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Structure, Chromosome Location, and Expression of the Human Smooth Muscle (Enteric Type) γ-Actin Gene: Evolution of Six Human Actin Genes

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Recombinant phages that carry the human smooth muscle (enteric type) γ-actin gene were isolated from human genomic DNA libraries. The amino acid sequence deduced from the nucleotide sequence matches those of cDNAs but differs from the protein sequence previously reported at one amino acid position, codon 359. The gene containing one 5′ untranslated exon and eight coding exons extends for 27 kb on human chromosome 2. The intron between codons 84 and 85 (site 3) is unique to the two smooth muscle actin genes. In the 5′ flanking region, there are several CArG boxes and E boxes, which are regulatory elements in some muscle-specific genes. Hybridization with the 3′ untranslated region, which is specific for the human smooth muscle γ-actin gene, suggests the single gene in the human genome and specific expressions in enteric and aortic tissues. From characterized molecular structures of the six human actin isoform genes, we propose a hypothesis of evolutionary pathway of the actin gene family. A presumed ancestral actin gene had introns at at least sites 1, 2, and 4 through 8. Cytoplasmic actin genes may have directly evolved from it through loss of introns at sites 5 and 6. However, through duplication of the ancestral actin gene with substitutions of many amino acids, a prototype of muscle actin genes had been created. Subsequently, striated muscle actin and smooth muscle actin genes may have evolved from this prototype by loss of an intron at site 4 and acquisition of a new intron at site 3, respectively.

Actins are ubiquitous proteins present in large amounts in all cells, and their amino acid sequences are highly conserved during evolution. Mammalian cells have at least six actin isoforms, which are classified as either muscle- or cytoplasmic-type actins (45–47). The muscle-type actins are essential components of the contractile apparatuses in muscle cells and are subdivided into two striated muscle actins (skeletal and cardiac α-actins) and two smooth muscle actins (aortic type α-actin and enteric type γ-actin), on the basis of predominantly expressed tissues. Two cytoplasmic actins, β- and γ-actins, are found in nonmuscle cells and participate in a variety of cell functions, such as cell motility and maintenance of the cytoskeleton. Expressions of individual actin isoforms are regulated with distinct patterns of both tissue and developmental stage specificity. However, each pair of the actin isoforms was coexpressed under some circumstances (10, 48).

Actins, like many contractile protein genes, are encoded by a multigene family. The high degree of sequence conservation among actin proteins suggests that the multigene family arose by divergence from a single common ancestral gene. The molecular structure analyses of the actin genes are useful for understanding the evolutionary processes of the actin gene family and identifying regulatory systems controlling the expression of individual actin genes.

So far, five of the six actin genes have been isolated from the human genome and mapped on different chromosomes (see Fig. 5). Although a cDNA clone has recently been reported for the smooth muscle (enteric type) γ-actin (27), its gene structure is not yet known.

This article describes the isolation and molecular structure of the human smooth muscle (enteric type) γ-actin gene. The gene contains one 5′ untranslated exon and eight coding exons in a 27-kb region and is located on human chromosome 2. Comparing genome structures, nucleotide sequences, and amino acid sequences among the six human actin genes, we proposed a hypothetical model of the evolutionary pathway of the actin gene family.

MATERIALS AND METHODS

Screening of human genomic libraries. The genomic DNA library of HUT14 cells, in vitro chemically transformed human fibroblast cells (17), was constructed by ligation of partially *Eco*RI-digested DNA to Charon 4A phage vectors. With a 0.85-kb fragment of pcDd actin ITL-1, a plasmid containing cDNA from *Dictyostelium discoideum* actin (8), the library was screened as described previously (44). One of clones which strongly hybridized with the probe, termed λHA-315, was used for further analysis. The second genomic DNA library, made by Lawn et al. (22), was screened with a 1.7-kb *Eco*RI-*Bam*HI DNA fragment of λHA-315, and λHA-3222 was obtained. The third genomic DNA library, made by Takiguchi et al. (39), which was constructed by ligation of partially *Sau*3AI-digested DNA from peripheral blood to *Bam*HI-digested EMBL4 phage vectors, was screened with a 122-bp *Apa*I-*Hpa*II fragment containing a 3′ untranslated region (UTR) of the human smooth muscle γ-actin cDNAs (27) or a 230-bp *Kpn*I-*Hin*cII fragment from intron 5. Clones

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λHACTSG-112 and λHACTSG-2, respectively, were obtained.

To map actin coding regions on the cloned inserts, DNAs from the recombinant phages or their subclones to plasmids were analyzed by Southern blot analysis. For detection probes, DNAs from cDNAs and exon regions of the smooth muscle α-actin gene (19, 44) or the cardiac α-actin gene (14) were prepared and nick translated with [α-^{32}P]dCTP by nick translation kit (Takara Shuzo Co., Kyoto, Japan).

cDNA cloning. Total RNA was prepared from human stomach by the guanidinium-CsCl method, and poly(A)$^+$ RNA was purified by oligo(dT) cellulose chromatography (23). The λgt11 cDNA library was constructed by oligo(dT) priming of poly(A)$^+$ RNA by using cDNA synthesis and λgt11 cloning systems (Amersham). The cDNA library was screened with a 100-bp *Dde*I-*Sma*I fragment of λHA-315, which includes the amino-terminal sequence of the actin coding region, and nucleotide sequences of the isolated cDNA clones were determined.

DNA sequencing. The human DNA inserts were digested with appropriate restriction enzymes and subcloned into either M13 phage or pUC plasmid vectors. DNAs were sequenced with [α-^{35}S]dATP with a T7 sequence kit (Pharmacia). Comparison of DNA sequence data was analyzed by UWGCG programs.

Southern and Northern (RNA) blot analyses. Genomic DNAs were isolated from human and mouse cultured cells, and 10 μg of *Eco*RI-digested DNAs were electrophoresed on 0.8% agarose gels and transferred to Hybond-N nylon membrane filters (Amersham). Total RNAs were extracted from various human tissues by the guanidinium-CsCl method. In the case of umbilical cord artery, poly(A)$^+$ RNA was collected. The RNAs were denatured with formaldehyde, electrophoresed on 1% agarose gels containing 2.5 M formaldehyde, and transferred to nylon filters. The 122-bp *Apa*I-*Hpa*II cDNA fragment including the 3′ UTR was nick translated and used as an actin isoform-specific probe. Southern blot hybridization was carried out as described previously (19) except that filters were washed with 2× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate) and 0.1% sodium dodecyl sulfate for 30 min twice at 60°C. Northern blot hybridization was carried out as described previously (19).

Cell hybrids and chromosome analysis. The isolation of rodent-human somatic cell hybrids and identification of human chromosomes within the hybrids have been described previously (2). High-molecular-weight DNAs isolated from 18 hybrid cells (10 μg each) were digested with *Eco*RI, separated on 0.7% agarose gels, and transferred to nitrocellulose filters. A 1.5-kb *Sac*I-*Eco*RI fragment from intron 8 of λHACTSG-112 was used as a hybridization probe.

Nucleotide sequence accession numbers. Nucleotide sequence accession numbers assigned by DDBJ-EMBL-GenBank are D00648 to D00654 and X16940 for the genome and cDNA of the human smooth muscle (enteric type) γ-actin gene, respectively.

RESULTS

A partial clone of the human smooth muscle (enteric type) γ-actin gene. The genomic library constructed from *Eco*RI partially digested human DNA was screened with the actin coding probe. An isolated recombinant clone, λHA-315, has 6.5- and 8.0-kb *Eco*RI fragments from the human DNA (Fig. 1). Several probes specific for the different regions of actin coding sequences demonstrated that λHA-315 was hybridized with probes having sequences upstream from codon 204 but not downstream from codon 204, e.g., probes including exon 7 of the smooth muscle α-actin gene (data not shown). A large number (more than 20) of actin-related sequences have been detected in the human genome, and many of them may represent pseudogenes (5). However, the actin gene encoded in λHA-315 seemed to have introns, because the actin coding regions were scattered in the cloned fragment. In addition, according to the restriction map, λHA-315 was different from previous clones including human actin genes (see Fig. 5).

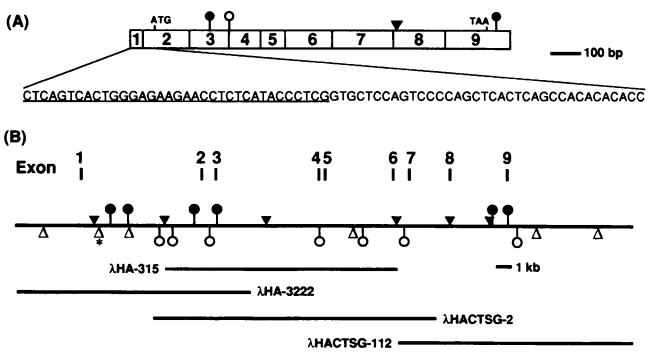
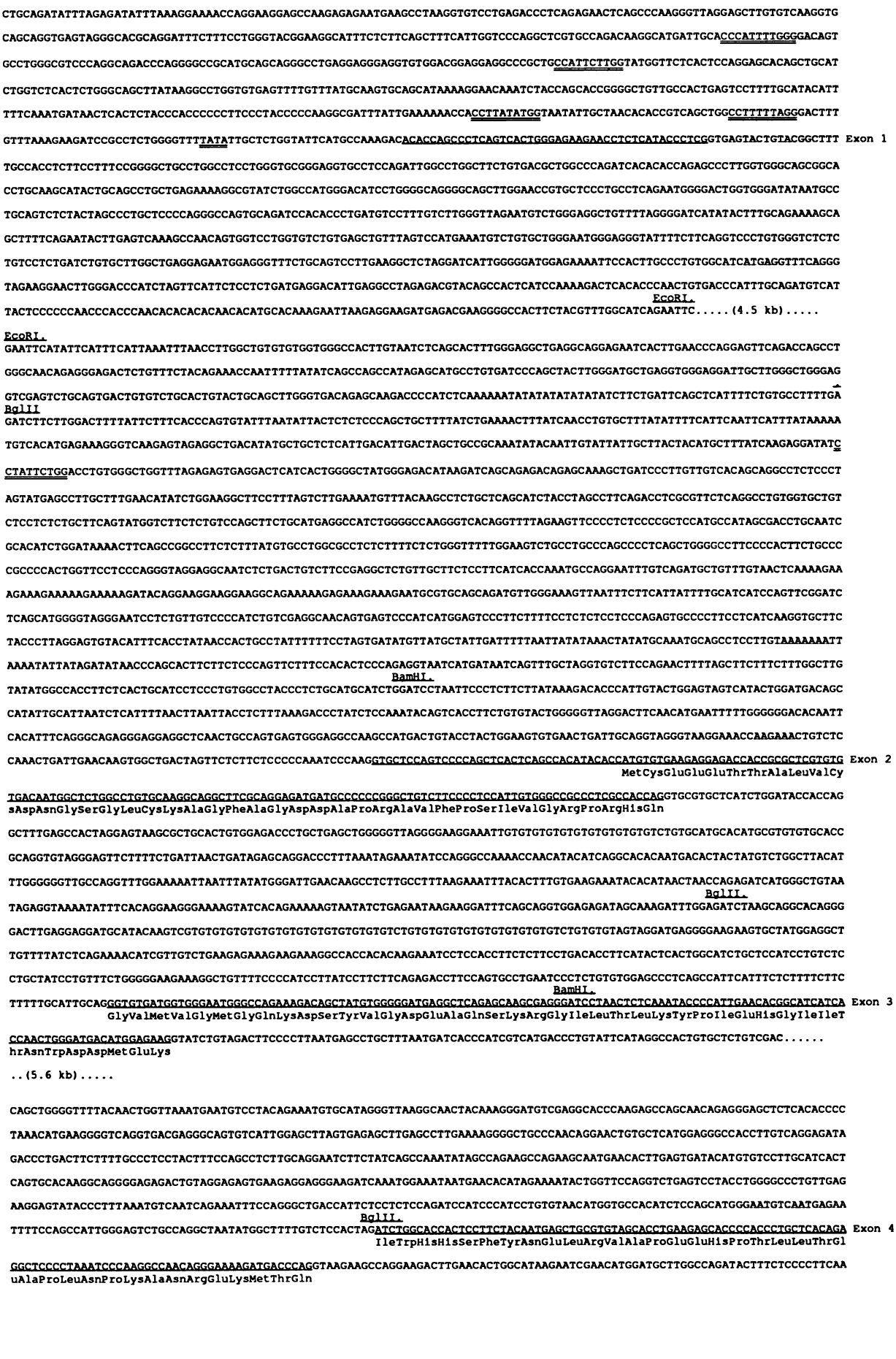


FIG. 1. Structures of human smooth muscle γ-actin cDNA and genomic clones. The locations of cleavage sites of *Bam*HI (closed circle), *Bgl*II (open circle), *Eco*RI (closed triangle), and *Hin*dIII (open triangle) are shown. (A) Schematic map of cDNA. The numbers in boxes represent corresponding exons. Initiation and termination codons are shown. The nucleotide sequence is the longest 5′ UTR among cDNA clones (pHSMγAc-1), and the underlined part corresponds to exon 1. (B) Restriction map and diagram of genomic clones. The black bars numbered 1 to 9 represent exons. A *Hin*dIII site, shown by the asterisk, was polymorphic (41).

To identify an actin isoform encoded by the cloned gene, the DNA fragment hybridized to the first coding exon region, the 1.5-kb *Bam*HI fragment, was sequenced, because the amino-terminal sequence is the most variable region of actin isoforms and the only difference between two smooth muscle actins, enteric type and aortic type (47). The deduced amino acid sequence after the initiation codon was Met-Cys-Glu-Glu-Glu-Thr-Thr (Fig. 2) and agreed with the amino-terminal sequence of smooth muscle (enteric type) γ-actin described previously (47) except that Met-Cys residues were not removed. Therefore, λHA-315 was suggested to include the human smooth muscle γ-actin gene.

Nucleotide sequences of other regions hybridized to the actin coding probes were also determined; λHA-315 contained five actin coding exons of the amino-terminal region, which encode the amino acid sequence up to codon 204 (Fig. 2). The deduced amino acid sequence was completely identical with that of the smooth muscle γ-actin. From these results, we concluded that λHA-315 was a partial clone of the human smooth muscle (enteric type) γ-actin gene.

5′ UTR in cDNA clones. cDNA clones of the human smooth muscle γ-actin gene were isolated from the human stomach cDNA library. One clone, pHSMγAc-5, which was 1,273 bp with 5′ UTR and 3′ UTR of 54 and 77 bp, respectively, has been reported elsewhere (27). Among the cDNA clones, the longest cloned 5′ UTR (pHSMγAc-1), whose sequence is shown in Fig. 1A, was 71 bp. In this sequence, the initial 35 nucleotides of the cDNA did not match with the sequence of the corresponding region of


Exon 1
EcoRI.
EcoRI.
BglII
BamHI.
Exon 2
MetCysGluGluGluThrThrAlaLeuValCy
sAspAsnGlySerGlyLeuCysLysAlaGlyPheAlaGlyAspAspAlaProArgAlaValPheProSerIleValGlyArgProArgHisGln
BglII.
Exon 3
GlyValMetValGlyMetGlyGlnLysAspSerTyrValGlyAspGluAlaGlnSerLysArgGlyIleLeuThrLeuLysTyrProIleGluHisGlyIleIleT
BamHI.
hrAsnTrpAspAspMetGluLys
..(5.6 kb).....
BglII.
Exon 4
IleTrpHisHisSerPheTyrAsnGluLeuArgValAlaProGluGluHisProThrLeuLeuThrGl
uAlaProLeuAsnProLysAlaAsnArgGluLysMetThrGln

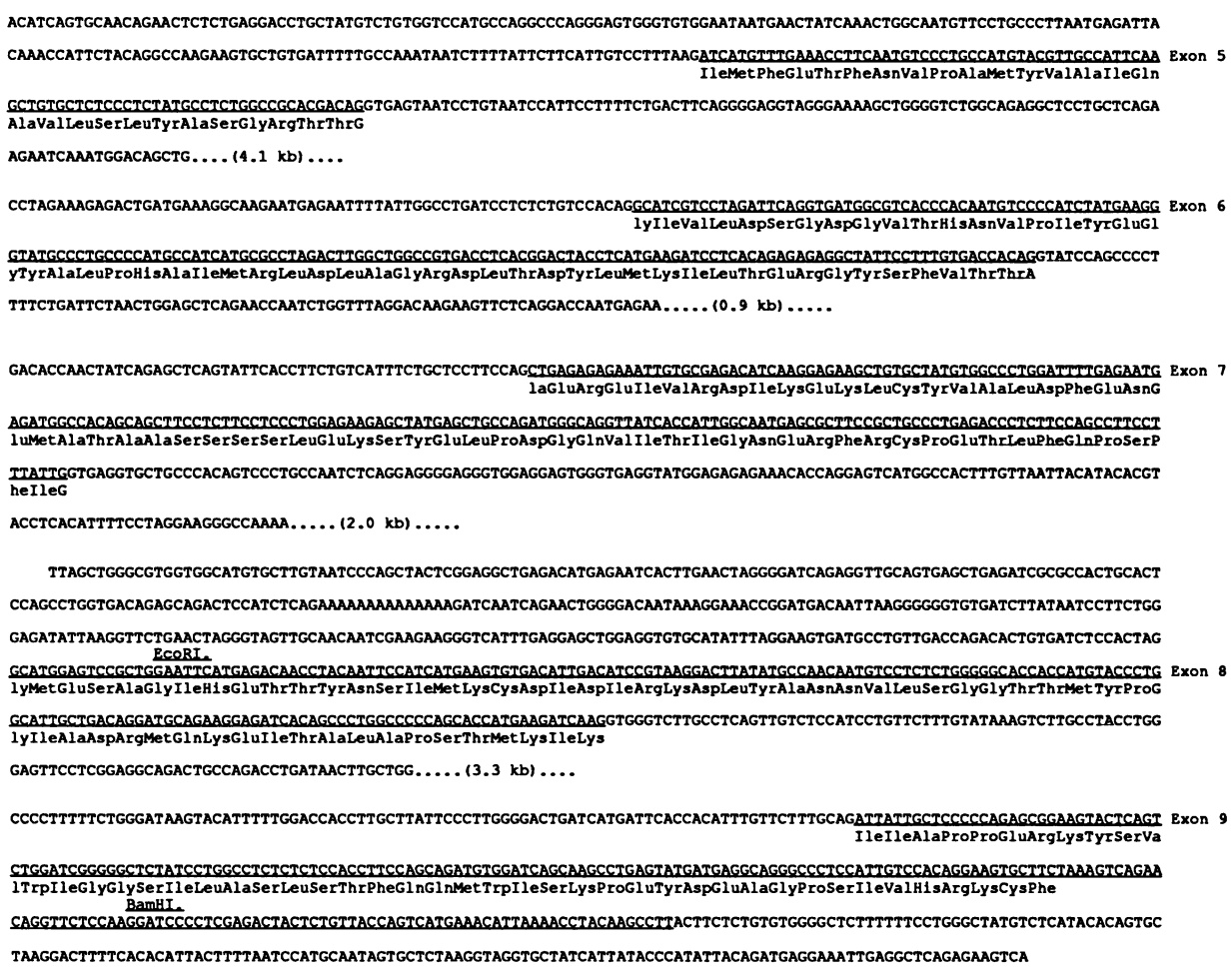


FIG. 2. Nucleotide sequences around nine exons encoding the human smooth muscle γ-actin gene and deduced amino acid sequences. Nucleotides corresponding to exons were determined by comparison with cDNA sequences (27) and are underlined. The TATA and CArG boxes are underlined twice. The cleavage sites of *Bam*HI, *Bgl*II, *Eco*RI, and *Hin*dIII are shown.

λHA-315 (underlined in Fig. 1A). There must be another 5′ side exon in the human smooth muscle γ-actin gene as in other actin isoform genes. To identify the first untranslated exon, the nucleotide sequence up to the end of the λHA-315 insert (2.1 kb from the initiation codon) was determined (Fig. 2), but we could not find the identical DNA sequence with the 35 nucleotides. Therefore, λHA-315 lacks the 5′ untranslated exon region.

Genomic clones including 5′ and 3′ parts of the human smooth muscle γ-actin gene. To clone the first exon, the second human genomic DNA library, obtained from T. Maniatis (22), was screened with the 1.7-kb *Eco*RI-*Bam*HI DNA fragment on the 5′ side of the λHA-315 insert. Clone λHA-3222, inserted with a 14.5-kb human DNA, was isolated and partially overlapped with clone λHA-315 (Fig. 1B). It was hybridized to an *Eco*RI-*Alu*I fragment including the 5′ UTR of pHSMγAc-1, whose sequence corresponded to all of exon 1 and 16 nucleotides from exon 2 (Fig. 1A). By sequencing the hybridized region, the identical DNA sequence with 35 nucleotides of the 5′ UTR was found at 0.8 kb upstream from a 5′ side *Eco*RI site (Fig. 2). Therefore, the first intron was 7.5 kb and interrupted the 5′ UTR 36 nucleotides upstream from the initiation ATG codon. Recently, we have obtained a new clone having a region up to 9 kb upstream from λHA-3222 (42).

Next, to clone a 3′ part of the human smooth muscle γ-actin gene, the 122-bp *Apa*I-*Hpa*II DNA fragment containing the 3′ UTR of pHSMγAc-5 was used for screening the genomic DNA library constructed from partially *Sau*3AI-digested human DNA. Clone λHACTSG-112, inserted with a 15-kb human DNA, was obtained (Fig. 1B). As demonstrated by hybridization with probes specific for the different regions of actin coding sequences, λHACTSG-112 contained three actin coding exons which correspond to the amino acid sequence downstream from codon 204 (Fig. 2). As the inserts in λHA-315 and λHACTSG-112 did not overlap each other, the same library was screened with the 230-bp *Kpn*I-*Hin*cII fragment derived from intron 5 to obtain a clone containing the gap region between these clones. A new clone, λHACTSG-2, indicated that the insert in λHACTSG-112 was just next to that in λHA-315 (Fig. 1B).

Restriction map and sequences of genomic clones. Figure 1B summarizes restriction maps of all of the genomic clones we isolated in this study. The smooth muscle γ-actin gene extends for about 27 kb. The nucleotide sequences around regions hybridized to the actin coding probes were determined, and those encoding the actin protein were identified by comparing cDNA sequences (27). Figure 2 shows the sequences of the entire protein coding portions as well as some 5′ and 3′ flanking sequences and intron sequences.

FIG. 3. Comparison of 3′ UTR sequences of the human smooth muscle γ-actin gene with mouse (20) and rat (24) cDNAs. These sequences begin at the termination codon and end at the nucleotide sequence before the polyadenylation site. The sequences have been aligned for maximal homology. Matches and gaps are indicated by bars and dots, respectively.

There are one 5′ UTR exon and eight coding exons. The sequences at the borders of the introns were in agreement with the consensus splice junction sequences GT-AG. Between the nucleotide sequences of cDNAs and the gene, there are three silent base substitutions in the coding region and one nucleotide substitution in the 5′ UTR (27). The existence of these sequence differences may reflect polymorphisms in the human population. We also noticed that one of the two *Hin*dIII sites in intron 1 was polymorphic (asterisk in Fig. 1B) (41). The coding region of this gene is interrupted by eight introns that are located at the same positions in the smooth muscle (aortic type) α-actin gene (19, 44). The two smooth muscle actin genes have a unique intron between codons 84 and 85, since such introns have never been found in any other actin genes. The deduced amino acid sequence is completely identical with that of the reported cDNA (27) and almost identical with that of smooth muscle γ-actin (46) except that Gln at codon 359 was substituted for Pro. The same substitution was also reported in mouse and rat smooth muscle γ-actin cDNAs (20, 24).

Sequences of 5′ flanking region and introns. A canonical TATA homology was observed at 73 bp upstream from a splicing junction of the first exon (Fig. 2), and usually transcripts of the gene were initiated 25 to 30 bp downstream from the TATA box, and the longest cDNA reached to 35 bp upstream from the splicing junction. In addition, we estimated the first exon to be about 45 bp by S1 digestion of RNA from human stomach, which was hybridized to a uniformly labeled single-strand DNA probe including the expected first exon region (data not shown). It has been observed that most, but not all, eucaryotic gene transcripts are initiated with an A residue. Therefore, although we have not yet precisely mapped the transcription start site, we chose the A nucleotide at 44 bp upstream from the splicing junction as the transcription start site.

As shown in Fig. 2, the sequence immediately upstream from the transcription start site indicated four potential CArG boxes, $CC(A/T)_6GG$ sequences (25, 29), at positions −73, −110, −338 and −434, respectively (numbers refer to base pairs upstream from the transcriptional start site at +1). The CArG boxes are transcriptional regulatory elements that are critical to the expression of actin genes and are found in multiple copies with the 5′ flanking and/or first intron regions. In the human smooth muscle γ-actin gene, we demonstrated that the second CArG had a positive transcriptional regulatory function and was a major binding site for nuclear factors (28). So far, we have found another potential CArG box in the intron 1 region, which is at 1.5 kb upstream from exon 2, but it did not show any functions (28). Since the nucleotide sequence of intron 1 is not complete, functional CArG boxes may be located there. The 5′ flanking region has several E boxes, CANNTG, which are binding sites of muscle differentiation factors, MyoD1 family proteins (1, 37). We demonstrated that, at least, the proximal E box at position −80 was a binding site for MyoD1 proteins in vitro (28). In addition, there are four AP2 binding site-like sequences between −397 and −506, although they are not completely identical with the original sequence (CCC CAGGC) (26) and we could not detect any nuclear factor binding activities.

Dinucleotide repeats (T and G residues) were found twice in intron 2. They are located at 100 and 530 bp, respectively, downstream from the end of exon 2. Another such sequence in exon 3 was observed by Southern blot analysis (data not shown), but its sequence is not yet known. This sequence can construct the left-handed Z-DNA in vitro (50) and affect gene expression in an enhancer-like manner (15). Since it has been reported that the human cardiac α-actin and smooth muscle α-actin genes had the T-G repeat sequences in introns or flanking regions (13, 19), such sequences might have some function in the expression of the actin gene family.

Southern blot analysis with the 3′ UTR. It has been reported that nucleotide sequences of 3′ UTRs of actin genes were isoform specific and conserved in evolution (7, 10, 19, 35, 36). According to cDNA data (27), the 3′ UTR of the smooth muscle γ-actin gene is 77 bp, and a putative polyadenylation signal ATTAAA is located 19 nucleotides upstream from the polyadenylation site. Its nucleotide sequence was compared with those of mouse and rat cDNAs (20, 24). As shown in Fig. 3, there is 75% sequence conservation between human and mouse cDNA, and 76% sequence conservation between human and rat cDNA. The 3′ UTRs of the smooth muscle γ-actin genes, as well as those of the smooth muscle α-actin genes (19), indicate lower sequence conservation than those of other actin isoform genes (about 85% homology). In addition, the 77-bp 3′ UTR is the shortest of those of the six human actin genes (see Fig. 5).

To demonstrate that the 3′ UTR of the smooth muscle γ-actin gene is also used as the isoform-specific probe, the 122-bp *Apa*I-*Hpa*II DNA fragment including the 3′ UTR was hybridized to human and mouse DNAs. A strong hybridized band was detected in the *Eco*RI-digested human DNA (Fig. 4A). Its size was about 12 kb and matched the size predicted from the restriction map of cloned DNAs. Therefore, there is one gene for the human smooth muscle γ-actin gene in the human genome. On other hand, since we could detect no band in the mouse DNA even under low-stringency conditions (Fig. 4A), the 3′ UTR of this gene could not hybridize to the mouse gene. According to these results, the 3′ UTR of the smooth muscle γ-actin gene, as well as that of the human smooth muscle α-actin gene, can recognize the corresponding gene in a human-specific manner. The reason the 3′ UTRs of two smooth muscle actin genes could not detect corresponding genes beyond species, whereas those of other four actins could, may be that these 3′ UTRs are shorter and have lower evolutionary conservation.

Northern blot analysis of human tissue RNAs. The smooth muscle γ-actin predominates in gastrointestinal tracts (47). To investigate the tissue-specific expression of this gene, we carried out Northern blot analysis of RNAs from several human tissues. The actin coding probe could detect 2.1- and/or 1.8-kb actin mRNAs in all tissues examined (19) (data not shown). As shown in Fig. 4B, however, the isoform-

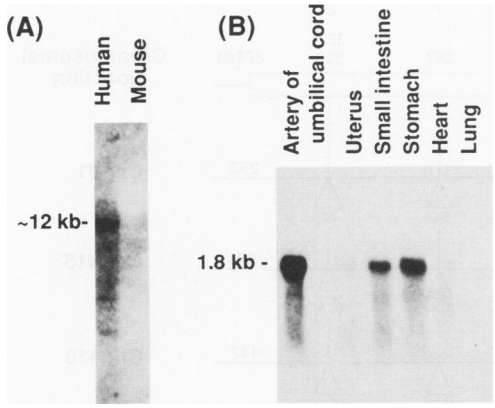


FIG. 4. Detection of the smooth muscle γ-actin gene and its expression in various human tissues. The 122-bp *Apa*I-*Hpa*II cDNA fragment including the 3′ UTR was used as a probe. (A) Southern blot analysis of *Eco*RI-digested DNAs from human and mouse cultured cells. The size of the fragment, as estimated from λ markers, is indicated to the left of the panel. Two fast-migrating bands in the human cell lane disappeared after high-stringency washing (0.2× SSC at 65°C). (B) Northern blot analysis of human tissue RNAs. Five micrograms of poly(A)$^+$ RNA from the artery of umbilical cord and 20 μg of total RNA from tissues (as indicated above their respective lanes) were denatured with formaldehyde and electrophoresed. The sizes of RNAs were calculated from positions of rRNAs, and denatured DNAs were detected by ethidium bromide staining of the gels.

specific probe including the 3′ UTR described above detected only the 1.8-kb RNA transcript in human stomach, small intestine, and artery of umbilical cord but hardly in uterus, heart, and lung. In addition, we could not detect any transcripts in skeletal muscle and brain (data not shown). The expression of smooth muscle γ-actin mRNA corresponded well to protein detection data (48). Since the artery of umbilical cord also expressed smooth muscle α-actin (19), the two smooth muscle actins are coexpressed in this tissue. Indeed, it has been reported that bovine aorta contains smooth muscle γ-actin-like protein as a minor component (48).

Chromosome location of the smooth muscle γ-actin gene. To determine the chromosome location of the human smooth muscle γ-actin gene, a panel of 18 rodent-human hybrid DNAs was analyzed by Southern blot analysis. When the 1.5-kb *Sac*I-*Eco*RI fragment from intron 8 was hybridized to the *Eco*RI-digested human DNA, a 2.3-kb band whose size corresponded to the size predicted from cloned DNAs was detected, whereas no band was obtained in rodent DNAs (data not shown). Thus, this intron fragment was used as the probe. Table 1 summarizes the distribution of the human smooth muscle γ-actin gene and human chromosomes in the hybrid cells. Since the signal for the human smooth muscle γ-actin gene was in good correlation with the presence of human chromosome 2, this gene must be located on chromosome 2. From the chromosome locations of the other five reported human actin genes (see Fig. 5), the six human actin genes are located on different chromosomes and no genetic linkage is seen among them.

DISCUSSION

Several lines of evidence indicated that the gene we cloned in this study was the only functional human smooth muscle γ-actin gene, although there are a large number of actin-related sequences detected in mammalian genomes. (i) By Southern blot analysis, this gene is unique in the human genome. (ii) This gene is transcribed into mRNA in the human enteric tissues, and the sequence of isolated cDNA clones was identical with that of the gene. (iii) The amino acid sequence deduced from the coding region is identical with that of smooth muscle γ-actin except for codon 359. (iv) All exon-intron boundary sequences are in good agreement with the GT-AG rule, and the introns are located at the same positions in the smooth muscle α-actin gene. (v) The relative positions of the putative TATA, CArG, and E boxes, which are transcriptional regulatory elements, correspond well to similar sequences of other actin genes.

TABLE 1. Distribution of the human smooth muscle (enteric type) γ-actin gene and human chromosomes in human-rodent cell hybrids

Hybrid[a]	ACTSG[b]	Presence of human chromosome[c]:																						
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
G35F5	−	±	−	+	+	−	+	−	−	+	−	+	−	+	+	−	+	+	+	+	±	+	+	+	−
G35C4	−	−	−	−	−	−	+	−	−	R	−	−	+	−	+	−	−	−	±	+	+	+	−	−	−
G35E4	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−
G35A4	−	+	−	+	+	−	+	−	+	+	−	−	−	−	+	−	+	−	−	+	+	+	−	+	−
G35A2	−	−	−	+	+	−	+	−	−	−	−	+	−	−	+	−	−	−	−	+	+	+	+	±	−
G35F1	−	#	−	−	+	+	−	−	+	−	+	−	+	−	+	−	−	−	+	+	+	+	−	+	−
G35A5	+	R	R	+	+	−	+	+	−	+	+	+	−	+	+	+	+	−	+	+	+	+	+	+	−
G95A4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	−
G24B2	−	−	−	+	−	+	−	+	−	#	+	−	−	+	+	+	+	−	−	+	#	+	+	+	−
G25A2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	−
G35F3	+	−	+	+	−	+	−	+	+	−	+	+	+	−	−	−	+	−	+	+	+	−	−	+	−
G24A9	+	−	+	−	+	−	+	−	+	+	+	−	−	#	+	+	−	+	+	#	+	+	−	+	−
G24A4	+	+	+	−	−	+	+	+	−	+	−	#	+	+	+	+	−	−	−	+	−	+	−	+	−
G1710	−	−	−	+	−	−	+	+	#	+	−	+	+	+	−	−	+	ND	−	+	−	+	#	ND	−
G1711	−	−	−	−	−	+	+	+	−	−	+	−	+	+	+	−	−	+	+	+	+	+	−	−	−
G1715	−	−	−	+	−	−	−	−	−	−	+	−	+	+	−	−	−	ND	+	+	−	+	−	+	−
RRP3-6	+	+	+	+	+	+	+	+	−	−	+	+	+	−	−	+	+	+	ND	R	−	+	−	+	+
G13C2	+	−	+	ND	+	+	−	+	−	#	+	+	−	+	+	+	+	ND	+	−	+	+	ND	+	ND

[a] G35F5 through G95A4 and G35F3 are human-Chinese hamster cell hybrids, and others are human-mouse cell hybrids.
[b] ACTSG, human smooth muscle (enteric type) γ-actin gene.
[c] R, rearrangement; #, less than 20%; ND, not determined.

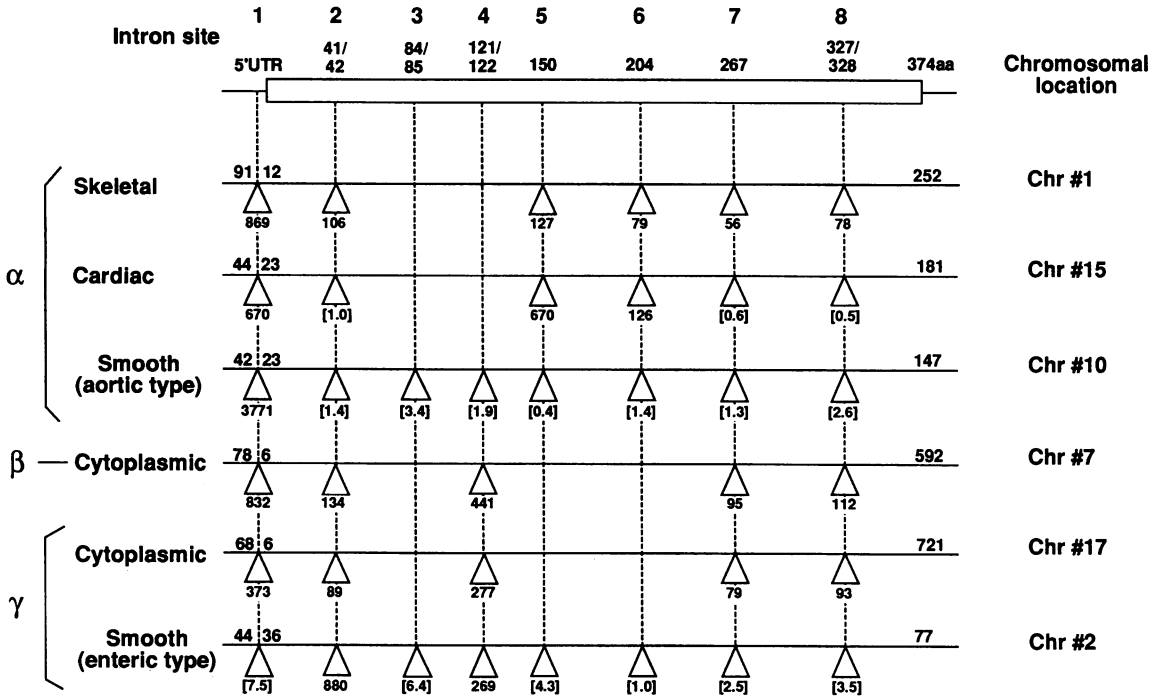


FIG. 5. Summary of the gene structures and chromosome locations of the six human actin genes; i.e., the skeletal α-actin (11, 40), cardiac α-actin (10, 11, 14, 25), smooth muscle α-actin (19, 31, 32, 43, 44), cytoplasmic β-actin (30, 33, 36), cytoplasmic γ-actin (6, 7), and smooth muscle γ-actin (this article; 27) genes. Triangles indicate intron positions. Numbers below them indicate intron sizes (base pairs or kilobases [in brackets]). Numbers above the lines indicate the sizes of the 5′ UTR in exon 1 and exon 2 and those of the 3′ UTR in the last exon.

Comparison of the six human actin genes. The isolated human smooth muscle γ-actin gene contains one 5′ untranslated exon and eight coding exons in an about 27-kb region. Since the DNA sizes of the other human actin genes, cytoplasmic γ-actin, cytoplasmic β-actin, smooth muscle α-actin, cardiac α-actin, and skeletal α-actin, are 2.8, 3.4, 17, 4.9, and 2.8 kb, respectively, the smooth muscle γ-actin gene is the largest actin gene in the human genome. The lengths and locations of introns of the six human actin genes are summarized in Fig. 5. A comparison of exon-intron organization of the human actin genes indicates that comparable structural regions are identically interrupted by introns, whereas the sizes of introns or untranslated regions are different. Introns at sites 1, 2, 7, and 8 are common in all the human actin genes. The two striated muscle actin genes have introns at sites 5 and 6, while the two cytoplasmic actin genes each have an intron at site 4. The two smooth muscle actin genes each have introns at both sites and a unique intron at site 3. This comparison gives a clue how these genes may have evolved.

The primary product translated from the smooth muscle γ-actin gene, encoding 376 amino acid residues, is one amino acid longer than two cytoplasmic actins and one amino acid shorter than three other muscle actins (Fig. 6). The presence of a Cys codon following the initiation codon, position −1, which is thought to be removed by the posttranslational processing, is unique to the four muscle actin genes. The amino acid at position 4 was deleted in smooth muscle γ-actin and two cytoplasmic actins. This difference is kept in mature proteins. Between two smooth muscle actins, there is one substitution at codon 359 except for the amino terminal regions. The same substitution was reported in mouse and rat smooth muscle γ-actins (20, 24). The significance of this substitution in smooth muscle γ-actin is not clear, but since it is the unique substitution beyond species, it might be useful for making an antibody specific for smooth muscle γ-actin.

When the nucleotide sequence of the smooth muscle γ-actin coding region was compared with those of other human actin genes, homologies of 82.4 to 85.8% were found. As shown in Fig. 6, most of the base changes in these sequences are the result of third-base substitutions and are silent substitutions that do not change amino acids. Therefore, the amino acid sequence differences between the smooth muscle γ-actin gene and other actin genes were less (93.6 to 99.5%) than those of the nucleotide sequences. They are summarized in the upper right portion of Table 2.

An evolutionary clock hypothesis predicts that the accumulation of silent or replacement substitutions is proportional to divergence time. To compare the coding sequences of the six actin genes, we used the 'divergence' method of UWGCG programs that calculates divergence for multiple events between two homologous sequences with the random substitution model (34). Numbers in the lower left portion of Table 2 showed percent divergences for silent and replacement substitutions between actin coding sequences.

Since the corrected percent divergence, which shows estimated divergence time, between two cytoplasmic actins is the lowest (44/1.4) among them, it is likely that these genes diverged the most recently. However, the corrected percent divergences of silent substitutions between the muscle and cytoplasmic actin sequences are generally lower (58 to 101%) than those between the muscle actin sequences (89 to 118%). Since other data demonstrate that estimated divergence time between muscle and cytoplasmic actin genes is greater than that between the muscle actin genes, the accu-

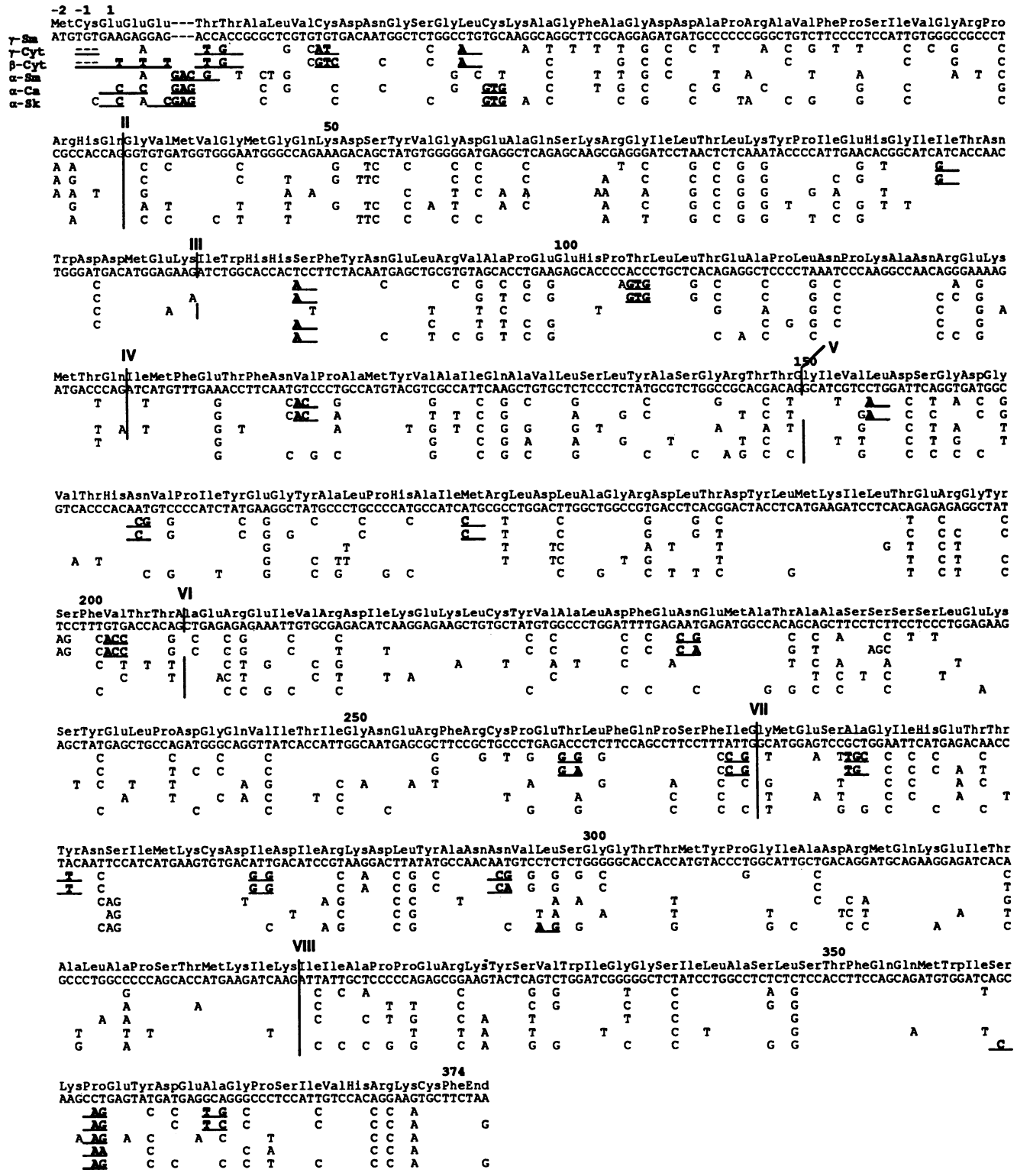


FIG. 6. Comparison of the six human actin coding sequences. The predicted sequence of the amino acid coding region of human smooth muscle γ-actin, γ-Sm (27), is compared with those of the human cytoplasmic γ-actin (γ-Cyt) (7), cytoplasmic β-actin (β-Cyt) (36), smooth muscle α-actin (α-Sm) (18), cardiac α-actin (α-Ca) (14), and skeletal α-actin (α-Sk) (16). In accordance with the previous numbering system (47), only 374 residues were numbered. Met and Cys are numbered as −2 and −1, respectively, and are absent from the mature protein through posttranslational cleavage. An extra Ser between positions 234 and 235 has been identified. The amino acid at position 4 is deleted in the smooth muscle γ-actin and two cytoplasmic actins and that at position −1 is deleted in two cytoplasmic actins, and they are shown by dashes. Codons that encode amino acids different from those in the smooth muscle γ-actin gene are underlined. Intron sites are shown by bars.

TABLE 2. Percent divergences of amino acid sequences, nucleotide sequences, and silent and replacement substitution sites in each codon among six human actin coding regions[a]

Actin	γ-Sm	γ-Cyt	β-Cyt	α-Sm	α-Ca	α-Sk
γ-Sm		94.4/82.4	93.6/84.0	99.5/85.1	98.7/85.8	98.7/84.4
γ-Cyt	101/6.6		98.9/91.3	94.7/82.6	94.1/83.0	93.6/85.8
β-Cyt	78/8.0	44/1.4		93.9/82.0	94.4/84.5	93.9/86.0
α-Sm	98/1.7	100/6.8	97/7.9		98.4/85.5	97.4/85.5
α-Ca	89/2.3	62/8.5	83/7.2	95/1.6		98.9/85.3
α-Sk	109/2.8	95/7.7	58/7.8	108/1.9	118/1.4	

[a] We calculated percent divergences in each pair of the actin coding regions shown in Fig. 6 in accordance with the University of Wisconsin Genetics Computer Group Bestfit and Divergence programs. The upper right portion shows the percent homologies in sequences of amino acids and nucleotides. The lower left portion shows the corrected percent silent substitution and replacement substitution. Abbreviations are the same as for Fig. 6.

mulation of silent substitutions might saturate because of the extremely conservative nature of actin coding sequences. Alternatively, since the actins are major components of total proteins in all tissues, tissue-specific codon usage may have some influence on the calculation. Indeed, there are some differences in codon usage among the six actin genes, e.g., in the smooth muscle γ-actin gene, codons ACC (15 of 25) and ACA (7 of 25) are used for Thr, whereas ACC (11 of 24) and ACT (10 of 24) are preferentially used in the smooth muscle α-actin gene.

In contrast to silent substitutions, the corrected percent divergence of replacement substitutions seems proportional to the divergence time. Numbers of replacements, however, are too small to calculate correct evolution periods, since there is also a strong evolutionary pressure to retain the functional protein structures. Therefore, replacements or silent substitutions cannot be used as evolutionary clocks for the actin coding sequences over long periods except for two cytoplasmic actins.

Evolution model. It is reasonable to postulate that the modern actin isoforms have one common ancestral actin gene and evolved from it. From data of the gene structures, the sequences of nucleotides and amino acids, and the tissue-specific expression, we propose here the model for actin gene evolution.

In this model, the intron positions found in modern actin genes are results of loss of some introns from and insertion of new introns into the ancestral actin gene. A limited number of intron positions has been identified and well conserved even in echinoderm actin genes; a starfish actin gene had introns at sites 2 and 4 through 7 (21). In addition, introns at sites 1 and 8 are common in all mammalian actin genes. Therefore, the ancestral actin gene was presumed to have at least seven introns at sites 1, 2, and 4 through 8.

Amino acid sequences of many species suggest two views about the ancestral actin gene. First, since actins present in the invertebrate muscle tissues resemble mammalian cytoplasmic actins rather than muscle actins (4, 49), the ancestral actin gene might have an amino acid sequence similar to those of the human cytoplasmic actin genes. Second, since Met-Cys sequences in the amino-terminal sites were also observed in some invertebrate actins (9, 38), the ancestral actin gene had the Cys residue following the initiation codon. In addition, it is likely that early vertebrates had only one muscle actin expressed in all striated muscles and that the smooth muscle actin arose later in evolution from early striated muscle actins (49).

Our proposed model of actin gene evolution is shown in Fig. 7. The cytoplasmic β- and γ-actin genes may have been created from the putative ancestral actin gene through loss of the introns at sites 5 and 6 and deletion at two amino acid sites. After duplication occurred recently in evolutionary time, four amino acids were substituted in the cytoplasmic β-actin gene.

According to the comparison of the amino acid sequences, muscle actins differ from cytoplasmic actins in 19 common amino acid substitutions, at positions 5, 6, 10, 16, 76, 103, 129, 153, 162, 176, 201, 225, 259, 266, 271, 278, 286, 296, and 364. Therefore, four muscle actins did not evolve independently from the common ancestral actin gene with cytoplasmic actins but must have one ancestral prototype of the muscle actin gene.

Subsequent to the divergence of the ancestral muscle and cytoplasmic actin genes, the muscle actin prototype gene had been duplicated to produce primitive striated muscle and smooth muscle actin genes. The two striated muscle actin genes may have evolved through the process in which the intron at site 4 was lost and two amino acids were substituted. After sequential duplication, they received some amino acid substitutions.

Since the two smooth muscle actin genes have the unique intron at site 3, this intron was in place in the primitive smooth muscle actin gene prior to its divergence. The intron at site 3 is found only in the two smooth muscle actin genes but never reported in other actins among either vertebrate or invertebrate genomes. If the ancestral actin gene and muscle actin prototype gene had carried the introns at site 3 as described by others (3), the excision of this intron would have occurred twice during cytoplasmic and striated muscle actin gene evolutions, according to our model. But, since the excision of introns seems a rare event, there is little possibility of this. Therefore, we think that the primitive smooth

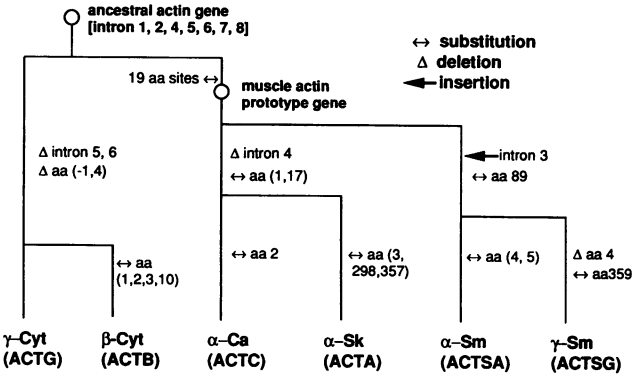


FIG. 7. A hypothetical family tree for the actin gene family. The vertical scale is not linear and merely represents relative evolutionary time. See Discussion in the text.

muscle actin gene may acquire the new intron at site 3. Although the intron at site 3 was inserted relatively late in evolution, the sizes of the introns in the two smooth muscle actin genes were very different and our Southern blot analysis did not show any homology between them (data not shown). The primitive smooth muscle actin gene was given an amino acid substitution at position 89 and then duplicated to the two smooth muscle actin genes. The gene products received some amino acid substitutions, and the smooth muscle γ-actin lost one amino acid at position 4.

According to this model, the deletion of the amino acid at position 4 had occurred twice, during the cytoplasmic and smooth muscle γ-actin evolutions. It is not clear, however, whether this deletion was an independent phenomenon. If not, the deletion of the amino acid at position 4 gives some advantage to actin proteins, because amino termini are important for interaction with myosin filaments, etc.

Significance of actin isoforms. Actins are important proteins in living cells. It is not clear why human cells have the six actin isoforms, because the actin isoforms seem to some extent interchangeable and the different actin isoforms confer no distinct functional advantage (12). Since actins interact with many ligands, the differences among the six actin isoforms might induce changes in cell structures through the alteration of interactions between actin filaments and cytoskeletal components. They must have evolved because of a regulatory requirement for multiple actin genes. It was reported that the actins are sometimes coexpressed in each isoform pair with variable relative levels. Since each pair of the actin genes represents the most recent gene duplication events in the evolution model, regions regulating gene expression may still keep cross-responsiveness. Now, since the 5′ flanking DNA sequences of all six of the human actin genes are known, we have made an effort to understand mechanisms of differential control of expression specific for actin isoform genes.

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