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Novel Chicken Actin Gene: Third Cytoplasmic Isoform

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We identified a novel chicken actin gene. The actin protein deduced from its nucleotide sequence very closely resembles the vertebrate cytoplasmic actins; accordingly, we classified this gene as a nonmuscle type. We adopted the convention for indicating the nonmuscle actins of the class Amphibia (Vandekerckhove et al., J. Mol. Biol. 152:413–426) and denoted this gene as type 5. RNA blot analysis demonstrated that the type 5 actin mRNA transcripts accumulate in adult tissues in a pattern indicative of a nonmuscle actin gene. Genomic DNA blots indicated that the type 5 actin is a single copy gene and a distinct member of the chicken actin multigene family. Inspection of the nucleotide sequence revealed many features that distinguished the type 5 gene from all other vertebrate actin genes examined to date. These unique characteristics include: (i) an initiation Met codon preceding an Ala codon, a feature previously known only in plant actins, (ii) a single intron within the 5′ untranslated region, with no interruptions in the coding portion of the gene, and (iii) an atypical Goldberg-Hogness box (ATAGAA) preceding the mRNA initiation terminus. These unusual features have interesting implications for actin gene diversification during evolution.

The actin genes represent a class of eucaryotic gene families whose members are differentially expressed both temporally (13, 15, 33, 40, 44, 48, 54) and spatially (4, 13, 61) during the development of the organism. Because of these characteristics, as well as a high degree of sequence conservation, the actin gene family is particularly well suited both for analysis of the relationship between gene structure and expression and for studying the evolutionary processes that lead to gene diversification. The actins found in plants (49, 50), protozoans (37), slime molds (31), and most invertebrate species (10, 12, 45) are very diverse, yet they tend to resemble the nonmuscle actins found in higher vertebrates. The appearance of striated actins coincides with the later emergence of the chordates, with a full complement of four different striated actins present in warm-blooded vertebrates (J. Vandekerckhove, Abstr. 3rd EMBO Workshop on Mol. Cell. Aspects of Myogenesis and Myofibrillogenesis in Cell Cultures of Normal and Diseased Muscle, Zurich, Switzerland, p. 75, 1983).

In the pioneering work of Vandekerckhove and Weber (58, 59), an amino acid sequencing technique was developed which permitted the detection of six different actin isoforms in birds and mammals. These actins include two nonmuscle isoforms (β- and γ-cytoplasmic), two striated isoforms (α-skeletal and α-cardiac), and two smooth muscle isoforms (α- and γ-smooth). However, it is possible that these six isoforms represent the minimal number of actins expressed in higher vertebrates since minor actins that are present at less than 5% of the total actin content would not be detected by amino acid sequencing techniques (58). This observation becomes particularly relevant in the case of the nonmuscle actin isoforms when one considers that at least five different nonmuscle actin types have been identified in cold-blooded vertebrates (57) and the broad distribution of actin isoforms found in many other eucaryotes. It is conceivable that a few nonmuscle actin gene sequences besides the β- and γ-cytoplasmic types might have been retained during the evolutionary development of the warm-blooded vertebrates. These nonmuscle actin genes may have remained undetected either because they became nonfunctional or because they are expressed at low levels.

The identification of a complete set of actin genes in an organism is possible, independent of their level of expression, by the application of recombinant DNA techniques and DNA sequence analysis. By this methodology, 6 of a potential 8 to 10 actin genes present in the chicken genome were identified in our laboratory (5). In this report, we show the complete nucleotide sequence of one of these isolated genes, including its flanking regions, and survey the accumulation of its RNA transcripts in different tissues. Interestingly, the sequence of this gene encodes a nonmuscle actin that has not been detected previously in warm-blooded vertebrates. Therefore, nonmuscle actin gene expression is likely more complex in birds than formerly thought. We adopted the convention devised by Vandekerckhove et al. (57) for indicating the different amphibian nonmuscle actin isoforms and designated this chicken nonmuscle actin gene as type 5. The structure and 5′ flanking region of this novel nonmuscle gene have many features which distinguish this gene from all other vertebrate actin genes examined to date. These unusual characteristics have interesting implications for the evolutionary history of the actin gene family.

MATERIALS AND METHODS

Materials. Restriction endonucleases, DNA polymerase I, DNA polymerase large fragment, T4 DNA ligase, and T4 polynucleotide kinase were obtained from New England BioLabs, Inc.; calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim Biochemicals; avian myeloblastosis virus reverse transcriptase was obtained from Life Sciences, Inc.; and ^{32}P-radionucleotides were obtained from Amersham Corp. Low-melting agarose was purchased from FMC Corp., Marine Colloids Div.

Strategy for sequencing the chicken type 5 nonmuscle actin gene. The type 5 nonmuscle actin gene was isolated from a λ Charon 4A chicken genomic library (5). A 6.8 kilobase (kb) *Eco*RI fragment containing the coding and flanking DNA sequences was gel purified from the genomic clone λAC12 and ligated into the *Eco*RI site of plasmid pBR322 DNA. The resulting subclone pAC1268 was mapped by single and

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double restriction enzyme digestions. Appropriate DNA fragments were dephosphorylated with calf intestinal alkaline phosphatase, 5'-end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, and sequenced by the technique of Maxam and Gilbert (29).

Isolation of nucleic acids. Nuclear DNA was isolated from frozen adult chicken liver tissue by the method of Lawson et al. (26), as modified by Chang et al. (5). Total polyadenylated [poly(A)$^+$] RNA was isolated from frozen chicken tissues, which included 3- to 4-week-old skeletal breast muscle, aorta, brain, gizzard, heart, liver, and whole 10-day chicken embryos, as described by Schwartz and Rothblum (47).

Chicken genomic DNA and poly(A)$^+$ RNA electrophoresis and blotting. Ten micrograms of purified chicken liver nuclear DNA was digested with *Eco*RI, *Bgl*I, or *Bam*HI, and the digestion products were fractionated by electrophoresis on a 0.8% agarose gel. The DNA was transferred onto two nitrocellulose filters by the bidirectional transfer technique of Smith and Summers (53). Hybridization with nick-translated ^{32}P-labeled DNA probes was performed at 68°C for 16 to 20 h. Filters were washed four times at 65°C in 500 ml of 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate for 15 min per wash, air dried, and then exposed to Kodak XAR-5 X-ray film at −70°C with a Cronex Lighting-Plus intensifying screen (E. I. du Pont de Nemours & Co.).

RNA electrophoresis and blotting was performed as described by Thomas (55). The RNA blots were prehybridized at 42°C for 4 h in 6× SSC–10× Denhardt buffer (without bovine serum albumin)–50 mM sodium phosphate (pH 7.0)–0.5% sodium dodecyl sulfate. The blots were hybridized at 42°C for 20 to 24 h with an excess of uniformly labeled single-stranded probes in hybridization buffer containing 50% formamide, 3× SSC, 5× Denhardt buffer (without bovine serum albumin), 25 mM sodium phosphate (pH 7.5), and 0.25% sodium dodecyl sulfate. The filters were washed four times at 42°C in 500 ml of 2× SSC–0.5% sodium dodecyl sulfate, rinsed once in 2× SSC, dried, and exposed to X-ray film as described above. The autoradiograms were scanned with a Kontes Fiber Optic Scanner (model 800), and the relative areas under the hybridization band peaks were determined with an integrator (model 3390A; Hewlett-Packard Co.).

Preparation of ^{32}P-labeled DNA probes. Double-stranded DNA fragments were isolated from low-melting agarose gels and labeled with α-^{32}P-deoxynucleotides (3,000 Ci/mmol) and DNA polymerase I as described by Rigby et al. (43). Single-stranded, uniformly ^{32}P-labeled DNA probes were produced as follows. Briefly, gel-purified restriction fragments, which are homologous to the 3' end of the type 5 actin gene, were made blunt ended by S1 endonuclease digestion (2); recessed 3' termini were filled with deoxynucleotides and DNA polymerase I (Klenow fragment) and subcloned into *Sma*I-digested M13 mp10 vector DNA (32); or both procedures were done. Single-stranded chimeric M13 DNA was hybridized with a 17-mer sequencing primer which was then extended with [α-^{32}P]dATP, -dTTP, and -dCTP (3,000 Ci/mol), 1 mM dGTP, and DNA polymerase I (Klenow) at room temperature for 15 min. The reaction was chased for 10 min with cold dNTP (1 mM). The extended ^{32}P-labeled DNA then was digested with an appropriate restriction enzyme which had a single recognition site within the M13 DNA sequences downstream from the sequencing primer site. The digested DNA was heat denatured at 90°C in 30% dimethyl sulfoxide buffer (30% [vol/vol] dimethyl sulfoxide, 1 mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue) and immediately fractionated in a 5% polyacrylamide gel. The ^{32}P-labeled fragment was located by autoradiography and purified from the gel as described by Maxam and Gilbert (29).

Primer extension analysis of the 5' leader sequence. Plasmid pAC1268 was digested with *Bgl*I, and the 3' protruding termini were removed with 20 U of S1 nuclease (2). A 838-base-pair (bp) fragment, which contained the 5' terminus of the coding region at one end, was further shortened by S1 endonucleolytic nibbling (52) to approximately 800 bp and cloned into the *Sma*I site of M13 and mp10 DNA. One M13 clone designated NM9 was properly oriented for primer extension analysis of chicken mRNA, because the 5' terminus of the coding region was juxtaposed to the sequencing primer cassette of the M13 DNA. The adjacent actin sequence began 63 bases downstream from the initiation methionine codon and continued about 770 bases upstream. A 10-ng amount of the 17-mer M13 sequencing primer was end labeled with [γ-^{32}P]ATP and polynucleotide kinase (28), annealed to 10 μg of the single-stranded NM9 DNA, and extended with 5 mM dGTP, dATP, dTTP, and dCTP and DNA polymerase I (Klenow) at room temperature for 15 min. The DNA was precipitated with ethanol, resuspended in restriction buffer, and digested with *Bst*NI as recommended by the supplier. The digested DNA was precipitated with ethanol, dissolved in 30% dimethyl sulfoxide, heat denatured, and fractionated by electrophoresis through a 5% polyacrylamide gel. A 91-base single-stranded fragment, which contained 55 bases of M13 DNA at its 5' end and 36 bases spanning positions 27 to 63 downstream from the first nucleotide of the ATG codon at its 3' end, was purified from the gel. The 91-base end-labeled primer was coprecipitated with 450 μg of 10-day chicken embryo poly(A)$^+$ RNA and redissolved in 40 μl of 400 mM NaCl–25 mM Tris hydrochloride (pH 7.5)–50% deionized formamide. The sample was heated at 68°C for 25 min, immediately transferred to 42°C for 22 h, precipitated, and pelleted by centrifugation. The pellet was dissolved in 300 μl of 0.3 M sodium acetate (pH 7.0) and precipitated again with ethanol. The pellet then was suspended in 100 μl of extension buffer (50 mM Tris-hydrochloride [pH 8.0], 120 mM KCl, 7 mM MgCl, 10 mM dithiothreitol, 0.5 mM each of dGTP, dATP, dTTP, and dCTP)–1 μl of RNasin (30 U/μl; Promega Biotech). A 3-μl volume of reverse transcriptase (20 U/μl) was added to the sample, and the mixture was incubated at 42°C for 1 h. After incubation, the sample was extracted once with phenol-chloroform-isoamyl alcohol (25:24:1[vol/vol]) and precipitated with ethanol. The precipitate was collected by centrifugation, dissolved in 99% formamide containing 0.5% xylene cyanol and 0.05% bromophenol blue, and fractionated in a 18-cm 5% polyacrylamide gel (5% [wt/vol] acrylamide, 0.26% *N*,*N*'-methylene bisacrylamide). The extension product fragment was recovered from the gel and sequenced by the method of Maxam and Gilbert (29).

Endonuclease S1 mapping. Uniformly ^{32}P-labeled single-stranded DNA fragments were precipitated with poly(A)$^+$ RNA, pelleted by centrifugation, suspended in 30 μl of hybridization buffer consisting of 15 mM PIPES [piperazine-*N*-*N*'-bis(2-ethanesulfonic acid); pH 6.4], 1 mM EDTA, and 400 mM NaCl, and incubated at 68°C for 12 h. After hybridization, 320 μl of ice-cold nuclease S1 buffer (300 mM NaCl, 50 mM sodium acetate [pH 4.5], 2 mM zinc acetate [pH 4.5]) containing 2,000 U of S1 nuclease (Miles Laboratories, Inc.) was added rapidly to the reaction, and the sample was mixed well and incubated at 37°C for 2 h. The reaction was stopped with the addition of 14 μl of stop buffer

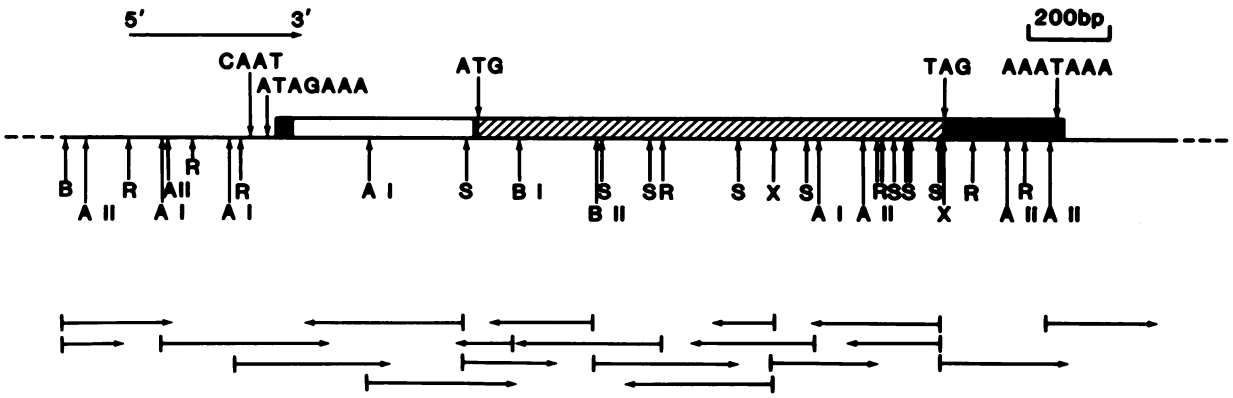


FIG. 1. Structural map and sequencing strategy for the chicken type 5 actin gene. Within a 2.6-kb region of the clone pAC1268 only those restriction sites are shown that were used for sequencing. Symbols for restriction enzymes: A 1, *Aval*; A 11, *Aval*I; B, *Bam*HI; B 1, *Bgl*I; B 11, *Bgl*II; R, *Rsa*I; S, *Sau*3AI; X, *Xba*I. The sequence was determined by the method of Maxam and Gilbert (29) in the region and direction represented by the arrows. The hatched area represents the protein coding region, the solid area represents the transcribed but untranslated region, the open bars represent the intron, and the thin line represents the flanking region. The putative regulatory signals CAAT and ATAGAA, the initiation codon ATG, the termination codon TAG, and the presumptive polyadenylic acid addition signal AAATAAA are indicated.

(0.5 M Tris-hydrochloride [pH 7.5], 0.25 M EDTA), extracted once with an equal volume of chloroform isoamyl alcohol (24:1 [vol/vol]) and precipitated with 875 μl of absolute ethanol. The precipitate was pelleted by centrifugation, redissolved in 300 μl of 0.3 M sodium acetate (pH 7.0), and precipitated again with ethanol. The precipitate was collected by centrifugation, dried under vacuum, and dissolved in 3 μl of 99% formamide containing 0.05% xylene and bromophenol blue, and the sizes of the protected fragments were determined by electrophoresis on polyacrylamide-urea sequencing gels (29).

RESULTS

Structure and nucleotide sequence of the type 5-like actin gene. The isolation of six different actin genes from a λ Charon 4A chicken genomic library was recently described (5). A 6.8-kb *Eco*RI restriction fragment isolated from one of these actin λ-clones which contained what was preliminarily identified as a nonmuscle actin gene was subcloned into the *Eco*RI site of pBR322 to produce the subclone pAC1268. The coding and flanking regions of the actin gene within pAC1268 were sequenced by the method of Maxam and Gilbert (29) (Fig. 1). The complete nucleotide sequence of 2,620 bp, which includes the encoded amino acid sequence and adjacent flanking DNA, is shown in Fig. 2.

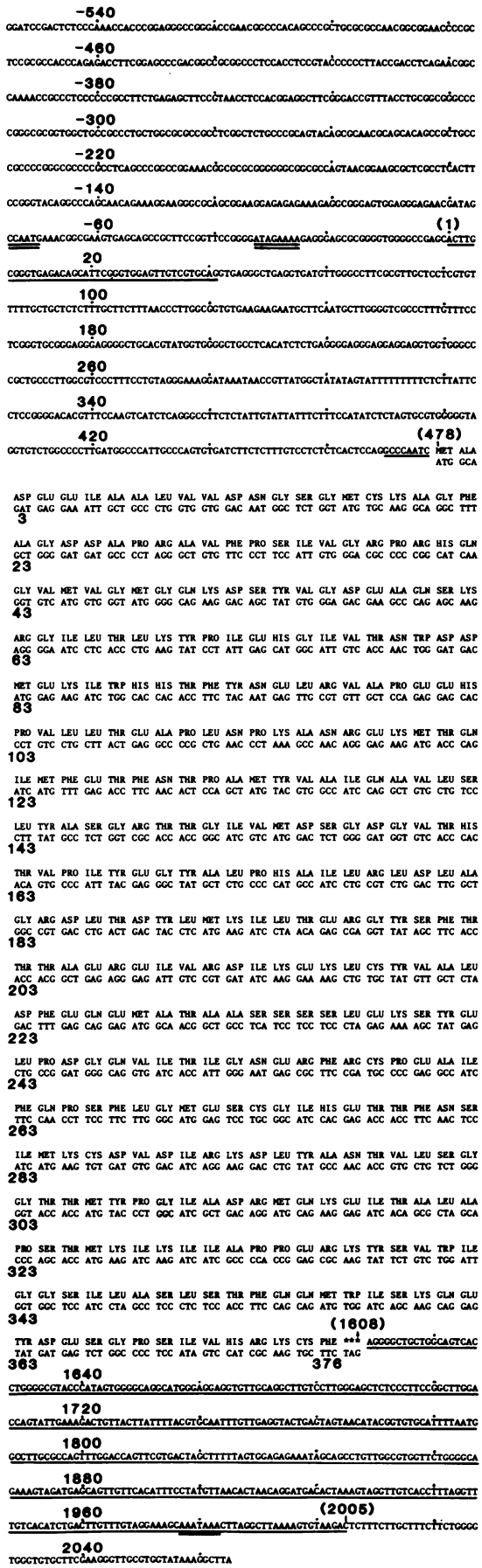
Analysis of the actin coding region. The nucleic acid sequence analysis revealed many features that distinguished this gene from all other vertebrate actin genes examined to date. First, this nonmuscle actin gene represents a new chicken actin isoform which had not been detected previously in warm-blooded vertebrates. Comparison of the encoded sequence with that of known chicken (24), rat (39), and bovine (60) β- or γ-cytoplasmic actins revealed only four amino acid replacements, one of which is a conservative substitution (Ile rather than Leu) in a nondiagnostic position (codon 262) in actin proteins (60). In contrast, comparison of this same region with avian and mammalian muscle-specific actins (11, 21, 60, 64) showed between 22 and 25 amino acid replacements.

The NH_2-terminal amino acid residues are the most reliable markers for identifying various actin isoforms (61). Examination of the NH_2-terminal region of this novel chicken nonmuscle actin gene showed that this gene most closely resembled the amphibian nonmuscle type 5 actin (Table 1). Because of this similarity, and in particular the identical combination of the NH_2-terminal acidic amino acid residues present in these two genes, we tentatively designated this gene as the chicken type 5 nonmuscle actin gene. The notation type 5 is adopted from the convention of Vandekerckhove et al. (57), who indicated the amphibian nonmuscle actins by arabic numerals which corresponded to a typical aspartate-glutamate variation at the NH_2-terminal polypeptide. The encoded protein of the nonmuscle gene also was found to be very similar to the cytoplasmic *Drosophilia* actins ACT-5C and ACT-42A at the amino-terminal region (Table 1).

Inspection of the NH_2-terminal amino acid residues of the chicken type 5 actin gene revealed a second unusual feature. Its initiation methionine codon was succeeded by an Ala codon. An Ala codon has not been previously found in the second amino acid position as examined by sequence analysis of actin genes from a variety of species across a broad evolutionary spectrum. A surprising exception is the plant actin genes (Table 1). Of the three separate and highly diverged plant actin genes examined to date, all have an amino-terminal Met-Ala dipeptide (49, 50). Except for the similarity of the NH_2-terminal dipeptides, the type 5 and plant actin genes do not closely resemble each other.

At the nucleotide level, the coding region of the type 5 actin gene differed from the same region of the chicken β-cytoplasmic (24), α-skeletal (11), and α-cardiac (K. S. Chang, K. N. Rothblum, and R. J. Schwartz, Nucleic Acids Res., in press) actin genes by 13, 17, and 20%, respectively. The coding region of the chicken β-cytoplasmic actin gene differed from the comparable regions of chicken α-skeletal and α-cardiac actin genes by 17 and 19%, respectively. Clearly, the higher degree of resemblance observed between the primary amino acid sequences encoded by the type 5 and β-cytoplasmic actin genes is reflected by the nucleic acid sequence.

Finally, the nucleotide sequence of the type 5 nonmuscle actin gene shows that its coding region is devoid of introns. None of the vertebrate actin genes examined to date, includ-



ing the α-skeletal (11, 64), α-cardiac (Chang et al., in press), α-smooth (56), and β-cytoplasmic (24, 39) actin genes, lack introns in their coding regions, except those actin genes that have been identified in human DNA as processed genes (35, 36). In contrast to these pseudogenes, the type 5 actin gene had no translational stop signals, deletions, or insertions in its coding sequence and did not have a polydeoxyadenylic acid track present approximately 20 nucleotides downstream from the putative AAATAAA polyadenylation signal (42), and the contiguous portion of the type 5 actin gene was not flanked by short direct repeats. However, the most definitive proof that the type 5 actin gene is not a processed gene was obtained by the characterization of the RNA transcripts expressed by this gene.

The 5′ untranslated and upstream flanking regions. To directly determine whether the type 5 nonmuscle actin gene is transcribed and concomitantly to define the 5′ terminus of its RNA transcript, primer directed synthesis of a DNA transcript of the 5′ end of its mRNA was performed and the labeled copy DNA was sequenced (Fig. 3). The DNA probe used for extension was homologous to a region encompassing nucleotides 23 to 63 downstream from the first nucleotide residue of the translation initiation codon.

The sequence analysis of the extension product unequivocally demonstrated that the type 5 nonmuscle gene is transcribed. It also revealed that the type 5 nonmuscle actin gene mRNA transcript was interrupted within its 5′ untranslated region by a single intron of 428 nucleotide residues. The extension product matches sequences encompassing 1 to 8 and 437 to 478 bp upstream from the coding portion of the type 5 actin gene (Fig. 2). The sequence at the junctions of the intron is in accordance with the consensus sequence for splice sites (3). The 3′ border of the intron also was confirmed by S1 mapping analysis (data not shown). The precise position of the cap site of the mRNA was precluded because of the frequent presence of a few bands of undegraded cDNA at the top of all lanes of a DNA sequencing gel (16). We chose the A residue shown in Fig. 2 as the 5′ cap site because of the observation that most eucaryotic gene transcripts are initiated with an A residue which is surrounded by pyrimidines (3).

Analysis of the sequences immediately upstream from the cap site of the mRNA showed two A-T-rich regions ATAGAA and CCAAT that begin 33 and 75 bp preceding the initiation site, respectively (Fig. 2). These sequences are very similar both in sequence and location to the consensus sequence CCAAT (1, 8) and the Goldberg-Hogness TATAA homology, which are frequently found upstream of the mRNA cap site of eucaryotic genes (3). Besides these two consensus sequences, no significant homology was observed between the 5′ flanking regions of the chicken type 5 actin gene and the other vertebrate actin genes examined to date. The G residue within the ATAGAA motif is unique to the

FIG. 2. Complete nucleotide sequence of the chicken type 5 actin gene. Numbers above the sequence indicate nucleotide sequence positions: negative numbers indicate nucleotide positions upstream from the transcription start site, and numbers within parentheses indicate the transcription start site, the translation start site, the terminal nucleotide of the coding region, and the putative polyadenylation site. Deduced amino acids of the actin protein are indicated above their respective codons and are numbered below the sequence with the initiation Met codon designated as the first codon. The 5′ and 3′ transcribed but untranslated regions are underscored. The CCAAT box, ATAGAA box, and putative polyadenylation signal AAATAAA are underscored twice.

type 5 gene among all other vertebrate actin genes previously studied.

The 3′ untranslated region. The size of the 3′ untranslated region of the type 5 actin gene was determined by S1 nuclease mapping analysis. Briefly, chicken poly(A)$^+$ RNA was hybridized with a single-stranded, uniformly ^{32}P-labeled genomic DNA probe homologous to about 1 kb of the 3′ flanking region immediately adjacent to the type 5 actin gene. This hybrid was digested with S1 nuclease and fractionated on a sequencing gel (Fig. 4A). Approximately 397 nucleotides of the DNA probe were protected by RNA from S1 nuclease digestion. To ascertain whether this probe was protected by a portion of DNA contiguous to the coding region of the type 5 gene, the DNA probe (Fig. 4A) was truncated 196 nucleotides by *Ava*II digestion and then used for S1 nuclease analysis. A DNA fragment of 201 nucleotides was protected from S1 nuclease digestion (Fig. 4B). This result confirms that an approximately 397-bp 3′ untranslated region is contiguous with the coding portion of the type 5 actin gene. Accordingly, the end of the protected region maps in the vicinity of position 2005 ± 3 bp (Fig. 2). A potential polyadenylation signal AAATAAA (45) is located 22 to 28 bp upstream from position 2005. Polyadenylic acid-RNA would not protect ^{32}P-labeled DNA probes in similar S1 mapping experiments (data not shown) which indicated that the type 5 actin RNA transcript is poly(A)$^+$. The combined data of the primer extension experiment, sequence analysis of the coding region, and the 3′ S1 mapping experiments specify a transcript of approximately 1,570 nucleotide residues in length [not including the poly(A)$^+$ tail]. This size is in agreement with the position at which the denatured type 5 mRNA transcript migrates in an agarose gel relative to that of the denatured chicken β-cytoplasmic [1,870 nucleotides excluding the poly(A)$^+$ tail; 24] and the chicken α-skeletal [1,476 nucleotides excluding the poly(A)$^+$ tail; 11] mRNA transcripts (see below). Together these results indicated that the 3′ untranslated region of the type 5 actin gene transcript is very likely about 397 nucleotide residues in length and is poly(A)$^+$ posttranscriptionally very near position 2005 on the gene map (Fig. 2).

Southern blot hybridization analysis of total cellular DNA. To determine the number of coding loci for the type 5 actin gene in the chicken genome, a Southern hybridization analysis of total chicken nuclear DNA was performed. Ten micrograms of chicken liver nuclear DNA was digested with restriction enzyme EcoRI, *Bgl*I, or *Bam*HI and fractionated by electrophoresis on a 0.8% agarose gel. The DNA was then bidirectionally blotted onto two nitrocellulose filters. One blot was hybridized with a 464-bp ^{32}P-labeled fragment encompassing the 3′ coding region of the type 5 actin gene (Fig. 5C) which revealed most, if not all, of the actin genes present in the chicken genome (Fig. 5A). The other Southern blot was hybridized with a ^{32}P-labeled, approximately 0.9-kb fragment containing the 3′ region juxtaposed to the coding region of the type 5 actin gene (Fig. 5C). None of the restriction enzymes used to digest the nuclear DNA had recognition sites within the region of the genomic DNA complementary to this probe (Fig. 5B). Only a single distinct hybridization band was recognized per lane. This pattern demonstrates that the type 5 actin gene is represented as a unique member of the chicken actin multigene family.

Tissue distribution of the type 5 actin mRNA. We originally classified the type 5 actin gene as a nonmuscle type because of the high degree of resemblance of its encoded protein to the β- and γ-cytoplasmic forms. To test this assignment, we examined the accumulation of the type 5 gene RNA transcripts within a variety of tissue types. Equal amounts of poly(A)$^+$ RNA from six different adult chicken tissue types and one embryonic stage were denatured with glyoxal, fractionated by agarose gel electrophoresis, and transferred to Pall nylon filters. These filters were hybridized with single-stranded, uniformly ^{32}P-labeled DNA probes which were either actin mRNA or type 5 mRNA specific. The relative abundance of the type 5 mRNA transcripts was measured by direct comparison of the intensities of the hybridization signals (Fig. 6).

Although these RNA blot analyses dealt with the mixed cell populations typical of a given organ, measurements of the relative steady-state levels of the type 5 actin mRNA in different tissues could be obtained. The type 5 mRNA transcripts accumulated to the greatest abundance in brain tissue relative to its steady-state levels in other adult tissues (Fig. 6). As determind by densitometric analysis of the autoradiogram (Fig. 6B) (and other films of replicate exper-

TABLE 1. Comparison of the deduced type 5 chicken actin terminal amino acid sequences with examples of vertebrate, invertebrate, fungal, plant, and protozoan actin sequences

Actin	Source	NH_2-terminal amino acid sequences[a]	References
Type 5 nonmuscle	Chicken	Met Ala Asp Glu Glu Ile Ala Ala Leu Val Val-	
β-Cytoplasmic	Chicken	*Met* Asp *ASP ASP* Ile Ala Ala Leu Val Val-	24
γ-Cytoplasmic	Human	*Met GLU* Glu Glu Ile Ala Ala Leu Val *ILE*-	20
α-Skeletal	Chicken	*Met CYS ASP GLU ASP* Glu *THR THR* Ala Leu Val *CYS*-	11
α-Cardiac	Human	*Met CYS ASP* Asp Glu Glu *THR THR* Ala Leu Val *CYS*-	21
α-Smooth	Chicken	*Met CYS GLU GLU* Glu *ASP SER THR* Ala Leu Val *CYS*-	5
Type 5	*Xenopus*	X Asp Glu Glu N N Ala Leu Val N-	57
SpG17	Sea urchin	Met *CYS* Asp *ASP ASP VAL* Ala Ala Leu Val *ILE*-	6
Type I-III	Nematode	Met *CYS* Asp *ASP* Glu *VAL* Ala Ala Leu Val Val-	10
ACT-5C and ACT-42A	*Drosophila*	Met *CYS* Asp Glu Glu *Val* Ala Ala Leu Val Val-	12
ACT-57E and ACT-87A	*Drosophila*	Met *CYS* Asp *ASP* Glu *VAL* Ala Ala Leu Val Val-	12
ACT-88F	*Drosophila*	Met *CYS* Asp *ASP ASP ALA GLY* Ala Leu Val Ile-	12
ACT-79	*Drosophila*	Met *CYS* Asp Glu Glu *ALA PHE SER* Leu Val Val-	12
Gene 8	*Dictyostelium*	Met *ASP GLY* Glu *ASP VAL GLU* Ala Leu X X-	31
Single type	Yeasts	*Met* Asp *SER* Glu *VAL* Ala Ala Leu Val *ILE*-	14, 38
SAc3	Soybean	*Met ALA ASP ALA* Glu *ASP* Ile *GLU PRO* Leu Val *CYS*-	49
MAc1	Maize	Met Ala Asp Glu *ASP* Ile *GLN PRO* Leu Val *CYS*-	50
Gene I	*Acanthamoeba*	*Met GLY ASP* Glu *VAL GLN* Ala Leu Val *ILE*-	37

[a] Amino acid differences from the type 5 sequence are indicated in capital italic letters. X, Amino acid not determined; N, neutral amino acid.

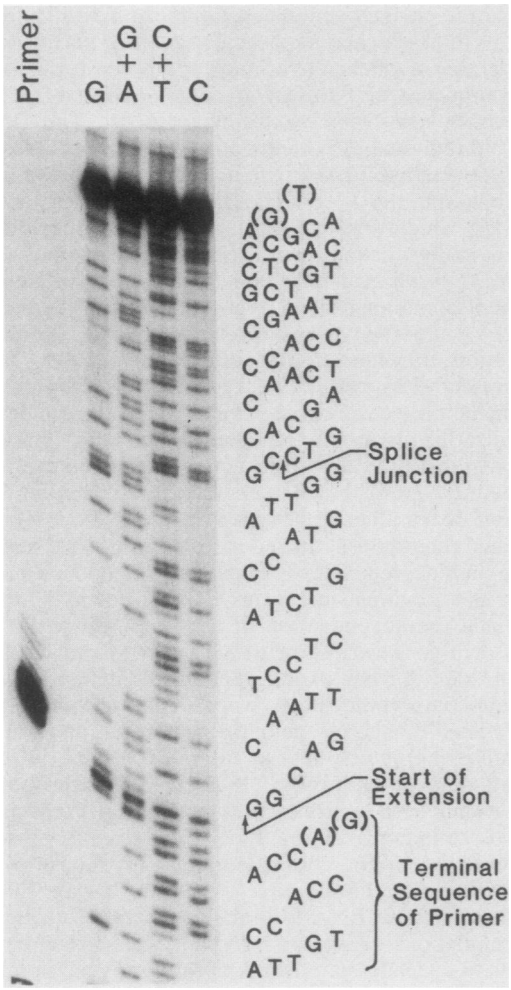


FIG. 3. Sequence of the 5′ primer extension product of the chicken type 5 actin gene mRNA. The autoradiograph of the sequencing gel is shown to the left with the nucleotide sequence indicated to the right. The sequence was determined by the method of Maxam and Gilbert (29). The end-labeled, unextended primer fragment is shown in the far left channel of the sequence gel. The terminal sequence of the primer DNA, the position from which extension of the primer fragment began, and the splice junction are indicated. Letters within parentheses represent nucleotides inferred from the gene sequence.

iments), the type 5 mRNA accumulated to levels approximately 2-, 4-, 7-, 10-, and 20-fold less in gizzard, heart, breast muscle, aorta, and liver tissues, respectively, relative to its steady-state level in brain tissue (Fig. 6C). The pattern of type 5 mRNA accumulation is similar to that observed for β-actin mRNA transcripts, which can be visualized in the RNA blot (Fig. 6A) (although the β-actin mRNA is more abundant in gizzard tissue than in brain tissue). These results were confirmed by stripping the hybridization probe from the RNA blot (Fig. 6B) and subsequently hydridizing the filter with a probe specific for 3′ β-actin mRNA (data not shown). In contrast to the type 5 and β-actin mRNAs, the α-skeletal transcripts accumulated in adult tissues in a muscle-specific pattern (Fig. 6A). It was only detected in breast muscle (visualized as a very prominent and intense hybridization signal), in heart tissue (the top band of the lower more-intense doublet), and to a lesser extent in aorta tissue but was not found in the other adult tissues. This observation was also confirmed by probing a mRNA blot similar to those in Fig. 6 with an α-skeletal 3′ specific DNA probe (data not shown). The pattern of the α-skeletal mRNA accumulation observed in this report is consistent with the results of Gunning et al. (19) and Mayer et al. (30), who demonstrated that the α-skeletal actin is expressed in both adult human skeletal muscle and heart in humans and rats, respectively. Therefore, unlike muscle-specific actin mRNA, the accumulation of the type 5 mRNA was not confined to muscle tissue types but rather was most abundant in brain tissue. Taken together, these results strengthen the conclusion that the type 5 actin gene is a nonmuscle type.

RNA blotting experiments also revealed the relative steady-state levels of type 5 mRNA at two different developmental stages. There was an approximately twofold greater amount of type 5 actin mRNA in whole 10-day embryo poly(A)$^+$ RNA than in adult brain. This may correlate with an increased expression of the type 5 actin gene in one or more tissue types at this stage of embryogenesis. This interesting possibility of developmental modulation is currently under investigation.

Relative steady-state levels in adult brain tissue of type 5 and β-cytoplasmic RNA transcripts. The results of the previous section established that both type 5 and β-cytoplasmic mRNA accumulated in the same tissues; however, their relative steady-state levels could not be compared since the actin mRNA probe NM35 showed stronger hybridization to the type 5 mRNA because it shares perfect sequence homology. Accordingly, we developed a RNA blot transfer analysis to compare the relative amounts of the type 5 mRNA and β-cytoplasmic mRNA in brain tissue. Different amounts of brain poly(A)$^+$ RNA were jointly hybridized to excess ^{32}P-labeled DNA probes specific for type 5 mRNA and β-cytoplasmic mRNA (Fig. 7). The relative abundance of the type 5 and β-cytoplasmic mRNA transcripts were measured by the direct comparison of the intensity of their hybridization signals. This was possible since each probe was uniformly ^{32}P-labeled in an identical manner (although the specific activity of the type 5 actin 3′ probe was up to threefold greater since it is three times longer in length than the β-cytoplasmic 3′ probe) and jointly hybridized to the same RNA blot. This comparison indicated that the accumulation of β-cytoplasmic mRNA in brain tissue is between three- and fourfold greater than that of the type 5 mRNA transcripts and probably represents the minimum relative difference at steady state. However, because the specific activity of the type 5 actin 3′ probe is greater than the β-cytoplasmic 3′ probe, the actual relative differences of mRNA accumulation may approach about 12-fold. These relative measurements are valid since the difference in intensities of the separate bands are proportional to the amount of RNA applied to each lane.

DISCUSSION

We identified a novel chicken actin gene. This gene which we designated type 5 was classified as a nonmuscle type because of the high degree of similarity between its coding region and those of the vertebrate cytoplasmic actin isoforms. The accumulation of the type 5 actin gene transcripts in adult tissues showed a pattern of expression characteristic of a nonmuscle, cytoplasmic type. This result reinforced our initial assignment of the type 5 actin gene as a nonmuscle isoform. Accordingly, this is the first report of the detection of a nonmuscle actin gene that exists in a warm-blooded vertebrate genome other than the β- and γ-cytoplasmic actin genes.

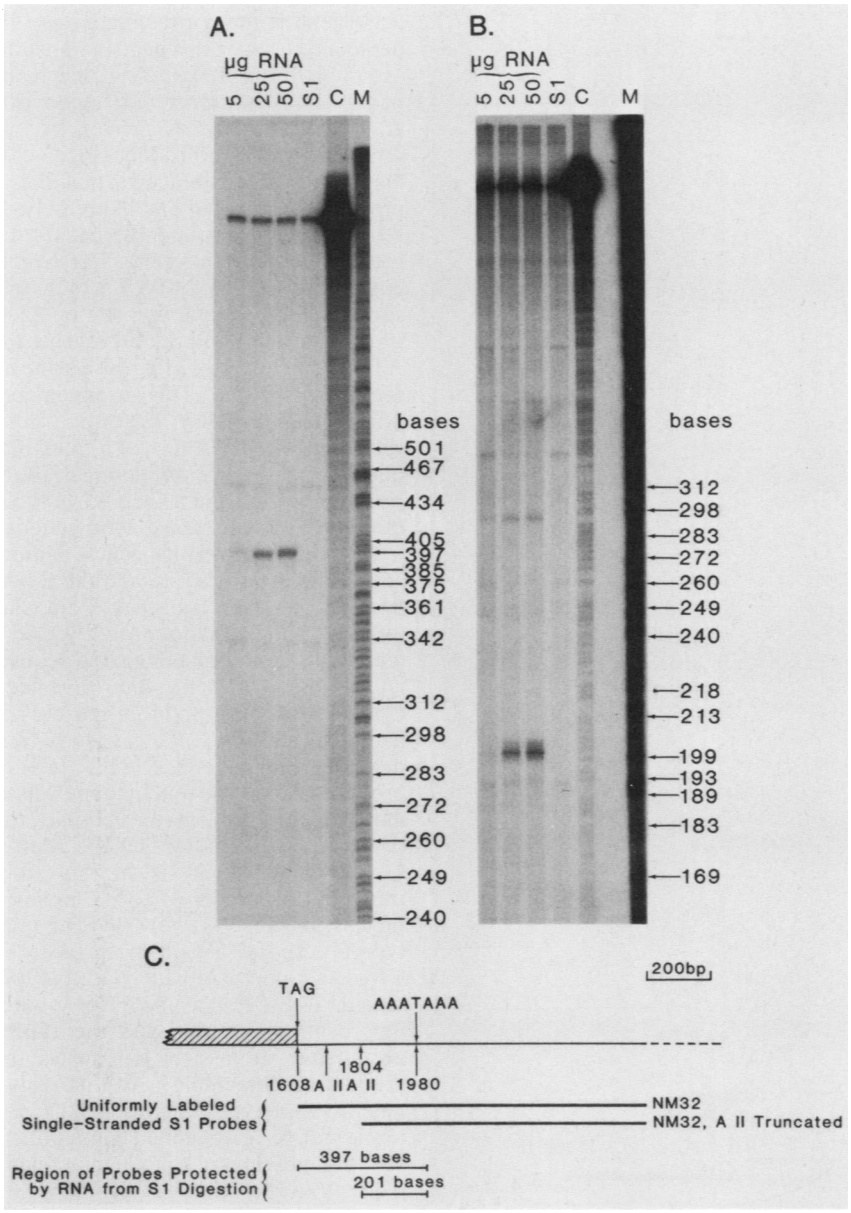


FIG. 4. Nuclease S1 mapping of the 3′ untranslated region of the type 5 actin gene. Hybrids formed between whole chicken embryo poly(A)$^+$ RNA and the 3′ uniformly labeled fragment NM32 (experiment A) or NM32 *Ava*II (A 11) truncated fragment (experiment B) were treated with 2,000 U of S1 nuclease as described in the text, with tRNA added as the carrier to bring the final RNA concentrations to 50 μg per reaction. Protected DNA fragments were separated on a 6% polyacrylamide sequencing gel in parallel with the dideoxy T reaction of an M13 chimera of known sequence which served as a length marker. (A and B) Autoradiograms of two separate S1 mapping experiments. The lanes of the autoradiogram indicate 5, 25, and 50 μg of poly(A)$^+$ RNA digested with S1 nuclease (5, 25, and 50); S1 nuclease digestion of 50 μg of *Escherichia coli* tRNA alone (S1); undigested S1 probe (C); and size markers (M). Numbers next to the arrows indicate marker fragment size. (C) Schematic diagram of 3′ portion of the chicken type 5 actin gene. The hatched area represents the coding sequence, and the thin line represents the 3′ flanking region. The protein coding termination signal (TAG) and presumptive polyadenylation signal (AAATAAA) are indicated. The numbers below the arrows indicate nucleotide sequence positions downstream from the transcription initiation site. The restriction sites for *Ava*II (A 11) are also shown below the arrows. The regions encompassed by the uniformly ^{32}P-labeled S1 probes are indicated below the diagram. NM32 is the M13 chimera from which the labeled probes were made. The region of the probes protected by the RNA from S1 nuclease digestion is indicated at the bottom.

Sequence analysis of the type 5 actin gene revealed that it lacked intron interruptions within its coding sequence and thus represents a unique exception among all vertebrate actin genes analyzed to date. One common characteristic of processed genes is that they lack introns (22). Moos and Gallwitz (35, 36) identified two β-cytoplasmic actin processed genes from a human genomic library. In contrast to these pseudogenes, the type 5 actin gene does not end with a short polydeoxyadenylic acid tract after the presumptive 3′ polyadenylation signal, it is not flanked by direct repeats, the integrity of its reading frame is not interrupted by nucleotide insertions or deletions, and there are no premature translation stop codons within its coding sequence. Also, unlike these and other human actin pseudogenes (27), the type 5

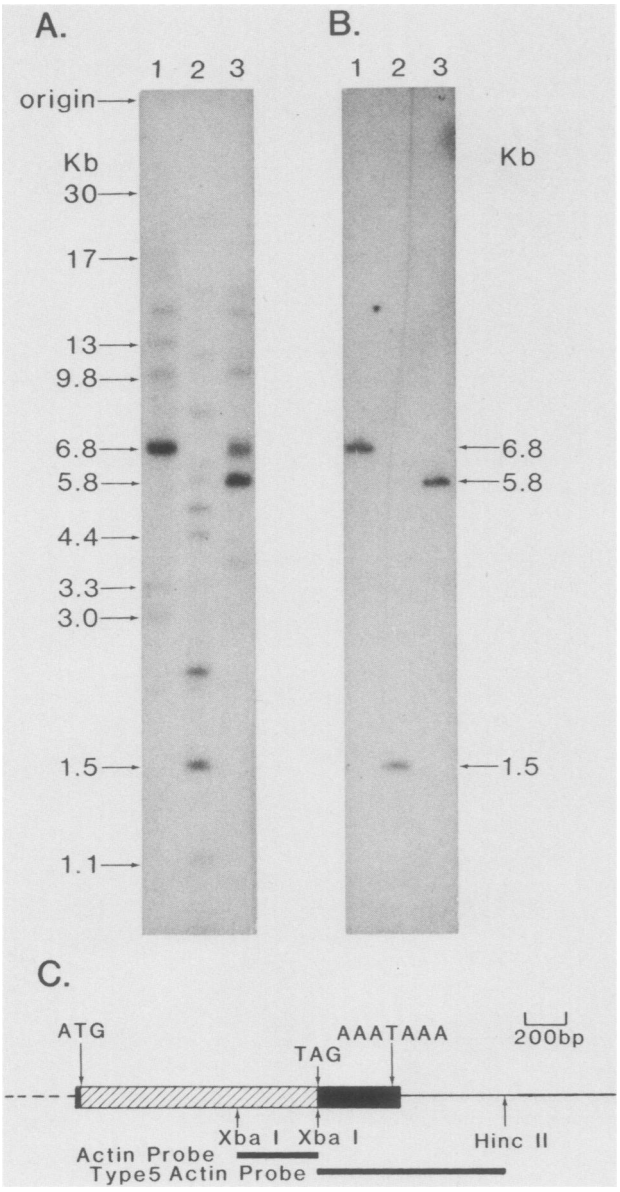


FIG. 5. Southern blot hybridization analysis of the type 5 nonmuscle actin gene in the chicken genome. Chicken nuclear DNA was digested to completion with *Eco*RI (lane 1), *Bgl*I (lane 2), or *Bam*HI (lane 3), electrophoresed on a 0.8% agarose gel, and bidirectionally blotted onto two nitrocellulose filters. The filters were hybridized to one of two nick-translated DNA fragments and washed as described in the text. (A and B) Autoradiograms of filters hybridized to an actin gene probe and a probe specific for the type 5 actin gene, respectively. The numbers next to the arrows indicate the size of fragments in kb. (C) Schematic diagrams of the contiguous portion of the type 5 actin gene and regions encompassed by probes used for hybridization analysis. Hatched area, coding region; solid area, transcribed but untranslated region; dashed line and thin line, intron and flanking region; solid bars, areas of type 5 gene from which probes were derived. The initiation (ATG) and termination (TAG) codons and the putative polyadenylation signal (AAATAAA) are indicated.

actin gene is unique in the chicken genome, probably represented only once per haploid genome as demonstrated by Southern blot analysis (Fig. 5).

Additional evidence for the conclusion that the type 5 actin gene is not a pseudogene are that RNA blot analysis demonstrates that this gene is transcribed and that its mRNA accumulates in a tissue-specific manner (Fig. 6). Furthermore, the 5′ untranslated region is processed by splicing after transcription, as proven by sequencing of a primer directed extension product (Fig. 3). Moreover, S1 nuclease mapping analysis indicates that the 3′ noncoding region is precisely terminated and likely poly(A)$^+$. Lastly, regulatory sequences present in most eucaryotic genes are also conserved in or near this gene. These include a putative polyadenylation signal AAATAAA (42), which precedes the presumptive polyadenylation site by 22 bp. Examination of the translation initiation region reveals that the initiation codon ATG is preceded by 3 bp by a purine and followed by 4 bp by a guanine residue. This arrangement is characteristic of a functional initiation site for translation by eucaryotic ribosomes (25). Within the 5′ flanking region are sequences that resemble or are identical to the Goldberg-Hogness TATAA box (3) and a CCAAT homology (1, 8). Thus, by all these criteria, the type 5 actin gene is not a processed gene.

Despite these results which demonstrate that the type 5 actin gene is transcribed and the RNA product is processed, there is no evidence that a translation product actually exists. Vandekerckhove and Weber (58, 61) developed a technique for radiolabeling and sequencing the amino-terminal residues of actin that revealed only the β- and γ-cytoplasmic actins and four muscle-specific actin isotypes in mammals and birds. However, this method is not capable of detecting minor actin species. It is quite possible that the product of the type 5 actin gene was not recognized since it may account for a small fraction of the total actin in the cell or tissue types studied. Of the adult tissue types examined by RNA blot analysis in this study, the type 5 RNA transcript accumulated to its highest level in brain. Nonetheless, our results show that the relative abundance of the type 5 actin mRNA in brain tissue is a minimum of 3- to 4-fold and more likely up to 12-fold less than the steady-state amount of β-cytoplasmic mRNA. Accordingly, it is possible that even in brain tissue the type 5 mRNA transcripts accumulate at a level so low as to make its translation product undetectable. Alternatively, low but detectable quantities of type 5 actin may be present in chicken brain tissue but have remained unidentified since this tissue has not been previously studied in actin typing studies. In earlier work, Vandekerckhove and Weber (59) identified only two nonmuscle actins β- and γ-cytoplasmic in bovine brain tissue. Perhaps the type 5 actin remained undetected because it occurs as a minor actin in bovine brain, or perhaps the type 5 actin gene is either conserved but not expressed or not conserved in mammals.

The very high conservation of the primary amino acid sequence between actins from a broad variety of distantly related organisms probably precludes the possibility of convergent evolution from separate primordial genes. Therefore, comparative studies of actin gene organization and structure may provide insight into the evolutionary history of the actin multigene families. Inspection of the type 5 gene sequence reveals a number of features that distinguish it from other members of the vertebrate actin multigene family and hence offers additional insights toward understanding the evolutionary relationships of the actin genes. One noticeable pecularity of the gene is that no introns were found within its coding region. This feature is in sharp contrast to the situation observed for other vertebrate and echinoderm actin genes and demonstrates that the similarity in number and location of introns within these deuterostomes is more

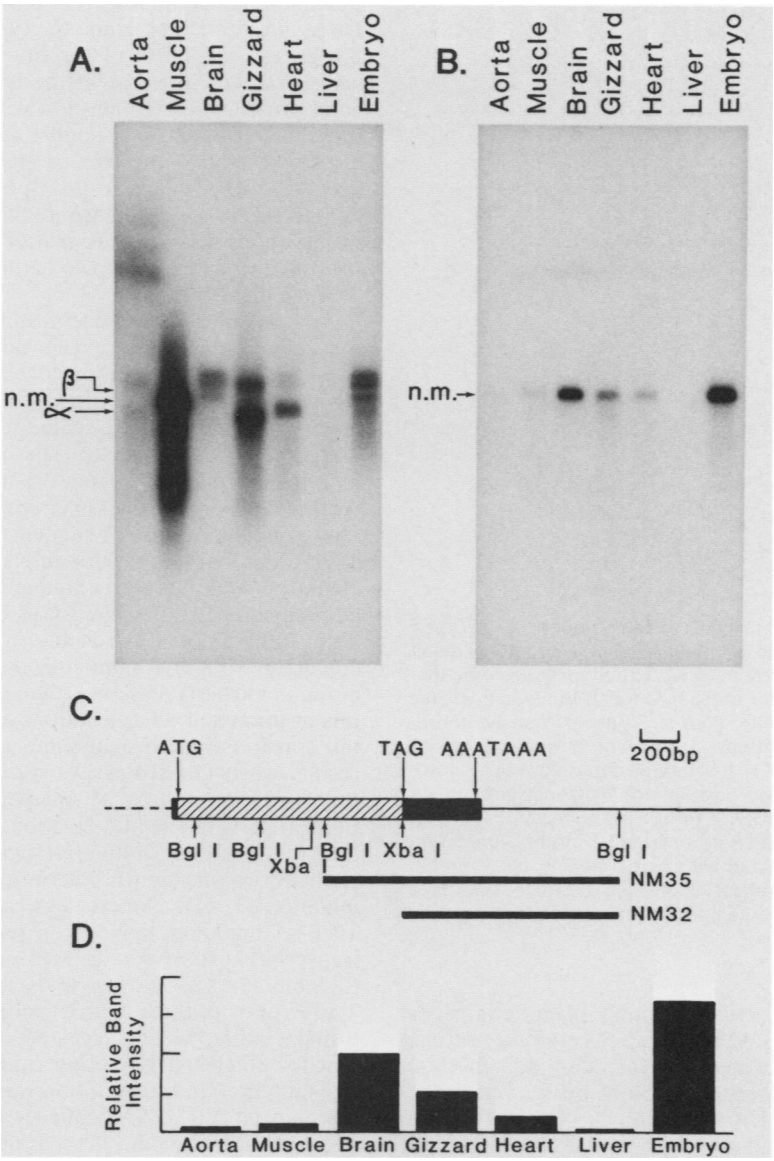


FIG. 6. RNA blot analysis of type 5 actin mRNA expression in chicken adult tissues and whole 10-day embryo. Samples (10 μg) of adult chicken tissue or whole 10-day embryo poly(A)$^+$ RNA were denatured with glyoxal and fractionated on 1% agarose gels. Gels were transferred to Pall Biodyne filters. The filters were hybridized to one of two uniformly ^{32}P-labeled single-stranded DNA probes and then washed as described in the text. (A and B) Autoradiograms of filters hybridized to an actin mRNA probe and a probe specific for type 5 mRNA, respectively. The letters next to the arrows indicate migration positions of β-cytoplasmic mRNA (β), type 5 nonmuscle mRNA (n.m.), and α-skeletal mRNA (α). (C) Schematic diagram of contiguous portion of the type 5 actin and regions homologous to probes used for hybridization analysis. The areas of the gene are represented as in Fig. 5. Hybridization probes were derived from M13 chimeras NM35 and NM32. (D) Lanes in (B) were scanned with a densitometer, and their relative areas under the hybridization band peaks were plotted. The autoradiogram in (B) was exposed to the filter for a period of time approximately three times longer than the filter in (A).

complex than initially thought. Previously, investigators noted that whereas there is no similarity in frequency and location of introns within actin genes found in species on highly divergent branches of the phylogenetic tree, on a smaller evolutionary scale among deuterostomes there appeared to be conservation of number and location of the actin genes (11, 64). For example, the vertebrate β-cytoplasmic actin gene and the α-skeletal and α-cardiac actin genes have five and six introns, respectively, which are located in patterns that are a subset of the eight intron positions of the α-smooth actin gene (56; unpublished data). Similarly, up to four of these eight introns occur at the same locations in the actin genes of a related invertebrate the sea urchin (6). Because of these features, intron placement has been used in an attempt to construct the evolutionary history of the actin family within the phylogenetic branch of the deuterostomes (11, 64). However, in terms of intron number and placement, the type 5 actin gene more closely resembles the ACT-5C actin gene member of the *Drosophila* multigene family. This is the first observation of a coincidence of intron patterns between the evolutionarily divergent groups of the protostomes (*Drosophila*) and deuterostomes.

A surprising feature of the type 5 actin gene is that its initiation Met codon is immediately followed by an Ala

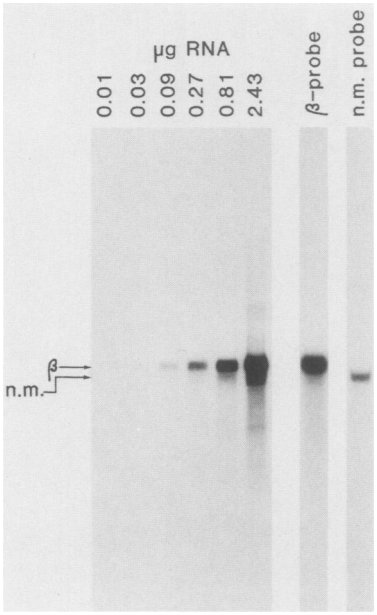


FIG. 7. Relative steady-state levels in adult brain tissue of type 5 and β-cytoplasmic RNA transcripts. Different amounts of adult chicken poly(A)$^+$ RNA were denatured with glyoxal and fractionated on a 1% agarose gel. After transfer to a Pall Biodyne filter, the filter was jointly hybridized to both a 3′ probe specific for β-cytoplasmic actin and a 3′ probe specific for nonmuscle type 5 mRNA. The amount of poly(A)$^+$ RNA applied to each lane is shown above each lane; the migration positions of β-cytoplasmic mRNA (β) and type 5 nonmuscle mRNA (n.m.) are indicated. β-probe, Poly(A)$^+$ RNA hybridized with β-cytoplasmic 3′ probe alone; n.m. probe, poly(A)$^+$ RNA hybridized with type 5 nonmuscle 3′ probe. The intensities of the hybridization signals between the three autoradiograms should not be compared since they were exposed for different lengths of time.

codon. Among all known actin genes only plant actin genes exhibit this feature (49, 50). Like the other vertebrate actins, the type 5 nonmuscle actin lacks a Cys codon after the Met codon. The Met-Cys codons are present in the vertebrate muscle-specific (11, 20, 21, 64), nematode (10), sea urchin (6), and six *Drosophila* (12) actin genes, whereas the more primitive *Dictyostelium* (31), *Acanthamoeba* (37), *Oxytricha* (23), and yeast (14, 38) actin genes do not have the Cys codon after the initiation Met codon. No amino-terminal Met or Cys codon has ever been detected in an actin protein yet sequenced; therefore, it is likely that the posttranslational removal of the Met-Cys dipeptide is necessary for normal actin function (20, 64). It remains to be established whether the Met-Ala dipeptide is posttranslationally cleaved from the presumptive mature polypeptide translation product of the type 5 actin gene for it to be a functional equivalent of other nonmuscle actins. It presently is not known whether the Met-Ala dipeptide is processed from the primary mRNA translation product of plant actin genes.

Gunning et al. (20) used the occurrence or absence of the 5′-terminal Met-Cys codons of actin genes to examine their evolutionary relationship. Assuming that their model is correct, revision may be required to include the presence of the 5′-terminal Met-Ala codons. The occurrence of the NH_2-terminal Met-Ala codons may show a closer evolutionary relatedness between the plant actin genes and the type 5 actin gene than among all the other vertebrate actin genes. Likewise, among the vertebrate actin genes, a closer evolutionary connection may exist between a majority of the *Drosophila* actin genes and the type 5 actin gene by virtue of the greater similarity of the first 18 amino-terminal amino acids (12) which represent the most variable region of the actin proteins (61). Consistent with this notion is that of all the chicken nonmuscle actins, the type 5 actin gene most closely resembles the two *Drosophila* actin genes ACT-5C and ACT-42A, whose transcripts accumulate in a pattern characteristic of cytoplasmic types (13). Together, these observations may indicate that of the vertebrate actin genes identified to date, the type 5 actin gene is the least diverged from an ancestral gene.

Analysis of the sequences within the 5′ flanking region of the type 5 actin gene reveals no significant homology between this gene and other vertebrate actin genes, except for signals associated with transcription of most structural eucaryotic genes. These include a CCAAT sequence (1, 8) and a ATAGAA sequence. The ATAGAA sequence occurs in a position upstream from the mRNA cap site that agrees with the location of the Goldberg-Hogness TATAA box (3). However, the ATAGAA sequence differs from the expected TATAA in that a G residue interrupts the A-T-rich oligonucleotides. This feature is unique among all vertebrate actin genes examined so far. Wasylyk et al. (63) have shown that a substitution of a G residue in the third position of the conalbumin TATAA homology resulted in a 10-fold decrease of the in vitro expression of that gene. Other substitutions in this position and other locations in the TATAA homology of the conalbumin and ovalbumin genes have yielded similar results when in vitro assays were used (46, 62). However, the combined results of independent experiments of the transcription of specific deletion mutations in vivo suggest that the TATAA Goldberg-Hogness box is the dominant element responsible for determining the precise start site of initiation (3, 51). Nonetheless, some mutations within the TATAA homology have been reported to reduce levels of transcription in vivo (e.g., sea urchin H2A [17] and rabbit β-globin [7, 18]). A single base modification within the TATAA box of the human β-globin gene is associated with a β-thalassemia, which suggested that this sequence is important for efficient RNA transcription, although the effect of this mutation on transcription was not studied (41). Perhaps the unusual ATAGAA sequence of the type 5 actin gene is one factor which controls the transcription of this gene. This possibility could be tested by generating appropriate point mutations and assaying the effect of the nucleotide alterations on transcription.

Our results show a previously undetected nonmuscle actin gene in chickens. Like amphibians (57), there are at least three cytoplasmic actin isotypes expressed in chicken nonmuscle cells. Thus birds reveal a greater spectrum of nonmuscle actin genes than have been identified in mammals. However, additional sequence analysis of the many actin related genes of humans (25 to 30 actin genes [9]) or mice (>20 actin genes [34]) may reveal additional types of actin genes, although many of these actin-related genes represent pseudogenes (27, 34).

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LITERATURE CITED

1. **Benoist, C., K. O'Hare, R. Breathnach, and P. Chambon.** 1980. The ovalbumin gene-sequence of putative control regions. Nucleic Acids Res. **8**:127–142.
2. **Berk, A. J., and P. A. Sharp.** 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell **12**:721–732.
3. **Breathnach, R., and P. Chambon.** 1981. Organization and expression of eucaryotic split genes coding for proteins. Annu. Rev. Biochem. **50**:349–383.
4. **Bruskin, A. M., A. L. Tyner, D. E. Wells, R. M. Showman, and W. H. Klein.** 1981. Accumulation in embryogenesis of five mRNAs enriched in the ectoderm of the sea urchin pluteus. Dev. Biol. **87**:308–318.
5. **Chang, K. S., W. E. Zimmer, Jr., D. J. Bergsma, J. B. Dodgson, and R. J. Schwartz.** 1984. Isolation and characterization of six different chicken actin genes. Mol. Cell. Biol. **4**:2498–2508.
6. **Cooper, A. D., and W. R. Crain, Jr.** 1982. Complete nucleotide sequence of a sea urchin actin gene. Nucleic Acids Res. **10**:4081–4092.
7. **Dierks, P., A. van Ooyen, M. D. Cochran, C. Dobkin, J. Reiser, and C. Wiessmann.** Three regions upstream from the cap site are required for efficient and accurate transcription of the rabbit β-globin gene in mouse 3T6 cells. Cell **32**:695–706.
8. **Efstratiadis, A., J. W. Posakony, T. Maniatis, R. M. Lawn, C. O'Connell, R. A. Spritz, J. K. DeRiel, B. G. Forget, S. M. Weissman, J. L. Slightom, A. E. Blechl, O. Smithies, F. E. Baralle, C. C. Shoulders, and N. J. Proudfoot.** 1980. The structure and evolution of the human β globin gene family. Cell **21**:653–668.
9. **Engel, J. N., P. W. Gunning, and L. H. Kedes.** 1981. Isolation and characterization of human actin genes. Proc. Natl. Acad. Sci. U.S.A. **78**:4674–4678.
10. **Files, J. G., S. Carr, and D. Hirsh.** 1983. Actin gene family of *Caenorhabditis elegans*. J. Mol. Biol. **164**:355–375.
11. **Fornwald, J. A., G. Kuncio, I. Peng, and C. P. Ordahl.** 1982. The complete nucleotide sequence of the chick α-actin gene and its evolutionary relationship to the actin gene family. Nucleic Acids Res. **10**:3861–3876.
12. **Fyrberg, E. A., B. J. Bond, N. D. Hershey, K. S. Mixter, and N. Davidson.** 1981. The actin genes of *Drosophila*: protein coding regions are highly conserved but intron positions are not. Cell **24**:107–116.
13. **Fyrberg, E. A., J. W. Mahaffey, B. J. Bond, and N. Davidson.** 1983. Transcripts of the six *Drosophila* actin genes accumulate in a stage- and tissue-specific manner. Cell **33**:115–123.
14. **Gallwitz, D., and I. Sures.** 1980. Structure of a split yeast gene: complete nucleotide sequence of the actin gene in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. U.S.A. **77**: 2546–2550.
15. **Garcia, R., B. Paz-Aliaga, S. G. Ernst, and W. R. Crain, Jr.** 1984. Three sea urchin actin genes show different patterns of expression: muscle specific, embryo specific, and constitutive. Mol. Cell Biol. **4**:840–845.
16. **Ghosh, P. K., V. B. Reddy, M. Piatak, P. Lebowitz, and S. M. Weissman.** 1980. Determination of RNA sequences by primer directed synthesis and sequencing of their cDNA transcripts. Methods Enzymol. **65**:580–595.
17. **Grosschedl, R., and M. L. Birnstiel.** 1980. Spacer DNA sequences upstream of the T-A-T-A-A-A-T-A sequence are essential for promotion of H2A histone gene transcription *in vivo*. Proc. Natl. Acad. Sci. U.S.A. **77**:7102–7106.
18. **Grosveld, G. C., E. deBoer, C. K. Shewmaker, and R. A. Flavell.** 1982. DNA sequences necessary for transcription of the rabbit β-globin gene *in vitro*. Nature (London) **295**:120–126.
19. **Gunning, P., P. Ponte, H. Blau, and L. Kedes.** 1983. α-Skeletal and α-cardiac actin genes are coexpressed in adult human skeletal muscle and heart. Mol. Cell Biol. **3**:1985–1995.
20. **Gunning, P., P. Ponte, H. Okayama, J. Engel, H. Blau, and L. Kedes.** 1983. Isolation and characterization of full-length cDNA clones for human α-, β-, and γ-actin mRNAs: skeletal but not cytoplasmic actins have an amino-terminal cysteine that is subsequently removed. Mol. Cell Biol. **3**:787–795.
21. **Hamada, H., M. Petrino, and T. Kakunaga.** 1982. Molecular structure and evolutionary origin of human cardiac muscle actin gene. Proc. Natl. Acad. Sci. U.S.A. **79**:5901–5905.
22. **Hollis, G. F., P. A. Hieter, O. W. McBride, D. Swan, and P. Leder.** 1982. Processed genes: a dispersed human immunoglobulin gene bearing evidence of RNA-type processing. Nature (London) **296**:321–325.
23. **Kaine, B. P., and B. B. Spear.** 1982. Nucleotide sequence of a macronuclear gene for actin in *Oxytricha fallax*. Nature (London) **295**:430–432.
24. **Kost, T. A., N. Theodorakis, and S. H. Hughes.** 1983. The nucleotide sequence of the chick cytoplasmic β-actin gene. Nucleic Acids Res. **11**:8287–8301.
25. **Kozak, M.** 1981. Possible role of flanking nucleotides in recognition of the AUG initiator codon by eukaryotic ribosomes. Nucleic Acids Res. **9**:5233–5252.
26. **Lawson, G. M., B. J. Knoll, C. J. March, S. L. C. Woo, M.-J. Tsai, and B. W. O'Malley.** 1982. Definition of 5′ and 3′ structural boundaries of the chromatin domain containing the ovalbumin multigene family. J. Biol. Chem. **257**:1501–1507.
27. **Leavitt, J., P. Gunning, P. Porreca, S.-Y. Ng, C.-S. Lin, and L. Kedes.** 1984. Molecular cloning and characterization of mutant and wild-type human β-actin genes. Mol. Cell Biol. **4**:1961–1969.
28. **Lillehaug, J. R., R. K. Kleppe, and K. Kleppe.** 1976. Phosphorylation of double-stranded DNAs by T4 polynucleotide kinase. Biochemistry **15**:1858–1865.
29. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific cleavage reaction. Methods Enzymol. **65**:499–560.
30. **Mayer, Y., H. Czosnek, P. E. Zeclon, D. Yaffe, and U. Nudel.** 1984. Expression of the genes coding for the skeletal muscle and cardiac actins in the heart. Nucleic Acids Res. **12**:1087–1100.
31. **McKeown, M., and R. A. Firtel.** 1981. Differential expression and 5′ end mapping of actin gene in *Dictyostelium*. Cell **24**:799–807.
32. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101**:20–78.
33. **Minty, A. J., S. Alonso, M. Caravatti, and M. E. Buckingham.** 1982. A fetal skeletal muscle actin mRNA in the mouse and its identity with cardiac actin mRNA. Cell **30**:185–192.
34. **Minty, A. J., S. Alonso, J. L. Guenet, and M. E. Buckingham.** 1983. Number and organization of actin-related sequences in the mouse genome. J. Mol. Cell. Biol. **167**:77–101.
35. **Moos, M., and D. Gallwitz.** 1982. Structure of a human β-actin related pseudogene which lacks intervening sequences. Nucleic Acids Res. **10**:7843–7849.
36. **Moos, M., and D. Gallwitz.** 1983. Structure of two human β-actin related processed genes one of which is located next to a simple repetitive sequence. Eur. Mol. Biol. Org. J. **2**:757–761.
37. **Nellen, W., and D. Gallwitz.** 1982. Actin gene and actin messenger RNA in *Acanthamoeba castellanni*. Nucleotide sequence of the split actin gene I. J. Mol. Biol. **159**:1–18.
38. **Ng, R., and J. Abelson.** 1980. Isolation and sequence of the gene for actin in *Saccharomyees cerevisiae*. Proc. Natl. Acad. Sci. U.S.A. **77**:3912–3916.
39. **Nudel, U., R. Zakut, M. Shani, S. Neuman, Z. Leavy, and D. Yaffe.** 1983. The nucleotide sequence of the rat cytoplasmic β-actin gene. Nucleic Acids Res. **11**:1759–1771.
40. **Ordahl, C. P., S. M. Tilghman, C. Ovitt, J. Fornwald, and M. T. Largen.** 1980. Structure and developmental expression of the chick α-actin gene. Nucleic Acids Res. **8**:4989–5005.
41. **Poncz, M., M. Ballantine, D. Solowiejczyk, I. Barak, E. Schwartz, and S. Surrey.** 1982. β-Thalassemia in a Kudish Jew. J. Biol. Chem. **257**:5994–5996.
42. **Proudfoot, N. J., and G. G. Brownlee.** 1976. 3′ non-coding region sequences in eukaryotic messenger RNA. Nature (London) **263**:211–214.
43. **Rigby, P. W., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specificity activity *in*

vitro by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
44. **Saborio, J. S., M. Segura, M. Flores, R. Garcia, and E. Palmer.** 1979. Differential expression of gizzard actin genes during chick embryogenesis. J. Biol. Chem. **254**:11119–11125.
45. **Scheller, R. H., L. B. McAllister, W. R. Crain, Jr., D. S. Durica, J. W. Posakony, T. L. Thomas, R. J. Britten, and E. H. Davidson.** 1981. Organization and expression of multiple actin genes in the sea urchin. Mol. Cell Biol. **1**:609–628.
46. **Schulz, T. Z., S. Y. Tsai, K. Itakura, X. Soberon, R. B. Wallace, M. Tsai, S. L. C. Woo, and B. W. O'Malley.** 1982. Point mutagenesis of the ovalbumin gene promoter sequence and its effect on *in vitro* transcription. J. Biol. Chem. **257**:11070–11077.
47. **Schwartz, R. J., and K. Rothblum.** 1980. Regulation of muscle differentiation: isolation and purification of chick actin messenger ribonucleic acid and quantitation with complementary deoxyribonucleic acid probes. Biochemistry **19**:2506–2514.
48. **Schwartz, R. J., and K. N. Rothblum.** 1981. Gene switching in myogenesis; differential expression of the actin multigene family. Biochemistry **20**:4122–4129.
49. **Shah, D. M., R. C. Hightower, and R. B. Meager.** 1982. Complete nucleotide sequence of a soybean actin gene. Proc. Natl. Acad. Sci. U.S.A. **79**:1022–1026.
50. **Shah, D. M., R. C. Hightower, and R. B. Meager.** 1983. Genes encoding actin in higher plants: intron positions are highly conserved but the coding seqences are not. J. Mol. Appl. Genet. **2**:111–125.
51. **Shenk, T.** 1981. Transcription control regions: nucleotide sequence requirements for initiation by RNA polymerase II and III. Curr. Top. Microbiol. Immunol. **93**:25–46.
52. **Shenk, T. E., C. Rhodes, P. W. J. Rigby, and P. Berg.** 1975. Biochemical method for mapping mutational alterations in DNA with S1 nuclease: the location of deletions and temperature-sensitive mutations in simian virus 40. Proc. Natl. Acad. Sci. U.S.A. **72**:989–993.
53. **Smith, G. E., and M. D. Summers.** 1980. The bidirectional transfer of DNA and RNA to nitrocellulose or diazobenzyloxymethyl-paper. Anal. Biochem. **109**:123–129.
54. **Storti, R. V., S. J. Horovitch, M. P. Scott, A. Rich, and M. L. Pardue.** 1978. Myogenesis in primary cell cultures from *Drosophila melanogaster*: protein synthesis and actin heterogeneity during development. Cell **13**:589–598.
55. **Thomas, P.** 1980. Hybridization of denatured RNA and small fragments of DNA transferred to nitrocellulose. Proc. Natl. Acad. Sci. U.S.A. **77**:5201–5205.
56. **Ueyama, H., H. Hamada, N. Battula, and T. Kakunaga.** 1983. Structure of a human smooth muscle actin gene (aortic type) with a unique intron site. Mol. Cell. Biol. **4**:1073–1078.
57. **Vandekerckhove, J., W. W. Franke, and K. Weber.** 1981. Diversity of expression of non-muscle actin in amphibia. J. Mol. Biol. **152**:413–426.
58. **Vandekerckhove, J., and K. Weber.** 1978. At least six different actins are expressed in a higher mammal: an analysis based on the amino acid sequence of the amino-terminal tryptic peptide. J. Mol. Biol. **126**:783–802.
59. **Vandekerckhove, J., and K. Weber.** 1978. Mammalian cytoplasmic actins are the products of a least two genes and differ in primary structure in at least 25 identified positions from skeletal muscle actins. Proc. Natl. Acad. Sci. U.S.A. **75**:1106–1110.
60. **Vandekerckhove, J., and K. Weber.** 1979. The complete amino acid sequence of actins from bovine aorta, bovine heart, bovine fast skeletal muscle and rabbit slow skeletal muscle. Differentiation **14**:123–133.
61. **Vandekerckhove, J., and K. Weber.** 1981. Actin typing on total cellular extracts. A highly sensitive protein-chemical procedure able to distinguish different actins. Eur. J. Biochem. **113**:595–603.
62. **Wasylyk, B., and P. Chambon.** 1981. A T to A base substitution and small deletions in the conalbumin TATA box drastically decrease specific *in vitro* transcription. Nucleic Acids Res. **9**:1813–1824.
63. **Wasylyk, B., R. Derbyshire, A. Guy, D. Molko, A. Roget, R. Téoule, and P. Chambon.** 1980. Specific *in vitro* transcription of conalbumin gene is drastically decreased by single-point mutation in T-A-T-A box homology sequence. Proc. Natl. Acad. Sci. U.S.A. **77**:7024–7028.
64. **Zakut, R., M. Shani, D. Givol, S. Neuman, D. Yaffe, and U. Nudel.** 1982. Nucleotide sequence of the rat skeletal muscle actin gene. Nature (London) **298**:857–859.