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Developmental Genetics and Somatic Differentiation of the Muscle Tissue

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Introduction

One of the most challenging problems of modern biology is to understand the development of higher organisms at a cellular and molecular level. Multicellular organisms comprise a large variety of tissues and organs which are gradually formed following the act of fecondation according to a very precise program. This problem has been under consideration since the 18th century, but even after the discovery of germinal cells (oocytes and spermatozoa) and the pioneering observations made by the 19th century embryologists concerning the major events accompanying the division of the egg, as well as the morphogenetic arrangements of the embryo and the foetus, many fantasmagoric explanations still prevailed for a long time regarding the reproduction of individuals (for example, the preformation theory).

Schematically, the problem of development can be tackled from two general angles:

— one, is to look at it from the standpoint of the genetic determinism underlying the developmental program.

— the other, is to focus on environmental factors as triggers and controlling devices of this program.

As far as the deterministic approach is concerned (namely what part in the development of higher organisms is hereditarily predetermined) one can list such classical achievements as the first description of spermatozoa by Anton van Leeuwenhoek, the early attempts to demonstrate the role of gametes in reproduction (ex. Spallanzani's experiments) and the historical saga leading from the Mendel's laws to the molecular biology of the gene and from these on to the contemporary studies on developmental genes whose action in pattern formation is already described by Dr L Wolpert at this Symposium.

But it is clear that in parallel to the logics arising from the study of genetic programs, there is another School of thought among developmental biologists which places a major emphasis on local or environmental signals and on signal-receptor interactions. It is nonetheless obvious that a comprehensive study of development requires that the problem be examined from the two complementary angles.

The muscle system is particularly suitable for this. It is one on which work from our laboratory has been at focus for almost two decades, and although the present report will more specifically address the genetic control of myogenic development, it is well known that many exogenous factors (hormonal, neural or even mechanical) also exert a major influence on the developmental fate of the skeletal muscle, not to forget the major role played by positional information at early embryonic stages.

The Muscle Model

The morphology and physiology of skeletal muscles is familiar to all of us. It is a tissue, made of the joining of many elongated subelements, the "myofibers" (Mf) which owe their contracile properties to the presence of a physically convertible network, the sarcomeric apparatus, whose major framework subunits are fibrous proteins, the main representatives of which are myosins, actins, tropomyosins and troponins. Contractility lies upon the properties of the myosin heavy chains whose globular heads behave like mechano-enzyme when combined to the actin filaments in the presence of Ca^{++} ions.

A simplified scheme illustrating how a muscle tissue is formed is depicted on figure 1. Skeletal muscles originate at least in some animals from a special embryonic territory, the para-axial mesoderm (Ms) a multipotent tissue which itself derives from the ectodermal layer following the interaction of the cap cells from the animal pole with cells from the vegetative pole a/ process particularly well studied in *Xenopus* embryos and involving FGF, activins and during which certain genes, such as "brachyury", become activated. From the para-axial mesoderm some clusters of cells, the *somites*, begin to form and it is from them that the first real precursors of the muscle, the myoblasts (Mb) do appear. The somites undergo various rearrangements and are the site of many cell migratory events, their differentiation proceeding according to a rostro-caudal gradients and giving rise successively to various compartments: the *sclerotome* (which will ultimately convert to bones and cartilages) plus the *dermo-myotome*. The latter will then generate two further mesodermal territories: the *dermatome* (skin, epidermis) and the *myotome*. Axial / or trunk muscle) precursors form inside the somites from the myotome, whereas limb muscles derive from a category of myoblasts which migrate away from the dermomyotome and colonize the limb buds. Myoblasts look, morphologically speaking like spindle-shaped cells with a large nucleus (figure 2). They divide rapidly but display no muscle characteristics: it is only after some time that the overt differentiation begins, myoblasts stop dividing, fuse their cytoplasm to form multinucleate structures, the myotubes (Mt) within which contractile proteins accumulate. Myotubes will then enter a long-lasting process of maturation involving hormonal and neural controls. The conversion of Mb to mature Mf can be observed in tissue culture *in vitro* which makes the muscle system one of choice to tackle various aspects of somatic differentiation:

As shown in this diagram, myogenesis can arbitrarily be subdivided into three main stages:

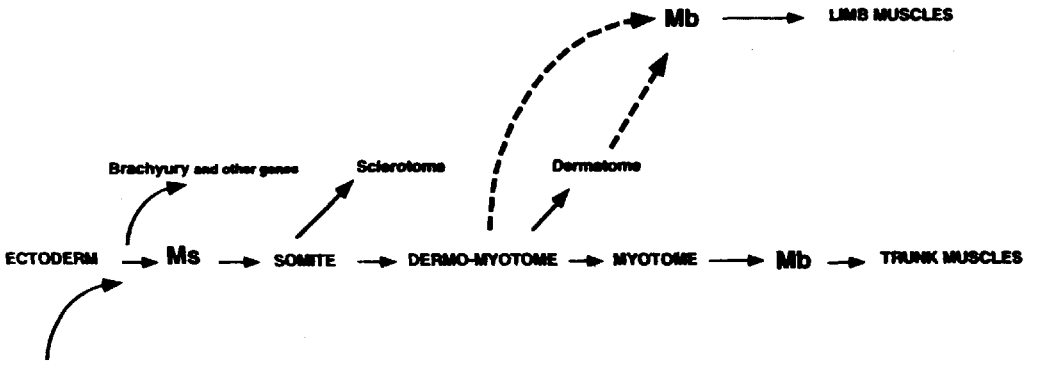

Figure 1. embryonic origin of skeletal muscles (see text for details)
Ms: para-axial mesoderm
Mb: myoblas (undifferentiated muscle cells)

(i) *determination*: during this stage myoblasts do form from the mesoderm and acquire the potentiality to differentiate into muscles

(ii) *differentiation*: during which Mb express the muscle specific program (i.e. begin to undergo morphogenetic changes, synthesize various muscle proteins, etc.)

(iii) *maturation*, or terminal morphogenesis

This apparently simple cascade does in fact involve the sequential activation of a plurality of genes and the synthesis of a large number of proteins plus many morphological rearrangements.

Therefore, the question, we and others have attempted to address is the following. Is this morphological and biochemical cascade controlled by special regulatory genes, and, if so, what is the nature of these genes?

As will be seen from the present report, a new category of regulatory genes has precisely been shown to command the muscle developmental pathway and it is on their mode of action that I wish to expand myself, in this lecture.

Myogenic Regulatory Genes—Main Characteristics

In 1987, Weintraub and his colleagues (Davis et al. 1987) succeeded in doing what proved to be a key experiment for it paved the way for the discovery of a family of master regulatory genes involved in the control of myogenesis. Taylor and Jones 1979 had shown that when a special pluripotent, mesoderm-like 10T1/2 cell line, is cultivated for several generations in the presence of 5-Azacytidine (a cytidine analog) this treatment considerably increases the rate of conversion of these cells into myoblasts (figure 3). From these 5-azacytidine-derived myoblasts Lassar and Weintraub (Davis et al. 1987) succeeded in cloning a gene whose complementary DNA, once recombined to a constitutive promoter and transferred into the precursor cells, was able to convert these cells into myoblasts with great efficiency. This was interpreted as the first characterization of a regulatory gene responsible for somatic determination. The name MyoD-1 (for myogenic determination) was onined and it was initially

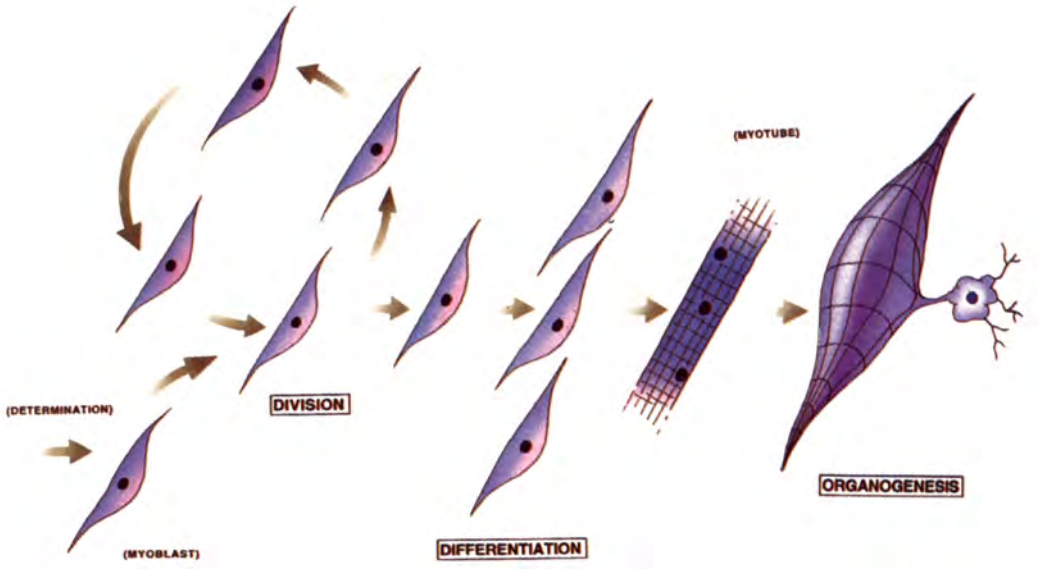

Figure 2. Scheme of muscle formation from dividing myoblasts to innervated muscle

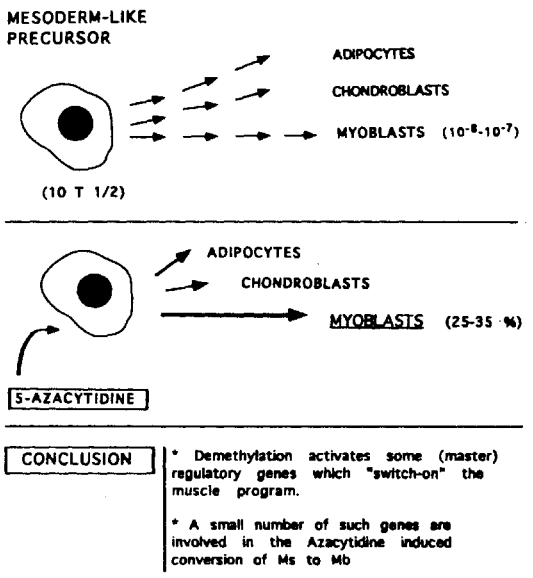

Figure 3. Myogenic conversion of mesoderm like precursor cells of the C3H10t1/2 cell line
Top panel: 10-8-10-7 refers the frequency of spontaneous conversion
Lower panel (25-35 %) : percentage of cells converted to the myogenic lineage following 5-azacytidine treatment

believed that the MyoD gene was the key gene whose activity can positively commit mesodermal precursors to enter the myogenic lineage thus behaving as a unique master gene. Later on, however 3 genes, with properties similar to those of MyoD, were characterized by other groups. At present, it is well established that myogenesis, in its early developmental stages, is under the control of a family of regulatory genes icluding, in addition to MyoD, genes which have been designated as: Myogenin, Myf5, MRF4 (Herçulin or Myf6) (Wright et al. 1989, Braun et al. 1989, 1990, Miner & Wold 1990, Rhodes & Konieczny 1989).

The discovery of these myogenic regulatory genes allows one to address the mechanism of muscle differentiation in more precise molecular terms.

Function of Myogenic Regulatory Genes

These genes behave like positive controlling devices. They code for regulatory factors which, by combining to a cis-sequence lying upstream of the promoters of muscle specific genes cause their trans-activation.

Of particular significance is the fact that the trans-activating factors do not bind by themselves to target sequences of mucle structural genes. They do so after combining with a ubiquitous factor, E_{12} (or E_{47}). It is the heterodimer which displays the capacity to bind, with great affinity to the muscle specific, cis-acting element. This special element comprises the sequence CANNTG (or E-box) (where N can be any of the 4 possible bases). Hence heterodimerization with E_{12} is a prerequisite to the function of myogenic regulatory genes. The clue to this type of interaction is found in the structures of the myogenic regulatory proteins themselves.

Accordingly, each of these proteins includes an N-proximal, acidic domain plus a basic region, as well as a special "motif" characterized by the existence of two α-helices joined by a non-helical, or loop region (this motif is usually referred to as HLH). C-terminal sequences differ in terms of length and composition with each protein of the family. The role of the HLH motif is essential for the activity of the regulatory factor,for it enables the protein to assemble with another HLH containing protein thus permitting heterodimerization, a phenomenon which increases binding affinity to the E-box, more than 50 fold. To complete this picture of myogenic regulatory proteins, it should be pointed out that some HLH containing factors can function like inhibitors of DNA binding rather than co-activators. Such is the case for example of Id (inhibitor of DNA binding) a protein described by Benezra et al. (Benezra et al. 1990) which can form an heterodimer with E_{12}, but, cannot attach to DNA, because it lacks a basic domain: as a consequence, E_{12} can exist, under at least three possible forms within the cell:

(i) free of all combinations
(ii) complexed with Id and thus inactive
(iii) complexed with any one of the 4 possi-

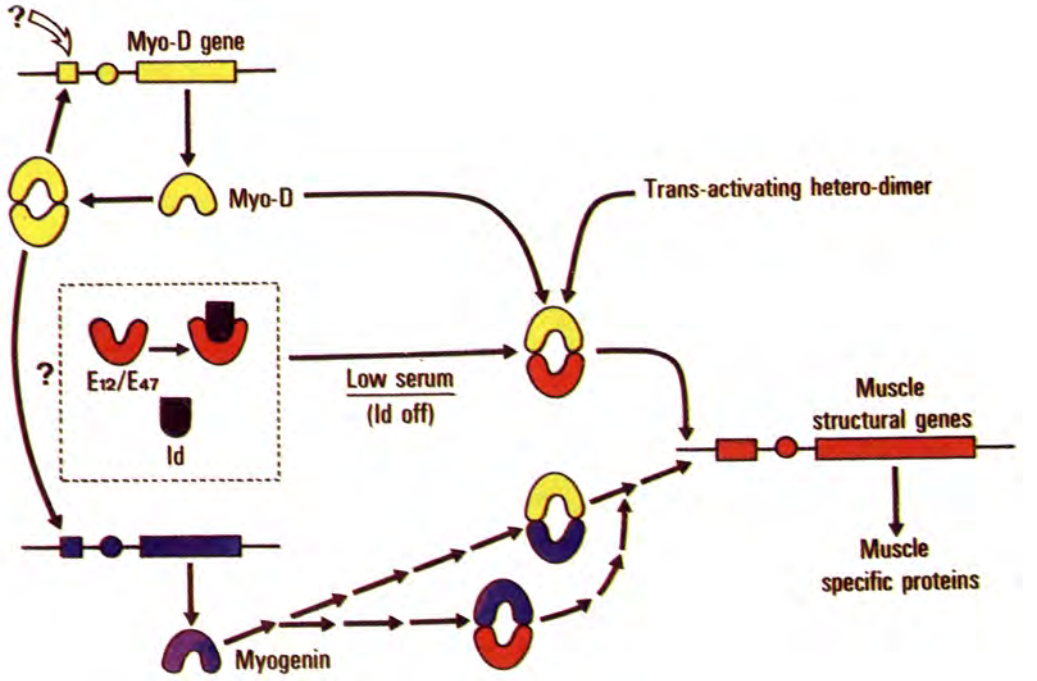

Figure 4. Transcriptional control of myogenesis of by MyoD and Myogenin (see text for details)

ble regulatory factors. From these considerations there is general agreement to believe that the following series of events is likely to occur when a myoblast differentiates into a myotube (figure 4).

At the myoblastic stage, E_{12}, would be requested under the form of an inactive complex with protein Id. The complex thus formed does not bind to DNA and most muscle specific genes remain inactive*.

When myoblasts stop dividing and enter a G1 phase, Id ceases to be formed, E_{12} becomes available to form a complex with MyoD and the MyoD-E_{12} dimer can attach to the E box, causing stimulated transcription of the corresponding gene.

Although this scheme accounts for the main genetic regulatory events during myogenesis, it only does so in a first approximation. Actually, the cascade involved in the developmental transition between actively dividing (and undifferentiated) and post-mitotic (differentiating) myoblasts is somewhat more complex and deserves the following comments (figure 5).

As long as mitogens (growth factors) are present in the medium, Mb proliferate but do not differentiate. This means that myogenic regulatory factors either do not function (i.e. cannot trans-activate muscle structural genes) or that their synthesis is impaired. The fact that myogenic factors do not function, is not only due to the sequestering effect of Id upon E_{12}. Other mechanisms of inhibition have also been reported in the literature which involve the action of various cellular oncogenes. Without going into details, figure 5 sums up many of the possible pathways according to which myogenic regulatory proteins are maintained in a state of transient blockade during the division of muscle prec-

*With the exception of a few genes like those encoding desmin (Li & Paulin 1993), or β-enolase (Lamande et al. 1989) whose activation does not involve interaction of myogenic factors with the Ebox at least in dividing myoblasts.

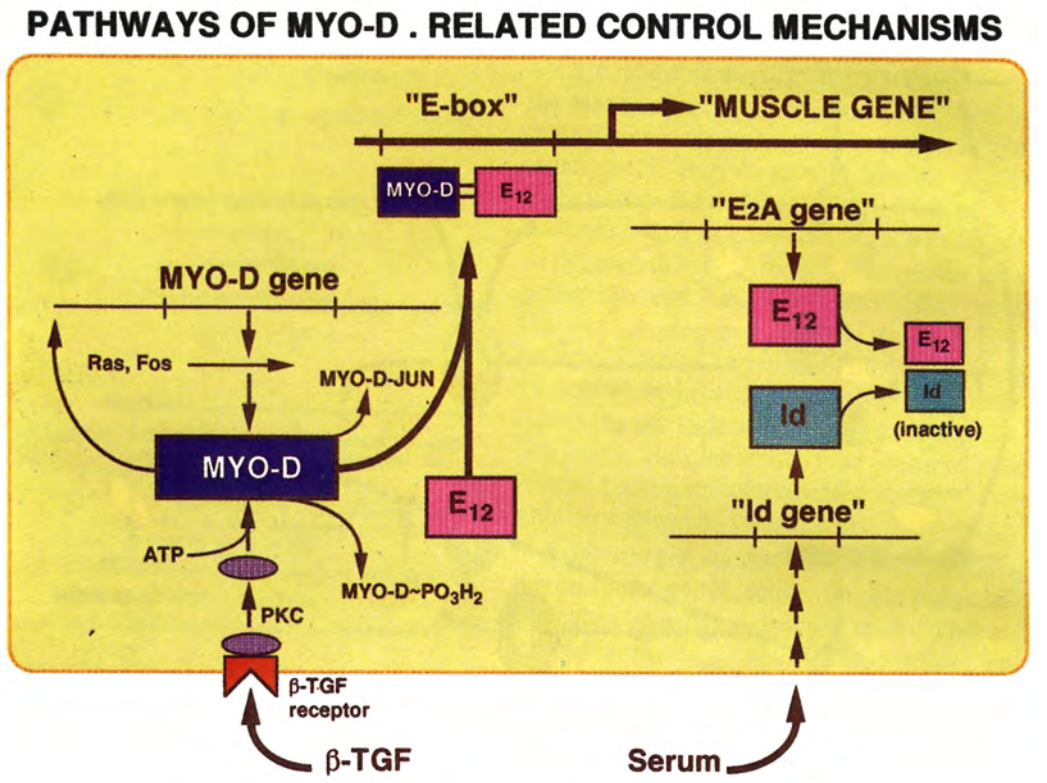

Figure 5. Pathways of MyoD. Related control mechanisms

ursor cells, a situation which is reverted when the exogenous level of growth factors decreases below a certain threshold. What is probably the most significant generalization to be drawn from these considerations is that, in eukaryotic cells, regulatory proteins can enter a large number of combinatorials, each one endowed with a rather strict specificity, a situation which is likely the result of evolution.

The Polymorphism of Myogenic Factors

An obvious question arises from the very existence of 4 myogenic regulatory genes. Are we dealing here with a purely gratuitous situation each factor being able to substitute the three others or does each myogenic factor exhibit a specificity vis-a-vis the target structural genes? To summarize (figure 6), although they all form dimers with E12 and bind to the E box, the HLH myogenic factors harbour some specificity in their mode of action: they do not stimulate the same groups of genes with the same efficiency and, thus, control different stages of myogenesis. For example, many arguments (Ott et al. 1991) (Montarras et al. 1991) support the view that, in mammals, Myf5 plays a special role at the onset of determination, being expressed just prior to the formation of Mb (in the dermomyotome) (Sassoon et al. 1989). MyoD is expressed at later stages within newly formed myoblasts. MRF4 is considered as a regulator of late developmental sequences of myogenesis (maturation?), at least, in the differentiation of limb muscles (the situation with trunk muscles being probably more complex (Bober et al. 1991, Hinterberger et al. 1991). Also, it is

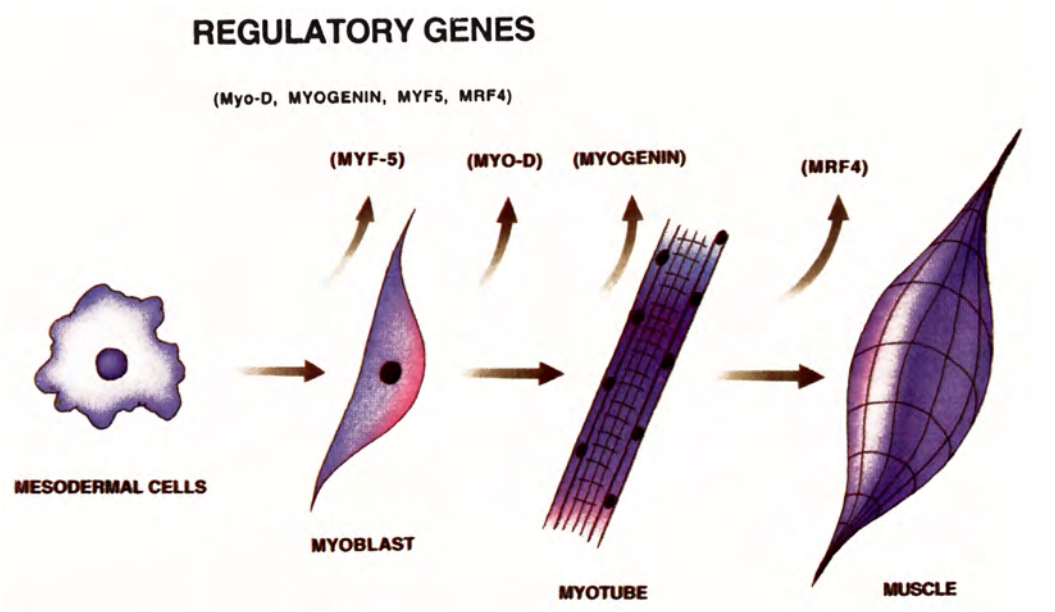

Figure 6. Developmental patterns in the expression of Myf5, MyoD, Myogenin and MRF4 during myogenesis

well established that Myf5 and MyoD expression are antagonistic. A high level of Myf5 expression is incompatible with a high level of MyoD expression and vice versa.

What Triggers the Activity of Myogenic Regulatory Genes?

One of the most intriguing question in developmental biology concerns the nature of the mechanisms which turn on the regulatory genes themselves. We have just seen that MyoD and MyoD-related genes behave like "master regulatory genes": they trigger the expression of most of the structural genes whose activity is characteristic of a muscle phenotype. But, however, important their role might be to determine the choice of mesodermal precursors when they enter the myogenic lineage, these genes are not functioning at all stage and some adequate signal might activate them at an early phase of development. It is unlikely that this signal would be associated with the activity of other regulatory genes, otherwise one would be dealing with an endless upstream cascade...

The same way as mitogenic factors can block the synthesis or activity of MyoD and other myogenic regulatory genes, some factors of general physiological relevance should exit which cause the myogenic regulatory genes to become active. Although it is likely that we are dealing here with a rather complex situation, involving various factors and including diffusible elements liberated through positional information, etc., results from our laboratory have recently shed light on the role of IGFs, the insulin growth factors as positive modulators of the myogenic pathway.

Possible Involvement of IGF

Florini and his coworkers were among the first authors who succeeded in showing that insulin-like growth factors (IGFs) although known as active mitogenic agents, behave like potent stimulators of myogenic differen-

tiation (Florini et al. 1991). This positive effect occurs via an autocrine secretion of these growth factors by myoblasts following transfer to a low serum "differentiation medium". The rate of spontaneous differentiation in several sublines of myogenic cells correlates with their level of expression of IGF-II. Addition of an antisense oligo-deoxyribonucleotide complementary to some codons of IGF-II inhibited myogenic differentiation in the absence but not in the presence of exogenous IGF-II. Finally, the authors gave suggestive evidence for an involvement of myogenin in the spontaneous (autocrine secretion mediated) differentiation.

In our laboratory the involvement of IGF in myogenic differentiation has been extensively reinvestigated. A first evidence came from the isolation in our laboratory of a myoblastic variant derived from the classical C2 line (Yaffé & Saxel 1977). In this variant the presence of IGF in the medium is required to enter differentiation since in the absence of exogenous IGF, C2 cells grow indefinitely as Mb. This requirement was by-passed when variant cells are stably transferred with MyoD-cDNAs (Montarras et al. 1989, 1991) suggesting that IGF was directly or indirectly involved in turning on the activity of MyoD and possibly other myogenic regulatory genes.

The second argument was based upon the use of antisense RNA to block the synthesis of IGF I or II, a situation resulting in an inhibition of muscle differentiation.

To allow for the accumulation of large amounts of anti-IGF messenger transcripts we have made use of an appropriate plasmid recombinant which was constructed by Prof. J. Ilan (Cleveland University) (Trojan et al. 1992). In this construct, the IGF-cDNA is placed, in an opposite orientation, in front of a metallothionine promoter such, that in the presence of an appropriate metallic inducer the transformed cells (which in most cases have integrated the plasmid) accumulate the IGF-anti-messenger.

Induction of the metallothionin promoter by Zn^{++} causes effective disappearance not only of endogenously made IGF mRNA but also of MyoD mRNA in a C2 mouse myoblastic cell line which synthesizes both IGF II

Figure 7. RNA blot analysis of the IGFII mRNA in proliferating cells

C2 cells were transfected by electroporation with 20 ug of a plasmid generating no antisense RNA (control cells) or 20 ug of the plasmid generating IGFI antisense RNA (anti I cells). Stably transfected cells were selected on the basis of their resistance to hygromycin. Total cellular RNAs from proliferating cells were prepared and analysed. In each case, 10 ug of total RNA were probed as indicated.

and MyoD constitutively. By contrast, and probably as a result of the antagonism between MyoD and Myf5 expression which we have already alluded to, anti IGF treated cells display considerable increase in the level of Myf5 transcript (Montarras et al. 1993) (figure 7).

These plasmid treated myoblasts with low MyoD and high Myf5 transcripts fail to differentiate. For example they do not express a muscle specific marker such as MLC2 and lack the capacity to form myotubes as probed by phase contrast examination or immuno-cyto-chemistry using an anti-Troponin T antiserum (Montarras et al. 1993, data not shown).

This inhibition is reversible since myogenic differentiation (formation of Mt) is restored upon addition of Insulin or IGF at low concentration. In recent experiments it was shown that IGF-mediated reversion of antisense inhibited Mb does not occur in the presence of cycloheximide, which suggests an indirect effect of IGF on myogenesis (Montarras & Pinset, unpublished observation). To account for the previous observations two alternative mechanisms are proposed:

IGF II whose synthesis is known to be stimulated many fold at the onset of myogenesis, for example when the dermomyotome begins to form (Lee et al. 1990) would indirectly trigger an increased synthesis of MyoD in a cell which is already precommitted by Myf5. This IGF dependent stimulation of MyoD would cause a reduction of Myf5 (since expression of the two regulatory genes is known to be antagonistic) below a threshold compatible with the arrest of division and the onset of myogenesis. An alternative model would imply that everything is under the control of mitogenic factors (here FGF is taken as an example). As long as the growth factor is present at high levels in the medium Myf5 expression remains high and this prevents the synthesis of IGF. The decrease of mitogens accompanying entry into G1 would cause both a large decrease in the level of Myf5 and a large increase in IGF synthesis and hence in MyoD production.

These hypotheses are currently under investigation. Preliminary observations have shown that transferring myoblasts (in which MyoD synthesis is blocked by anti-MyoD transcripts) from a high to low serum containing medium causes a rapid down-shift in the level of Myf5 expression supporting part of model II.

However, complicated the regulatory cascade might look, it is clear that myogenesis offers developmental biologists a model to approach the mechanisms of tissue determination at a molecular level and this might have much bearing on the strategy to address the pathology of neuromuscular diseases.

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