Office de la Propriété Intellectuelle du Canada
Un organisme d'Industrie Canada

Canadian Intellectual Property Office
An agency of Industry Canada

CA 2059137 C 2005/04/19

(11)(21) 2 059 137

(12) BREVET CANADIEN
CANADIAN PATENT

(13) C

(86) Date de dépôt PCT/PCT Filing Date: 1990/07/02
(87) Date publication PCT/PCT Publication Date: 1990/12/31
(45) Date de délivrance/Issue Date: 2005/04/19
(85) Entrée phase nationale/National Entry: 1991/12/18
(86) N° demande PCT/PCT Application No.: US 1990/003729
(87) N° publication PCT/PCT Publication No.: 1991/000287

(51) Cl.Int.^{5}/Int.Cl.^{5} C12N 15/12, A61K 48/00, C12N 15/85, C12N 5/10

(72) Inventeurs/Inventors:
HUGHES, STEPHEN H., US;
SUTRAVE, PRAMOD, US

(73) Propriétaire/Owner:
THE UNITED STATES OF AMERICA, REPRESENTED BY THE SECRETARY, U.S. DEPARTMENT OF COMMERCE, US

(74) Agent: GOWLING LAFLEUR HENDERSON LLP

(54) Titre : DEVELOPPEMENT DE LA MUSCULATURE DES ANIMAUX
(54) Title: ENHANCEMENT OF MUSCULATURE IN ANIMALS

(57) Abrégé/Abstract:
The present invention relates to DNA segments encoding chicken c-ski protein, to DNA constructs comprising the DNA segments and to cells transformed therewith. The present invention further relates to transgenic animals having increased muscle size. In addition, the present invention relates to methods of stimulating muscle growth and preventing degeneration of muscle, and to methods of treating muscle degenerative diseases.

2059137

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C07H 21/00, C12N 15/00, 5/00

A1

(11) International Publication Number: WO 91/00287

(43) International Publication Date: 10 January 1991 (10.01.91)

(21) International Application Number: PCT/US90/03729

(22) International Filing Date: 2 July 1990 (02.07.90)

(30) Priority data:
373,864 30 June 1989 (30.06.89) US

(71) Applicant: UNITED STATES OF AMERICA, represented by THE SECRETARY, UNITED STATES DEPARTMENT OF COMMERCE [US/US]; Washington, DC 20231 (US).

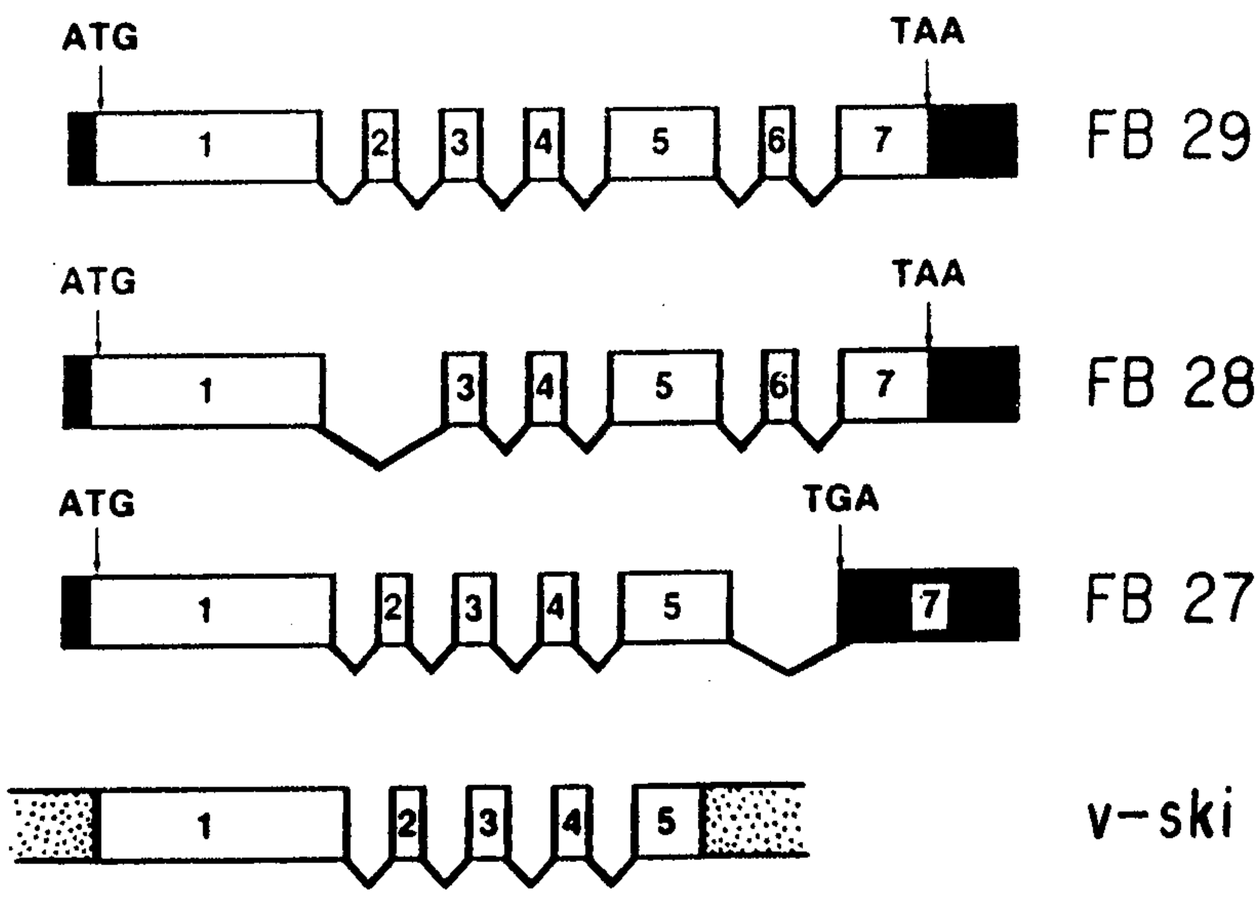
(72) Inventors: HUGHES, Stephen, H. ; 13405 Loy Wolfe Road, Smithsburg, MD 21783 (US). SUTRAVE, Pramod ; 1211 Rutledge Place, #2D, Frederick, MD 21701 (US).

(74) Agent: OLIFF, James, A.; Oliff & Berridge, 277 S. Washington Street, Alexandria, VA 22314 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent)*, DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ENHANCEMENT OF MUSCULATURE IN ANIMALS



(57) Abstract

The present invention relates to DNA segments encoding chicken *c-ski* protein, to DNA constructs comprising the DNA segments and to cells transformed therewith. The present invention further relates to transgenic animals having increased muscle size. In addition, the present invention relates to methods of stimulating muscle growth and preventing degeneration of muscle, and to methods of treating muscle degenerative diseases.

* See back of page

ENHANCEMENT OF MUSCULATURE IN ANIMALS

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates the c-*ski* gene. In particular, the present invention relates to DNA segments encoding chicken c-*ski* protein, to DNA constructs comprising the DNA segments and to cells transformed therewith. The present invention further relates to animals having increased muscle size.

Background Information

Viruses that contain the v-*ski* oncogene are not only capable of causing morphological transformation in vitro, but also can induce myogenic differentiation (Stavnezer et al., 1981, J. Virol. *39*, 920-934; Li et al., 1986, J. Virol. *57*, 1065-1072; Stavnezer et al., 1986, J. Virol. *57*, 1073-1083; Colmenares and Stavnezer, 1989, Cell *59*, 293-303). Viruses that carry and express c-*ski* cDNAs also induce foci and myogenic differentiation (Sutrave et al., 1990, Mol. Cell. Biol. *10*, 3137-3144). This suggests the possibility that the *ski* oncogene is bifunctional since the two known functions of *ski*, transformation and differentiation, would appear to be contradictory properties. Using a v-*ski* probe, genomic clones for c-*ski* have been isolated and partially sequenced (Stavnezer et al., 1989, Mol.

Cell. Biol. *9*, 4038-4045). Comparisons of the properties of two forms of c-*ski* that are related by alternative splicing, and of several v-*ski* and c-*ski* deletion mutants have shown that the portions of *ski* required for transformation and differentiation are quite similar. These results suggest that the ability of c-*ski* and v-*ski* to cause transformation and induce differentiation may be related aspects of a single property of *ski* rather than two separate functions.

Relatively little is known about the biochemical functions of the ski proteins. All of the biologically active forms of c-*ski* and v-*ski* that have been studied are localized primarily in the nucleus (Barkas et al., 1986, Virology *151*, 131-138; Sutrave et al., 1990, Mol. Cell. Biol. *10*, 3137-3144). When the c-*ski* proteins are overexpressed in chicken cells, different forms of c-*ski* differ in their subnuclear localization; however, the significance of these differences, if any, is as yet unclear. When chromatin condenses for cell division, the over-expressed c-*ski* proteins are associated with the condensed chromatin (Sutrave et al., 1990, Mol. Cell. Biol. *10*, 3137-3144). Biochemical studies have also shown that at least one form of c-*ski* can bind to DNA in the presence of other proteins (Nagase et al., 1990, Nucl. Acids Res. *18*, 337-343).

None of the available data make it possible to infer the normal function of c-*ski* either in terms of its role in growth and development (if any) or to have any direct insight into its mode of action.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide an isolated and characterized c-*ski* cDNA.

It is another object of the present invention to provide a gene that increases the muscle size in animals.

It is another object of the present invention to provide domestic livestock with increased muscle size and decreased fat tissue.

It is a further object of the present invention to provide a treatment for patients suffering from serious muscle injury or muscle degenerative diseases.

Various other objects and advantages of the present invention will be apparent from the drawings and the following description of the invention.

In one embodiment, the present invention relates to a DNA segment encoding a chicken c-*ski* protein or a DNA fragment complementary to said segment.

In another embodiment, the present invention relates to a DNA construct comprising a DNA segment encoding a chicken c-*ski* protein and a vector. In a further embodiment, the present invention relates to a DNA construct comprising a DNA segment encoding a truncated chicken c-*ski* protein having the function of c-*ski* and a vector. The present invention also relates to host cells stably transformed with either to of the two DNA constructs described above, in a manner allowing expression of the protein encoded in the construct.

In yet another embodiment, the present invention relates to a animal having increased muscle size, all of whose cells contain a DNA

construct comprising a DNA segment encoding a ski protein and a vector, introduced into the animal, or an ancestor of the animal. The DNA segment may encode the entire protein or a truncated version thereof.

In further embodiment, the present invention relates to an animal having increased muscle size, all of whose cells contain a DNA construct comprising a DNA segment encoding a truncated *ski* protein having the function of *ski* and a vector, introduced into the animal, or an ancestor of the animal.

In another embodiment, the present invention relates to a method of stimulating muscle growth or preventing muscle degeneration comprising delivering a DNA construct of the present invention to the muscle under conditions such that the protein of the construct is expressed and muscle growth induced.

In a further embodiment, the present invention relates to a method of treating a muscle degenerative disease comprising delivering a DNA construct of the present invention to the effected muscle under conditions such that the protein of the construct is expressed and treatment effected.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. The Structure of the c-*ski* cDNA clones.

The lengths of the cDNAs are drawn to scale and the restriction sites indicated. v-*ski* is shown for comparison. The dotted boxes in v-*ski* represent the *gag* region of the *gag-ski* fusion in the acutely transforming virus SKV. A, B, C and D represent the regions used for generating single-strand probes for S1 nuclease protection analysis.

The numbers next to the arrows correspond to the exon number.

Figure 2. Complete coding sequence and the potential coding region of a cDNA of the FB29 type.
This assumes that the 5' end sequences of the FB29 are similar to the 5' ends of the FB28 and CEL clones. ↑ indicates the site where FB28 and CEL diverge. The 25 bases found only in the CEL clones are not shown. ↓ indicates the boundaries of v-*ski*. The exon boundaries are numbered and the alternately spliced exons are boxed. The single base and amino acid change between c-*ski* and v-*ski* is also boxed with a dashed line. The translation termination codon is boxed in thick lines. The potential polyadenylation signals are densely underlined. The AT rich region containing ATTTA sequences that might be involved in mRNA stability is underlined with dashed lines.

Figure 3. Diagrammatic illustration of the alternate mRNAs generated for c-*ski* locus as deduced from the cDNA sequence analysis.
The exons are not drawn to scale. The c-*ski* mRNAs are shown in relation to v-*ski*. The dark areas are noncoding regions while the open boxes are the protein coding regions of the cDNA. The dotted boxes on both ends of v-*ski* are the *gag* regions of the *gag-ski* fusion in transforming *ski* viruses. The relative positions of the putative translational initiation codon and the translation termination codons are also shown.

Figure 4. S1 nuclease protection analysis of total RNA. Figure 4A shows the uniformly labeled single-strand probes used for hybridization are shown schematically below each picture, the thick lines represent the cDNA sequence while the thin lines represent M13 sequence. The overall length of probes and expected lengths of protected fragments are also shown. The RNA hybridization is indicated at the top of each lane. The numbers (8, 10, 12, 15 or 17) above the lane indicates the age of the embryo from which the RNA was prepared. Figure 4B shows Probe A (see Figure 1) contains a *Kpn*I-*Hind*III fragment of the FB27/29 type. This probe produced a fragment of 645 base pairs (bp) and two smaller fragments of 262 and 272 bp, shown by arrows. Figure 4C shows Probe B (see Figure 1) contains a *Kpn*I-*Hind*III fragment of the FB28 type. This probe produced a fragment of 534 bp as shown by the arrows. Smaller fragments were not detected. Figure 4D shows Probe C (see Figure 1) contains a 497-bp *Hind*III fragment of the FB27 type linked to M13 sequences. The probe yielded a 497-bp fragment and two smaller fragments of 243/254. Only the 479-bp fragment is marked by an arrow. Figure 4E shows Probe D (see Figure 1) is 1116 bp in length containing a *Hind*III fragment of FB28/29 type. Probe D produced a 799-bp fragment which is marked by an arrow.

Figure 5. Sequence homology between c-*ski* and the p19 region of *gag* from avian leukosis virus. The c-*ski* sequences are from positions 218 to 242 and the p19 sequence of *gag* region are from positions 633 to 658. The homologous regions are boxed.

Figure 6. The c-*ski* expression cassette.
The *Pvu*I to *Nru*I segment shown in the drawing was isolated by gel electrophoresis following double digestion of the plasmid. The linear DNA was used to create the transgenic mice by microinjection of fertilized eggs.

Figure 7. A transgenic mouse that expresses c-*ski* and a normal litter mate.
The c-*ski* transgene appears to segregate normally in crosses. The photograph shows a heterozygous mouse that displays the muscular phenotype (foreground) and a DNA-negative litter mate. Double blind DNA analyses confirmed that the muscular phenotype segregates with the transgene.

Figure 8. Northern transfer analysis of transgene expression.
Panel A shows the analysis of RNA isolated from various tissues of a mouse of the line TG 8566. The upper part of the panel shows an autoradiogram after hybridization with chicken c-*ski*. The expected position of migration of the c-*ski* message appropriately transcribed from the transgene is 2.5 kb. The position corresponding to 2.5 kb is marked (*ski*). The lower panel shows an autoradiogram from the same filter following hybridization to a chicken β-actin cDNA. The β-actin cDNA will hybridize not only with β-actin mRNA but also with other actin messages. The expected position of migration of both β-actin and α-actin mRNAs are indicated on the right of the panel.
Panel B. The autoradiograms shown in panel B are similar to those shown in panel A except that the RNAs derive from three other transgenic lines. The lines used to prepare the RNAs are indicated

at the top of the figure. The filters shown in panel B were done at the same time; those in panel A were done on a different day.

Figure 9. RNase protection of RNA from the transgene.

The top of the figure shows an autoradiogram of the gel. The first lane contains the antisense RNA probe, without RNase digestion. The next two lanes show the results of digestion following hybridization of the probe either without added RNA or with tRNA. The next eight lanes show the results of hybridization to RNA isolated either from the hearts (heart) or the skeletal muscle (SK muscle) of the four transgenic lines. The next lanes show the results of hybridizing the probe to RNA from skeletal muscle of a mouse that does not carry the transgene (control SK muscle). The last lane contains molecular weight markers.

Below the autoradiogram is a diagram that shows a drawing of the MSV LTR c-*ski* expression cassette in relation to the antisense RNA probe. The T7 transcript begins in the middle of the c-*ski* coding region and goes entirely through the MSV LTR into adjacent sequences that derive from pBR322 (marked pBR). If the transcripts deriving from the transgene initiate appropriately, then a fragment of 984 bases should be protected.

Figure 10. chicken c-*ski* protein expression in transgenic mice.

Extracts were made from the liver (Liv) or skeletal muscle (Sk.M) of control mice (control) or mice carrying and expressing the c-*ski* transgene. The positions of migration of radioactively labeled molecular weight standards are shown to the left of the figure.

Figure 11. Cross sections made precisely through the middle of the plantaris muscle: (a) from control mouse and (b) from a mouse of line TG 8566. Both illustrations are at the same magnification, the size marker is 200 μ. (c) Higher power illustration from the plantaris of control and (d) from the plantaris of an affected mouse. Size markers in (c) and (d) are 100 μ.

Figure 12. Distribution of fiber diameters in selected muscles from normal and transgenic Mice Panel A. The diaphragm appears normal in transgenic mice that express c-*ski*. A diaphragm from a mouse that has the muscular phenotype (TG 8566) and a diaphragm from a normal control mouse were sectioned and the number of individual muscle fibers of a given cross-sectional area tallied. Panel B. The anterior tibial muscle is grossly enlarged in mice from the line TG 8566. Transverse sections were prepared from both a transgenic mouse and a control mouse. The number of fibers of each given cross-sectional area were tallied. This muscle is composed of two distinct types of fibers, some of which are smaller, others larger, than the fibers found in the controls (see also Figure 11).

Figure 13. Transgene expression in specific muscles from TG 8566.

20 μg of total RNA was fractionated by electrophoresis and transferred to nitrocellulose membranes. The transfers were first probed with chicken c-*ski*, then the filters were stripped and reprobed with a chicken β-actin cDNA (Cleveland et al., 1980, Cell *20*, 95-105). The RNA was isolated from the diaphragm, the soleus, or from bulk

skeletal muscle (sk muscle).

Figure 14. Immunofluorescence staining of sections made through the middle of the Rhomboideus capitis muscle of an affected mouse. (a) staining with monoclonal antibody NOQ7 5 4D, specific for slow MHC. Slow fibers are not hypertrophied. (b) staining with monoclonal antibody SC 711 specific for IIa MHC. IIa fibers are not hypertrophied. (c) is with monoclonal antibody 2G3 which reacts with all fast MHC isoforms. All hypertrophied fibers stain with this antibody. (d) is with m/a BF-F3 specific for IIb MHC. Many, but not all, hypertrophied fibers stain. Magnification x 230.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a DNA segment encoding all, or a unique portion, of a chicken c-*ski* protein. The DNA segment may encode one of several chicken c-*ski* proteins, for example, FB29, FB28 and FB27. A "unique portion" as used herein is defined as consisting of at least five (or six) amino acids or correspondingly, at least 15 (or 18) nucleotides. The invention also relates to DNA constructs containing such DNA segments and to cells transformed therewith.

The present invention relates to DNA segments that encode the amino acid sequence of exon 6 given in Figure 1 or the amino acid sequence of exon 7 given in Figure 1. The present invention also relates to DNA segments that in addition to exon 7 further comprise at least four exons selected from the group consisting of: exon 1, exon 2, exon 3, exon 4, exon 5 or exon 6, given in Figure 1. Examples of such DNA segments

include FB29, FB28, and FB27.

DNA segments to which the invention relates also include those encoding substantially the same proteins as those encoded in the exons of Figure 1 which includes, for example, allelic forms of the Figure 1 amino acid sequences. The invention also relates to DNA fragments complementary to such sequences. A unique portion of the DNA segment or the complementary fragment thereof of the present invention can be used as probes for detecting the presence of its complementary strand in a DNA or RNA sample.

The present invention further relates to DNA constructs and to host cells transformed therewith. In one embodiment, the DNA constructs of the present invention comprise a DNA segment encoding a c-*ski* protein of the present invention and a vector, for example, pMEX *neo*. In another embodiment, the DNA constructs comprise a DNA segment encoding a truncated c-*ski* protein having the function of c-*ski* (such as, for example, ΔFB29) and a vector (for example, pMEX *neo*). The DNA construct is suitable for transforming host cells. The host cells can be procaryotic or, preferably, eucaryotic (such as, mammalian).

The present invention further relates to animals, such as, for example, domestic livestock, having increased muscle size. The development of such strains of domestic livestock with increased muscles continues to be a major goal of conventional breeding schemes. (Domestic livestock as used herein refers to animals bred for their meat, such as, for example, pigs, chickens, turkeys, ducks, sheep, cows and fish, particularly, trout and catfish).

Introduction of various genes into the germ lines of mice and of some types of domestic

2059137

livestock is relatively routine to those skilled in the art. The present inventors have produced mice having increased muscle size by introducing a DNA construct comprising ΔFB29 and the pMEX *neo* vector into fertilized eggs. Resulting founder mice and their offspring have the DNA construct in all their cells, somatic and germ.

Introduction of DNA constructs encoding a *ski* protein (such as, for example a c-*ski* protein), into fertilized eggs of animals (such as, by microinjection) results in strains of animals having increased muscle development and decreased fatty tissue. As one skilled in the art will appreciate, the animals with increased muscle size of the present invention can also be produced using DNA encoding a *ski* protein from various species (chicken being just one such example). Furthermore, animals of the present invention can be produced using a DNA construct encoding protiens related to *ski*, such as, for example, *sno* gene.

The DNA segment ΔFB29 generated by a frameshift mutation results in a truncated protein. However, as one skilled in the art will appreciate, the transgenic animals of the present invention can also be generated by DNA constructs containing DNA segments encoding a full length *ski protein, a portion of a* ski protein, such as, one or two exons or a biological active deletion derivative, such as, for example, v-*ski*, which represents a truncated c-*ski* fused to a viral protein. Further, it is also recognized that the selective expression of the protein in muscle tissue may result from DNA constructs created in vectors other than pMEX *neo*.

The present invention also relates to a method of stimulating muscle growth and preventing muscle

degeneration in an animal, such as for example, a human. A possible treatment for injuries resulting in loss of muscle tissue and neurological injuries resulting in degeneration of the muscle would be to stimulate muscle growth. In the case of lose of muscle this would involve stimulating regrowth of the tissue. Whereas in the case of neurological injuries, the muscle growth would need to be rendered independent of the missing nerve stimulus. According to the present invention, muscle growth could be stimulated by delivering a DNA construct encoding a *ski* protein to the muscle tissue under conditions such that the protein encoded in the construct is expressed. The construct can be targeted and delivered to the muscle using standard methods known to those skilled in the art.

The present invention further relates to a method of treating a muscle degenerative disease such as, for example, muscular dystrophy and amyotrophic lateral sclerosis (also known as Lou Gehrig disease). Treatment would comprise delivering a DNA construct of the present invention to the effected muscle under conditions such that the protein encoded in the construct is expressed and treatment effected.

Examples

Screening of cDNA Library

Two chicken cDNA libraries were screened with a v-*ski* probe using standard protocols (Maniatis et al., 1982, Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). One library was made from poly A$^+$ mRNA isolated form the body wall of 10-day embryos and the other from mRNA isolated from AEV-

2059137

transformed chicken erythroblasts. Four distinct c-*ski* cDNAs were isolated; three were from the body wall library (these cloned were designated FB27, FB28 and FB29) and one cDNA clone from the erythroblast library (designated CEL). These cDNAs included sequences extending both 5' and 3' of the portion of *ski* present in the virus. The cDNAs demonstrate that v-*ski* derives from a single cellular gene and suggest that multiple c-*ski* mRNAs, encoding distinct *ski* proteins, are produced from the c-*ski* locus by alternate splicing (Leff et al., 1986, Ann. Rev. Biochem. 55, 1091-1117), adding to a growing list of oncogenes known to produce multiple mRNAs in this fashion (Ben-Neviah et al., 1986, Cell *44*, 577-586; Levy et al., 1987, Mol. Cell. Biol. 7, 4142-4145; Martinez et al., 1987, Science *237*, 411-415; McGrath et al., 1983, Nature *304*, 501-506).

Structure of the cDNA clones

The structure of all of the c-*ski* cDNA clones that have been characterized by DNA sequencing (Sanger et al., 1977, Proc. Natl. Acad. Sci. USA *74, 5463-5467*) and their relationships to v-*ski* are presented in Figure 1.

Only FB28 and CEL have sequences 5' of the v-*ski* sequences; both FB27 and FB29 are truncated at the 5' end. Assuming that the missing sequences at the 5' end of FB29 are similar to those in FB28 and CEL, a composite nucleotide sequence and the deduced amino acid sequence for a cDNA of the FB29 type is shown in Figure 2. The first ATG with a substantial downstream open reading frame is located at nucleotide position 168. Upstream of this ATG no reading frames are open, suggesting that these sequences represent the 5' untranslated region.

Based on the cDNA sequence analysis and comparisons with the positions of the splice donor and acceptor sites known from the genomic sequence (Stavnezer et al., 1989, Mol. Cell. Biol. 9, 4038-4045), exon boundaries have been derived (see Figure 3). As seen in the figure, c-*ski* sequences are distributed over seven exons. FB29 contains all seven exons; FB28 and FB27 lack exon 2 and exon 6, respectively. This differential splicing affects the protein coding potential of the three cDNAs.

Differential splicing of exon 2 deletes 37 amino acids without affecting the coding potential of the open reading frame downstream. Differential splicing of exon 6, however, affects the coding potential of exon 7. If exon 5 is spliced to exon 7 (seen in FB27 cDNA), a translation termination codon is generated at the splice junction and exon 7 becomes a noncoding exon. However, if exon 5 is spliced to exon 6 and exon 6 to exon 7, as in FB28/29, then the open reading frame continues in exon 7 for 417 nucleotides encoding an additional 129 amino acids.

Assuming that the missing 5' ends of the FB29 and FB27 mRNAs contain sequences identical to those present in CEL and FB28, then translation of mRNAs corresponding to the three different types of cDNAs would lead to the generation of three proteins, one containing 750 amino acids (from FB29), the second 713 amino acids (from FB28), and a third protein containing 510 amino acids (from FB27).

The CEL clone is missing 3' sequences. cDNAs that derive from the body wall (FB) library have long 3' untranslated regions that contain a 95-base pair (bp) AT-rich region from nucleotide 2803

to 2898. Within this region there are two copies of a sequence ATTTA that has been implicated in mRNA destabilization in a variety of transiently induced mRNAs including c-*myc*, interferon, c-*jun*, and c-*fos* (Meijlink et al., 1985, Proc. Natl. Acad. Sci. USA *82*, 4987-4991; Ryder et al., 1985, Proc. Natl. Acad. Sci. USA *85*, 1487-1491; Shaw et al., 1986, Cell *46*, 659-667). Addition or deletion of these sequences have also been shown to affect the transformation potential of c-*fos* (Meijlink et al., 1985, Proc. Natl. Acad. Sci. USA *82*, 4987-4991).

The c-*ski* cDNAs contain two potential poly(A) signals (AATAAA) located at positions 3348 and 4167. Although all three clones isolated from the FB library end at the same position, none has a poly(A) tail; therefore, it is likely that the 3' ends of the c-*ski* mRNAs are not contained in these clones. The 4.2-kb cDNAs that were isolated and characterized are smaller than the 5.7 -8.0 and 10.0-kb mRNAs detected by Northern transfer analysis (Li et al., 1986, J. Virol. 57, 1065-1072). This discrepancy has not been explained, however, it is suggested that the clones isolated so far, which lack poly(A) tails, also lack sequences from the 3' ends of the mRNAs.

The 5' End of c-*ski* mRNA(s)

Sequence comparisons at the 5' end reveals that both FB28 and CEL are colinear up to a position 89 bp upstream of the putative translational initiation ATG codon. FB28 has an additional 76-bp while CEL has 25 bp that are different from those found in FB28. This region where the two sequences diverge has been compared with sequences from genomic clones (Stavnezer et al., 1989, Mol. Cell. Biol. *9*, 4038-4045) and the

upstream sequences in the FB28 are colinear with the genomic sequences.

Examination of the sequences in the genomic DNA at the point of divergence of CEL and FB28 does not reveal consensus sequences for donor or acceptor splice sites. In order to confirm the authenticity of the clones, S1 nuclease protection analysis was carried out. The results demonstrated that the sequences present in FB28 are expressed as mRNA in normal embryos. Similar S1 analyses have provided no evidence for the presence in mRNA of the 25 bp at the extreme 5' end of the CEL clone. It is suggested that the first 25 bases of the CEL clone are the result of a cloning artefact, and that the sequences found in the FB28 clone are expressed in c-*ski* mRNA(s).

In an attempt to determine how much of the 5' untranslated region of the c-*ski* mRNA(s) are contained in FB28, primer extension analysis was carried out. Copying the 5' segment present in FB28 should give an extension product of about 280 bases. However, a primer extension product of 220 bases was seen. S1 analyses data have shown that this segment is expressed in RNA. It is possible that the observed primer extension product is the result of premature termination; however, it is also possible that there are multiple 5' ends for the c-*ski* mRNAs.

Organization of the Internal Exons

Sequence comparisons in the central portion of FB27, FB28 and FB29 and CEL cDNAs reveal that FB28 lacks a small region of 111 bp (exon 2) from 1079-1191 that would eliminate 37 amino acids. The genomic sequence (Stavnezer et al., 1989, Mol. Cell. Biol. *9*, 4038-4045) in the corresponding region reveals the existence of consensus splice

donor and acceptor sequences at the boundaries of the deletion suggesting that the cDNA derives from a differentially spliced mRNA.

In order to confirm the existence of mRNAs of the FB28 and of the FB27/29 type, S1 analysis was carried out on total RNA. Total RNA was isolated from 8, 10, 12, 15 and 17-day-old chicken embryos using standard protocols (Chirgwin et al., 1979, Biochemistry *18*, 5294-5299). Approximately 20 to 30 μg of total RNA was used for nuclear S1 analysis using standard procedures (Maniatis et al., 1982, Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

A uniformly labeled single-stranded probe spanning the region between the *Kpn*I and *Hind*III sites of FB29/27 or FB28 (probes A and B of Figure 1) was hybridized with total cellular RNA. As shown in Figure 4B, hybridization to mRNA and subsequent S1 digestion of a probe derived from FB29 produced protected fragments of 645 bp indicating hybridization to mRNA of the FB27/29 type and the 262/272 bp fragments expected if the probe hybridized with mRNA of FB28 type. FB28 (probe B) protected a fragment of 534 bp (see Figure 4C). Smaller fragments from the hybridization of the FB28 probe to mRNAs of the FB27/29 type were not observed; however, S1 does not always cleave a DNA probe efficiently opposite a looped out region of RNA. These experiments indicate that mRNA corresponding to both the FB27/29 type, containing the second exon, and FB28 type, missing the second exon, are expressed in normal cells.

Organization of the 3' Coding Exons

Sequence comparison of the three fibroblast

clones demonstrated that FB28 and FB29 contain a segment (exon 6) that is absent in FB27. Examination of the genomic DNA at a position corresponding to the position where the cDNAs diverge reveals a consensus splice donor (Stavnezer et al., 1989, Mol. Cell. Biol. *9*, 4038-4045). The two cDNAs appear to derive from splicing events that either include or exclude exon 6. Downstream of this alternate exon the 3' portion of all three cDNAs are identical.

To investigate whether both types of cDNAs represent normal cellular mRNA, S1 analysis was carried out. Uniformly labeled single-stranded probes were generated from an 799-bp *Hind*III fragment (see probe D, Figure 1) from the 3' end of FB28 and a 497-bp *Hind*III fragment from the 3' end of FB27 (probe C) subcloned in M13mp18. Single-stranded probes were prepared and were hybridized to 20 μg of total RNA prepared from whole chicken embryos of different ages. As seen in Figure 4E, hybridization of the RNA to probe D (FB28) followed by S1 treatment produced a 799-bp fragment that would be expected if mRNA of FB28/29 type was present. Smaller fragments that would be generated by the FB28 probe hybridizing with mRNA of the FB27 type were not detected. S1 digestion of the FB27 probe (probe C) produced a fragment of 497-bp and also two smaller fragments of 243 to 254 bp (see Figure 4D). It is likely that these smaller fragments derive from S1 cleavage of the FB27 probe at the site where the probe differs from mRNAs of the FB28/29 type. These data confirm the existence of the FB28/29 mRNA, but suggest that, if FB27 mRNA exists, it is likely to be present at a lower level than FB28/29.

Differential Splicing

A schematic illustration of the differential splicing of three c-*ski* mRNAs as deduced from the nucleic acid sequence analysis of the cDNA clones is shown in Figure 3. S1 analysis of total RNA derived from chicken embryos has confirmed the existence of two classes of mRNAs, those that do, and those that do not, contain exon 2. Using a similar protocol, the existence of mRNA that contains exon 6 was confirmed. Utilizing this technique, the existence of mRNA lacking exon 6 which would correspond to FB27 cDNA, could not be demonstrated; however, several lines of argument suggest that the FB27 cDNA is not a simple cloning artefact. The sequences absent from FB27 are bounded by apparent splice junctions (as judged by an examination of both the cDNA sequences and the available genomic DNA sequence). Although cDNAs are occasionally obtained that contain one or more introns, presumably because a partially processed mRNA was reverse transcribed, isolating cDNA artefactually missing an exon is less likely on theoretical grounds, and seems to occur rarely, if at all, in the manufacture of a cDNA library. For these reasons, the interpretation that FB27 represents a real, if relatively rare, c-*ski* mRNA is currently favored.

Comparison of c-*ski* with v-*ski*

Transduction of c-*oncs* by retroviruses often results in truncation, deletion, or point mutation(s) in the c-*onc* (Bishop, J. M., 1983, Ann Rev. Biochem. *52*, 301-354). Comparing c-*ski* and v-*ski* sequences shows that the v-*ski* gene is truncated at both 5' and 3' ends and represents only part of the c-*ski* coding region (see Figure 1 and 3). v-*ski* sequences begin at position 244 (the putative initiator ATG is at position 168)

and end at position 1541 (see Figure 3). The biological significance of the 5' and 3' truncations are unknown. There is only one base change in the v-*ski* relative to the c-*ski*, at position 1284 where v-*ski* has a 'C' and c-*ski* has a 'T'. This base change alters the amino acid from Trp in c-*ski* to Arg in v-*ski*. Deletion analysis of v-*ski* implies that this amino acid change does not play a significant role in the transforming potential of v-*ski*. The biological activities of the cDNAs are being evaluated by expressing the coding regions from the cDNAs using replication-competent retroviral vectors (Hughes et al., 1987, J. Virol. *61*, 3004-3012).

As shown in Figure 5, 18 of 20 bp are identical between c-*ski* and the p19 region of *gag* in the parental ALV. This region of homology contains the 5' junction between viral sequences and v-*ski*. With such a long stretch of homology, it is impossible to assign the 5' recombinational joint precisely. No substantial homology can be seen precisely at the 3' *ski*/ALV joint. However, just downstream of the 3' junction, there is an ALV sequence that is closely homologous to a segment of c-*ski* found just 3' of the v-*ski*/AVL junction. It is possible to invoke this region of homology in the alignment of the nucleic acids involved in the recombination event.

Transduction mechanism

The exact mechanism(s) by which retroviruses acquire cellular oncogenes remains uncertain. It has been suggested that as a first step, the viral DNA is integrated next to or within a cellular oncogene (Bishop, J. M., 1983, Ann Rev. Biochem. 52, 301-354). The retroviral and cellular sequences can then be fused by DNA deletion events

(Bishop, J.M. 1983, Ann. Rev. Biochem. *52*, 301-354; Czernilofsky et al., 1983, Nature *301*, 736-738) or RNA read through (Herman et al., 1987, Science *236*, 845-848; Nilsen et al., 1985, Cell *41*, 1719-726); however, homology is not known to be involved in either of these processes. In the case of *ski*, a comparison of c-*ski* sequences with the p19 coding region implies that some event in the generation of the *ski* viruses involved homologous recombination. However, the homologous recombination event may be secondary. If the original event were nonhomologous and generated a viral genome that contained a direct repeat, it would be expected, as has been observed for Rous sarcoma virus, that sequences between the repeats could be lost by recombination during viral passage, presumably through copy choice during reverse transcription. It is possible, however, to propose models in which the oncogene is acquired by homologous recombination between the c-*onc* and the replication competent viruses. Short stretches of homologies have been observed at both ends of viral oncogenes (Banner et al., 1985, Mol. Cell. Biol. *5*, 1400-1407; Van Beveren et al., 1983 Cell *32*, 1241-1255) or only at the left hand or 5' recombination joint (Besmer et al., 1986, Nature (London) *320*, 415-421; Roebroek et al., 1987, J. Virol. *61*, 2009-2016). The present data does not allow the determination of whether the 5' homologous recombination event was primary or secondary.

Transgene and Generation of the Transgenic Mice

The largest form of the isolated cDNAs, that is FB29 which contains sequences that derive from all seven coding exons of c-*ski*, and judged by DNA sequence, encodes a c-*ski* protein of 750 amino

acids, was used to create the derivative ΔFB29. ΔFB29 has a frameshift mutation at position 1475 in the fifth coding exon (one C in a run of five Cs was lost in the frameshift mutant), and is predicted to give rise to a protein of 448 amino acids of which the first 436 are identical to the first 436 amino acids of the FB29 form of c-*ski* (the last 12 amino acids are past the frameshift mutation and thus differ from those of the FB29 form of c-*ski*). ΔFB29 used in the generation of a the transgene is shown schematically in Figure 6.

The construction of the *ski* portion of the transgene is already described (Sutrave and Hughes, 1989, Mol. Cell. Biol. *9*, 4046-4051; Sutrave et al., 1990, Mol. Cell. Biol. *10*, 3137-3144). Briefly, a truncated chicken c-*ski* cDNA called ΔFB29 had been previously cloned into the adaptor plasmid Cla12Nco. The ΔFB29 segment was released from the adaptor plasmid by *Cla*1 digestion and the 5' overhangs filled in using the Klenow fragment of *E. coli* DNA polymerase I and all four dNTPs. This blunt-ended fragment was ligated to the pMEX *neo* vector which have been digested with *Eco*R1 restriction enzyme and blunt-ended with the Klenow fragment. Clones were selected that had inserts in the correct orientation and were digested with both *Pvu*I and *Nru*1 restriction endonucleases. These enzymes release a segment that contains the ΔFB29 cDNA flanked by an MSV LTR and the SV40 polyA signal (see Figure 6). This fragment was gel purified and used to inject fertilized mouse eggs [Hogan et al., 1986, Manipulating the mouse embryo. A Laboratory Manual. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory)].

The ΔFB29 clone was placed in the pMEX expression plasmid in such an orientation that the

truncated c-*ski* cDNA between an MSV LTR and an SV40 polyadenylation site (see Figure 6). The plasmid was digested with *Pvu*I and *Nru*I to release the expression cassette. The expression cassette was purified by gel electrophoresis and introduced into fertilized mouse eggs by microinjection. Forty-four founder mice were obtained after two independent injections. The mice were identified by dot blot analysis of DNA isolated from tail clips. This analysis was confirmed by Southern transfer. Three of the 44 founder mice showed a distinct muscle phenotype (TG 8566, TG 8821, and TG 8562). These three founders and a single mouse that contained unrearranged copy of the complete transgene but did not show any phenotype (TG 8542) were used to generate lines. Southern transfer analysis of DNA from TG 8566, TG 8821, TG 8562, and TG 8542 suggests that the site of integration of the transgene in each line is different and the copy number varies from approximately 5-35 copies per genome.

DNA positive mice from the three lines (TG 8566, TG 8821 and TG 8562) had a similar distinct appearance resulting from abnormal muscle growth. Although the three lines of mice carry an oncogene, none of the lines appears to have an increased incidence of tumors. This result is not totally unexpected, since the v-*ski* virus is not tumorigenic in chickens unless the birds are injected with infected cells (E. Stavnezer, 1988, in The Oncogene Handbook, E. P. Reddy, A. Skalka, and T. Curran, eds. (Amsterdam: Elsevier Science Publishing Co.), pp. 393-401). The three strains of mice do not express high levels of the transgene except in skeletal muscle. This is consistent with the interpretation that c-*ski* affects skeletal muscle cells directly and not as

a secondary consequence of altered motor neuron function. The majority of the skeletal muscles are involved. Mice with this phenotype can be readily identified by looking for enlarged limb and jaw muscles (Figure 7). Since the phenotype was obtained with three separate founders, the most reasonable explanation is that the phenotype was caused by the chicken c-*ski* transgene. Therefore, all four lines of mice were examined, the three with the muscular phenotype and the one line that did not have an observable phenotype, for the expression of the transgene.

The expression cassette was also introduced into fertilized pig eggs using standard procedures known to those in the art. Founder pigs show the expected muscle specific selection of expression of the gene and accordingly, it is expected that the pigs will have the same muscle phenotype as that seen in the founder mice.

DNA/RNA Analysis

Total cellular DNA and RNA were isolated by standard procedures. For RNA isolation, tissues were frozen in liquid nitrogen immediately following dissection and homogenized in RNAzol (Cinna Biotex) and processed according to the manufacturer's recommendation. For Northern transfer analysis, approximately 20 μg of total RNA from different tissues was fractioned by electrophoresis on 1.5% agarose gels containing 2.2 M formaldehyde. The RNA was transferred to nitrocellulose membranes and probed either with a nick-translated chicken *ski* cDNA or a chicken β-actin cDNA. The coding region of the β-actin cDNA cross reacts with the messages for the other actions and can be used to validate the quantity and quality of RNA from most tissues.

Total RNA from spleen, lung, brain, kidney, liver, stomach, heart, and leg (skeletal) muscle was isolated as described and the results are shown in Figure 8. All three lines with the phenotype (TG 8566, TG 8821 and TG 8562) expressed a 2.5-kb chicken c-*ski* specific transcript at high levels in skeletal muscle; however, some lines of mice showed low levels of chicken c-*ski* RNA in other tissues. The TG 8562 line has RNA from the transgene in the heart, although at a lower level than in skeletal muscle. Histopathology of hearts from TG 8562 mice showed that there is no significant effect on this tissue. Line TG 8542, which does not show any phenotype, had a much lower level of RNA from the transgene in muscle than did the lines that showed the phenotype. The observation that expression was restricted to muscle was unexpected since the MSV LTR has been shown to express in a variety of tissues when linked to other genes (Khillan et al., 1987, Genes Dev. *1*, 1327-1335).

To determine whether the transcript was initiated at the proper site in tissues expressing the *ski* transgene, RNase protection assays were carried out as described by Melton (Melton et al., 1984, Nucl. Acids Res. *12*, 7035-7036). A 1.8-kb *Pvu*1 to *Bgl*1 fragment was subcloned in Bluescript KS vector and used to generate radioactively labeled RNA from the T7 promoter. Approximately 10 μg of total RNA was hybridized with 5 x 10^5 cpm of probe. The hybridizations were carried out overnight at 50°C in 80% formamide and 1x buffer (5x hybridization buffer is 0.2 M Pipes, pH 6.4, 2 M sodium chloride, 5 mM EDTA). After hybridization, the samples were diluted in ribonuclease digestion buffer (10 mM Tris Cl, pH 7.5, 0.3 M sodium chloride, 5 mM EDTA) and treated

with RNase T1 at a concentration of 1 u/μl for 60 min at 30°C. The RNase digestions were stopped by adding 10 μl of 20% SDS and 4 μl of Proteinase K (stock 10 mg/ml) and incubating at 37°C for 15 min. The digested samples were extracted with phenol chloroform (1:1 mixture) and ethanol precipitated with carrier tRNA. The pellet was rinsed once with 70% ethanol, dried and dissolved in formamide containing bromophenol blue and xylene cyanol dyes. The samples were denatured at 100°C and separated on 6% polyacrylamide gels containing 7.5 M urea.

Uniformly labeled antisense RNA was generated by T7 RNA polymerase from a fragment that spans the MSV LTR and c-*ski* (see Figure 9). Using RNA from the three positive transgenic lines, a protected fragment of approximately 980 bases was seen, which is the expected size if the transcript is initiated at the authentic initiation site within the MSV LTR (Figure 9). This analysis also gives a more quantitative estimate of the level of transgene RNA in the heart and skeletal muscle of both the phenotypically positive and the phenotypically negative lines of mice. Figure 9 shows that the level of transgene RNA in the heart of TG 8821 is much lower (estimated at perhaps 1/10-1/20) than the level found in the skeletal muscle. In addition, this analysis shows that the phenotypically negative line, TG 8542, has a low but detectable level of transgene RNA in skeletal muscle. These data suggest not only that the muscular phenotype is associated with the expression of the chicken c-*ski* transgene, but also that a minimum threshold level of c-*ski* RNA must be reached to produce the muscular phenotype. In fact, these data suggest that the minimal threshold level to see an effect of the transgene

is high, probably several thousand-fold the levels of endogenous c-*ski* expression in the chicken tissues examined (Sutrave and Hughes, 1989, Mol. Cell. Biol. *9*, 4046-4051).

Protein Analysis

The underlying assumption is not that the expression of the c-*ski* RNA gives rise directly to the muscular phenotype, but rather that the phenotype results from the presence of the c-*ski* protein. Accordingly, the c-*ski* protein was looked at in Western transfer assays. Although rabbit antisera have been prepared that specifically recognize the 50-kd form of c-*ski* (Sutrave et al., 1990, Mol. Cell. Biol. *10*, 3137-3144), these antisera do not work well in Western transfer assays. Mouse monoclonal antibodies that recognize c-*ski* and that work well in Western transfer assays have been developed, however, the use of these reagents presents a technical problem. These monoclonals were not available in sufficient quantity to permit direct labeling. Indirect labeling procedures using, for example, labeled rabbit anti mouse detect not only the anti *ski* monoclonal but also the endogenous mouse heavy chain, which comigrates with the 50-kd form of c-*ski* made from the transgene. To avoid this problem, extracts of muscle and control tissue (liver) from normal controls and from the transgenic mice were prepared. The endogenous mouse antibodies were removed from these extracts by precipitation with rabbit anti mouse antibody as described below.

For detection of *ski* protein in tissues from the transgenic and control mice, 1-5 mg of tissue was homogenized in 1 ml of RIPA buffer, 20 mM Tris Cl pH 7.5, 150 mM NaCl, 0.5% SDS, 0.5% NP40, 0.5%

sodium deoxycholate, 1 mM EDTA, 1 mM PMSF, and 35 μ/ml of aproteinin. The homogenate was clarified by centrifugation at 10,000 rpm for 10 min. Mouse IgG was removed from 100 μl of the supernatant by incubation with 10 μl of 1 mg/ml rabbit anti mouse IgG (in PBS) for 2 hr on ice. The complex was removed by adding 100 μl of 40% protein A sepharose beads in RIPA buffer. The resulting supernatant was collected and 20 μl was fractionated on 10% SDS polyacrylamide gels. The proteins were transferred to nitrocellulose membranes overnight in buffer containing 0.125 M Tris Cl, 0.092 M Glycine and 20% Methanol, pH 8.3. The filters were blocked with 4% dry nonfat milk in TBS buffer (0.5 M Tris Cl, pH 7.4 and .2 M sodium chloride) for 2 hr at room temperature and incubated with a mixture of three anti-*ski* monoclonal antibodies at a dilution of 1:3000 for 2 hr at room temperature and were then washed 3x with TBS. Secondary incubations with rabbit anti mouse IgG were done for 2 hr at room temperature (1:2000 dilution from a 1 mg/ml stock). The filter was washed as described above and finally incubated with 5 μCi of ^{125}I protein A (Amersham, sp. act. 30 mCi/mg) for 2 hr at room temperature. The filter was washed 3x with TBS and exposed to XAR Kodak film at -70°C for 6 days.

Only affected tissues (skeletal muscle) from the transgenic animals contain the 50-kd c-*ski* protein (Figure 10). These data also suggest that there may be differences in the level of the c-*ski* protein in the muscles of the three positive lines; however, the complexities of the manipulations in this experiment make quantitative interpretation a questionable proposition.

Histology

For histology, selected muscles from the line TG 8566 were isolated so that they remained attached at their origin and insertion and they were then fixed in 2% formaldehyde, 2% gluteraldehyde. Fixed muscle were transected precisely through the middle of the muscle belly and embedded in JB4 plastic (Polysciences, PA).

For immunocytochemistry, tissues were snap frozen in isopentane cooled in liquid nitrogen. The procedure for immunochemical staining was as outlined in Narusawa et al. [(1987), J. Cell. Biol. *104*, 447-459].

The results showed that not only is expression of the transgene and its effect restricted to muscle, the expression and the effects appear, at least in the line TG 8566, to be confined to certain muscles, and apparently to specific fiber types. However, the affected fibers are not all of the same type.

The myocardium was normal and there were no abnormalities of visceral smooth muscle in these animals. Figures 11a and 11b compare cross sections made precisely through the middle of the plantaris muscle from mature male controls and transgenic mice. Cross sectional area of the control is 2.7 μ^2 and that of the TG 8566 mouse is 9.4 μ^2, more than twice the control value. This massive growth is generalized. Comparable increases in cross section were found in almost all axial and appendicular muscles throughout male and female mice of line TG 8566. Only three muscles were found that appear to be normal: the tongue, the diaphragm, and the soleus; these are the same size in transgenic as in control muscles (see Figure 12). RNA was isolated from the diaphragm and soleus muscles of TG 8566 mice. Northern transfer analysis shows that the level of

chicken c-*ski* RNA is much lower in these two phenotypically normal muscles than in the affected muscles from the same line (Figure 13).

The most obvious additional gross morphologic abnormality is that transgenic animals were almost totally devoid of fat whereas control animals contained substantial amounts of subcutaneous and intraperitoneal fat. For this reason, there is little difference in weights between control and TG 8566 mice. There are also skeletal abnormalities; the tibia of transgenic animals is normal in size but was bowed cranially, apparently as an adaptation to accommodate the more than two-fold increase in size of the anterior tibia and extensor digitorum muscles.

The muscles in control mice are made up of fibers with a range of cross sectional areas. The range of sizes is greatly extended in TG 8566 mice (Figures 11 and 12). Not all fibers are affected; the hypertrophy is limited to a select population of fibers (Figure 11d). Hypertrophy of these fibers apparently accounts for the increase in muscle mass in line TG 8566. No evidence was found that the numbers of fibers is significantly increased in individual muscles. For example, the population of fibers in the control plantaris is 912 ± 111 (n=3) and in TG 8566 is 991 ± 87 (n=3). Similarly, no difference in the total number of fibers in the extensor digitorum longus muscle of TG 8566 and control mice was found.

To investigate which types of fibers are affected in the line TG 8566, three monoclonal antibodies were used that specifically recognize slow myosin heavy chains (MHC) (NOQ7 5 4D, Narusawa et al., 1987, J. Cell Biol. *104*, 447-459), all fast MHCs (2G3, Narusawa et al., 1987, J. Cell Biol. *104*, 447-459) type II a fast MHC (SC

7 11, Schiaffino et al., 1989, J. Muscle Res. and Cell Motil. *10*, 197-205) and type IIb fast MHC (BF-F3, Schiaffino et al., 1989, J. Muscle Res. and Cell Motil. *10*, 197-205). Figure 14 shows immunofluorescent staining of sections from the rhomboideus capitis muscle with these three antibodies. No evidence was found that slow fibers are enlarged (Figure 14a) and the total number of slow fibers in the rhomboideus capitis from a TG 8566 mouse (120) approximates the number found in rhomboideus capitis from control mice (117). Type IIa fibers are also not affected (Figure 14b). Many of the type IIa fibers lie between hypertrophied fibers and are frequently distorted in shape as if compressed by the expansion of their neighbors (Figure 14b).

The small, fast IIa fibers and all of the hypertrophied fibers stain with the monoclonal antibody 2G3 (Figure 14c) indicating that all hypertrophied fibers are fast. In the rhomboideus capitis, most, but not all, large fibers also stain with the monoclonal antibody BF-F3 that is specific for IIb MHC (Figure 14d). In the plantaris, there is more variation in reactivity and only 50% of hypertrophied fibers stain with BF-F3. These results show that the hypertrophic modification of fibers in TG 8566 mice involves at least two types of fast fibers. One of these is type IIb. By exclusion, we suggest that the others are IIx fibers (Schiaffino et al., 1989, J. Muscle Res. and Cell Motil. *10*, 197-205; Termin et al., 1989, Histochemistry *92*, 453-457; Gorza, 1990, J. Histochem. Cytochem. *38*, 257-265).

Variation in staining of hypertrophic fibers with the actomyosin ATPase histochemical reaction after acid pre-incubation, DPNH staining for mitochondrial enzyme activity and PAS staining all

support the conclusion that more than one fast fiber type is affected in this line of transgenic mice. These results also support the interpretation that both IIb and IIx fibers are hypertrophied.

Occasional necrotic and regenerating fibers were found in some, but not all, muscles of line TG 8566 mice. In the hind limb, these appeared to be most prevalent in the anterior tibial muscle; they were never found in the rhomboideus capitis, a superficial muscle of the neck.

While the foregoing invention has been described in some detail for the purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention and appended claims.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED

1. A DNA molecule encoding a c-*ski* protein, said DNA molecule having the ability to produce an increase in muscle mass when operably linked to a promoter effective for expression in muscle cells, wherein said DNA segment comprises exon 6 or exon 7 or both exon 6 and exon 7 as shown in Figure 2, with the proviso that said DNA segment does not encode an arginine residue at amino acid 373 of the protein as numbered in Figure 2.

2. A DNA molecule comprising exon 6 (nucleotides 1699-2000) as defined in Figure 2, having the ability to produce an increase in muscle mass when operably linked to a promoter effective for expression in muscle cells.

3. A DNA molecule comprising exon 7 (nucleotides 2001-2420) as defined in Figure 2, having the ability to produce an increase in muscle mass when operably linked to a promoter effective for expression in muscle cells.

4. The DNA molecule according to claim 3, further comprising at least four exons selected from the group consisting of: exon 1 (nucleotides 168-1079), exon 2 (nucleotides 1080-1190), exon 3 (nucleotides 1191-1316), exon 4 (nucleotides 1317-1432), exon 5 (nucleotides 1433-1698) and exon 6 (nucleotides 1699-2000), as defined in Figure 2.

5. A DNA construct comprising:

i) said DNA molecule according to claim 1;

and

ii) a vector.

6. The construct according to claim 5 wherein said vector is pMEX *neo*.

7. A DNA construct comprising:

i) a DNA molecule comprising at least 20 continuous base pairs of the nucleotide sequence of Figure 2, wherein said DNA molecule encodes a truncated c-*ski* protein having the ability to produce an increase in muscle mass; and

ii) a vector.

8. The DNA construct according to claim 7, wherein said DNA molecule is ΔFB29.

9. The DNA construct according to claim 7, wherein said vector is pMEX *neo*.

10. A DNA molecule comprising at least 20 continuous base pairs of the nucleotide sequence of Figure 2 having the ability to produce an increase in muscle mass when operably linked to a promoter effective for expression in muscle cells.

11. A host cell stably transformed with said DNA construct according to claim 5, in a manner allowing expression of said protein encoded in said DNA construct.

12. A host cell stably transformed with said DNA construct according to claim 7, in a manner allowing expression of said protein encoded in said DNA construct.

13. The host cell according to claim 11 or claim 12 which is a mammalian cell.

14. An animal cell comprising the construct according to claim 5, in a manner allowing expression of said protein encoded in said construct.

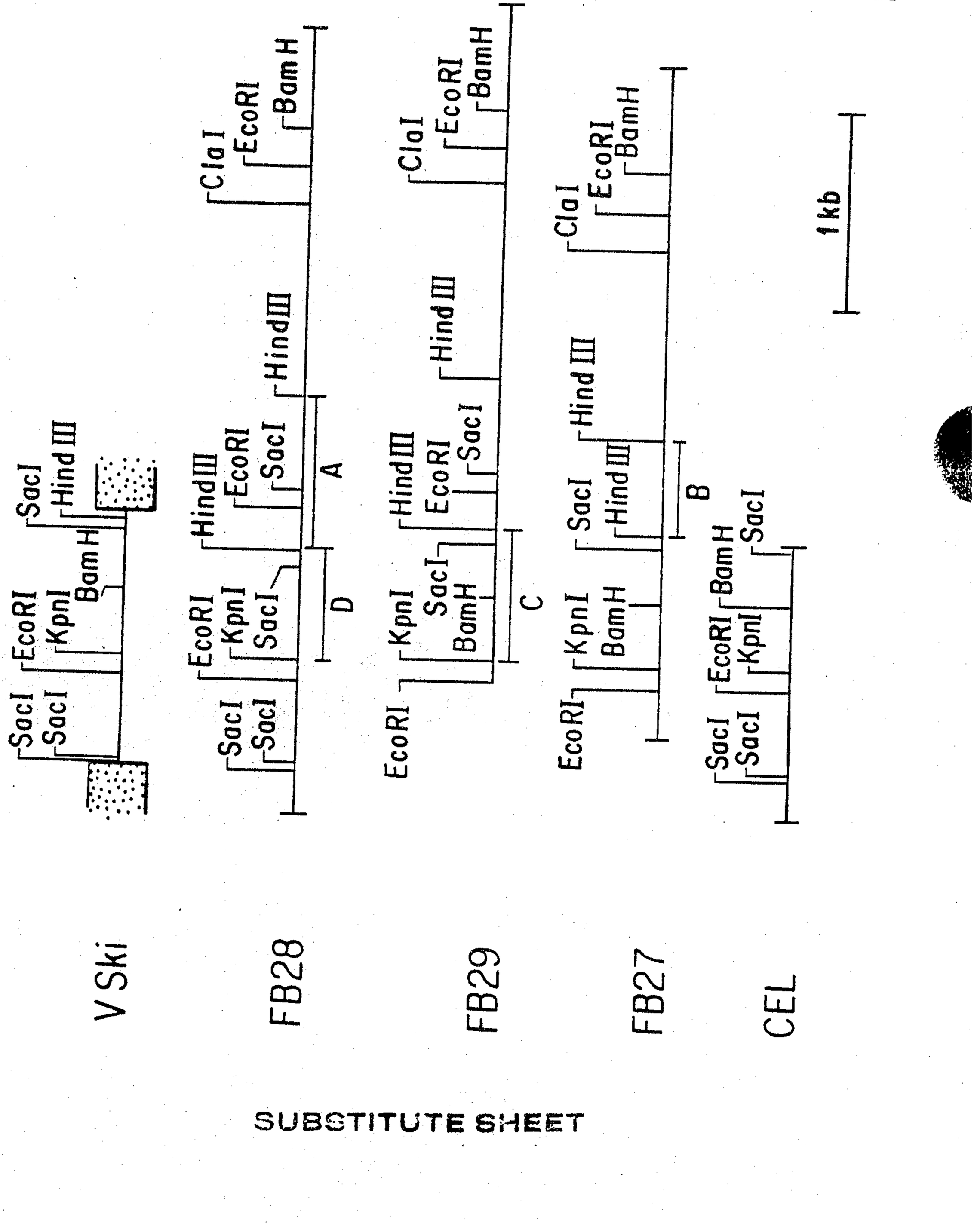
15. An animal cell comprising the construct according to claim 7, in a manner allowing expression of said protein encoded in said construct.

16. The use of the DNA construct of claim 5, for stimulating muscle growth or preventing muscle degeneration.

17. The use of the DNA construct of claim 5, for treating a muscle degenerative disease.

18. The use of the DNA construct of claim 17 wherein said muscle degenerative disease is muscular dystrophy or amyotrophic lateral sclerosis.

FIG. 1


ClaI
EcoRI
BamH
HindIII
SacI
KpnI
1 kb
A
B
C
D
V Ski
FB28
FB29
FB27
CEL

```
 TC CTC GTC GTC TGT GGA TTG CTA AAC CTG AGT GGG AAG GGG GGG GAA
                                  ↓
AAA AAA AAG GGT GGG TTG TTG TTT TGT TTA AAA AAA GAA AAA ATC CCT TAA      98

GTG GAT TTG TAC CAG CGT GGA AGA TAA CTG GGG ATT TTT GTT GTT TGT
                                 ┌──→1
TTT GGG AAT AGA AAC TAA AAA┌ATG GAG ACT GTA AGT AGA AGC AGC TTC CAG     197
                           └Met Glu Thr Val Ser Arg Ser Ser Phe Gln      10
                                  ↓                    Vski
CCT CAT CCA GGA CTG CAG AAG ACC TTG GAA CAG TTT CAT CTG AGC TCT
Pro His Pro Gly Leu Gln Lys Thr Leu Glu Gln Phe His Leu Ser Ser
                ↓
ATG AGC TCC CTG GGT GGC CCT GCT GCT TTC TCA GCG CGA TGG GCA CAG GAG     296
Met Ser Ser Leu Gly Gly Pro Ala Ala Phe Ser Ala Arg Trp Ala Gln Glu      43

ATG TAC AAG AAA GAC AAT GGC AAA GAC CCA GCG GAA CCT GTA CTG CAT
Met Tyr Lys Lys Asp Asn Gly Lys Asp Pro Ala Glu Pro Val Leu His

CTG CCC CCT ATC CAG CCC CCC CCG GTG ATG CCT GGT CCC TTC TTC ATG CCC     395
Leu Pro Pro Ile Gln Pro Pro Pro Val Met Pro Gly Pro Phe Phe Met Pro      76

TCG GAC AGA TCC ACT GAG AGG TGC GAG ACC ATC CTG GAA GGG GAA ACC
Ser Asp Arg Ser Thr Glu Arg Cys Glu Thr Ile Leu Glu Gly Glu Thr

ATC TCC TGC TTC GTG GTG GGT GGG GAA AAG CGC CTT TGC TTG CCC CAG ATC     494
Ile Ser Cys Phe Val Val Gly Gly Glu Lys Arg Leu Cys Leu Pro Gln Ile     109

CTG AAC TCG GTG CTC AGG GAC TTC TCC CTG CAG CAG ATC AAT TCG GTG
Leu Asn Ser Val Leu Arg Asp Phe Ser Leu Gln Gln Ile Asn Ser Val

TGC GAT GAG CTA CAC ATT TAC TGC TCC AGA TGC ACC GCT GAC CAG CTG GAG     593
Cys Asp Glu Leu His Ile Tyr Cys Ser Arg Cys Thr Ala Asp Gln Leu Glu     142

ATC CTC AAA GTC ATG GGC ATC TTG CCC TTC TCT GCC CCC TCC TGC GGG
Ile Leu Lys Val Met Gly Ile Leu Pro Phe Ser Ala Pro Ser Cys Gly

CTG ATC ACT AAA ACT GAT GCT GAG AGG CTT TGC AAT GCC TTG CTT TAT GGT     692
Leu Ile Thr Lys Thr Asp Ala Glu Arg Leu Cys Asn Ala Leu Leu Tyr Gly     175

GGC ACC TAT CCT CCC CAC TGC AAG AAG GAA TTC TCT AGC ACG ATT GAG
Gly Thr Tyr Pro Pro His Cys Lys Lys Glu Phe Ser Ser Thr Ile Glu

CTG GAG CTT ACA GAG AAG AGC TTC AAG GTG TAC CAC GAG TGC TTT GGG AAG     791
Leu Glu Leu Thr Glu Lys Ser Phe Lys Val Tyr His Glu Cys Phe Gly Lys     208

TGT AAG GGA CTC CTG GTA CCA GAG CTT TAC AGT AAC CCC AGC GCA GCC
Cys Lys Gly Leu Leu Val Pro Glu Leu Tyr Ser Asn Pro Ser Ala Ala

TGT ATC CAG TGC TTG GAC TGC AGG CTC ATG TAC CCG CCT CAC AAA TTT GTG     890
Cys Ile Gln Cys Leu Asp Cys Arg Leu Met Tyr Pro Pro His Lys Phe Val     241

GTC CAC TCT CAC AAA TCC CTG GAA AAC AGG ACT TGC CAC TGG GGC TTT
Val His Ser His Lys Ser Leu Glu Asn Arg Thr Cys His Trp Gly Phe
```

FIG. 2

```
GAC TCT GCA AAC TGG AGG TCC TAC ATC CTC CTT AGC CAG GAT TAC ACT GGG      989
Asp Ser Ala Asn Trp Arg Ser Tyr Ile Leu Leu Ser Gln Asp Tyr Thr Gly      274

AAA GAG GAG AAA GCT AGG CTG GGC CAG CTC TTA GAT GAA ATG AAA GAA
Lys Glu Glu Lys Ala Arg Leu Gly Gln Leu Leu Asp Glu Met Lys Glu
                                                    ---> 2
AAA TTT GAC TAT AAC AAC AAA TAC AAG AGG AAA GCC CCC AGG AAC CGT GAG      1088
Lys Phe Asp Tyr Asn Asn Lys Tyr Lys Arg Lys Ala Pro Arg Asn Arg Glu      307

TCT CCT AGA GTT CAG CTC CGC CGG AAC AAA ATG TTC AAG ACA ATG CTG
Ser Pro Arg Val Gln Leu Arg Arg Asn Lys Met Phe Lys Thr Met Leu

TGG GAT CCA GCT GGA GGT TCA GCG GTA CTG CAG CGT CAG CCA GAT GGA AAT      1187
Trp Asp Pro Ala Gly Gly Ser Ala Val Leu Gln Arg Gln Pro Asp Gly Asn      340
          ---> 3
GAG GTC CCT TCA GAT CCT CCT GCT TCC AAG AAA ACC AAA ATA GAC GAC
Glu Val Pro Ser Asp Pro Pro Ala Ser Lys Lys Thr Lys Ile Asp Asp
                                                              C---
TCC GCT TCC CAA TCT CCA GCT TCT ACT GAG AAG GAA AAG CAG TCC AGT TGG      1286
Ser Ala Ser Gln Ser Pro Ala Ser Thr Glu Lys Glu Lys Gln Ser Ser Trp      373
                                                                Arg
                                        ---> 4
TTA CGG TCC TTA TCC AGT TCA TCT AAT AAG AGC ATT GGC TGT GTC CAT
Leu Arg Ser Leu Ser Ser Ser Ser Asn Lys Ser Ile Gly Cys Val His

CCC CGT CAG CGT CTC TCA GCT TTC CGG CCC TGG TCC CCT GCT GTA TCA GCA      1385
Pro Arg Gln Arg Leu Ser Ala Phe Arg Pro Trp Ser Pro Ala Val Ser Ala      406

AAT GAG AAA GAG CTC TCA ACC CAT CTT CCT GCA TTG ATC CGA GAC AGC
Asn Glu Lys Glu Leu Ser Thr His Leu Pro Ala Leu Ile Arg Asp Ser
                                                              ---> 5
AGT TTT TAC TCC TAC AAA AGC TTT GAG AAT GCT GTG GCC CCC AAC GTG GCA      1484
Ser Phe Tyr Ser Tyr Lys Ser Phe Glu Asn Ala Val Ala Pro Asn Val Ala      439

CTC GCA CCT CCT GCC CAA CAG AAA GTT GTG AGC AAC CCA CCC TGT GCC
Leu Ala Pro Pro Ala Gln Gln Lys Val Val Ser Asn Pro Pro Cys Ala
      Vski
ACA GTG GTG TCC CGG AGC AGC GAA CCG CCG AGC AGC GCT GCG CAG CCA CGG      1583
Thr Val Val Ser Arg Ser Ser Glu Pro Pro Ser Ser Ala Ala Gln Pro Arg      472

AAA AGA AAA CAT GCT GCA GAA ACC CCG GCT GTC CCA GAG CCA GTG CCC
Lys Arg Lys His Ala Ala Glu Thr Pro Ala Val Pro Glu Pro Val Ala

ACG GTT ACT GCC CCT GAA GAG GAT AAG GAA TCA GAA GCA GAA ATT GAA GTA      1682
Thr Val Thr Ala Pro Glu Glu Asp Lys Glu Ser Glu Ala Glu Ile Glu Val      505
                                                    ---> 6
GAG ACC AGG GAG GAA TTC ACC TCC TCC TTA TCC TCG CTC TCC TCC CCA
Glu Thr Arg Glu Glu Phe Thr Ser Ser Leu Ser Ser Leu Ser Ser Pro

TCC TTT ACT TCA TCC AGC TCT GCA AAG GAC ATG AGC TCA CCT GGG ATG CAA      1781
Ser Phe Thr Ser Ser Ser Ser Ala Lys Asp Met Ser Ser Pro Gly Met Gln      538

GCC CCA GTC CCA GTC AAC AGT TCA TAT GAG GTT GCA GCA CAT TCT GAC
Ala Pro Val Pro Val Asn Ser Ser Tyr Glu Val Ala Ala His Ser Asp
```

FIG. 2 (continued)

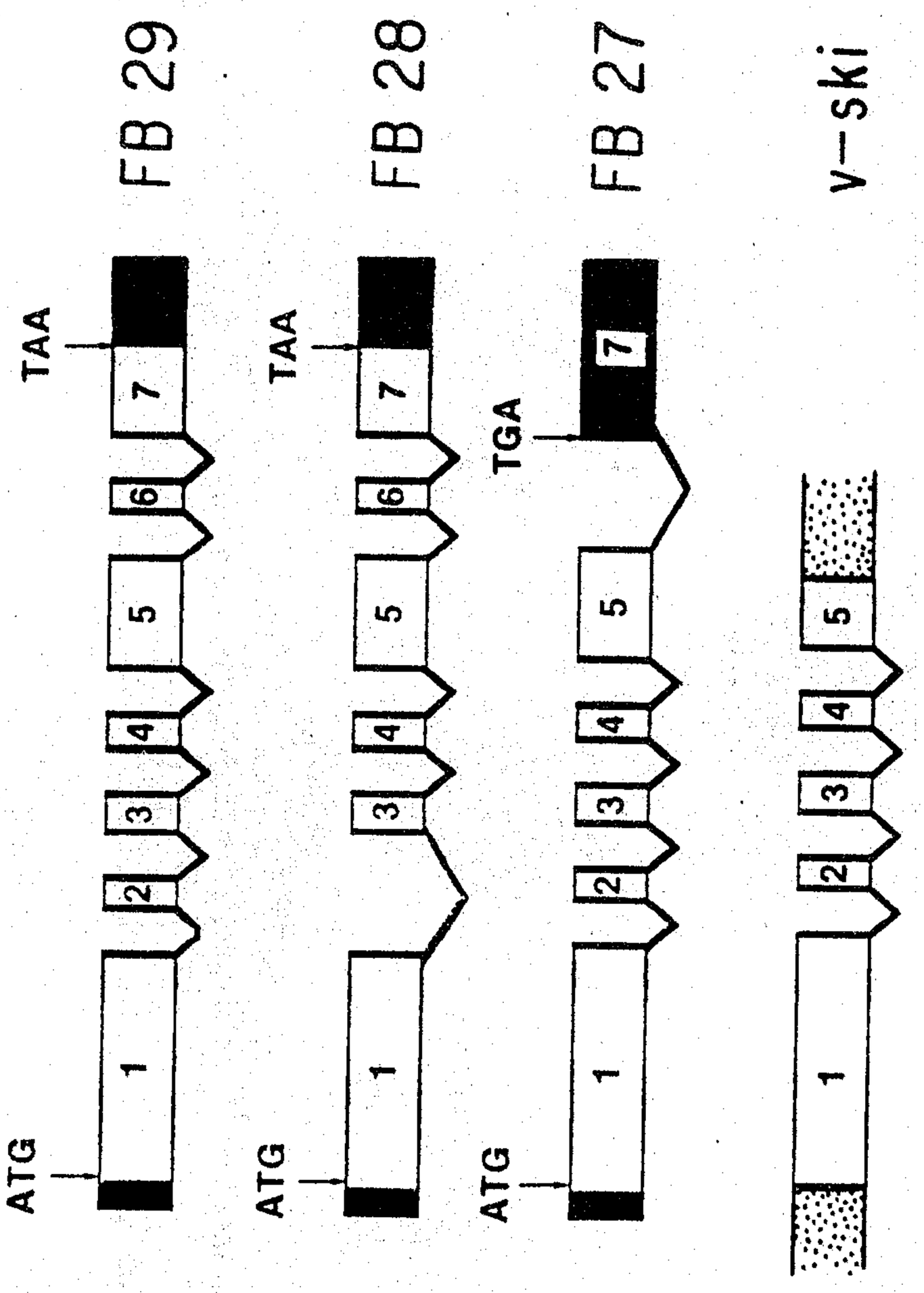
```
TCT CAC AGC AGT GGG TTG GAA GCT GAG CTG GAG CAC CTA AGG CAG GCC CTG      1880
Ser His Ser Ser Gly Leu Glu Ala Glu Leu Glu His Leu Arg Gln Ala Leu       571

GAC AGT GGC CTA GAT ACA AAA GAA GCC AAA GAA AAA TTC CTC CAT GAA
Asp Ser Gly Leu Asp Thr Lys Glu Ala Lys Glu Lys Phe Leu His Glu

GTT GTT AAA ATG AGA GTG AAG CAG GAA GAG AAG CTA AAT GCT GCC TTG CAA      1979
Val Val Lys Met Arg Val Lys Gln Glu Glu Lys Leu Asn Ala Ala Leu Gln       604

GCC AAA CGC AGC CTA CAT CAG GAG CTG GAG TTC CTC AGA GTG GCA AAG
Ala Lys Arg Ser Leu His Gln Glu Leu Glu Phe Leu Arg Val Ala Lys

AAG GAG AAA CTG AGA GAA GCA ACG GAG GCA AAA CGC AAC TTA AGG AAA GAG      2078
Lys Glu Lys Leu Arg Glu Ala Thr Glu Ala Lys Arg Asn Leu Arg Lys Glu       637

ATT GAG CGT CTG AGA GCT GAG AAT GAG AAG AAA ATG AAG GAA GCA AAC
Ile Glu Arg Leu Arg Ala Glu Asn Glu Lys Lys Met Lys Glu Ala Asn

GAG TCT CGG ATA CGG CTA AAG AGG GAA CTG GAA CAA GCC AGG CAG ATC CGG      2177
Glu Ser Arg Ile Arg Leu Lys Arg Glu Leu Glu Gln Ala Arg Gln Ile Arg       670

GTT TGC GAC AAG GGT TGT GAA GCT GGC AGG CTT CGG GCC AAG TAC TCT
Val Cys Asp Lys Gly Cys Glu Ala Gly Arg Leu Arg Ala Lys Tyr Ser

GCC CAG ATT GAG GAC CTA CAG GTT AAG CTT CAG CAT GCA GAG GCT GAC AGG      2276
Ala Gln Ile Glu Asp Leu Gln Val Lys Leu Gln His Ala Glu Ala Asp Arg       703

GAG CAG CTC CGA GCT GAC CTG ATG CAT GAG AGG GAG GCT CGA GAA CAC
Glu Gln Leu Arg Ala Asp Leu Met His Glu Arg Glu Ala Arg Glu His

TTG GAA AAA GTA GTC AAG GAA CTT CAG GAA CAG CTG TGG CCT AAA TCA AGC      2375
Leu Glu Lys Val Val Lys Glu Leu Gln Glu Gln Leu Trp Pro Lys Ser Ser       736

AGT CAA TCC AGC AGT GAA AAC ACA ACG AGC AAC ATG GAG AAT TAA ACC
Ser Gln Ser Ser Ser Glu Asn Thr Thr Ser Asn Met Glu Asn End

ACG TCG TCT AAT ACA ACA GAA TGA CAT ATA TGC ACA GTA AGG GAG GAT GGG      2474
                                                                          751

TGGGGTACGT GTGTAAGTGC ATGTGTGAGT AGTTGTGTCT TAACACACAG ATCTAGGAAT
ATGGATTCTT ATTAGTTGGA AGGCAAATGT TACTCTTTAT AACAGAAGCA CTGAATTACG      2594

CCTCTTTTTT TTTCCAATCC ATATAGCACA ACATCTTACT GTGCCTATAA AACACAAATG
TGTTTATAAA CAAAATACTT TTAAGTCCAC AGCAAATTTT CTACTGGCAA ACTCCAAGCA      2714

AGCAGCATCC TCCAACTAGA ATCAGAGTAA AAGGCAAGCA TGGCAGTGTT TTCATGTTGC
CCTTCTGCCT GTCGGAACAT TTTGGAATTT AAAAACAAAC TTTTCTTATA AGCTATTTAA      2834

AGTAATTCAT TACACAGACT TGGTATTAAA AAAAATTAAC AAGATTTTTT ATAACGAACC
TTTAAAAGCA AAACAAAAAC CTTCGATGCA CAATTTTTAC GACTTGTTAA AGGCTTTGGG      2954

ATTCTTACTG CAGAAGCCCT TTGGTGATGA TGCCATTTCA TTAGCAGTTT TTTTTAATCC
TGTCCTGTGG TTGTATGAGA ATTTCAGAGT GCTTTTCAAA GTTGATTTTT TTCCTTAGAA      3074
```

FIG. 2 (continued)

```
ACAATCACCT TCATTTCCTG TCCTGAACAC AAGAAGAAAG GAAGATGCAG GACTGTAAGG
GCGTGGGGGA GGGCAGGAAG AGAAGATGGA CGCTTTGGAA TTATAAACCC AGCCTTACAG      3194

ACTTCAGTGT TTCAAATCAC GCCATGTTTT CTAAAGACGT CTTCATTAAT CGATGTGTTC
AAAAGACTCA CTTCATCCAA GAGCACTTCA GCTTTAGGAA AAGAAAGAAG GAAGTAAAGG      3314

AAGGAAATGG ATGACCTGTT AAGTTGGTTG AGAAATAAAG CAGAAGATGT GTTTTGAAGT
CATTCTGAAA TCTTCGCGTC AGCTTTCAGT TCTCTGGAAA ACTCATCTTT GTTGCACCAT      3434

CTTACCATAG AATTCAGTAT TTACCTACTT CTATTCTGAA CTGTTTGTCA GGATTTCTGT
GCCCAAGGAG AGTGCAACAC CGCATTATTG GATACTACAG AAAAGAAAAA CCACGTTTTT      3554

GCTGCTGTGA ATAAGCCTAC ATCTTTTTTA AAAGAAAAAC TTCTGTTTTT AAGAATAGAA
ATTACTTTAA TTTTGGGATC CGAGCCGCAG CCCTGGAATA GAAATGCAGC CTACCATCAC      3674

TCTGTCTTAC TACCATTGTT AGCGTCGTCG TTCATTTTTT TTTAAACTGC ACTTTGTCAG
AACCTCACTC TGCATTTTAT TCCATATTTT GGAAGTTTAC AAGTTCAGCA TTCTCGATTC      3794

TGCTCTGCAG ATGTTAAAAT CATCACCACC ATTTTCCACC ACGCGACACC TCGGCCGTCA
TTTCCATGTA TGCAAAAGAA GAACTCAGTG GGTACAGAAT GCTACCAAAT ACAAAGGCAG      3914

CAGAGCAGCG TGCTGCTGGT TGGGTTTCAC AGCTGCGCTG CACGGCTGTG GCTGTCGAGG
CTGGGAAGTG CTCAAATACA GTTGGTGCTT TACTGAATGA GAGAGGAGTT ATTTTCACCC      4034

ACACACACTC ACCTCTGATA CACTCAAGCT CAGTGAAAAG TTGATCTGGG GCTGCAGTTG
TGCCTTCCAG CTCATTTTTC CTCTCAGCAT CTTCTATAGG CAATGCTGAC ACTTTTTTTT      4154

TAAACCTTAA AGAATAAAAA G   4175
```

FIG. 2 (continued)

FIG. 3



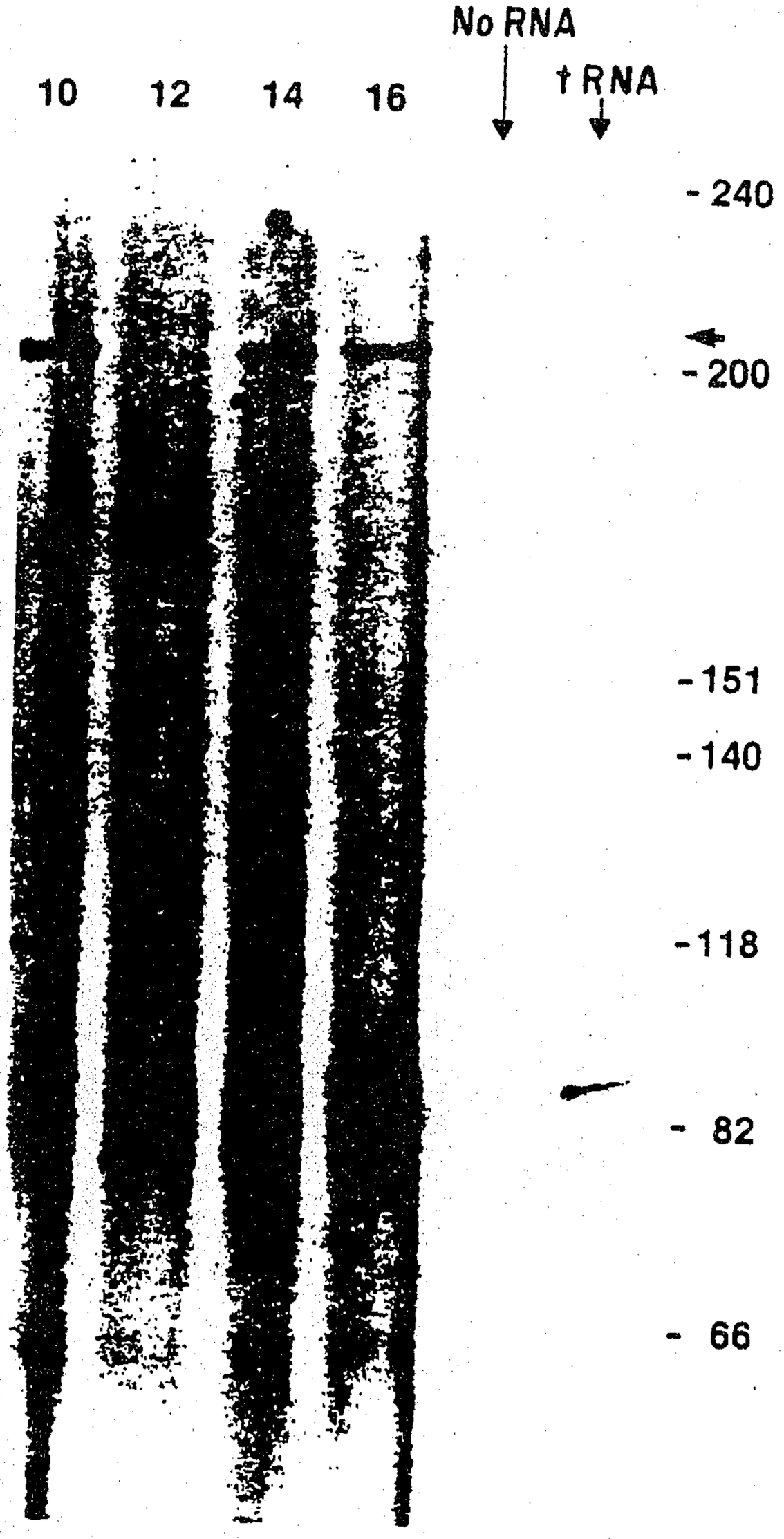


FIG. 4A

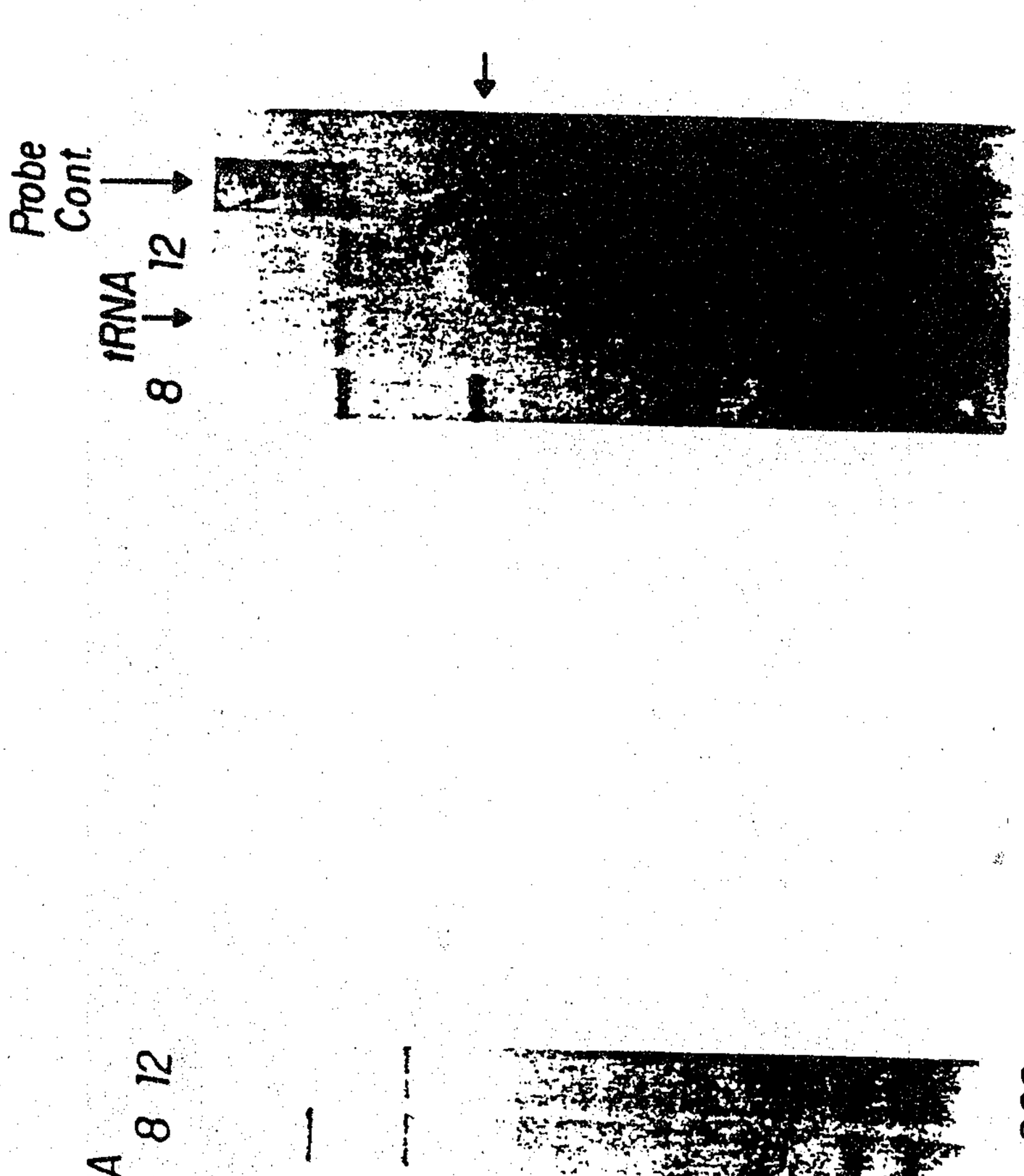

Probe
Cont
tRNA
8 12
851
534
272/262

FIG. 4C

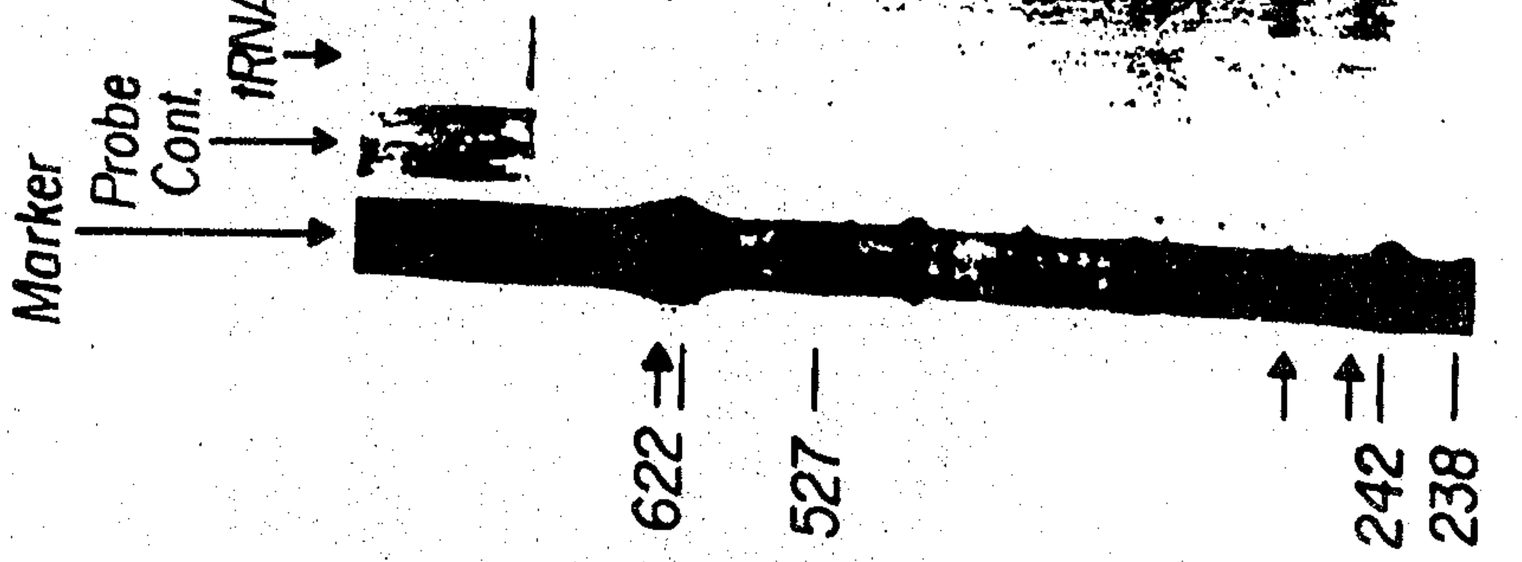

Marker
Probe
Cont.
tRNA
8 12
962
645
272/262
622
527
242
238

FIG. 4B

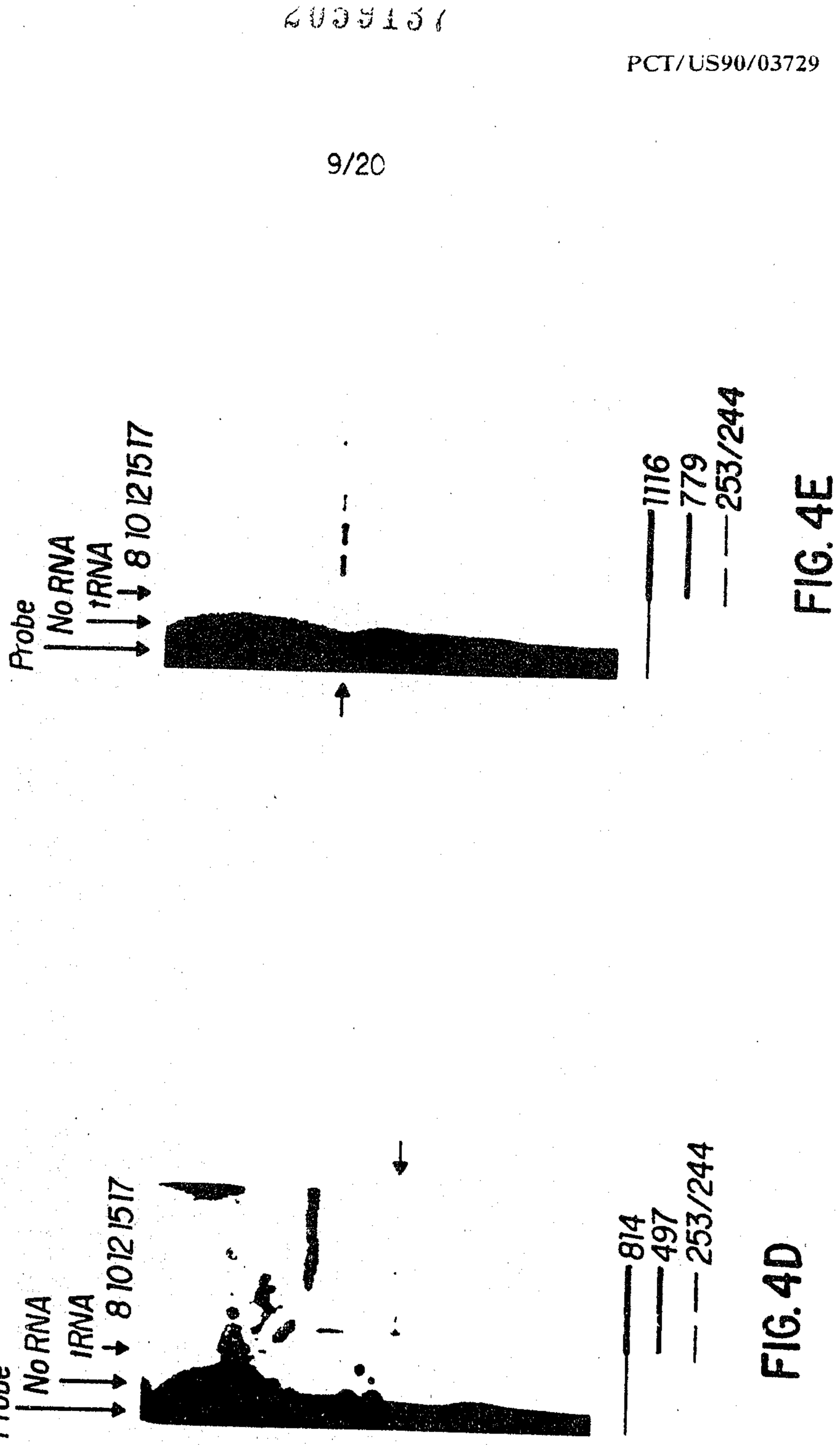

Probe
No RNA
tRNA
8 10 12 15 17
1116
779
253/244

FIG. 4E

Probe
No RNA
tRNA
8 10 12 15 17
814
497
253/244

FIG. 4D

```
ALV   GAGA GGAACAG G TT A CATCTGAGC AA
c-ski CCTT GGAACAG T TT - CATCTGAGC TC
```

FIG. 5

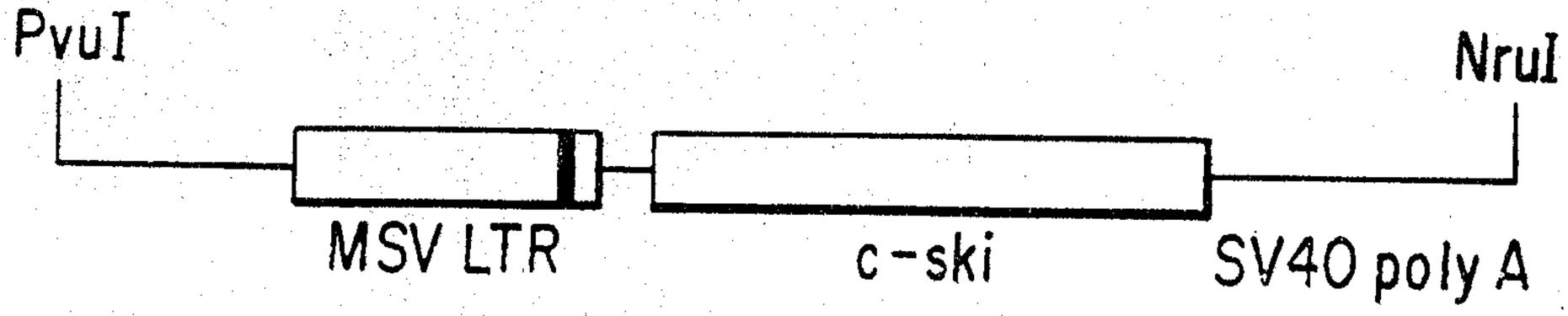


FIG. 6

FIG. 7

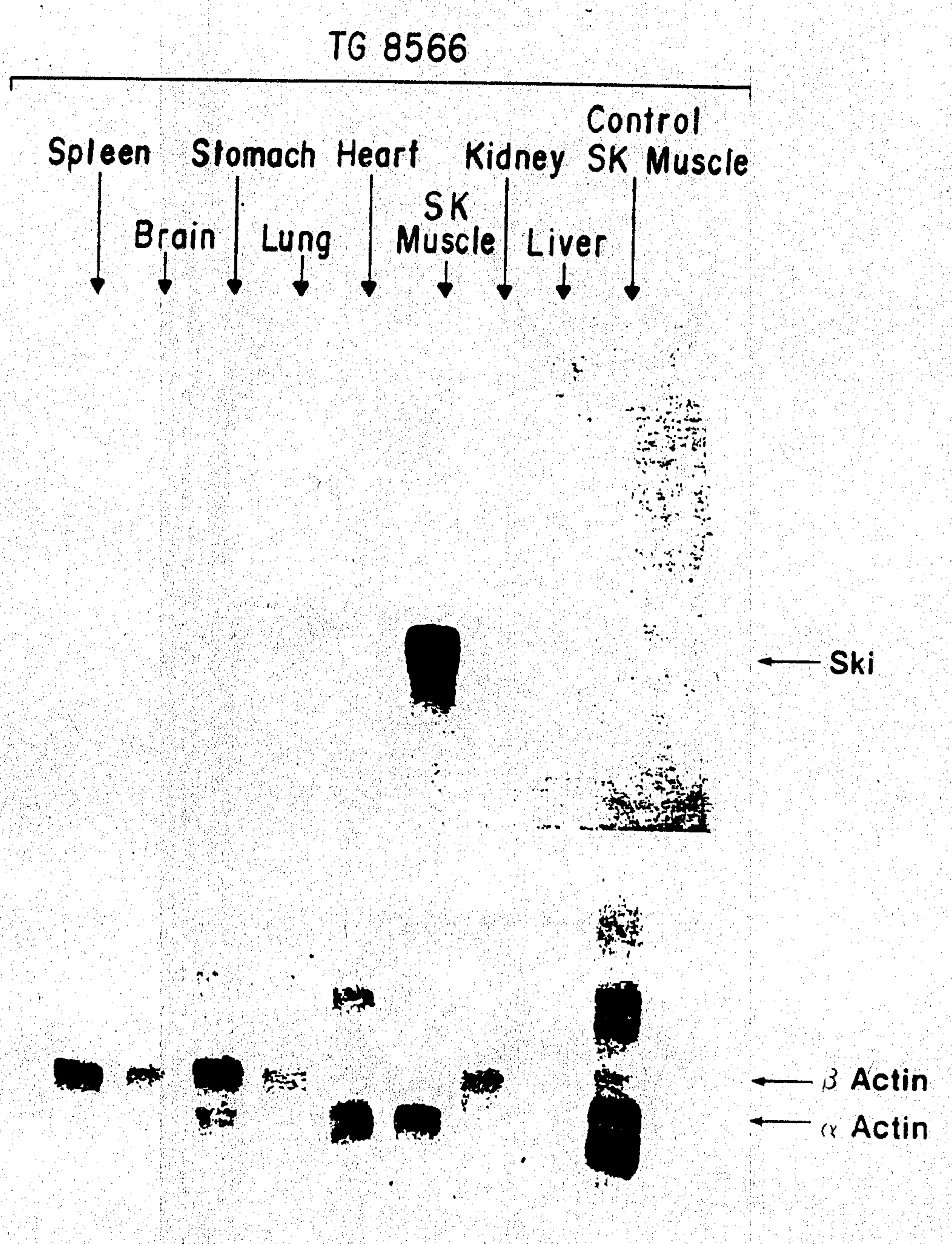

TG 8566
Spleen
Brain
Stomach
Lung
Heart
SK Muscle
Kidney
Liver
Control SK Muscle
Ski
β Actin
α Actin

FIG. 8A

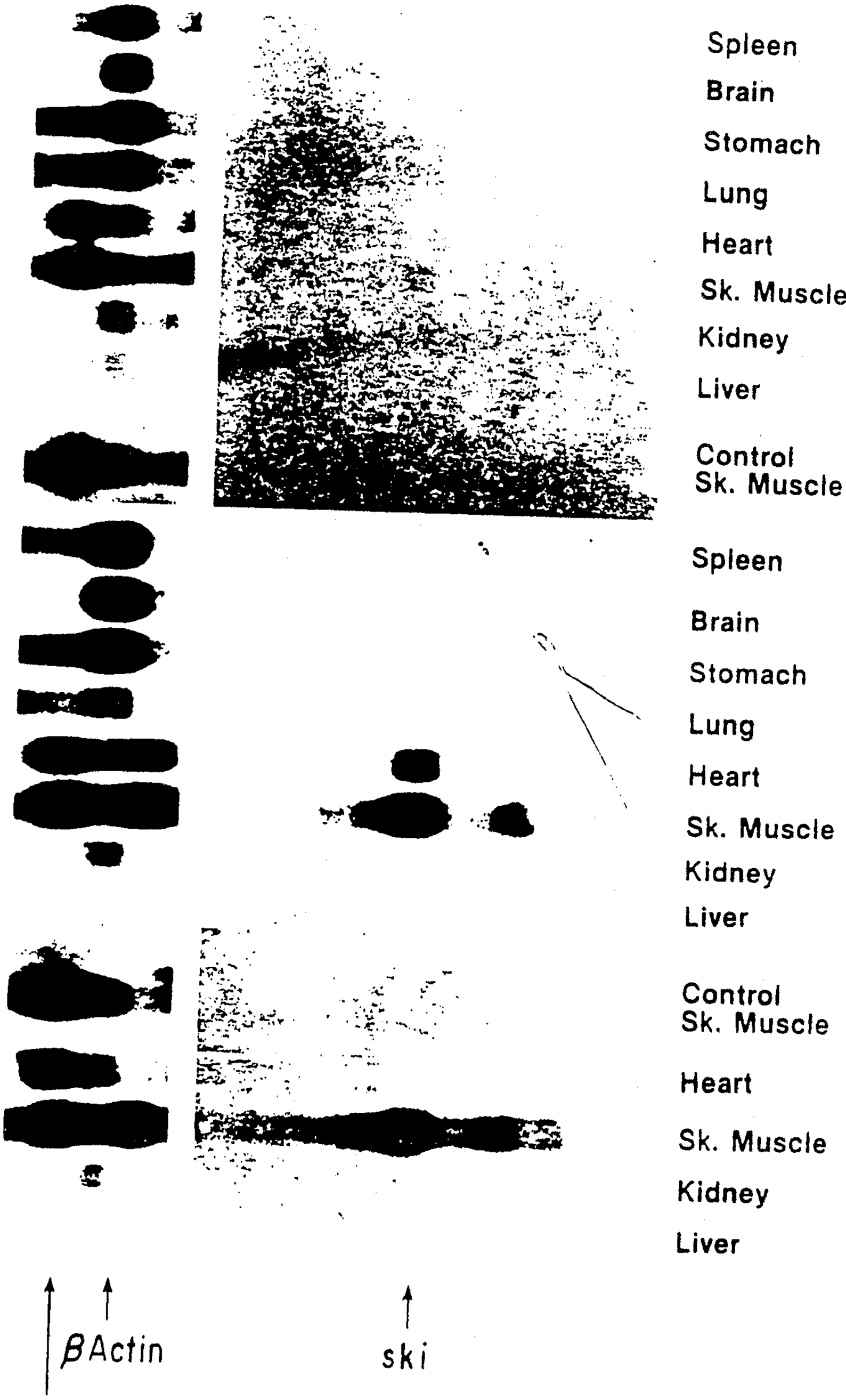

Spleen
Brain
Stomach
Lung
Heart
Sk. Muscle
Kidney
Liver
Control Sk. Muscle
TG 8542
Spleen
Brain
Stomach
Lung
Heart
Sk. Muscle
Kidney
Liver
TG 8821
Control Sk. Muscle
Heart
Sk. Muscle
Kidney
Liver
TG 8562
βActin
αActin
ski

FIG. 8B

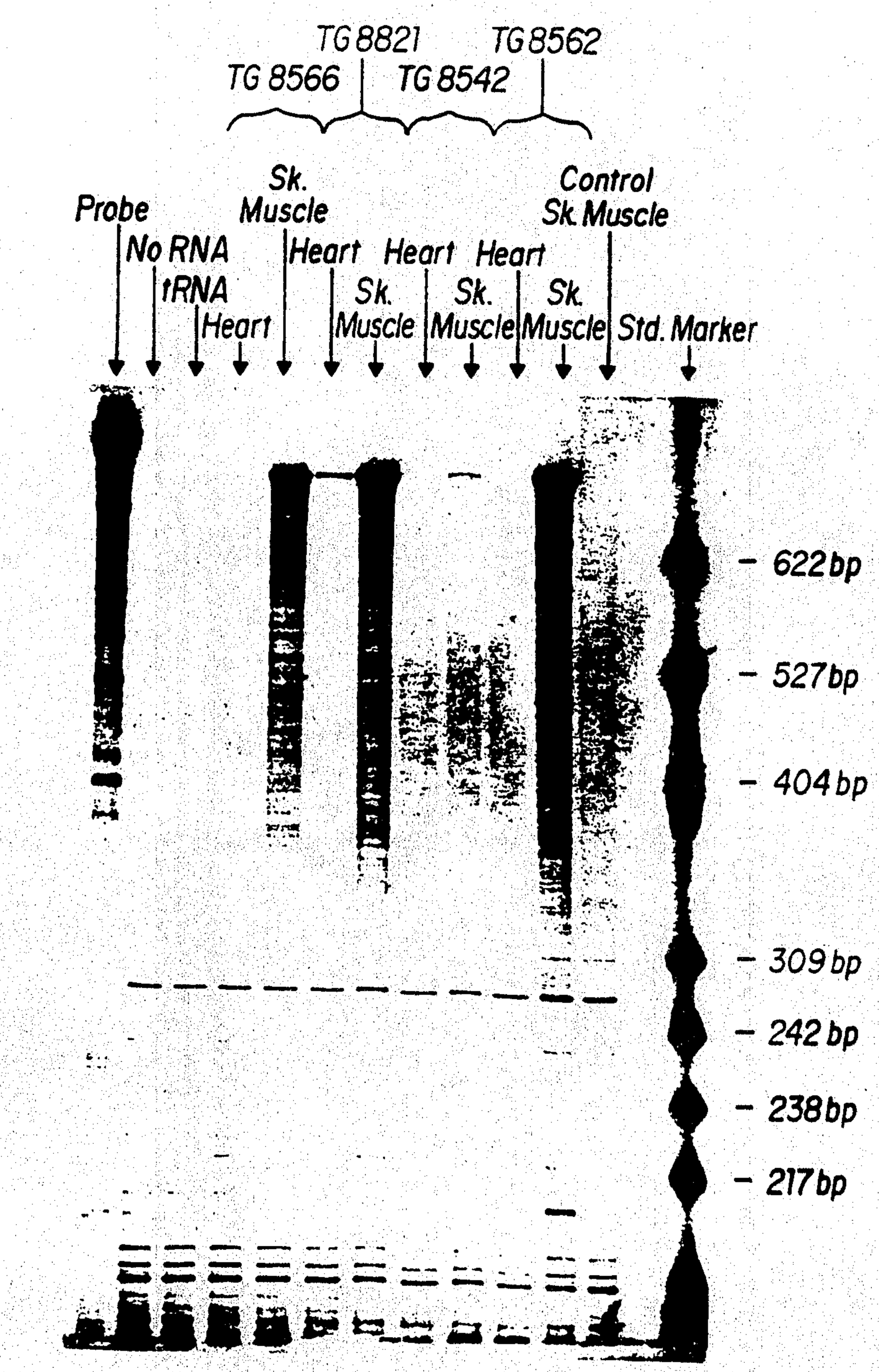

TG 8821
TG 8562
TG 8566
TG 8542
Probe
No RNA
tRNA
Heart
Sk. Muscle
Heart
Heart
Sk. Muscle
Heart
Sk. Muscle
Heart
Sk. Muscle
Control Sk. Muscle
Std. Marker
622 bp
527 bp
404 bp
309 bp
242 bp
238 bp
217 bp
SKI
1820 bp
T7 pol Transcript
984 bp
Protected Fragment

FIG. 9

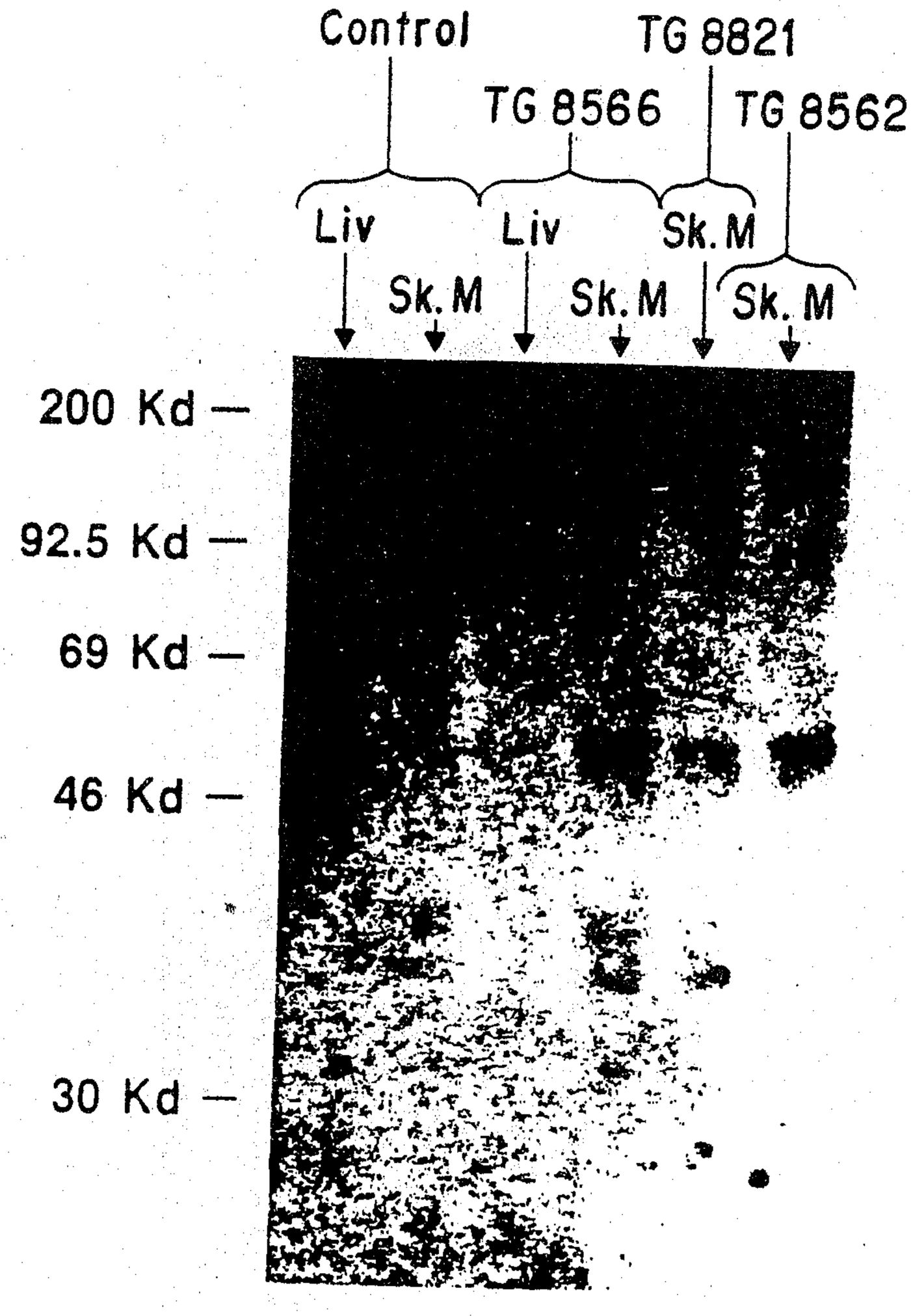

Control
TG 8821
TG 8566
TG 8562
Liv
Liv
Sk.M
Sk.M
Sk.M
Sk.M
Sk.M
200 Kd
92.5 Kd
69 Kd
46 Kd
30 Kd

FIG. 10

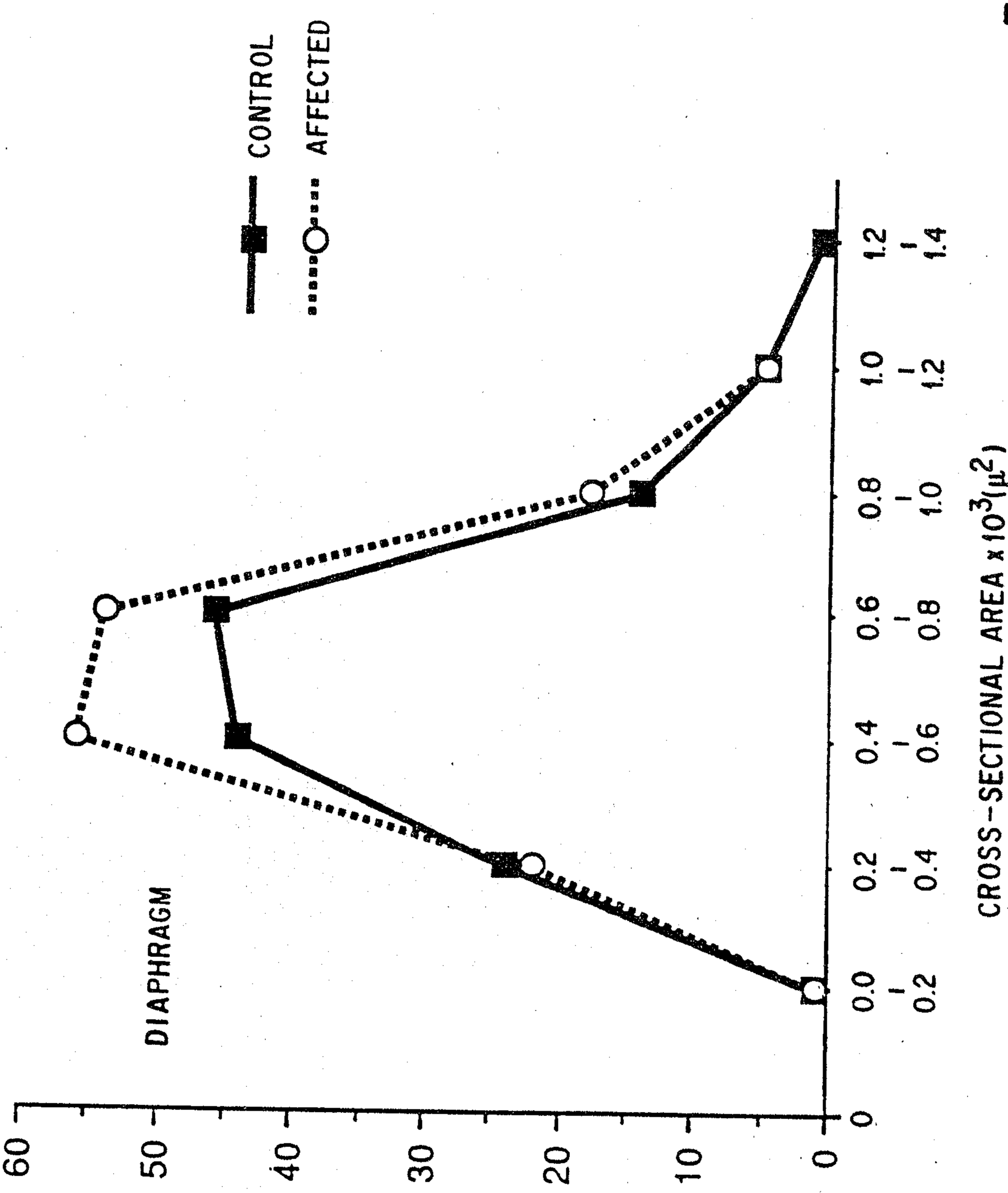
FIG. 11C

FIG. 11D

FIG. 11A

FIG. 11B

FIG. 12A


CONTROL
AFFECTED
DIAPHRAGM
OF FIBERS
60
50
40
30
20
10
0
0.0
0.2
0.2
0.4
0.4
0.6
0.6
0.8
0.8
1.0
1.0
1.2
1.2
1.4
CROSS-SECTIONAL AREA x $10^3(\mu^2)$

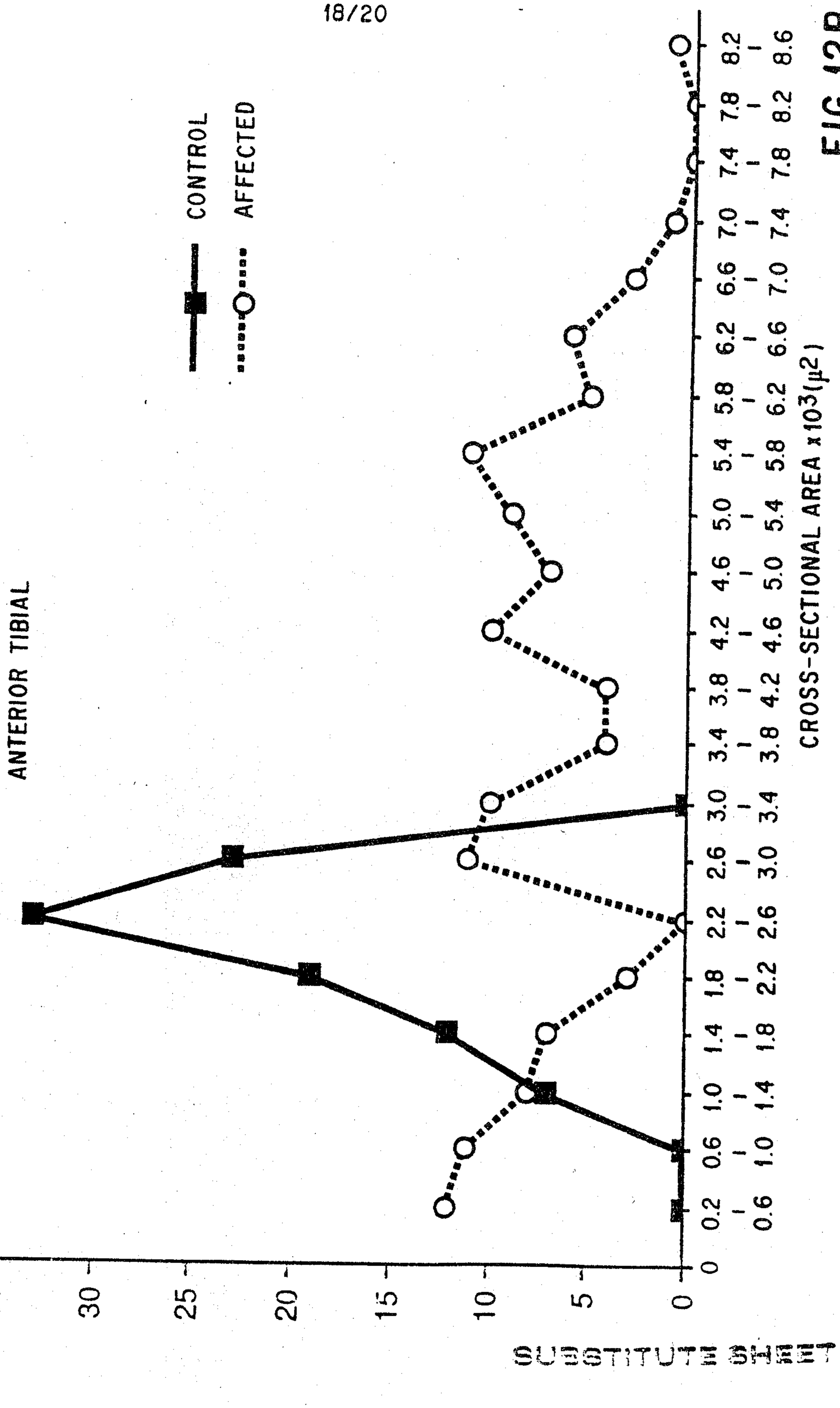

CONTROL
AFFECTED
ANTERIOR TIBIAL
OF FIBERS
CROSS-SECTIONAL AREA x10³(μ2)

FIG. 12B

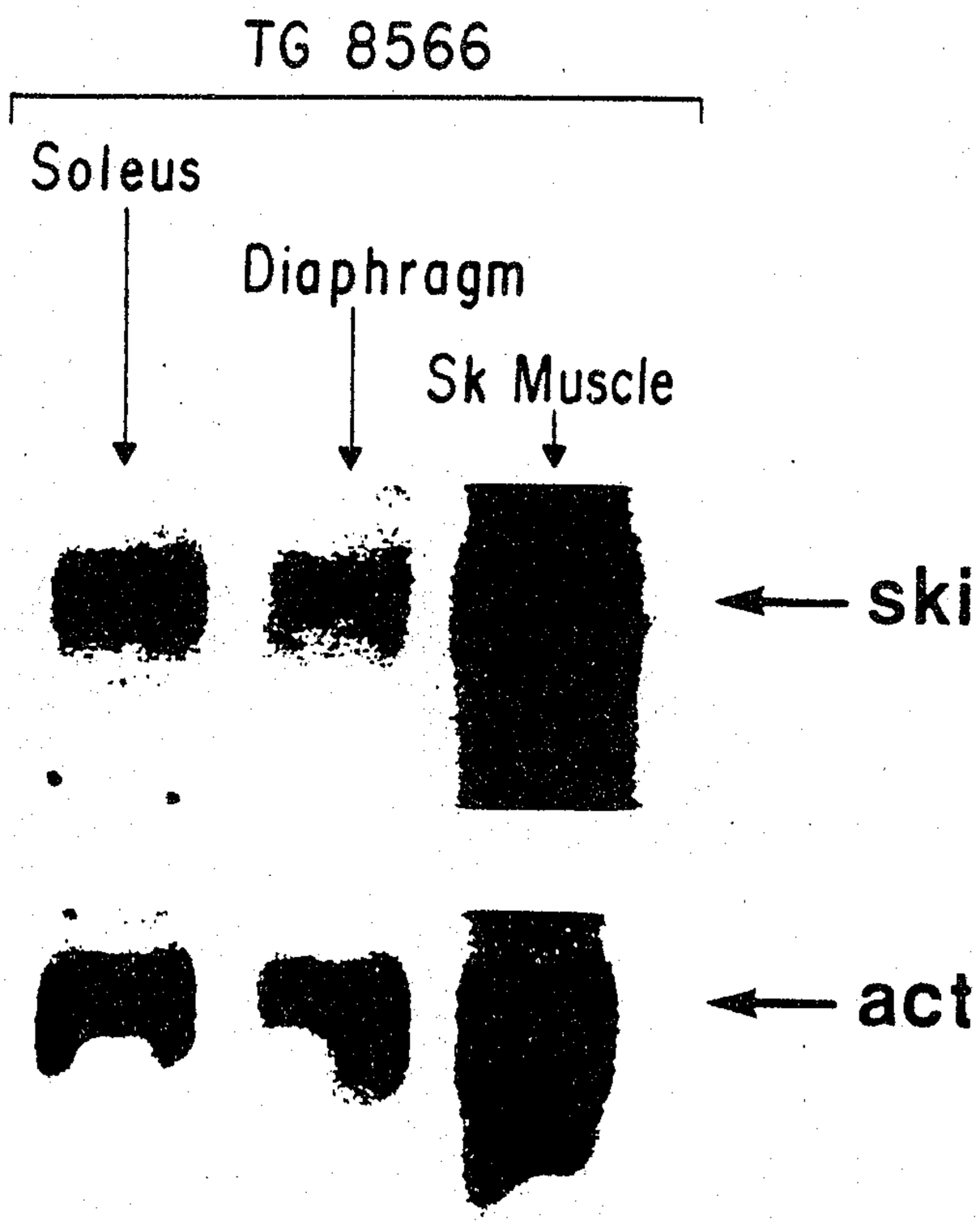


FIG. 13

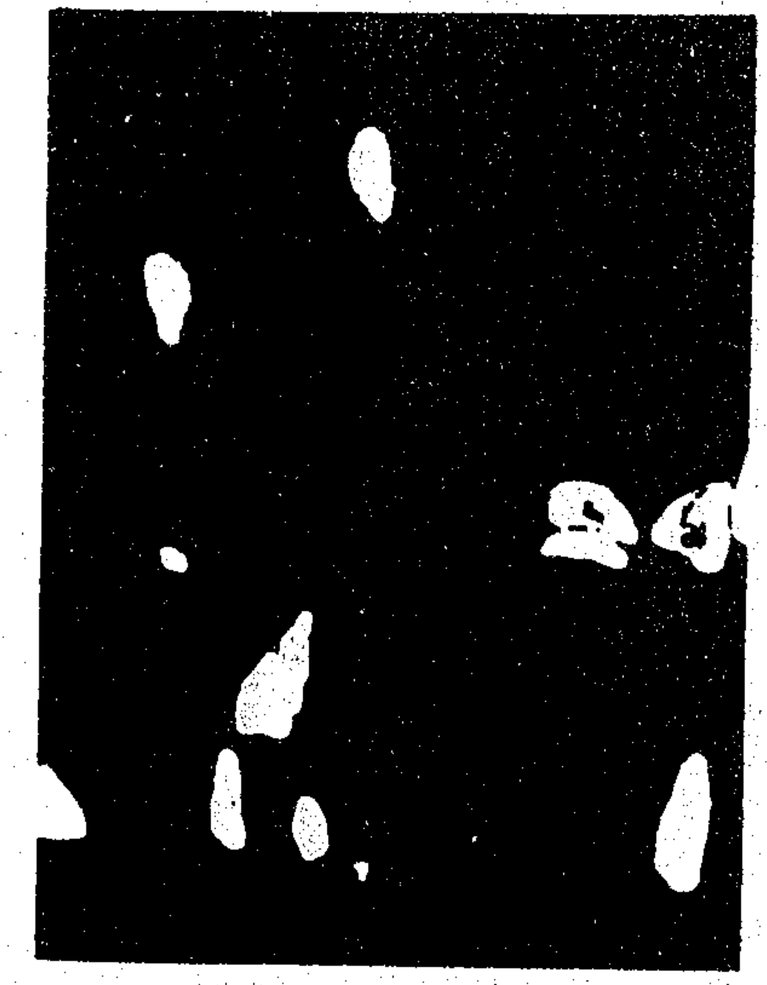


FIG. 14B

FIG. 14D

FIG. 14A

FIG. 14C