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Novel Muscle-Specific Enhancer Sequences Upstream of the Cardiac Actin Gene

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A DNase I-hypersensitive site analysis of the 5′-flanking region of the mouse α-cardiac actin gene with muscle cell lines derived from C3H mice shows the presence of two such sites, at about −5 and −7 kb. When tested for activity in cultured cells with homologous and heterologous promoters, both sequences act as muscle-specific enhancers. Transcription from the proximal promoter of the α-cardiac actin gene is increased 100-fold with either enhancer. The activity of the distal enhancer in C2/7 myotubes is confined to an 800-bp fragment, which contains multiple E boxes. In transfection assays, this sequence does not give detectable transactivation by any of the myogenic factors even though one of the E boxes is functionally important. Bandshift assays showed that MyoD and myogenin can bind to this E box. However, additional sequences are also required for activity. We conclude that in the case of this muscle enhancer, myogenic factors alone are not sufficient to activate transcription either directly via an E box or indirectly through activation of genes encoding other muscle factors. In BALB/c mice, in which cardiac actin mRNA levels are 8- to 10-fold lower, the α-cardiac actin locus is perturbed by a 9.5-kb insertion (I. Garner, A. J. Minty, S. Alonso, P. J. Barton, and M. E. Buckingham, EMBO J. 5:2559–2567, 1986). This is located at −6.5 kb, between the two enhancers. The insertion therefore distances the distal enhancer from the promoter and from the proximal enhancer of the bona fide cardiac actin gene, probably thus perturbing transcriptional activity.

When myoblasts fuse to form myotubes in culture, numerous muscle marker transcripts begin to appear (5), including those for α-cardiac actin, which accumulate rapidly (1). In the mouse embryo, as skeletal muscle first forms in the myotome, muscle-specific transcripts accumulate more asynchronously (4), but in this situation also, α-cardiac actin is one of the first muscle genes to be expressed (43). It is the major striated actin isoform in embryonic skeletal muscle, as well as in the heart, and is gradually replaced by α-skeletal actin as skeletal muscle matures (30). Nuclear run-on assays have shown that regulation of the α-cardiac actin gene is primarily transcriptional (9, 10).

Mainly as a result of experiments with skeletal muscle cells in culture, the initial stages of muscle gene activation have begun to be understood in molecular terms. Key regulatory sequences and, in a number of cases, the transcriptional factors which interact with them have been identified for many muscle genes (35). The E box motif (CANNTG, where N is any nucleotide), which is the binding site for the MyoD family (MyoD, myogenin, myf-5, and MRF4) of skeletal muscle-specific transcriptional factors (34, 48), together with A/T-rich consensus sequences, which bind MEF-2 (22) and MHox (12) proteins, which are abundant in striated muscle, are important in conferring muscle specificity to these genes. Other regulatory elements (8, 35) have also been implicated in the transcriptional activity of muscle genes, such as the serum response element or CArG box, which binds several ubiquitous proteins (47), and the MCAT box (28).

In the case of the α-cardiac actin gene, studies have concentrated on the proximal promoter region. Extensive work with the human promoter has focused on four CArG box elements present in this region (31, 32). The first 117 bp upstream from the transcription start site, which contains the first CArG box, are sufficient to confer muscle-specific expression on a heterologous reporter gene (41, 42). It has been shown that this depends on the presence of the CArG box (at −100), an SpI site (at −70), and an E box (at −50). The last is transactivated by MyoD. The proximal promoter region (−320) is also sufficient to direct muscle-specific expression of the mouse α-cardiac actin gene (14), and given the sequence conservation (18) with the human gene, it is very probable that the mouse promoter is also regulated by these sequences. However, our observations on the BALB/c inbred mouse line suggested that there might be another level of regulation operating on this gene (18, 19).

In BALB/c mice, the level of mature α-cardiac actin gene transcripts is reduced in both cardiac and skeletal muscles by 8- to 10-fold compared with that in C3H mice, for example, and this reduction is due mainly to an effect at the transcriptional level. The only apparent difference between the two loci is that there is a 9.5-kb insertion at 6.5 kb upstream of the cardiac actin gene in the genome of BALB/c mice. It is possible that this perturbs regulatory elements in the 5′-flanking region of the gene. We carried out a DNase I-hypersensitive site analysis in order to detect nucleosome-free chromatin regions, which are frequently associated with regulatory sequences (3). We demonstrate the presence of two such sites in the 5′ upstream region of the α-cardiac actin locus in muscle cells derived from C3H mice. This DNA was cloned, and the sites were shown, by functional analysis in cell cultures, to correspond to muscle-specific enhancer sequences situated just distal and proximal of the duplication insertion point in BALB/c mice. More extensive analysis of the distal enhancer demonstrates the presence of multiple E boxes, one of which is essential for enhancer function. There was no detectable transactivation of the en-

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hancer by the MyoD family of regulatory factors, even though MyoD and myogenin can bind to the functionally important E box. Other sequences are also required for enhancer function, and these are apparently activated by a mechanism which is independent of regulation by the MyoD family.

MATERIALS AND METHODS

Cell lines and cell culture. C2/7 is a skeletal muscle cell line subcloned from the original C2 cell line derived from adult C3H mice (7). P28 cells are derived from C3H mouse fetal muscle maintained in culture, after enrichment for myoblasts by dexamethasone treatment (14). J774 cells are a mouse macrophage cell line (38), and C3H10T1/2 cells are an embryonic mouse fibroblast line (39). L cells are a thymidine kinase-negative (TK^-) fibroblast-like cell line derived from mouse connective tissue (24). Cos cells are fibroblast-like cells derived from the CV1 monkey kidney cell line transformed by a simian virus 40 mutant (20). C2/7 and p28 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal calf serum (FCS) at low density and induced to differentiate with DMEM–2% FCS. C3H10T1/2 and L cells were grown in DMEM–10% FCS. Cos cells were grown in DMEM–7% FCS.

DNase I hypersensitivity experiments. Nuclei from p28 and C2/7 myoblasts and myotubes and J774 cells were prepared by the protocol of Mellon and Borghee (29) and digested with 0 to 50 U of DNase I (Boehringer) per mg of DNA for 10 min at 37°C. Digestion was almost total with 50 U of enzyme per mg of DNA (data not shown). DNA was extracted, digested with restriction enzymes, and blotted on Hybond N^+ (Amersham) by alkaline transfer. Hybridization was carried out at 65°C in 5× SSC–0.1% sodium dodecyl sulfate (SDS)–100 μg of salmon sperm DNA per ml–50 mM Na_2HPO_4 (pH 7.2)–5× Denhardt's solution with the probe described below (50× Denhardt's solution is 5 g of Ficoll 400, 5 g of polyvinylpyrrolidone, and 5 g of bovine serum albumin in 500 ml of distilled water; 1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Washes were carried out in 0.1× SSC–0.1% SDS at 60°C.

Probe. The probe used for the detection of hypersensitive sites and for the cloning of the corresponding sequences was a *Sau*3A-*Eco*RI fragment of 950 bp derived from clone λIG10, which was isolated from a library made with a *Sau*3A partial digest of BALB/c DNA (18) and corresponds to the −1630 to −680 region of the α-cardiac actin promoter. This probe hybridizes to a single 12-kb fragment in *Eco*RI-digested C3H DNA.

Construction of the C3H *Eco*RI library. C3H/Hej DNA (2 μg) was restricted with *Eco*RI and ligated for 2 h at room temperature with lambda phage EMBL4 digested with *Eco*RI and *Bam*HI (Stratagene). The ligated products were packaged by using Gigapack Gold (Stratagene). A total of 10^6 phage were plated on *Escherichia coli* NM646. Filters were hybridized with the *Sau*3A-*Eco*RI probe at 42°C in 50% formamide–0.1% SDS–5× SSC–5× Denhardt's solution–100 μg of salmon sperm DNA per ml. Positive clones were purified by several rounds of screening, and the DNA was isolated. The restriction map of one of these clones (λ5) was established by hybridizing partial digestions of the insert with the *Sau*3A-*Eco*RI probe.

Clonings. The vectors used for cloning are pBLCat2 (27), which contains the chloramphenicol acetyltransferase (CAT) coding sequence under the control of the thymidine kinase promoter, and pox, a pBLCat3 plasmid (27) in which the CAT coding sequence is under the control of the BALB/c α-cardiac actin promoter (−660 to +119). A partial *Xba*I digest of the λ5 clone was subcloned into pBLCat2 to give the pHSd2.5(+) and pHSd2.5(−) and the pHSp5.0 clones. A *Hin*dIII fragment was generated from pHSp5.0 after partial digestion with *Xba*I, restriction with *Hin*dIII and *Bgl*II, and religation to give a shorter sequence containing the proximal hypersensitive site (HSp). This was subcloned as pHSp1.9. Subfragments of these inserts were cloned into pBLCat2 and pox to give clones 1 to 16 B or P, respectively (see Fig. 5).

Transfection experiments. Cells were grown until about 30% confluent in 60-mm-diameter dishes (Falcon) in their respective growth media. Transfections were carried out by the calcium phosphate method (21). The medium was changed 2 to 4 h before transfection, and precipitates were allowed to form for 30 min at room temperature. Cells were transfected with 10 μg of a CAT reporter plasmid and 1 μg of a plasmid with the luciferase gene under the control of the Rous sarcoma virus promoter (RSV luciferase) (46) to normalize the transfection efficiency. Cells were incubated with the precipitate overnight and rinsed twice with DMEM on the following day. They were then fed with their respective growth media for 24 h for Cos cells, 10T1/2 cells, L cells, and C2/7 myoblasts or induced to differentiate over 48 to 72 h for C2/7 myotubes. Cells were then collected in 100 μl of a solution containing 40 mM Tris (pH 7.5), 150 mM NaCl, and 1 mM EDTA; 30% of the extract was used for the luciferase assay, and up to 30% was used for the CAT assay.

CAT assays. CAT assays were carried out by the butyryl coenzyme A method (44). The reaction took place in 100 μl of 250 mM Tris (pH 7.5) in the presence of 0.53 mM butyryl coenzyme A (Sigma B1508) and 0.04 μCi of [^{14}C]chloramphenicol (50 to 60 mCi/mmol; Amersham catalog no. CFA 754) for 1 h. Butyrylated products were extracted with 200 μl of tetramethylpentadecane-xylene (2:1), and the radioactivity in 170 μl of the organic phase was counted in organic counting scintillant (Amersham). Counts were normalized to the corresponding luciferase assay data to take into account the variability in transfection efficiency.

Luciferase assays. Samples were diluted to a final volume of 100 μl with LUC buffer (250 mM Tris phosphate [pH 7.5], 8 mM $MgCl_2$, 1 mM EDTA, 2 mM dithiothreitol), and luciferase activity was measured with a luminometer (LUMAT LB 9501; Berthold), which injects 100 μl of reaction mix (0.2 mM luciferine [sodium] and 4 mM ATP in LUC) into each sample.

Transactivation experiments. Each cotransfection was carried out with 13 μg of total DNA consisting of 1 μg of RSV luciferase vector (46), 10 μg of a CAT reporter construct, and 2 μg of the pEMSV αscribe expression vector with or without a myogenic factor sequence. The myogenic cDNAs cloned into this vector have been described before (6). 10T1/2 cells were transfected by the calcium phosphate method already described. After 24 h, cells were rinsed and cultured in DMEM–10% FCS for another 24 or 48 h before being collected.

Sequencing strategy. The version 2.0 sequencing kit (U.S. Biochemicals catalog no. 70770, Sequenase version 2.0) was used with either commercial primers (−40 or M13 reverse primer) or purified oligonucleotides complementary to the thymidine kinase promoter sequence or the enhancer sequence itself. Both strands were sequenced by using Bluescript and pBLCat2 plasmids containing the cloned insertions corresponding to clones 5, 8, and 16 shown in Fig. 5.

Mutagenesis experiments. Four single E boxes (numbers iv, v, vi, and vii) were mutated by using the Transformer site-directed mutagenesis kit (Clontech, Palo Alto, Calif.). For each E box, the CANNTG consensus was changed to GGNNCT, leaving the central two nucleotides unaltered. A single *Xba*I site in the wild-type plasmid was used for the selection of mutated plasmids according to the manufacturer's

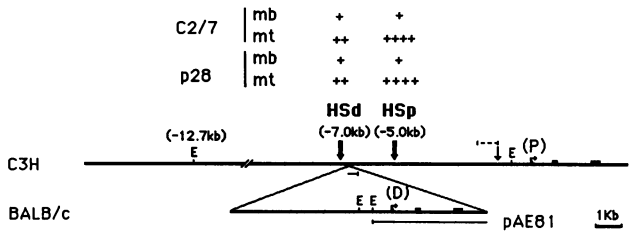


FIG. 1. 5′-Flanking region of the α-cardiac actin gene in C3H and BALB/c mice. The upstream region of the gene in C3H mice is shown, and the approximate position (−6.5 kb) of the partial duplication, which contains the promoter region and first three exons of the gene, in BALB/c mice is shown. Exons are shown as black boxes; transcription start sites for the duplicated (D) and normal (P) promoters are indicated with bent arrows. E, *Eco*RI restriction site. An additional *Eco*RI site present only in BALB/c DNA is marked (↓); the dashed line between brackets adjacent to the arrow indicates the *Sau*3A-*Eco*RI fragment used as a probe. HSd and HSp, distal and proximal hypersensitive sites, respectively. Above these sites, the relative intensities of the subbands generated by DNase I digestion in myoblasts (mb) or myotubes (mt) of the C2/7 and p28 muscle cell lines are indicated. ⊢ and ⊣ delineate the insert contained in pAE81 described in reference 16 which was used to generate the probe used for precise mapping of the insertion point.

instructions. A plasmid containing only this selection mutation (TCTAGA changed into ACTAGG) was used as a control in transfection assays.

Electrophoretic mobility shift assays. Myotube nuclear extracts were prepared as previously described (15). The double-stranded probes were as follows: E box iv, CATTCTTGGTT TCAGCTGTTAGCTAAAGATG; E box iv$^-$ (mutated E box iv), same as E box iv but with the E box (underlined) mutated to GGGCCT; and E box vi, CCTCCGTCCAGCAGCTGTA AGTAGATACAA. The binding buffer consisted of 50 mM Tris-HCl (pH 7.9), 60 mM KCl, 5 mM dithiothreitol, 4% polyethylene glycol 8000, 5% glycerol, 1 mM EDTA, and 100 μg of bovine serum albumin per ml. Standard reaction mixtures included about 2 μg of myotube extract, 20 pmol of labeled oligoprobe, and 2 μg of nonspecific competitor DNA [poly(dI-dC) · poly(dI-dC)]. Where indicated, 1 μl of serum or different amounts of competing oligonucleotide were added simultaneously with other reagents. Incubation was performed for 20 min at room temperature in a final volume of 16 μl. Samples were then run at 120 V on a native 5% polyacrylamide gel with 0.5× TBE (10× TBE is 0.9 M Tris base, 0.9 M borate, 20 mM EDTA [pH 8.3]). A MyoD polyclonal antibody was kindly provided by A. Lassar and H. Weintraub (45). Monoclonal antibodies for myogenin were the gift of W. Wright (51).

RESULTS

Identification of two DNase I-hypersensitive sites in the 5′ upstream region of the α-cardiac actin gene. The 5′-flanking region of the α-cardiac actin gene in C3H mice is shown in Fig. 1, and the insertion point for the partial duplication of the locus present in BALB/c mice is indicated (18). In order to look for DNase I-hypersensitive sites in the region of the duplication, we used as a probe a *Sau*3A-*Eco*RI fragment located at the distal end of a 12-kb *Eco*RI fragment, upstream of the proximal promoter of the gene (Fig. 1). Two muscle cell lines, C2/7 (7) and p28 (14), were used for these experiments. Both are derived from skeletal muscle of C3H mice and express the endogenous α-cardiac actin gene upon differentiation.

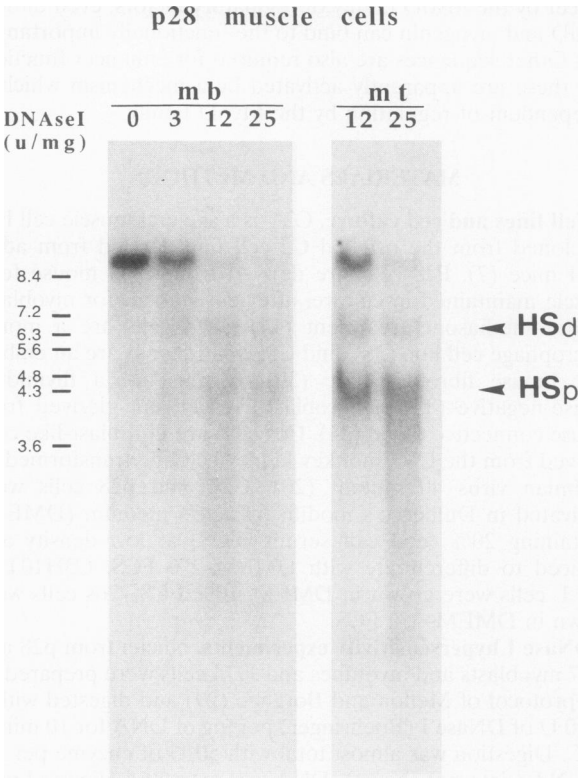


FIG. 2. DNase I-hypersensitive sites in the 5′-flanking region of the α-cardiac actin gene in C3H mice. DNA from myoblasts (mb) or myotubes (mt) of the p28 muscle cell line was digested with different concentrations of DNase I, as indicated. DNA was extracted and analyzed by Southern blotting after *Eco*RI restriction. The two subbands generated as a result of DNase I digestion and revealed by hybridization with the *Sau*3A-*Eco*RI probe shown in Fig. 1 arise as a result of a distal (HSd) and a proximal (HSp) hypersensitive site in the 5′-flanking region of the α-cardiac actin gene in C3H muscle cells. The size of the original *Eco*RI fragment is 12 kb. The subbands migrate as fragments of 6.3 kb for HSd and 4.3 kb for HSp, corresponding to sites −7.0 and −5.0 kb, respectively, from the transcription start site of the C3H cardiac actin gene. Sizes are shown in kilobases.

Nuclei from myoblasts and myotubes were isolated and digested with a range of DNase I concentrations. DNA was extracted, digested with *Eco*RI, and analyzed by Southern blotting. The two cell lines gave similar results: after hybridization with the *Sau*3A-*Eco*RI probe, two subbands were visible on the blot below the 12-kb *Eco*RI band, corresponding to a distal site (HSd) located at about −7.0 kb and a proximal site (HSp) located at about −5.0 kb (Fig. 2) from the transcription start site of the C3H α-cardiac actin gene. Both sites were detected in myoblasts, but the subbands that they generate were more intense in myotubes, particularly in the case of the proximal site (see Fig. 2). Neither hypersensitive site was detected in the mouse nonmuscle macrophage cell line J774 (38).

Cloning of the *Eco*RI genomic fragment containing both hypersensitive sites. A library of fragments obtained by digesting C3H DNA with *Eco*RI was prepared in a lambda EMBL4 vector. Three independent clones were isolated on the basis of hybridization with the *Sau*3A-*Eco*RI probe (Fig. 1) and shown to contain a 12-kb fragment of genomic DNA. One clone, λ5, was selected and characterized by Southern blotting. It proved

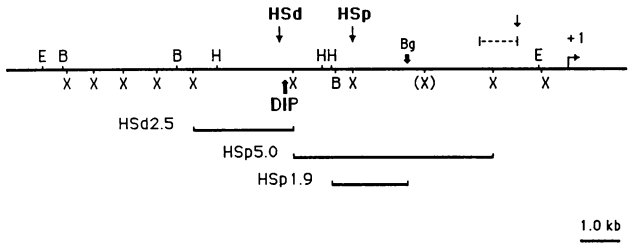


FIG. 3. Restriction map of the 5'-flanking region of the α-cardiac actin gene in C3H mice. The data presented for the 12-kb *Eco*RI fragment are based on restriction mapping of cloned C3H DNA. Cloned BALB/c DNA in the proximal promoter region has a restriction map similar to that seen for C3H DNA by Southern blotting of genomic DNA except for the additional *Eco*RI site indicated by an arrow (↓). The positions of the proximal (HSp) and distal (HSd) hypersensitive sites are indicated. The point where the duplication is inserted in BALB/c mice is also indicated (DIP). The dashed line between brackets indicates the position of the *Sau*3A-*Eco*RI probe generated from BALB/c DNA. The bent arrow indicates the transcription start site of the gene (+1). Fragments of the insertion in λ5 were subcloned into CAT expression vectors as a 2.5-kb *Xba*I fragment (HSd2.5) and an adjacent 5-kb *Xba*I fragment (HSp5.0), as indicated. A subclone of this (HSp1.9) contained a 1.9-kb HindIII-BglII fragment. Abbreviations for restriction enzymes: E, *Eco*RI; B, *Bam*HI; X, *Xba*I; H, *Hin*dIII; Bg, *Bgl*II. The λ5 phage was not systematically mapped for *Bgl*II sites, and the site in question is indicated with an arrow. (X), *Xba*I site which can be cut in genomic DNA but not after amplification in the bacterial strain used (SURE; Stratagene).

	C2/7 muscle cells		non muscle cells		
	mb	mt	L	Cos	10T1/2
pBLCat2	1	1	1	1	1
pHSp 5.0 (+)	0.5	6	0.5	-	-
pHSp 1.9 (+)	0.5	60	-	1	0.5
pHSd 2.5 (+)	1	70	1	1	1
pHSd 2.5 (-)	-	70	-	-	-

FIG. 4. Enhancer activity of fragments containing the HSd and HSp sites. The fragments described in the legend to Fig. 3 were introduced upstream of the thymidine kinase promoter in pBLCat2 and transfected into myoblasts (mb) or myotubes (mt) of the C2/7 mouse muscle cell line or into L, Cos, or 10T1/2 nonmuscle cell lines. Results are shown as levels of CAT activity for a given quantity of extract, normalized for transfection efficiency with luciferase activity in a control RSV luciferase plasmid cotransfected with the pBLCat2 vector and assayed on the same sample. The activity of the vector alone is taken as 1 for each cell type tested. The results represent the averages of at least two experiments done in duplicate. -, not done.

to have the same restriction map as the endogenous gene and had therefore not undergone rearrangement.

A restriction map of the 5' region of the C3H cardiac actin locus is presented in Fig. 3. The map of the proximal promoter region (−1.1 kb) is based on data from Southern blotting with C3H genomic DNA. It is identical to that of the cloned BALB/c DNA except for an *Eco*RI site present only in the latter. This is the only restriction polymorphism between the two inbred mouse lines which we have detected in this region. We cannot, of course, rule out the possibility of subtler changes between the loci.

Each hypersensitive site correlates with a muscle-specific enhancer activity. After partial digestion of the cloned 12-kb *Eco*RI fragment with *Xba*I, fragments containing each hypersensitive site were subcloned into pBLCat2 (27), an enhancer test plasmid in which CAT expression is under the control of the thymidine kinase gene promoter. HSd was subcloned as a 2.5-kb *Xba*I fragment to give pHSd2.5(+), in its normal orientation, and pHSd2.5(−), in the reverse orientation. A 5.0-kb *Xba*I fragment containing the HSp was subcloned to give pHSp5.0(+), and a 1.9-kb *Hin*dIII-*Bgl*II fragment derived from it, also containing the HSp site, was subcloned to give pHSp1.9(+) (Fig. 3). When transfected into C2/7 muscle cells, all fragments showed enhancer activity in myotubes but not in nonmuscle L (24), Cos (20), and 10T1/2 (39) cells (Fig. 4) or in C2/7 myoblasts. The 5.0-kb fragment containing HSp was not very active, but a subclone of it, pHSp1.9(+), showed a very high level of enhancement, suggesting that there may be a sequence which is inhibitory in skeletal myotubes in the 5' region of HSp5.0(+). HSd showed high-level muscle-specific activity in both orientations, in keeping with the classical definition of an enhancer. The proximal enhancer is currently under investigation. However, given the probable importance of the distal enhancer in vivo, as indicated in experiments with the BALB/c mouse, we first concentrated our efforts on the fine analysis of this regulatory element.

Dissection of the sequence containing the distal site. The 2.5-kb *Xba*I genomic fragment present in pHSd2.5 was cut with different restriction enzymes and subcloned into pBLCat2 (27) or into pox, a plasmid containing the CAT reporter gene under the control of the proximal promoter of the α-cardiac actin gene (−660 to +119) (18). These constructions were transfected into C2/7 muscle cells. The results are summarized in Fig. 5.

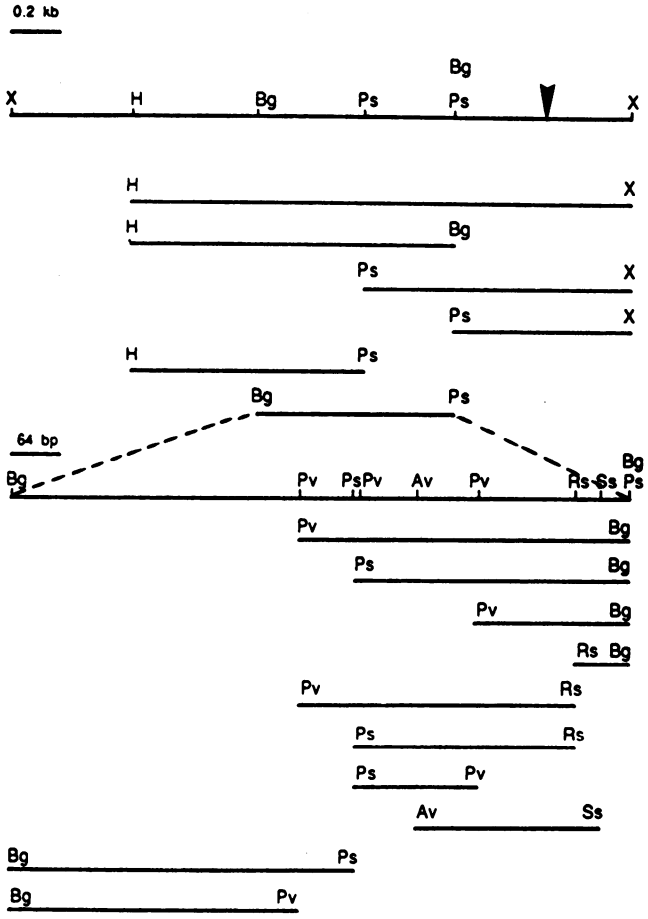
The enhancer activity seen in myotubes is contained in a 0.8-kb *Bgl*II fragment (clone 6). This fragment is highly active with the α-cardiac actin promoter. The addition of 5' or 3' sequences does not enhance but rather reduces the signal. The 0.8-kb *Bgl*II fragment is also very active with the thymidine kinase proximal promoter present in pBLCat2. In this case, 5' sequences appear to increase the activity by two- or threefold.

Further dissection of the 0.8-kb *Bgl*II fragment shows that it probably contains a mosaic of regulatory elements; no single subfragment gives full enhancement with either the homologous or the heterologous promoter. Part of the activity is retained in a 360-bp *Pst*I-*Bgl*II fragment (clone 8) corresponding to the 3' half of the 0.8-kb sequence. Further dissection of this fragment results in considerable loss of activity, suggesting that the combination of different sequences within the fragment is necessary for full enhancement (see clones 8 to 14 in Fig. 5).

Low activity is also retained by the 5' *Bgl*II-*Pvu*II fragment (clone 16). Comparison of clones 7 and 8 and of clones 11 and 12 suggests that a negative regulatory element is present in the central 80-nucleotide *Pvu*II-*Pst*I fragment. However, comparison of clones 15 and 16, containing the 5' part of the enhancer with and without this fragment, shows much less of a negative effect. In the complete enhancer (clone 6), this negative effect is avoided.

No enhancer activity was detected in myoblasts with any of the fragments tested.

Fine mapping of the duplication insertion point in BALB/c mice. Since the distal enhancer is very close to the point of insertion of the duplication in BALB/c mice (Fig. 1), we determined whether this mapped within or immediately 3' of



Clone	C2/7 mt		C2/7 mb	
	pox (P)	TK (B)	pox (P)	TK (B)
Without insert	1	1	1	1
1	81	93.5	1	
2	105	89		
3	18	7.5		
4	-	1		
5	-	2.4		
6	173.5	37.1	1.2	0.7
6	173.5	37.1	1.2	0.7
7	7.8	2.3		
8	35	18		
9	4	0.6		
10	2			
11	0.5	2	0.1	
12	8	1		
13	3.5	1		
14	4	-		
15	4	5		
16	6.8	6		

FIG. 5. Dissection of the enhancer activity associated with the HSd site. Different fragments of pHSd2.5(+), clones 1 to 16, subcloned into pBLCat2 with the TK promoter (B) or into pox (P), which contains the CAT coding sequence under the control of the α-cardiac actin gene promoter (−660 to +119) (16), were transfected into myoblasts (mb) or myotubes (mt) of the C2/7 muscle cell line. CAT activities were normalized for transfection efficiency with the luciferase vector and assayed on the same sample. The activity of the plasmid without an additional fragment was taken as 1 in each series of transfections and each cell type. The results shown are averages of at least two independent transfections done in duplicate. Restriction enzyme abbreviations: X, *Xba*I; H, *Hin*dIII; Bg, *Bgl*II; Ps, *Pst*I; Pv, *Pvu*II; Av, *Avi*II; Rs, *Rsa*I; Ss, *Ssp*I. The precise site of insertion of the partial duplication in BALB/c mice is shown (▼). -, not done.

the active fragment. The duplicated locus present in BALB/c genomic DNA, corresponding to 9.5 kb and spanning the partially duplicated α-cardiac actin gene and adjacent 3′ sequences, was cloned into a lambda vector with *Eco*RI linkers, and an *Eco*RI subfragment containing the junction was subcloned into pBR322 to give pAE81 (18) (Fig. 1). The third intron was sequenced across the position where it differs from that of the bona fide gene, giving 140 nucleotides of sequence downstream of the insertion point in BALB/c mice. This sequence has no homology with the distal enhancer (Fig. 6). It contains a *Pvu*II site, corresponding to one of those mapped in the pHSd2.5(+) plasmid. The location of the insertion point 350 nucleotides downstream of the active 0.8-kb *Bgl*II fragment (clone 6) was demonstrated by hybridization of the junction region to the clones shown in Fig. 5 (data not shown). We therefore conclude that the insertion in BALB/c DNA lies just downstream of the distal enhancer element (see Fig. 5). The duplication therefore includes the proximal enhancer together with the promoter and part of the cardiac actin gene.

Sequence of the distal enhancer. The DNA sequence of the *Bgl*II fragment (clone 6) which contains full enhancer activity is shown in Fig. 6. Two complementary decamers are present, one overlapping the *Pvu*II site that is disrupted in clones 7 and 11 and the other present in the 360-bp 3′ fragment. It is possible that full enhancer activity depends on loop formation between these two complementary sequences (clone 6). Loop formation may neutralize the negative effect of the 80-bp *Pvu*II-*Pst*I fragment.

When the enhancer sequence was scanned for known protein-binding sites, it was immediately striking that it contains seven E boxes (CANNTG), potential binding sites for members of the helix-loop-helix family of transcriptional regulators, which includes the MyoD family of skeletal muscle-specific transcription factors (34, 48). The consensus sequence for a myogenin heterodimer present in myotube nuclear extracts is TTGCACCTGTTNNTT (51); that for a MyoD heterodimer with E47, a partner similar to the E protein probably present in the extracts, is TCA(C/G)(C/T)TGT (2). The E47 protein itself preferentially binds to CA(C/G)(C/T)TGA (2).

Of the E boxes present in the HSd enhancer sequence, iv and vi have a core sequence which most resembles that preferred by a MyoD or myogenin heterodimer. The sequence

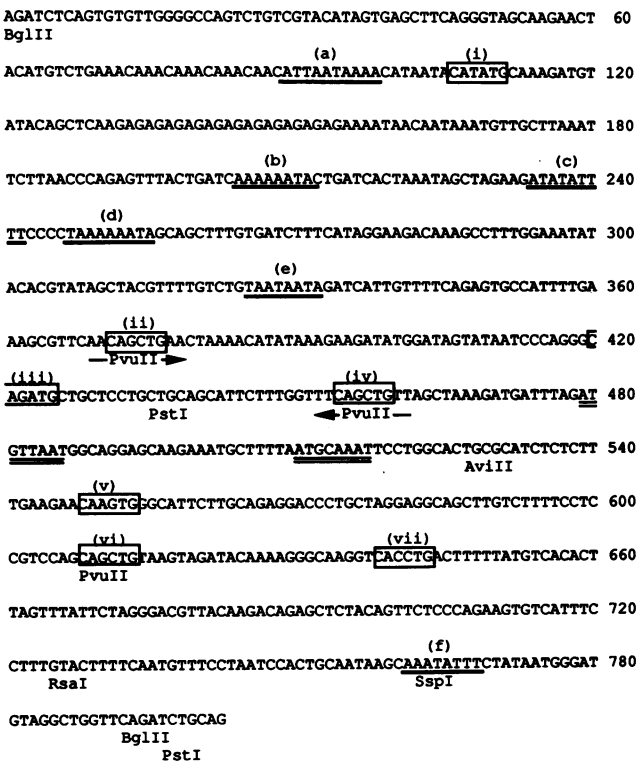


FIG. 6. Sequence of the distal enhancer. The sequence of the 800-bp *Bgl*II-*Pst*I fragment is shown. Restriction sites used for subcloning are indicated. E box sequences are boxed and numbered i to vii. A/T-rich sequences are underlined with a bold line and labeled a to f. The complementary decamers are underlined with an arrow. The Oct-1 binding sites are shown with a double underline; the second is a perfect Oct-1 consensus. Nucleotide numbers are shown on the right.

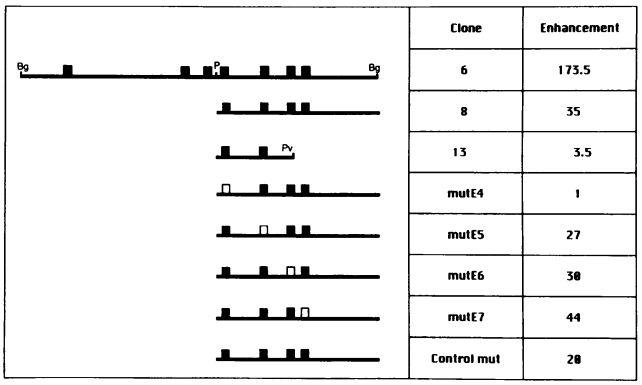


FIG. 7. Mutagenesis experiments. The 800-bp distal enhancer is shown (clone 6). Shorter constructs containing either wild-type (black boxes) or mutated (open boxes) E boxes are represented. Clones 6, 8, and 13 are identical to those illustrated in Fig. 5. Clones containing a mutation in one of the E boxes are named mutE4 to mutE7 and also contain a selection mutation. Control mut is identical to clone 8 except for the selection mutation (which affects the polylinker). The data in the last column represent the enhancement factor for the α-cardiac actin promoter in C2/7 myotubes for each construct. The results shown are averages of at least two independent experiments done in duplicate. Bg, *Bgl*II; P, *Pst*I; Pv, *Pvu*II.

of E box ii is closer to the consensus on the other DNA strand, and that of E box vii is closer to the E47 binding consensus. No NF1, COMP1, or MEF-2 sites are present; these sequences have been found to occur adjacent to myogenin-binding sites when casting experiments are performed with random oligonucleotides and myotube nuclear extracts (17).

The 5′ half of the enhancer (clone 16) containing site i has some activity. Sites iv to vii are present in the active 3′ fragment (clone 8), and progressive loss of these sites correlates with loss of activity (Fig. 5). Loss of optimum enhancer activity occurs when the 0.8-kb *Bgl*II-*Pst*I fragment is cut at the central *Pvu*II site, with consequent destruction of site ii. The 80-bp fragment which inhibits activity of the 3′ region also contains a consensus binding site iii for a helix-loop-helix protein.

Of the other potentially interesting binding sites, a perfect Oct-1 binding sequence (ATGCAAAT) (16) and a second, less well conserved Oct-1 consensus sequence (ATGTTAAT) are present in the active 3′ fragment (clone 8). A number of A/T-rich sequences are present, particularly in the 5′ part of the enhancer, although none of these correspond to the target sites for MEF-2 [consensus (C/T)T(A/T)(A/T)AAATA(A/G) (22)] or MHox [consensus T(A/T)ATAAT(A/T)A) (12)]. There are no CarG box (32), MCAT (28), HF-1a or HF-1b (33), or Sp1 (42) sites and no other sequence motifs in common with known cardiac or skeletal regulatory elements (8, 35). By analogy with other muscle-specific enhancers, it seemed very probable that the activity of this enhancer in skeletal muscle cells is regulated by the MyoD family of transcriptional factors.

Mutagenesis of potential regulatory sequences. In order to find out whether the E boxes were important for activity, we mutated E boxes iv to vii of the most active subfragment (clone 8). The mutated constructs were transfected into C2/7 cells, and CAT activity was assayed (Fig. 7). In myotubes, the control plasmid (see Materials and Methods) gives a twofold reduction of the original clone 8 enhancement. Compared with this value, the disruption of E boxes v to vii does not have any effect on the enhancer activity. However, enhancer activity is completely abolished when E box iv is mutated. This E box is not sufficient for activity, since clone 13P (see Fig. 7) is barely active, indicating that additional sequences are required, probably located in the 3′ 66 nucleotides of clone 8 (clone 10).

Are myogenic factors involved in activation of the distal enhancer? Myogenic factors regulate the activation of skeletal muscle genes by interaction with E box elements. It therefore seemed probable that this occurs with the distal enhancer, since E box iv is essential for activity in C2/7 myotubes, which accumulate MyoD and myogenin. Cotransfection experiments were performed with some of the CAT reporter constructs described above and expression vectors for MyoD, myogenin, myf-5, and MRF4 sequences in 10T1/2 embryonic fibroblasts (Fig. 8). The proximal promoter of the mouse α-cardiac actin gene, like that of the human gene (42), showed transactivation, as expected (Fig. 8A). This was most striking with MyoD (fivefold) but was also seen with the other factors. The proximal promoter with the distal enhancer (clone 6P) showed no additional transactivation by the myogenic factors (Fig. 8A).

These experiments were also carried out with the distal enhancer in combination with the TK promoter since, particularly in the case of MyoD, transactivation of the proximal promoter may mask an additional effect of the enhancer. The distal enhancer with MyoD (Fig. 8B) showed no transactivation. As a control enhancer sequence with the TK promoter, we used a 900-bp 3′-flanking fragment of the mouse ML1F/MLC3F gene (37) containing an enhancer sequence similar to

A

Construct		MyoD	myogenin	myf-5	MRF4
pox	-	1	1	1	1
	+	5.1	2.6	3.2	2.6
Clone6P	+	5.6	2.1	3.0	2.7

B

Construct		MyoD
pBLCat2	-	1
	+	1
pMLC1/3E	-	1
	+	10
pHSd2.5(+)	-	1
	+	0.3

FIG. 8. Transactivation experiments. In these experiments, 10 μg of each CAT construct was cotransfected into 10T1/2 cells with 2 μg of pEMSV expression vector with (+) or without (−) the myogenic factor sequence, as indicated. (A) Enhancement (fold) of CAT activity under the control of the proximal cardiac actin promoter (pox) alone or this promoter with the 800-bp *Bgl*II fragment of the distal enhancer (clone 6P; see Fig. 4), in the presence or absence of each myogenic factor. The basal activity of the pox construct alone was taken as 1. (B) Enhancement (fold) of CAT activity under the control of the TK promoter alone (pBLCat2) or of this promoter with the mouse myosin light-chain 3′ enhancer (pMLC1/3 E) (38) or with the distal enhancer of the cardiac actin gene [pHSd 2.5(+)] (Fig. 3), in the presence or absence of MyoD. The basal activity of the pBLCat2 construct alone was taken as 1. Values are the averages of at least three independent experiments.

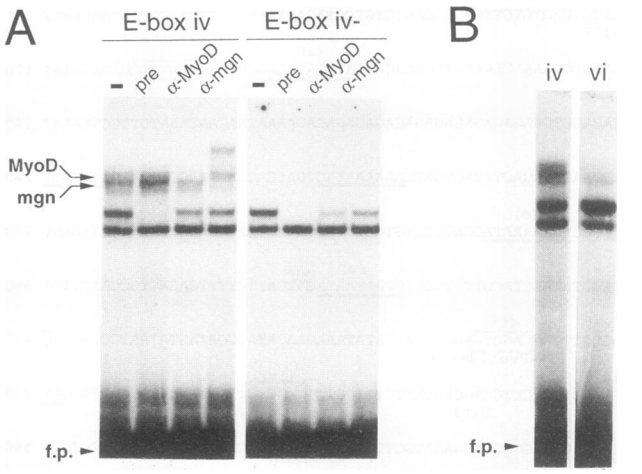


FIG. 9. Binding properties of E boxes iv and vi. (A) Electrophoretic mobility shift assays of E box iv. Wild-type E box iv or mutant E box iv$^-$ oligonucleotides were used to detect binding activities in myotube nuclear extracts. Signals corresponding to MyoD and myogenin (mgn) binding were identified with specific antibodies to MyoD (α-MyoD) and myogenin (α-mgn), respectively. A preimmune serum (pre) was used as a control for specificity. Lanes —, nuclear extract alone. f.p., free probe. (B) Comparison of binding activities on E box iv and E box vi present in myotube nuclear extracts.

that described for the rat gene (49). This gave the expected high level of enhancement when introduced into pBLCat2 and transfected into C2/7 myotubes and when cotransfected into nonmuscle cells with MyoD (Fig. 8B), myogenin, or myf-5 expression vectors but not with MRF4 (results not shown).

E box iv binds MyoD and myogenin from C2/7 myotubes. Having identified E box iv as essential for enhancer activity, we investigated whether MyoD or myogenin, the major myogenic factors present in C2 myotube extracts, actually bind to it. Five retarded complexes could be separated in bandshift assays (Fig. 9). The two slowest-migrating bands were clearly E box specific. They appear with the wild-type probe (E box iv) but not with the mutated probe (E box iv$^-$). With specific antibodies, the two E box-specific bands could be attributed to binding of MyoD and of myogenin (Fig. 9A). The anti-MyoD antibody completely eliminated the shift caused by MyoD, whereas the antimyogenin antibody induced a supershift of the myogenin-containing complex. Direct comparison of adjacent bands as well as competition experiments (data not shown) indicated that these two myogenic factors bind E box iv with affinities similar to that seen on the right-hand MyoD binding site in the muscle creatine kinase enhancer. The faint band just below the myogenin-containing complex was weaker on the mutated E box track but detectable after a longer exposure (result not shown). The two strong faster-migrating binding activities which were not affected by the E box mutation and are therefore not directly related to its transcriptional function were clearly present in Cos and HeLa cell nuclear extracts but barely detectable in myoblast nuclear extracts (data not shown).

In order to verify that the binding of MyoD and myogenin to E box iv seen in the bandshift assays was related to function, we examined whether similar complexes form on another nonfunctional E box in the enhancer. For this comparison, we chose E box vi because it has the same core sequence (CAGCTG) as E box iv. In fact, E box vi binds MyoD and myogenin from C2 myotubes only very poorly (Fig. 9B). In competition experiments (not shown), a 100-fold molar excess of unlabeled E box vi does not eliminate MyoD and myogenin binding to the labeled E box iv. In contrast, the shifts were abolished at a 50-fold molar excess of unlabeled over labeled E box iv oligonucleotide. Thus, E box iv binds MyoD and myogenin with a significantly higher affinity than E box vi, although the core E box sequence is identical. Taken together, the results from the mutagenesis and bandshift experiments show that binding of myogenic factors is required for activity of the enhancer. The transactivation data clearly show that it is not sufficient.

DISCUSSION

We have demonstrated that two DNase I-hypersensitive sites are present approximately 5 and 7 kb upstream of the transcription start site of the mouse α-cardiac actin gene. DNase I-hypersensitive sites have been identified in and around many different genes (for a review, see reference 3), and they have been found to correspond to different types of regulatory regions, some of which play a role in the transcriptional activity of the gene. These may be silencers or activators, binding tissue-specific or more general transcription factors (3, 23). They are not systematically associated with gene activity but rather reflect an open state of the chromatin.

In the case of the α-cardiac actin locus, the two hypersensitive sites are detected in DNA from both myoblast and myotube cells in culture, although they are more evident once the cells have differentiated. This is particularly true for the proximal site, which may be associated with a further change in chromatin configuration occurring at the terminal differentiation step. Cloned DNA fragments from both regions have strong myotube-specific enhancer activity, suggesting that at least one factor required for their function is present only as the cells begin to differentiate.

Two other muscle genes have been analyzed for DNase I-hypersensitive sites. The chicken myosin light-chain gene MLC2A (50), expressed mainly in the heart, exhibits four heart-specific hypersensitive sites, one of which (−1.5 kb) has been shown to correspond to an element which can act as a silencer in muscle cells. Several DNase I-hypersensitive sites have been found around the mouse δ and γ acetylcholine receptor genes (11) in cultured muscle cells. The functional activity of these sites has not yet been characterized, but since the three muscle-specific sites map to the proximal promoters of the γ (G1 and G2) and δ (D1) subunit genes, they lie within regions involved in transcriptional regulation. For the muscle-specific enhancers identified so far (35), no analysis which examines DNase I hypersensitivity has been reported.

A number of muscle-specific enhancer sequences located at different sites in and around muscle genes have been characterized, including those of the 3′ enhancer of the myosin light-chain MLC1F/3F gene (49), and 5′ enhancer of the muscle creatine kinase gene (25), and the intronic enhancer of the quail troponin I gene (52). They are required for high-level activity of the proximal promoters of these genes in muscle cells. In some cases, such as the chicken acetylcholine receptor gene encoding the α-subunit (36), an enhancer is present in the proximal promoter region. The proximal promoter of the mouse α-cardiac actin gene, like that of the human gene, is sufficient to direct the expression of a reporter gene in myotubes. The level of expression in C2 myotubes is relatively low, similar to that obtained with the MLC3F promoter without the 3′ enhancer sequence, and much lower than that obtained with the promoter of the α-subunit of the acetylcholine receptor (14). It is therefore perhaps not surprising to find that high-level expression of the α-cardiac actin gene also requires enhancer elements.

The proximal enhancer has not yet been analyzed in detail. The 5-kb fragment contains a more active 1.9-kb fragment, suggesting that a negative element may be present in the longer sequence which, at least with a heterologous promoter, reduces the enhancement seen in C2 myotubes. The distal enhancer activity is contained within an 800-bp fragment. Further deletions result in reduced activity, and the functional analysis demonstrates that the enhancer is a complex element. However, much of the activity is retained in the 3′ 360-bp fragment (clone 8). An 80-bp *Pvu*II-*Pst*I sequence immediately 5′ to this fragment exerts a strong negative effect. This fragment contains an E box which may bind a negative regulator (40) when not in the normal configuration. We suggest that this may arise because a loop structure in this region fails to form when the upstream sequences are absent or masked. Six other E boxes are present in the 800 bp, four of them in the more active 3′ region. None of these correspond exactly to the consensus binding site for MyoD or myogenin (TTGCACCTGTTNNTT) heterodimers with E12-like proteins (2, 51), although some (E boxes iv and vi) are a better match than others. However, the E box (CCAACTGC) in the proximal promoter of the cardiac actin gene, which is clearly activated by MyoD, also does not correspond to this consensus (42). Although A/T-rich sequences are present in the enhancer, these do not correspond to consensus sequences for efficient binding of the MHox (12) and MEF-2 (22) muscle transcription factors, nor are there consensus sequences for general factors known to be important in skeletal muscle gene transcription, such as the CArG or MCAT sequences (35), or in cardiac muscle (8).

Site-directed mutagenesis of the E boxes in the distal enhancer followed by functional analysis in transfection experiments demonstrates that E box iv plays an essential role in enhancer activity. Mutation of this E box results in a 20-fold reduction in CAT reporter expression levels. Bandshift assays with myotube nuclear extracts showed that MyoD and myogenin are able to bind to an E box iv-containing probe. The mutation in the E box iv$^-$ probe specifically disrupts these complexes. In contrast, E box vi, which is not functionally important, does not bind MyoD or myogenin efficiently, although it has the same core sequence. This observation points to the importance of flanking sequences in determining E box binding efficiencies. Given the functional importance of E box iv and the fact that it binds myogenic factors, it seemed probable that this enhancer would be activated via this E box by the MyoD family of regulatory factors, as in the case of other well-characterized muscle-specific enhancers (49). However, cotransfection experiments with expression vectors for the myogenic factors showed no significant transactivation of the distal enhancer sequence.

Transfection data show that other sequences are required, notably a 66-bp fragment 3′ to the E box sequences (see Fig. 6). This element is not functional on its own, but its deletion eliminates activity. No evident consensus sequences are present in the 66-bp fragment, and experiments are in progress to identify the regulatory element(s) and factor(s) implicated in its activity. What is striking about the α-cardiac actin distal enhancer is the lack of transactivation by myogenic factors. We propose that this is due to the requirement for the additional 3′ sequence which may interact with at least one essential component not present in 10T1/2 cells expressing myogenic factors. Unlike proteins, such as MEF-2, that are required for other muscle enhancers (13, 26), this component is apparently not encoded by a gene which is activated by the myogenic factors.

In the BALB/c mouse, cardiac actin gene transcription is reduced in both developing skeletal and cardiac muscle. In the adult heart of normal mice, cardiac actin is a major constituent. Interestingly, the BALB/c mouse shows no cardiomyopathy, at least under laboratory conditions. This may be due to a compensatory increase in α-skeletal actin, since this represents 43% of the total actin in BALB/c mouse hearts, whereas in the heart of C3H mice, this isoform accounts for only 8% of the α-actin (18, 19).

Fine mapping of the insertion duplication point in BALB/c mice places it just distal to the upstream enhancer. The insertion contains a partial duplication of 6.5 kb of 5′-flanking sequence, including the proximal promoter, together with the first three exons and corresponding introns of the α-cardiac actin gene. Both promoters are active, but the upstream promoter is much more efficient (10-fold) than the downstream one, as estimated from nuclear pre-mRNA levels (18). Transcripts from the upstream promoter are terminated, presumably as a result of cryptic termination sites in the intervening DNA, before reaching the downstream promoter and gene, ruling out direct transcriptional interference.

It seems likely that the high-level activity of the upstream promoter and the reduced activity of the downstream one in BALB/c mice are due to their relative distances from the distal

enhancer. The truncated upstream gene has the distal and proximal enhancers in the correct configuration in relation to the promoter, whereas the intact gene has only the proximal enhancer at the normal position. Since the proximal promoter regions of mutant BALB/c and normal C3H mice are very similar, it is probable that the displacement of the distal enhancer by the insertion in BALB/c mice is responsible for the relatively lower transcription of the α-cardiac actin gene in these mice. The correct juxtaposition of the two enhancers which is perturbed in the BALB/c locus may also be very important for high-level expression of the gene. More generalized effects on chromatin structure as a result of the insertion may also affect transcription. A detailed analysis of hypersensitive sites upstream of the cardiac actin gene in cardiac and skeletal muscles from the BALB/c mouse should provide additional information on this point. The fact that cardiac actin transcription is reduced in both cardiac and skeletal muscle in BALB/c mice points to the potential role of these enhancers in the heart as well, where the genes of the MyoD family are not expressed. The activity of the enhancers and E box iv in cardiac cells is currently under investigation.

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