were added. The DNA was slowly eluted in a clean container, and was precipitated by adding 3 ml of sodium acetate and two volumes of ethanol. Following a 30 min centrifugation, the plasmid DNA was resuspended in 1 ml TE, and was stored at -20°C.

2.3 RNA Preparation and Analysis

2.3.1 Isolation of Total Cellular RNA

RNA was purified from cultured cells after the method of Auffray and Rougeon (1980). All the manipulations were done with cold solutions, at 0°C. Briefly, after a wash with PBS, cells were lysed by adding 2 ml of 8M urea/3M LiCl solution to

each dish. After a 5 min incubation, the lysate was transferred to a tube, and was homogenized for 30 to 60 seconds at speed # 7 of a Polytron tissue homogenizer. The solution was stored overnight at 0°C to allow RNA precipitation. The RNA was pelleted by centrifugation (10,000 rpm, 30 min), and was washed twice with 3M LiCl. The pellet was resuspended in 500 μ l of diethyl pyrocarbonate (DEPC)-treated water, and was stored at -70°C.

2.3.2 Formaldehyde Agarose Gel Electrophoresis

RNA was electrophoresed in denaturing horizontal 1% agarose gels in a buffer consisting of 9% formaldehyde, 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA (pH 7.0). The RNA samples were ethanol precipitated and resuspended in 30 μ l of buffer (50% formamide, 10% formaldehyde, 20 mM MOPS, 5 mM sodium acetate and 1 mM EDTA in DEPC water). After heating at 65°C for 20 min and cooling 10 min on ice, the RNA samples were loaded on a gel with a 1/10 volume of loading buffer (0.1% xylene cyanol, 30% glycerol and 0.05 μ g/ml ethidium bromide). The gel was photographed after completion of the electrophoresis using a 302 nm wavelength U.V. light.

2.3.3 Northern Transfer and Hybridization

The RNA which was size-fractionated in the formaldehyde agarose gel, was transferred to a Hybond-N filter (Amersham) as described by Thomas (1980). The filter was blotted for 24 hours and was U.V. irradiated for 1 min. For the prehybridization, the filter was incubated at 42°C for at least one hour in a solution of 50% formamide, 5X Denhardt's solution, 0.3% SDS, 0.25 mg/ml denatured salmon sperm DNA, and 5X SSC (from a 20X stock consisting of 3M NaCl and 0.3 M sodium citrate). The radiolabelled probe (see section 2.3.4) was added to the prehybridization solution, and was incubated at 42°C overnight. The filter was then washed with 2X SSC, 0.2% SDS for 30 min at room temperature, followed by 0.2X SSC, 0.2% SDS for at least 15 min at 65°C. To visualize hybridization, Kodak XAR-5 films were exposed to the filter for at least one day at -70°C in a cassette with 2 Lanex regular screens.

In order to reprobe filters, the original probe was removed by incubating the blot for 1-2 hours at 65°C in 0.005 M Tris-Cl pH 8.0, 0.002 M EDTA, 0.1X Denhardt's solution.

2.3.4 Multiprime Labelling of DNA

DNA fragments purified from agarose gels by electroelution were labelled with ^{32}P by multiprime reaction (Feinberg and Vogelstein, 1983) using the Amersham multiprime DNA labelling kit. Denatured DNA (25 ng) was incubated for one hour at 37°C in a reaction mixture consisting of 50 mM Tris-Cl (pH 7.8), 10 mM 2-mercaptoethanol, 20 μM each of dATP, dGTP and dTTP, 5 mM $MgCl_2$, 50 μCi of [α - ^{32}P]dCTP (with a specific activity of 3,000 Ci/mM), random hexanucleotides and 2 units of the Klenow fragment of DNA polymerase I. After removal of unincorporated nucleotides by ethanol precipitation, the radiolabelled DNA was heat denatured and used in Northern hybridization experiments described in section 2.3.3.

2.4 Plasmid Construction

Plasmids were constructed by standard molecular biology techniques, most of which were described by Maniatis (1982). All constructs were verified by restriction endonuclease digestion and size-fractionation in 1% to 2% agarose gels, or in 2% to 3% NuSieve GTC agarose gels (FMC Bioproducts, Rockland, ME, USA), which permitted the resolution of as little as 10 bp in the range of 250 to 500 bp fragments.

2.4.1 Restriction Endonuclease Digestion and Modification of DNA Fragments

Plasmid DNA was digested with restriction endonucleases under the conditions suggested by the manufacturer. To do partial digests, the plasmid DNA was incubated from 4 to 10 min with 1 u of enzyme per μg of DNA. To generate blunt end termini from 5' overhangs, the digested DNA (usually 0.5 to 10 μg in 20 μl) was incubated

for 30 min at room temperature with 2 u of the Klenow fragment of DNA polymerase I (Klenow), deoxynucleosides triphosphate (0.1 mM of each one), and buffer (0.05 M Tris-Cl pH 7.2, 0.01 M $MgSO_4$, 0.1 mM dithiothreitol, 50 $\mu g/ml$ bovine serum albumin). To generate blunt ends from 3' termini, the digested DNA was first incubated with 2 u Klenow for 2 min at 37°C, followed by 10 min at 37°C in the presence of the deoxynucleosides triphosphate (0.1 mM of each one).

Restriction fragment termini were dephosphorylated by adding the appropriate amount of 10X phosphatase buffer (0.05 M Tris-Cl pH 9.0, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1mM spermidine), and 1 u of calf intestinal phosphatase (phosphatase). For 5' overhangs, the reaction mixture was incubated twice for 30 min at 37°C, with the addition of a second aliquot (1 u) of phosphatase after the first 30 min incubation. For 3' overhangs or blunt ends, the reaction mixture was incubated twice for 15 min at 37°C and for 15 min at 56°C, with the addition of 1 u of phosphatase after the first 30 min of incubation.

2.4.2 Addition of Linkers to Restriction Fragments

Blunt end restriction endonuclease fragments were first phenol and chloroform extracted. After an ethanol precipitation, the DNA was resuspended in a total volume of 25 μl in the presence of 1 u of T4 DNA ligase, 2 μg of synthetic linkers, and 5X ligase buffer (0.25 M Tris-Cl pH 7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM dithiothreitol, 25% w/v polyethylene glycol-8000). Following an overnight incubation at room temperature, the mixture was phenol and chloroform extracted, and was ethanol precipitated. Excess linkers were removed by digesting the DNA for at least 3 hours in the presence of 50 to 100 u of the restriction endonuclease which recognized the linker sequence.

2.4.3 Isolation and Ligation of Restriction Fragments

The digested and modified DNA fragments contained in loading buffer (as in section 2.3.2) were size-fractionated on 0.6% low melting point agarose gels (BRL Ultra Pure) in Tris-borate buffer (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M

EDTA). The DNA was visualized with a long-wave U.V. light. The bands of interest were excised out of the agarose with a sharp blade, and placed in an Eppendorf tube. After diluting the gel fragment in 1/3 volume of water, the gel was melted by heating at 65°C. Ligations were done by mixing the appropriate amounts of vector and insert (usually at a 1:1 or 1:2 ratio) with T4 DNA ligase (1u/10 µl of reaction mixture), and 5X ligase buffer (see section 2.4.2). The ligation mixture was incubated overnight at room temperature and used the following day to transform bacteria after diluting to a total volume of 300 µl.

2.4.4 Preparation and Transformation of *E. coli*

Competent DH5 *Escherichia coli* (*E. coli*) were prepared exactly as described by Hanahan (1985), and were stored at -70°C. For the transformation of bacteria, 50 to 80 µl of ligation mixture (corresponding to about 200 ng of vector) were added to 200 µl of freshly thawed competent *E. coli*. The bacteria were stored on ice for 30 min. Following a 50 seconds heat-shock at 42°C, the bacteria were returned to the ice for 5 min. "Terrific broth" (Tartof et al., 1987) was added to the bacteria, which were next incubated for 1 hour at 37°C. Bacteria (200 µl) were plated onto prewarmed ampicillin dishes (100 µg/ml ampicillin, 15 g agar/l of "Terrific Broth"), and were grown overnight at 37°C.

2.5 Culture Conditions for EC Cells

P19 EC cells were maintained and induced to differentiate as described by Rudnicki and McBurney (1987).

2.5.1 Routine Culture

EC cells were cultured in a 5% CO₂ incubator at 37°C in the presence of α-modified minimal essential medium (Gibco, Mississauga, Ont., Canada) supplemented with 7.5% calf serum and 2.5% fetal calf serum (Bocknek Laboratories, Rexdale, Ont., Canada). The cells were passaged at least every two days. Briefly, cells were washed

with PBS (0.8% NaCl, 0.02% KCl, 0.02% KH_2PO_4 , and 0.115% Na_2HPO_4 , pH 7.4), and were incubated for 5 min with a small volume (0.5 ml in a 60 mm dish) of trypsin-EDTA (1 mM EDTA and 0.025% w/v trypsin in PBS). The cells were dispersed by vigorously pipetting with a Pasteur pipette, and were resuspended in medium before being transferred to tissue culture dishes at a density of approximately 10^5 cells/ml of medium. If necessary, cells were counted with the aid of an electronic particle counter (Coulter Counter, Coulter Electronics Inc.).

2.5.2 Conditions for Differentiation

P19 EC cells were induced to differentiate into cultures containing cardiac and skeletal muscle, by culturing them in petri grade dishes for 5 days in the presence of 1% DMSO (Sigma). The medium was replenished every two days, and on the fifth day (day 5 after the initiation of differentiation) the aggregates were plated onto tissue culture dishes without DMSO in the medium. For growth until day 10, the medium was changed every two days.

Differentiation into neurons, glia, and fibroblasts, was done as described above, except that the DMSO was replaced by 5×10^{-7} M retinoic acid (Eastman Kodak).

2.6 Transfection and Selection of EC cells

Transfections of EC cells with plasmid DNA were performed according to the protocol of Chen and Okayama (1987). Highly purified plasmid DNA for transfections was prepared as described in Section 2.2.2. The day preceding the transfection, P19 cells were seeded at a density of 500,000 per 60 mm dish. The following day, the medium (5ml) was changed several hours before adding DNA to the dishes. Plasmid DNA (10-20 μg) was diluted to a total volume of 450 μl with sterile water in an Eppendorf tube. Sterile 2.5 M $CaCl_2$ (50 μl) was first added to the tube, followed by 500 μl of 2X BES buffer (50 mM N,N-bis 2-hydroxyethyl-2-aminoethane-sulfonic acid pH 6.90, 280 mM NaCl, 1.5 mM Na_2HPO_4). The solution was vortexed and

stored 5 to 10 min at room temperature. The precipitated DNA was added dropwise to the cells and remained in the dish for 14 to 18 hours. The medium was then changed, and the cells were allowed to grow for 24 hours. The cells were next either harvested if a transient assay was being done, or plated in the presence of the antibiotic G418 if clones were selected. For the selection of the transfected clones, 500,000 cells were plated onto a 100 mm dish with 10 ml of medium, or 1.1×10^6 cells were plated onto a 150 mm dish with 22 ml of medium. The selective medium for the neo gene product consisted of regular medium supplemented with 400 $\mu\text{g}/\text{ml}$ G418 and β -mercaptoethanol from a 100X stock which was prepared with 7 μl of pure β -mercaptoethanol in 10 ml of medium. The medium was usually changed 3 days after the beginning of selection. It was also changed later if a high number of dead cells was present in the medium during the 10 to 14 days of selection.

To ensure that all plasmid preparations were equivalent, several preparations of certain constructs were used in transient and permanent transfections. Levels of expression from the reporter genes in the transfected plasmids were independent of the plasmid preparation. To ensure that there was efficient transfection, an internal standard (pRSVL or pRSVCAT for transient transfections, and pPGKneo-ActCAT for permanent transfections) was included along with the test plasmid. Samples with CAT or luciferase activities comparable to the activities found in untransfected P19 cells were not considered in the analysis of the data.

2.7 Detection of β -gal Activity

2.7.1 Histochemical Staining

In situ staining for β -gal activity was performed following the method developed by Pearson et al., (1963). Briefly, the cells were fixed in 0.2% glutaraldehyde (in PBS) for 5 min at 4°C. After washing twice with cold PBS, the cells were incubated overnight at 37°C in staining solution which consisted of 0.1 M phosphate buffer (pH 7.3), 2% X-gal in dimethyl formamide (55X stock), 1mM $MgSO_4$ and ferrowerri mix (11X stock contained 21 mg of potassium ferrocyanide and 16.4 mg of potassium

ferricyanide dissolved in 1 ml of water). Stained cells were observed with an inverted microscope, and were photographed using Kodak Ektachrome 50 color film or Kodak Panatomic-X 32 black and white film.

2.7.2 Spectrophotometric Assay

β -gal activity was measured using the spectrophotometric assay developed by Norton and Coffin (1985). After three washes in PBS, cells were harvested in a sucrose solution (0.25 M sucrose, 10 mM Tris-Cl, 10 mM EDTA) by scraping. Cells were lysed by three freeze/thaw cycles, and the debris were pelleted by centrifugation for 10 min in a microcentrifuge. Total protein concentration in each cell extract was determined using the Bio-Rad Protein Assay solution, based on the Bradford method (Bradford, 1976). The reaction mixture for the β -gal assay consisted of 800 μ l of assay buffer (100 mM $NaPO_4$ pH 7.0, 1mM $MgSO_4$, 100 mM β -mercaptoethanol), 200 μ l of stock ONPG (4 mg/ml ONPG in assay buffer), and 400 μ g of proteins from the cell extracts. Final volumes were equalized by addition of sucrose solution. The reaction at 28°C was monitored by measuring the change in absorbance at a wavelength of 420 nm during 60 to 120 min.

One unit of β -gal activity is the activity that permits the hydrolysis of 10^{-9} moles of ONPG /min, in the assay conditions described above.

2.7.3 Immunofluorescence

Immunofluorescence to detect the presence of β -gal or of other intracellular antigens, was performed as described by Rudnicki and McBurney (1987). After a wash in PBS, cells grown on gelatin-coated coverslips (0.1% gelatin in water) were fixed in -20°C methanol for 20 min. The air-dried coverslips were rehydrated in PBS for 10 to 20 min. The primary antibody was added at the appropriate dilution into 60 μ l of PBS per coverslip. After incubating 45 min at room temperature in a humidity chamber, the coverslips were washed three times for 5 min each in PBS. The coverslips were then incubated for 30 min in the humidity chamber with a fluorochrome-conjugated secondary antibody diluted 1:50 in PBS. After three 5 min

washes in PBS, the coverslips were placed on a slide in a drop of mounting medium (50% glycerol, 0.5% paraphenylene diamine buffered with PBS). The fluorescence was visualized using a microscope equipped with epifluorescent optics. Photography was done with Kodak Tri-X pan 400 black and white film or Kodak Ektachrome 400 color slide film. The primary antibodies used in these studies are listed in Table 1.

2.8 Immunocytochemistry

The immunocytochemistry procedure was similar to the immunofluorescence one except in the last few steps. Cells in 100 mm dishes were fixed in -20°C methanol for 20 min. The rehydrated cells were incubated at room temperature for 45 min with 3 ml of mouse monoclonal primary antibody diluted in PBS. After three PBS washes of 5 min each, the cells were incubated for 30 min in the presence of 2.5 ml of anti-mouse biotinylated species-specific antibody diluted 1:50 in PBS. After washing again three times with PBS, the cells were incubated for 15 min with 2.5 ml of streptavidin-biotinylated horseradish peroxidase (Amersham) diluted 1:1,000 in PBS containing 1% w/v of bovine serum albumin. Finally, after washing three times with PBS, 3 ml of diaminobenzidine solution (0.5 mg/ml in PBS containing 0.02% hydrogen peroxide) were added to the cells. After 30 min, this solution was replaced by PBS. Cells were photographed using the same films as those for X-gal stained cells photography.

2.9 CAT Assay

CAT activity was assayed by measuring the acetylation of chloramphenicol by ^3H acetyl CoA in a modified version of the Sleigh protocol (1986). Cell extracts were prepared in a sucrose solution as described in section 2.7.2 (β -gal assay). Total protein concentration in the cell extract was determined with the Bio-Rad Protein Assay solution (see section 2.7.2). Cell extracts (200 μg of proteins) were first heated at 65°C for 10 min. Following centrifugation, the supernatant was incubated for

Table 1: Antibodies used in immunodetection of muscle antigens

Name	Type	Specificity	Reference
B4	mouse monoclonal	smooth and striated muscle actins	Lessard, 1988
β -gal	rabbit polyclonal	β -galactosidase	Cappel, Cooper Biomedical Malvern, PA, USA
CGA7	mouse monoclonal	smooth muscle actins	Gown et al., 1985
MF20	mouse monoclonal	muscle myosin heavy chain	Bader et al., 1982
SM-1	mouse monoclonal	smooth muscle α -actin	Skalli et al., 1986

one hour at 37°C in the presence of 1.6 mM chloramphenicol, 0.4 μ Ci of tritiated acetyl CoA (4.3 Ci/mmol), 0.25 M Tris-Cl pH 7.8 in a total volume of 200 μ l. The reaction mixture was extracted twice with 400 μ l of cold ethyl acetate, and the amount of tritium in the supernatant was measured with the scintillation counter.

2.10 Luciferase Assay

Luciferase activity was measured according to the protocol of de Wet et al (1987). After three washes in PBS, cells were harvested by scraping in 200 μ l of extraction buffer (100 mM KH_2PO_4 , 1 mM dithiothreitol, pH 7.8). Cells were lysed by three freeze/thaw cycles, and the debris were pelleted by centrifugation for 10 min in a microcentrifuge. The reaction mixture consisted of 20 μ l of cell extract and of 350 μ l of assay buffer (25 mM glycylglycine, 15 mM $MgSO_4$, 0.2 mM luciferin, pH 7.8) placed in a luminometer cuvette. The reaction was initiated by injecting 50 μ l of 0.04 M ATP in the chamber containing the cuvette. The amount of light emitted during the reaction was measured and integrated over a period of 45 seconds starting 15 seconds after the beginning of the reaction. Activities were corrected for the amount of protein which was measured as described in section 2.7.2.

Chapter 3

Regulated Cardiac Muscle Gene Expression

3.1 Introduction

Myogenesis is characterized by the activation of a large set of muscle-specific genes which encode contractile proteins required for the formation of the sarcomere. Increases in gene expression are controlled primarily at the level of transcription (reviewed in Buckingham, 1985). Transfection of muscle genes into cultured skeletal myoblasts allowed the identification of several *cis*-acting DNA sequences required for transcription in muscle cells. Regulatory elements and enhancers that permit muscle-specific activation were specifically identified in the 5'-flanking regions of the genes encoding troponin T, myosin heavy chain, myosin light chain-2, cardiac actin and skeletal actin (Bouvagnet et al., 1987, Braun et al., 1989a, Mar and Ordahl, 1988, Minty and Kedes, 1986b, Muscat and Kedes, 1987). In addition, elements located in the first intron are also required for the regulated expression of the troponin I and the muscle creatine kinase gene (Yutzey et al., 1989, Sternberg et al., 1988). Although these various genes are all induced during muscle differentiation, no common DNA sequence was discovered. Instead, many different sequences seem to be involved, some of which govern the tissue-specificity while others control the quantitative aspect of transcription. Similarly, *trans*-acting factors that bind to the

cis-acting regulatory sequences seem to be numerous, and they are not necessarily restricted to muscle (Boxer et al., 1989, Braun et al., 1989a, Buskin and Hauschka, 1989, Walsh, 1989). Muscle-specific gene expression thus seems to involve the complex interaction of diverse factors at specific DNA sequences.

Cardiac actin is one of the earliest markers for myogenic differentiation. This actin isoform is found not only in the developing heart, but also in the myotomal regions of somites where skeletal muscle subsequently forms (Sassoon et al., 1988). The cardiac isoform of actin predominates in embryonic skeletal muscle, although it is later replaced by skeletal actin (Minty et al., 1982). This developmental and tissue-specific regulation of muscle actins occurs mainly at the level of gene transcription.

Previous studies have identified DNA sequences referred to as CarG boxes, which are required for the high-level expression of the human cardiac α -actin promoter in C2C12 skeletal myocytes (Miwa and Kedes, 1987). The muscle-specific transcription of the cardiac actin gene is also directed by the CarG motif in microinjected *Xenopus* embryos (Mohun et al., 1989). In another report however, the same element negatively regulates the expression from the smooth muscle α -actin promoter, while it enhances transcription from the β -actin gene (Carroll et al., 1988, Kawamoto et al., 1988). In addition, the CarG box sequence and the serum responsive element of the *c-fos* protooncogene are structurally similar, and both seem to be the binding site of an identical factor (Boxer et al., 1989). Muscle-specific gene expression is therefore probably not governed by the CarG box element alone since this element has multiple actions, which are not restricted to cardiac and skeletal muscle cells.

The mode of expression of vertebrate muscle genes was mostly studied in skeletal myocytes, and consequently, the regulatory elements required for cardiac muscle-specific transcription are unknown. Transgenic mice experiments have shown that an exogenous skeletal actin gene is developmentally regulated in both the cardiac and skeletal muscle of mice (Shani, 1986). Studies with muscle creatine kinase chimeric genes introduced into the mouse germ line, indicated the presence of several sequences required for the differential expression in cardiac and skeletal muscle (Johnson et al., 1989). In a different approach, tissue-specific expression in cardiac

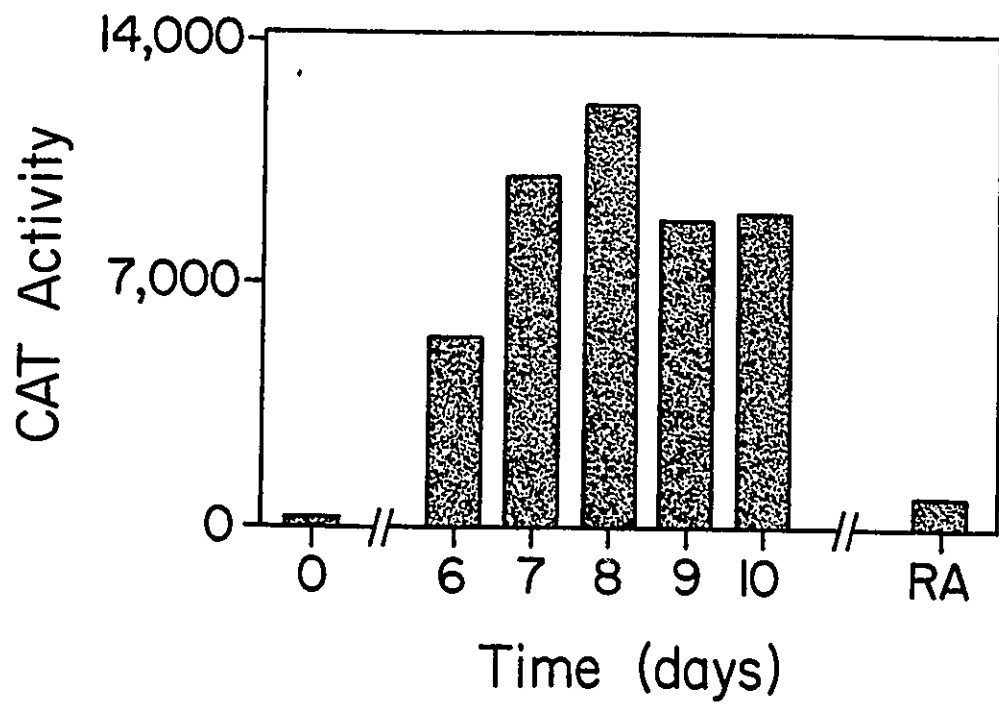
muscle was studied with the mouse P19 EC cell line. When exposed to DMSO, aggregates of P19 cells differentiate into a variety of cell types which include cardiac and skeletal muscle (McBurney et al., 1982a). Furthermore, it was shown that a transfected human cardiac α -actin gene is expressed with the appropriate developmental and cell type specificity during the differentiation of P19 cells (Rudnicki et al., 1988). To further define the *cis*-acting regulatory elements involved in this tissue-specific expression, we constructed a series of linker-scanning and deletion mutations of the human cardiac actin promoter region, and we tested their transcriptional activity during the differentiation of pooled populations of stably transfected P19 cells. Sequences between -237 and +6 bp upstream of the cap site of the cardiac actin promoter were sufficient to direct high levels of expression during the DMSO-induced differentiation of P19 cells. The sequences located between -237 and -227 bp upstream of the cap site, are almost identical to the CarG box consensus sequence, and were required for the high level of expression during myogenic differentiation. A second region of the promoter located between -227 and -158 bp may be responsible for the tissue-specific expression, since in sequential deletions, the removal of this region resulted in the loss of expression in muscle-containing cultures.

3.2 Regulated Expression of the Human Cardiac α -actin Promoter during the Differentiation of P19 Cells

Previous work from this laboratory has shown that the 1500 bp region between approximately -750 and +740 bp relative to the cap site of the human cardiac α -actin gene is sufficient to mediate muscle-specific expression of the thymidine kinase reporter gene during the differentiation of P19 cells (Rudnicki et al., 1988). To further delineate sequences required for this tissue-specific expression, we deleted the first exon and the first intron sequences, and established whether the 5'-flanking region appropriately activated expression in differentiated cells. Thus the region

between -750 and +6 bp of the human cardiac α -actin gene was placed upstream of the reporter gene lac Z in the construct pP/B ActlacZ (as shown in Appendix B). The corresponding β -gal activity was quantitated using a spectrophotometric assay or was visualized by *in situ* staining or by immunofluorescence. We also tested the muscle-specific expression of an actin-CAT construct (pPGKneo-ActCAT) which contains the human cardiac α -actin promoter region between -440 and +6 bp. This plasmid with a different reporter gene was used subsequently in the deletion and mutation experiments, as an internal standard for transfection efficiency and cellular differentiation. We therefore cotransfected pP/B ActlacZ, pPGKneo-ActCAT, pPGK, and pCA1035 in P19 cells. The last plasmid contains the PGK promoter directing the transcription of the neo gene, which confers resistance to the antibiotic G418. The PGK gene in pPGK was included for its enhancing effect on the transcription of the PGK promoter, which results in this case in a higher number of G418-resistant colonies (M. McBurney, personal communication). Following transfection and selection, the colonies were pooled and differentiated either with DMSO or RA. As shown in Figure 1, the expression from both the CAT and the β -gal chimeric genes is low in undifferentiated cells, and it increases as cells differentiate into muscle during the 10 day regime with DMSO. These changes in expression parallel those previously reported for the endogenous cardiac actin gene in differentiated P19 cells (Rudnicki et al., 1988). Highest activities are found between days 6 and 8, concomitant with the appearance of spontaneously beating cardiac muscle. By days 9 and 10, cultures become confluent, and a decrease in the activity of the reporter genes is observed. As expected, the muscle-specific actin promoter is not activated in differentiated cultures containing neurons, glia, and fibroblast cells (see RA in Figure 1). Results with the two different reporter genes were similar, but due to the elevated background CAT activity (of up to 5,000 cpm) in P19 cells, levels of expression from this reporter gene were not as precise as those from the lac Z gene. However, since the activity with both chimeric genes was restricted to muscle-containing cultures, the 5'-flanking region between -750 and +6 bp of the human cardiac α -actin promoter appropriately directs tissue-specific expression in stable transformants of P19 cells.

Figure 1: Muscle-specific expression of transfected human cardiac α -actin genes during differentiation of EC cells. P19 cells were transfected with 5 μ g of pP/B ActlacZ, 5 μ g of pPGKneo-ActCAT, 2.5 μ g of pCA1035, and 5 μ g of pPGK. Over 400 G418-resistant transformants were pooled and differentiated (Section 2.5.2). Day 0 corresponds to undifferentiated cells. From days 0 to 5, cells were aggregated in medium containing 1% DMSO. On the fifth day, cells were allowed to reattach onto tissue-culture grade dishes, and were harvested at daily intervals for the 5 following days. RA corresponds to differentiated cells, 6 days after RA treatment. Neurons could be observed in this case, while with DMSO, beating cardiac muscle was detected. Shown here are results from 1 of at least 5 experiments. The top panel shows β -gal activities for each cell extract (in u/mg of protein $\times 10$) determined using the spectrophotometric assay described in Section 2.7.2, while the bottom panel shows CAT activities (in cpm per 200 μ g of proteins) which are measured as the acetylation of chloramphenicol with 3H acetyl CoA. The background β -gal and CAT activities from untransfected P19 cells were subtracted from each of the values.



To visualize which cells of the differentiated aggregates express the chimeric actin gene, we performed *in situ* staining with the chromogenic substrate X-gal on the pooled populations of cells stably transfected with the cardiac actin-lacZ gene. β -gal activity which was detected as a blue color in the cells, was found in very few undifferentiated cells, and in RA-treated cultures, positive cells were seen even less frequently (Figures 2a and b). In contrast, muscle-containing cultures contained high numbers of β -gal-positive cells (Figure 2c). Most of the blue cells in these cultures had a relatively large and flat morphology as compared to P19 cells, and were distributed at the periphery of the aggregates (Figure 2d). Some of the cells were located within areas where beating muscle was observed before fixation and X-gal staining. Skeletal muscle cells, which represent only a small fraction of the striated muscle in the cultures, also expressed the actin-lacZ chimeric gene (Figure 2e). These results therefore indicate that the muscle-specific gene is expressed in cardiac and skeletal muscle, as well as in other unidentified cells which could be progenitor cells of cardiac or skeletal myocytes.

To confirm the identity of the cells expressing the transfected cardiac actin gene, we performed indirect immunofluorescence experiments using antibodies reacting with β -gal, or with endogenous muscle-specific gene products listed in Table 1 (of Chapter 2). The β -gal protein and muscle myosin heavy chain were detected with the Cappel β -gal antibody and the MF20 antibody respectively, in DMSO-treated cultures which were transfected with the actin-lacZ gene. As previously shown (Edwards et al., 1983), the MF20 antibody brightly stained skeletal and cardiac muscle cells in cultures differentiated with DMSO. In double labelling experiments, we observed colocalization of the β -gal protein and of the MF20 antigen in many cells that appeared to be cardiac or skeletal muscle cells (Figure 3). The chimeric actin gene is therefore expressed with muscle myosin heavy chains in striated muscle cells.

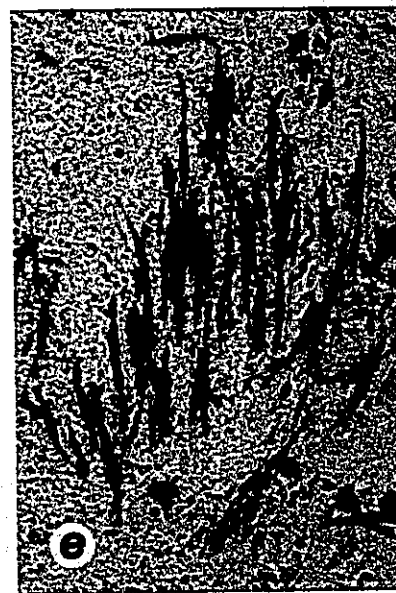
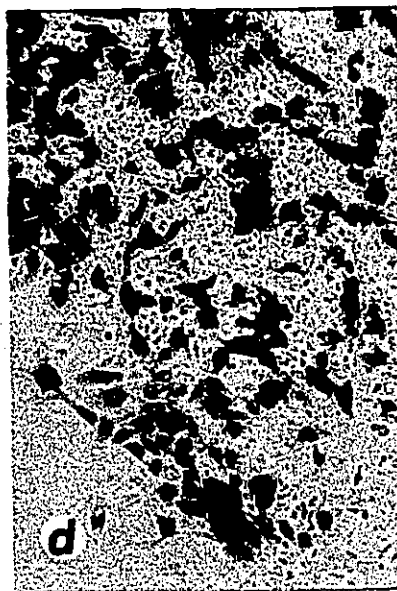
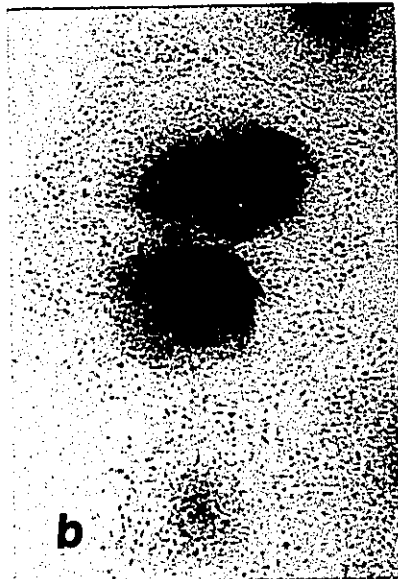
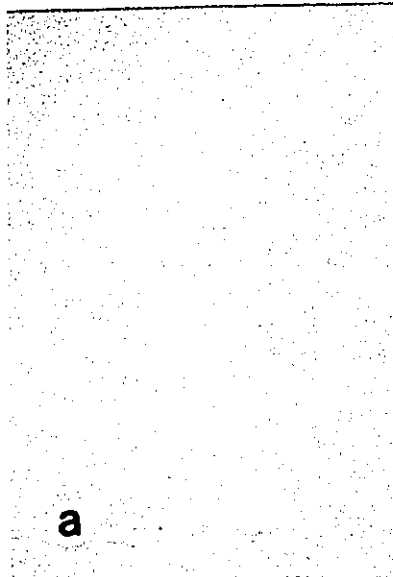
In the previous immunofluorescence experiment, many MF20-positive cells did not stain with the β -gal antibody, probably because not all transformants have the capacity to express the transfected muscle-specific gene. Unexpectedly, we also observed that many cells containing β -gal did not react with the MF20 antibody.

These cells were located in regions containing MF20-reactive cells, or at the periphery of aggregates as found for the X-gal stained cells of Figure 2c. The failure to detect muscle MHC in cells expressing the cardiac actin chimeric gene can be explained by the delayed activation of the genes: MHC mRNA accumulation peaks at day 9, rather than at day 6 for cardiac actin in DMSO-treated P19 cells (Rudnicki et al., accepted in Dev. Biol.). Similarly in mouse embryos, the accumulation of cardiac actin precedes the activation of other muscle-specific genes which occurs during the terminal differentiation of the cells (Sassoon et al., 1988). It is therefore possible that the cells expressing the cardiac actin chimeric gene are embryonic cardiac myocytes which have not yet accumulated other muscle-specific gene products.

To determine whether muscle-specific isoforms of the actin protein were co-expressed with the cardiac actin chimeric gene, we performed experiments using several antibodies reacting with various isoforms of actin (Table 1). The B4 antibody reacts with all four striated and smooth muscle actins, but shows preferential reactivity toward enteric smooth muscle γ -actin (Lessard, 1988). In the DMSO-treated cultures of P19 cells, this antibody used at a low concentration (10 μ g/ml) mostly stained the peripheral cells of the cultures which included β -gal reactive cells (data not shown). Filaments arranged as stress fibres were very brightly stained within fibroblast-like cells located at the edges of the aggregates. This is consistent with a previous report demonstrating the expression of smooth muscle actins in DMSO-treated P19 cells (Rudnicki et al., accepted in Dev. Biol.).

To confirm that smooth muscle actins were indeed expressed in the β -gal-positive cells, we made use of the CGA7 and the SM-1 antibodies. The mouse monoclonal antibody CGA7 is reactive with vascular smooth muscle α -actin and with enteric smooth muscle γ -actin, while the SM-1 antibody is only reactive with vascular smooth muscle α -actin (Gown et al., 1985, Skalli et al., 1986). In double labelling experiments with each of these mouse monoclonal antibodies and with the rabbit polyclonal β -gal antibody, we observed that the fibroblast-like β -gal-positive cells distributed at the periphery of the aggregates were often CGA7-reactive, as well as SM-1-reactive (Figures 4 and 5). In fact, the edges of the DMSO-treated aggregates were usually entirely stained with the smooth muscle-specific antibody. The

Figure 2: Localization of cells expressing the actin-lacZ chimeric gene in differentiated P19 cultures. Cells were transfected with pPv/B ActlacZ and a selectable plasmid as described in the legend of Figure 1. Pooled populations of the stably transfected cells were stained for β -gal activity using the chromogenic substrate X-gal, which produces a blue color upon cleavage by the enzyme (Section 2.7.1). Very few undifferentiated cells (a), or cells differentiated with RA (b), express the actin-lacZ gene. In panel c, the differentiation of the same pooled population in the presence of DMSO resulted in a high number of blue cells. These cells are found mostly at the periphery of aggregates (d). The actin-lacZ gene is also expressed in bipolar multinucleate cells which are characteristic of skeletal muscle (e). Bar, 250 μ m.




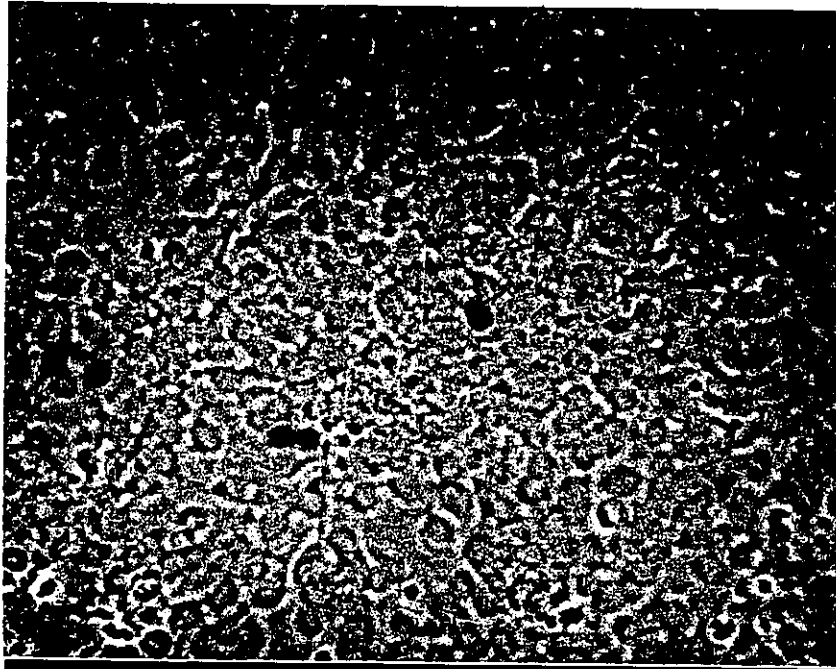


Figure 3: Localization of the β -gal protein in muscle cells of actin-lacZ transformants differentiated with DMSO. Cells were transfected with a chimeric cardiac actin-lacZ gene and differentiated in the presence of DMSO until day 6, as previously described (Figure 1). Panel **a** is a phase contrast field of cells stained with the mouse MF20 monoclonal antibody reactive with muscle myosin (**b**), and with the rabbit polyclonal β -gal antibody (**c**). Indirect labelling was with rhodamine-conjugated anti-mouse IgG, and with an anti-rabbit biotinylated species-specific antibody followed by a fluorescein-streptavidin complex. Bar, 50 μ m.

a



b



c

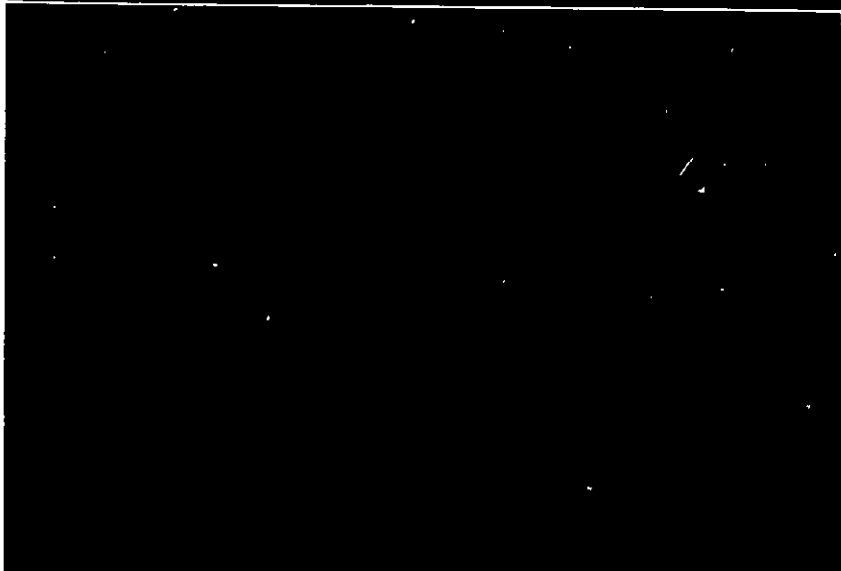
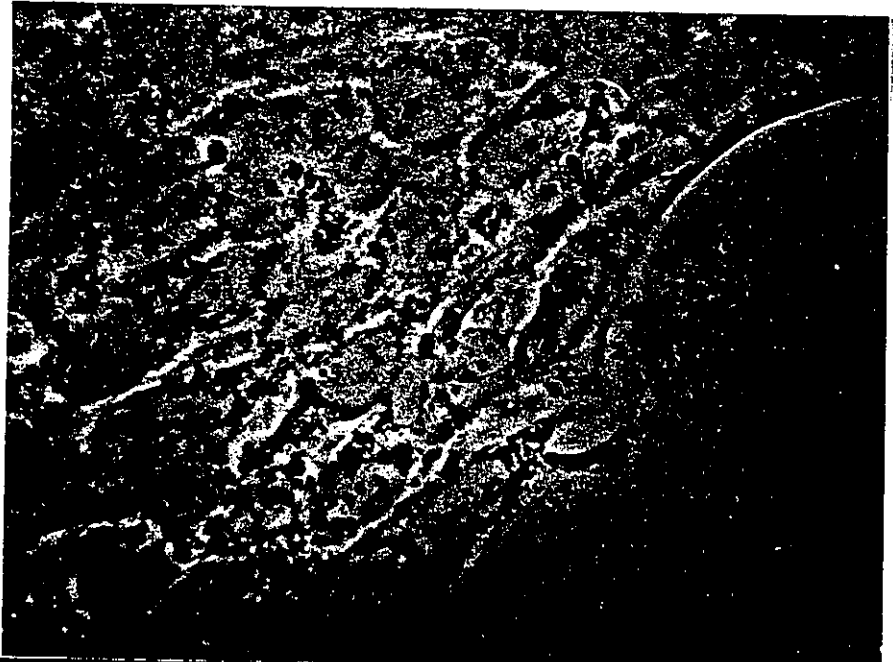


Figure 4: Colocalization of β -gal and of smooth muscle actins in the peripheral cells of DMSO-treated aggregates containing the cardiac actin-lacZ gene. P19 transformants containing pPv/B ActlacZ were differentiated until day 6 of the DMSO regime (Section 2.5.2). Panel a is a phase contrast field of cells stained with the mouse CGA7 monoclonal antibody (b), and with the rabbit β -gal polyclonal antibody (c). Indirect labelling was as previously described (Figure 3). Bar, 20 μ m.

a



b



c

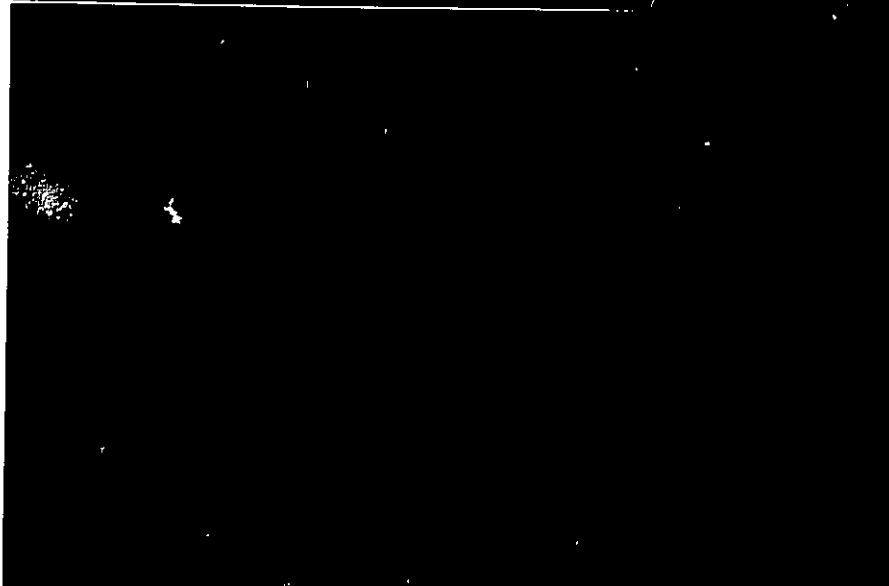
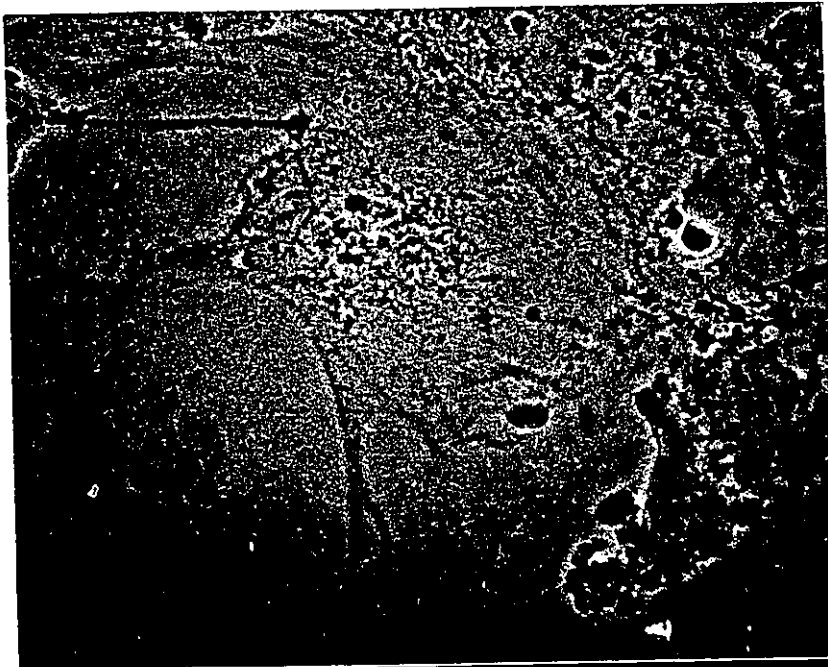


Figure 5: Localization of β -gal in peripheral cells of DMSO-treated aggregates expressing the smooth muscle α isoform of actin. P19 transformants containing pPv/B ActlacZ were differentiated until day 6 of the DMSO regime (Section 2.5.2). Panel **a** is a phase contrast field of cells at the periphery of aggregates which were stained with the SM-1 antibody (**b**), and with the β -gal antibody (**c**). Indirect labelling was as previously described (Figure 3). Bar, 20 μm .

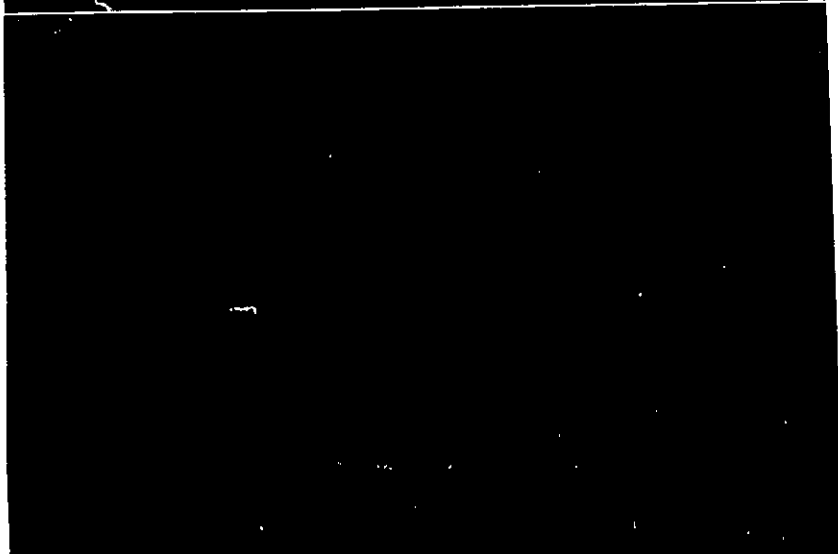
a



b



c



chimeric cardiac actin gene is therefore expressed in cells which contain smooth muscle actins.

The expression of endogenous cardiac and skeletal actins in these cells was difficult to visualize with an anti-muscle actin antiserum which is specific toward cardiac and skeletal actin (Bulinski et al., 1983). This antibody showed some non-specific nuclear binding, but it also stained some filaments arranged as stress fibres within the peripheral cells of DMSO-treated aggregates (data not shown). (It should be noted however, that these results could not be verified due to the unavailability of this polyclonal antibody.) Taken together, these results demonstrate that the peripheral cells expressing the transfected cardiac actin-lacZ gene generally contain smooth muscle actin, and possibly endogenous striated actin isoforms.

3.3 Cardiac Actin Gene Sequences Required for the Expression in EC-Derived Cardiac Muscle

To define in more detail the regions of the human cardiac α -actin promoter which confer the developmental and tissue-specific regulation of transcription, we constructed a series of linker-scanning mutants. Each construct contains an 8 bp synthetic oligonucleotide with the sequence CCTCGAGG located within the -440 to +6 bp region of the cardiac actin promoter sequence, which is linked to the lac Z reporter gene. The mutant plasmids were cotransfected in P19 cells with pPGK, and pCA1035 as previously described (Section 5.2). Selected stable transformants were pooled and differentiated with RA or DMSO. β -gal activities in the transfected cells containing the different mutant actin-lacZ genes, all followed the same general pattern (Table 2). Low β -gal activities were present in undifferentiated cells. RA-treatment of the same cells usually reduced the β -gal activities to near background levels as the cells differentiated along the neuroectodermal lineage. In contrast, the expression from the mutant cardiac actin-lacZ constructs increased as cells differentiated into muscle-containing cultures following DMSO-treatment.

Table 2: Regulated expression of actin-lac Z linker mutants during the differentiation of P19 cells

Linker position ^a	β -gal activity ^b			
	undiff.	RA	DMSO day 6	DMSO day 10 ^c
no linker	2.1	2.1	38.4	16.7
"	2.6	2.1	18.2	9.1
418	4.5	4.1	16.1	8.9
363	7.3	2.6	10.0	5.2
312	2.7	3.8	34.8	10.3
237	1.1	0.4	19.3	6.4
227	14.5	8.9	18.0	8.8
158	2.1	3.4	28.3	11.1
128	5.5	2.3	14.4	7.4
82	18.0	6.4	25.7	11.2
21	8.6	6.0	16.7	17.2
9	14.1	3.2	28.4	15.8
Δ 158-128	2.1	2.6	11.4	8.9

(a) Cells were stably transfected with 5 μ g of linker mutant plasmid, 5 μ g of pCA1035, and 5 μ g of pPGK as described in Section 2.6. Linker mutant plasmids contain the CCTCGAGG linker sequence at the various indicated positions within the -440 to +6 region of the human cardiac α -actin promoter. The control plasmid which lacks a linker sequence, pPv/B ActlacZ, is shown in Appendix B. The plasmid with the linker at the position Δ 158-128 contains an internal deletion at the linker insertion site between -158 and -128 bp in the actin promoter. Following transfection and selection, at least 500 G418-resistant colonies were pooled and differentiated (Section 2.5.2).

(b) β -gal activities were determined using the spectrophotometric assay described in Section 2.7.2, and are expressed in u/mg of proteins $\times 10$. The background β -gal activity of untransfected P19 cells was subtracted from each measurement.

(c) β -gal activities were measured in undifferentiated cells (undiff.), in RA-treated cells at day 6 (RA), and in DMSO-treated cells at days 6 and 10 (DMSO day 6,

The magnitude of this increase varied from one experiment to the other probably as a result of different transfection efficiencies and of variable proportions of muscle cells in the differentiated cultures. In almost all cases however, the expression from the actin-lacZ gene was highest on day 6 of the DMSO-treatment, a time at which spontaneously beating muscle appears. Taken together, these results show that the various mutated actin promoters were appropriately regulated during differentiation. The various linker insertions therefore did not disrupt any important element involved in the tissue-specific and developmental regulation of the cardiac actin promoter.

To further analyze the cardiac actin promoter, we performed sequential 5' deletions from the various linker mutation sites between -750 to -47 bp upstream of the cap site, as shown in Figure 6. Deletion plasmids of actin-lacZ genes were co-transfected into P19 cells with pPGKneo-ActCAT, pPGK, and pCA1035. The two latter plasmids are required for the selection of a high number of G418-resistant colonies as previously explained (Section 5.2), while pPGKneo-ActCAT is included as an internal standard. Therefore, levels of CAT activity from the actin-CAT gene in the various muscle-containing cultures of transfected cells are indicative of the transfection efficiency, and of the capacity of the cells to differentiate with DMSO, which might vary depending on how the cells recover from the transfection and selection steps. β -gal activities in turn represent the transcriptional activity of the deleted actin promoter constructs. Results shown in Figure 7 demonstrate that the deletion of sequences between -750 and -237 bp, or of the first exon and intron sequences between +6 and +740 bp, does not alter the DMSO-induced activation of the actin promoter which is linked to the reporter gene lac Z. The actin-lacZ gene with 237 bp of 5'-flanking region is thus expressed at high levels when cells differentiate into cardiac and skeletal muscle-containing cultures. In contrast, low levels of β -gal activities can be observed in RA-treated cells, while undifferentiated cells have a basal level of activity which corresponds to $\sim 10\%$ of the maximal activity observed in DMSO-treated cultures. Minimal effects of sequences upstream of -237 can not be detected in these experiments due to the inherent variation in the expression of the transfected chimeric genes in P19 cells (data not shown).

Figure 6: Schematic representation of actin-lac Z deletion constructs and of control plasmids. Details of construction are given in Appendix B. The parental plasmid pP/B ActlacZ consists of the human cardiac actin promoter from -750 to +6 (thin line) placed upstream of the lac Z gene (open box). The black arrows represent the positions of CarG box homologies (Minty and Kedes, 1986b), while open triangles represent the positions of C-rich regions. Coordinates indicate the nucleotide positions of the deletion endpoints in each construct, relative to the mRNA cap site at +1. The control expression vector pCH126A2 lacks promoter sequences, while pPGKlacZ contains the 520 bp pgk-1 promoter sequence (Xba I-Taq I fragment) shown here as an undulate line. The actin-lacZ hybrid gene in pHalacZ includes the actin first exon (hatched box) and the first intron, fused to the lac Z coding sequence.

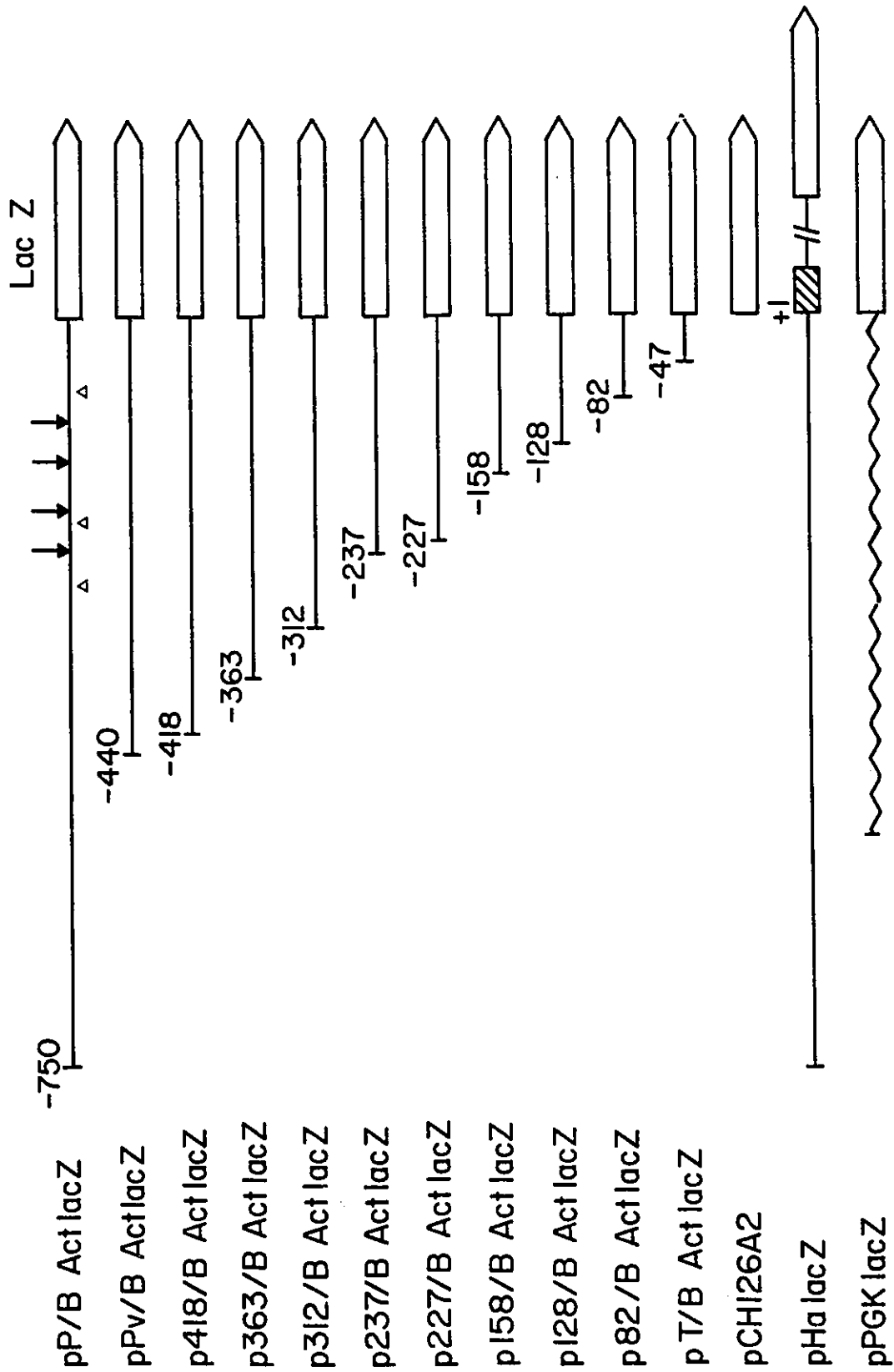
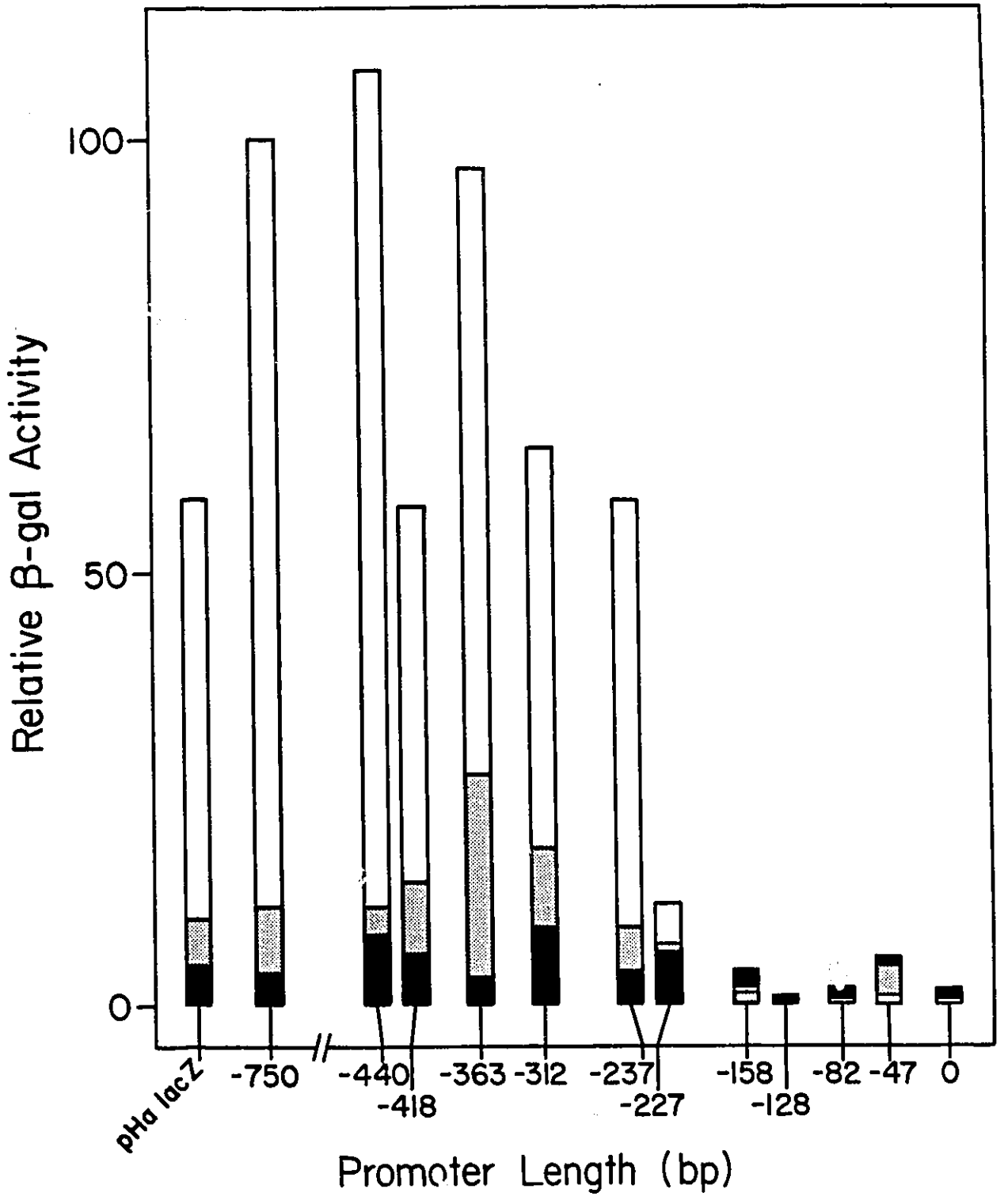


Figure 7: **Transcriptional activity of 5'-flanking regions of the human cardiac α -actin gene in differentiated cultures of stably transfected P19 cells.** β -gal activities were measured in over 600 pooled transformants derived after transfection with 5 μ g of actin-lacZ deletion plasmid, 5 μ g of pPGKneo-ActCAT, 5 μ g of pPGK, and 2.5 μ g of pCA1035. Cells were differentiated with DMSO or RA for 6 days as previously described (Section 2.5.2). Relative levels of β -gal activities in undifferentiated cultures (stippled bars), or in the same cultures after RA-treatment (black bars) or DMSO-treatment (open bars), were measured using the spectrophotometric assay detailed in Section 2.7.2. The plasmid with the longest 5'-flanking region, from -750 to +6, was assigned an activity of 100, which corresponds to a measured β -gal activity of $6.19 \times 10^2 u/mg$ of total protein. Promoter lengths indicate the region of 5'-flanking DNA present in the constructs, relative to the transcription initiation site of the cardiac actin promoter. The plasmid with the promoter length of 0 corresponds to the expression vector, pCH126A2, without any promoter sequence, while pHalacZ contains the actin promoter with the first intron and exon sequences, from -750 to +700 bp. Results of 1 of 3 experiments are shown here. Transfections were monitored with the internal standard pPGKneo-ActCAT.



However, the deletion of a 10 bp sequence between -237 and -227 bp reproducibly lowered the expression of the actin promoter, from 100% to ~10%. The promoter with the actin region of 227 bp still seemed to retain muscle-specific inducibility, since cells treated with DMSO expressed higher levels of lac Z than those treated with RA, or than undifferentiated cells. These results thus suggest that the -237 to -227 sequence which contains a CarG box consensus sequence, contains a positive regulatory element for the expression in cardiac muscle-containing cultures.

Further 5' deletions in the cardiac actin promoter revealed the presence of a sequence with regulatory functions located between -227 and -158. As shown in Figure 7, deletion of this region results in the entire loss of regulated expression. In the case of DMSO-treated cells, lac Z expression is reduced from ~10% to ~1%. Similarly, expression in undifferentiated cells is also reduced from ~10% to ~1%. Expression in RA-treated cells remained unchanged since the cardiac actin promoter is never activated in cells differentiating along the neuroectodermal lineage. In additional 5' deletions, activities from the actin promoter were all near background levels, as was observed with the construct containing the 158 bp promoter region. Sequences located between -237 and -158 bp thus seem to be involved in the tissue-specific transcriptional activation of the cardiac actin gene in cultures containing cardiac muscle.

To test whether the regulatory sequences of the cardiac actin promoter could enhance the activity of a heterologous promoter, we subcloned the -650 to -86 bp region of the cardiac actin gene in both orientations upstream of the mouse hsp 68 promoter. Similarly, the cardiac actin first intron sequence was placed upstream of the hsp 68 promoter (Appendix B, Figure 10 of Chapter 4). The chimeric genes were permanently cotransfected in P19 cells as previously described (Figure 7). β -gal activities from the various constructs were similar in both undifferentiated and DMSO-treated cultures (data not shown), therefore indicating that the cardiac actin sequences did not stimulate muscle-specific expression of an heterologous promoter.

3.4 Discussion

In this study, we have transfected P19 cells with chimeric genes consisting of various lengths of the human cardiac actin promoter linked to the lac Z reporter gene encoding β -gal. Pooled populations of stable transformants expressed high levels of β -gal only following differentiation into cardiac muscle-containing cultures. An analysis of the cardiac actin promoter showed that sequences between -237 and +6 bp were sufficient to permit expression of the chimeric actin-lacZ gene in muscle-containing cultures. The deletion of regions between -237 and -227 bp, and between -227 and -158 bp, was associated with the loss of muscle-specific expression. The regulatory regions identified in this study were different from the ones previously identified in transient assays with a skeletal muscle cell line (Minty and Kedes, 1986b) suggesting the presence of multiple regulatory elements which modulate the developmental and tissue-specific expression of the cardiac actin gene.

High levels of transcription of the cardiac actin-lacZ chimeric gene in P19 stable transformants occurred 6 days after the beginning of the DMSO regime, as was previously reported for the endogenous cardiac actin gene (Rudnicki et al., 1988). Immunofluorescence experiments showed that many cells expressing the transfected cardiac actin gene contained muscle MHC, which was recognized by the MF20 antibody. Most β -gal-positive cells also stained with the B4 antibody which reacts with cardiac actin, in addition to the other muscle actin isoforms. Smooth muscle α -actin was detected in these cells with the SM-1 antibody. Similarly, previous studies demonstrated the coexpression of cardiac and smooth muscle actins in cardiac myocytes of neonatal mice (R. Low, personal communication). The expression of the cardiac actin chimeric gene and of other muscle-specific genes in these studies is therefore consistent with the embryonic nature of the cardiac myocytes in differentiated P19 cultures, as was also indicated in previous studies of these cells (Rudnicki et al., accepted in Dev. Biol.).

Transcription of muscle-specific genes has been investigated in various skeletal muscle cell lines. Several regions involved in transcriptional activation were localized in 5'-flanking sequences of the myosin heavy chain gene, the muscle creatine kinase

gene, the skeletal actin gene, and the cardiac troponin T gene (Bouvagnet et al., 1987, Jaynes et al., 1988, Mar and Ordahl, 1988, Muscat and Kedes, 1987). A common *cis*-acting DNA element responsible for the muscle-specific transcription of all these genes has not been identified. Instead, a combination of multiple promoter regions seems to be required for the appropriate activation of the genes in skeletal muscle. Similarly, our results with the cardiac actin promoter in differentiated P19 cells are consistent with the role of more than one region of the promoter for the expression in cardiac muscle. Diversity of expression in the fetal liver and gastrointestinal tract has also been shown to be generated by the cooperative action of several enhancers in the α -fetoprotein gene (Hammer et al., 1987).

The activity of the human cardiac actin promoter has previously been analyzed in the C2C12 skeletal myoblast cell line. It was specifically shown that two CarG box domains located at -140 and -100 bp (CarG box 1 and 2) play the major role as *cis*-acting DNA sequences required for transcription in C2C12 cells (Miwa and Kedes, 1986). In contrast, regions between -237 and -227 bp, and between -227 and -158 bp, are required in differentiated P19 cells for the cardiac-specific expression of the same gene. The requirement of different regulatory sequences in these experiments is probably a consequence of the differences between the biological assays used to analyze the cardiac actin promoter. Different cell types are found in each cell culture system: muscle cells in differentiated P19 cultures consist mainly of cardiac myocytes, while in the C2C12 cell line, they consist of skeletal myocytes. Since the expression of the cardiac actin gene differs in cardiac and skeletal muscle (Section 1.4.1), distinct elements are probably involved in the transcriptional activation in each cell type. Recent studies with the MCK gene in transgenic mice indicated that certain sequences of the promoter are responsible for the tissue-specific expression in one cell type while others direct high levels of expression in both muscle cell types (Johnson et al., 1989). A similar mechanism of transcriptional regulation for the cardiac actin gene could therefore account for the differences in *cis*-acting DNA regulatory sequences required for expression in differentiated P19 cells and C2C12 cells.

The P19 cell line further differs from C2C12 cells in the events required for expression of a transfected muscle-specific gene. In P19 cells as in transgenic mice, chimeric genes must first become integrated in the genome where they are not expressed and assume an inactive configuration. Following induction to differentiate, the gene must thus undergo changes to an active configuration that allow transcription to occur when the appropriate proteins bind to certain *cis*-acting DNA regulatory sequences. In contrast, expression from the transiently transfected cardiac actin gene in C2C12 cells only involves a subset of the regulatory events since the muscle-specific gene is already in an open configuration and in the presence of muscle-specific factors. Expression in these cells may therefore not be representative of the transcriptional activation which occurs during muscle development, as in the P19 cell culture system. Indeed, transfected human and chicken cardiac actin chimeric genes were not regulated in the same fashion as the endogenous gene in C2C12 cells (Minty and Kedes, 1986a and b, Seiler-Tuyns et al., 1984). Results from analyses of the human cardiac actin promoter in C2C12 cells and in P19 cells may therefore differ due to the different biological activities measured in each assay.

In conclusion, the experiments reported here show that two regions of the human cardiac actin gene located between -237 and -158 bp are involved in regulating the tissue-specific expression during differentiation of transfected P19 cells into cardiac muscle-containing cultures. These regions differ from those previously reported in other biological test systems, thus indicating the role of cell background in modulating expression. Our results further indicate that the transcriptional activation of cardiac actin during differentiation of EC cells involves multiple sequences that are likely to represent target sites for various DNA-binding regulatory proteins.

Chapter 4

MyoD-induced Muscle Gene Expression

4.1 Introduction

MyoD and MyoD-related proteins can activate the transcription of certain genes that lead to the differentiated phenotype of cells. Following the stable transfection of various nonmuscle cell lines, the conversion to the skeletal muscle cell type was observed in cells expressing MyoD (Davis et al., 1987). In EC cells, many different cell types which include cardiac as well as skeletal muscle, form in cells transfected with a MyoD expression vector (Chapter 5). Although the mechanism of action of MyoD has not yet been determined, it must involve the direct or indirect activation of certain genes via DNA-protein interactions since MyoD is a sequence-specific DNA binding protein (Lassar et al., 1989).

The activation of muscle-specific gene expression by MyoD has been demonstrated in transient transfections of various cell lines including 10T1/2 fibroblasts, melanoma cells, and neuroblastoma cells. Transcription from endogenous muscle genes, as well as from muscle-specific promoters linked to a reporter gene, was stimulated in these cells transfected with a MyoD expression vector (Weintraub et al., 1989). Similarly, a MyoD-related protein, myogenin, was shown to activate transcription from the muscle creatine kinase promoter 156 bp 5' enhancer sequence

(Edmonson and Olson, 1989). This DNA segment is known to be the binding site of three nuclear factors that are expressed with kinetics similar to those of the MyoD-related protein during differentiation of skeletal myocytes (Kelvin et al., in preparation). In fact, it was recently discovered that MyoD binds to the MCK enhancer sequence as well as to enhancers of a number of other muscle-specific genes (Lassar et al., 1989). In other studies, factors have been shown to bind to tissue-specific elements in the cardiac actin, skeletal actin, and myosin light chain-2 genes (Braun et al., 1989a, Miwa et al., 1987a, Walsh and Schimmel, 1987). However, the understanding of the relationship between these factors and the MyoD-like proteins awaits the isolation and purification of the proteins that interact with the muscle-specific elements.

In this study, we analyzed the effect of MyoD on the activation of different promoters in P19 cells. Transient expression of the MyoD cDNA did not activate the pgk-1 housekeeping gene promoter, but was sufficient to activate the human cardiac actin gene promoter linked to the reporter gene lac Z. Endogenous muscle genes such as sarcomeric actins and myosin heavy chains were not stimulated in these transfections. Sequences which are necessary for the MyoD-induced activation of the human cardiac actin gene are located within the -312 and -47 bp region of the promoter, and include several conserved cytidine-rich regions.

4.2 The MyoD Gene Product Activates Transcription from the Human Cardiac Actin Promoter in P19 Cells

In stable transfections of nonmuscle cells with the MyoD expression vector, many changes in gene expression directly or indirectly caused by MyoD are required to produce the characteristic phenotype of a muscle cell. To test whether MyoD can also activate muscle genes of EC cells in transient assays, we analysed the expression from the muscle-specific human cardiac α -actin promoter (in pP/B ActlacZ) following cotransfection with the MyoD expression vector (pPGKMyoD). β -gal activities

from the actin-linked reporter gene were measured 48 hours following transfection, a time at which no morphological changes due to MyoD were detectable in the P19 cell culture. In control transfections with a sequence unrelated to MyoD (in pPGK-neo), the expression from the cardiac actin promoter was low, as indicated by the β -gal activities which were close to the background level present in P19 cells. In the presence of MyoD, the expression from the cardiac actin promoter increased ten-fold (Table 3). However, expression from the muscle-specific promoter was not stimulated by the protooncogene *c-myc* (in pPGKcmyc) although this protein shares a region of homology with MyoD which is important in the biological effect of both proteins (Murre et al., 1989b). *In situ* staining for β -gal activity with the chromogenic substrate X-gal, showed that the number of cells expressing high levels of the transfected actin-lacZ gene increases 5 to 8-fold in the presence of MyoD (Table 4). Therefore MyoD activates transcription from the cardiac actin-lacZ gene in P19 cells where the chimeric gene is usually not expressed.

Duplicates of the previous transiently transfected cultures containing MyoD were used to analyze expression from the endogenous, as well as from the transfected gene. The Northern analysis of Figure 14 in Chapter 5 showed specifically that although the exogenous cardiac actin expression was greatly stimulated by MyoD (Tables 3 and 4), endogenous expression of muscle actins was undetectable in EC cells expressing MyoD. As expected, levels of cytoskeletal actins were similar in all the transfected cells. Double immunofluorescence with the muscle myosin MF20 antibody and the β -gal antibody also showed that MyoD could not activate endogenous muscle-specific genes in transient transfections. Thus, a high number of cells cotransfected with the MyoD expression vector and the actin-lacZ chimeric gene stained with the β -gal antibody, but not with the MF20 antibody (data not shown). Together, these results show that MyoD has the capacity to *trans*-activate, either directly or indirectly, expression from a transiently transfected muscle actin gene, but not from endogenous muscle genes present in EC cells.

Different promoters were compared to determine whether the MyoD transcriptional activation described above represented a generalized effect on transfected promoters, or a specific interaction with muscle-specific elements. Chimeric genes

Table 3: Activation of the human cardiac α -actin gene promoter in P19 cells transiently transfected with the MyoD expression vector

Plasmid ^a	Relative β -gal activity (\pm SEM) ^b
pPGKMyoD	10.4 (\pm 1.0)
pPGKc-myc	1.3 (\pm 0.2)
pPGKneo	1.0 (\pm 0.4)

(a) Transfections in P19 cells were performed with 9 μ g of test plasmid listed in the Table, plus 9 μ g of pPv/B ActlacZ. After 48 hours cells were either harvested for β -gal assays (Section 2.7.2), or fixed for *in situ* staining with X-gal (Section 2.7.1).

(b) The relative β -gal activity for each plasmid is an average of results from 6 experiments, with the corresponding standard error of the mean (SEM). Values were normalized to the average β -gal activity of the negative control, pPGKneo. Experiments included an internal standard (pRSVCAT) for the transfection efficiency.

Table 4: MyoD-induced increase in the number of P19 cells expressing the actin-lacZ gene

Plasmid ^a	% of β -gal positive cells ^b	
	I	II
pPGKMyoD	1.7	6.6
pPGKc-myc	0.4	1.2
pPGKneo	0.2	0.8

(a) see note in Table 3.

(b) Cells were stained for β -gal activity as described in Section 2.7.1. β -gal positive cells, as detected by their blue color, were counted. Results of two experiments (I and II) are shown as a percentage of blue cells per total number of cells in each transfection. At least 10,000 cells were scored for β -gal activity in each sample.

containing the pgk-1 constitutive promoter or the muscle-specific actin promoter (as shown in Figure 6 of Chapter 3) were each transiently transfected with or without the MyoD expression vector in P19 cells. β -gal activities from the linked reporter gene showed that MyoD stimulated the muscle-specific promoter, but not the house-keeping gene promoter which was expressed at a high constitutive level (Figure 8a). Transient activation of gene expression by MyoD thus seems to be restricted to muscle-specific promoters in P19 cells, as reported for other cell lines (Weintraub et al., 1989).

4.3 Several Regions of the Cardiac Actin Promoter are Required for MyoD Transcriptional Activation

Expression of the human cardiac α -actin gene in the mouse C2C12 skeletal muscle cell line has been shown to be dependent on two 5'-flanking elements located at -100 and -140 bp upstream of the cap site and referred to as CarG boxes (Minty and Kedes, 1986b). To determine the role of the CarG boxes in the MyoD-dependent activation of muscle genes, we examined the ability of MyoD to stimulate expression from the human cardiac actin promoter containing different linker mutations or deletions. The transcriptional activity in each of the various mutant promoters was measured as a β -gal activity from the reporter gene lacZ. P19 cells were first cotransfected with the MyoD expression vector (or with pPGKneo as a negative control), and cardiac actin-lacZ chimeric genes containing an 8 bp linker mutation at a specific position within the actin promoter region from -440 to +6 bp upstream of the cap site. As shown in Table 5, none of the linker insertions affected the MyoD-induced *trans*-activation of the actin promoter, therefore indicating that the mutations did not disrupt any critical element required for transcription. Similarly, the deletion of CarG box 2 (as defined by Minty and Kedes, 1986b) in the construct p Δ 158-128 ActlacZ did not result in a decrease of the MyoD-induced transcription. In contrast, an internal deletion of the TATA box region (in p Δ 82-21 ActlacZ), or

the entire removal of sequences upstream of the TATA box from -47 bp (in pT/B ActlacZ), abolished transcription of lac Z, an effect not reversed by MyoD. These results indicate that the TATA box region is necessary but not sufficient for the MyoD induction of transcription. Additional element(s) conferring MyoD-specific expression are therefore located between -440 and -47 bp upstream of the cap site, and exclude the muscle-specific CarG box located at -148 bp.

To further define the sequences required for MyoD *trans*-activation, varying lengths of the 5'-flanking regions of the human cardiac actin promoter were fused to the lac Z reporter gene as shown in Figure 6 in Chapter 3. Results of cotransfections with MyoD in Figure 8b showed first that deletion of sequences upstream of -312 bp had no effect. A major loss in transcriptional activity (from 100% to ~45%) accompanied the deletion of sequences between -312 and -237 bp upstream of the cap site. It is of interest to note that upon deletion of this sequence, the background level of transcriptional activity of the cardiac actin promoter in P19 cells (in the absence of MyoD) was also reduced from ~10% to near 0%. The analysis of the sequences located in the -312 to -237 region revealed the presence of a cytidine (C)-rich region which is conserved in other muscle-specific promoters (Figure 9), and which may therefore have a regulatory function in the transcription of muscle genes.

In other 5' deletions, minor decreases in activity with MyoD were observed with the sequential removal of sequences between -237 and -227, -227 and -158, -158 and -128, -128 and -82, and -82 and -47 (Figure 8b). The latter decreases in β -gal activity were relatively small but reproducible in the various experiments. The inclusion of a longer downstream sequence containing the actin first exon and intron had a negative effect on the MyoD induction, as seen with the decrease from 100 to ~50% in transcriptional activity (Figure 8a). However, other experiments have shown that the reduced transcription from this construct is due to improper splicing of the hybrid transcript (W. Colledge, personal communication). These data therefore indicate that the human cardiac actin gene contains several positive regulatory sequences between -312 and -47 bp, which are necessary for the MyoD-induced transcriptional activation.

Table 5: MyoD-induced expression of actin-lac Z linker mutants in transient transfections

Linker position ^a	Relative β -gal activity ^b	
	+ MyoD (\pm SEM)	- MyoD (\pm SEM)
(pPv/B ActlacZ)	100 (\pm 0)	12 (\pm 2)
(pT/B ActlacZ)	4 (\pm 2)	2 (\pm 1)
418	113 (\pm 30)	10 (\pm 2)
363	121 (\pm 33)	10 (\pm 3)
312	140 (\pm 28)	9 (\pm 2)
237	91 (\pm 17)	6 (\pm 3)
227	95 (\pm 24)	8 (\pm 2)
158	104 (\pm 28)	6 (\pm 2)
128	84 (\pm 11)	8 (\pm 1)
82	71 (\pm 4)	4 (\pm 2)
21	118 (\pm 28)	9 (\pm 4)
9	127 (\pm 25)	12 (\pm 7)
Δ 158-128	141 (\pm 32)	6 (\pm 2)
Δ 82-21	5 (\pm 1)	2 (\pm 1)

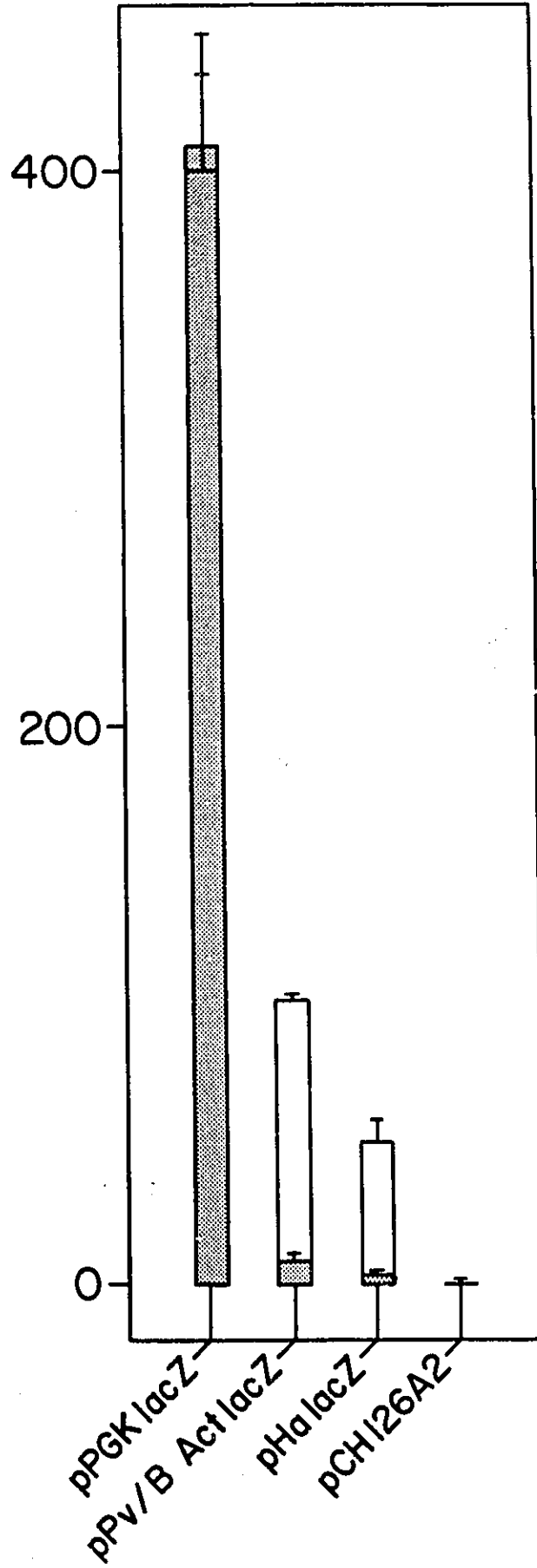
(a) Cells were transiently transfected with 10 μ g of linker mutant plasmid and 10 μ g of pPGKMyoD (+MyoD) or of pPGKneo (-MyoD). Linker mutant plasmids are identical to the control plasmid pPv/B ActlacZ, except for the sequence CCTCGAGG inserted in the actin promoter region between -440 to +6. The position of the linker as indicated in the Table, is relative to the transcription initiation site of the human cardiac α -actin gene. The mutants p Δ 82-21 ActlacZ and p Δ 158-128 ActlacZ contain deletions between the indicated positions, while the negative control pT/B ActlacZ contains the minimal promoter sequence between -47 and +6.

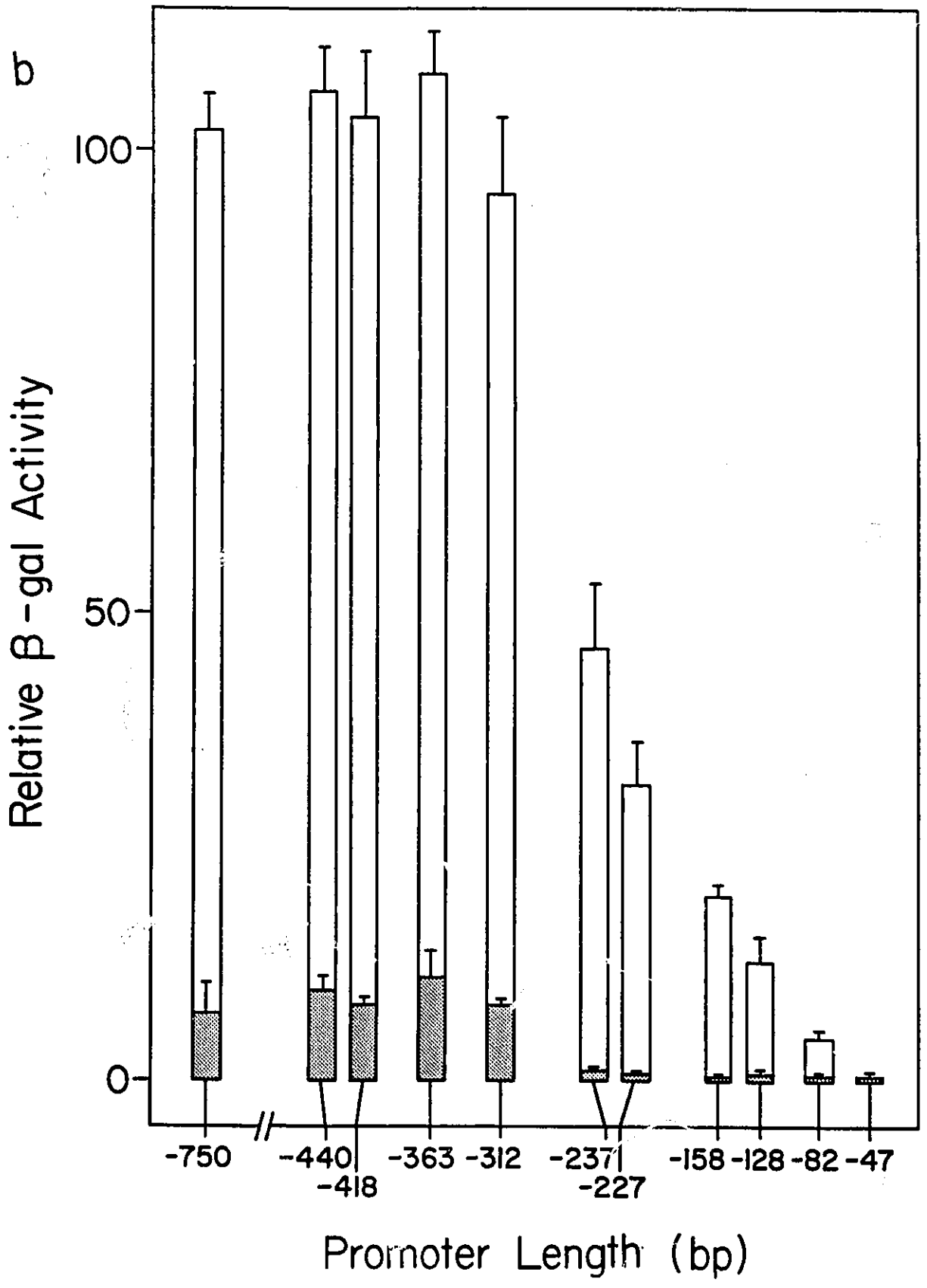
(b) In each experiment, β -gal activities were expressed relative to the activity of the intact promoter in pPv/B ActlacZ, which was assigned the value of 100. Shown here are averages of 4 experiments with the standard errors of the means (SEM) calculated from the normalized β -gal activities. Transfections efficiencies were monitored with an internal standard (pPSVCM).

Figure 8: Transcriptional activity of upstream regions of the human cardiac actin promoter in P19 cells. Transfection of actin-lacZ constructs either with or without the MyoD expression vector, were performed as described in Table 3. The average activity of pPv/B ActlacZ and pP/B ActlacZ was assigned a value of 100. Data shown here represent averages of 5 to 9 experiments with the standard error of the mean (thin lines) determined from the normalized values. Open bars in the graph correspond to transfections done in the presence of pPGKMyoD, while stippled bars correspond to transfections done with a control vector, either pPGKmyc or pPGKneo, both of which gave similar results. Experiments included an internal standard (pRSVCAT or pRSVL). Panel a includes a comparison of two different promoter sequences (from the cardiac actin and the pgk-1 gene), as well as the expression from the vector pCH126A2 lacking promoter sequences. Panel b shows the expression from the cardiac actin promoter with progressive deletions of 5'-flanking DNA. Schemes of the constructs are found in Figure 6.

a

Relative β -gal Activity





Mouse c. actin	(-279)	CCCC g t CCCCCaCCCC
Human c. actin	(-276)	CCCC t g CCCCCt a CCC
Mouse c. actin	(-81)	CCCCCaCCCCt g CCCCC
Human c. actin	(-83)	CCCCCaCCCCt g CCCCC
Mouse c. actin	(-211)	CCCCt CCCC t t CCt t
Human c. actin	(-214)	CCCCt CCCC t t CCt t
Mouse skel. actin	(-669)	CC t CCt CCCCCt Ct tC
Mouse MCK	(-1113)	g CCt CaCCCCCaCCCC
Rat MCK	(-1113)	CCCCCaCCCCCaCCCC
Mouse MLC _V	(-162)	t CCCCt CCCCCaCCCa
Mouse MLC _A	(-76)	g g CCCt CCCC a g CCCC

Figure 9: **Sequence similarities between promoter regions of muscle-specific genes.** Mouse and rat muscle creatine kinase (MCK) sequences are respectively from Jaynes et al. (1988), and Horlick and Benfield (1989). Atrial and ventricular myosin light chain-1 (MLC_A and MLC_V) sequences are from Cohen et al. (1988). Human and mouse cardiac actin sequences are respectively from Minty and Kedes (1986b), and Garner et al. (1986), while mouse skeletal actin sequences are from Chien-Tsung Hu et al. (1986). Numbers in parenthesis indicate the nucleotide positions upstream of the cap site of each gene, while capital letters show the C-rich sequences.

To determine whether the MyoD regulatory sequences were capable of conferring MyoD-inducibility on a heterologous nonmuscle promoter, a fragment of the cardiac actin upstream region from -650 to -86 bp was placed upstream of the 800 bp heat-shock protein 68 (hsp) promoter, in either orientation. The actin upstream region was replaced in other constructs by the actin first intron as shown in Figure 10. These chimeric genes were cotransfected in P19 cells either with the MyoD expression vector, the c-myc expression vector, or an unrelated sequence (which is the neo expression vector). Promoter activity from the lac Z reporter gene was measured as usual at approximately 48 hours following transfection. As shown in Table 6, the addition of actin sequences in the various constructs did not result in an increase in the transcription of the hsp promoter in the presence of MyoD. Instead, an unpredicted result of this experiment was that MyoD reproducibly decreased the expression from the hsp promoter since for all the constructs tested, β -gal activities were always lowest with the MyoD expression vector as compared to the control plasmid or to the c-myc expression vector. Additionally, we observed that the activity of the various actin-containing constructs was generally higher than that of the parental plasmid pCH126PHS. These higher activities may perhaps be due to the increase in the distance between the hsp promoter and the prokaryotic plasmid sequences; the latter sequences were shown to mediate a *cis* negative effect on expression from eukaryotic genes (Peterson et al., 1987). However, the magnitude of this effect can not be quantitated precisely due to the inherent variability in the expression of the hsp promoter. This promoter is induced by stress factors, and thus possibly by the transfection procedure. The background activity of the unstimulated hsp promoter which was assigned a value of 100 in Table 6, was still very low in comparison to the full activity of the promoter following a heat-shock, as seen by the 35-fold increase in transcription. It therefore seems that MyoD inhibits the hsp promoter activity, an effect which can not be reversed by the addition of MyoD-specific cardiac actin regulatory sequences.

Figure 10: Schematic representation of chimeric genes with cardiac actin regions upstream of the heat-shock promoter. The parental plasmid pCH126PHS (R. Kothari et al., 1989) consists of the mouse heat-shock protein 68 (hsp) promoter (dark box) linked to the lac Z gene (open box). Other constructs shown here contain actin regions (thin lines), in both orientations (arrows) upstream of the hsp promoter. pAE and pAEinv contain the human cardiac actin region from -650 to -86 bp upstream of the cap site, while pINT and pINTinv contain the actin first intron. from approximately +15 to +740 bp downstream of the cap site.

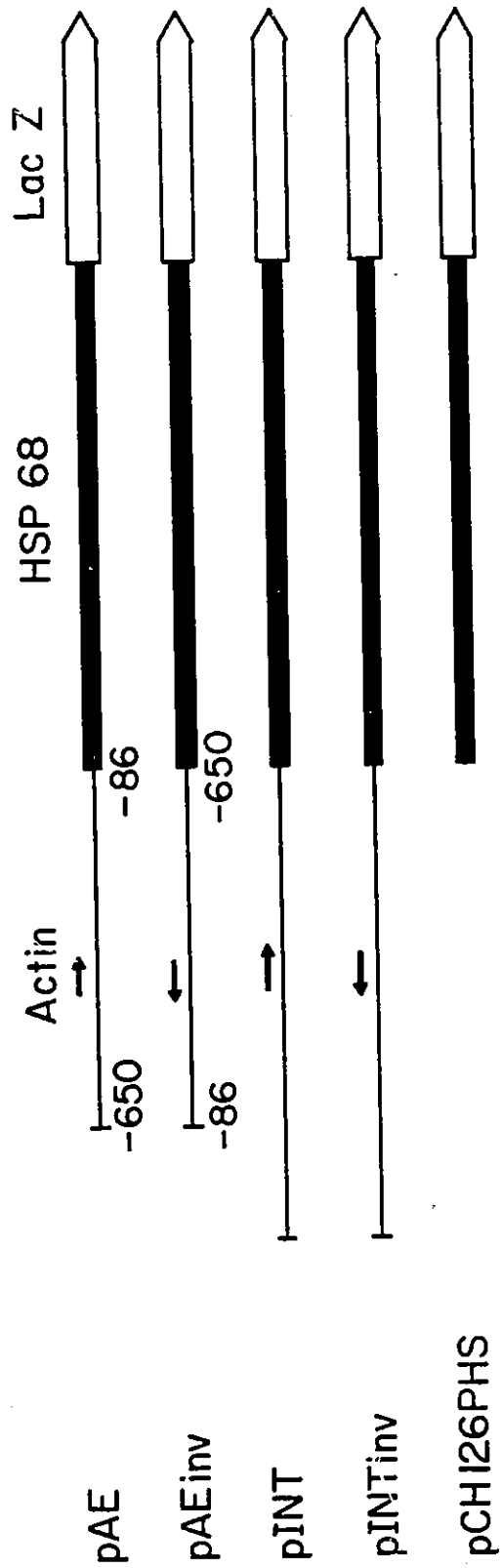


Table 6: Failure of the MyoD responsive sequence to enhance transcription from the hsp 68 promoter

Plasmid ^a	Relative β -gal activity ^b		
	+MyoD	+c-myc	+neo
pAE	176	246	257
pAEinv	109	154	276
pINT	86	258	243
pINTinv	81	197	256
pCH126PHS	27	121	100
			+HS3670

(a) Cells were transiently transfected with 9 μ g of test plasmid (shown in Figure 9), 3 μ g of pRSVCAT, and 9 μ g of pPGKMyoD (+MyoD), of pPGKcmyc (+c-myc), or of pPGKneo (+neo). pRSVCAT was included as an internal standard for the transfection efficiency.

(b) These data are results of 1 of 3 experiments. β -gal activities were normalized to the CAT activity in each sample, and were expressed relative to the activity of pCH126PHS, which was assigned value of 100. The value with ^{+HS} for pCH126PHS indicates the activity of the promoter following a 30 min heat-shock at 42°C.

4.4 Discussion

In this study, P19 cells were transiently cotransfected with a MyoD expression vector and a cardiac actin-lacZ chimeric gene. Expression from the actin promoter in EC cells increased approximately 10-fold in the presence of MyoD, in contrast to endogenous muscle-specific genes which remained inactive. In addition, MyoD did not stimulate transcription from the hsp 68 inducible promoter and from the pgk-1 constitutive promoter in the same transient transfections. Deletion analysis of the cardiac actin promoter revealed that multiple 5'-flanking regions are required for the MyoD-induced transcriptional activation. Taken together, our results are consistent with a role for MyoD as a muscle-specific transcriptional enhancer which acts by way of DNA-protein interactions involving several sequences in muscle promoters.

MyoD and MyoD-related proteins have previously been shown to stimulate the transcription from the muscle myosin heavy chains (MHC), desmin, and muscle creatine kinase (MCK) promoters in transient transfections of various cell lines (Davis et al., 1987, Edmondson and Olson, 1989, Weintraub et al., 1989). In P19 EC cells, the transfection of a cardiac actin chimeric gene in the presence of a MyoD expression vector also resulted in the activation of the muscle-specific gene. In the same assay, expression from the pgk-1 housekeeping gene promoter linked to the reporter gene lac Z was not affected by MyoD, while the expression from the inducible heat-shock promoter in the absence of heat-shock was inhibited by the MyoD expression vector. The regulatory activity of MyoD may therefore not be restricted to muscle-specific promoters, as was also indicated by the weak positive effect of MyoD on the Rous Sarcoma Virus promoter and by the 2-4 \times stimulatory effect on the SV40 promoter (Edmondson and Olson, 1989, Weintraub et al., 1989). Whether these minimal effects play a role in cellular gene expression is not clear since the transient assays with the MyoD expression vehicle involve unusually high levels of MyoD expression. Therefore our results indicate that MyoD is specifically a strong activator of the cardiac actin promoter, an effect that can probably be applied to other muscle-specific promoters as was indicated in other studies with different cell culture systems.

Expression from endogenous muscle actins and myosin heavy-chains was not activated in P19 cells transiently transfected with the MyoD expression vector, as seen by immunofluorescence or by Northern analysis. In contrast, previous reports have shown that MyoD is able to activate endogenous muscle myosin heavy chain and desmin genes in transient transfections of many cell lines, including cells of mesodermal origin and differentiated cells derived from ectodermal and endodermal germ layers (Tapscott et al., 1988, Weintraub et al., 1989). P19 cells which are of embryonic origin therefore differ from more mature cells of different germ layer origin in the control of gene expression. It is possible that genes encoding proteins for differentiated functions are repressed by mechanisms such as methylation, chromatin structure modifications, or by the presence of negative regulatory factors in P19 cells. Although it is not clear what mechanism(s) repress the transcription of the endogenous cardiac actin gene in undifferentiated cells, our results indicate that the activity of MyoD depends on other factors which may control accessibility to the gene during the differentiation of a cell.

Serial 5' deletions of the cardiac actin promoter showed that at least 5 different regions which are located between -312 and -47 bp are involved in MyoD *trans*-activation. The deletion of each of these regions resulted in one third to one half decreases in the β -gal activity (Figure 8b). The transcription induced by MyoD was still at least 10 times higher than without MyoD in all cases, except for the -47 bp construct (Figure 8b). In addition, an internal deletion of the TATA box region in p Δ 82-21 ActlacZ was sufficient to abolish the MyoD-inducible activity of the promoter, thus indicating the predicted requirement of a TATA box for this RNA polymerase II gene. However, the TATA box region alone is insufficient for MyoD *trans*-activation as seen with the -47 bp deletion construct (Figure 8b). The transcription of the cardiac actin gene induced by MyoD therefore involves many *cis*-acting regulatory DNA sequences located downstream of -312 bp in the promoter region, where probably various nonmuscle and muscle-specific factors interact.

The TATA box region is necessary for appropriate initiation of transcription by RNA polymerase II, while DNA sequences upstream of the TATA box permit the

attachment of factors which regulate the levels of expression during muscle development (Maniatis et al., 1987, Gustafson et al., 1988). Specific interactions between the factors binding to the cardiac actin TATA box region and to the upstream sequences seem to be necessary, since the upstream region alone (from -650 to -86) was unable to activate expression from the hsp 68 promoter linked to the lac Z reporter gene. Similarly, previous studies have shown the lack of enhancer potential of the cardiac actin 5'-flanking region placed upstream of another heterologous promoter (Minty and Kedes, 1986b). The upstream muscle-specific regulatory sequences are therefore only functional in the context of the cardiac actin TATA box region with its associated proteins.

In EC cells lacking the MyoD expression vector, the transfected cardiac actin promoter was expressed at a low level which corresponded to ~10% of the maximal activity with MyoD. Deletion of sequences between -312 and -237 bp abolished this expression in EC cells, and also reduced by more than 50 % the activity of the gene in the presence of MyoD (Figure 8b). This result thus suggests the presence of a positive regulatory element which is not MyoD-specific, and which can be activated by factors present in P19 cells. The inappropriate expression of cardiac actin in undifferentiated cells may therefore have resulted from the binding of positive regulatory factors to the -312 to -237 bp region of the transfected gene, combined with the absence of control mechanisms which normally repress endogenous muscle gene expression in P19 cells. The cardiac actin gene therefore seems to contain not only MyoD-responsive sequences but also a region between -312 and -227 bp which acts as a general positive regulator of transcription.

In previous studies, MyoD was shown to be localized in nuclei of both myoblasts and myotubes of the C2C12 cell line (Tapscott et al., 1988). It was also demonstrated that the muscle-specific transcription of the human cardiac actin gene in C2C12 skeletal myoblasts depends on the presence of two proximal CarG domains (CarG box 1 and 2) in addition to a distal region located between -443 and -393 bp (Miwa and Kedes, 1987). In contrast, our studies revealed that MyoD *trans*-activation of cardiac actin chimeric genes involves various regions located between the proximal CarG boxes and the distal region defined by Kedes et al. Moreover, the

deletion of CarG box 2 had no effect on MyoD *trans*-activation while that of CarG box 1 only caused a minor decrease in expression in the same experiments. These differences in the requirement of *cis*-acting DNA sequences suggest that MyoD alone is insufficient for the expression of cardiac actin in the C2C12 skeletal muscle cell line. Other factors which perhaps interact with the proximal CarG boxes must be present in C2C12 cells to activate the chimeric actin gene.

Sequence analysis of the cardiac actin regions involved in MyoD *trans*-activation revealed significant homology with C-rich regions present in the muscle creatine kinase (MCK) gene, the myosin light-chain-1 gene, and the skeletal actin gene, as shown in Figure 9. In the cardiac actin gene, this motif is found in 3 regions of the promoter which are involved in the MyoD induction of transcription. In the MCK gene, the C-rich sequence is located within the tissue-specific enhancer element of this gene, and it represents the binding site of nonmuscle factors (Jaynes et al., 1989, Horlick and Benfield, 1989). Recent studies have also demonstrated that MyoD and myocyte-specific factors interact with the MCK enhancer (Lassar et al., 1989, Buskin and Hauschka, in press, Kelvin et al., in preparation). It is therefore possible that in the cardiac actin gene and in the MCK gene, the C-rich sequences are involved in tissue-specific expression modulated by muscle factors such as MyoD. However, a role for these specific sequences or for others, awaits the detailed analysis of the cardiac actin *cis*-acting DNA sequences and of the factors interacting with them.

In conclusion, we demonstrated that the MyoD-induced *trans*-activation of the cardiac actin gene involves several regulatory sequences which probably interact with various factors, some of which are found in nonmuscle cells. Differences in the regulation of the transfected cardiac actin gene, of its endogenous counterpart, and of the same gene in transfected C2C12 cells, indicates that MyoD is insufficient for the activation of this gene in the different cell types. This suggests that factors other than MyoD participate in the regulation of transcription of muscle-specific genes, as was also indicated by the absence of MyoD during the development of cardiac myocytes (Chapter 5).

Chapter 5

MyoD-induced Differentiation of EC Cells

5.1 Introduction

Differentiation of multipotential stem cells into skeletal muscle includes the stages of determination into myoblasts, followed by differentiation into myocytes which express various muscle-specific genes. Five genes which play a role in the determination and differentiation events have recently been described (Braun et al., 1989b, Davis et al., 1987, Lin et al., 1989, Pinney et al., 1988, Wright et al., 1989). Pinney and co-workers (1988) demonstrated that transfection of Myd, a human genomic DNA cloned into a cosmid vector, induced myogenesis in 10T1/2 fibroblasts. Davis et al. (1987) isolated the MyoD1 cDNA which also converts nonmuscle cells to myoblasts, when expressed under the control of a strong viral promoter. This myogenic function was shown to be provided by a region of MyoD which shares homology with a region of the myc protein, of the immunoglobulin kappa chain enhancer binding proteins, and of the *Drosophila daughterless*, *achaete-scute* and *twist* gene products. The homologous protein segments can potentially form an amphipathic helix-loop-helix motif which in turn could be important for both dimerization and DNA-binding properties (Murre et al., 1989b). MyoD may thus be a member of a

family of transcription factors involved in the control of cell growth and differentiation.

Additional regulatory genes are involved in muscle differentiation as was shown with the discovery of three cDNAs referred to as myogenin, Myf-5, and CMD1 which all show a high degree of sequence identity of MyoD1 (Wright et al., 1989, Braun et al., 1989b). Expression of the MyoD-related cDNAs induces myogenesis in various nonmuscle cell types, and activates muscle-specific promoters in permanent and transient transfections of these cells. Therefore, although the mechanism of action of the various MyoD-related proteins is not yet known, evidence with cell culture systems suggests a role in development for these gene products.

In this study, we used the P19 embryonal carcinoma cell system to investigate the role of MyoD in differentiation, and more specifically, in the activation of muscle-specific genes. When exposed to DMSO, P19 multipotent cells differentiate along the mesodermal lineage to form cultures containing cardiac muscle, and in a lower proportion, skeletal muscle (McBurney et al., 1982a, Rudnicki et al., accepted in Dev. Biol.). We were unable to detect any expression of the endogenous MyoD gene during the *de novo* differentiation of P19 cells into muscle. However, upon transfection of the MyoD1 cDNA under the control of the strong constitutive phosphoglycerate kinase promoter, a significant proportion of the P19 transformants became differentiated into various cell types which included cardiac and skeletal muscle, in the absence of DMSO.

5.2 Absence of MyoD Expression during the DMSO-induced Differentiation of P19 cells

MyoD expression *in vivo* is restricted to neonatal and adult skeletal muscle (Davis et al., 1987). Our initial interest was to investigate whether MyoD was also expressed during the *de novo* differentiation of EC cells into cultures of cardiac and skeletal muscle. P19 cells were differentiated in the presence of DMSO as described in section 2.5.2, to form muscle-containing cultures with mostly cardiac myocytes. By

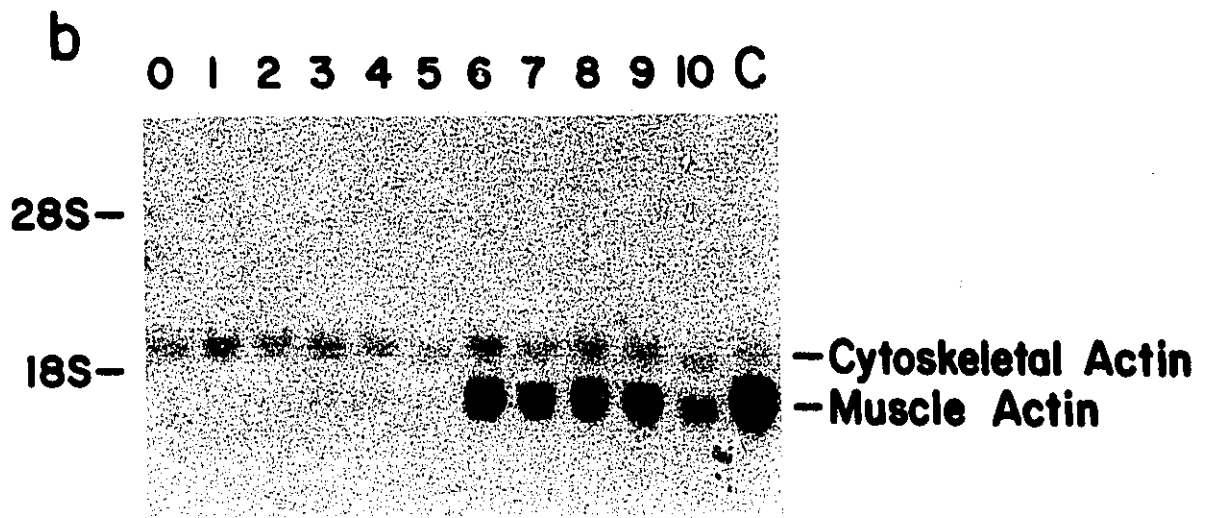
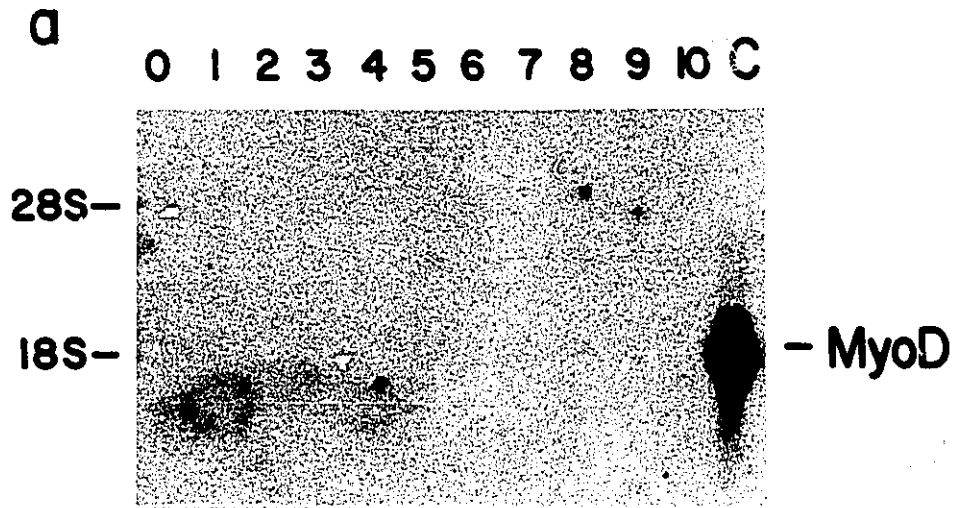
Northern analysis, we were unable to detect MyoD mRNA during the ten days of differentiation of P19 cells along the myogenic lineage (Figure 11a, lanes 0 to 10) although MyoD mRNA was present in the control differentiated MR322 cells, which contain large amounts of skeletal muscle (Figure 11a, lane C). As expected, the levels of cytoskeletal actin mRNA were relatively constant, while induction of muscle actins was concomitant with the appearance of spontaneously beating muscle on day 6 (Figure 11b). Differentiation of P19 cells into cardiac muscle-containing cultures therefore does not seem to involve MyoD expression.

5.3 Induction of Differentiation by MyoD in P19 Cells

We next tested whether MyoD could induce muscle differentiation when expressed at high levels in P19 EC cells. To achieve this, the MyoD1 cDNA was placed under the control of the pgk-1 promoter. Constitutive expression from this promoter was previously found to be comparable to that of the SV40 promoter in P19 cells (M. McBurney and C.N. Adra, personal communication). To produce stable transformants expressing MyoD, we cotransfected the MyoD1 cDNA (in pPGKMyoD), or an unrelated sequence (in pPGKhyg), together with a plasmid (pCA1035) carrying the neo selectable marker gene which confers resistance to the antibiotic G418. After ten days of G418 selection in growth-promoting medium, stable clones resistant to the antibiotic were isolated. In the control transfections which lacked the MyoD expression vector, we observed that most G418-resistant colonies consisted of EC cells, or of EC cells with a few nonmuscle cells, as was found in previous stable transfections (data not shown). Skeletal and cardiac muscle were only rarely detected in these transfections. In contrast, G418-resistant transformants containing the MyoD expression vector were often differentiated into various cell types. Spontaneously beating muscle, and large areas with bipolar multinucleate skeletal muscle cells were readily observed in many of these transformants. We fixed and scored these colonies for the presence of cells expressing striated muscle isoforms of myosin

Figure 11: **MyoD is not expressed during differentiation of P19 cells into cardiac myocytes.** Total RNA was isolated from P19 cells at daily intervals throughout the ten day DMSO-induced differentiation (section 2.5.2). Day 0 corresponds to undifferentiated EC cells. Cells were cultured as aggregates in Petri-grade dishes from day 0 to 5, and were transferred to tissue-culture dishes on day 5. Spontaneously beating cardiac muscle was visible on day 6. Lanes 0 to 10 in the Northern analysis contain 10 μ g of RNA from cells of day 0 to 10 during the differentiation regime, while the control lane C contains 10 μ g of RNA from differentiated MR322 cells (Rudnicki et al., accepted in Dev. Biol.), a cell line capable of skeletal myogenesis.

- (a) The Northern blot was probed with the entire MyoD1 cDNA sequence from pEMC11.
- (b) The blot was stripped and hybridized with the 0.6 kb 3' end Pst I fragment of the human cardiac α -actin gene to detect actin transcripts.



heavy-chain, which are recognized by the monoclonal antibody MF20 (Table 1). As shown in Table 7, higher numbers of colonies with muscle cells, as detected with the MF20 antibody, were observed when the MyoD cDNA was included in the transfections. Some colonies in the control transfections also contained muscle cells but they differed considerably from the MyoD-containing colonies. In the former, only a few cells were differentiated into cardiac muscle (Figure 12a and b) while in the presence of MyoD, differentiation of entire colonies could be observed (Figure 12c, d, and e). Furthermore, skeletal muscle was not detected in control transfections, while in the presence of MyoD, we observed colonies containing a large number of skeletal muscle cells (Figure 12e). Nonmuscle differentiated cell types were also present in MyoD-containing colonies as seen by the lack of MF20 reactivity in cells which were morphologically different from P19 EC cells (data not shown).

The effect of MyoD expression on the induction of differentiation in P19 cells was confirmed by Northern analysis of a pooled population of G418-resistant cells containing either pPGKMyoD, or a control plasmid with an unrelated sequence (pPGKhyg). As shown in Figure 13, the presence of muscle-specific α -actin mRNAs is correlated with the expression of MyoD, while levels of cytoskeletal actin mRNAs remain unchanged in the experiment. Taken together, these observations indicate that high levels of MyoD expression can induce differentiation of P19 cells into various cell types which include cardiac and skeletal muscle.

5.4 Discussion

In this study, we have transfected P19 EC cells with the MyoD cDNA under the transcriptional control of the strong constitutive promoter of *pgk-1*. Stable transformants that were isolated contained not only skeletal muscle but also various differentiated cells, and in particular, large amounts of cardiac muscle. Differentiation into these cell types was due to the high levels of expression of MyoD, since cells transfected with a plasmid lacking the MyoD sequence remained mostly undifferentiated. We also have analyzed the expression from the endogenous MyoD gene in untransfected P19 cells that were treated with DMSO to differentiate along the

Table 7: Muscle development in EC cells stably transfected with the MyoD expression vector

Experiment ^a	% of colonies containing muscle cells ^b	
	+MyoD	-MyoD
I	27	3
II	48	16
III	32	21

(a) Transfections were performed with the following plasmids:
 Experiment I: 10 μ g pPGKMyoD (or pGEM4) plus 5 μ g pCA1035;
 Experiments II and III: 10 μ g pPGKMyoD (or pPGKhyg) plus 1 μ g
 pCA1035 plus 5 μ g pPGK.

Note that pCA1035 contains the neo gene, while pPGK contains the pgk-1 gene, which is included to increase the number of G418-resistant colonies (M. McBurney, personal communication).

(b) Any colony containing one or more myosin-positive cells as detected by horseradish peroxidase-linked immunostaining with the MF20 antibody (Table 1) was scored as positive. Results shown here are percentages of colonies with muscle cell(s) per total number of G418-resistant colonies for each transfected plasmid. At least 100 G418-resistant colonies were scored in each case.

Figure 12: Differentiated phenotype of P19 colonies transfected with pPGKMyoD. The distribution of cardiac and skeletal muscle in G418-resistant colonies transfected either with or without the MyoD expression vector (as described in Table 7) was determined by horseradish peroxidase-linked immunocytochemistry using the primary antibody MF20, which is reactive with muscle myosin (Table 1). Control transfections (without MyoD) yielded colonies with no MF20-positive cells (a), or with a few cardiac muscle cells (b). Following cotransfection with pPGK-MyoD, many colonies were extensively differentiated, and contained cells which had differentiated along the myogenic lineage, as seen by the presence of spontaneously beating cardiac muscle (c and d), and of bipolar multinucleate skeletal muscle (e). Bar, 250 μm .

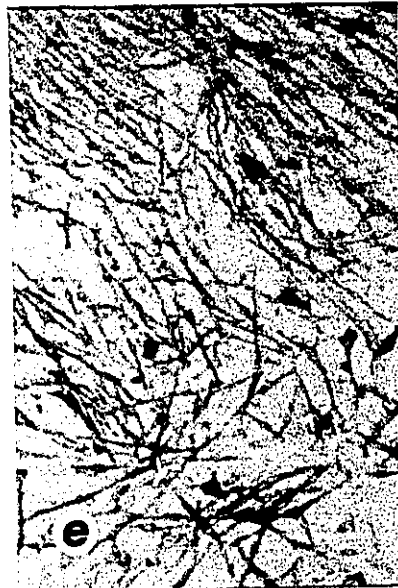
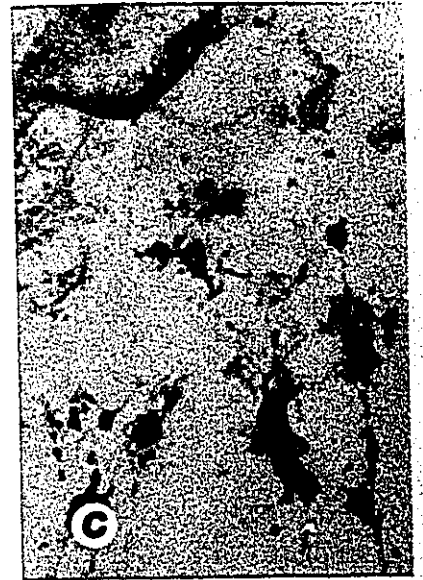
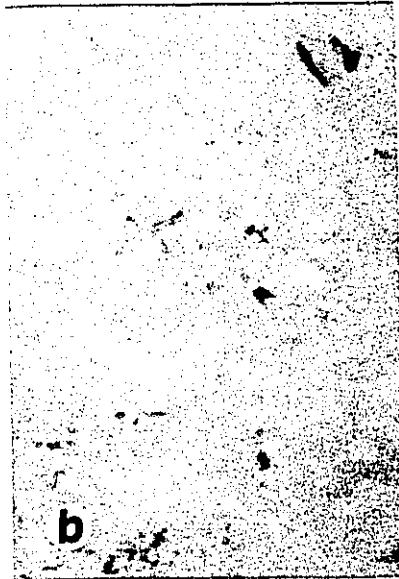


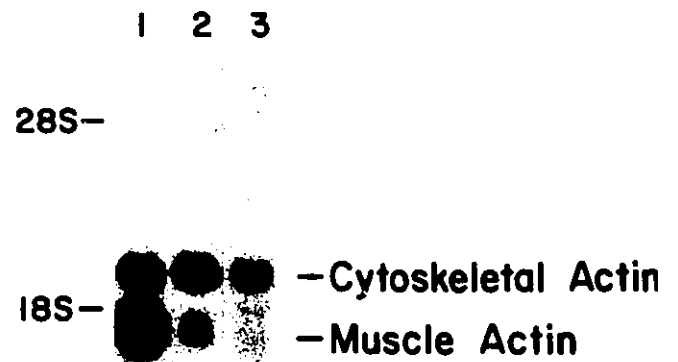
Figure 13: **Expression of MyoD and of muscle-specific actin mRNAs in stable transformants of P19 cells carrying the MyoD expression vector.** P19 cells were transfected with pCA1035 along with pPGKMyoD (lane 2), or pPGKhyg (lane 3) as described for Experiments II and III in Table 7. After 14 days of selection in G41S, at least 100 colonies were pooled, and total RNA was extracted and analysed by Northern hybridization. 10 μ g of RNA were loaded into each lane. Lane 1 which contains RNA from differentiated MR322 cells (M. Rudnicki et al., accepted in Dev. Biol.) is included as a positive control for the expression of MyoD and muscle actins. The Northern blot was probed for MyoD RNA (a), and was subsequently stripped and re-probed for actin RNAs (b) as described in Figure 11.

Figure 14: **Muscle-specific mRNAs from endogenous genes do not accumulate in transient transfections of P19 cells with the MyoD expression vector.** (see Chapter 4) Total RNA was isolated from P19 cells following a transient transfection with 10 μ g of pPv/B ActlacZ plus 10 μ g of pPGKMyoD (lane 1), of pPGKcmv (lane 2), or of pPGKneo (lane 3). 10 μ g of RNA were loaded in each lane. Shown here are two experiments (I and II) which were done in duplicate for X-gal staining (see Table 4). Undifferentiated (lane 4) and DMSO-differentiated (lane 5) P19 cells are included as respectively negative and positive controls for the expression of muscle actins. In lane 6, differentiated MR322 cell are included as a positive control for the expression of MyoD. The Northern blot was first hybridized with a MyoD probe (a), then stripped and re-hybridized for actin mRNAs (b) as described in Figure 11. The approximate size of the transcripts were: MyoD, 1.9 kb; cytoskeletal actin, 2.0 kb; and muscle actin, 1.6 kb.

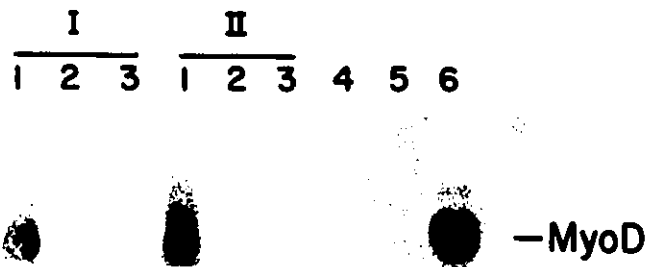
13 a



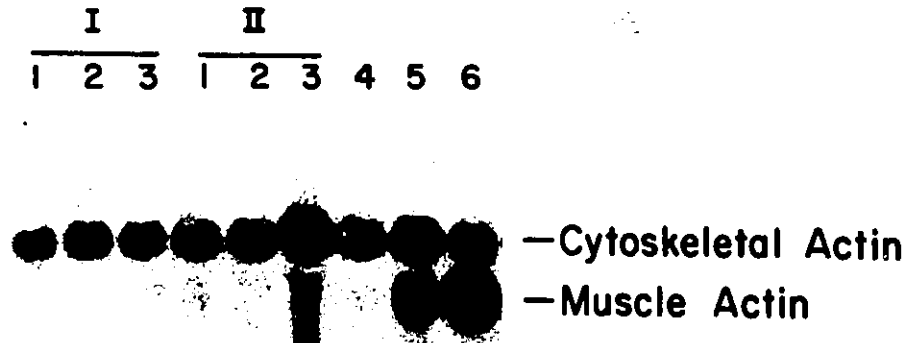
b



14 a



b



myogenic lineage. MyoD transcripts were absent during the *de novo* differentiation of these cells into cultures containing spontaneously beating cardiac muscle. Hence, MyoD does not play a role in the normal differentiation of cardiac muscle even though its inappropriate expression in EC cells leads to differentiation into this cell type.

Previous studies have indicated that MyoD was involved in skeletal muscle differentiation (Davis et al., 1987). This nuclear phosphoprotein which is normally found in myocytes, activates transcription of muscle-specific genes (Tapscott et al., 1988, Weintraub et al., 1989). In stable transfections, the expression of MyoD results in the conversion of nonmuscle cells to skeletal myoblasts that can differentiate into myotubes. In EC cells however, MyoD expression is insufficient to quantitatively convert EC cells into skeletal muscle since various cell types including cardiac muscle were derived from cells containing the MyoD expression vector. Developmental programs for muscle formation therefore do not seem to rely on a single master regulatory protein but rather on several proteins which must appropriately interact to permit the expression of specific differentiated functions.

The forced expression of MyoD in pigment, nerve, fat, liver, and fibroblast cell lines results in the myogenic conversion of these cells, as determined by the expression of endogenous muscle-specific markers (Weintraub et al., 1989). The failure to observe a similar myogenic conversion in P19 cells may be due to the different developmental stage of these cells. P19 cells have a broad spectrum of differentiation, and may have to first become committed to the mesodermal lineage before undergoing determination and differentiation into muscle. Certain factors which are present in differentiated cell types, and are necessary for MyoD action may be absent or inactive in EC cells. Indeed, AP-1 activity which is involved in mediating transcriptional changes associated with cell differentiation and development, is absent in EC cells. The protooncogene *c-fos* that encodes a protein participating in complexes with the transcription factor AP-1, is not expressed in P19 cells but is activated as the cells differentiate into muscle (Edwards and Adamson, 1986, Halazonetis et al., 1988, Curran and Franza, 1988). Evidence for regulatory factors that can induce or repress the expression of differentiated functions has also been

provided by heterokaryons experiments with myocytes and nonmyogenic cells (Blau et al., 1985, Hardeman et al., 1986). Studies on the DNA and protein interactions of MyoD may thus lead to the discovery of factors with a general activity linked to differentiated functions.

MyoD was not expressed in undifferentiated P19 cells or in cells treated with DMSO to differentiate into cardiac muscle. Similarly, previous reports showed that MyoD mRNA was absent from mouse neonatal and adult cardiac muscle (Davis et al., 1987). The induction of cardiac cell development is therefore not the normal role played by this gene product. This activity of MyoD may be due to its abnormally high expression in cells which usually do not express the MyoD gene, namely undifferentiated P19 EC cells. The MyoD gene product has sequence-specific DNA-binding properties, and is a member of a family of proteins which share similarities in the region required for DNA binding and for myogenic conversion of nonmuscle cells. In addition, the conserved region of one of the related proteins was shown to function as a dimerization domain (Murre et al., 1987). Consequently, high levels of expression of MyoD in stably transfected EC cells could lead to the loss of subtle specificities of DNA-protein interactions that might be present at more physiological levels of MyoD. MyoD could thus mimic related proteins and interact with DNA or proteins at sites of reduced affinity which modulate differentiation to cardiac muscle or to other cell types. Alternatively, it is possible that the presence of MyoD in vast excess may saturate repressors that normally inhibit the activity of MyoD or of MyoD-like proteins in undifferentiated EC cells; the supplementary MyoD in these cells may thus be able to activate differentiation pathways which are normally not accessible to MyoD in EC cells. The analysis of the specific sequences which are required for the MyoD-induced transcriptional activation of muscle genes may provide some information on the sites with which MyoD interacts directly or indirectly, and thus give some indication on how induction of myogenesis normally occurs.

In conclusion, the experiments reported here demonstrated that induction of differentiation by a regulatory protein expressed at high levels in a cell culture system does not necessarily reproduce the differentiation events that normally occur

during development. MyoD expressed at high levels in EC cells leads to differentiation into various cell types, in addition to the previously reported skeletal muscle. Our finding thus indicates that MyoD is not sufficient to mediate myogenic conversion of EC cells. This strongly suggests a role for other regulatory proteins which cooperate with MyoD in the developmental events leading to the differentiation of skeletal muscle.

Chapter 6

General Conclusions

6.1 The Role of Multiple Regulatory Regions in Muscle-Specific Transcription

Human cardiac actin chimeric genes are subject to developmental and tissue-specific regulation during the differentiation of P19 cells into cardiac muscle-containing cultures (Rudnicki et al., 1988, Chapter 3). By deletional analysis, we have demonstrated that multiple 5'-flanking regions of the human cardiac actin promoter are involved in the regulated expression in cardiac muscle (Chapter 3), and in the MyoD-induced transcription of the gene (Chapter 4). The sequences which were important in the cardiac-specific expression differed from those required in the MyoD-induced *trans*-activation. The differences may result from the fact that transient transfections only assay the effect of a single muscle-specific protein, MyoD, while permanent transfections and differentiation into muscle involve many developmental events and thus diverse regulatory factors. Alternatively, differences in cardiac and in MyoD-induced transcription may be explained by the fact that MyoD participates in skeletal myogenesis but not in cardiac myogenesis (Chapter 5). The requirement of different regulatory sequences for the expression of the cardiac actin gene in the presence of MyoD and in cardiac muscle may therefore reflect the use of distinct mechanisms of expression in the cardiac and skeletal muscle cell types. Indeed,

studies of the muscle creatine kinase gene in transgenic mice provided evidence for *cis*-acting DNA elements which have differential effects on transcription in cardiac versus skeletal muscle (Johnson et al., 1989).

Previous studies of the human cardiac actin gene transiently introduced into skeletal myocytes have shown the importance of the CarG box element, a sequence which is recognized by nonmuscle-specific proteins. Although four CarG box sites are present in the cardiac actin 5'-flanking region, only the two proximal ones were necessary for transcription in the C2C12 skeletal muscle cell line (Minty and Kedes, 1986b). This indicates that the CarG box sequence alone does not dictate a specific regulatory function. Indeed, the CarG box sequence in the *c-fos* protooncogene or in the β cytoskeletal actin gene have different regulatory functions in each context (Kawamoto et al., 1988, Prywes and Roeder, 1986).

Our studies with the human cardiac actin gene have shown that the sequence between -237 and -227 bp containing CarG box 4, played an important role in the expression of the gene in cardiac muscle or with MyoD (see Figures 7 and 8), while CarG box 2 which was involved in the expression in C2C12 cells, did not significantly contribute to the cardiac actin gene expression. It is of interest to note that although the deletion of a 10 bp sequence containing the CarG box 4 resulted in an important decrease in expression, the transcription from the deleted cardiac actin promoter remained inducible in cardiac muscle, or in the presence of MyoD. CarG box elements therefore act as positive regulators of transcription while other sequences probably control the MyoD-induction and tissue-specificity of the expression. It was recently shown that the CarG box 4 of the cardiac actin gene is the binding site for the serum responsive factor (SRF) and for another distinct protein whose footprint was contained within the SRF binding site (Gustafson and Kedes, 1989). Purification of the CarG box binding proteins and their characterization should reveal their mode of interaction with other proteins which direct the tissue-specific expression.

At least six other factors in addition to the CarG box binding factors (CBF) were shown to recognize different regions in the cardiac actin promoter (Gustafson and Kedes, 1989). In particular, the regions between -312 and -237 bp, and between

-82 and -47 bp, which are both involved in MyoD-induced *trans*-activation (Chapter 4) contain respectively three and one Sp1 binding sites. Additional factors which are not muscle-specific also bind to other upstream regions of the promoter, some of which are involved in transcription with MyoD or in cardiac muscle. From these results, it is clear that transcription of the cardiac actin gene involves complex interactions of proteins at many sites in the promoter.

6.2 A Model for Muscle-specific Expression

Based on these data and on other analyses of muscle-specific promoters, we can interpret our results in terms of a model in which upstream regions of the human cardiac actin gene are differentially used in cardiac and skeletal muscle development. Some common regulatory proteins such as the CBF and Sp1 factors may act as general positive activators of transcription. These nonmuscle-specific proteins may also have some regulatory functions restricted to muscle-specific expression. Two levels of control can be envisaged to explain how ubiquitous factors may participate in muscle-specific regulation. A primary level of control on tissue-specificity may involve activation at the level of the chromatin structure, which could be required to permit subsequently the interaction of the gene with transcriptional activators. At a second level of regulation, the nonmuscle-specific proteins may be able to stimulate muscle-specific transcription following some post-translation modification or when combined with other nuclear factors which are restricted to muscle cells. This cooperative mode of regulation would allow a different pattern of expression of the same gene in cardiac and skeletal myocytes, providing that differences are present in the amount and/or in the type of transcriptional activator in the two cell types.

6.3 The Role of Multiple Regulatory Proteins in Muscle-specific expression

Certain muscle-specific factors have been shown to regulate the transcription of genes which are expressed in muscle cells (Braun et al., 1989b, Davis et al., 1987, Wright et al., 1989). In particular, a nuclear factor referred to as MEF-1 which is present in skeletal myocytes, has been shown to bind to two muscle-specific enhancers, E1 and E2, in the muscle creatine kinase (MCK) gene (Buskin and Hauschka, 1989). Preliminary results have shown that MEF-1 is identical to the putative myogenic determination factor MyoD1 (Lassar et al., submitted). Our results are also consistent with the action of MyoD at sequences which are homologous to the MCK E2 element in the cardiac actin gene (Chapter 4). Gel mobility shift assays with various fragments of the cardiac actin 5'-flanking region in the presence of the MyoD protein would permit to determine whether MyoD directly binds to the E2-like sequences in the cardiac actin gene.

In Chapter 5, we demonstrated that the expression of MyoD is not sufficient to convert P19 EC cells to skeletal myocytes. Other regulatory factors must therefore act with MyoD to modulate the activation of the skeletal muscle differentiation program. Recent studies have shown that MyoD could form heterodimers with other nuclear proteins, the E12 and E47 proteins, which are present in many mammalian tissues (Murre et al., 1989b). The heterodimers had higher affinity than the homodimers for the specific DNA binding sites. The ectopic expression of MyoD in P19 cells may therefore induce differentiation by forming heterodimers whose affinities for their respective DNA sequences may be significantly altered. The *myc* protein which shares the dimerization domain with MyoD, did not form heterodimers capable of binding to DNA (Murre et al., 1989b). Additionally, the expression of *c-myc* declines rapidly as cells undergo terminal differentiation (Davis et al., 1987, Lachman and Skoultchi, 1984). The *myc* protein may negatively regulate differentiation by binding with MyoD and consequently preventing its action on muscle-specific promoters. Another possibility is that the *myc* protein binds to the E12 or E47 proteins thus making them unavailable for MyoD dimerization. In this situation

the MyoD homodimer which predominates in the cells, may not have sufficient affinity to interact with the *cis*-acting regulatory sequences that are responsible for the activation of muscle-specific genes. A similar mode of regulation has been discovered with the *c-fos* protooncogene and the AP1 factor. Dimerization of the *fos* and the *jun* proteins in AP1 results in an increased DNA-binding affinity to certain regulatory elements in genes (Halazonetis et al., 1988). The same type of interaction between MyoD and the *myc* protein could be investigated *in vivo* by cotransfections of a muscle-specific chimeric gene with expression vectors encoding both regulatory proteins. This experiment would test whether the presence of the *myc* protein affects the MyoD-induced *trans*-activation of muscle-specific genes. *In vitro*, immunoprecipitation experiments with the *myc* and MyoD proteins would permit to determine whether the factors can form dimers.

Additional factors play a role in the activation of muscle-specific genes as indicated by the repression of myogenic differentiation by specific growth-related factors. It was recently demonstrated that myogenic cultures which were inhibited from differentiating by treatment with fibroblast growth factor or with transforming growth factor β , contained reduced levels of MyoD mRNA. Inhibition still occurred in cultures engineered to constitutively express MyoD mRNA, thus indicating that additional regulatory pathways important in myogenesis are affected (Vaidya et al., 1989). Evidence for a role of negative regulators has also been provided with the initial transfection experiments performed with the MyoD expression vector. The efficient myogenic conversion of various cell types by MyoD or by MyoD-related proteins required not only the stable expression of the transfected cDNA but also the withdrawal of serum from the culture medium (Braun et al., 1989b, Davis et al., 1987, Weintraub et al., 1989). This procedure is associated with a decrease in levels of expression of the *fos* oncogene. In addition, cotransfection of *fos* and MyoD expression vectors with a MCK-CAT chimeric gene resulted in a decrease of the MyoD-induced CAT transcription thus suggesting a negative role for the oncogene (Lassar et al., Cell, in press). Other growth regulating proteins including adenovirus E1A, the *ras* oncoprotein and proliferin have also been found to inhibit myogenesis (Webster et al., 1988, Olson et al., 1987, Wilder and Linzer, 1989).

This suggests that expression of differentiated functions initially depends on the interplay of numerous factors which control proliferation and development of cells, and perhaps also the activity of MyoD. The cloning and characterization of the activity of other regulatory factors that mediate the response to growth-regulated factors may develop a clear picture of the cellular events that permit the initiation of muscle differentiation.

Expression in cardiac muscle involves different factors than those present in skeletal muscle since MyoD and the MyoD-related proteins are absent in cardiac myocytes. The experimental approach to isolate a regulatory gene which directs cardiac-specific expression, could first involve DNA-binding studies with the region of the cardiac actin promoter that is important in cardiac-specific expression, namely the sequences between -237 and -158 bp. Gel mobility shift assays could be performed using P19 cell extracts prepared after different periods of time following treatment with DMSO to induce differentiation. This will permit the detection of cardiac-specific DNA-binding activities that may appear during differentiation into cardiac muscle. One approach to clone the cardiac-specific factor would involve the subtracted hybridization of cDNAs derived from mRNA of undifferentiated P19 cells and of DMSO-treated P19 cells which were at the specific stage where the cardiac-specific DNA-binding activity is present (as previously determined by gel mobility shift experiments). Common muscle-specific transcripts of contractile proteins may also be subtracted using RNA isolated from a fully differentiated skeletal muscle cell line. Finally screening of the subtracted cDNAs which were cloned in an expression vector, may be done with an oligonucleotide that includes the DNA-binding site of the cardiac-specific factor (Kadonaga et al., 1987). Alternatively, the identification of a cDNA encoding a transiently expressed muscle-specific factor, may be done using the subtracted cDNA as a probe to screen the viral plaques of the library. The library should also be screened with a negative probe consisting of RNA from P19 cells, to eliminate sequences that were not removed during the subtraction procedure. Plaques showing a differential hybridization with the two types of probes can be isolated and used to probe a "Northern" blot with RNA from DMSO-treated P19 cells at different stages, to determine the pattern of expression

of the cloned cDNA. *In vivo* studies similar to the transient and permanent transfections assays that were performed in this study with P19 cells, would permit to assess the role of this protein. These experimental approaches will lead to the identification of factors involved in cardiac-specific activation of transcription; the study of the protein-protein and protein-DNA interactions of the isolated factors may help in elucidating the overall mechanisms that govern the activation of differentiated functions during development.

Appendix A

List of Plasmids

Plasmid	Description
pSP64-T,P/B Act	Actin Pst I-Ban II fragment (-750 to +6) in pSP64.
pGEM4-P/B Act	Actin Pst I-Ban II fragment(-750 to +6) in pGEM4.
pGEM4-Pv/B Act	Actin Pvu II-Ban II fragment (-440 to +6) in pGEM4.
pGEM4-H x	pGEM4-Pv/B Act with linker in Hae III site at position - x (pGEM4-H21, pGEM4-HS2, pGEM4-H227).
pGEM4-M x	pGEM4-Pv/B Act with linker in Mnl I site at position - x . (pGEM4-M9, pGEM4-M128, pGEM4-M158, pGEM4-M237, pGEM4-M312, pGEM4-M363, pGEM4-M418).
pGEM4- $\Delta x - y$	pGEM4-Pv/B Act with a deletion (- x to - y) in the actin region.
pP/B ActlacZ	Actin Pst I-Ban II fragment (-750 to +6) upstream of lac Z.
pT/B ActlacZ	Actin Tth 111I-Ban II fragment (-47 to +6) upstream of lac Z.
pPv/B ActlacZ	Actin Pvu II-Ban II fragment (-440 to +6) upstream of lac Z.
p x /B ActlacZ	Actin x -Ban II fragment (- x to +6) upstream of lac Z ($x=418, 363, 312, 237, 227, 158, 128, \text{ or } 82$).
pM x ActlacZ	pPv/B ActlacZ with a linker at a Mnl I site (position - x where $x=418, 363, 312, 237, 158, \text{ or } 128$).
pH x ActlacZ	pPv/B ActlacZ with a linker at a Hae III site (position - x

	where $x=227$ or 82).
$p\Delta x - y$ ActlacZ	pPv/B ActlacZ with an internal deletion ($-x$ to $-y$) in the actin region upstream of lacZ.
pAE	Cardiac actin region (-650 to -86) upstream of the heat-shock promoter-lacZ gene.
pAEinv	Identical to pAE except for the orientation of the actin region.
pINT	Cardiac actin first intron upstream of the heat-shock promoter-lacZ gene.
pINTinv	Identical to pINT except for the orientation of the actin intron.
pPGKneo	Mouse PGK promoter (Xba I-Taq I fragment) upstream of the neo gene.
pGEM4-ActCAT	Human cardiac actin promoter (-440 to +6) upstream of the CAT gene.
pPGKneo-ActCAT	Cardiac actin promoter-CAT gene and PGK promoter-neo gene.
pPGKMyoD	Mouse PGK promoter (Xba I-Taq I fragment) upstream of the MyoD1 cDNA.

Additional Plasmids

Plasmid	Description	Reference
pCA1035	Mouse PGK promoter upstream of the neo gene.	C. Adra, U. of Ottawa
pCH126A2	Lac Z coding region without promoter.	R. Kothari et al., 1989
pCH126PHS	Mouse heat-shock protein 68 promoter upstream of lac Z.	R. Kothari et al., 1989

APPENDIX A. LIST OF PLASMIDS

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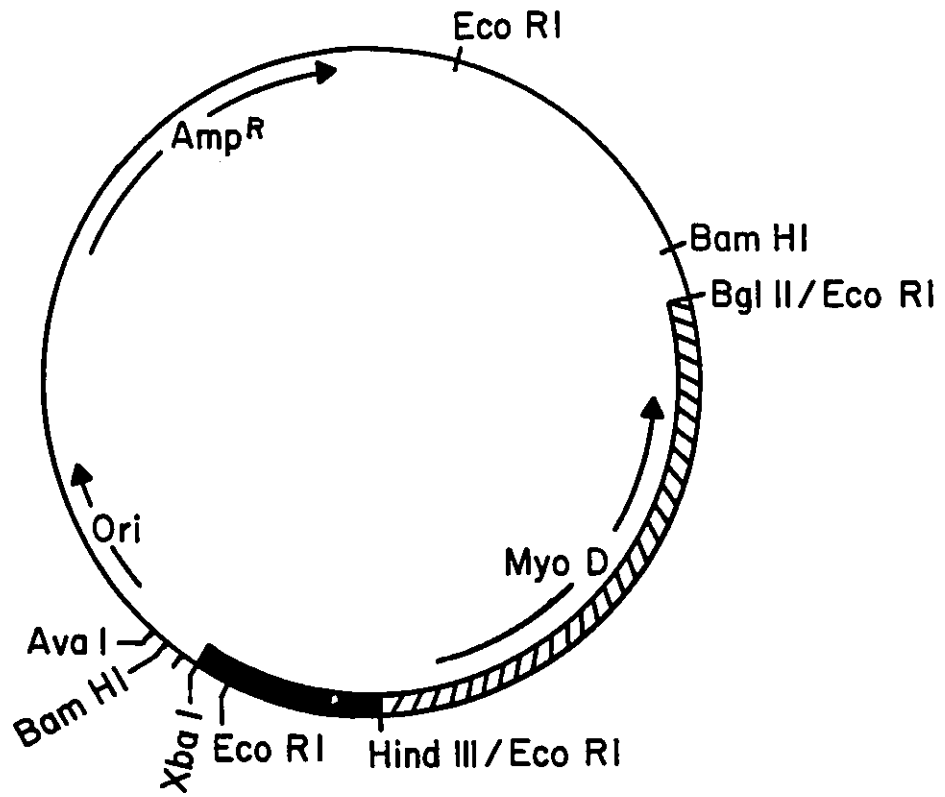
pHalacZ	Human cardiac actin promoter (-750 to +750) upstream of lac Z.	W. Colledge, U. of Ottawa
pPGK	Mouse PGK gene.	C. Adra, U. of Ottawa
pPGKlacZ	Mouse PGK promoter upstream of lac Z.	C. Adra, U. of Ottawa
pPGKcmyc	Mouse PGK promoter upstream of the mouse c-myc gene.	R. St-Arnaud, U. of Ottawa
pPGKhyg	Mouse PGK promoter upstream of the hygromycin B gene of <i>E. coli</i> .	R. Hawley, U. of Ottawa
pPGKluc	Mouse PGK promoter upstream of the luciferase gene.	K. Jardine, U. of Ottawa
pRSVCAT	Rous Sarcoma Virus promoter upstream of the CAT gene.	C. Gorman et al. 1982

Appendix B

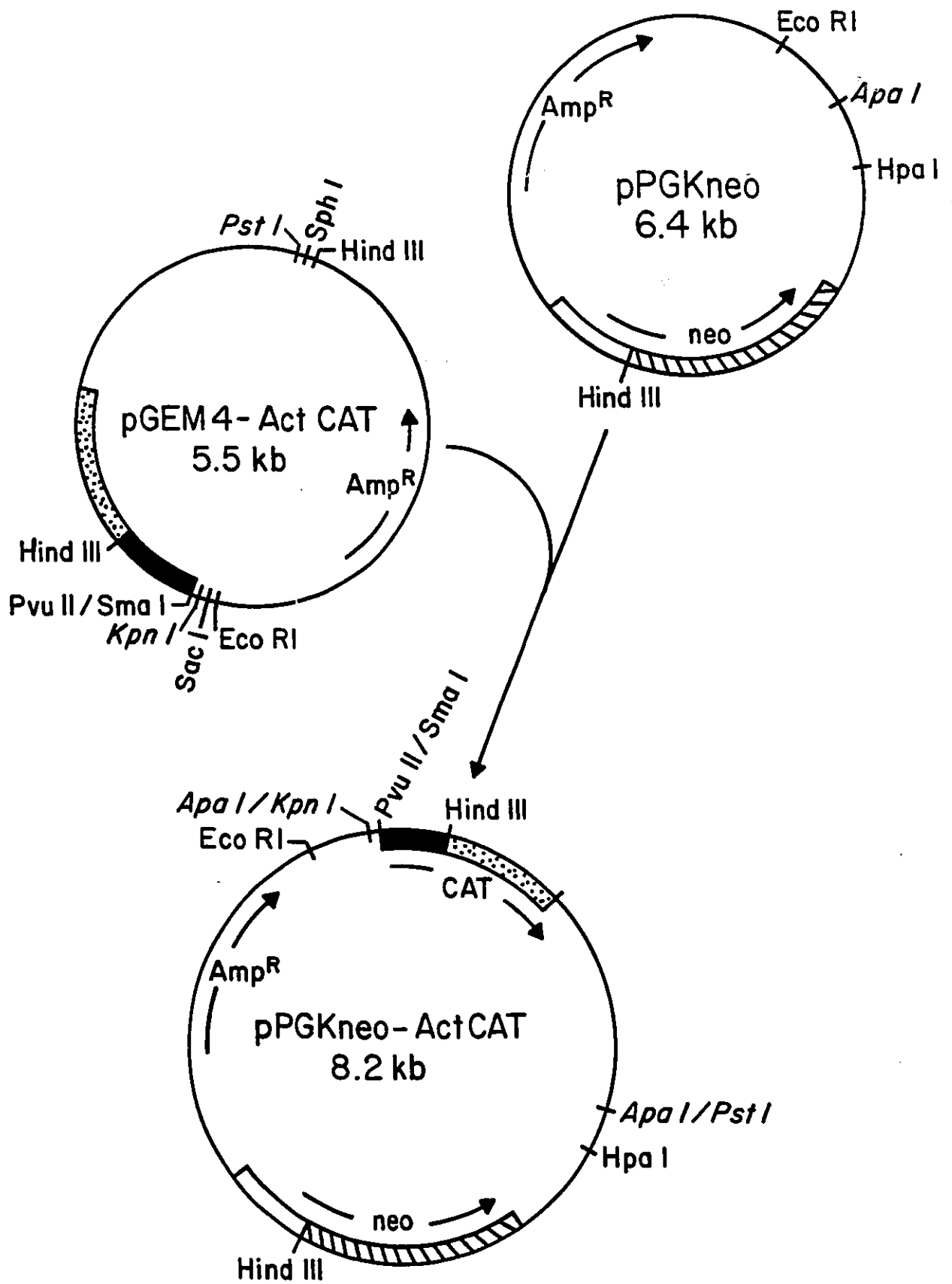
Maps and Construction of Plasmids

The Recombinant pPGKMyoD. This construct consists of the MyoD1 cDNA inserted downstream of the mouse pgk-1 promoter region (Xba I-Taq I fragment), and terminated by the SV40 polyadenylation site (in the Hpa I-Eco RI fragment). The vector pTB4 (provided by T. Bladon, University of Ottawa) was digested with Bgl II and Hind III, was blunt-ended, and was dephosphorylated (see section 2.4.1 for details on these modifications of the DNA). The MyoD1 cDNA was isolated as a 1.7 kb Eco RI fragment from pEMC11 (Davis et al., 1987), and was blunt-ended before ligating with the vector pTB4. The filled and hatched boxes in the figure represent respectively the pgk-1 promoter and the MyoD1 cDNA, while the arrow indicates the direction of transcription.

pPGKMyoD
(5.5 kb)

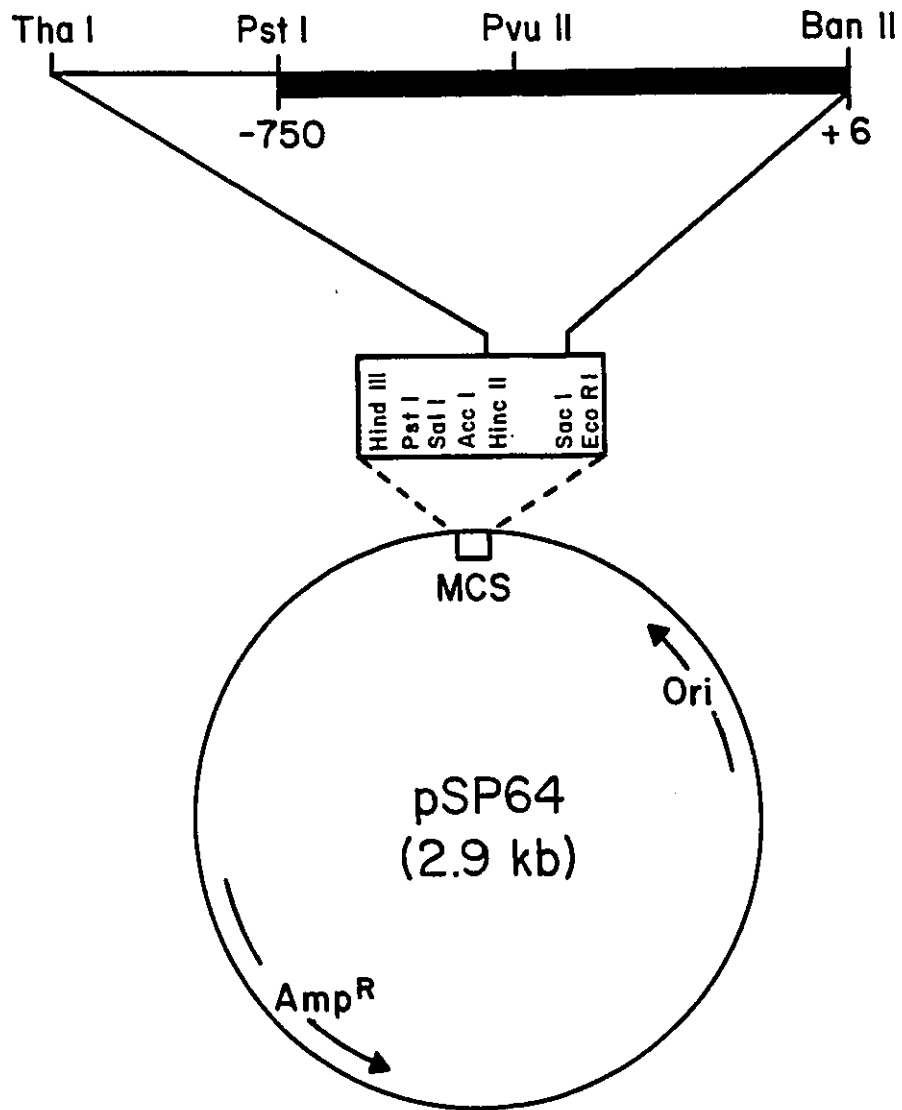


The Recombinants pPGKneo, pGEM4-ActCAT, and pPGKneo-ActCAT. pPGKneo was constructed by inserting the neo Hind III-Hpa I fragment of pSV2neo (Southern and Berg, 1982) into the Hind III, Hpa I-digested pPGKgpt (provided by C. Adra, University of Ottawa). The open box in the figure represents the pgk-1 promoter while the hatched box represents the neo gene which is transcribed in the direction of the arrow. pGEM4-ActCAT was prepared by ligating together three DNA fragments: the 2.2 kb Pst I-Hind III fragment from pSV2CAT (CAT gene and SV40 polyadenylation site), the 450 bp human cardiac α -actin promoter Pvu II-Hind III fragment from pGEM4-P/B Act, and the Pst I-Sma I phosphatase-treated pGEM4 vector. The filled and stippled boxes in the figure represent respectively the actin promoter region and the CAT gene. pPGKneo and pGEM4-ActCAT were used to prepare pPGKneo-ActCAT. pGEM4-ActCAT was digested with Kpn I and Pst I, and was blunt-ended. The ActCAT fragment was next ligated to the ApaI-digested, blunt-ended pPGKneo. Transcription of the two chimeric genes was in opposite direction as indicated by the arrow in the figure, and in each case was terminated by the SV40 polyadenylation region.



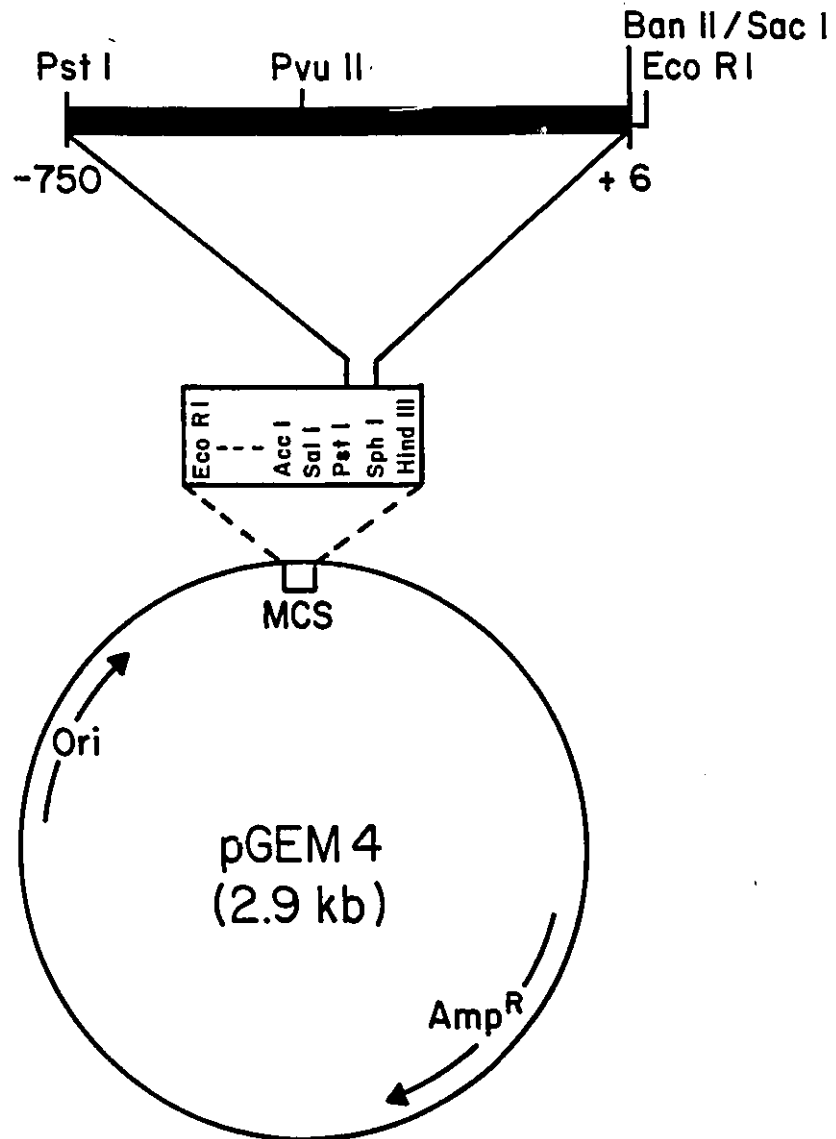
The Recombinant pSP64-T,P/B Act. It contains the human cardiac α -actin promoter region which was isolated by digesting pATA15 (actin 1.4 kb Pst I fragment in pBR325, provided by M. Ruben and M. Rudnicki, University of Ottawa) to completion with *Tha* I and partially with *Ban* II. The actin fragment was ligated at the *Hinc* II and *Sac* I multiple cloning sites (MCS) of pSP64 (Promega, Mississauga, Canada). The filled box in the figure indicates the actin promoter region between Pst I (-750) and *Ban* II (+6). The region of the insert between *Tha* I and Pst I consists of pBR325 sequences which were removed in the construct pGEM4-P/B Act.

pSP64-T,P/B Act



The Recombinant pGEM4-P/B Act. It consists of the actin Pst I-Ban II fragment (-750 to +6) subcloned in the multiple cloning site (MCS) of pGEM4 (Promega, Mississauga, Canada). pSP64-T,P/B Act was digested with Eco RI, was blunt-ended, and was finally digested with Pst I. The actin fragment was inserted in pGEM4 which was digested with Sph I, was blunt-ended, and digested with Pst I. This construct was used to subclone the actin promoter in pP/B ActlacZ and in pPv/B ActlacZ.

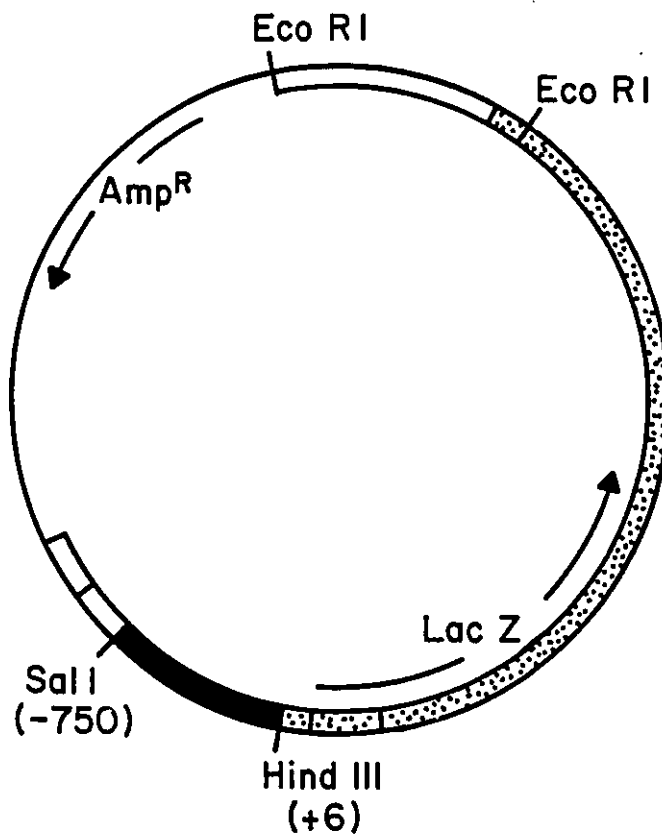
pGEM4-P/B Act



The Recombinant pP/B ActlacZ. It contains the actin promoter region between -750 and +6, subcloned upstream of the lac Z reporter gene. The plasmid was constructed by digesting pGEM4-P/B Act with Hind III and Sal I. The actin fragment was next ligated to the Hind III, Sal I-digested expression vector pCH126A2 (Kothary et al., 1989).

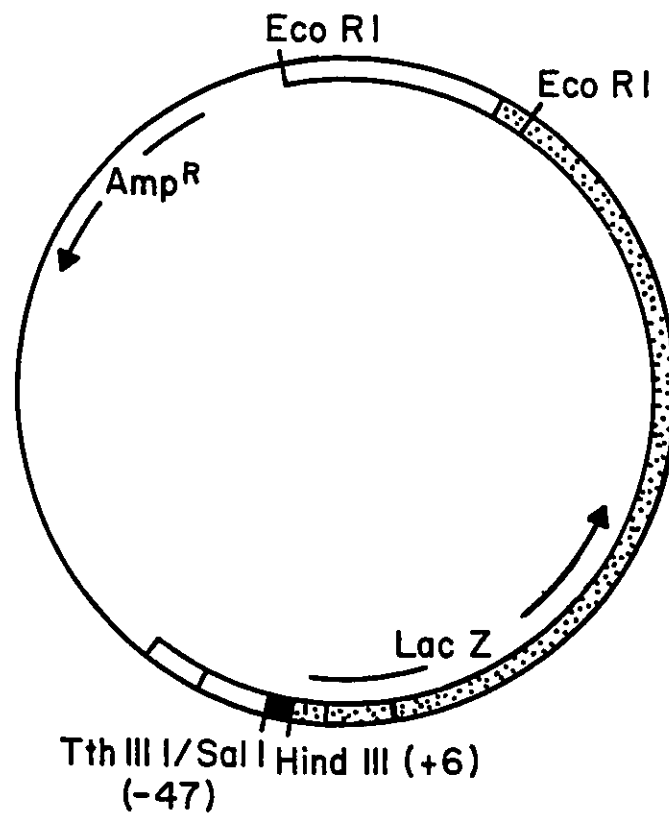
The chimeric gene shown in the figure thus consists of the actin promoter region (filled box) directing the transcription of lac Z (stippled box) which terminates with the SV40 polyadenylation site (open box). The two open boxes upstream of the actin promoter represent two SV40 polyadenylation sites which are present to prevent cryptic promotion from the prokaryotic sequences.

pP/B Act lac Z
(8.0 kb)



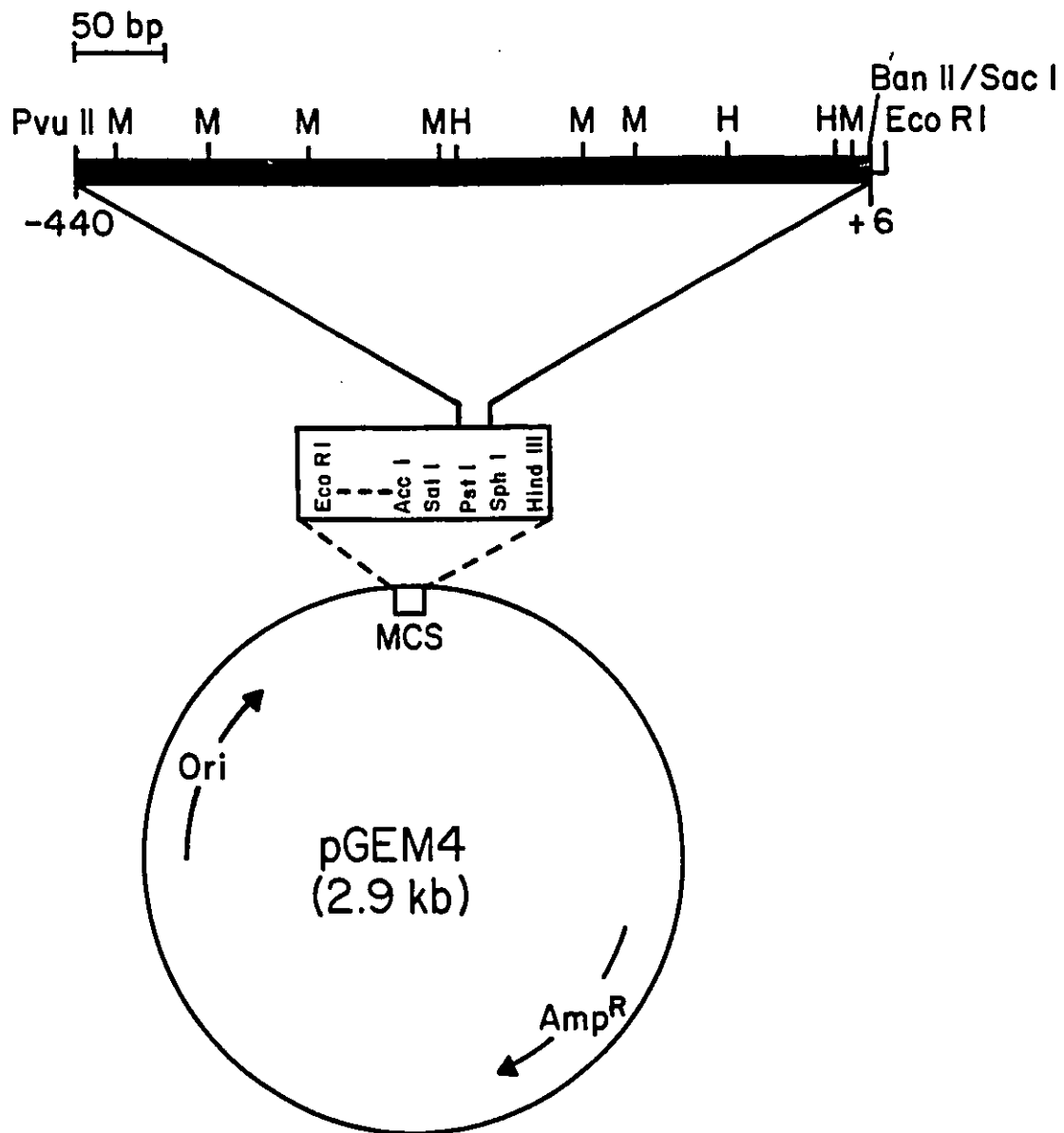
The Recombinant pT/B ActlacZ. It contains the actin promoter region between -47 and +6, subcloned upstream of the lac Z reporter gene. The construct was prepared from pP/B ActlacZ by digesting it to completion with Sal I, partially with Tth 111 I, blunt-ending with Klenow, and religating. The legend for the map of the chimeric gene is as described for pP/B ActlacZ.

pT/B Act lac Z
(7.3 kb)



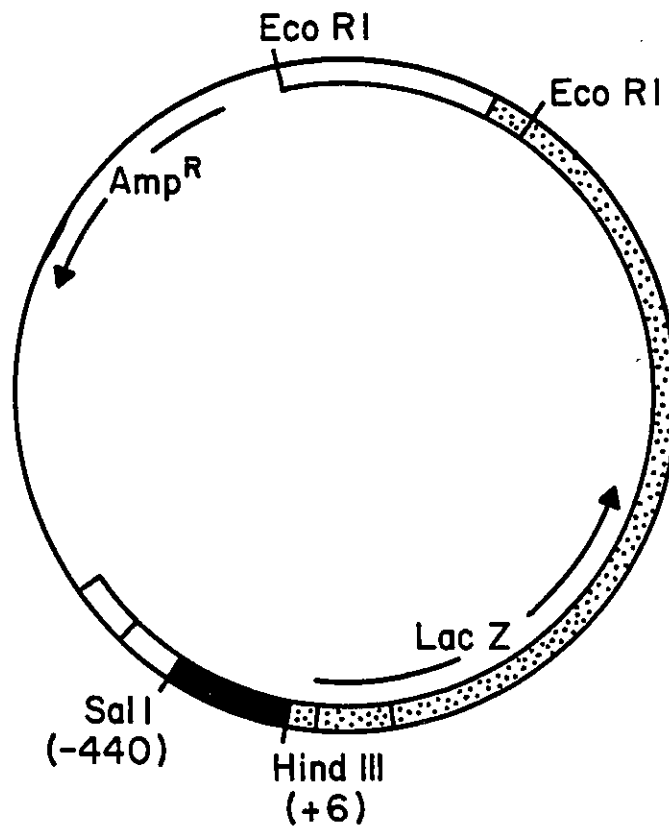
The Recombinant pGEM4-Pv/B Act. This construct was prepared from pGEM4-P/B Act by digesting it to completion with Pst I, blunt-ending with Klenow, digesting partially with Pvu II, and finally religating the plasmid on itself. This plasmid was used to produce the linker mutations in the -440 to +6 region of the actin promoter (filled box in the figure). The Mnl I and Hae III sites where Xho I linkers were inserted are indicated on the figure as M and H respectively. (Sites present in the vector sequence were not included.)

pGEM4 - Pv/B Act



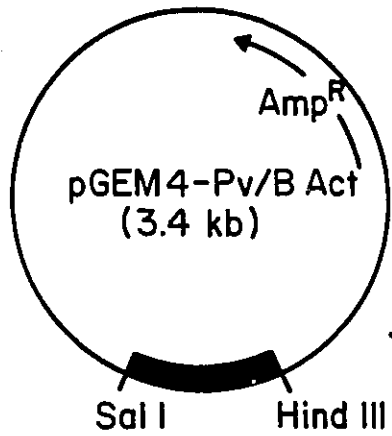
The Recombinant pP_v/B ActlacZ. It contains the actin promoter region between -440 and +6, subcloned upstream of the lac Z reporter gene. The construct was made by ligating the Hind III-Sal I actin fragment of pGEM4-P_v/B Act with the Hind III, Sal I-digested expression vector pCH126A2. The legend for the map of the chimeric gene is as described for pP/B ActlacZ.

pPv/B Act lacZ
(7.7 kb)

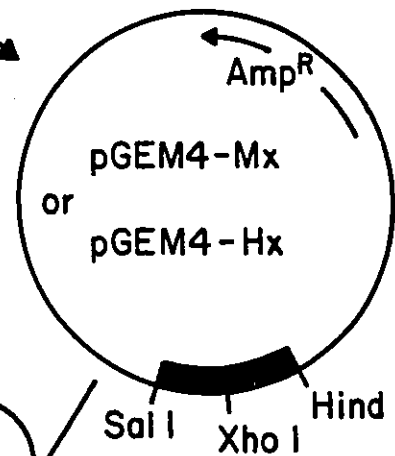


Construction of Linker Mutants pM x ActlacZ and pH x ActlacZ. A linker-scanning library of the actin promoter between -440 to +6 was produced by partially digesting pGEM4-P v /B Act with Mnl I or Hae III, which cut respectively 22 and 17 times in the plasmid. Xho I linkers were ligated to the digested plasmid. In the case of Mnl I, a Klenow treatment was necessary to permit ligation of the linkers. Following the digestion of the excess linkers by Xho I, the linear plasmids were isolated in a low melting agarose gel. The plasmids were self-ligated, were introduced into bacteria, and were next analysed by restriction endonuclease digestions for the presence of a Xho I linker in the actin promoter region. Recombinants pGEM4-M x and pGEM4-H x contained a Xho I linker at respectively a Mnl I and a Hae III site at position - x upstream of the cap site. The various linker mutants are listed in appendix A, and linker sites are shown on the map of pGEM4-P v /B Act.

pM x ActlacZ and pH x ActlacZ contain the -440 to +6 region of the actin promoter with a Xho I linker at position - x , subcloned in the expression vector pCH126A2. The plasmids were constructed by ligating the Hind III, Sal I actin fragment of pGEM4-M x or of pGEM4-H x , with the Hind III, Sal I-digested pCH126A2. The various linker mutants are listed in appendix A, and they are identical to pP v /B ActlacZ except for the presence of a Xho I linker. The legend for the map of the chimeric gene is as described for pP/B ActlacZ.

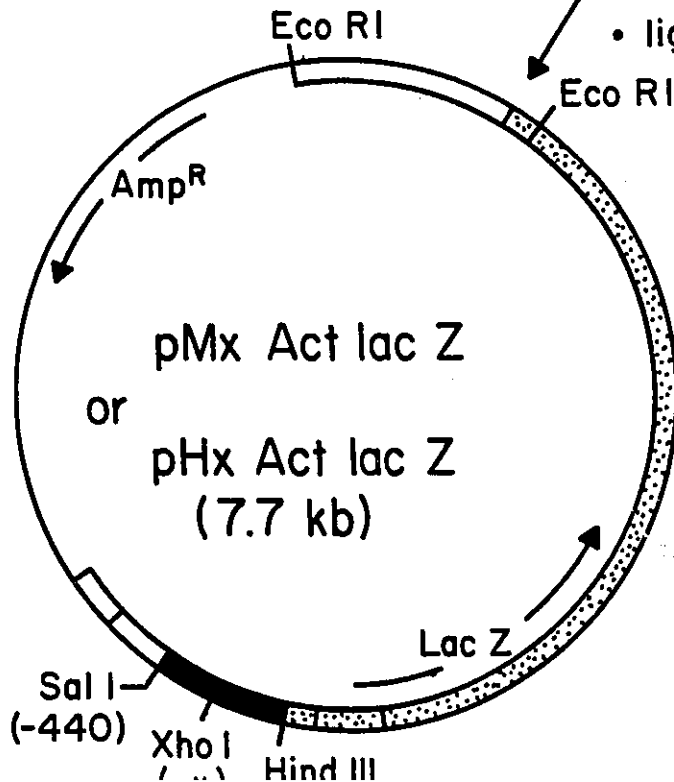


- partial Mnl I or Hae III
- Xho I linker + ligase
- Xho I
- ligase



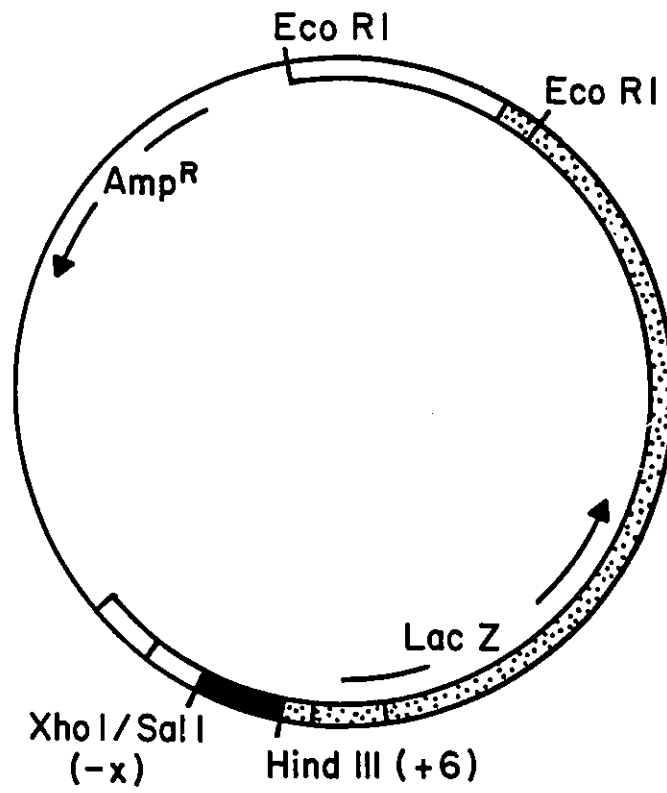
pCHI26A2

- Hind III, Sal I
- ligase



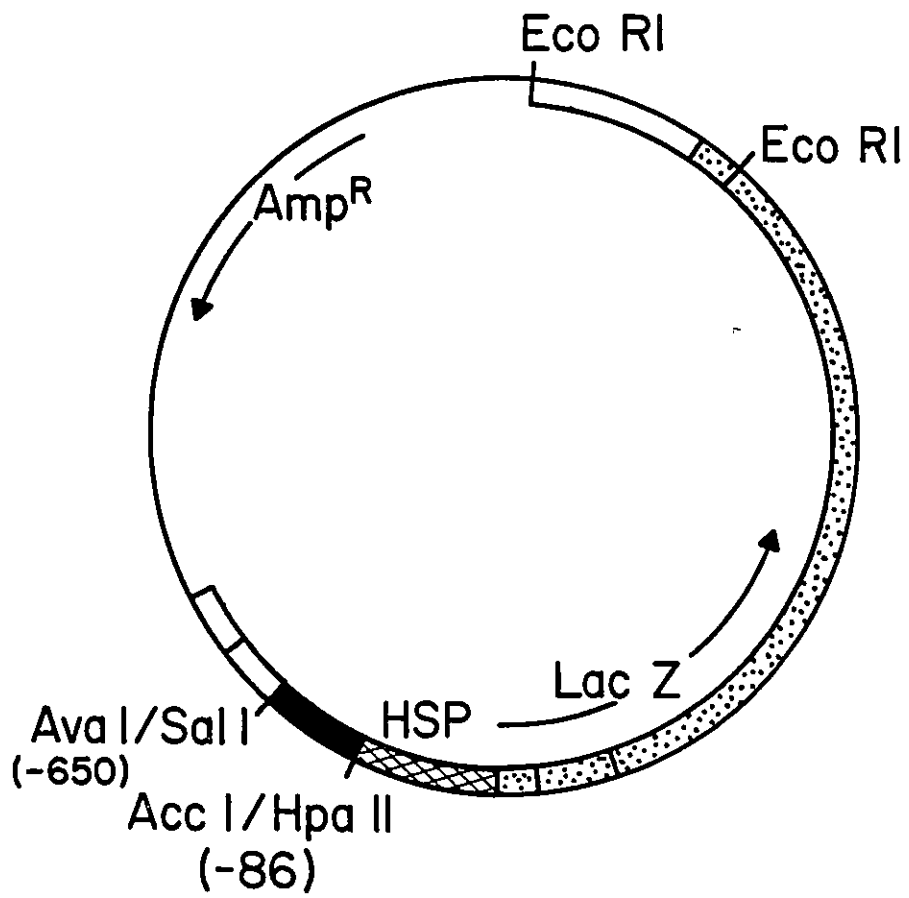
The Recombinants p*x*/B ActlacZ. These deletion plasmids consist of the actin promoter region between $-x$ and $+6$, subcloned upstream of the reporter gene lac Z. The constructs were prepared by digesting the linker mutants pM*x* ActlacZ or pH*x* ActlacZ with Sal I and Xho I, and self-ligating the plasmid, thus deleting the actin sequences 5' to the linker site at position $-x$ upstream of the cap site. The various deletion plasmids are listed in appendix A. The legend for the map of the chimeric genes is as described for pP/B ActlacZ.

px/B Act lac Z
(7.3 to 7.7 kb)



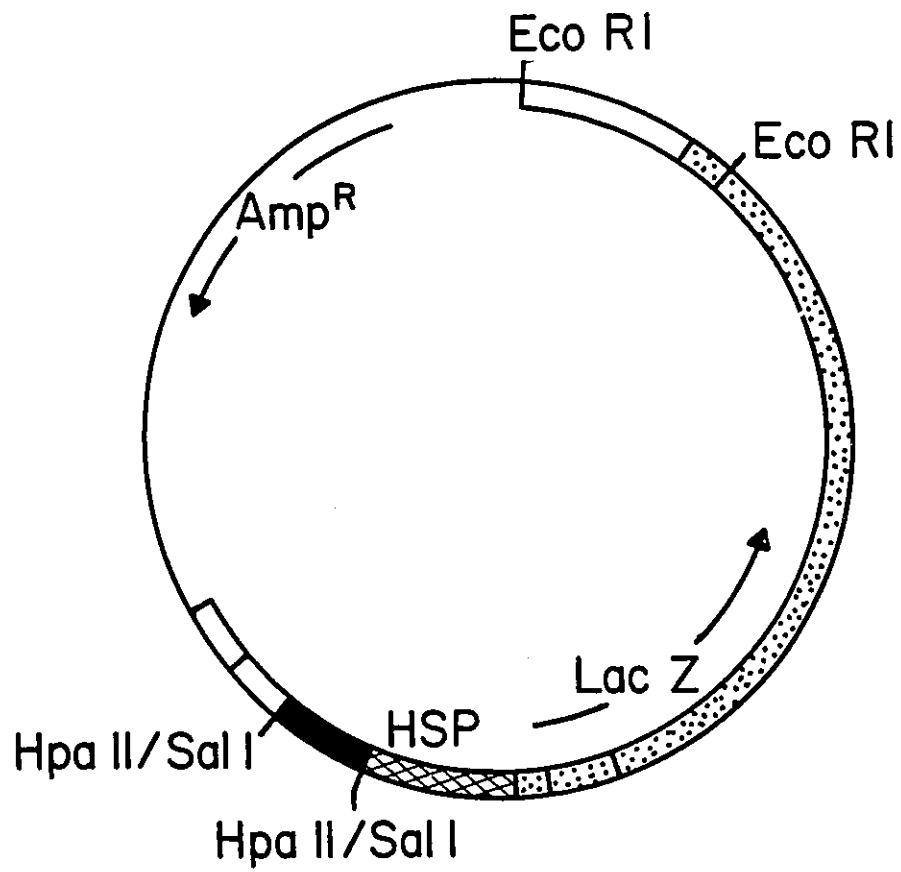
The Recombinants pAE and pAEinv. These plasmids consist of the actin *Ava*I-*Hpa* II fragment from -650 to -86 subcloned upstream of the mouse heat-shock protein 68 promoter, which directs the transcription of *lac Z*. To produce these chimeric genes, the actin fragment was isolated from pGEM VII (J. Abrahamson, University of Ottawa) by digesting with *Ava* I and blunt-ending. This fragment was ligated to the *Sal* I-digested and blunt-ended pCH126PHS (Kothary et al., 1989). The filled, hatched and stippled boxes in the figure represent respectively the actin upstream region, the heat-shock promoter and the *lac Z* reporter gene. The open boxes include SV40 polyadenylation sequences. The orientation of the actin fragment in pAE follows the direction of transcription of *lac Z* as indicated by the arrow. pAEinv is identical to pAE except for the opposite orientation of the actin insert.

pAE
(8.6 kb)



The Recombinants pINT and pINT_{inv}. These plasmids contain the actin first intron subcloned upstream of the mouse heat-shock protein 68 promoter, directing the transcription of lac Z. The constructs were prepared by digesting with Hpa II and blunt-ending the 0.7 kb actin first intron sequence from pATA15 (M. Ruben and M. Rudnicki, University of Ottawa). This fragment was ligated to the Sal I-digested and blunt-ended pCH126PHS. The filled, hatched and stippled boxes in the figure represent respectively the actin first intron, the heat-shock promoter and the lac Z reporter gene. The open boxes include SV40 polyadenylation sequences. The orientation of the actin fragment in pINT, follows the direction of transcription of lac Z as indicated in the figure. pINT_{inv} is identical to pINT except in the orientation of the actin first intron.

pINT
(8.9 kb)



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