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Human Actin Genes Are Single Copy for α-Skeletal and α-Cardiac Actin but Multicopy for β- and γ-Cytoskeletal Genes: 3′ Untranslated Regions Are Isotype Specific but Are Conserved in Evolution

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We have constructed isotype-specific subclones from the 3′ untranslated regions of α-skeletal, α-cardiac, β-cytoskeletal, and γ-cytoskeletal actin cDNAs. These clones have been used as hybridization probes to assay the number and organization of these actin isotypes in the human genome. Hybridization of these probes to human genomic actin clones (Engel et al., Proc. Natl. Acad. Sci. U.S.A. 78:4674–4678, 1981; Engel et al., Mol. Cell. Biol. 2:674–684, 1982) has allowed the unambiguous assignment of the genomic clones to isotypically defined actin subfamilies. In addition, only one isotype-specific probe hybridizes to each actin-containing gene, with a single exception. This result suggests that the multiple actin genes in the human genome are not closely linked. Genomic DNA blots probed with these subclones under stringent conditions demonstrate that the α-skeletal and α-cardiac muscle actin genes are single copy, whereas the cytoskeletal actins, β and γ, are present in multiple copies in the human genome. Most of the actin genes of other mammals are cytoplasmic as well. These observations have important implications for the evolution of multigene families.

The actins comprise a group of proteins which are highly conserved in evolution. There are six distinct actin isotypes found in mammals (21). These isotypes differ from each other by only a few amino acids; most of these differences are clustered at the amino terminus. Two smooth muscle isotypes (22) and two striated muscle isotypes, α skeletal and α cardiac (21), are integral components of the highly ordered contractile apparatus of muscle fibers. The cytoskeletal actins, β and γ, are part of the complex network of filaments that make up the cytoarchitecture of every nonmuscle cell (20). There may be additional actins present in low abundance which could so far have escaped detection. In this regard, we have previously reported the presence of greater than 30 actin-coding fragments in the human genome (7, 8), although some of these have recently been found to be actin pseudogenes (15a, 19; J. E. Engel, doctoral dissertation, Stanford University, Palo Alto, Calif.). It is not clear what selective advantage, if any, is associated with the organization of actin genes as a multigene family.

We have begun a systematic survey of the structure, organization, and evolution of actin genes and mRNAs to improve our understanding of the functional significance of actin heterogeneity. We have previously described the isolation of numerous actin genes from a human genomic library (7, 8). Recently, we have constructed nearly full-length cDNA clones for four human actin isotypes, α skeletal, β cytoskeletal, γ cytoskeletal (10), and α cardiac (P. Gunning, P. Ponte, H. Blau, and L. Kedes, Mol. Cell. Biol., in press) (referred to here as α, β, γ, and cardiac). In this work, we describe the construction of 3′ untranslated (3′-UT) region subclones from each of these actin cDNAs. We demonstrate that these subclones have no homology to one another, and thus define isotype-specific markers. These clones have permitted the unambiguous classification according to isotype of different actin genes and mRNAs which previously could only be distinguished by differential hybridization to actin-coding region probes. We have used the isotype-specific clones as probes in RNA and DNA blot hybridization experiments to characterize the members of the four isotypically defined actin subfamilies. We conclude that although genes for α and cardiac actins are probably single copy, there are multiple copies of DNA segments of heterogeneous size which contain sequences which are highly homologous to the cytoplasmic actin probes. Additionally, we find that the 3′-UT regions of

the actin genes are conserved in vertebrates, in disagreement with previous data, which suggested that the 3'-UT regions of actin genes diverge rapidly (6). By using this cross-species isotype-specific hybridization, we show that the relative ratios of the actin genes for different isotypes in other mammals parallel that seen in humans.

MATERIALS AND METHODS

General methods. Growth and transformation of *Escherichia coli*, colony hybridization (9), and purification of plasmid DNA followed standard protocols as previously described (5). Preparation of Charon phage recombinant DNA and phage gels, blots, and hybridization conditions were as previously described (7). Genomic DNA preparation and digestion and DNA gels, blots, and hybridization in the presence of dextran sulfate were performed as previously described (17) with the following exception: to improve the signal to noise ratio when genomic Southern blot hybridizations were performed in the presence of dextran sulfate, a gel-purified fragment containing the sequences of interest, instead of whole plasmid, was the substrate for nick translation (18). When the insert was short (less than 300 base pairs), it was concatenated by self-ligation with T4 ligase before nick translation. HeLa cells were grown and maintained as previously described (7). For RNA analysis, pure populations of primary human myoblasts were grown in tissue culture as previously described (3). Preparation of RNA (4) from HeLa and human myoblast cells, gel electrophoresis of glyoxal-treated RNA samples (14), and blot conditions and filter hybridizations (1) were all performed as previously described (17).

Construction of actin subclones. The strategy for the construction of actin isotype-specific 3'-UT region subclones was devised from DNA sequence data which indicated the location of restriction sites relative to the coding and noncoding regions of the actin cDNA clones (P. Ponte and P. Gunning, unpublished data; J. E. Engel, doctoral dissertation; Gunning et al., in press).

We subcloned the 3'-UT region of the α-actin cDNA by creating a deletion in the original cDNA clone (see Fig. 1A). The α-actin cDNA clone, pHMαA-1 (10), was first cut with both *Hin*dIII and *Xba*I. The larger of the two resulting fragments contains the 3'-UT region of the insert starting at the *Xba*I site in the termination codon. The resulting assymetric ends of this fragment were filled in by incubation with *E. coli* DNA polymerase (Klenow fragment) in the presence of 50 mM Tris-hydrochloride (pH 7.5); 50 μM each of dATP, dCTP, dGTP, and TTP; 1 mM dithiothreitol; and 5 mM $MgCl_2$ at 16°C for 2 h. The DNA was then diluted to <1 μg/ml to minimize interstrand contacts and was religated as described previously (10). We transformed *E. coli* (HB101) with this ligation mixture and screened Amp^r colonies for the absence of actin-coding sequences and for the regeneration of the *Xba*I restriction site. Candidate clones were further characterized by their ability to hybridize to the original cDNA insert.

A similar deletion subcloning approach was employed to construct a cardiac actin 3'-UT-region clone (Fig. 1B). The cardiac actin cDNA, pHMcA-1 (Gunning et al., in press), was linearized with *Hin*dIII and subjected to partial digestion with *Rsa*I. The DNA digest was analyzed by electrophoresis on a 1% agarose gel. The band corresponding to a deletion from the *Hin*dIII site of the simian virus 40 linker to the *Rsa*I site at amino acid 361 was identified by coelectrophoresis with size standards. DNA was isolated from the appropriately sized gel slice, the *Hin*dIII end filled in, and the plasmid recircularized as described above. Bacterial transformants were screened for the absence of actin-coding sequences, for the presence of appropriately sized restriction fragments, and by their ability to hybridize to the original cDNA insert.

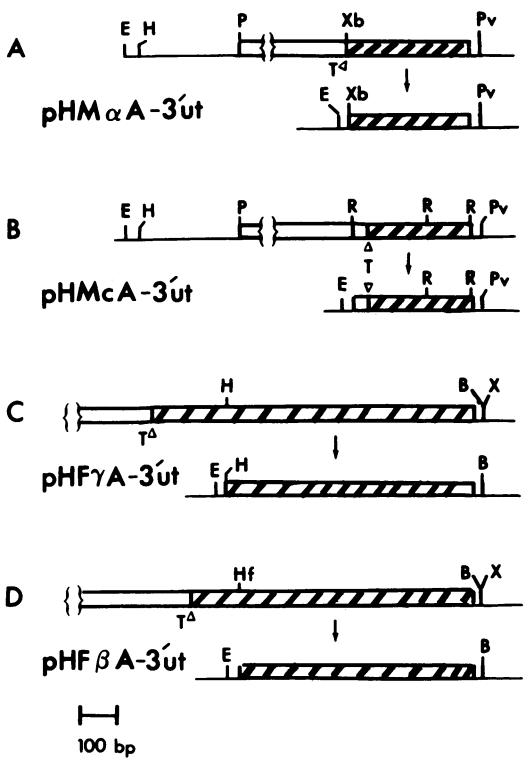

FIG. 1. Construction of actin 3'-UT-region subclones. A detailed description of the construction is given in the text for α (A), cardiac (B), γ (C), and β (D) actin. In each case, the upper portion of the figure represents the parent actin cDNA clone, and the lower portion shows the 3'-UT-region subclone. The name of the subclone is given to the left of each figure. A single line indicates a vector region, open-boxed space indicates actin-coding region, and hatched-boxed space delimits the 3'-UT region. The position of the termination codon is marked by a T. Other letters indicate restriction endonuclease sites: E, *Eco*RI; H, *Hin*dIII; Hf, *Hin*fI; X, *Xho*I; Xb, *Xba*I; P, *Pst*I; Pv, *Pvu*II; R, *Rsa*I; B, *Bam*HI.

The 3'-UT regions of β- and γ-actin cDNAs (10) were both subcloned by first isolating the desired DNA fragment from the appropriate clone. This fragment was then used to replace the 404-base pair *Hin*dIII-*Bam*HI fragment of pBR322.

To clone the 3'-UT region of γ actin, pHF1 was

digested with *Hin*dIII and *Bam*HI (see Fig. 1C). A 700-base pair *Hin*dIII-*Bam*HI fragment so obtained corresponds to the 3′ terminus of the cDNA insert. An equimolar amount of vector and cDNA fragments were then ligated as previously described. Plasmid DNA isolated from colonies of *E. coli* transformed with this mixture was characterized by the presence of a 700-base pair *Bam*HI-*Hin*dIII fragment and by their ability to hybridize to the original cDNA insert.

A 665-base pair *Hin*fI-*Bam*HI fragment was isolated from the β-actin clone HF5 (see Fig. 1D). This segment contains the 3′ terminus of the cDNA insert. Equimolar amounts of this fragment and the pBR322 fragment were ligated at the *Bam*HI site. Next, the ends were filled with Klenow fragment and deoxynucleotide triphosphates as described earlier. The blunt ends were ligated, and the DNA was used to transform *E. coli*. Plasmid DNA from selected transformants was characterized by the presence of a 700-base pair *Eco*RI-*Bam*HI fragment and by their ability to hybridize to the original cDNA insert.

A cardiac intron clone, pHRL83-IVS-IV, was constructed in the following manner: the 14-kilobase (kb) actin-containing *Eco*RI fragment of HRL83, a human genomic actin clone previously shown to encode a cardiac actin gene (8, 11), was isolated and digested with endonuclease *Pst*I. Among the fragments produced, there is a 0.4-kb *Pst*I fragment derived totally from intron IV of this gene (11). The digested DNA was size fractionated by electrophoresis on an agarose gel. The 0.4-kb fragment was electroeluted from the gel and ligated into the *Pst*I site of pBR322. *E. coli* were transformed with the resulting plasmid, and plasmid DNA from transformed colonies was assayed by the size of the *Pst*I insert contained and by the ability to hybridize to HRL83.

RESULTS

Construction of isotype-specific DNA subclones from the 3′-UT regions of actin-cDNA clones. We have previously isolated and characterized cDNA clones encoding four different actin isotypes (α, β, γ [10], and cardiac [Gunning et al., in press]). The DNA sequence of most of each of the clones has been determined. Although the coding regions are all highly homologous, there is no significant homology in the 3′-UT regions (P. Ponte and P. Gunning, unpublished data; J. E. Engel, doctoral dissertation). Therefore, we constructed 3′-UT-region subclones of each actin cDNA for use as isotype-specific probes. Figure 1 indicates the region of each cDNA which was used. (A complete description of the details of the subclone construction and of the clone characterization is described above). The α-actin subclone (Fig. 1A) contains the complete 253-base pair 3′-UT region, followed by an 83-base pair polydeoxyadenylic acid tail (P. Gunning, unpublished data). The cardiac actin subclone (Fig. 1B) contains the 3′ terminal 44 base pairs of actin-coding region, the 183-base pair 3′-UT region flanked by a 49-base pair polydeoxyadenylic acid tail (Gunning et al., in press). The γ-actin subclone (Fig. 1C) includes 540 of the 750-base pair 3′-UT region, ~130 base pairs of polydeoxyadenylic acid, and 30 base pairs of simian virus 40 linker (J. E. Engel, doctoral dissertation). The β-actin subclone (Fig. 1D) contains 460 of the 595-base pair 3′-UT region, 175 base pairs of polydeoxyadenylic acid, and 30 base pairs of simian virus 40 linker (P. Ponte, unpublished data).

Size of human actin mRNAs. The actin subclones were used to assess the size of human actin mRNAs in blot hybridization experiments. Total HeLa cell RNA (10 μg) or total human myoblast RNA (10 μg) was electrophoresed on a 1.2% agarose gel, transferred to diazobenzyloxymethyl-paper, and hybridized with ^{32}P-labeled γ-actin-specific clone. The resulting autoradiogram shows that this probe detects an actin mRNA of 2,150 bases (Fig. 2, lanes 1 and 4). The probe was then removed from the filter, and the filter was rehybridized with a β-actin-specific probe. In this case, an mRNA of 1,950 bases is detected (Fig. 2, lanes 3 and 6). To demonstrate that the β and γ probes detect two closely migrating but distinct actin mRNAs, the filter was then rehybridized to a mixture containing both probes. The autoradiogram shows two bands of actin hybridization (Fig. 2, lanes 2 and 5) coincident with the β- and γ-actin mRNAs. Thus, contrary to previous observations, human β- and γ-actin RNAs can be distinguished by

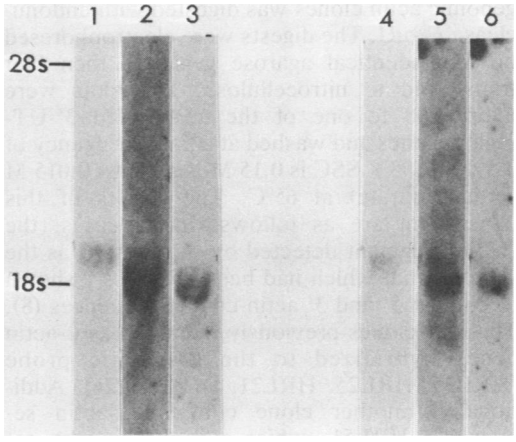

FIG. 2. Sizes of human cytoplasmic actin mRNAs. HeLa cell (lanes 1, 2, and 3) and human myoblast (lanes 4, 5, and 6) RNA (10 μg) was treated with glyoxal, electrophoresed on a 1.2% agarose gel, transferred to diazobenzyloxymethyl-paper, and hybridized to a γ-specific probe (lanes 1 and 4), a β-specific probe (lanes 3 and 6), or both (lanes 2 and 5). The sizes of the RNAs were calculated from the positions of RNAs (indicated to the left of the figure) detected by ethidium bromide staining of the gel.

size under denaturing conditions. This size difference is seen in two very different cell types, a transformed line and a primary cell culture. In contrast, α- and cardiac actin probes hybridize to mRNAs in human adult skeletal muscles which are indistinguishable by size (1,700 base pairs) (Gunning et al., in press).

Classification of genomic clones as members of actin subfamilies. We have previously characterized 12 distinct, non-allelic human actin-containing genomic clones (7, 8). The clones were preliminarily classified (i) by their ability to hybridize to both 5′ and 3′ chick actin DNA probes and (ii) on the basis of the thermal stabilities of RNA-DNA hybrids. By using these criteria, we were able to classify nine of the clones into three types (β, β or γ, and α-like), yet several unresolved questions remained. Could the 3′-UT-region clones by used to assign unambiguously the genomic clones as members of isotypically defined subfamilies of actin genes? Did actin genes of the same isotype have homologous 3′-UT regions or are these regions unique for each expressed gene? Could different isotype-specific probes hybridize to the same actin-containing genomic clone? Since the isotype-specific 3′-UT-region clones shared no sequence homology, hybridization of more than one of these probes with a genomic clone could suggest close linkage or clustering of different actin genes in the genome. Alternatively, it might suggest whether recombination among actin genes or pseudogenes occurs.

To address these questions, DNA from the 12 genomic actin clones was digested with endonuclease *Eco*RI. The digests were electrophoresed on four identical agarose gels and then blot transferred to nitrocellulose. The blots were hybridized to one of the ^{32}P-labeled 3′-UT-region clones and washed at a final stringency of 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C. The results of this experiment are as follows. In all cases, the *Eco*RI fragment detected by the probe was the same as that which had been previously shown to contain 5′ and 3′ actin-coding sequences (8). The four clones previously identified as β-actin genes hybridized to the β-specific probe (HRL35, HRL25, HRL21, and HRL24). Additionally, another clone containing actin sequences, HRL51, which was previously not classified because it did not hybridize to actin mRNA in a positive mRNA selection experiment, showed strong hybridization to the β-actin-specific probe. The β probe did not hybridize to any other of the 12 actin genomic clones. Four clones (HRL34, HRL84, HRL23, and HRL45) hybridized exclusively to the γ-specific clones. Previously, these clones could only be identified as either β or γ clones on the basis of positive mRNA selection and thermal melt experiments.

These data are presented in a more compact form in Fig. 3. This figure shows the results of an experiment in which DNA from β- and γ-actin genomic clones was coelectrophoresed to demonstrate the specificity of the β- and γ-actin subclones. DNA from β- and γ-genomic clones was digested with endonuclease *Eco*RI. DNA from at least one β- and one γ-genomic clone was coelectrophoresed in a single gel lane. Duplicate blots were made, and these were hybridized with either a β-specific probe (Fig. 3, lanes 1, 3, and 5) or a γ-specific probe (Fig. 3, lanes 2, 4, and 6). Lanes 1 and 2 of Fig. 3 contain DNA from HRL25, HRL34, and HRL35; lanes 3 and 4 contain DNA from HRL21, HRL23, and HRL84; lanes 5 and 6 contain DNA from HRL24 and HRL45. No hybridization of β probe to γ genes (or vice versa) is seen.

As expected, the genomic clone known to encode cardiac actin (11; Gunning et al., in press), HRL83, hybridized to the cardiac probe (Fig. 3, lane 7). DNA sequencing of both the cardiac actin cDNA and HRL83 have conclusively shown that the cDNA derives from mRNA transcribed from this gene (Gunning et al., in press). Surprisingly, HRL51, which shows strong homology to the β-actin probe (Fig. 3, lane 8), also hybridizes to the cardiac actin probe (Fig. 3, lane 9). This result is not due to the presence of 44 base pairs of actin-coding region in the cardiac actin 3′-UT-region clone,

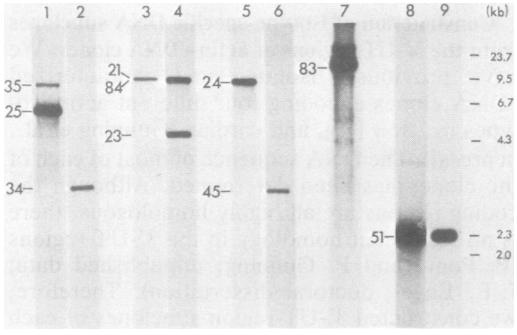

FIG. 3. Hybridization of genomic actin-containing phage DNA to isotype-specific probes. Phage DNA (0.5 μg) was digested with *Eco*RI, electrophoresed on a 0.8% agarose gel, blot transferred to nitrocellulose, and hybridized to an isotype-specific 3′-UT-region actin probe. The number of the genomic clone contained in each lane or duplicate set of lanes is indicated to the left. Size markers are indicated to the right of the figure. The probes hybridized to each lane are as follows: β, lanes 1, 3, 5, and 8; γ, lanes 2, 4, and 6; cardiac, lanes 7 and 9. The additional bands of hybridization seen in lane 7 are the result of incomplete digestion of clone HRL83 DNA.

since none of the other 10 genomic actin genes shows any hybridization to this probe even after long exposures (data not shown). This curious clone contains 5′ and 3′ actin-coding sequences as well as β- and cardiac actin 3′-UT-region sequences on a single 2.3-kb *Eco*RI fragment. Since 2.3 kb of DNA is barely large enough to accommodate a single actin gene with no introns, we suspect that this clone contains an actin pseudogene.

The α-actin-specific clone did not hybridize to any of the 12 genomic clones. This result was expected, since only HRL83, the cardiac actin gene, was capable of selectively hybridizing to α-actin mRNA in positive mRNA selection and thermal melt experiments.

Two of the actin clones, HRL54 and HRL65, did not hybridize to any of the 3′-UT-region probes. Under stringent hybridization conditions, these clones also do not hybridize to actin mRNA from a pool containing α-, β-, and γ-actin mRNA (8). Possibly, these clones contain smooth muscle actin genes.

In conclusion, each genomic actin clone which hybridizes to a cytoplasmic or skeletal muscle actin mRNA under stringent conditions can be definitively assigned to a specific actin isotype by hybridization to the 3′-UT-region probes described above. Therefore, the 3′-UT regions of actin genes define distinct subfamilies of the actin gene family. With one exception, hybridization of an isotype-specific probe to a genomic clone precludes hybridization to any other genomic clone, suggesting that the multiple actin genes are not closely linked in the human genome.

Copy number of specific actin genes. We had previously estimated that more than 30 actin-coding segments are present in the human genome (7). To determine precisely the number of α-, β-, γ-, and cardiac actin genes present in the human genome, we used the isotype-specific probes in DNA blot hybridization experiments.

To analyze the number of DNA segments containing sequences which hybridize to the α-actin clone, we digested HeLa cell DNA with each of five restriction endonucleases. Four of the enzymes do not cut the α-actin cDNA clone. A *Sac*I site bisects the α-actin 3′-UT region (P. Gunning, unpublished data). The digested genomic DNA was electrophoresed on an agarose gel, blot transferred to nitrocellulose, and analyzed for the size and number of fragments hybridizing to an α-actin-specific probe. A single strong band of hybridization is seen in each lane (Fig. 4, lanes 1, 2, 4, and 5), except for two bands of hybridization seen in the lane containing *Sac*I-digested DNA (Fig. 4, lane 3). We conclude from this experiment that the α-actin gene is single copy.

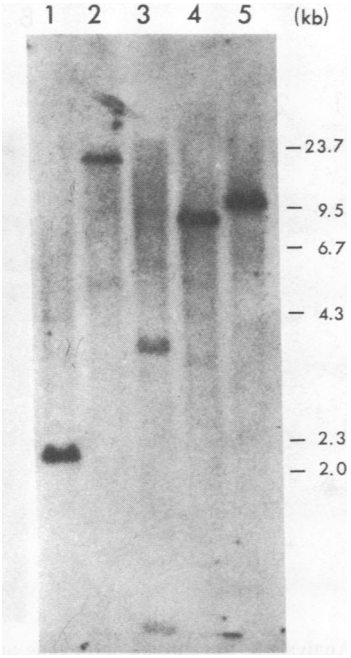

FIG. 4. Detection of the α-actin gene in the human genome. DNA (8 μg) was digested with *Bgl*II (1), *Eco*RI (2), *Sac*I (3), *Pvu*II (4), or *Hin*dIII (5), size fractionated on a 0.8% agarose gel, and blot transferred to a nitrocellulose filter. The filter was hybridized in the presence of dextran sulfate to 10^6 dpm per lane of α-actin-specific probe for 24 h and then washed extensively. The final wash was at 65°C in 0.5× SSC. Size markers are indicated to the right of the figure.

To determine the number of copies of the cardiac actin genes in the human genome, the same experiment was performed by using the cardiac actin-specific 3′-UT region as a hybridization probe. When the filter was washed at 65°C in 0.1× SSC, only one band of hybridization to the cardiac actin probe was detected (Fig. 5A, lanes 1 through 5). The 14-kb *Eco*RI fragment which hybridizes at this stringency (Fig. 5A, lane 2) is presumably the same *Eco*RI fragment contained in the genomic clone HRL83 described above. As a second measure of cardiac gene number, we constructed an actin intron subclone from HRL83. The construction and characterization of this intron subclone are described above. There is an endonuclease *Pvu*II site in the intron fragment; the other four enzymes used to digest the genomic DNA do not cut the intron DNA. The intron probe was hybridized to an identical blot of genomic DNA digested with these five enzymes. The nitrocellulose filter was washed at a slightly lower stringency (65°C in 0.5× SSC). The results of this experiment (Fig. 5B) demonstrate that only clone HRL83 contains cardiac intron sequences. However, when a replica filter which has been

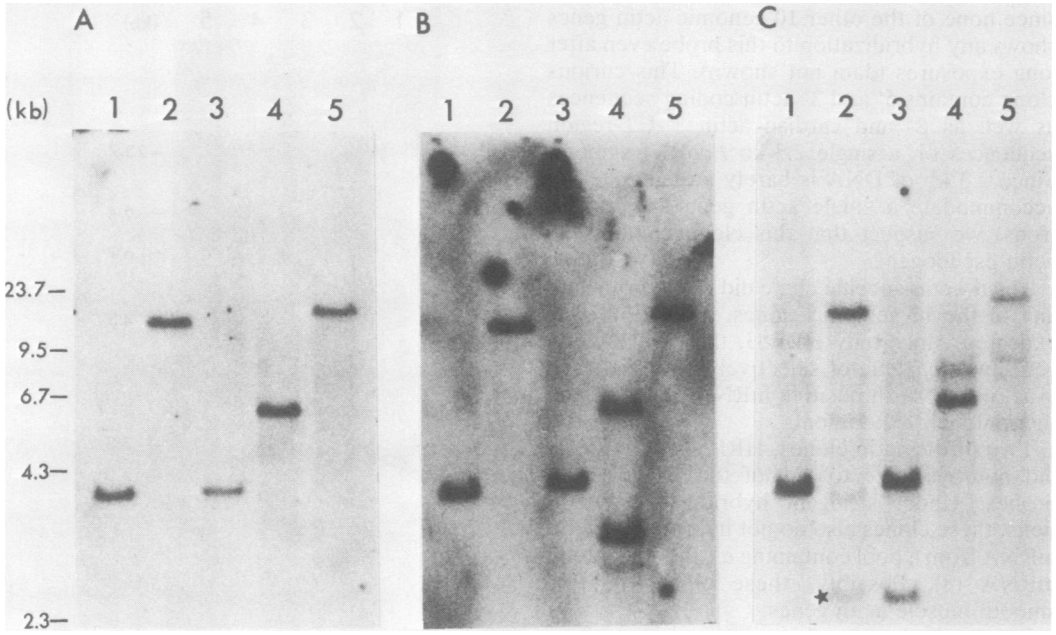

FIG. 5. Analysis of the cardiac actin gene and related sequences. The three filters shown in the figure were blotted from gels identical to that described to Fig. 4. Panels A and C were hybridized to the cardiac 3'-UT-region probe but were washed at two different final stringencies: A, 65°C in 0.1× SSC; C, 65°C in 0.5× SSC. Panel B was hybridized to the cardiac intron probe described in the text and washed at the same stringency as C.

hybridized to the cardiac actin 3'-UT-region probe is washed at this lower stringency, several additional bands of hybridization are detected (Fig. 5C, lanes 1 through 5). These bands cannot result from hybridization to coding region sequences contained in the cardiac subclone, since at the stringency used none of the other actin-containing cloned genes hybridizes to this probe (see above). In genomic DNA digested with endonuclease *Eco*RI, one of the bands which hybridizes to the cardiac actin probe is presumably the actin-containing 2.3-kb *Eco*RI fragment of the genomic clone, HRL51 (Fig. 5C, lane 2, indicated by an asterisk). This is the suspected pseudogene clone which is detected by both the cardiac- and β-actin-specific probes when DNA blots of actin-containing genomic clones are washed at this stringency. Thus, these other hybridizing segments share limited homology with the cardiac actin gene (HRL83) expressed in human heart and skeletal muscle (Gunning et al., in press), and no detectable homology to an intron derived from that gene. However, we cannot exclude that these other weakly hybridizing segments are cardiac actin-related genes expressed in tissues other than heart and skeletal muscles.

Since an alteration of the filter-washing conditions served to distinguish the cardiac actin gene from related DNA segments, we attempted to identify the cytoplasmic actin parent genes by a similar scheme. *Eco*RI-digested HeLa DNA was electrophoresed on parallel lanes of an agarose gel. The gel was blotted, and the resulting filters were hybridized to β- or γ-actin isotype-specific probes. Individual filter strips were then washed at increasingly stringent conditions. X-ray film was exposed to each strip such that exposures of approximately equal intensity were obtained. The results of this experiment for the γ-actin-specific probe are shown in Fig. 6A. At the lowest stringency used (65°C in 0.5× SSC), this probe hybridizes with a range of intensities to approximately 15 distinct *Eco*RI fragments (Fig. 6A, lane 1). Surprisingly, even at wash stringencies as high as 75°C in 0.1× SSC, essentially all of these bands are still detectable after a long exposure (Fig. 6A, lane 4). The relative intensity of the hybridization shows only a small amount of variation from band to band.

A similar result is produced when the β-actin probe is used (Fig. 6B). Greater than 20 bands of hybridization can be discerned. One of these, the 2.3-kb DNA fragment, corresponds to HRL51.

These results confirm our prediction that the majority of the actin sequences in the human genome are cytoplasmic-like. However, unlike the results of experiments with α- and cardiac isotype-specific probes (Fig 4. and 5), we were

unable to identify which of the hybridizing genomic fragments gave rise to the β- and γ-actin mRNAs represented by our cDNA clones. The implications of this result for the evolution of the human actin gene family are described below.

Actin genes in other mammals. The genomes of other mammals also contain many more actin-containing DNA segments than would be anticipated from the known number of actin protein isotypes (6, 14a, 16). We have observed that there is a surprisingly high degree of homology between the 3′-UT-region sequence of each particular human actin isotype and its analog in other vertebrate species (manuscript in preparation). The isotype-specific cross-species hybridization of the 3′-UT regions could therefore be used to determine whether isotype distribution of the actin multigene families in the genomes of other mammals paralleled the distribution seen in humans. We investigated this question by using the human actin 3′-UT-region clones as hybridization probes in DNA blot experiments. A representative example with mouse thymus DNA is shown in Fig. 7. *Eco*RI-digested DNA (8 μg) was electrophoresed on a 0.8% agarose gel, blot transferred to nitrocellulose, and hybridized to an actin isotype-specific probe. The resulting autoradiogram shows that the genes containing cytoplasmic actin sequences (Fig. 7: γ, lane 1; β, lane 2) comprise the bulk of the mouse actin genes, whereas the α- (lane 3) and cardiac (lane 4) actin probes hybridize to very few bands. Similar results were obtained when rat actin DNA was assayed (data not shown). Thus, we conclude that the tremendous increase in the number of actin-containing DNA segments which has occurred since the avian-mammalian divergence has been significantly biased towards the amplification of cytoplasmic genes.

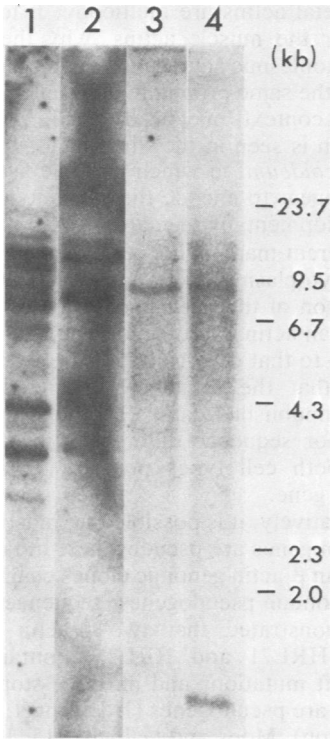

FIG. 7. Conservation of the 3′-UT region of actin isotypes in the mouse genome. Mouse thymus DNA (8 μg) was digested with *Eco*RI, size fractionated on an agarose gel, blot transferred to nitrocellulose, and hybridized to a human isotype-specific actin probe. The filter was washed at a final stringency of 65°C in 0.5× SSC. Probes were as follows: 1, γ; 2, β; 3, α; 4, cardiac.

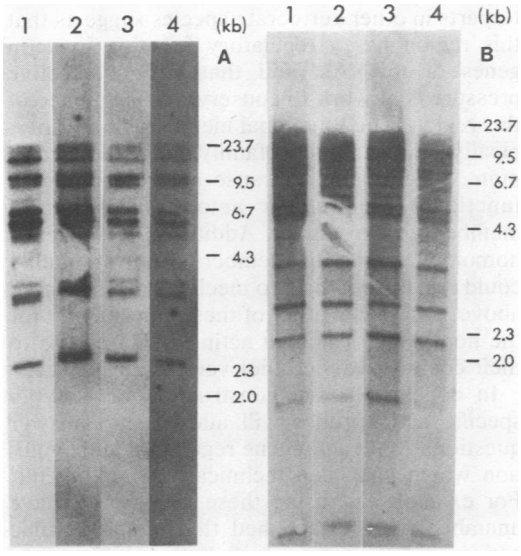

FIG. 6. Copy number of human β- and γ-actin genes. DNA (8 μg) was digested with EcoRI and electrophoresed on an 0.8% agarose gel. The filter shown in (A) was hybridized to the γ-actin-specific probe and that in (B) to the β-actin-specific probe (as described in Fig. 4). The filters were washed under increasingly stringent conditions with lanes being removed from the wash steps as follows: 1, 65°C in 0.5× SSC; 2, 65°C in 0.1× SSC; 3, 70°C in 0.1× SSC; 4, 75°C in 0.1× SSC. Film was exposed to the filter strips from times ranging from 6 h (lane 1) to 48 h (lane 4) to obtain approximately equal exposures of each lane.

DISCUSSION

The development of isotype-specific actin clones has allowed us to establish that the genes for human α and cardiac actins are essentially single copy, whereas the genes encoding the

cytoskeletal actins are multicopy. If few genes serve for the muscle actins, why then do so many cytoplasmic actin genes exist? The need to regulate the same protein in different spatial and temporal contexts may be involved. This type of regulation is seen in the slime mold, *Dictyostelium discoideum*, in which multiple actin genes which appear to encode the same actin isotype are developmentally regulated (13). It is possible that different mammalian cell types express different cytoplasmic actin genes. However, a comparison of the partial sequence of a cDNA clone of an actin expressed in human epidermal cells (12) to that of our fibroblast β-actin cDNA reveals that the sequences are identical (P. Ponte, unpublished data). Since one would expect minor sequence differences in duplicated genes, both cell types probably express the identical gene.

Alternatively, it is possible that most of the β- or γ-actin genes are pseudogenes. In fact, all of the human β-actin genomic clones characterized to date contain pseudogenes; sequence analysis has demonstrated that two β-actin genomic clones, HRL21 and HRL25, contain many frameshift mutations and in-frame stop codons and thus are pseudogenes (J. E. Engel, doctoral dissertation). Moos and Gallwitz (15, 15a) have also recently described the isolation and characterization of two "processed" or "reverse transcript" type β-actin pseudogenes, distinct from the two pseudogenes mentioned above. If the cytoskeletal actin genes are represented by many pseudogenes, then why is this not also the case for sarcomeric actin genes? One important clue might be that α and cardiac actin are expressed in high levels only in differentiated cells, whereas β and γ actins are expressed in germ cells. Only rarely should the reverse transcripts of mRNAs expressed primarily in somatic cells become fixed in the genome after incorporation of this DNA into somatic cell DNA. On the other hand, reverse transcripts of mRNAs expressed in germ cells, like β and γ actin, are more likely to generate heritable pseudogenes. Indeed, other mammalian multigene families expressed in germ cells also contain a large proportion of reverse transcript type pseudogenes (12a, 19). Scarpulla and Wu, for example, have sequenced 4 of the approximately 25 rat cytochrome *c* genes and found that only 1 of these can code for a functional mRNA, whereas 3 are processed pseudogenes (19). Similar results have been obtained from the sequence of a gene (and three processed pseudogenes) for a human β-tubulin isotype expressed in germ line cells (12a). Although these correlations are intriguing, it remains to be determined whether expression of a gene in mammalian germ line cells is related to the generation of an unusually large number of pseudogenes. In any event, to our knowledge, this is the first time that the gene copy numbers have been determined for the members of a mammalian multigene family which are differentially expressed in germ line and somatic cells.

Why is there such a high degree of homology between the multiple cytoskeletal actin genes or pseudogenes or both and the corresponding 3′-UT-region β- or γ-actin-specific clones (as evidenced by the stability of the cDNA probe-genomic DNA hybrids shown in Fig. 6)? The sequences of the intronless actin pseudogenes described above are highly homologous (>96%) to the β-actin cDNA over long stretches (P. Ponte, unpublished data). Duplexes formed between these pseudogenes and the cDNA would be as stable as duplexes formed with the parent gene under the hybridization conditions employed in the experiments shown in Fig 6. This sequence homology suggests that either the pseudogenes have been generated in the recent evolutionary past or their homology to the functional parent gene is being maintained by a mechanism such as gene conversion, which is known to occur between the members of a multigene family (see reference 2 for review).

Our observation that human actin 3′-UT regions share significant homology to their counterparts in other vertebrate species suggests that this region has a regulatory function in actin genes or mRNAs, and that strong selective pressure is at work to conserve the sequence of this region. The functional members of an isotypically defined actin subfamily might share common 3′-UT regions because of some common functional requirement among the different members of that family. Additionally, sequence homology among the members of the subfamily could result from the two mechanisms discussed above, although neither of these can account for the homology of human actin 3′-UT regions to their counterparts in other vertebrate species.

In conclusion, the construction of isotype-specific actin probes will allow us to answer questions about actin gene regulation and evolution which had been technically very difficult. For example, by using these probes, we have unambiguously established that α and cardiac actins are coexpressed in both adult human heart and skeletal muscle (Gunning et al., in press). Therefore, these probes will be particularly useful tools for studying the kinetics of actin mRNA accumulation and turnover, for distinguishing between and quantitating the different actin isotypes, and for determining when they are coexpressed.

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