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α-Skeletal and α-Cardiac Actin Genes Are Coexpressed in Adult Human Skeletal Muscle and Heart

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We determined the actin isotypes encoded by 30 actin cDNA clones previously isolated from an adult human muscle cDNA library. Using 3′ untranslated region probes derived from α-skeletal, β- and γ-actin cDNAs and from an α-cardiac actin genomic clone, we showed that 28 of the cDNAs correspond to α-skeletal actin transcripts. Unexpectedly, however, the remaining two cDNA clones proved to derive from α-cardiac actin mRNA. Sequence analysis confirmed that the two skeletal muscle α-cardiac actin cDNAs are derived from transcripts of the cloned α-cardiac actin gene. Direct measurements of actin isotype mRNA expression in human skeletal muscle showed that α-cardiac actin mRNA is expressed at 5% the level of α-skeletal actin. Furthermore, the α-cardiac actin gene expressed in skeletal muscle is the same gene which produces α-cardiac actin mRNA in the human heart. Of equal surprise, we found that α-skeletal actin mRNA accounts for about half of the total actin mRNA in adult heart. Comparison of total actin mRNA levels in adult skeletal muscle and adult heart revealed that the steady-state levels in skeletal muscle are about twofold greater, per microgram of total cellular RNA, than those in heart. Thus, in skeletal muscle and in heart, both of the sarcomeric actin mRNA isotypes are quite abundant transcripts. We conclude that α-skeletal and α-cardiac actin genes are coexpressed as an actin pair in human adult striated muscles. Since the smooth-muscle actins (aortic and stomach) and the cytoplasmic actins (β and γ) are known to be coexpressed in smooth muscle and nonmuscle cells, respectively, we postulate that coexpression of actin pairs may be a common feature of mammalian actin gene expression in all tissues.

Actin is a highly conserved protein which participates in a wide variety of cellular functions in eucaryotes including muscle contraction, amoeboid movement, cytokinesis, and mitotic division (19). Within mammals, six different actin isotypes have been identified (28). Two of these, cytoplasmic β- and γ-actin, are coexpressed in all mammalian nonmuscle cells studied (see reference 28). Similarly, the two smooth-muscle actins are coexpressed in smooth muscle and have not been detected in other tissues. The relative amounts of these two smooth-muscle isotypes vary significantly in different smooth-muscle cells (29). In contrast, α-skeletal actin is thought to be the only actin expressed in adult skeletal muscle, and α-cardiac actin is thought to be the only actin expressed in adult heart muscle (27). Hereafter, we will refer to α-skeletal actin and α-cardiac actin as skeletal actin and cardiac actin, respectively.

The determination of actin isotype expression in different mammalian tissues is difficult because these proteins are nearly identical. Only three of the different actins can be resolved by two-dimensional gel electrophoresis (28). Because of very small differences in their isoelectric points, when one actin isotype greatly predominates, resolution becomes even more difficult. In response to this, Vandekerckhove and Weber (27, 29) developed a method for radiolabeling and sequencing the amino-terminal residues of actins which allows identification of all six mammalian actins. However, this method is neither readily adaptable for routine analysis nor capable of sensitive discrimination between skeletal and cardiac actin. A minor actin present at about 5% of the total actin content would not be detected (27).

On the other hand, measurement of actin mRNA expression is a particularly sensitive assay for actin isotype expression. Although the sizes of the six mammalian actin mRNAs fall into only two length classes (24), the discovery by Cleveland et al. (3) that chicken β- and γ-actin mRNAs have isotype-specific 3′ untranslated regions allowed investigators to develop cloned DNA probes specific for a particular actin mRNA. Thus far, probes specific for skeletal

actin have been constructed from chicken (17), rat (24), and mouse (14). In addition, Minty et al. (13) have been able to use a fragment derived from the protein-coding region of a mouse cardiac actin cDNA clone to detect its expression. In the latter case, however, the lack of absolute actin isotype specificity diminishes the sensitivity of the probe.

In this report, we describe the unexpected isolation of cardiac actin cDNA clones from an adult human skeletal muscle cDNA library. We derived 3′ untranslated region fragments that hybridize specifically to cardiac actin DNA and RNA sequences. Similarly, we developed a 3′ untranslated region probe from a skeletal actin cDNA (6) which is skeletal actin specific. We used these isotype-specific probes to measure the steady-state levels of actin isotype mRNAs in human adult skeletal muscle and in human adult heart. Cardiac actin mRNA accounts for about 5% of adult muscle actin mRNA. The cardiac actin gene expressed in adult muscle is the same as that expressed in human heart. Skeletal actin mRNA accounts for essentially all of the remainder of the actin mRNA in skeletal muscle. To our surprise we found that in adult human heart muscle, skeletal actin mRNA represents about 50% of the total actin mRNA. The unexpected finding that these two actin isotypes are coexpressed in human sarcomeric muscles now suggests that most, if not all, tissues (sarcomeric muscle, smooth muscle, and nonmuscle tissues) express a minimum of two actin isotypes, albeit at widely differing relative levels.

MATERIALS AND METHODS

RNA and DNA isolation. Total cellular RNA was isolated from HeLa cells by the guanidine hydrochloride procedure as previously described (2). Adult human skeletal leg muscle was obtained after surgical amputation from patients with gangrenous toes. Dissected muscle fragments were washed with phosphate-buffered saline (pH 7.0) before extraction of total RNA with phenol-chloroform (18). A 10-g piece of an adult human heart ventricle was obtained from the heart of a patient undergoing heart transplant surgery. The heart was kept in phosphate-buffered saline (pH 7.00) at room temperature for 1 h while it was photographed and examined. A piece of ventricle was extracted for RNA as described for skeletal muscle.

Screening of cDNA library. The 30 actin cDNA clones previously isolated from an adult human skeletal muscle cDNA library (6) were screened with nick-translated DNA fragments (21).

General methods. Plasmid DNA preparation, restriction enzyme digestion conditions, agarose gel electrophoresis, isolation of DNA fragments, transfer of DNA to nitrocellulose paper, and hybridizations were performed exactly as described previously (4). Minilysate DNA preparations followed the protocol of Holmes and Quigley (9).

Subcloning of DNA from the 3′ untranslated region of the human cardiac actin gene was performed as previously described (4). A DNA fragment was generated by digestion of the cardiac actin gene clone with the restriction endonucleases *Pst*I and *Hae*III (Fig. 1). This fragment was inserted into the *Pst*I-*Eco*RI cleavage sites of plasmid pBR322.

DNA sequence analysis. We determined the DNA sequence of the cardiac actin cDNA clone and genomic subclone by the method of Maxam and Gilbert (11).

Isolation and labeling of DNA fragments. We used purified DNA fragments isolated from a set of actin 3′ untranslated region cDNA subclones (20) as probes in all RNA blot analyses. The plasmid bearing the 3′ untranslated region subclone of skeletal actin (pHMα-A-3′UT) was digested with restriction endonucleases *Xba*I and *Pvu*II. A 366-base-pair (bp) DNA fragment was then isolated from this digest mixture. This fragment extends from the termination codon through the 253-bp 3′ untranslated region and 83-bp polydeoxyadenylic acid [poly(dA)] tail to the 30-bp simian virus 40 primer fragment (20). The plasmid carrying the corresponding cardiac actin 3′ untranslated region (pHMcA-3′UT) was digested with restriction endonucleases *Eco*RI and *Pvu*II. This digest yielded a 306-bp fragment extending from the last 44 bp of coding region through the 183 bp of 3′ untranslated region and 49-bp poly(dA) tail to the 30-bp simian virus 40 primer fragment (20). A DNA fragment encoding the carboxyl terminus of β-actin was prepared from a chicken β-actin cDNA as described previously (4). DNA fragments were labeled by nick translation (21). Labeled fragments were added to all hybridization solutions at a concentration of 10^6 dpm/ml. For quantitative Northern blot and dot-blot analyses, 1 μg of each of the similarly sized skeletal and cardiac actin DNA fragments were nick translated in parallel to give similar specific activities of 10^8 dpm/μg.

RNA gel analysis, dot blots, and hybridization to DNA probes. RNA denatured by treatment with 1 M glyoxal was electrophoresed on 1.2% agarose gels (12). After visualization of the RNA with ethidium bromide, the RNA was transferred to diazobenzyloxymethyl-paper by blotting (1). The diazobenzyloxymethyl filters were first washed with a prehybridization solution and then hybridized with radiolabeled DNA fragments in 50% formamide–50 mM Na_2HPO_4 (pH 7.0)–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt solution as described by Alwine et al. (1). Hybridization for 48 h at 42°C was followed by extensive washing of the filters in 0.5× SSC–0.1% sodium dodecyl sulfate (SDS) at 50°C for 1.5 h and finally in 0.1× SSC–0.1% SDS at 60°C for 30 min.

Serial dilutions of RNA preparations were spotted directly onto nitrocellulose paper (dot blots) exactly as described by Thomas (26). The filters were prehybridized and hybridized according to Thomas's method (26). After washing in 0.5× SSC–0.1% SDS at 50°C for 1.5 h, the filters were subjected to a final wash for 30 min under conditions indicated in the text and figure legends.

RESULTS

Two actin mRNA isotypes in human muscle. We have previously described the isolation of 30 actin-encoding cDNA clones and subsequent

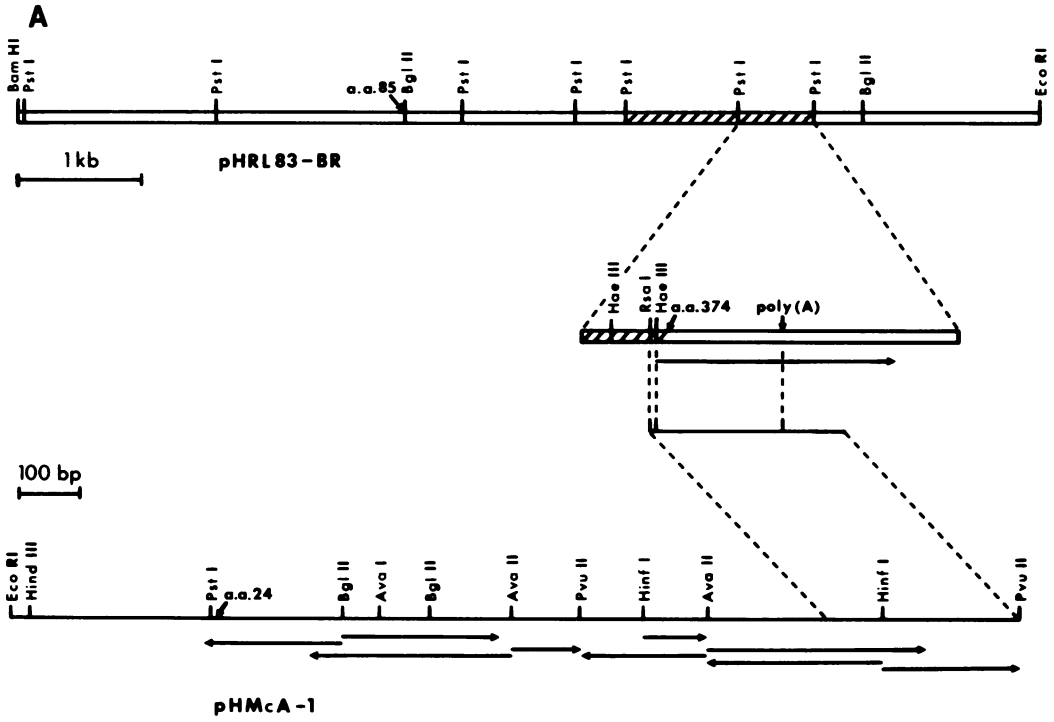

FIG. 1. Restriction map of human cardiac actin genomic clone and cDNA clone. (A) A number of restriction endonuclease cleavage sites were located in the human actin-coding genomic subclone pHRL83-BR. The DNA fragments excised by digestion with the enzyme *Pst*I and containing the 3′ end of the actin-coding region were identified by their ability to hybridize to a probe corresponding to the carboxy-terminal coding region of chicken β-actin cDNA (shown cross-hatched) (4). The 0.6-kilobase DNA fragment generated by cleavage with the restriction endonuclease *Pst*I contained the 3′ end (shown on the expanded scale). A more detailed restriction map of this fragment was determined. A DNA fragment excised by digestion with restriction endonucleases *Hae*III and *Pst*I contains the last 31 bp of coding region plus the 3′ untranslated region and flanking sequences. This fragment was subcloned by inserting it between the *Pst*I and *Eco*RI cleavage sites of PBR322 (pHRL83-3′UT). The *Eco*RI site was regenerated, and the DNA sequence of the inserted fragment was determined from this site. (B) Restriction endonuclease cleavage sites on the human muscle cardiac actin cDNA clone pHMcA-1 were determined by standard mapping techniques. The part of the cDNA clone retained in the 3′ untranslated region subclone pHMcA-3′UT (20) is delineated by the dashed lines and is flanked by *Rsa*I and *Pvu*II cleavage sites. The arrows beneath pHMcA-1 show the direction and extent of DNA sequencing reactions used to determine the nucleotide sequence of this clone. The locations of the poly(A) addition sites are shown for both clones.

identification of a skeletal actin clone from an adult human skeletal muscle cDNA library (6). Preliminary restriction enzyme analysis indicated that all the clones had similar sites for infrequently cutting enzymes. However, to ascertain whether all 30 clones were derived from an identical mRNA type, we took advantage of a unique property of the cDNA cloning method used. All clones constructed by the method of Okayama and Berg (15) extend 5′ from the polyadenylic acid [poly(A)] tail of the mRNA. Accordingly, all clones which contain an actin-coding region must also contain the 3′ untranslated region of the mRNA. Since the 3′ untranslated regions of skeletal acin mRNAs in chicken, rat, and mouse are isotype specific (3, 14, 17, 24), we subcloned this region of the human skeletal actin cDNA clone (20) and used it to probe the remaining 29 human actin isolates. All but two of the actin-coding cDNA clones hybridized strongly. Our probes prepared from similar subclones derived from human β- and γ-actin cDNA clones (20) failed to hybridize to any of the muscle actin isolates. We therefore concluded that these two remaining muscle actin clones encoded neither skeletal actin, β-actin, nor γ-actin.

Adult human muscle expresses a cardiac actin gene. We next demonstrated that the two non-skeletal actin cDNA clones are homologous to a cardiac actin gene contained in a subclone, pHRL83, previously described (4). First, we constructed a 3′ untranslated region subclone from this gene (Fig. 1A) and used it to probe the

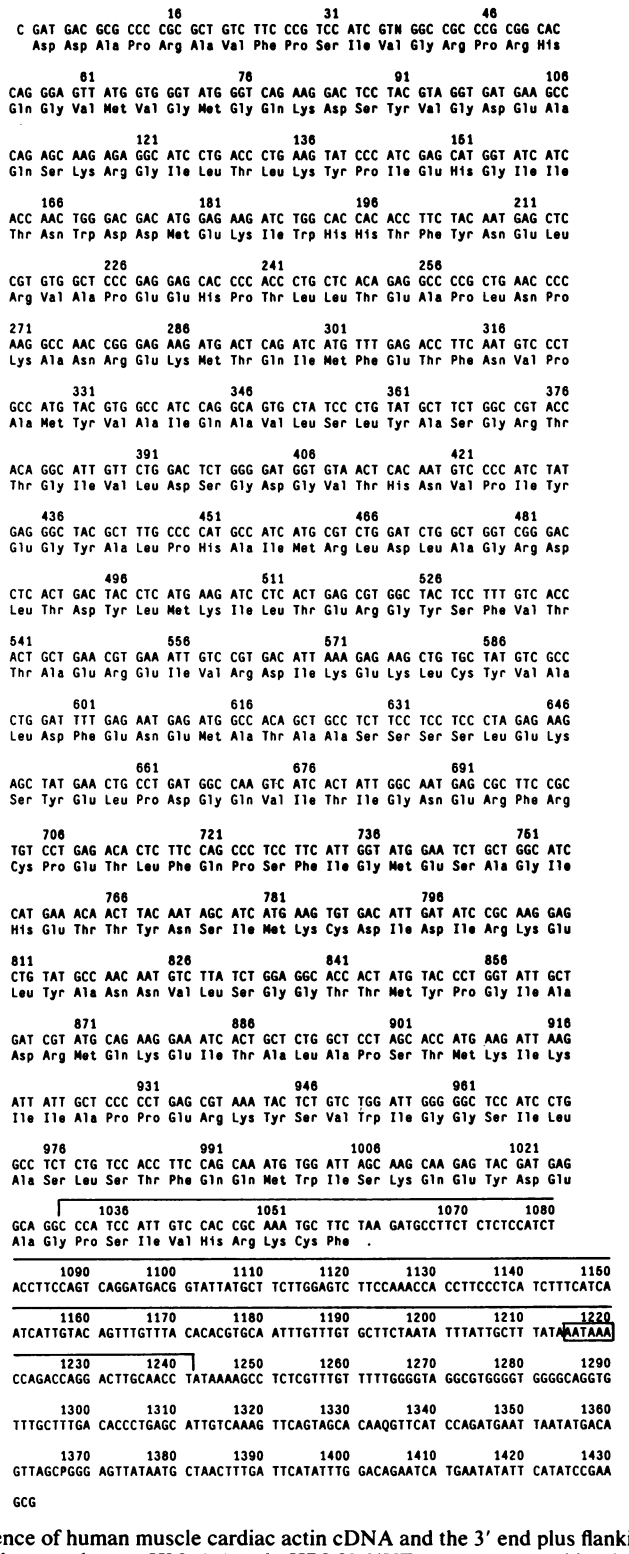

FIG. 2. DNA sequence of human muscle cardiac actin cDNA and the 3′ end plus flanking region of a cardiac actin genomic clone. The two clones pHMcA-1 and pHRL83-3′UT were sequenced by the strategy displayed in Fig. 1. The two sequences are shown as a continuous merged sequence; the region of overlap between the

30 muscle actin cDNAs. The two non-skeletal actin clones hybridized very strongly, whereas no hybridization was detectable to any of the remaining 28 skeletal actin cDNAs. A map of restriction endonuclease cleavage sites within the longer of the two cardiac actin cDNA clones, pHMcA-1, is shown in Fig. 1B. The entire cDNA segment within this clone and the 3′ untranslated region subclone derived from the genomic clone (pHRL83-3′UT) were sequenced by using the strategies described in the legend of Fig. 1.

The cDNA segment in the plasmid pHMcA-1 commences at its 5′ end with the codon for amino acid 24, extends through the remainder of the coding sequence and 3′ untranslated region, and terminates with a 49-bp poly(dA) tail (Fig. 2). The derived amino acid sequence demonstrates that the clone encodes a protein identical to bovine cardiac actin and is thus unambiguously distinguished from the other five mammalian actin isotypes (Fig. 2; 28). The 183-bp 3′ untranslated region of this cardiac actin cDNA contains a single polyadenylation signal sequence, AATAAA, starting 27 bp upstream from the poly(dA) tail.

The 3′ untranslated region of the cardiac actin gene cloned in plasmid pHRL83-3′UT starts with the codon for amino acid 366 and is base-for-base identical to that of the cDNA through the final 10 codons and the entire 3′ untranslated region (Fig. 2). The identity of the gene and the cDNA identifies HRL83 as carrying the gene which gave rise to the mRNA expressed in adult skeletal muscle. Comparison of the cDNA and genomic sequences confirms the absence of intervening sequences within the 3′ untranslated regions of all actin genes studied to date (30). Further sequencing of the genomic subclone 190 bp downstream of the polyadenylation site failed to detect potential alternative polyadenylation signals (except for a AATTAA sequence at position 1347 [Fig. 2] that is similar to known polyadenylation signals).

We have compared both of our sequences with that recently published by Hamada et al. (8) for a human cardiac actin gene. The sequences are identical apart from a few third-base position changes and two differences in the untranslated region (Fig. 2). These differences most likely are due to sequencing errors or reflect allelic polymorphisms.

Specificity of 3′ untranslated region subclones. The construction of 3′ untranslated region subclones of the human β-, γ-, skeletal, and cardiac actin cDNAs has been described elsewhere (20). Computer analysis has demonstrated that none of the 3′ untranslated region DNA sequences are homologous (unpublished data). This has also been confirmed by direct cross-hybridization experiments (20). An example of such an experiment is shown in Fig. 3 and demonstrates that no cross-hybridization is detectable between cardiac and skeletal actin 3′ untranslated probes. Similarly, the cardiac and skeletal actin 3′ untranslated probes do not cross-hybridize to the total skeletal and cardiac actin cDNAs, respectively (Fig. 3).

Expression of skeletal and cardiac actin genes in human muscle and heart. Although the cardiac actin cDNA cloned from adult human muscle establishes that the cardiac actin gene is expressed in that tissue, this does not determine the degree of expression of skeletal relative to cardiac actin genes in muscle. Accordingly, we used quantitative RNA blot transfer analysis to examine the expression of skeletal and cardiac actin genes within adult human skeletal muscle. Different amounts of total muscle RNA were coelectrophoresed in adjacent wells of a 1.2% agarose gel. After transfer to diazobenzyloxymethyl-paper, the blots were hybridized to the skeletal actin probe, and a single RNA of approximately 1.7 kilobases in size (lanes 1 through 3, Fig. 4) was detected. This result indicates that our skeletal actin cDNA clone, pHMαA-1, is close to full length. If one allows for a poly(A) tail of 200 bp, then the skeletal actin cDNA, which has 103 bp of 5′ leader, 1,134 bp of coding sequence, and 253 bp of 3′ untranslated region, predicts a minimum mRNA size of 1,690 bp (6). The cardiac actin probe also hybridized to a single RNA of approximately 1.7 kilobases in size (lanes 5 through 7, Fig. 4). From our direct DNA sequencing data we know that the 3′ untranslated region of the cardiac

sequences of the two clones was identical and is indicated in the figure by overlining. The cDNA clone pHMcA-1 sequence starts at nucleotide 1 and extends to nucleotide 1241. This is followed in the cDNA clone by 49 adenine (A) residues. The polyadenylation site is at nucleotide 1241 or 1242. The nucleotide sequence of the genomic subclone pHRL83-3′UT starts at position 1030 and extends to nucleotide 1433. The derived amino acid sequence is shown below the nucleotide sequence. The poly(A) addition signal (AATAAA) is shown boxed at nucleotides 1215 to 1220. The *Rsa*I cleavage site used to generate the 3′ untranslated subclone of pHMcA-1 is located between nucleotides 1016 and 1017. There are several differences between these sequences and that of the human cardiac actin gene sequenced by Hamada et al. (8). These occur at nucleotides 220, 638, 640, 682, 968, 1122, and 1227 and were reported as T, T, G, C, T, C, and T, respectively, by Hamada et al. (8).

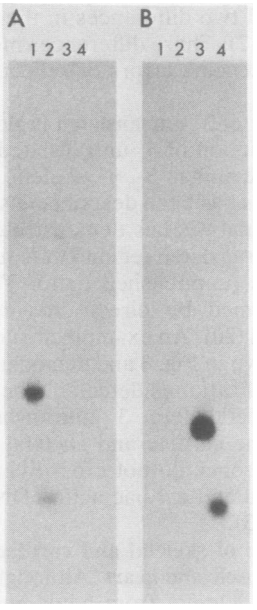

FIG. 3. Hybridization specificity of human muscle skeletal and cardiac actin 3′ untranslated region probes. The skeletal and cardiac actin cDNA clones (pHMαA-1 and pHMcA-1) plus the 3′ untranslated region subclones (20) were digested with appropriate restriction enzymes to generate cDNA fragments free of vector. After electrophoresis on a 1% agarose gel, the fragments were transferred to nitrocellulose and hybridized with a nick-translated skeletal actin 3′ probe (A) or cardiac actin 3′ probe (B), and the filters washed at a final stringency of 0.1× SSC–0.1% SDS at 55°C. The DNA digests for the two blots were identical. Lane 1, pHMαA-1 (see Fig. 1 of reference 6) cut with *Pvu*II plus *Hin*dIII. This digestion generates cDNA insert segments of 1,000 bp (5′ end) and 850 bp (3′ end). Only the 850-bp fragment hybridizes to the probe in (A). Lane 2, pHMαA-3′UT (see reference 20) cut with *Pvu*II plus *Xba*I generates a 400-bp insert that hybridizes to the probe in (A). Lane 3, pHMcA-1 (see Fig. 1) cut with *Pvu*II plus *Hin*dIII generates cDNA insert segments of about 850 bp (5′ end) and 660 bp (3′ end). Only the latter hybridizes in (B). Lane 4, pHMcA-3′UT (see reference 20) cut with *Pvu*II plus *Eco*RI generates a 380-bp insert that hybridizes to the probe used in (B).

actin mRNA is 70 bp shorter than that of skeletal actin mRNA. This suggests that the sum of the lengths of the poly(A) tail and the 5′ untranslated leader segment of the cardiac actin mRNA is slightly longer than the corresponding sum within the skeletal actin mRNA. As expected, no hybridization was observed with either probe to the HeLa cell RNA (lanes 4 and 8, Fig. 4).

The relative abundance of the two mRNAs in the muscle RNA preparation can be measured from a direct comparison of the intensities of hybridization signals. This is possible because the specific activities of the nick-translated cardiac and skeletal actin probes and the conditions used to hybridize them with this RNA blot were virtually identical (see above). The intensities of hybridization seen in the autoradiograph presented in Fig. 4 show that the amount of cardiac actin mRNA present in 20 μg of muscle RNA (lane 7) is very similar to the amount of skeletal actin mRNA present in 1 μg of the same RNA (lane 1). This means that the steady-state level of cardiac actin mRNA is about one-twentieth that of the level of skeletal actin mRNA in adult human muscle. This ratio is similar to the ratio of actin mRNA isotypes among the clones in the muscle cDNA library: 2 cardiac actin clones to 28 skeletal actin clones. Since the muscle RNA preparations used to construct the cDNA library and perform the blot analysis were isolated from different individuals, the ratio of ~20:1 may be common in adult humans.

The expression of cardiac actin mRNA in adult human muscle raises several interesting

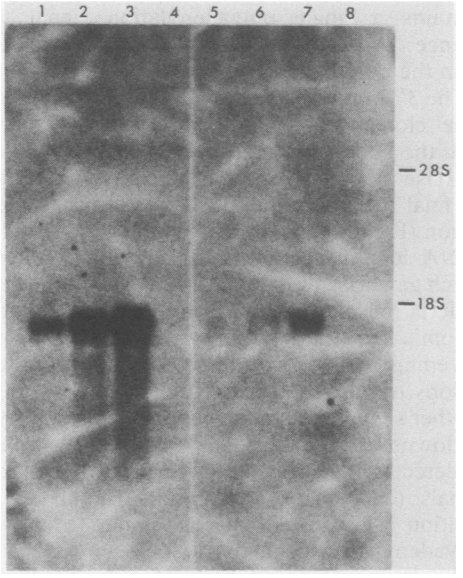

FIG. 4. RNA blot analysis of skeletal actin and cardiac actin mRNA expression in adult human muscle. Samples of adult human skeletal muscle and HeLa cell total RNA were denatured with glyoxal and size fractionated on a 1% agarose gel. After transfer to diazobenzyloxymethyl-paper, the filters were allowed to hybridize to either the skeletal actin (lanes 1 through 4) or the cardiac actin (lanes 5 through 8) 3′ probe. The two probes were isolated as described in the text and nick translated to virtually identical specific activities. The final washing of the filters was conducted in 0.1× SSC–0.1% SDS at 60°C. The human 28S and 18S rRNAs were visualized with ethidium bromide, and their migration positions are shown to the right of the blot. Lanes 1, 2, 3, 5, 6, and 7 contained 1, 5, 10, 5, 10, and 20 μg of adult skeletal muscle RNA, respectively. Lanes 4 and 8 each contained 20 μg of HeLa cell RNA.

questions. (i) Since actins are a multigene family with at least 30 human members (4, 5, 10), is the cardiac actin gene expressed in human muscle the same as or different from the one expressed in the human heart? (ii) Is there a reciprocal expression of skeletal actin mRNA in human heart tissue, and if so, (iii) is it transcribed from the same gene that generates skeletal actin mRNA in muscle?

Although we were fortunate to obtain a sample of adult human heart from a heart transplant patient, the resulting RNA preparation was slightly degraded, possibly because of a 1-h examination of the excised heart in 37°C physiological saline. We therefore chose to use dot-blot analysis rather than Northern blot analysis of the heart RNA to address these questions. Serial dilutions of RNA isolated from adult human skeletal muscle and heart and from HeLa cells were spotted onto quadruplicate dot-blot panels in quantities ranging from 5 to 0.25 μg. After baking and prehybridization treatment, the filters were hybridized with either isotype-specific skeletal or cardiac actin radiolabeled nucleic acid probes or with the non-isotype-specific chicken β-actin-coding region probe. After washing, the filters were dried and autoradiographed (Fig. 5). Figure 5A shows that the skeletal actin probe hybridized not only to skeletal muscle RNA as expected, but to heart RNA as well. The probe did not hybridize with HeLa cell RNA. The intensity of skeletal actin mRNA hybridization in 0.5 μg of skeletal muscle RNA was almost identical to that seen with 2.5 μg of heart RNA. This indicates that skeletal actin mRNA, as a fraction of total RNA, is expressed at significant levels in adult heart: about one-fifth of the level at which it is expressed in adult skeletal muscle. The cardiac actin mRNA probe also hybridized to both skeletal muscle and heart RNA, but not to HeLa cell RNA (Fig. 5B). The intensity of its hybridization to 1.0 and 0.5 μg of heart RNA was comparable to that seen with 5 and 2.5 μg, respectively, of skeletal muscle RNA. Thus, we conclude that cardiac actin is expressed in adult skeletal muscle at about one-fifth of the level at which it is expressed in heart.

The use of skeletal and cardiac actin nucleic acid probes of similar specific radioactivities also allows us to measure directly the relative expression of these two isotypes in each RNA preparation. The intensity of hybridization of the skeletal actin probe to 0.25 μg of skeletal muscle RNA (Fig. 5A) was slightly greater than that observed with the cardiac actin probe to 5 μg of the same RNA (Fig 5B). This result confirms the Northern blot data that cardiac actin mRNA is expressed at about 5% of the level of skeletal actin mRNA in adult human skeletal muscle (Fig. 4). Conversely, each sam-

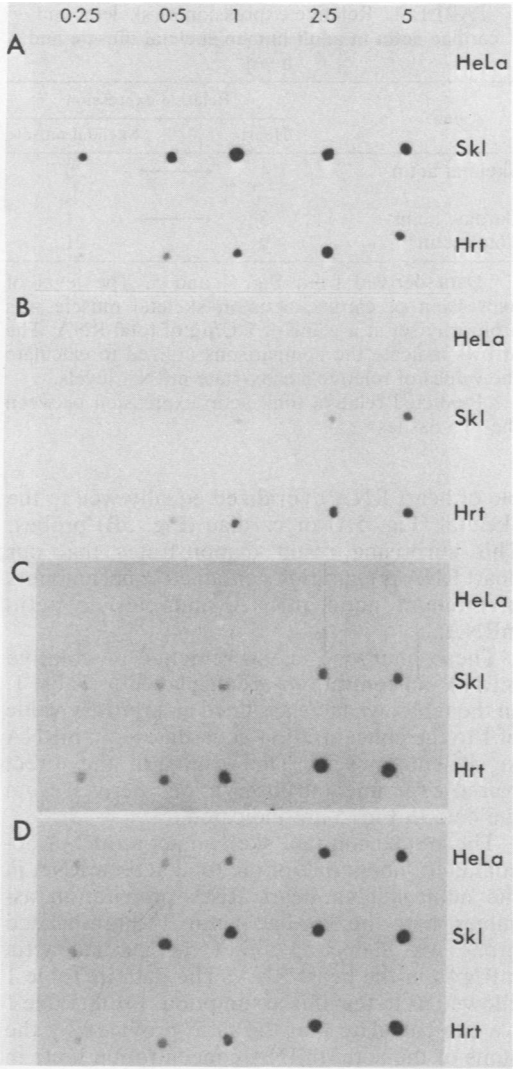

FIG. 5. RNA dot hybridization measurement of expression of skeletal actin and cardiac actin mRNAs in adult human skeletal muscle and heart. Serial dilutions of RNA isolated from adult human skeletal muscle (Skl) and heart (Hrt) and from HeLa cells were spotted onto nitrocellulose paper in quantities ranging from 5 to 0.25 μg as indicated. DNA fragments corresponding to the skeletal actin 3′ probe (A), the cardiac actin 3′ probe (B and C), and the chicken β-actin 3′ coding region probe (D) were nick translated and allowed to hybridize to the indicated panels. Final washing conditions were 0.1× SSC–0.1% SDS at 55°C for (A) and (B), the same salts at 65°C for (C), and 0.5× SSC–0.1% SDS at 50°C (D). The skeletal actin and cardiac actin probes were radiolabeled by nick translation to virtually indistinguishable specific activities, and thus quantitative comparisons between the intensity of autoradiographic exposures between (A) and (B) are a direct measure of relative concentrations of the specific mRNA moieties.

TABLE 1. Relative expression of skeletal and cardiac actin in adult human skeletal muscle and heart[a]

Gene	Relative expression		
	Heart		Skeletal muscle
Skeletal actin	4	⟷	20
			↕
Cardiac actin	5	⟷	1
Total actin[b]	9		21

[a] Data derived from Fig. 4 and 5. The level of expression of cardiac actin in skeletal muscle was arbitrarily set at a value of 1 U/μg of total RNA. The arrows indicate the comparisons utilized to calculate the values of relative steady-state mRNA levels.

[b] Predicted relative total actin expression between the two tissues.

ple of heart RNA hybridized equally well to the skeletal (Fig. 5A) or cardiac (Fig. 5B) probes. This surprising result demonstrates that our heart RNA preparation contained equal amounts of skeletal actin mRNA and cardiac actin mRNA.

These pairwise measurements of relative mRNA concentrations are displayed in Table 1. In the table, we have assigned an arbitrary value of 1 to the concentration of cardiac actin mRNA in skeletal muscle. The results of the direct paired experimental observations derived from the data in Fig. 5 are indicated.

The conclusion that skeletal actin mRNA accounts for about half of the total actin mRNA in the adult human heart RNA preparation assumes that the cardiac actin 3′ untranslated probe was detecting all of the cardiac actin mRNAs in the heart RNA. The data in Table 1 allowed us to test this assumption. From Table 1 we can calculate that the ratio provided by the sums of the actin mRNA concentration units in muscle (20 + 1 = 21) to the actin mRNA concentration in heart (4 + 5 = 9) is 21/9 = 2.3. This calculated ratio predicts that the total concentration of actin mRNA in skeletal muscle RNA is twice that in heart muscle mRNA. We tested this prediction directly by comparing the total actin mRNA levels in muscle and heart. We used a chicken β-actin carboxyl terminal coding region fragment as a probe. This fragment has been shown to hybridize strongly to all human actin genes (4, 5). The data in Fig. 5D demonstrate that the hybridization intensity observed with 0.25, 1.0, and 2.5 μg of muscle RNA closely paralleled that observed with 0.5, 2.5 and 5 μg, respectively, of heart RNA. We conclude that total actin mRNA levels, as a fraction of total RNA, are indeed about twofold higher in adult skeletal muscle than in adult heart. This value indicates that cardiac actin transcripts homologous to the clone pHMcA-1 are the major cardiac actin-encoding mRNAs of adult heart and confirms the nearly identical quantities of skeletal actin mRNA and cardiac actin mRNA in heart muscle.

Number of expressed actin genes in muscle and heart. We next attempted to establish whether or not the expression of skeletal and cardiac actin in the skeletal muscle and heart tissues reflects the transcription of two single-copy genes. Analysis of human genomic DNA has demonstrated that the skeletal actin gene is single copy (20), and thus the skeletal actin mRNA expressed in both muscle and heart must be the product of the same gene. In contrast, when the cardiac 3′ untranslated region is used as a probe in genomic blotting experiments, it shows weak homology to three other genomic loci in addition to the corresponding transcribed gene (20). Under high-stringency washing conditions of 0.1× SSC at 65°C, the hybridization of the cardiac probe to these other genomic loci is totally eliminated (20). Thus, we reasoned that if the hybridization of the cardiac actin probe to both heart and skeletal muscle RNA was unaffected by the stringency of hybridization and wash conditions, then the cardiac mRNAs in both tissues were probably products of the same gene. Accordingly we hybridized a dot-blot panel with the cardiac probe and washed it under high stringency. Figure 5C shows that the relative expression of cardiac actin between skeletal muscle and heart was unaffected by this procedure. The intensity of hybridization observed with 0.5 and 0.1 μg of heart RNA was again very similar to that obtained with 2.5 and 0.5 μg, respectively, of skeletal muscle RNA. This result suggests that the same cardiac actin gene is expressed in both tissues.

DISCUSSION

Cardiac actin gene expression in adult human tissues. In contrast to the findings of Vandekerckhove and Weber (27) in nonprimate mammals, we have demonstrated that in adult humans the cardiac actin gene is not expressed in heart tissue exclusively but is also significantly expressed in skeletal muscle. Results from two independent experimental approaches demonstrate that cardiac actin mRNA accounts for about 5% of total actin mRNA in skeletal muscle. Furthermore, the skeletal muscle cDNA library which yielded two cardiac actin clones from a total of 30 actin clones was constructed with RNA from a different patient from the one who provided the sample for quantitative Northern blot analysis.

Recently, Minty et al. (13) described the isolation of a cardiac actin cDNA clone from a neonatal mouse skeletal muscle cDNA library. They further demonstrated that skeletal and

cardiac actin mRNAs are coinduced during early myogenesis in mouse. In contrast to skeletal actin, cardiac actin gene expression was observed to decline to undetectable levels (≤5%) in adult mouse skeletal muscle. This observation does not necessarily conflict with our data since we are using a more sensitive assay. Whereas they used a cardiac actin-coding region sequence to measure cardiac versus skeletal actin mRNA concentrations (13), we have used isotype-specific 3′ untranslated region probes.

Does the low level of expression of cardiac actin in human skeletal muscle reflect a truly physiological level of regulated expression or rather a persistent "leakage," perhaps resulting from initially high expression during fetal myogenesis? Although 5% of total actin mRNA may seem to be a relatively low quantity of cardiac actin mRNA, comparison of this to actin expression in heart suggests that this is not the case. As a fraction of total RNA, cardiac actin is expressed in skeletal muscle at 20% of its level in adult heart. Thus, the level of cardiac actin mRNA expression in skeletal muscle, when considered in absolute terms (i.e., per microgram of RNA), is quite significant when compared with total actin expression in heart. It is, rather, the exceptionally high level of expression of skeletal actin mRNA in skeletal muscle that overshadows cardiac actin mRNA levels. Whether these mRNA ratios of relative expression are directly comparable to ratios of relative steady-state protein levels is unknown, and such measurements may prove particularly difficult (29).

The human cardiac actin gene previously described by Engel et al. in phage HRL83 (4) has been demonstrated by DNA sequence analysis of the 3′ untranslated region subclone (pHRL83-3′UT) to be the same gene recently sequenced and reported by Hamada et al. (8). Thus, the mRNA used as the template for pHMcA-1 must be a transcript of the gene carried in HRL83. This establishes this gene as the major if not sole cardiac actin gene expressed in adult human heart and skeletal muscle. It is thus the first human actin gene, of the more than 30 which exist in the human genome, unequivocally identified as an expressed gene.

We have recently demonstrated the presence of human genomic DNA fragments, in addition to the 13-kilobase *Eco*RI fragment represented in the clone HRL83, that have limited homology to the 3′ untranslated region of the cardiac actin cDNA (20). A similar observation was also made by Hamada et al. (8), using a probe derived from their genomic cardiac actin clone. These observations raise questions concerning the number of human cardiac actin genes and their potential for expression. Thus the possibility remains that the human genome contains functional cardiac actin genes other than the one found in HRL83.

Skeletal actin gene expression in adult human tissues. The skeletal actin mRNA accounts for about 95% of the total actin mRNA level of adult skeletal muscle. This measurement is based upon direct Northern blot analysis and parallels the findings of others in avian (23) and mammalian skeletal muscle systems (13, 24). However, the detection of skeletal actin mRNA expression in human heart, particularly at levels approaching 50% that of cardiac actin mRNA, is at considerable variance with other mammalian studies. Shani et al. (24) first reported that probes derived from the 3′ untranslated region of a rat skeletal actin gene hybridize to adult rat heart RNA at about one-fiftieth the level of rat muscle RNA. Minty et al. (13) performed a similar experiment in mouse cells and concluded that skeletal actin mRNA is expressed at less than or equal to 2% of cardiac actin mRNA levels in mouse heart. The essential difference between the observations lies to the extent to which skeletal actin mRNA is expressed relative to cardiac actin mRNA in rodent heart muscle versus human heart muscle. The difference may, at least in part, reflect the pathophysiological status of the tissue analyzed. In our case, the human RNA sample was obtained from a diseased heart, whereas the rats and mice analyzed were presumably healthy. It is an intriguing and testable possibilty that alterations in cardiac physiology may be associated with an alteration in the relative expression of skeletal and cardiac actin mRNA. Thus, although skeletal actin expression may be a general feature in mammalian adult hearts, the level of its expression many show species differences, physiological regulation, or both. In any case, our data clearly demonstrate that human heart is capable of expressing skeletal actin mRNA at a level similar to that of cardiac actin mRNA.

Role of actin isotypes. The spectrum of actin isotype expression within different mammalian tissues is now more complex with the advent of more sensitive isotype-specific assays. There is no longer any mammalian tissue known to express only a single actin isotype. Furthermore, it now appears that the actins are usually expressed in isotypic pairs: the two cytoplasmic actins in nonmuscle cells, the two smooth-muscle actins in smooth muscle, and the two sarcomeric actins (skeletal and cardiac) in skeletal and heart muscle. In addition, some tissues may be able to express up to four actin isotypes. Rubenstein and Spudich (22) reported the detection of an α-like actin protein in chicken embryo fibroblasts in addition to the two cytoplasmic actins. More recently, it has been demonstrated that this α-like actin, corresponds to aortic smooth-muscle actin (29). Ordahl et al. (17)

reported the transitory appearance of an α-like actin mRNA in developing chicken brain. Hall et al. (7) have demonstrated the presence of a cytoplasmic actin in the postsynaptic densities of adult rat skeletal muscle fibers. Finally, Vandekerckhove and Weber (29) have recently reported the coexistence of both the two smooth-muscle and the two cytoplasmic actins within rat aorta and vena cava and the tenia coli of rat and cow. Most of these studies have the shortcoming that they deal with mixed cell populations characteristic of organs. Nevertheless, it seems likely that, with the development of additional actin isotype-specific mRNA probes, detection of minor actin mRNA isotypes will increase.

What is the possible significance of paired actin gene expression? The partners of each of the three actin gene pairs represent the most recent gene duplication events as evidenced by the fact that the partners are more closely related to each other than to members of either of the other two pairs (28). Accordingly, genomic regions regulating gene expression may have partially diverged between partners since the duplication events occurred. If this is the case, then there may still be residual cross-responsiveness (leakiness) of such promoter regions of paired members to cellular or environmental stimuli. Preliminary data have demonstrated that the human skeletal actin and cardiac actin genes are on separate autosomes (T. Shows, P. Gunning, P. Ponte, and L. Kedes, unpublished data). Thus it is not the case that these two genes are reciprocally activated because they are in close chromosomal proximity.

Coexpression of these gene pairs may be physiologically relevant rather than purely coincidental. Both rats and mice express skeletal actin mRNA at a level less than 2% that of cardiac actin mRNA in adult heart (13, 24). In a pathophysiologically abnormal human heart we found that the skeletal actin expression was much greater and approached that of cardiac actin. Whether this was due to the intrinsic disease process or is true of human heart in general remains to be determined. In either case, these observations suggest that the relative ratios of this actin pair may be under regulation. This might reflect subtle differences between the different actin gene products. At the protein level, the differences between the six actin isotypes might allow cells to change their cytoarchitecture through the alteration of interactions between actin filaments and other cytoskeletal components. This possibility is supported by the observation that the amino terminus of actin is both the site of major actin-myosin interactions (25) and the location of most of the amino acid differences between actin proteins (28).

Alternatively, differences in the untranslated segments of the actin mRNAs might be related to physiological regulation. There is striking conservation in evolution of parts of the 3′ untranslated regions of skeletal actin mRNAs even between species as distant as human and chicken (16, 20). The same conservation holds for the 3′ untranslated region of cardiac mRNA (P. Gunning, P. Ponte, H. Blau, and L. Kedes, manuscript in preparation). Such conserved regions might confer specific selectable properties on the mRNAs that could, for example, affect their stability, translational capacity, or intracellular location. At this time, there are no conclusive data to preferentially support any one of these possibilities. Nevertheless, the role of differentially expressed isotypes is one of the central problems of actin biology, and the resolution of this question will provide insight not only into the function of these genes but also into the evolutionary significance of multigene families.

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