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# The Development Expression of the Rat α-Vascular and γ-Enteric Smooth Muscle Isoactins: Isolation and Characterization of a Rat γ-Enteric Actin cDNA

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**We have isolated and characterized two cDNA clones from whole rat stomach, pRVαA-19 and pREγA-11, which are specific for the α-vascular and γ-enteric smooth muscle isoactins, respectively. The rat γ-enteric smooth muscle actin contains a single amino acid substitution of a proline for a glutamine at position 359 of the mature peptide when compared with the chicken gizzard γ-actin sequence (J. Vandekerckhove and K. Weber, FEBS Lett. 102:219, 1979). Sequence comparisons of the 5′ and 3′ untranslated (UT) regions of the two smooth muscle actin cDNAs demonstrate that these regions contain no apparent sequence similarities. Additional comparisons of the 5′ UT regions of the two smooth muscle actin cDNAs to all other known actin sequences reveal no apparent sequence similarities for the rat γ-enteric isoactin within the 15 base pairs of sequence currently available, while the rat α-vascular isoactin contains two separate sequences which are similar to sequences within the 5′ UT regions of the human and chicken α-vascular actin genes. A similar comparison of the 3′ UT regions of the two smooth muscle actins demonstrates that the α-vascular isoactins do not contain the high degree of cross-species sequence conservation observed for the other isoactins and that the γ-enteric isoactin contains an inverted sequence of 52 nucleotides which is similar to a sequence found within the 3′ UT regions of the human, chicken, and rat β-cytoplasmic isoactins. These observations complicate the apparent cross-species conservation of isotype specificity of these domains previously observed for the other actin isoforms. Northern blot analysis of day 15 rat embryos and newborn, day 19 postbirth, and adult rats demonstrates that the day 15 rat embryo displays low to undetectable levels of smooth muscle isoactin mRNA expression. By birth, the stomach and small intestine show dramatic increases in α-vascular and γ-enteric actin expression. These initially high levels of expression decrease through day 19 to adulthood. In the adult rat, the uterus and aorta differ in their content of smooth muscle isoactin mRNA. These results demonstrate that the γ-enteric and α-vascular isoactin mRNAs are coexpressed to various degrees in tissues which contain smooth muscle.**

The actin multigene family provides a useful model system to examine the mechanisms involved in gene regulation during embryonic development. Prior studies (43, 44) have identified at least six highly conserved vertebrate actin isoforms which include two striated muscle isoactins (α-skeletal and α-cardiac), two smooth muscle isoactins (α-vascular and γ-enteric), and two cytoplasmic isoactins (β-cytoplasmic and γ-cytoplasmic). In the species which contain multiple actin isoforms, it has been demonstrated that the various isoactins are differentially expressed during embryonic development in a tissue-specific manner (12, 14, 20, 24, 27, 38). In addition, these various actin isoforms appear to be coexpressed so that the striated muscle, smooth muscle, and cytoplasmic isoactins are always paired (17, 20, 24, 42, 46). This phenomenon is more pronounced during development, but it is also evident in adult tissues, in which one specific isoform of the pair usually predominates within a given cell type.

The demonstration that the various isoactin mRNAs contain domains within their 3′ untranslated (UT) regions which are isotype specific makes it possible to generate actin-isoform-specific probes. At present, cDNA fragments have been identified which are specific for the α-skeletal, α-cardiac, β-cytoplasmic and γ-cytoplasmic isoactins (8, 10, 18, 31). In addition, two independent groups have recently reported the isolation and partial characterization of α-vascular actin cDNAs which also contain α-vascular actin-specific domains (21, 22). No specific cDNA clone is currently available for the γ-enteric smooth muscle actin isoform. Interestingly, sequence analysis has demonstrated that these isotype-specific 3′-UT-region domains tend to be highly conserved across widely divergent species (16, 29, 31, 32, 34, 47, 48). This observation has implicated these conserved regions in the regulation of expression of the various isoactins in which they are found (16, 47, 48). Similarly conserved domains found within the 3′ UT regions of several other mRNA species have been shown to be essential for the proper expression of these messages (3, 35). Recently, DePonti-Zilli et al. (9) have shown that a 40-base-pair (bp) sequence in the 3′ UT region of the β-actin gene is responsible for transcriptional regulation of this gene during myogenesis. However, similar functions have not been demonstrated for the other conserved domains found within the 3′ UT regions of the remaining actin isoforms.

The lack of well-characterized smooth muscle actin-specific clones has hampered the developmental studies involving smooth muscle tissues. Even so, previous studies (24, 44–46) have shown that the γ-enteric and α-vascular actins are coexpressed in smooth muscle tissues and that their relative proportions differ from tissue to tissue. Additional studies have demonstrated that the expression of smooth muscle isoactins are regulated by development (22, 24), hormones (21), pathological conditions such as atheroscle-

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rosis (13, 36), and specific culture conditions (1, 39, 41). The complete nucleotide sequence of the chicken α-vascular actin gene and a partial nucleotide sequence of the human α-vascular actin gene have been reported (5, 41). Recently, Carroll et al. (6) identified a *cis*-acting repressor element within the core promoter of the chicken α-vascular actin gene.

In an attempt to further define the developmental expression of the two smooth muscle actins, we isolated and characterized a rat γ-enteric smooth muscle actin cDNA designated pREγA-11. This γ-enteric actin cDNA contains a single amino acid substitution from the chicken gizzard γ-actin sequence reported by Vandekerckhove and Weber (45) and represents the first report of a γ-enteric smooth muscle actin cDNA. In addition, we have isolated and recently reported the nucleotide sequence of a rat α-vascular smooth muscle actin cDNA designated pRVαA-19 (26). Sequence comparisons of these two rat smooth muscle actin cDNAs demonstrate that their 3′ UT regions do not possess the high degree of cross-species, isotype-specific sequence similarities observed for the other actin isoforms. Isotype-specific domains of both the γ-enteric and α-vascular actin cDNAs were utilized to examine the developmental expression of the smooth muscle actins in various tissues of embryonic, neonatal, day 19 postbirth, and adult rats.

## MATERIALS AND METHODS

**Production and screening of a rat stomach cDNA library.** Double-stranded cDNAs synthesized from whole rat stomach poly(A)$^+$ RNA by the method of Gubler and Hoffman (15) and size fractionated from 1.0 to 3.2 kilobases (kb) were kindly provided by G. Schull, University of Cincinnati College of Medicine. Exactly 100 ng of these double-stranded cDNAs were poly(C) tailed, annealed to poly(G)-tailed pBR322 (Bethesda Research Laboratories, Inc.), and used to transform competent *Escherichia coli* RR1 cells. The resulting cDNA library was replicated as outlined by Hanahan and Messelson (19) and screened for sequences complementary to actin by using the actin probe pHMαA-PX (10), which hybridizes to all known actins. The actin probe pHMαA-PX was labeled by the random primer method (11) to a specific activity of at least $10^8$ cpm/μg, and hybridizations were carried out under the conditions described by Erba et al. (10). Colonies displaying a duplicated positive signal to pHMαA-PX were chosen for further analysis.

**Identification of cDNAs containing stomach-specific actin sequences.** Purified plasmid DNA (25) isolated from putative positive colonies was digested with *Pst*I, electrophoresed on a 1% agarose gel, and prepared for Southern blot analysis (37). The blot was probed with a mixed pool of four 3′-UT-specific actin probes: pHMαA-3′ UT-Fnu, pHMcA-3′ UT-DB, pHFβA-3′ UT-HF, and pHFγA-3′ UT-Fnu (Table 1). The clones which displayed no hybridization with any of the four 3′-UT-specific probes were chosen for subclassing. Subclassing was performed by digesting purified plasmid DNA from each of the clones with *Sau*3AI or *Hin*fI. The digests were Southern blotted, as above, and reprobed with pHMαA-PX. Clones showing similar fragmentation patterns were classed together. The largest clone of each class was digested with a series of restriction enzymes under the conditions designated by the supplier (Bethesda Research Laboratories). Each restriction map generated was cross-referenced to the other clones and to all known rat-specific actin restriction maps. Two novel actin clones designated pRVαA-19 and pREγA-11 were chosen for complete sequence analysis, and two additional actin cDNA clones designated pREγA-5 and pREγA-10 were chosen for partial sequence analysis.

**M13 cloning and sequencing.** Specific actin cDNA restriction fragments were subcloned into the M13 cloning vectors mp18 or mp19 for DNA sequence analysis. The M13 constructs were used to transform *E. coli* JM109 (*recA*). Single-stranded M13 templates were sequenced by using the quasi-end-labeling technique (4). DNA sequences were recorded and analyzed by using the computer programs of Queen and Korn (33) on an IBM AT computer.

**Isolation and characterization of smooth muscle actin-specific probes.** α-Vascular- and a γ-enteric-actin-specific cDNA probes were generated by isolating a *Dde*1-*Pst*1 3′-UT-region restriction fragment from the 3′ UT region of pRVαA-19 (26) and an *Xmn*I-*Pst*I 3′-UT-region restriction fragment from the 3′ UT region of pREγA-11, respectively. The *Dde*I end of the pRVαA-19 restriction fragment was specifically blunt-ended by using the Klenow fragment at 14°C for 2 h under standard conditions (Bethesda Research Laboratories). Both restriction fragments were subcloned into *Sma*I-*Pst*I-digested Bluescript SK as described by the supplier (Stratagene). Direct sequence analysis was performed on insert DNA by using single-stranded Bluescript SK DNA under the conditions for sequencing described above. Specificity of the subclones, designated pRVαA-3′ UT-DP and pREγA-3′ UT-XP, was determined by probing DNA dot blots containing 2 μg of purified plasmid DNA from pHMαA-PX, pHFγA-3′ UT-Fnu, pHFβ-3′ UT-HF, pHMcA-3′ UT-DB, pHMαA-3′ UT-Fnu, pHFαA-1, pHFβA-1, clone 11, clone 19, pBR322, and Bluescript SK with each of

TABLE 1. Summary and specificity of actin cDNAs used in this study

| Clone (reference) | Description | Specificity |
|---|---|---|
| pRVαA-19 (26) | Rat α-vascular actin cDNA | All actins from higher eucaryotes |
| pREγA-11 (this paper) | Rat γ-enteric actin cDNA | All actins from higher eucaryotes |
| pHFβA-1 (17) | Human β-cytoplasmic actin cDNA | All actins from higher eucaryotes |
| pHMαA-1 (17) | Human α-skeletal actin cDNA | All actins from higher eucaryotes |
| pHMαA-PX (10) | Human α-skeletal actin cDNA from amino acids 202–374 | All actins from higher eucaryotes |
| pRVαA-3′ UT-DP (this paper) | Rat α-vascular actin 3′ UT region from bp 1229–1319 | Rat and mouse α-vascular actin |
| pREγA-3′ UT-XP (this paper) | Rat γ-enteric actin 3′ UT region from bp 1134–1237 | Rat γ-enteric actin |
| pHMαA-3′ UT-Fnu (18) | Human α-skeletal actin 3′ UT region from bp 115–251 | Human and rodent α-skeletal actin |
| pHMcA-3′ UT-DB (18) | Human α-cardiac actin 3′ UT region from bp 1–171 | Human and rodent α-cardiac actin |
| pHFβA-3′ UT-HF (10) | Human β-cytoplasmic actin 3′ UT region from bp 133–537 | Human and rodent β-cytoplasmic actin |
| pHFγA-3′ UT-Fnu (10) | Human γ-cytoplasmic actin 3′ UT region from bp 345–710 | Human and rodent γ-cytoplasmic actin |

these 3'-UT-region domains. The dot blots were prehybridized in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt–1% sodium dodecyl sulfate–100 mM sodium phosphate, pH 6.5–250 μg of salmon sperm DNA per ml at 65°C for 2 h. Denatured probe was added directly to this mixture, and the membrane was hybridized overnight at 65°C. The hybridization solution was discarded, and the membranes were washed three times for 5 min each time and one time for 20 min at room temperature in 2× SSC–0.2% SDS. The membranes were then washed once for 20 min at room temperature and once for 1 h at 65°C with vigorous agitation in 0.2× SSC–0.2% SDS. The membranes were then exposed to film for an appropriate period of time.

**Northern blot analysis.** Total cellular RNA was isolated from 1 to 2 g of tissue by a procedure based on the methods of Chirgwin et al. (7). The tissues examined included whole rat embryos at day 15 of gestation which had been trisected into a head, gut, and tail region; skeletal muscle, heart, stomach, intestine, kidney, liver, and brain of newborn, day 19, and adult rats; and uterus and aorta of adults rats. Northern blot (RNA blot) analysis was performed with 20 μg of total cellular RNA by the method of Thomas (40) by using Biotrans nylon membrane (ICN Pharmaceuticals Inc.). The Northern blots were probed with the pRVαA-3' UT-DP- and pREγA-3' UT-XP-specific fragments under the prehybridization, hybridization, and wash conditions outlined above. These same Northern blots were then stripped and reprobed with pHMαA-PX under the conditions suggested by Erba et al. (10). The resulting blots were exposed to Kodak (X-Omat) AR film for an appropriate period of time. Multiple exposures of the smooth muscle actin Northern blots were scanned by using a Hoefer Scientific Instruments GS-300 transmittance/reflectance scanning densitometer. The data were analyzed by using an IBM PC-AT computer, and the exposures which displayed linearity were averaged and reported as a specific fold increase or decrease over the baseline value.

## RESULTS

The screening of approximately 50,000 clones from a rat stomach cDNA library yielded two primary groups of stomach-specific actin cDNAs. One class of these clones contained the cDNA designated pRVαA-19 which has been previously described (26) and shown to be an α-vascular-actin-specific cDNA. The second class of clones contained three cDNAs, one of which was designated pREγA-11. A partial restriction map of this clone and the series of subclones generated to sequence it are shown in Fig. 1. The complete nucleotide sequence of pREγA-11 is shown in Fig. 2. The entire clone is 1,237 bp in length with 5' and 3' UT regions of 15 and 91 bp, respectively. The amino acid sequence encoded by pREγA-11 is in agreement with that proposed by Vandekerckhove and Weber (44) for the chicken gizzard γ-actin, except for a single amino acid substitution of a proline for a glutamine at position 359 of the mature peptide. The first amino acid of the mature actin protein-coding region is preceded by the previously reported Met-Cys initiator sequence (18). A possible polyadenylation signal and poly(A)$^+$ tail are observed at bp 1203 and 1222, respectively.

Since previous studies (2, 6, 9) have shown that both the 5' and 3' UT regions of the various isoactins are involved in the regulation of expression of these isoactins, we compared the sequences of these specific regions of our two smooth muscle actin cDNAs with each other and with all other known actin sequences. When the 3' UT regions of pRVαA-19 and pREγA-11 were compared with each other, no apparent sequence similarities were found. A similar comparison of the 3' UT regions of pRVαA-19 and pREγA-11 with the remaining actin isoforms yielded several interesting observations. First, the γ-enteric actin 3' UT region contained an inverted sequence of 52 bp which displayed 77, 66, and 61% similarity to a sequence within the 3' UT region of the β-cytoplasmic actin human cDNA, chicken gene, and rat gene, respectively (Fig. 3). Second, the α-vascular actin 3' UT region showed no sequence similarities to the chicken or human α-vascular actin genes but showed 91% sequence similarity to the mouse α-vascular actin 3' UT region (A. Strauch, personal communication). As previously reported (26), this sequence similarity does not begin until after the initial 35 bp of the GC-rich segment which is found only in the rat α-vascular actin 3' UT region. The significance, if any, of this rat-specific, highly repetitive GC-rich segment is currently under investigation.

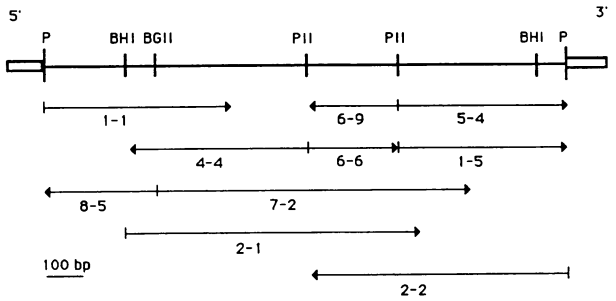


FIG. 1. Partial restriction map and sequencing strategy for the rat γ-enteric smooth muscle actin cDNA. The open boxes represent pBR322, and the solid line represents the insert pREγA-11. Each labeled arrow depicts the designation, origin, length, and direction of sequencing for an individual subclone. Arrows pointing to the right indicate sequencing on the upper strand, and arrows pointing to the left indicate sequencing on the lower strand. Restriction endonuclease sites: BHI, *Bam*HI; BGII, *Bgl*II; P, *Pst*I; PII, *Pvu*II.

A similar series of comparisons were made with the 5' UT regions of both pRVαA-19 and pREγA-11. No apparent sequence similarities were observed for the γ-enteric actin 5' UT region, but only 15 bp of this region are presently available for this analysis. However, a comparison of the α-vascular actin 5' UT region with the human and chicken α-vascular actin genes showed two separate regions of significant sequence similarity (Fig. 4). The human α-vascular actin gene contains a 26-bp stretch of 80% similarity in the 5'-flanking sequence immediately preceding the initiation codon, but the corresponding sequence in the chicken α-vascular smooth muscle actin gene shows only 60% similarity. The chicken α-vascular actin gene contains a second sequence of 19 nucleotides further upstream which displays 74% similarity to the corresponding region in the rat. This segment is absent from the human α-vascular smooth muscle actin gene.

Prior studies (18, 27, 30, 31, 33, 45, 46) have demonstrated that specific domains within the 3' UT regions of the various isoactins are isotype specific. To analyze these regions of pRVαA-19 and pREγA-11, we generated two subclones designated pRVαA-3' UT-DP and pREγA-3' UT-XP, which contain the 3' UT regions of the rat α-vascular and γ-enteric isoactins, respectively. Cross-hybridization studies with pRVαA-3' UT-DP and pREγA-3' UT-XP demonstrate that neither 3' UT region showed any marked cross-reactivity

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                                        30                                  60
TCC ACC GCG CCC ACC ATG TGT GAA GAA GAG ACC ACC GCC CTT GTG TGT GAC AAT GGG TCT
                    Met Cys Glu Glu Glu Thr Thr Ala Leu Val Cys Asp Asn Gly Ser
                                        90                                 120
GGC CTG TGC AAG GCA GGC TTT GCA GGA GAC GAC GCT CCC AGG GCT GTC TTT CCC TCC ATT
Gly Leu Cys Lys Ala Gly Phe Ala Gly Asp Asp Ala Pro Arg Ala Val Phe Pro Ser Ile
                                       150                                 180
GTG GGC CGC CCT CGG CAT CAG GGC GTG ATG GTG GGA ATG GGC CAG AAA GAC AGC TAT GTG
Val Gly Arg Pro Arg His Gln Gly Val Met Val Gly Met Gly Gln Lys Asp Ser Tyr Val
                                       210                                 240
GGG GAC GAA GCC CAG AGC AAG CGT GGG ATC CTG ACC CTC AAA TAC CCC ATT GAG CAC GGC
Gly Asp Glu Ala Gln Ser Lys Arg Gly Ile Leu Thr Leu Lys Tyr Pro Ile Glu His Gly
                                       270                                 300
ATC ATC ACG AAC TGG GAT GAC ATG GAG AAG ATC TGG CAC CAC TCC TTC TAC AAC GAG CTG
Ile Ile Thr Asn Trp Asp Asp Met Glu Lys Ile Trp His His Ser Phe Tyr Asn Glu Leu
                                       330                                 360
CGA GTA GCA CCA GAA GAG CAC CCG ACC CTG CTC ACA GAG GCC CCC CTA AAC CCT AAA GCC
Arg Val Ala Pro Glu Glu His Pro Thr Leu Leu Thr Glu Ala Pro Leu Asn Pro Lys Ala
                                       390                                 420
AAC AGG GAG AAG ATG ACC CAG ATC ATG TTC GAA ACC TTC AAT GTT CCT GCC ATG TAT GTT
Asn Arg Glu Lys Met Thr Gln Ile Met Phe Glu Thr Phe Asn Val Pro Ala Met Tyr Val
                                       450                                 480
GCC ATT CAA GCT GTG CTC TCG CTC TAT GCA TCT GGC CGC ACC ACA GGC ATC GTC CTG GAT
Ala Ile Gln Ala Val Leu Ser Leu Tyr Ala Ser Gly Arg Thr Thr Gly Ile Val Leu Asp
                                       510                                 540
TCA GGG GAT GGC GTC ACC CAC AAT GTC CCC ATC TAC GAG GGC TAT GCA CTG CCC CAT GCC
Ser Gly Asp Gly Val Thr His Asn Val Pro Ile Tyr Glu Gly Tyr Ala Leu Pro His Ala
                                       570                                 600
ATC ATG CGT CTT GAC CTG GCT GGA CGG GAT CTC ACA GAC TAC CTC ATG AAA ATT CTC ACA
Ile Met Arg Leu Asp Leu Ala Gly Arg Asp Leu Thr Asp Tyr Leu Met Lys Ile Leu Thr
                                       630                                 660
GAA AGA GGC TAT TCC TTT GTG ACC ACA GCT GAG AGA GAA ATT GTG CGA GAC ATC AAG GAG
Glu Arg Gly Tyr Ser Phe Val Thr Thr Ala Glu Arg Glu Ile Val Arg Asp Ile Lys Glu
                                       690                                 720
AAG CTG TGC TAC GTA GCC CTG GAT TTT GAG AAT GAG ATG GCC ACG GCG GCT TCG TCT TCT
Lys Leu Cys Tyr Val Ala Leu Asp Phe Glu Asn Glu Met Ala Thr Ala Ala Ser Ser Ser
                                       750                                 780
TCC CTG GAG AAG AGC TAC GAG TTG CCT GAT GGG CAG GTC ATC ACG ATT GGC AAT GAG CGC
Ser Leu Glu Lys Ser Tyr Glu Leu Pro Asp Gly Gln Val Ile Thr Ile Gly Asn Glu Arg
                                       810                                 840
TTC CGC TGC CCG GAG ACC CTG TTC CAG CCT TCC TTC ATT GGC ATG GAG TCA GCT GGA ATT
Phe Arg Cys Pro Glu Thr Leu Phe Gln Pro Ser Phe Ile Gly Met Glu Ser Ala Gly Ile
                                       870                                 900
CAT GAG ACA ACA TAC AAT TCC ATC ATG AAG TGT GAC ATT GAC ATC CGC AAG GAT TTG TAT
His Glu Thr Thr Tyr Asn Ser Ile Met Lys Cys Asp Ile Asp Ile Arg Lys Asp Leu Tyr
                                       930                                 960
GCT AAC AAT GTC CTC TCT GGG GGC ACT ACC ATG TAC CCT GGG ATT GCT GAC AGG ATG CAG
Ala Asn Asn Val Leu Ser Gly Gly Thr Thr Met Tyr Pro Gly Ile Ala Asp Arg Met Gln
                                       990                                1020
AAG GAG ATC ACA GCC TTG GCT CCC AGC ACC ATG AAG ATC AAG ATC ATC GCT CCT CCG GAG
Lys Glu Ile Thr Ala Leu Ala Pro Ser Thr Met Lys Ile Lys Ile Ile Ala Pro Pro Glu
                                      1050                                1080
CGG AAG TAC TCA GTC TGG ATT GGA GGC TCC ATC CTG GCT TCT CTC TCC ACC TTC CAG CAA
Arg Lys Tyr Ser Val Trp Ile Gly Gly Ser Ile Leu Ala Ser Leu Ser Thr Phe Gln Gln
                                      1110                                1140
ATG TGG ATC AGC AAG CCA GAG TAT GAT GAA GCG GGG CCC TCC ATT GTC CAC AGG AAA TGC
Met Trp Ile Ser Lys Pro Glu Tyr Asp Glu Ala Gly Pro Ser Ile Val His Arg Lys Cys
                                      1170                                1200
TTC TAA AGT CAC AGG GCC TTC TCT GGG GAT CCC TGC AAG ACT GCT GTC ACC AGT CAC AGA
Phe End

TCA TTA AAA CCT TCA AGC CTT AAA AAA AAA AAA AAA A 1237
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FIG. 2. Complete nucleotide sequence of the γ-enteric smooth muscle actin cDNA pREγA-11. The deduced amino acid sequence is shown by the three-letter code. The nucleotide sequence is numbered consecutively, starting with the first base of the cDNA. The single amino acid substitution mentioned in the text is underlined.

with any of the actin cDNAs listed in Table 1. On the basis of these observations and the sequence comparison data, it appears that these two 3′-UT-region fragments are isotype-specific for the α-vascular and γ-enteric isoforms of actin.

Northern blot analysis using the α-vascular actin-specific subclone demonstrated an intense band below the 18S standard which predicts an mRNA size of approximately 1.4 kb (Fig. 5). The day 15 rat embryo demonstrated low levels of α-vascular actin mRNA expression, with the gut region displaying the highest level of α-vascular actin mRNA expression, followed by the tail and then the head regions. The skeletal muscle and heart displayed low levels of α-

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RAT BETA        155TGTTTTGTTTTGGCGCTTTTGACTCAAGGATTTAAAAACTGGAACGGTGA-AGGCGACCGCAGTT218
                    :    :  ::   ::::    ::::    :   :::  : :  : :    ::  :   :     :
RAT GAMMA      1229TTTTTTTTTAAGGC----TTGA----AGG-TTT--TAA-T-GATCTGTGACTGGTGACAGCAGTC1178
                         ::          :::    :   :::  : :  : :    ::
HUMAN BETA     1283TTTTTTTTTTTGGC----TTGACTC-AGGATTTAAAAACTGGAACGGTGA-AGGTGACAGCAGTC1341
                      :  ::   ::      :::    :  :::: : :  :::    ::    :     :
CHICKEN BETA   2650TTTTTTCTTTTGGCGC--TTGACTC-AGGATTAAAAAACTGGAATGGTGA-AGGTGTCAGCAG-C2710
```

FIG. 3. Sequence comparison of the 3′ UT regions of the rat γ-enteric smooth muscle cDNA pREγA-11 and the rat, human, and chicken β-cytoplasmic actins (23, 28, 32). The appropriate nucleotide positions are indicated as superscripts. The rat γ-enteric sequence represents an inverted sequence and is numbered accordingly. Mismatches with the rat γ-enteric sequence are indicated by colons. Percent sequence similarities: rat/rat, 61%; rat/human, 77%; rat/chicken, 66%.

vascular actin mRNA expression at birth, day 19, and adulthood. The kidney, liver, and brain showed extremely low levels of α-vascular actin mRNA expression at birth and day 19, and α-vascular actin mRNA expression was undetectable by adulthood. The stomach displayed high levels of α-vascular actin mRNA expression at birth, followed by a 4.8-fold decrease in expression by adulthood. The small intestine also demonstrated a relatively high level of α-vascular actin mRNA expression at birth. These levels were maintained through day 19, followed by a 3.7-fold decrease in expression by adulthood. Interestingly, the content of α-vascular actin mRNA in the small intestine was significantly less than that observed in the stomach at all time points examined: 5.1-fold at birth, 1.5-fold at day 19, and 3.5-fold by adulthood. In the adult rat, the uterus displayed a level of α-vascular actin mRNA expression which was 2.1-fold less than that of the adult stomach, while the aorta contained a 7.8-fold higher level of α-vascular actin mRNA expression than did the adult stomach. It is important that on longer exposure a second higher-molecular-weight mRNA species of 5.1 kb was observed in the tissues that displayed high levels of α-vascular actin mRNA expression.

As with the α-vascular actin Northern blots, Northern blot analysis using the γ-enteric-actin-specific subclone identified a primary mRNA transcript of approximately 1.4 kb (Fig. 6). The day 15 rat embryo contained no detectable levels of γ-enteric actin mRNA. In a similar manner, the skeletal muscle, heart, kidney, liver, and brain displayed undetectable levels of γ-enteric actin mRNA expression from birth through adulthood. In contrast, the stomach demonstrated extremely high levels of γ-enteric actin mRNA expression at birth. These high levels of γ-enteric actin mRNA expression had decreased 2.2-fold by adulthood. The small intestine also displayed a high level of γ-enteric actin mRNA expression at birth, which decreased 3.0-fold by adulthood. The content of γ-enteric actin mRNA found in the small intestine was consistently less than that observed in the stomach: 3.9-fold at birth, 1.8-fold at day 19, and 5.3-fold by adulthood. These results are similar to those observed for the expression of the α-vascular actin mRNA. In the adult rat, both the uterus and aorta displayed low levels of γ-enteric actin mRNA expression, with the uterus and aorta showing a 10.6-fold and an 8.6-fold decrease in γ-enteric actin mRNA expression, respectively, when compared with the adult stomach. As in the α-vascular actin Northern blot, a second high-molecular-weight mRNA species was observed at approximately 5.1 kb in the tissues that displayed the highest levels of γ-enteric actin mRNA expression.

Figure 7 shows the same blot shown in Fig. 5 and 6 stripped and reprobed with the actin probe pHMαA-PX, which has been shown to hybridize to all known actin isoforms found in higher eucaryotes. An examination of this blot shows that various levels of actin expression were present in each of the tissues examined at the different time points during development. In addition, this blot is shown in its entirety from origin to dye front to demonstrate that no excessive mRNA degradation is present in any of the lanes. The higher-molecular-weight band of approximately 2.6 kb represents the position at which the two cytoplasmic isoactins migrate. The lower-molecular-weight bands of approximately 1.6 and 1.4 kb indicate the positions of the two striated muscle isoactins and the two smooth muscle isoactins, respectively. A direct comparison of this control blot with the two smooth muscle actin Northern blots shown in Fig. 5 and 6 demonstrates that the α-vascular and γ-enteric actin mRNA bands are coincident with the smooth muscle actin bands identified by this blot. The same apparent nonspecific binding at the 28S rRNA band observed in the smooth muscle blots is also present in the control blot. Ethidium bromide staining of these gels demonstrates that similar quantities of total cellular RNA were added to all lanes.

## DISCUSSION

This study represents the first reported isolation and characterization of a γ-enteric smooth muscle actin cDNA. Sequence analysis of this clone reveals that the γ-enteric smooth muscle actin cDNA contains the previously observed Met-Cys initiator dipeptide found at the amino terminus of the other muscle isoactins and demonstrates that this dipeptide is common to all four of the muscle actin isoforms. In addition, the rat γ-enteric actin contains a proline at residue 356 and thus differs from the reported

```
Human                    -25AGAATCCT-GTGAAGCAGCTCCAGCT-1
                               : :   : :     :
Rat          -42CCAGCCAGTCGCCATCAGGAACCTCGAGAAGCTGCTCCAGCT-1
                        :: : ::
Chicken      27CCAGCCAAGCACTGTCAGG45
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FIG. 4. Sequence comparison of the 5′ UT regions of the rat α-vascular smooth muscle actin pRVαA-19 and the human and chicken α-vascular actin genes (5, 41). The appropriate nucleotide positions are indicated as superscripts. Mismatches are indicated by colons.

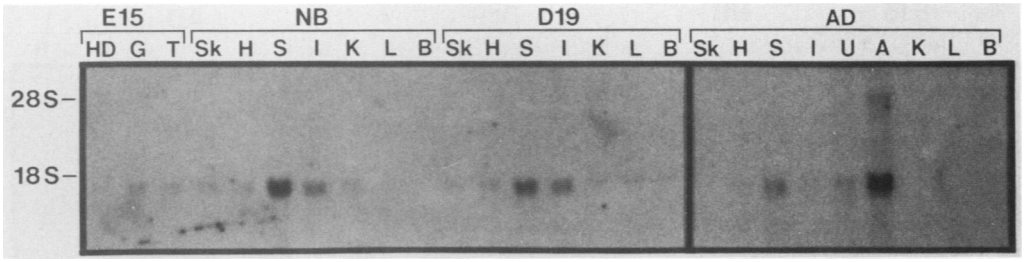


FIG. 5. Northern blot analysis using the α-vascular-actin-specific subclone pRVαA-3′ UT-DP. Approximately 20 μg of total cellular RNA was blotted from the head (HD), gut (G), and tail (T) regions of day 15 rat embryos (E15) and from the skeletal muscle (Sk), heart (H), stomach (S), intestine (I), kidney (K), liver (L), and brain (B) of newborn (NB), day 19 postbirth (D19), and adult (AD) rats. In addition, RNA from the uterus (U) and aorta (A) of adult rats was also blotted. The positions of the 28S and 18S standards are indicated.

sequence of chicken gizzard γ-actin, which has a glutamine at this position. The significance of this substitution is not known, but it is unlikely that this clone represents a third putative smooth muscle actin isoform. This assumption is supported by the fact that two additional rat γ-enteric actin cDNAs, pREγA-5 and pREγA-10, which were partially sequenced both contained this amino acid substitution. In addition, the 3′ UT region of pREγA-11 identifies a major actin component found primarily in the rat gut and uterus. Consequently, this amino acid substitution most likely represents a species- and/or tissue-specific difference observed in the rat.

The sequence comparisons of pRVαA-19 and pREγA-11 indicate that there is a lack of sequence conservation between the 3′ UT regions of the two smooth muscle isoactins and that the 3′ UT region of the α-vascular actin does not display the extensive evolutionary conservation previously observed for the other actin isoforms. This observation extends the results of Carroll et al. (5), which indicate a lack of conserved 3′ UT sequences between the α-vascular actins of chickens and humans. In addition, the γ-enteric 3′ UT region contains an inverted sequence which is similar to a sequence found within the 3′ UT region of the β-cytoplasmic isoactin. This observation, as well as the fact that the two cytoplasmic isoactins and the γ-enteric isoactin all contain 1 less amino acid than the remaining three muscle actin isoforms, may provide us with a clue to the evolutionary divergence of the actin multigene family. Additional genomic information regarding the structure and organization of the rat γ-enteric smooth muscle actin gene will help to resolve this issue. All of these results are in direct contrast to the 3′-UT-region comparative sequence data obtained for the other actin isoforms. Since these regions have been implicated in the regulation of expression of these various isoforms of actin, the biological significance of these observations is not clear. However, for the smooth muscle isoactins, it would appear that the 3′ UT regions are more complex and species specific than the 3′ UT regions of the other actin isoforms. Thus, these sequences may not play a significant role in the regulation of expression of the smooth muscle isoactins.

Northern blot analysis demonstrates several interesting features about the developmental and tissue-specific expression of the γ-enteric and α-vascular smooth muscle isoactins. First, the two smooth muscle actins are represented by a primary mRNA transcript of 1.4 kb. These results are consistent with those previously reported for the α-vascular actin isoform (21, 22) and indicate that there does not appear to be multiple α-vascular actin mRNA species in the rat as was previously reported for the chicken (5). However, additional genomic and S1 nuclease data are needed to confirm these results. The higher-molecular-weight mRNA species of 5.1 kb appears to be the result of nonspecific binding of the probe, since this band comigrated with the 28S ribosomal RNA band and was also observed in the control blot. Second, the day 15 rat embryo displayed low levels of α-vascular actin mRNA expression, but there were no detectable levels of γ-enteric actin mRNA expression. These low levels of α-vascular actin mRNA expression appear to be the result of the high degree of neovascularization occurring during embryogenesis, since tissue sections of day 15 rat embryos probed with an α-vascular-actin-specific monoclonal antibody (36) showed only staining of the developing blood vessels and heart (N. Sawtell, personal communication). Consequently, the low levels of α-vascular actin mRNA expression observed within the skeletal muscle, heart, kidney, liver, and brain of newborn, day 19, and adult rats are assumed to represent the high degree of neovascularization which occurs during organ and tissue development. These results demonstrate that the smooth muscle

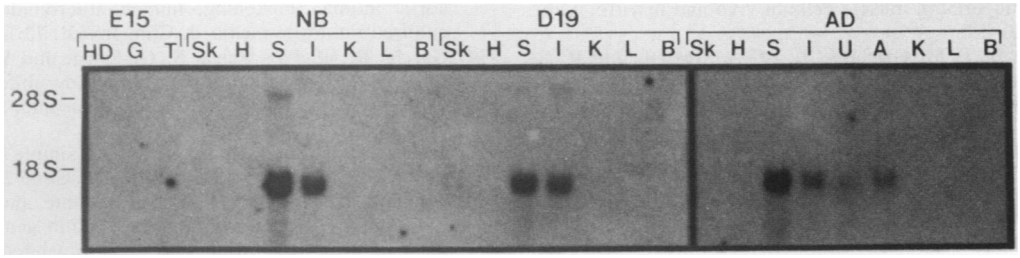


FIG. 6. Northern blot analysis using the γ-enteric-specific subclone pREγA-3′ UT-XP. Approximately 20 μg of total cellular RNA was blotted from the head (HD), gut (G), and tail (T) regions of day 15 rat embryos (E15) and from the skeletal muscle (Sk), heart (H), stomach (S), intestine (I), kidney (K), liver (L), and brain (B) of newborn (NB), day 19 postbirth (D19), and adult (AD) rats. In addition, RNA from the uterus (U) and aorta (A) of adult rats was also blotted. The positions of the 28S and 18S standards are indicated.

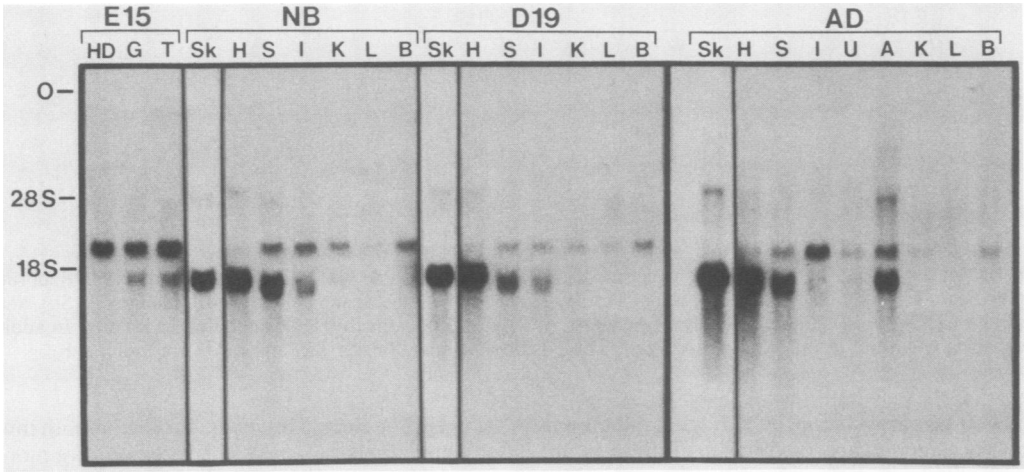


FIG. 7. Northern blot analysis of the same blot shown in Fig. 5 and 6 stripped and reprobed with the actin-coding region clone pHMαA-PX. Samples represent head (HD), gut (G), and tail (T) region of day 15 rat embryos (E15) and the skeletal muscle (Sk), heart (H), stomach (S), intestine (I), kidney (K), liver (L), and brain (B) of newborn (NB), day 19 postbirth (D19), and adult (AD) rats. In addition, DNA from the uterus (U) and aorta (A) of adult rats was also blotted. The positions of the 28S and 18S standards and gel origin (O) are indicated. Since skeletal muscle contains a high concentration of actin mRNA, the skeletal muscle lanes (Sk) were only exposed for 1 h, whereas the remainder of the gel was exposed for 6 h.

isoactins are not highly expressed in day 15 rat embryos and tissues which are not smooth muscle specific. Third, the stomach and small intestine displayed high levels of coexpression of the two smooth muscle isoactins during development of these tissues. The precise levels of expression for the γ-enteric and α-vascular actin isoforms varied in these two tissues at birth and were subsequently down-regulated in a tissue-specific manner through day 19 into adulthood. These results are similar to those observed for the coexpression of the striated muscle and cytoplasmic isoactins during development. Finally, in the adult rat, the stomach, small intestine, uterus, and aorta all displayed various levels of γ-enteric and α-vascular actin expression. These various levels of mRNA expression appear similar to the various levels of isoactin protein expression originally reported for these tissues (44–46).

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