

Structure of a Human Smooth Muscle Actin Gene (Aortic Type) with a Unique Intron Site

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Received 25 January 1984/Accepted 14 March 1984

A recombinant phage containing an actin gene (λ Ha201) was isolated from a human DNA library and the structure of the actin gene was determined. The amino acid sequences deduced from the nucleotide sequences of λ Ha201 were compared with those of six actin isoforms; they matched those of bovine aortic smooth muscle actin, except for codon 309, which was valine (GTC) in λ Ha201 and alanine (GCN) in bovine aortic smooth muscle actin. Southern blot hybridization experiments showed that the gene of normal human cells did not have the *TaqI*-sensitive site around position 309, whereas half of the genes of HUT14 cells did. These results indicate that one allele of the aortic smooth muscle actin gene in HUT14 cells has a transition point mutation (C \rightarrow T) at codon 309 and that the amino acid sequences of normal human aorta and bovine smooth muscle actins are probably identical. In addition to the five introns interrupting exons at codons 150, 204, and 267, and between codons 41 and 42 and 327 and 328, which are common to skeletal muscle and cardiac muscle actin genes, the smooth muscle actin gene has two more intron sites between codons 84 and 85 and 121 and 122. The previously unreported intron site between codons 84 and 85 is unique to the smooth muscle actin gene. The intron site between codons 121 and 122 is common to β -actin genes but is not found in other muscle actin genes. A hypothesis is proposed for the evolutionary pathway of the actin gene family.

Six different actin isoforms have been identified in, i.e., vertebrates by amino acid sequencing skeletal muscle, cardiac muscle, two smooth muscles (enteric and aortic), and two cytoplasmic actins (β and γ) (25). Their amino acid sequences are very similar and well conserved through evolution. For example, skeletal and cardiac muscle actins differ by only four amino acids (25). Even skeletal muscle actin and cytoplasmic β -actin differ by only 25 amino acids out of their total of 374 amino acids (25). However, their functions are different, and their expression is distinctly controlled during differentiation. Therefore, it will be helpful to know the structure of their genes (nucleotide sequences and the location of introns, etc.) to understand the regulatory mechanisms of their expression in different tissues and their evolutionary origin. There have been reports on the structure of skeletal muscle actin genes in rats (26) and chicks (6) and of cardiac muscle actin genes in humans (10), but not on smooth muscle actin genes. We previously constructed a DNA library from a chemically transformed human fibroblast cell line (HUT14 [13]) to study the alterations of gene structure associated with neoplastic transformation (15). Using a recombinant plasmid DNA containing a cDNA copy of actin mRNA from *Dictyostelium discoideum* (5) as a probe, we have isolated four different actin genes; one of them (λ Ha101) was characterized as the cardiac muscle actin gene, because its detailed restriction map was identical to that of the human cardiac muscle actin gene which we had isolated from another gene library constructed from DNA obtained from β -thalassemia patient cells (10). Here we report the isolation and structural characterization of the human aortic type of smooth muscle actin gene. The gene has two more intron sites than do skeletal and cardiac muscle actin genes, between codons 84 and 85 and 121 and 122. The intron site between codons 84 and 85 has never been found in any actin gene of vertebrates or nonvertebrates. On the other hand, the intron between

codons 121 and 122 was also found in the β -actin gene of rats (20, 21). We also found that this gene has a transition point mutation in position 309, substituting cytosine with thymine.

MATERIALS AND METHODS

Materials. pcDd actin ITL-1, a recombinant plasmid DNA containing a cDNA copy of actin mRNA from *D. discoideum*, was obtained from Firtel (5). HUT14 cells are an in vitro, chemically transformed human fibroblast cell line (13). The *Escherichia coli* strain used for the cloning of DNA was HB101. Various restriction endonucleases were obtained from Bethesda Research Laboratories and used under the recommended conditions specified by the supplier. T4 ligase, for the ligation of DNA fragments to a vector DNA, pBR322, and pBR325 were also obtained from Bethesda Research Laboratories. Alkaline phosphatase (lyophilized, from calf intestine) and polynucleotide kinase were from P-L Biochemicals, Inc. [α - 32 P]dCTP (>400 Ci/mmol) for nick translation was from Amersham Corp., and [γ - 32 P]ATP (>2,000 Ci/mmol) for the labeling of the 5' end was purchased from ICN Pharmaceuticals, Inc.

Construction of DNA library and screening for actin genes. The DNA gene library of HUT14 cells was constructed by ligation of partially *EcoRI*-digested DNA (ca. 20 kilobase pairs [kb]) to Charon 4A phage vector by the method of Maniatis et al. (17). The library was screened by the procedure described by Benton and Davis (1). The probe used for screening was the *Sau96I* 0.85-kb fragment of pcDd actin ITL-1 and was labeled with [α - 32 P]dCTP by nick translation (18). Plaque hybridization was carried out under the same conditions as described before (10). Seven different clones which strongly hybridize with the probe were obtained. DNA was isolated from one of these clones, termed λ Ha201, by the method of Lawn et al. (14) and used for further analysis. All experiments involving recombinant phages and plasmids were carried out in accordance with the federal guidelines for recombinant DNA research.

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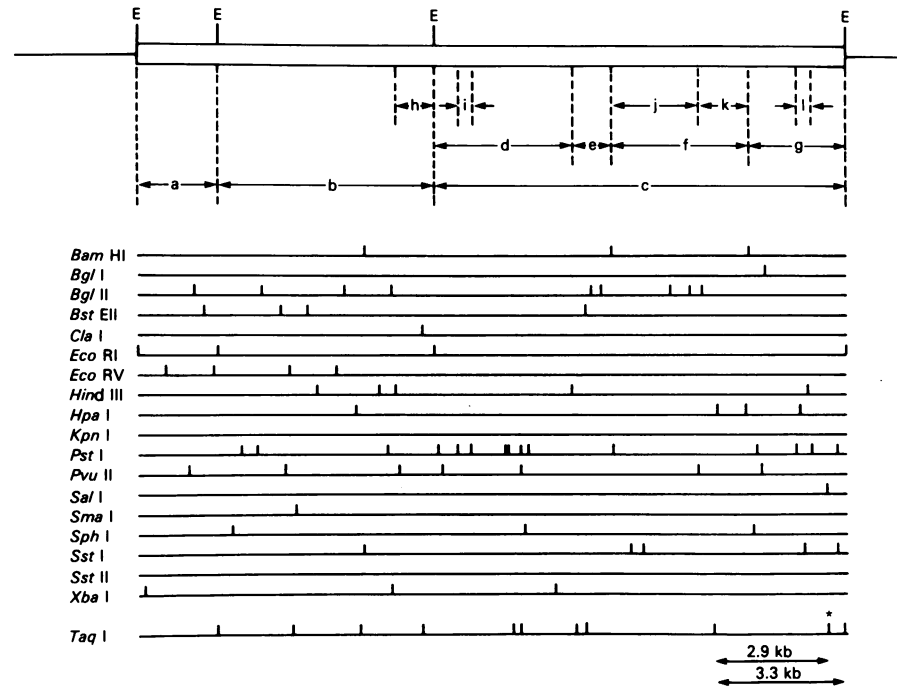


FIG. 1. Map of the inserted DNA in the λ Ha201 gene and the strategy of subcloning. E, *Eco*RI sites. Fragments a, b, and c were inserted into the *Eco*RI site of pBR325 DNA to prepare subclones 201pEco_{2.0}, 201pEco_{5.5}, and 201pEco_{10.5}, respectively. Fragments d, e, f, and g were ligated to pBR22 DNA to make subclones 201pEH_{3.5}, 201pHB_{1.0}, 201pBam_{3.5}, and 201pBE_{2.5}, respectively. Fragments h, i, j, and k were subcloned with pBR322 for convenience in the sequencing of DNA, yielding 201pHE_{1.0}, 201pPst_{0.3}, 201pBV_{2.2}, and 201pVB_{1.3}, respectively. Fragment l was subcloned into pBR322 to make a probe for Southern blot hybridization analysis (Fig. 4). *, Site where a point mutation was suggested to have occurred. Open box at top, Inserted DNA; line, phage vector DNA.

Subcloning of λ Ha201 gene and detection of exons. Since the DNA inserted into Charon 4A phage is long (Fig. 1, about 18 kb) and has two more *Eco*RI sites in it, it was first divided into three subclones. Each of the three *Eco*RI-digested fragments of λ Ha201 DNA was ligated into the *Eco*RI site of pBR325. The subclones obtained were called 201pEco_{2.0}, 201pEco_{5.5}, and 201pEco_{10.5} (Fig. 1). Since the inserted DNA of 201pEco_{10.5} was still long (10.5 kb), it was further divided into four subclones. They were constructed by ligating fragments of 201pEco_{10.5} cut with *Eco*RI-*Hind*III, *Hind*III-*Bam*HI, *Bam*HI, or *Bam*HI-*Eco*RI to pBR322 DNA, which had been cut by the same combination of enzymes and treated with alkaline phosphatase. The final subclones prepared were called 201pEH_{3.5}, 201pHB_{1.0}, 201pBam_{3.5}, and 201pBE_{2.5} (Fig. 1). These subclone DNAs were digested with various restriction endonucleases, electrophoresed on a 1% agarose gel (in 40 mM Tris-acetate [pH 7.8]-2 mM EDTA), and then transferred to a nitrocellulose filter (Schleicher & Schuell, Inc.) by the method of Southern (23). After baking at 70°C for 2 h, the filters were subjected to prehybridization (at 65°C for 5 h) and then to hybridization with either of two probes nick translated with [α -³²P]dCTP. The probes were one of the *Taq*I-digested fragments (corresponding to codons 10 through 72) and one of the *Hin*fI-digested fragments (corresponding to codons 50 through 155) of 204pVB_{2.1}, a subclone of λ Ha204, which carries a pseudo- β -actin gene lacking introns. After hybridization, in a solution containing 0.9 M NaCl-0.09 M sodium citrate-0.08% polyvinylpyrrolidone-0.08% Ficoll-50 μ g of *E. coli* tRNA per ml-50 μ g of *E. coli* DNA per ml-0.5% sodium dodecyl sulfate (SDS)-³²P-labeled probe, for 18 h at 65°C, the filter

was washed twice with 0.3 M NaCl-0.03 M sodium citrate-0.1% SDS at 50°C for 15 min and then twice with 0.03 M NaCl-0.003 M sodium citrate-0.5% SDS at room temperature for 15 min. After drying, the filter was exposed to an X-ray film and kept at -70°C for 1 to 2 days. Positive DNA fragments detected by the autoradiogram were subjected to subcloning for the sequencing of DNA (see below).

Sequencing of DNA. Recombinant DNA of interest (20 μ g) was digested with an appropriate restriction endonuclease and then treated with alkaline phosphatase (1 U) for 1 h at 37°C. After inactivation of the phosphatase by boiling, the fragments were labeled at the 5' end with [γ -³²P]ATP by polynucleotide kinase at 37°C for 30 min. The labeled fragments were separated by 6% polyacrylamide gel electrophoresis (in 40 mM Tris-acetate [pH 7.8]-2 mM EDTA) either immediately or after redigestion by another restriction endonuclease. In the former case, isolated DNA fragments from the gel were redigested by a secondary enzyme, and singly end-labeled fragments were obtained by a second polyacrylamide gel electrophoresis. The extraction of DNA fragments from polyacrylamide gels consisted of three successive steps: extraction from smashed gels with the above gel buffer containing 0.1% SDS, column chromatography on hydroxyapatite (Bio-Rad Laboratories), and dialysis to remove potassium phosphate in the eluate. The sequencing of DNA was carried out by the chemical base modification cleavage procedure of Maxam and Gilbert (19). Four reactions were done for each end-labeled sample (G, A + C, T + C, and C). Autoradiograms were read by two people independently.

Southern blot hybridization analysis of total cellular DNA.

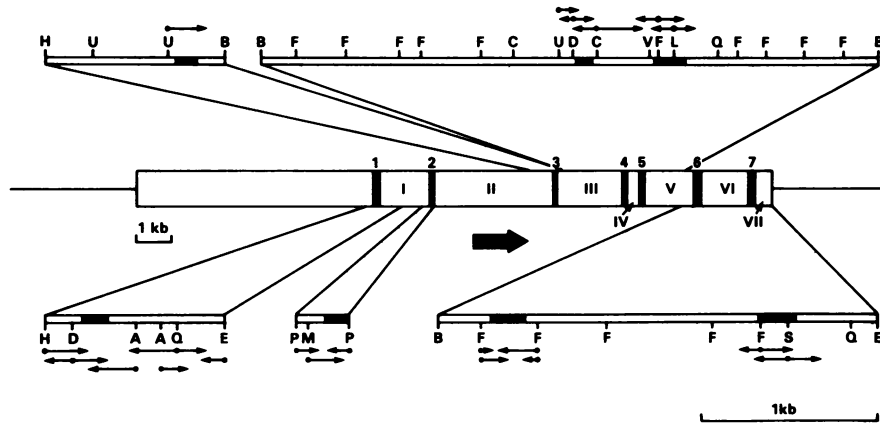


FIG. 2. Structure of human smooth muscle actin (aortic type) gene and strategy for sequencing of DNA. Center, Structure of human smooth muscle actin (aortic type) gene. Arrow, Direction of transcription. Line, Charon 4A DNA. Seven exons and seven introns are designated 1 through 7 and I through VII, respectively, from the 5' end to the 3' end. Top and bottom, Strategy for the determination of DNA sequences. Arrows, Direction and extent of sequence determination of each fragment analyzed. The proportion determined from both strands is 52% for total sequences and 77% for exons. Restriction enzyme cleavage sites: H, *Hind*III; D, *Dde*I; A, *Acc*I; Q, *Taq*I; E, *Eco*RI; P, *Pst*I; M, *Mbo*II; U, *Sau*96I; B, *Bam*HI; F, *Hin*I; C, *Hinc*II; V, *Pvu*II; L, *Bgl*II; and S, *Sal*I. Solid areas, coding regions whose locations were determined by the sequencing of DNA.

High-molecular-weight DNA isolated from HUT14 cells and KD cells (from which HUT14 cells were derived [13]) by the method of Blin and Stafford (2) was digested with *Taq*I, electrophoresed on a 1% agarose gel, and transferred to a nitrocellulose filter. Hybridization experiments were carried out as described above with salmon sperm DNA (Sigma Chemical Co.) instead of *E. coli* DNA. The probe used was the 0.4-kb *Pst*I fragment from intron VI, cloned into pBR322 (Fig. 1l). The washing procedures were the same as above, except that the temperature was 60°C for the second series of washes. The filter was subjected to autoradiography for 1 week at -70°C.

RESULTS

Structure of the inserted DNA in λHa201 gene. Figure 1 shows a restriction map of the human DNA inserted into the *Eco*RI site of the Charon 4A phage vector. Since the DNA isolated from HUT14 cells was partially digested with *Eco*RI and ligated to the vector, the inserted DNA of λHa201 gene has two more *Eco*RI sites. The clone has no sites for *Kpn*I or *Sst*II. The total length of the inserted DNA is 18 kb. Figure 1 also shows the derivation of the 12 subclones (a through l) of λHa201.

Detection of coding regions. To know where exons are embedded, we performed Southern blot hybridization with two kinds of probes. One of the *Taq*I-digested fragments of 204pVB_{2.1} DNA, corresponding to codons 10 through 72, was used for the detection of N terminus coding regions. The fragment hybridized to two subclones, 201pEco_{5.5} and 201pEH_{3.5}, suggesting that there may be one intron site between codons 10 and 72. Since more detailed hybridization analysis showed that the probe hybridized to the 1.0-kb *Hind*III-*Eco*RI fragment (Fig. 1h) of 201pEco_{5.5}, the fragment was then subcloned into the *Eco*RI-*Hind*III sites of pBR322 DNA, yielding subclone 201pHE_{1.0}. At the same time, the probe also hybridized to the 0.3-kb *Pst*I fragment (Fig. 1i) of 201pEH_{3.5}, which was then subcloned into the *Pst*I site of pBR322 DNA, yielding subclone 201pPst_{0.3}. The other probe, one of the *Hin*I-digested fragments of 204pVB_{2.1} DNA corresponding to codons 50 through 152, was found to hybridize not only to 201pEH_{3.5} (201pPst_{0.3})

but also to 201pHB_{1.0} and to 201pBam_{3.5}, suggesting that there may be two intron sites between codons 50 and 152. Since 201pBam_{3.5} has one *Pvu*II site, it was divided into two subclones by using this site in pBR322. They were called 201pBV_{2.2} (for 2.2-kb *Bam*HI-*Pvu*II fragment; Fig. 1j) and 201pVB_{1.3} (for 1.3-kb *Pvu*II-*Bam*HI fragment; Fig. 1k). From these data, the direction of transcription and the location of some of the exons were determined.

Strategy for the sequencing of DNA. Figure 2 shows the overall structure of the human DNA insert in the λHa201 gene and the strategies for DNA sequencing. For exon 1, *Hind*III, *Dde*I, *Acc*I, *Taq*I, and *Eco*RI sites were used for labeling the 5' end. For exon 2, *Pst*I and *Mbo*II sites were used for labeling. *Sau*96I sites were adopted for exon 3. For exon 4, *Hin*I sites were first used for labeling, but even a tail of the coding region was discovered. *Hinc*II, *Sau*96I, and *Dde*I sites were then tried, and this time codons 122 through 150 were found. *Hin*I and *Bgl*II sites were used to determine the nucleotide sequences of exon 5. *Hin*I, in combination with *Dde*I or *Bgl*II as the second enzyme to give singly end-labeled fragments, was used for exon 6 (codons 204 through 267). *Hin*I and *Sal*I sites were used for exon 7.

Structure and nucleotide sequences of human smooth muscle actin gene (aortic type). Figure 3 shows the nucleotide sequences of the exons and their adjacent intron regions. From codon 1 to the end of this clone is ca. 11.3 kb. Since the region for codons 328 through 373 is missing in this clone, the entire length of the functional gene is estimated to be more than 12 kb. The lengths of introns I through VI are ca. 1.4, 3.4, 1.9, 0.4, 1.4, and 1.3 kb, respectively. The exon-intron junctions assigned according to the GT-AG rule (GT at the 5' and AG at the 3' termini of each intron [3]) are codons 150, 204, and 267 and are between codons 41 and 42, 84 and 85, 121 and 122, and 327 and 328. The amino acid sequences predicted from the nucleotide sequences matched those reported for smooth muscle actin (bovine aortic type [25]) except for codon 309, in which the sequences determined were GTC (valine codon) instead of GCN (alanine codon). Because these sequences are a part of the *Sal*I site (GTCGAC), which we used for end labeling in the sequencing of DNA, it is not a reading mistake of GCC. Because

HUT14 cells, from which this gene was picked up, were transformed by a single treatment with 4-nitroquinoline-1-oxide (13), it was considered possible that the mismatch at codon 309 might be derived from a transition point mutation from C to T, induced by 4-nitroquinoline-1-oxide. Another point mutation has been found in β -actin of this transformed line (24). Since the *SalI* site includes a *TaqI* site (TCGA), the possibility of a point mutation was examined by Southern blot hybridization analysis of *TaqI*-digested DNA. The probe used was the 0.4-kb *PstI* fragment, which was located in intron VI and hybridized to the 2.9-kb *TaqI* fragment of λ Ha201 insert DNA (Fig. 1). Only one hybridizable band, corresponding to the 3.3-kb fragment, was detected in human diploid fibroblasts (KD cells) (Fig. 4), indicating that KD cells do not have the *TaqI* site within the 3.3-kb *TaqI* fragment. On the other hand, HUT14 cell DNA gave two bands corresponding to 3.3- and 2.9-kb fragments (Fig. 4, lane b). It is likely that human cells have only a single copy of the aortic type of smooth muscle actin gene and that HUT14 cells are heterozygous at this *TaqI* site; that is, one haploid genome has a point mutation at codon 309 in aortic smooth muscle actin gene, and the other haploid genome does not.

DISCUSSION

The amino acid sequences deduced from the nucleotide sequences were identical to those reported for bovine aortic smooth muscle actin (25), except for codon 309, in which codon GCN for alanine was replaced by codon GTC for valine. Because the DNA library from which this gene was picked was constructed from DNA of HUT14 cells, one of the chemically transformed human fibroblast cell lines (KD cells [13]), this codon difference was likely due to a point mutation. However, it was also possible that human and bovine smooth muscle actins (aortic type) differ by one amino acid, because the amino acid sequences of human smooth muscle actin have not been reported. The results of Southern blot hybridization experiments with *TaqI*-digested DNAs of KD and HUT14 cells showed that normal human fibroblasts do not have the *TaqI* site near codon 309, whereas the transformed cells are heterozygous; one haploid has the *TaqI*-sensitive site, and the other haploid does not. These results indicate that the alteration of codon 309 is due to a point mutation from G · C to A · T. This is in agreement with the report (12) that 4-nitroquinoline-1-oxide causes mainly GC \rightarrow AT transitions.

New findings are presented in this paper on the evolutionary origin of actin genes. Since the amino acid sequences are very similar among actins, it is reasonable to postulate that they have one ancestral gene. However, there is much argument about whether the ancestral gene had many introns and lost some of them during its evolution or whether introns have been inserted into an intronless ancestral gene (16). Skeletal, cardiac, and two smooth muscle actins can be classified into an α -actin family, and the structures of their genes are known (6, 10, 26); this paper and our unpublished data. To our surprise, smooth muscle actin genes (both aortic and enteric types) have two more intron sites than do skeletal and cardiac muscle actin genes. One of them is located between codons 84 and 85 and has never been reported before among actin genes, including those of vertebrates and nonvertebrates, which suggests that this intron was inserted late in the evolutionary process. The other new intron site is located between codons 121 and 122. This intron site is common not only to the β -actin gene of rats (21), but also the sea urchin actin gene (4). Other intron sites

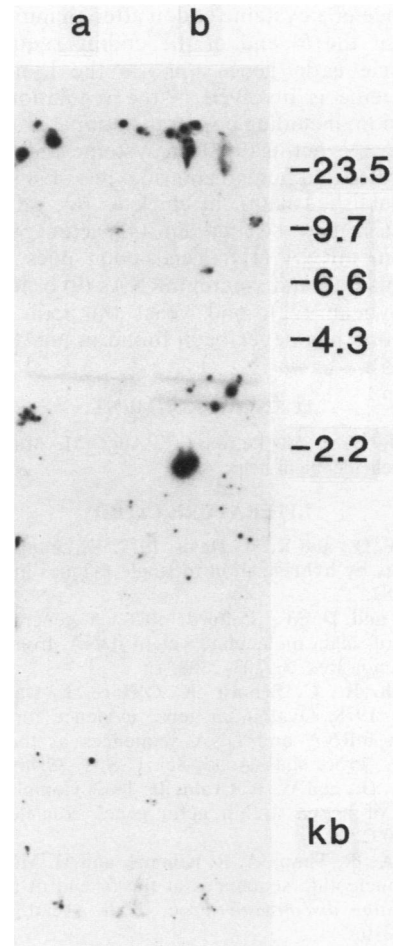


FIG. 4. Southern blot hybridization analysis of the smooth muscle actin gene (aortic type) in a human genome. Human DNAs from KD cells (a) and HUT14 cells (b) (20 μ g each) were completely digested with *TaqI*, electrophoresed on a 1% agarose gel, and transferred to a nitrocellulose filter. Hybridization experiments were carried out, with the 32 P-labeled *PstI* 0.4-kb fragment as probe, for 18 h at 65°C. The filter was washed twice with 0.3 M NaCl-0.03 M sodium citrate containing 0.5% SDS at 50°C and twice with 0.03 M NaCl-0.003 M sodium citrate containing 0.5% SDS at 60°C. The filter was dried and exposed to an X-ray film for 1 week at -70°C.

at codons 150 and 204 are not present in the β -actin gene (21), but they are present in all α -actin family genes (6, 10, 26; this paper and our unpublished data). The intron at codon 150 was also found in soybean actin genes (22), and the one at codon 204 was found in sea urchin actin genes (4). The other intron sites at codon 267 and between codons 41 and 42 and 327 and 328 are common between α -actin family genes and the β -actin gene. These findings suggest that the ancestral actin gene had introns interrupting codons at least at codons 150 and 204 and between codons 121 and 122. Through the process in which the intron interrupting between codons 121 and 122 was lost, skeletal and cardiac muscle actin genes may have been generated. The smooth muscle actin gene may have evolved from the ancestral actin gene through its obtaining a new intron between codons 84 and 85. On the other hand, the β -actin gene may have been created from the ancestral actin gene through its losing the introns interrupting at codons 150 and 204. Such a hypothesis gives both deletion and insertion as origins of introns.

The presence of a cysteine codon after an initiator methionine codon at the 5' end of the coding sequence in the smooth muscle actin gene supports the hypothesis that encoded cysteine is involved in the regulation of muscle actin expression, including post-translational processing, but not of cytoplasmic actins (9). This cysteine codon was found in human cardiac and human enteric types of smooth muscle (10; our unpublished data), in chick α - (6), rat α - (26), six *Drosophila* (7), and sea urchin (4) actin genes and in human α -actin mRNA (11). This codon does not exist in human fibroblast β - and γ -actin mRNAs (9) or in *D. discoideum* (5), soybean (22), and yeast (8) actin genes. The cysteine residue has never been found in any type of actin molecules (25).

ACKNOWLEDGMENT

We thank J. Hare, M. Petrino, C. Augl, M. Mullinix, and L. Nischan for their technical help.

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