IL-2 protein surrounding the Cys^{58} residue may be involved in the interaction with the IL-2 receptor on cell surfaces, and therefore slight perturbations of the amino acid sequence (cysteine → serine) in that region may adversely affect the receptor binding capability of the protein. To demonstrate that Cys^{58} is directly involved in receptor binding, it is necessary to study the structure-function relationship of the surrounding amino acids, either by site-specific mutagenesis, in order to identify the effects of these changes on receptor binding, or by coupling of the protein to the receptor, in order to identify the amino acids involved in the direct interaction with the receptor.

Our results clearly demonstrate that Cys^{125} of the human IL-2 protein is not involved in a disulfide bridge and is not necessary for the interaction with the IL-2 receptor. However, both Cys^{58} and Cys^{105} are necessary for biological activity and therefore may be involved in a disulfide bridge that holds the IL-2 protein in a biologically active conformation. In addition, the phenotype of the Ser^{58} IL-2 suggests that Cys^{58} may also be involved in receptor binding. Purification of these three mutant IL-2 proteins should help to determine in what ways their protein conformations differ from that of the wild-type protein.

Using the same technique to replace a free cysteine residue in human fibroblast interferon while retaining biological activity helped us to overcome difficulties encountered during the purification of the recombinant interferon from *E. coli* (*11*). The purified recombinant wild-type interferon protein had a low specific activity and poor stability because of the preferential formation of incorrect disulfide bridges between the three cysteine residues of the protein (*11*). Substituting a serine for one of the cysteines stabilized the recombinant fibroblast interferon, so that it had a specific activity comparable to that of native interferon (*11*). In the case of recombinant human IL-2, we have found that the purified protein is unstable in the reduced form and readily oxidizes into oligomeric forms with loss of biological activity. After controlled oxidation, the purified IL-2 protein had a higher specific activity and greater stability, but the oxidized product is heterogeneous and all possible disulfide bridges are formed (*17*). Therefore, the ability to replace one of the cysteine residues with a serine residue while retaining biological activity is likely to help generate a recombinant IL-2 that is both molecularly more homogeneous and has a reproducibly higher specific activity. On the basis of our present results and our results on human fibroblast interferon (*11*), we suggest that this could be a general method for preparing biologically active "muteins" (*18*) from proteins containing nonessential cysteine residues.

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9. The M13 phage vector was used as a source of a single-stranded DNA template (*10, 11*). A Hind III–Pst I DNA fragment containing the entire coding region of des-alanyl IL-2 (*8*) was cloned into the Hind III and Pst I sites of the phage M13mp9 (*12*), and single-stranded phage DNA (M13-IL2) was used as template for site-specific mutagenesis. Ten picomoles of the synthetic oligonucleotide, DM27, was phosphorylated and hybridized with 2.6 μg of single-stranded M13-IL2 DNA and incubated with 5 units of DNA polymerase I Klenow fragment at 37°C for 2 hours as described (*10, 11*). The reaction was terminated by heating to 80°C, and the reaction mixture used to transform competent *E. coli* K12 strain JM103 cells (*13*), plated onto agar plates and incubated overnight to obtain phage plaques. Plates containing mutagenized M13-IL2 plaques, as well as two plates containing untreated M13-IL2 phage plaques, were chilled to 4°C and phage plaques from each plate were transferred onto two nitrocellulose filter circles. The filters were prepared for hybridization as described (*10, 11*). Labeled probes were prepared by phosphorylating the oligonucleotide primer with ^{32}P-labeled adenosine triphosphate. The filters were hybridized to ^{32}P-labeled primer (1.0×10^5 cpm/ml) in DNA hybridization buffer (2.5 ml per filter) at 55°C for 12 hours. The filters were washed twice at 55°C for 10 minutes each time in washing buffers containing 0.1 percent SDS and double-strength standard saline citrate (SSC), and once at 55°C for 10 minutes with 0.1 percent SDS and single-strength SSC. The filters were air dried and autoradiographed at −70°C for 2 to 3 days. The oligonucleotide primer DM27 was designed to create a new Pst I restriction site. Therefore, RF DNA (*14*) from plaques that hybridized to this primer were screened for the presence of a new Pst I site. In a similar fashion, the primers DM28 and DM29 were used to create site-specific mutations. The DNA from all three of these mutagenized clones were sequenced (*15*) to confirm that the UCU codon for cysteine was converted to a UGU codon for serine.
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Identification and Location of Brain Protein 4.1

Abstract. *Protein 4.1 is a membrane skeletal protein that converts the low-affinity interaction between spectrin and actin into a high-affinity ternary complex of spectrin, protein 4.1, and actin that is essential to the structural stability of the erythrocyte. Pig brain was shown to contain an 87-kilodalton immunoreactive analog of protein 4.1 that has partial sequence homology with pig erythrocyte protein 4.1 and the same location as spectrin in the cortical cytoplasm of neuronal and glial cell types of the cerebellum.*

Protein 4.1, spectrin, and actin are major components of a fibrous meshwork of proteins located on the cytoplasmic surface of the erythrocyte membrane, which is now commonly referred to as the membrane skeleton. The membrane skeleton is a strong, flexible, elastic structure that appears to be responsible for the maintenance of the erythrocyte shape, reversible deformability, and membrane structural integrity (*1*). Erythrocyte protein 4.1 from human, pig, and mouse is a phosphoprotein of about 80,000 daltons (80 kD) (*2*) that binds to the terminal ends of the fibrous spectrin $(\alpha\beta)_2$ tetramer (α = 240 kD, β = 220 kD) and thereby stimulates the weak, end-on, bivalent binding of spectrin tetramer to F actin probably through formation of a ternary complex of spectrin,

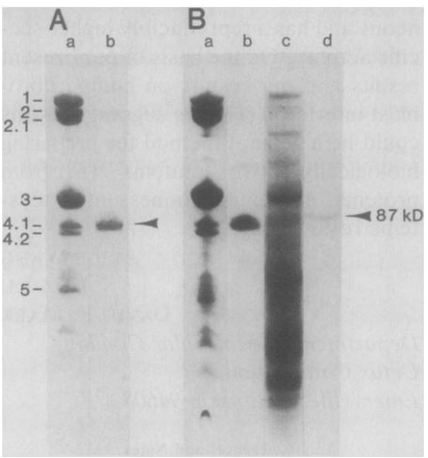

Fig. 1. Characterization of pig rbc protein 4.1 and IgG to pig rbc protein 4.1. (A) Pig rbc membranes and protein 4.1 were isolated in the presence of 0.4 m*M* diisopropylfluorophosphate (*22*). Pig rbc membrane protein (64 μg) (lane a) and pig protein 4.1 (10 μg) (lane b) were subjected to SDS-PAGE. Gels were stained with Coomassie blue and then destained (*28*). (B) Immunoautoradiography by gel overlay with 56 μg of affinity-purified IgG to pig rbc protein 4.1 and 2.5 μCi of ^{125}I-labeled protein A was performed (*10*). (Lane a) Coomassie blue–stained pig rbc membrane protein (80 μg). (Lane b) Matching autoradiogram for lane a. (Lane c) Coomassie blue–stained preparation of crude pig brain membrane protein (100 μg) (*15*). (Lane d) Matching autoradiogram for lane c. Autoradiography was performed with Kodak X-OMAT XAR film and an intensifying screen at −20°C for 1 day (lane b) or 5 days (lane d).

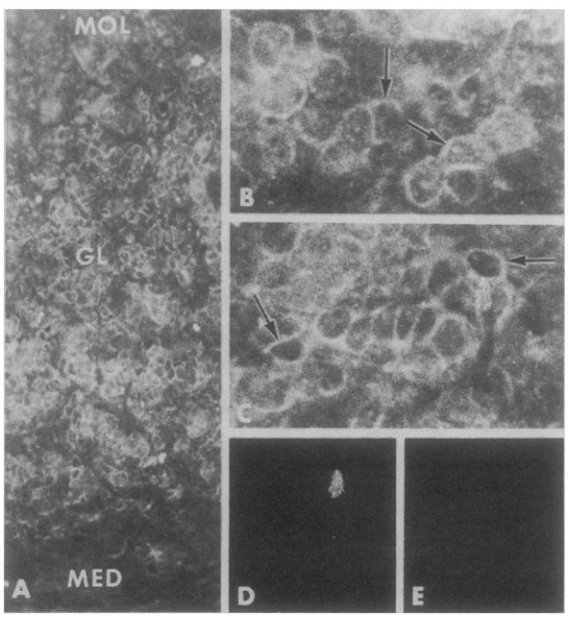

Fig. 2. Location of an immunoreactive analogue of protein 4.1 in pig cerebellum. Tissues were prepared from a male pig anesthetized with pentobarbital (35 mg per kilogram of body weight) and perfused (carotid arteries) at a pressure of 120 mmHg with phosphate-buffered saline. The cerebellum was frozen in Freon-12, and frozen sections (10 μm) were prepared for indirect immunofluorescence. (A) A low magnification picture (×250) of the cerebellum stained with IgG to pig rbc protein 4.1 (*15*). Bright staining of the internal granule layer (*GL*) and low to moderate staining of the medullary (*MED*) and molecular (*MOL*) layers is shown. (B) Granule neurons in the GL are immunoreactive to IgG to pig rbc protein 4.1. Note the brightly fluorescent cortical cytoplasm (arrows) encircling a nonfluorescing nucleus (×875). (C) Sections of granule neurons in the GL stained with IgG to pig rbc spectrin have a staining profile identical to that observed with IgG to pig rbc protein 4.1 (×875). Control sections were stained with nonimmunized IgG (D) or IgG to pig rbc protein 4.1 that had been absorbed with five times pig rbc protein 4.1 (E). Both photomicrographs are of the internal granule layer (identified by phase optics) and times of exposure and printing correspond to those in (B) (×875).

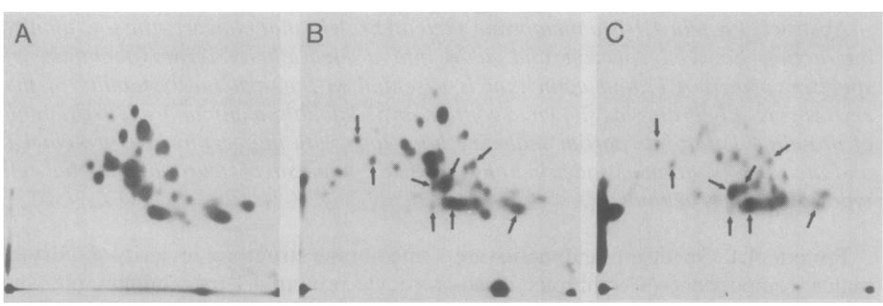

Fig. 3. Tryptic peptide analysis of pig erythrocyte and brain protein 4.1. Affinity-purified IgG to pig rbc protein 4.1 (28 μg) was used to immunoprecipitate protein 4.1 from pig rbc and brain membranes solubilized in Triton X-100 at high ionic concentration (*26*). Immunoprecipitated pig brain and rbc protein 4.1 as well as total pig brain and rbc membrane proteins were separated by SDS-PAGE (*28*). Two-dimensional tryptic peptide mapping analysis of peptides labeled with ^{125}I within the gel slice was performed (*29*). (A) Tryptic map of pig rbc protein 4.1 excised from a gel containing total ghost protein (see lane a in Fig. 1A). (B) Tryptic map of immunoprecipitated pig rbc protein 4.1. (C) Tryptic map of immunoprecipitated pig brain protein 4.1. Some of the common tryptic peptides are indicated (arrows).

protein 4.1, and actin (*3*). The importance of this complex to the structural integrity of the erythrocyte is best shown by two pathological red cell disorders: hereditary spherocytosis (HS), in which the spherical, osmotically fragile red cells are in some instances due to a defective interaction between the HS spectrin and protein 4.1 that leads to a defective ternary complex (*4, 5*); and homozygous mild hereditary elliptocytosis (HE), in which the elliptical and fragile red cells are in some cases due to a lack of protein 4.1 (*6*). In addition to its function in the ternary complex, protein 4.1 has been shown to have its own high-affinity binding site on the erythrocyte membrane (*1, 3, 7, 8*) and therefore serves as a linkage site between spectrin, actin, and the bilayer core.

A major advance in understanding of cytoskeleton-membrane interactions was the discovery that spectrin-like molecules, which are both immunologically and structurally related to erythrocyte spectrin, are present in diverse nonerythroid cells (*9, 10*). Subsequent immunofluorescence studies have shown the widespread occurrence of nonerythroid, spectrin-like molecules in the cortical cytoplasm of nearly every mammalian and avian cell or tissue studied (*11–13*). Brain is relatively rich in an immunoreactive and structural analog of spectrin (brain spectrin, also known as fodrin, brain actin–binding protein, or calspectin), which has become the best studied member of the nonerythroid spectrin family of molecules (*1*). Brain spectrin from pig, mouse, and chicken is a large, asymmetric $(\alpha\beta)_2$ tetramer (~ 10^6 daltons, ~ 11*S*) that constitutes 2 to 3 percent of the total crude brain membrane protein (*11, 13–15*). Rotary shadowed images of brain spectrin showed a long flexible rod (~ 2000 Å in contour length) with the strands woven into a tight double helix, a morphology that is similar to erythrocyte spectrin tetramer (*11, 14*). Brain spectrin shares with erythrocyte spectrin the following additional properties: (i) a phosphorylated β subunit (*16*); (ii) a binding site for the spectrin-binding protein syndein (or ankyrin) (*13, 14*); (iii) a binding site for calmodulin (*17, 18*); and (iv) the ability to cross-link F actin (*11, 13, 14, 17, 19*). Electron microscopy of the interaction of brain spectrin and actin showed a bivalent brain spectrin molecule binding laterally to F actin in an end-on orientation (*19*), an image identical to the interaction of erythrocyte spectrin tetramer and F actin. Two other investigations have shown that erythrocyte protein 4.1 can strengthen the interaction of brain spectrin and actin in

vitro, suggesting that brain spectrin also contains a protein 4.1 binding site (*20, 21*). The significance of these observations depends on the existence of a protein 4.1–like analog in brain. We now demonstrate that pig brain contains an 87-kD immunoreactive analog to protein 4.1 that shows partial sequence homology with erythrocyte protein 4.1 and has the same location as spectrin in mammalian brain.

Homogenous protein 4.1 isolated from pig red blood cells (rbc) (*22*) (Fig. 1A) was used as an immunogen in New Zealand white male rabbits. Affinity-purified immunoglobulin G (IgG) to pig rbc protein 4.1 was isolated and characterized by immunoautoradiography by gel overlay (Fig. 1B). The protein 4.1 antibody reacted with the 80-kD protein 4.1 band when total pig erythrocyte membrane protein separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was treated with the antibody and ^{125}I-labeled protein A. Faint staining of 135- and 63-kD polypeptides was also observed. Minor immunoreactive polypeptides of larger and smaller molecular size than protein 4.1 in human erythrocyte membranes have also been observed (*3, 23*), and the results of peptide-mapping analysis have led to the conclusion that a family of protein 4.1 sequence-related proteins exists in erythrocyte membranes (*3*). The results of preliminary peptide mapping analysis suggest that the minor 135- and 63-kD immunoreactive polypeptides also share sequence homology with pig rbc protein 4.1 (data not shown). The IgG to pig rbc protein 4.1 was used to determine whether mammalian brain contains a protein 4.1 analog. The staining of total pig brain membrane protein with the antibody was specific for a single immunoreactive polypeptide with an apparent molecular size of 87 kD migrating slightly slower than erythrocyte protein 4.1 (Fig. 1B). Since the pig rbc antibody was specific for the 87-kD protein 4.1–like polypeptide, it could reasonably be used to locate this protein by indirect immunofluorescence of pig brain tissue.

The location of the brain 4.1–like protein in pig and mouse brain tissue was identical to the location of spectrin in mouse neural tissue (*15, 16, 24, 25*) and pig neural tissue (Fig. 2). As an example, indirect immunofluorescence studies of pig cerebellum stained with pig rbc protein 4.1 IgG and rhodamine-conjugated IgG to rabbit revealed an intense fluorescence of the cortical cytoplasm at or near the plasma membrane of granule neurons (Fig. 2, A and B) but revealed no staining of cell nuclei. Similar staining was recorded for other neuronal cell types (for example, Purkinje cells). Glial cells showed a similar but less intense ringlike staining of their cell soma. In the medullary layer immunofluorescent strands or tubelike profiles were observed occasionally, but this did not appear to be consistent with the Protargol-silver staining of axons (not shown). Experiments with control specimens revealed that staining was specific for the brain 4.1–like protein since neither brain slices stained with IgG from a nonimmunized rabbit (Fig. 2D) nor with rbc protein 4.1 IgG that had been incubated with a fivefold excess of pig rbc protein 4.1 (Fig. 2E) exhibited fluorescence. Staining of pig neural tissue with IgG to mouse rbc spectrin, which we have shown to be specific for the 240- and 235-kD subunits of brain spectrin (*16, 24, 25*), revealed identical staining of the cerebellum described above for IgG to pig rbc protein 4.1. As an example, the identical ringlike staining pattern of the cortical cytoplasm of granule cell neurons was seen with antibodies to erythrocyte spectrin (Fig. 2C) and protein 4.1 (Fig. 2B).

To determine whether the pig brain 4.1-like protein was structurally related to pig erythrocyte 4.1, we immunoprecipitated protein 4.1 from solubilized brain and erythrocyte membranes with affinity-purified IgG to pig protein 4.1 (*26*) and performed two-dimensional tryptic peptide analysis on the 87-kD (brain) and 80-kD (erythrocyte) ^{125}I-labeled polypeptides (Fig. 3). The peptide maps of erythrocyte protein 4.1 excised from a gel containing total pig erythrocyte membrane protein (Fig. 3A) or immunoprecipitated pig erythrocyte protein 4.1 (Fig. 3B) were nearly identical, with 80 to 90 percent spot homology. The peptide maps of immunoprecipitated erythrocyte (Fig. 3B) and brain protein 4.1 (Fig. 3C) showed partial sequence homology, with 50 percent of the tryptic peptides overlapping. Final determination of the extent of homology between erythrocyte and brain protein 4.1 will require amino acid sequence data. The protein detected in brain was not due to contaminating erythrocytes in the brain preparation because (i) it had a molecular size of 87 kD whereas that of erythrocyte protein 4.1 was 80 kD, (ii) it had only partial sequence homology to erythrocyte protein 4.1 by two-dimensional tryptic peptide analysis, and (iii) it could be detected by indirect immunofluorescence as a component of neural cells.

Proteins immunologically related to erythrocyte protein 4.1 have also been reported to be present in platelets, polymorphonuclear leukocytes, and fibroblasts (*23, 27*). However, in cultured fibroblasts protein 4.1 has been found along the stress fibers or actin filament bundles (*23*), whereas spectrin is found on the cytoplasmic surface of the plasma membrane, suggesting that the role of protein 4.1 might differ in erythrocytes and fibroblasts. Our results show that brain protein 4.1 is immunologically and structurally related to erythrocyte protein 4.1 and has the same location as spectrin in the cortical cytoplasm of neural cells. These observations raise the possibility that actin may be associated to the neural cell membranes as part of a ternary complex of spectrin, protein 4.1, and actin, and by analogy to the erythrocyte model this ternary complex may be essential for maintenance of the shape and membrane structural integrity of neuronal and glial cell types.

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α-Cardiac Actin Is the Major Sarcomeric Isoform Expressed in Embryonic Avian Skeletal Muscle

Abstract. *A primer extension assay that is diagnostic for the messenger RNA's (mRNA's) transcribed from the β-cytoplasmic, α-cardiac, and α-skeletal actin genes of the chicken was used to measure the mRNA levels for these actin isoforms. Measurements were made in chicken breast muscle during myogenesis in vivo and in vitro. α-Cardiac actin mRNA accounts for more than 90 percent of the sarcomeric actin transcripts expressed in avian embryonic breast muscle. Five weeks after hatching, α-skeletal actin mRNA is the only detectable sarcomeric actin transcript.*

Analysis of the various actin isoforms poses a problem since there is little divergence in their protein sequences (*1*). The pioneering work of Vandekerckhove and Weber (*1, 2*) has shown that there are at least six actin isoforms in adult mammalian tissues: two sarcomeric forms (α-cardiac and α-skeletal muscle actin), two smooth muscle forms (α- and γ-smooth muscle actin), and two cytoplasmic forms (β- and γ-cytoplasmic nonmuscle actin). The conservation in amino acid sequence is striking in that the α-skeletal muscle isoform differs in 4 of 375 amino acids from α-cardiac actin, 6 of 375 amino acids from the γ-smooth isoform, 8 of 375 amino acids from the α-smooth isoform, and 24 or 25 of 375 amino acids from the β- and γ-cytoplasmic actins. In order to avoid possible problems due to homology between the various isoforms, a primer extension assay was used in place of RNA dot blots or Northern blots (*3*) to determine the level of expression of the different actin genes during myogenesis. This assumes that each actin gene initiates transcription at a point that yields a primer-extended fragment of unique length for each gene.

The sequence of the 5′ coding exon for each of the actin genes used in this study is given in Fig. 1. Both the sarcomeric actins encode the amino terminal methionine-cysteine dipeptide which is not present in the β-cytoplasmic actin gene. However, the cysteine residue is absent from the processed sarcomeric actin (*1, 2*). The function of this cysteine residue is not clear, but similar results have been reported for other vertebrate sarcomeric actin genes (*4–7*). The encoded amino acid sequence for the amino terminus of each actin gene agrees exactly with the diagnostic sequence characteristic for each isoform (*1, 2*). The primer sequences used in this study are underlined in Fig. 1.

The lengths of the extended primers (Fig. 2A) indicate that the β-cytoplasmic, α-cardiac, and α-skeletal actin messenger RNA's (mRNA's) have 5′ noncoding leader sequences of approximately 90, 60, and 75 base pairs (bp), respectively. Our results agree with the reported leader length for α-skeletal actin mRNA (*4*).

The β-actin isoform represents more than 90 percent of the total actin transcripts measured in dividing myoblasts 36 hours after plating (lane a in Fig. 2A and Table 1). The low level of expression of the α-cardiac isoform in 36-hour cultures (lane b in Fig. 2A) represents the small percentage of myoblasts (5 to 10 percent) that have undergone fusion (*8, 9*) and are expressing the differentiation-specific cardiac isoform. No α-skeletal actin transcripts are detectable in the prefusion cultures with this exposure time. After most of the cells have fused (approximately 70 to 80 percent), there is a marked reduction in the expression of β-actin mRNA (lane d in Fig. 2A and Table 1) and a concomitant increase in the expression of the α-cardiac (lane e in Fig. 2A and Table 1) and α-skeletal actin transcripts (lane 1 in Fig. 2B and Table 1). However, in the latter case, α-skeletal actin represents less than 5 percent of the sarcomeric actin transcripts expressed in differentiated cultures.

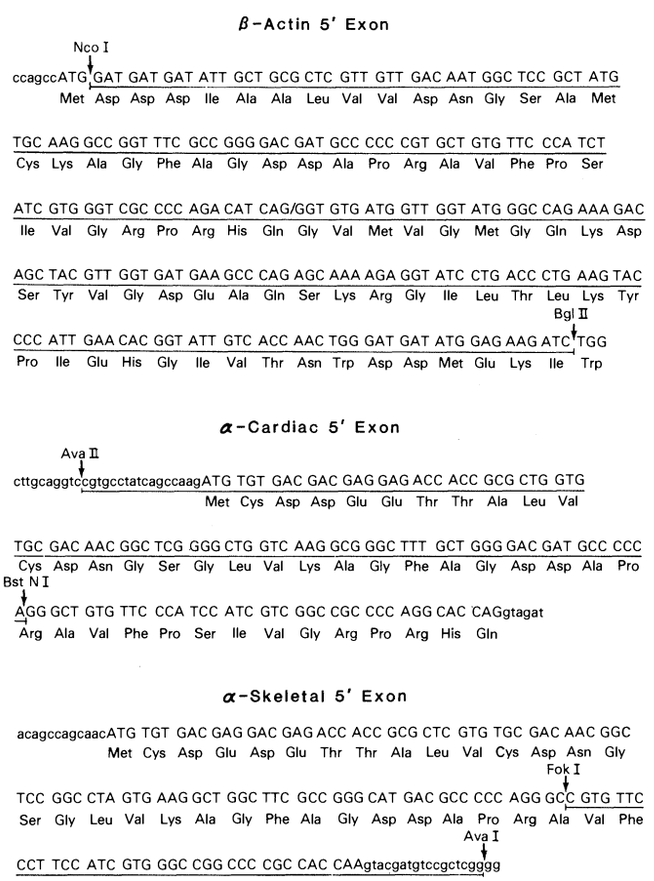

Fig. 1. The nucleotide sequence of the 5′ coding exon for the β-actin, α-cardiac actin, and α-skeletal actin genes in the chicken. The primer fragments are underlined in the sequence, the 3′ end of the primer being on the 5′ side of the depicted sequence. The lengths of the primers are as follows: β-actin, 252 bp; α-cardiac actin, 105 bp; and α-skeletal actin, 54 bp. The β-actin primer was prepared from the full size β-actin cDNA clone, pA-1, constructed by Cleveland *et al.* (*12*); the genomic sequence data obtained in our laboratory were used to select the restriction enzyme sites. The sarcomeric actin primers were cut from the appropriate subclones of the complete genomic fragments prepared as described earlier (*11*). Lower-case letters indicate flanking or intron sequence positions. The slash in the β-actin sequence is the position of the first intron within the coding portion of the genomic sequence. The recognition site for Fok I is 13 bp to the 5′ side of the cut site. The sequence data reported here for the α-skeletal actin agree with those reported previously (*4*).