Characterization of a Cardiac-specific Enhancer, Which Directs α-Cardiac Actin Gene Transcription in the Mouse Adult Heart*

Expression of the mouse α-cardiac actin gene in skeletal and cardiac muscle is regulated by enhancers lying 5’ to the proximal promoter. Here we report the characterization of a cardiac-specific enhancer located within −2.354/−1.36 kbp of the gene, which is active in cardiocytes but not in C2 skeletal muscle cells. In vivo it directs reporter gene expression to the adult heart, where the proximal promoter alone is inactive. An 85-bp region within the enhancer is highly conserved between human and mouse and contains a central AT-rich site, which is essential for enhancer activity. This site binds myocyte enhancer factor (MEF2) factors, principally MEF2D and MEF2A in cardiac nuclear extracts. These results are discussed in the context of MEF2 activity and of the regulation of the α-cardiac actin locus.

α-Cardiac actin is a member of a highly conserved multigene family, found in all eukaryotic cells, of which the isoforms are differentially expressed in a tissue-specific and developmentally regulated manner (1). In mouse, α-cardiac actin is expressed in the early myocardium and in somites from embryonic day 8.5, as the skeletal muscle of the myotome begins to form (2). After birth, the α-cardiac actin gene remains expressed at a high level in the heart and is down-regulated in skeletal muscles at the same time as α-skeletal actin remains expressed in skeletal muscle and is down-regulated in the heart (3–5).

Several lines of genetic evidence suggest that α-cardiac actin is essential for normal structure and function of adult cardiac myocytes. In BALB/c mice, a 5’-partial duplication of the α-cardiac actin gene is associated with the down-regulation of the α-cardiac actin mRNA and protein (6), leading to enhanced expression of α-skeletal actin mRNA and protein after birth through a compensatory mechanism (5). However, the hearts of adult BALB/c mice present functional alterations with increased contractility (7). When a null mutation is introduced into the α-cardiac actin gene, the mice either do not survive to term or die within 2 weeks of birth because of extensive loss of thin filaments and sarcomere disorganization leading to heart failure (8). Attempts to rescue the deficient cardiocytes by transgenic expression of a non-cardiac actin in such mutant mice causes heart enlargement and dysfunction (8) resembling human idiopathic dilated cardiomyopathy. In humans, missense mutations of the α-cardiac actin gene predispose affected cardiocytes to mechanical injury leading to idiopathic dilated cardiomyopathy (9) and eventual cell death. Taken together, these results indicate that both the type of actin expressed in the adult heart and the levels at which it is expressed are important for correct contractile function.

Several regulatory sequences involved in the expression of the α-cardiac actin gene during muscle cell differentiation in vitro and the development of mammalian striated muscles in vivo have been identified. Transcriptional activation in differentiating skeletal muscle cells depends on critical sites in the proximal promoter of the human and mouse genes, notably a CArG-box binding serum response factor (10, 11), an E-box binding myogen regulatory factors (10–13), and an Sp1 site (10, 13). In cardiocyte cultures, the same sites seem to be involved (14, 15), although the role of the E-box is controversial (12). Interestingly, it has been shown that the multiple CArG-boxes found in the α-cardiac actin promoters of several species (16, 17) are sites of serum response factor and Nkx-2.5 interaction (18) acting to promote high levels of activity (19). However, previous observations on the BALB/c mouse line suggested that there might be other levels of regulation operating on this gene (6).

Two DNase I hypersensitive sites (HSp1 and HSp2) were identified upstream of the mouse-α-cardiac actin gene in the C2 skeletal muscle cell line. When these sequences were tested for activity in different cell lines with homologous and heterologous promoters they were shown to act as striated muscle-specific enhancers (20). Analysis of the more distal sequence, at −7 kbp from the gene, showed that its activity depends on an E-box, a target of myogenic regulatory factors, and also on an AT-rich 3’ sequence, which binds the homeomain protein Emb in interaction with MEF2D and p300 (21). This complex potentially plays a role in opening chromatin at the α-cardiac actin locus, rendering the E-box and downstream regulatory regions accessible; in transgenic mice when this sequence is present robust expression is seen (22). The proximal promoter itself is a weak regulatory element directing expression to embryonic skeletal muscle and to the embryonic heart in a few transgenic lines. The distal sequence HSp3 acts as a skeletal muscle enhancer in vivo. Expression in the adult heart is seen with transgenes containing −5 kbp of α-cardiac actin DNA, which includes the proximal DNase I hypersensitive site, con-

* This work was supported by the Pasteur Institute and the CNRS. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† The abbreviations used are: HSp, proximal DNase I hypersensitive site; HSp3, distal DNase I hypersensitive site; MEF, myocyte enhancer factor; POX, proximal promoter of the α-cardiac actin gene; TkCAT, thymidine kinase promoter driving the chloramphenicol acetyltransferase gene; TkLaCZ, thymidine kinase promoter driving the β-galactosidase gene; BSvLuc, Rous sarcoma virus long repeat driving the luciferase gene; MCK muscle creatine kinase; contig, group of overlapping clones; EnC, new enhancer region; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; n, nuclear localization sequence.
sistent with a role for this proximal enhancer region in the transcription of the gene in cardiac muscle.

In this study, we have examined in more detail this aspect of \(\alpha\)-cardiac actin expression. We report on a third enhancer located within \(\sim 2.354\) kb from the site of the gene that directs transgene expression in the adult heart but not in skeletal muscle. In the embryo, it directs expression in the skeletal muscle of the somites, as well as the heart. However, unlike the previously described distal and proximal enhancers, it is not active in the C2 skeletal muscle cell line derived from postnatal muscle but only in cardiocytes. Its activity depends on a MEF2 site.

**Experimental Procedures**

**Reporter Constructs and Site-directed Mutagenesis—**A 2.43-kbp BamHI fragment derived from the clone 1G10 of the murine \(\alpha\)-cardiac actin gene (6) containing the \(\sim 2.354, \sim 0.119\)-kb region of the gene was inserted into the BamHI site of the promoterless plasmid, pBLCAT6 (23) generating the construct Enc proximal promoter region (POX). Deletions in this sequence were generated either by using convenient restriction sites in the 5’ region or by using partial digestion. Fragments were also cloned into a CAT construct (20) in which the POX was inserted into the BamHI site of the promoterless plasmid, pBLCAT6. Mutations of the MEF2 site were introduced separately, by site directed mutagenesis (transformation site-directed mutagenesis kit or QuikChange site-directed mutagenesis, Stratagene), in the \(-2.3\) to \(-1.4\)-kbp region cloned in BlueScript. The two mutations substituted for the native MEF2 site were, respectively, TTTCTGACCGGAGGTCGT (m1) and TTTCGACCGGAGGTCGT (m2). Bold characters indicate mutated nucleotides. Mutated DNA fragments were subsequently sequenced and transferred into POX (\(-0.669\) kb) or TkCAT. Multimerized sequences of the native or mutated \(\alpha\)-cardiac actin MEF2 box were cloned into TkLaCl at a SfiI site introduced previously (provided by F. Relax), and confirmed by sequencing.

**Cell Culture, DNA Transfection, and Assays—**The C2/7 line, a subclone of the C2 cell line derived from the skeletal muscle of adult C3H mice, was grown as reported previously (24). For DNA transfection, the cells were cultured in 6-cm diameter dishes with 20% fetal calf serum and allowed to differentiate in 2% fetal calf serum. The embryonic mouse fibroblast cell line C3H10T1/2 was grown in 10% fetal calf serum (24).

Ventricular cardiocytes were prepared from hearts of 18 embryonic day (E18) rat fetuses according to the protocol of (25). Hearts were trimmed of the atria and outflow tract and the ventricular cells were dissociated under gentle shaking in 1x trypsin (T4674 Sigma) in ADS medium complemented with glucose. Trypsin was inhibited by adding decompplemented newborn calf serum (Invitrogen). Cells were recovered by centrifugation and kept at 37°C in newborn calf serum. Fresh enzyme solution was added to the tissue until complete dissociation was reached. Cells were plated in 6-cm dishes in plating medium and grown in 5% CO₂.

Cardiocytes and myogenic cells were transfected by the calcium phosphate transfection technique (26). The embryonic fibroblast cell line, C3H10T1/2, was transfected with superfect (Qiagen) under the conditions described by the manufacturer. 9 µg of CAT reporter construct and 0.5 µg of a reporter gene containing the Rous sarcoma long terminal repeat linked to the luciferase gene (RsvLuc) were added to each dish. Myotubes were rinsed in phosphate-buffered saline and collected after being maintained 48 h in Dulbecco’s modified Eagle’s medium supplemented with 2% fetal calf serum as reported elsewhere (24). Cardiocytes were rinsed with phosphate-buffered saline for 60 h following transfection, collected in 40 ml Tris-HCl (pH 7.5), 150 mM NaCl, and 1 mM EDTA, and lysed by three freeze-thaw cycles.

Co-transfection was carried out with 15.5 µg of total DNA consisting of 0.5 µg of RsvLuc, 10 µg of a TkLaClZ construct regulated by a concatamerized MEF2 site (x4) from the mouse muscle creatine kinase (MCK) or \(\alpha\)-cardiac actin genes and 5 µg of MEFP2, MEFLC2, or MEF2D expression vectors (27). As a control an empty expression vector was cotransfected.

CAT activity was measured by the simple phase extraction procedure (28), and luciferase activity was measured as described previously (20).

**Preparation of Nuclear Extracts and Gel Mobility Shift Assays—**Nuclear extracts from ventricles of adult rat hearts were prepared according to the procedure of Mal and Ordahl (29) with slight modifications. Hearts were trimmed of the atria and outflow tract in washed sterile phosphate-buffered saline and homogenized in a Tisse Masser for 12 s at half-power in homogenization buffer (10 mM Hepes, pH 7.6, 25 mM KCl, 1 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 0.4 mM phenylmethysulfonyl fluoride, 1.8 mM sucrose, 5% glycerol), with added protease inhibitor mixture (Roche Applied Science, number 1697498). Liberation of nuclei was checked with trypsin blue dye, and the homogenate was centrifuged on a pad of homogenization buffer at 4°C for 60 min. Nuclei were transferred to a Dounce homogenizer in lysis buffer (10 mM Hepes, pH 7.5, 100 mM KCl, 0.1 mM EDTA, 3 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 10% glycerol), with added protease inhibitor mixture. Following 6 strokes with an A pestle, NaCl was added to 0.5 M final volume. The homogenate was left for 30 min on a slow rotating table and then spun for 60 min at 35,000 rpm. The supernatant was dialyzed against 25 mM Hepes, pH 7.5, 100 mM KCl, 1 mM EDTA, 3 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 10% glycerol, with added protease inhibitor mixture, and stored in liquid nitrogen. Protein concentration was determined using the Bio-Rad protein assay.

**Generation of Transgenic Mice, Screening of the Transgenic Lines, and Histochemical Staining for \(\beta\)-Galactosidase—**The 2.4-kbp BamHI fragment was inserted at the BamHI site of pAC1SDKnLacZ (\(\alpha\) represents a nuclear localization sequence) (22). The insert was extracted by cutting with SacII and XhoI, eluted, purified, resuspended, and injected into the pronuclear stage of fertilized mouse eggs. The transgenic line shown here had a copy number of 2–3. Transgenic mice were obtained as reported elsewhere (33) and (34). The transgenic mice were genotypically determined using a 2-kbp cognate sequence of the gene as the transgenic probe. Two methods were used with the DNA Strider TM 1.3b program. DNA matrix analysis was performed with variable windows and stringencies. DNA alignment was performed by the blocks method with variable mismatch and gap penalties.
An α-Cardiac Actin Enhancer Active in the Adult Heart

Fig. 1. Sequences upstream of the promoter of the α-cardiac actin gene regulate its expression in striated muscle cells. The 5′ region of the α-cardiac actin gene is schematized, showing the approximate position of the two DNaseI hypersensitive sites (HSd, HSp), which correspond to distal and proximal regions with enhancer activity in C2/7 skeletal muscle cells (20) of the cardiac enhancer (EnC) region and the minimum promoter (POX). The CAP site (transcription start site) is indicated by an arrow. The borders (a) of the distal enhancer (HSd site) were determined by alignment of the partial published sequence within this fragment (20) with the mouse genomic sequence (locus AC 133157 NT 039209.1M). The borders (b) of the proximal enhancer (HSp site) were determined by sequence alignment and restriction sites of our sequenced fragment with the mouse genomic sequence. The borders of the EnC enhancer (c1 and c2) were also determined by sequence alignment. In the case of c1, there was a restriction polymorphism such that the BamH1 site was absent in the mouse genomic sequence (locus AC 133157 NT 039209.1M). These fragments have been tested individually for their ability to enhance activity of the minimum promoter POX (−0.669, +0.119 kbp (20)), after transient transfection in primary cultures of cardiocytes derived from dissected ventricles and in differentiated myotubes of the C2 muscle cell line. The results are expressed relative to the activity of the plasmid POX set at 1 for both cell types. The pRSVLuc plasmid was included in all transfections as an internal control to correct for transfection efficiencies. Values are the average of three experiments with different DNA preparations.

|                     | HSd | HSp | EnC | POX |
|---------------------|-----|-----|-----|-----|
| Cardiocytes         | 1.4 | 8.5 | 14.0| 1.0 |
| C2/7 Myotubes       | 81.0*| 8.1 | 1.3 | 1.0 |

* from Biben et al., 1994

RESULTS

Identification of a Cardiac Muscle Enhancer—The 5′-flanking region of the mouse α-cardiac actin gene was re-examined for cardiac as well as skeletal muscle regulatory elements by transfection experiments in primary cultures of cardiac muscle cells (cardiocytes), as well as the C2 skeletal muscle cell line, previously used to identify muscle enhancers (20). The POX of −770 bp is active in both cell types but is a weak regulatory element in vivo where it does not direct transgene expression to the adult heart. The activity of the CAT reporter gene driven by POX was taken as 1 to compare the effect of upstream sequences, cloned in front of the POX transgene (Fig. 1). The distal enhancer region (HSd), which is active in differentiated skeletal muscle myotubes, shows very low activity in cardiocytes. The proximal enhancer, HSp, is active in cardiocytes as well as in C2/7 myotubes. In this deletion analysis a new enhancer region (EnC), which is active only in cardiocytes, is now identified in the −2.354- to −0.669-kbp region immediately 5′ of the proximal promoter.

We had previously shown that −5.7 kbp of α-cardiac actin upstream sequence (construction T3, (22)) contained the necessary regulatory elements to direct nlacZ transgene expression to the adult heart. We now tested whether −2.354 kbp was sufficient for this transcriptional activity in vivo. Of four founder mice, one had a rearranged transgene, as shown by Southern blotting, and one was infertile, the two others gave rise to transgenic lines, which expressed the nlacZ transgene in the adult heart. In one case β-galactosidase activity was patchy in both atria and the ventricles, with prior expression in the embryonic heart also (results not shown); this expression was lost in subsequent generations. In the other line, all aspects of transgene expression were stable over the four generations examined. Between embryos/mice within a single litter, the pattern was also maintained, with minor variations in the extent of labeling in the right ventricle. Strong expression was seen in the embryonic (Fig. 2, a and b) and adult (Fig. 2, c and d) heart. In the adult both atria show intense X-gal staining.

Fig. 2. Expression of nlacZ in transgenic animals under the control of a −2.354- to +0.119-kbp fragment upstream of the α-cardiac actin gene. a and b, an embryonic day 10.5 embryo shown from the right (a) and from the left (b). Staining of the heart (H) is observed together with staining of somitic muscle (S). Some ectopic expression is present in the head as reported before for other α-cardiac actin transgenes (20). c and d, an adult heart in ventral (c) and dorsal (d) views showing strong X-gal staining of the right and left atria (RA and LA) and most of the left ventricle (LV) with partial staining of the right ventricle (RV).

Most of the left ventricular myocardium is labeled. Part of the right ventricle is positive, with negative areas of myocardium ventrally. This was also the case at birth and during postnatal development (3 weeks, 3 months, results not shown). This is
similar to the expression profile of the T3 transgenic lines, where -5.746 kbp, which included the HSp and EnC enhancers, was driving the transgene. These sequences were compared using the program DNA matrix alignment (DNA Strider TM 1.3B), which allows for comparison of long sequences. The human sequence (~8 kbp upstream of the CAP site) is on the ordinate, and the CAP site of the gene is indicated by a horizontal arrow. The mouse sequence (10 kbp) is on the abscissa, with the CAP site of the mouse gene indicated by a vertical arrow. The window is 19 and stringency 17 for the diagram shown. Dots indicate the hits obtained showing conserved sequences in the region of the Hsd site, the HSp site, the EnC, and the proximal promoter, b. DNA alignment, blocks method of Martinez (DNA Strider TM 1.3B), which is convenient for short sequences. The penalty was set at large (3), and the gap penalty was set at medium (2). The mouse sequence (~1.432 kbp (XbaI), ~1.350 kbp (SpeI)) is on the upper line (capital letters), and the human sequence (~1.435 kbp (XbaI), ~1.350 kbp is on the lower line. Nucleotide identity in blocks is shown by #, out of blocks is shown by \. Gaps are represented by a dash in the mouse sequence and by a = in the human sequence.

**Sequence Conservation in the 5’-Flanking Region of the α-Cardiac Actin Gene—Comparison of DNA genomic sequences located 8.6 kbp 5’ to the CAP site of the mouse and human**

*Fig. 3. Sequence homology between mouse and human DNA upstream of the CAP site of the α-cardiac actin gene. a, the human (locus AC012271, clone RP11-6403) and the mouse (locus AC 133157 NT 039209.1M) contigs were extracted from data banks by probing with a fragment of the known sequences of the promoter of the human and the mouse gene, respectively. These sequences were compared using the program DNA matrix alignment (DNA Strider TM 1.3B), which allows for comparison of long sequences. The human sequence (~8 kbp upstream of the CAP site) is on the ordinate, and the CAP site of the gene is indicated by a horizontal arrow. The mouse sequence (10 kbp) is on the abscissa, with the CAP site of the mouse gene indicated by a vertical arrow. The window is 19 and stringency 17 for the diagram shown. Dots indicate the hits obtained showing conserved sequences in the region of the Hsd site, the HSp site, the EnC, and the proximal promoter, b. DNA alignment, blocks method of Martinez (DNA Strider TM 1.3B), which is convenient for short sequences. The penalty was set at large (3), and the gap penalty was set at medium (2). The mouse sequence (~1.432 kbp (XbaI), ~1.350 kbp (SpeI)) is on the upper line (capital letters), and the human sequence (~1.435 kbp (XbaI), ~1.350 kbp is on the lower line. Nucleotide identity in blocks is shown by #, out of blocks is shown by \. Gaps are represented by a dash in the mouse sequence and by a = in the human sequence.*
An α-Cardiac Actin Enhancer Active in the Adult Heart

α-cardiac actin genes was carried out using the DNA matrix program (Fig. 3a). A genomic contig of 150 kbp was identified in which the human gene was located. The sequence comparison shows regions of homology at −8 to −7 kbp and −5 to −4 kbp, where the HSd and HSb sites are located, respectively. It also identifies a conserved region lying within −2 kbp from the CAP site potentially corresponding to the new enhancer region (EnC), in addition to sequences in the POX, which have been shown previously to play a role in its activity (16). The mouse −2 kbp fragment does not display significant homology with unrelated fragments of the human contig, using the matrix program (results not shown). Similar results were obtained using a larger (15 kbp) fragment of human genomic DNA (results not shown). We then focused on the conserved region at about −1.4 kbp from the CAP site. This relative location is conserved between human and mouse, and the homology is maintained at high stringency. Using the blocks method of Martinez (51), DNA alignment shows that the conserved sequence is located between −1.432 and −1.350 kbp from the CAP site, with 85% identity and two gaps, and with most mismatches located at the extremities of the region (Fig. 3b). The functional characteristics of the conserved 82-bp sequence were tested by transfection experiments in primary cardiocyte cultures, in myotubes of the skeletal muscle C2/7 cell line and in the C3H 10T1/2 embryonic fibroblast line (Fig. 4). Upstream sequences were introduced in front of the POX directing a CAT reporter sequence. The activity of POX in the different cell cultures was taken as 1. As reported in Fig. 1 the complete −2.354-kbp sequence upstream of the α-cardiac actin CAP site was active in cardiocytes only. A series of deletions demonstrate that activity in cardiocytes depends on sequences lying 5’ to the SpeI site at −1.35 kbp from the gene. The BamHI/SpeI fragment (−2.354 to −1.364 kbp) was cloned in front of the Tk promoter driving the CAT reporter and shown to be active in cardiocytes, with some activity also in C2/7 myotubes (Table I). Activity in cardiac muscle cells was retained in the reverse orientation, and we therefore concluded that the sequence acts as an enhancer. Full activity is retained with a subfragment, which extends from the SpeI site to the HincII site a further 500 bp upstream (Fig. 4). The highly conserved 85-bp region alone does not exert more than about 2-fold activity, with incremental rises in activity as sequences extending to the HincII site are included. Between the HincII and the XbaI site, there is an Ebox, and its mutation results in some loss of activity, but we have not identified any single site that is essential for activity in contrast to the MEF2 site, which is essential for activity in the context of the larger fragment (BamHI/SpeI fragment). There are potential sites for cardiac regulators, some of which had a minor effect on activity.
The MEF2 site is required for enhancer activity

The 2.4-kb fragment (B-B) was cloned into KS+ (Stratagene), and a fragment (S/B-S) (~2.35 kb, ~1.35 kb) was isolated including the (B/S) fragment of the gene plus a linker of 6 bp. This (S/S) fragment was cloned at a XbaI site in front of the Tk promoter in sense (S/B-S) and antisense (S/B-S) orientation. The mutated fragment (B/m2/S mutation m2, 5-`TTTTCTATTTTAATCGTGGTG-3`) was also cloned in front of the Tk promoter. Constructs were transiently transfected into primary cultures of cardiocytes derived from dissected ventricles, and into C2/7 myotubes.

Oligonucleotides containing the native or mutated (mutation m2) MEF2 site were coamplified and cloned at an XbaI site introduced in front of the Tk promoter driving the LacZ gene. Constructs were transfected into primary cultures of cardiocytes derived from dissected ventricles and into C2/7 myotubes and into NIH3T3 cells. Native oligonucleotide, 5-`CAGTTTTCTATTTTAATCGTGGTG-3' mutated oligonucleotide, 5-`CAGTTTTCTATTCTAATCGTGGTG-3` Mutated nucleotides are indicated in bold. The pHSVLac plasmid was included in all transfections as an internal control to correct for transfection efficiencies. CAT, Luc, and β-galactosidase activities were quantitated as indicated under "Experimental Procedures." Values are the average of three experiments with different DNA preparations. In all experiments, activity of the Tk construct was taken as 1. ND, not determined; B, BamH1; S, SpeI.

|                  | Cardiocytes | C2/7 Myotubes | Fibroblasts |
|------------------|-------------|---------------|-------------|
| TkCAT            | 1           | 1             | ND          |
| (/S)/B-S) sense  | 17.1        | 5.5           | ND          |
| (S-B/S)na.sense  | 19          | ND            | ND          |
| (B/m2/S)/TkCAT   | 4.7         | 0.7           | 0.2         |
| Tk LacZ          | 1           | 1             | 1           |
| (Native) 4× MEF2 site | 7.8      | 0.9           | 0.7         |
| Tk LacZ          | 1.3         | 0.7           | 0.8         |
| (Mutated) 3× MEF2 site | 1.3      | 0.7           | 0.8         |

Activity of the Cardiac Enhancer Depends on a Critical MEF2 Site—We focused our attention on the highly conserved region, which contains an AT-rich sequence, CTATTTTCTAC, which is a potential binding site for the MEF2 family of transcription factors (consensus CTA/AAT4 TAG/A (33)). When this sequence is mutated (m1, m2) in the context of the BamH1/Spe1 fragment all enhancer activity is lost, both with the POX (Fig. 4) and Tk (Table 1) promoters. The low level activity seen with the conserved Xbal/Spe1 fragment is also eliminated when the central MEF2 site is mutated (Fig. 4). These results demonstrated that the activity of the cardiac enhancer depends on this critical MEF2 site. The functional role of this site was further illustrated by experiments in which it was cloned as a multimer (4 × 24 bp of site and flanking sequences) in front of the Tk promoter driving a lacZ reporter. This resulted in a 7.8-fold increase in activity over the Tk lacZ transgene alone, specifically in cardiocytes (Table 1). The fact that this multimer is not active in C2/7 myotubes suggests that a cardiac-specific complex is involved. A larger fragment with the Tk promoter shows some activity in C2 myotubes, which is lost when the site is mutated, consistent with a role for MEF2 in skeletal muscle cells.

The binding of MEF2 isoforms to the sequence is shown in Fig. 5. The same 45-bp sequence, radioactively labeled, was tested by gel mobility shift assay. Nuclear extracts from adult cardiac muscle form a complex, which is competed by excess cold oligomer and by the standard MEF2 site present in an enhancer of the MCK gene but not by the mutated α-cardiac actin oligomer (m2) (Fig. 5A). When different antibodies to MEF2 isoforms, mHox or Oct1, which also bind AT-rich sequences, are added to the nuclear extract, the presence of MEF2 in the complex is demonstrated (Fig. 5B). A complete band shift is seen with a MEF2 antibody and also to a large extent with an antibody to the MEF2D isoform. A MEF2A antibody also shifts part of the complex. MEF2B does not appear to be involved. The extent of the shift with the MEF2D (and 2A) antibody suggests that if MEF2C, for which specific antibodies are not available, is also part of the complex, it is probably a minor component.

DISCUSSION

We have identified a novel enhancer (EnC) situated in the −2.354 to −1.364-kb region upstream of the mouse α-cardiac actin gene. In cell culture experiments this sequence is only active in cardiocytes, not in differentiated skeletal muscle or non-muscle cells. In this respect it differs from the previously identified enhancers at −7 and −5 kb from the gene, which are both active in skeletal myotubes. The latter also has some activity in cardiocytes and may contribute to the expression of α-cardiac actin in the heart. However we now show that a transgene containing only the proximal enhancer (−2.354 kb of 5'-flanking sequence) will direct expression in the adult heart, which the promoter alone (~669 bp) has never been shown to do (22). Regulation of the α-cardiac actin gene is therefore orchestrated by at least three enhancers, with different striated muscle specificities. Two sets of results suggest that the HSd may exercise control over the accessibility of the locus (see Ref. 21), possibly in conjunction with the HSp and EnC regions. The complete −8.6-kb transgene gives robust, reproducible expression and is not subject to silencing in vivo. In BALB/c mice, a partial duplication of the gene (6) separates this distal enhancer from more proximal regulatory sequences, including the EnC, which is not modified.2 In these mice, expression of the α-cardiac actin gene is observed in the adult heart but at a reduced level (6). The combination of enhancers with different muscle specificities is a feature of muscle gene regulation. The desmin (34) or MCK (35) genes are controlled by skeletal and cardiac enhancers, whereas the myosin MLC1F/3F gene is regulated by distinct skeletal muscle specific enhancers active at embryonic and fetal stages of development (36). An extreme example of the fine control exercised by many distinct regulatory elements is provided by Myo5, where multiple sequences, extending over 100 kb upstream of this key myogenic regulatory gene, control its spatiotemporal expression and hence the onset of skeletal muscle formation at different sites in the embryo (see Ref. 37).

The EnC is active in primary cultures from perinatal ventricular muscle and directs expression of the nlacZ transgene in the adult heart. Although the atria are strongly labeled by X-gal staining, not all cardiocytes in the ventricles are β-galactosidase positive. This is particularly evident in the adult right ventricle. A similar profile was seen with −5.746 kb of 5'-flanking sequence (22) and is therefore not because of the absence of the HSp region. In the presence of the full −8.6-kb flanking sequence more complete cardiac expression is seen, suggesting that in the absence of the HSd element, shorter transgenes are more susceptible to this effect. Other muscle transgenes also show regionalized transcription in the heart, potentially reflecting the modular nature of cardiac gene regulation (see Ref. 38). In the case of a myosin light chain, MLC1F, transgene expression in the left ventricle, but not the right, appears to reflect a phenomenon of in vivo chromatin silencing not seen in transitory transfection experiments (25).

The EnC extends over 1 kb and contains potential regulatory sites some of which, when mutated, reduced enhancer activity (results not shown). However the main region of sequence conservation between man and mouse localizes to the −1.432 to −1.350-kb region that surrounds the MEF2 site. Mutation of this site confirmed that it is critically important for enhancer activity. Concatemers of this site with its flanking

2 M. Lemonnier, unpublished observations.
region retain cardiac-specific activity. Because MEF2 is also a regulator of skeletal muscle, this would suggest that the flanking sequences are implicated in the binding of a complex, which is cardiac specific. Gel mobility shift assay experiments show that MEF2D binds to the sequence, with some additional interaction with MEF2A and possibly MEF2C. MEF2, and particularly MEF2C have been implicated in the regulation of a number of cardiac muscle genes, such as \( \alpha \)-myosin heavy chain (39, 40) or myosin light chain, MLC2V (41), and desmin (42).

The regionalization of ventricular expression is difficult to equate with the MEF2 site alone particularly because the desmin transgene, which depends on MEF2, is expressed in the right, but not the left ventricle (42). This is also the case for an MLC2V transgene (43). A potentially critical role for MEF2C in the formation of the left ventricle is indicated by the loss of this cardiac compartment in MEF2C mutant mice (44).

The enhancer described here is remarkable for its activity in adult cardiac muscle. The role of MEF2 isoforms in the adult heart has been questioned because of a report that MEF2 proteins were not detectable (45) and that MEF2 activity was shown to fall as the heart matures (46). In the gel mobility shift assay experiments reported here we show that nuclear extracts from the ventricular muscle of adult mouse hearts do contain MEF2 binding activity, in this case principally MEF2D. Functionally it has been shown that a transgene containing multimerized MEF2 sites from the enhancer of the \( \alpha \)-cardiac actin gene is mainly active in the embryonic heart (47). However low, but detectable, levels of expression persisted into adulthood. Indeed a detectable level of MEF2C has been reported in adult hearts and is reduced in the failing hearts of diabetic patients accompanied by a down-regulation of MEF2 target genes (48).

Furthermore in mice that lack MEF2A, which binds the MEF2 site in EnC, there is an adult cardiac phenotype (49). Interestingly in these mice \( \alpha \)-cardiac actin transcripts increase, possibly because of a stress response, which leads to an increase in MEF2D (49). This may also be the case for adult cardiocytes. The MEF2D mutant phenotype has not been published. However there are suggestions that this isoform is important for regulation of cardiac genes as shown by studies on the regulation of the human \( \alpha \)-skeletal actin gene (50). Furthermore it is MEF2D that is implicated in the complex with Emb, a member of the POU domain family of proteins, which forms on the AT-rich HSd site of the \( \alpha \)-cardiac actin gene (21).

We conclude from our analysis that the activity of the cardiac-specific enhancer, that we have identified as one of at least three enhancers regulating the mouse \( \alpha \)-cardiac actin gene, depends on a MEF2 site, which in adult cardiomyocytes binds the MEF2D isoform. The striking sequence conservation...
around this site suggests that in this case MEF2D may participate in a cardiac-specific regulatory complex.

Acknowledgments—We thank Philippe Daubas for his help in the preparation of the whole mount photographs, Christian Mareck (C.E.A., Saclay, France) for providing the DNA Strider TM 1.3f8 program, R. Prywes for providing the MEF2A/C and MEF2D antibodies, and C. Bodin for technical assistance. We also thank Stefano Schiaffino and Simonetta Ausoni (University of Padova, Italy) for teaching us to culture cardiocytes.

REFERENCES

1. Vandekerckhove, J., and Weber, K. (1979) Differentiation 14, 123–133
2. Sassoon, D., Garner, I., and Buckingham, M. (1988) Development (Camb.) 104, 155–164
3. Gunning, P., Ponte, P., Blau, H. M., and Kedes, L. (1983) Mol. Cell. Biol. 3, 1985–1995
4. Buckingham, M. E. (1985) Essays Biochem. 20, 77–109
5. Garner, I., Sassoon, D., Vandekerckhove, J., Alonso, S., and Buckingham, M. E. (1989) Dev. Biol. 134, 236–245
6. Garner, I., Minty, A. J., Alonso, S., Barton, P. J., and Buckingham, M. E. (1986) EMBO J. 5, 259–267
7. Hewett, T. E., Grupp, I. L., Grupp, G., and Robbins, J. (1994) EMBO J. 13, 740–746
8. Kumar, A., Crawford, K., Close, L., Madison, M., Lorenz, J., Doetchman, T., Babinet, C., and Paulin, D. (1993) Neuronasal Dysfunct. Disord. 3, 423–427
9. Olson, E. N. (1994) Neuronasal Dysfunct. Disord. 3, 423–427
10. Sartorelli, V., Webster, K. A., and Kedes, L. (1990) Gene Dev. 4, 1811–1822
11. Tui, D., Clergue, N., Montarras, D., Pinsot, C., Kahn, A., and Phan-Dinh-Tuy, F. (1990) J. Mol. Biol. 213, 677–686
12. Skerjanc, I. S., and McBurry, M. W. (1994) Dev. Biol. 163, 125–132
13. Biesiada, E., Hamamori, Y., Kedes, L., and Sartorelli, V. (1999) Mol. Cell. Biol. 19, 2577–2584
14. Pari, G., Jardine, K., and McBurry, M. W. (1991) Mol. Cell. Biol. 11, 4796–4803
15. Sartorelli, V., Hong, N. A., Bishopric, N. H., and Kedes, L. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 4047–4051
16. Minty, A., and Kedes, L. (1986) Mol. Cell. Biol. 6, 2125–2136
17. Mohun, T. J., Garrett, N., and Gurdon, J. B. (1986) EMBO J. 5, 3185–3189
18. Chen, C. Y., and Schwartz, R. J. (1995) J. Biol. Chem. 270, 15628–15633
19. Chen, C. Y., and Schwartz, R. J. (1996) Mol. Cell. Biol. 16, 6372–6384
20. Biben, C., Kirschbaum, B., Garner, I., and Buckingham, M. (1994) Mol. Cell. Biol. 14, 3504–3513
21. Molinari, S., Relaix, F., Lemonnier, M., Kirschbaum, B., Schafer, B., and Buckingham, M. (2004) Mol. Cell. Biol. 24, 2944–2957
22. Biben, C., Hadchouel, J., Tajbakhsh, S., and Buckingham, M. (1996) Dev. Biol. 173, 200–212
23. Bosshart, M., Kluppel, M., Schmidt, A., Schütz, G., and Luckow, B. (1992) Gene (Amst.) 110, 129–130
24. Catala, F., Wanner, R., Barton, P., Cohen, A., Wright, W., and Buckingham, M. E. (1995) Mol. Cell. Biol. 15, 4585–4596
25. Kelly, R. G., Lemonnier, M., Zaffran, S., Munk, A., and Buckingham, M. E. (2003) J. Cell Sci. 116, 5005–5013
26. Graham, F. L., and Van der Eb, A. J. (1973) Virology 52, 456–467
27. Martin, J. F., Miano, J. M., Husted, C. M., Copeland, N. G., Jenkins, N. A., and Olsen, E. N. (1994) Mol. Cell. Biol. 14, 1847–1856
28. Seed, B., and Shenk, J. Y. (1986) Reviews (Amst.) 67, 271–277
29. Mar, J. H., and Ordahl, C. P. (1990) Mol. Cell. Biol. 10, 4271–4283
30. Han, T. H., and Prywes, R. (1995) Mol. Cell. Biol. 15, 2907–2915
31. Maitrise, T., Sambrook, J., and Fritsch, E. F. (1989) Molecular Cloning, pp. 52–55, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
32. Sanes, J. R., Rubenstein, J. L. R., and Nicolas, J.-P. (1986) EMBO J. 5, 3133–3142
33. Gossett, L. A., Kelvin, D. J., Sternberg, E. A., and Olsen, E. N. (1989) Mol. Cell. Biol. 9, 5022–5033
34. Li, Z., Coluci, E., Babinet, C., and Paulin, D. (1993) Neuronasal Dysfunct. Disord. 3, 423–427
35. Amaecher, S. L., Buskin, J. N., and Hauensch, S. D. (1993) Mol. Cell. Biol. 13, 2753–2764
36. Kelly, R., Alonso, S., Tajbakhsh, S., Cosso, G., and Buckingham, M. (1995) J. Cell Biol. 129, 383–396
37. Hadchouel, J., Carvajal, J., Daubas, P., Bailard, L., Chang, T., Rocancourt, D., Cex, D., Tajbakhsh, S., Rigby, P., and Buckingham, M. (2003) Development (Camb.) 130, 3415–3426
38. Kelly, R., Zammit, P., and Buckingham, M. (1999) Trends Cardiovasc. Med. 9, 3–10
39. Molkentin, J. D., and Markham, B. E. (1993) J. Biol. Chem. 268, 19512–19520
40. Lee, Y., Nadal-Ginard, B., Mahdavi, V., and Izumo, I. (1997) Mol. Cell. Biol. 17, 4047–4057
41. Navankasattusas, S., Zhu, H., Garcia, A. V., Evans, S. M., and Chien, K. R. (1992) Mol. Cell. Biol. 12, 1469–1478
42. Kuisk, I. R., Li, H., Tran, D., and Capetanaki, Y. (1996) Dev. Biol. 174, 1–13
43. Zou, Y. M., and Chien, K. R. (1995) Mol. Cell. Biol. 15, 2972–2982
44. Lin, Q., Scharzw, J., Bucana, C., and Olsen, E. N. (1997) Science 276, 1404–1407
45. Sahamianian, S. V., and Nadalginard, B. (1996) Mech. Dev. 57, 103–112
46. Kolodieiezcyk, S. M., Wang, L., Balazsi, K., DeRepentigny, Y., Kohary, R., and Megeney, L. (1999) Curr. Biol. 9, 1203–1206
47. Naya, F., Wu, C., Richardson, J., Overbeek, P., and Olson, E. (1999) Development (Camb.) 126, 2045–2052
48. Razeghi, P., Young, M., Cockrill, T., Fraizer, O., and Tegtmeyer, H. (2002) Circulation 106, 407–411
49. Naya, F., Black, B., Wu, H., Bassel-Duby, R., Richardson, J., Hill, J., and Olson, E. (2002) Nat. Med. 88, 1303–1309
50. Slepak, T., Webster, K., Zang, J., Prentice, H., O'Dowd, A., Hicks, M., and Bishopric, N. (2001) J. Biol. Chem. 276, 7575–7585
51. Martinez, H. M. (1983) Nucleic Acids Res. 11, 4629–4634