deleterious effects on the phagocytosis or oxygen burst activities, the morphology, the adherence to glass, or the density of cells within monolayers of either infected or uninfected macrophages. Since clomipramine is a lipophilic compound, it would most likely be absorbed through the macrophage surface membrane, and thus the values given in Fig. 1 probably represent an underestimation of its effective intracellular cidal concentrations.

Clomipramine appears to be more toxic to *L. donovani* amastigotes within macrophages than to extracellular promastigotes ($LD_{50} = 0.6 \pm 0.08$ and 2.3 ± 0.35 μM, respectively) (Fig. 1). Since clomipramine uncouples oxidative phosphorylation in mammalian mitochondria (*6*), it is possible that this drug also disrupts the $\Delta\mu_{H^+}$ across the surface membrane of *L. donovani*. As a consequence, both promastigotes and amastigotes would cease to maintain *p*H homeostasis as well as the $\Delta\mu_{H^+}$-driven processes (for example, active transport of solutes such as L-proline and D-glucose), which would result in cell death. Further, amastigotes that reside in the extremely acidic environment of macrophage secondary lysosomes, would be rendered more sensitive to the effects of clomipramine than would promastigotes.

Imipramine (≤ 50 μM) had essentially no influence on proline transport of *L. donovani* promastigotes (Table 3). Clomipramine, however, at low concentrations such as 10 μM (20 pmol per 1×10^6 cells) inhibited the transport of proline by 28 percent, whereas at 100 μM (0.20 nmol per 1×10^6 cells), transport was almost completely inhibited. Clomipramine is a more potent inhibitor of serotonin transport (*11*) and oxidative phosphorylation (*6*) than is imipramine. The ionophores valinomycin and nigericin were used to assess whether clomipramine inhibited L-proline transport by uncoupling the $\Delta\mu_{H^+}$ (Table 3). Addition of either valinomycin (0.5 μM) or nigericin (2 μM) to promastigotes resulted in partial inhibition of L-proline transport, which was related to the effect of valinomycin on the membrane potential ($\Delta\psi$) and of nigericin on the *p*H gradient (ΔpH), (*3*). However, concomitant addition of nigericin (2 μM) and clomipramine (10 μM) resulted in a synergistic effect on the inhibition of proline transport. A similar significant synergistic inhibition of L-proline transport (70 percent) was observed when valinomycin (0.5 μM) and clomipramine (10 μM) were used together. These results suggest that clomipramine affects both $\Delta\psi$ and ΔpH. However, it remains to be determined whether clomipramine affects $\Delta\mu_{H^+}$ by inhibiting a surface membrane proton pump (for example, H^+ adenosinetriphosphatase), or by having an ionophore-like characteristic.

One of the objectives in the design of chemotherapeutic agents is to take advantage of physiologic differences between an infectious organism and its mammalian host. The current results with *L. donovani* have demonstrated that this approach is feasible. As we suggested (*3*), energy transduction processes similar to those of *L. donovani* may also occur in various other parasitic protozoa. Therefore, clomipramine and compounds with a similar mode of action might prove useful as antiparasitic agents.

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4 April 1984; accepted 27 July 1984

A Single Troponin T Gene Regulated by Different Programs in Cardiac and Skeletal Muscle Development

Abstract. *A cloned complementary DNA derived from a messenger RNA transiently present at low abundance levels in early chick embryonic skeletal muscle hybridizes to a messenger RNA present at high abundance levels in cardiac muscle. Genomic DNA hybridization and nucleotide sequence identity of complementary DNA's from both heart and skeletal muscle demonstrate that the messenger RNA's from both sources are encoded by the same gene. The encoded polypeptide is a troponin T sequence which is probably a cardiac isoform. This single copy troponin T isogene is governed by different regulatory programs in heart and skeletal muscle differentiation.*

The contractile proteins of muscle are members of large families of related isoproteins. In some cases the different members of an isoprotein family are so closely related that amino acid sequences differ in only small regions. For example, within the six-member actin protein family found in birds and mammals there are only 27 amino acid differences between the most widely diverged members, and the difference between muscle isoforms of actin can be due to as few as three amino acids (*1*). Despite this similarity in primary structure, each member of a contractile protein family is expressed in a highly tissue-specific, or developmental stage-specific, manner. During skeletal muscle differentiation, both the light chains (*2*) and heavy chains (*3*) of myosin as well as other muscle proteins (*4*) undergo complex, stage-specific programs of isoform switching. Thus, the expression of contractile protein isoforms appears to be precisely regulated and is often tightly coupled to specific developmental stages. Many of these switches occur within single muscle fibers (*5*), which presents an interesting problem in understanding the assembly and turnover of the myofibrillar apparatus.

Caplan *et al.* (*6*) have argued that isoprotein switching during muscle development is functionally analogous to globin isoform switching and that each successive isoprotein provides a slightly altered function which is essential to the developmental progression of muscle. While no obvious functional differences have been detected between closely related contractile protein isoforms (*7*), there may be subtle differences which have not been discerned. An alternative hypothesis would place the significance of isoformic diversity at the level of the gene rather than at the level of the protein (*8*). In this model, duplication of contractile protein genes allows them to

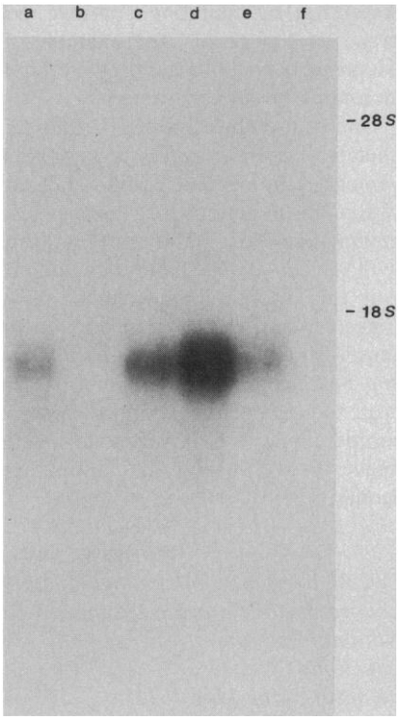

Fig. 1. Northern blot hybridization of 106A4. Each lane contains 2 μg polyadenylated [poly(A)] RNA separated on formaldehyde agarose gels and blotted onto Genescreen (New England Nuclear). RNA was extracted and sequences containing poly(A) selected as described (*13*). The 3′ untranslated cDNA insert (Hpa II fragment underlined in Fig. 2) was labeled by nick-translation and hybridized to the blot (*13*). (Lane a) Thigh muscle, day 10; (lane b) thigh muscle, day 18; (lane c) cardiac muscle, day 10; (lane d) cardiac muscle, day 18; (lane e) breast muscle, day 10; and (lane f) breast muscle, day 18. Days 10 and 18 represent development in ovo.

be placed under different developmental and tissue-specific regulatory programs. Divergence in isoform primary structure would be a secondary by-product of the duplication of the genes reflecting mutational drift.

Some contractile isoproteins previously thought to be cardiac-specific may be expressed as embryo-specific isoforms in skeletal muscle as suggested in rat myosin heavy chain (*9*), chick myosin light chain (*10*), and chick troponin T (*11, 12*). For each example, immunological or peptide mapping criteria show that the isoprotein present in adult heart is indistinguishable from that present transiently in embryonic skeletal muscle. In these cases a single gene appears to be governed by two different regulatory programs in different tissues and, therefore, gene duplication is not required to bring isoform genes under different regulatory programs.

In the above examples, it is possible that the proteins detected in adult heart and embryonic skeletal muscle are not identical but are the products of closely related but distinct genes because only the expressed proteins have been analyzed. Different isoforms of myosin heavy chain can share common antigenic determinants [Benfield *et al.*, in (*3*)]. There is no satisfactory way to rule out this possibility without directly determining that the same gene encodes the same protein in both tissues. In this report, we demonstrate that the gene encoding a heart troponin T isoform is also expressed transiently during the early stages of in vivo skeletal muscle development. Thus a single contractile protein isogene is governed by two different developmental regulatory programs in different tissues.

The complementary DNA (cDNA) clone 106A4 was isolated from chicken embryonic muscle polyadenylated RNA by a method designed to obtain cDNA clones of developmentally regulated, low-abundance messenger RNA's (mRNA's) (*13*). Briefly, a combination of colony hybridization and RNA blot hybridization was used to identify cDNA clones representing RNA's present at early but not late stages of leg muscle development. The clone 106A4 hybridizes to a message that is present at early stages of leg muscle development but is absent from limb mesenchyme progenitor cells and terminally differentiated leg muscle and nonmuscle tissue. Further RNA blot hybridizations (*14*) showed that the 106A4 mRNA is present in embryonic and adult heart. At day 10 in ovo, leg, breast, and heart muscle all contain low levels of the 106A4 mRNA (Fig. 1). At late embryonic stages (day 18 in ovo) the 106A4 mRNA disappears from leg and breast skeletal muscle but its relative abundance increases in heart muscle (Fig. 1). Dot blot analysis (*14*) indicated that by day 18 of embryonic development (3 days before hatching) the relative abundance of the 106A4 mRNA was reduced at least 10- to 20-fold in thigh muscle, reduced to undetectable levels in breast muscle, and induced 10- to 20-fold in cardiac muscle (data not shown).

Fig. 2. Nucleotide sequence of cardiac muscle 106A4 cDNA. Restriction endonuclease cleavage sites: P, Pst I; S, Sac I; H, Hind III; X, Xma III. The first codon of the open reading frame and the termination codon are marked by overbars and designated as 1 and t, respectively. The Hpa II 3′ untranslated fragment used as a hybridization probe is underscored. The T (thymine) of the putative poly(A) additional signal is at position 908. To isolate a cardiac muscle cDNA library, unlabeled oligo-dT (dT, deoxythymidylate) primed double-stranded cDNA was generated from day 18 heart muscle poly(A) RNA. Portions of the double-stranded cDNA were digested with restriction endonucleases that generate cohesive ends compatible with the single restriction sites of pBR322. The digested cDNA's were separated electrophoretically, blotted onto nitrocellulose, and probed with the 106A4 cDNA. It was determined that Hpa II generated a cDNA fragment of 700 base pairs (bp), which hybridized to the skeletal muscle cDNA. Hpa II-digested cardiac muscle cDNA was ligated into the Cla I site of pBR322. The resulting cDNA library produced seven clones which hybridized to the skeletal muscle 106A4 cDNA; six contained the 139-bp Hpa II fragment (underscored) while a seventh cDNA was a 697-bp Hpa II fragment overlapping and extending the skeletal muscle clone by 570 bp. To confirm the identity of the cardiac and skeletal cDNA's a second cDNA library was constructed by means of addition of synthetic linkers to double-stranded cDNA. Three of the 106A4-specific clones were selected from this library, and all matched the sequence of the clones obtained from the Hpa II library. All sequence determinations were done with the M 13 system (*21*).

Many muscle-specific genes are members of strongly conserved gene families and in some cases cDNA probes for one mRNA have been shown to cross-hybridize with mRNA's from related genes (for example, cardiac and skeletal muscle α-actin cDNA's will cross-hybridize). It is important, therefore, to determine if the mRNA's detected by 106A4 cDNA in heart and skeletal muscle are encoded by the same gene or by two different, but related, genes.

We first compared the nucleotide sequence of the cardiac and skeletal mRNA's by identifying and sequencing 106A4-specific cDNA's from a heart muscle cDNA library (Fig. 2). Cardiac 106A4 cDNA has a single open reading frame terminating at position 707 and a 3′ untranslated segment of 222 nucleotides (Fig. 2). The entire original skeletal muscle 106A4 cDNA sequence precisely matches the 3′ terminal 358 nucleotides (from positions 570 to 927) of the cardiac sequence. Six independently isolated cardiac cDNA's were sequenced to confirm the identity of the cardiac 106A4 mRNA with the skeletal muscle 106A4 mRNA. The sequence identity between the mRNA's from both tissues indicates that they are transcribed either from the same gene or from two different genes which are identical in the 45 codons which produce the COOH-terminal end of the protein and their entire 3′ untranslated regions. The latter possibility is unlikely because even closely related mRNA's, such as those encoding cardiac and skeletal α-actins, are divergent in their 3′ untranslated regions (see references in *12*).

To demonstrate unequivocally that the same gene encodes the 106A4 mRNA in both tissues we determined that there is only one 106A4 locus in the chicken genome by Southern blot hybridization (*15*). Chicken genomic DNA digested with restriction endonucleases that cleave outside the coding region was electrophoresed, blotted, and hybridized with a labeled cDNA fragment from the 3′ untranslated region (Fig. 3). In each case only a single hybridizing band was detected, as expected for a gene present once per haploid genome (*16*). This result, taken together with the identity of the 3′ untranslated regions of the cardiac and skeletal 106A4 mRNA's, demonstrates that a single gene encodes the mRNA from both sources.

The identification of the 106A4-encoded polypeptide as a troponin T isoform was made by translating the nucleotide sequence of the cDNA and comparing the resulting amino acid sequence with contractile protein sequences from the Dayhoff sequence bank (*17*). The open reading frame shown in Fig. 4 encodes a polypeptide whose amino acid sequence matches that of rabbit skeletal muscle troponin T (*18*) in 133 out of 236 amino acids (56 percent similarity). We therefore conclude that the 106A4 protein is a cardiac isoform of troponin T (*19*). Most of the amino acid identity between rabbit troponin T and the 106A4 protein occurs between amino acids 49 to 137 (88 residues) and amino acids 165 to 207 (42 residues) of the rabbit sequence. These regions have 77 and 90 percent similarity, respectively (Fig. 4). Such strong conservation of amino acid sequence not only identifies the 106A4 protein as troponin T, it also indicates regions of troponin T whose primary structure is potentially important for function in these systems. The differences observed may be due to evolutionary drift or may represent differences between heart and skeletal forms of troponin T.

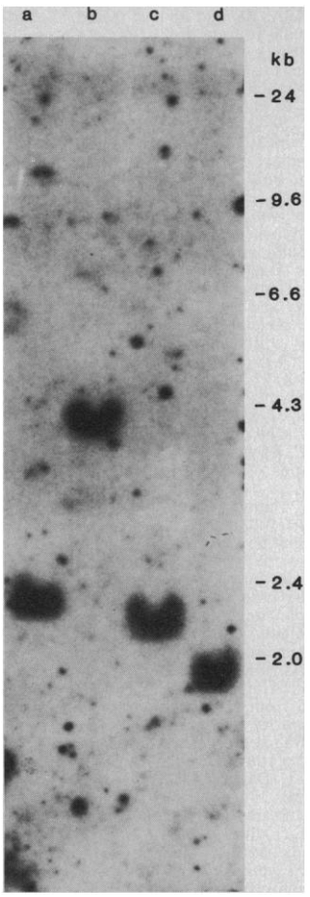

Fig. 3. Hybridization of cDNA 3′ untranslated probe to genomic blot. Each lane contains 10 μg of chicken genomic DNA digested with the indicated restriction enzyme and blotted onto nitrocellulose. Bound DNA was hybridized to the nick-translated 3′ untranslated fragment indicated in Fig. 2. This blot was deliberately overexposed to demonstrate that only a single band is detectable in each lane. (Lane a) Bam HI; (lane b) Hind III; (lane c) Pvu II; and (lane d) Sac I.

In skeletal muscle development the cardiac troponin T gene is first expressed at early stages when other skeletal muscle specific genes such as α-actin, creatine kinase, and myosin heavy chain are also expressed. However, the cardiac troponin T gene is down-regulated during late skeletal muscle development at the same time that the other contractile protein genes are strongly up-regulated. It seems likely, therefore, that the cardiac troponin T gene will share with skeletal muscle-specific genes some regulatory components involved with its initial turn-on in early skeletal muscle development but will have additional components required for its down-regulation at late developmental stages. Toyota and Shimada have recently shown that down-regulation of cardiac

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106A4 REQEPGEGESKPKPKPFMPNLVPPKIPDGERLDFDDIHRKRMEKDLNELQALIEAHFESRKKEEEELISLKDRIEQRRA
                          *  **** **  *****  **  *** ******  *** ********  **********
Troponin-T EPAPEHVVPEEVHEEEKPRKLTAPKIPEGEKVDFDDIQKKRQNKDLMELQALIDSHFEARKKEEEELVALKERIEKRRA
                                    |                                              |
                                   50                                            100

           ERAEQQRIRSEREKERQARMAEERARKEEEEARKKAEKEARKKKAFSNMLHFGG-YMQKSEKKGGKKQTEREKKKKILS
           ********* * * *** * *** ** *** *   **    **** * *      * *   * ***** ** ***** *
           ERAEQQRIRAEKERERQNRLAEEKARREEEDAKRRAEEDLKKKKALSSMGANYSSYLAKADQKRGKKQTAREMKKKILA
                                              |
                                             150

           ERRKPLNIDHLSEDKLRDKAKELWQTIRDLEAEKFDLQEKFKRQKYEINVLRNRVSDHQKVKGSKAARGKTMVGGRWK
           ***********  ********** *   **  **   ** ***** *   * **    *        *  ******
           ERRKPLNIDHLSDEKLRDKAKELWDTLYQLETDKFEFGEKLKRQKYDIMNVRARVEMLAKFSKKAGTTAKGKVGGRWK
                              |                                              |
                             200                                            250
```

Fig. 4. Comparison between rabbit skeletal muscle troponin T (*18*) and chicken 106A4 protein. The protein sequences are paired to produce the maximum number of matched residues. The 106A4 protein is the upper sequence. Note that the first residue of the 106A4 open reading frame corresponds to amino acid 24 of rabbit troponin T. The dash represents a gap introduced in the sequence of 106A4 to optimize homology. Asterisks indicate positions of homology. Abbreviations: A, alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

troponin T in embryonic skeletal muscle in vitro is under neurogenic control (*20*). It will be interesting to determine if the neurogenic regulation of cardiac troponin T occurs at the level of transcription.

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Modulation of the Metastatic Activity of Melanoma Cells by Laminin and Fibronectin

Abstract. *Metastatic mouse melanoma cells have a high affinity for the basement membrane and the ability to degrade it; these properties may allow tumor cells to invade the membrane and disseminate. In this study it was found that the metastatic potential of mouse melanoma cells varied when the cells were exposed in culture to fibronectin or laminin. After removal of fibronectin or exposure to laminin, the cells had an increased affinity for basement membrane collagen, were more invasive of basement membranes in vitro, and produced more lung colonies in vivo. These changes are correlated with and may be due to an increase in the laminin-binding capacity of the tumor cell surface.*

Metastasis is one of the major causes of mortality in cancer. The propensity to metastasize is found only in a minority of the cells in a malignant tumor (*1*). To metastasize, tumor cells must enter and then leave the circulatory system, evade host defenses, and proliferate in a secondary site. Lines of tumor cells with increased metastatic potential have been obtained in vivo by various techniques, including serial transfer of cells from metastatic lesions, cloning cells from metastatic lesions, and culturing the cells that penetrate the walls of isolated organs in vitro (*2*). The metastatic activity (*3*) and certain phenotypic characteristics, such as plasminogen activator (*4*), that are associated with invasiveness are reduced by culture in vitro and reexpress themselves when tumor cells are transferred to a host. Such findings indicate that the metastatic phenotype of tumor cells is genetically constituted but subject to modulation by as yet unidentified factors encountered in the host and in culture.

Histopathological studies indicate that metastatic cells are able to traverse basement membranes (*5*). This is not unexpected, since basement membranes represent the major physical barrier blocking passage of cells across blood vessel walls and movement of cells from one tissue compartment to another. Basement membranes are continuous extracellular matrices that separate epithelial cells from the underlying stroma. They are composed of several unique components, including type IV collagen; glycoproteins such as fibronectin, laminin, and entactin; and a heparan sulfate proteoglycan. Tumor cells will attach to a variety of substrates. However, metastatic cells show a higher affinity for type IV collagen than for other collagens using laminin as an attachment factor (*6, 7*). Highly malignant murine fibrosarcoma cells have increased amounts of laminin on their cell surface, whereas far less laminin is found on cells of low malignancy (*8*). This would favor binding of the cells to basement membranes. Furthermore, the metastatic tumor cells produce a collagenase that attacks type IV collagen, whereas nonmetastatic tumor cells do not (*9*). However, such activity is also found in normal cells that are able to traverse basement membranes (*10*). Local degradation of basement membranes is probably required for cells to cross these barriers.

Fibronectin and laminin are glycoproteins used by cells to bind to collagenous matrices (*7, 8, 11*). Metastatic tumor cells may be exposed to these proteins at various times during their transport in the body and during their colonization of target tissues. Fibronectin is present in serum and other body fluids (*12*) as well as in connective tissues, whereas laminin is found exclusively in basement membranes (*13*). Since both proteins influence the adhesion, growth, motility, and differentiation of cells (*11*), we examined the metastatic potential of mouse melanoma cells after culturing them in medium containing laminin or fibronectin. We found that fibronectin suppressed invasive activity, whereas laminin increased invasiveness. These changes were correlated with the ability of the cells to bind to and invade basement membranes in vitro and with their ability to bind laminin.