Chicken Nonmuscle Myosin Heavy Chains: Differential Expression of Two mRNAs and Evidence for Two Different Polypeptides

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Abstract. Two different mRNAs encoding two different nonmuscle myosin heavy chains (MHCs) of ~200 kD have been identified in chicken nonmuscle cells, in agreement with the results of Katsuragawa et al. (Katsuragawa, Y., M. Yanagisawa, A. Inoue, and T. Masaki. 1989. *Eur. J. Biochem.* 184:611–616). In this paper, we quantitate the content of mRNA encoding the two MHCs in a number of different tissues using RNA blot analysis with two specific oligonucleotide probes. Our results show that the relative content of mRNA encoding MHC-A and MHC-B differs in a tissue-dependent manner. Thus the ratio of mRNA encoding MHC-A versus MHC-B varies from >9:1 in spleen and intestinal epithelial cells, to 6:4 in kidney and 2:8 in brain. The effect of serum on MHC mRNA expression was studied in serum-starved cultures of chick embryo fibroblasts. Serum stimulation results in a threefold increase in the mRNA encoding MHC-A and a threefold decrease in mRNA encoding MHC-B.

Using SDS polyacrylamide gels, we have separated two nonmuscle MHC isoforms (198 and 196 kD) that can be distinguished from each other by two-dimensional peptide mapping of chymotryptic digests. We provide preliminary evidence that the MHC-A mRNA encodes the 196-kD polypeptide and that the MHC-B mRNA encodes the 198-kD polypeptide.

MYOSIN is a ubiquitous protein that demonstrates force-generating ATPase activity when it interacts with actin. Myosin is found in all eukaryotic cells and serves as an integral part of the contractile apparatus in muscle cells. Although best studied in muscle contraction, myosin in nonmuscle cells appears to be involved in diverse cellular motile processes (see reference 33 for review), including cytokinesis (7, 22), capping of surface receptors (10, 16, 28) and chemotaxis (10). In vertebrates, conventional myosin molecules consist of a pair of heavy chains (200 kD) and two pairs of light chains (15–28 kD). In addition to the 480-kD myosin molecules, *Acanthamoeba*, *Dictyostelium* (see references 1, 17 for review) and the brush border of vertebrate intestinal epithelial cells (6, 12) contain a smaller myosin-like molecule that preserves many of the structural and functional properties of the globular domain, but lacks the rod portion.

Whereas myosins from vertebrate muscle and nonmuscle cells share common subunit composition and native structure, sarcomeric (skeletal and cardiac muscle) and nonsarcomeric (smooth muscle and nonmuscle) myosins from different sources have distinct features (see references 31, 35 for review). Chemical, immunological, and molecular genetic studies have demonstrated multiple isoforms of the myosin heavy chain (MHC)[1] and suggest that sarcomeric and nonsarcomeric MHCs constitute a distinct family of isoforms (14, 27). Vertebrate sarcomeric MHC isoforms are known to be encoded by a highly conserved multigene family (27) and expression of sarcomeric MHC isoforms is regulated developmentally, hormonally and in a tissue-specific and muscle fiber type-specific manner (13, 18, 23, 36, 37, 39; see reference 34 for review).

On the other hand, the complexity of MHC isoforms found in vertebrate smooth muscle and nonmuscle cells and the factors regulating the expression of these MHC isoforms are less well understood. SDS-PAGE has provided evidence for two different MHC isoforms in smooth muscle cells (M_r = 204 and 200 kD) (4, 8) and isolation of cDNA (26) and genomic clones (2) indicates that these two MHC isoforms are generated by alternative splicing of mRNA. In addition, a developmental difference in the expression of these two MHC mRNAs in rabbit aorta has also been reported (19).

For vertebrate nonmuscle cells, the existence of multiple MHC isoforms was first suggested on the basis of chemical cleavage experiments (4). Recently, evidence for the presence of two vertebrate nonmuscle MHC genes was obtained by isolating two different cDNA clones (2.8 and 0.9 kb) which encode part of the chicken fibroblast MHC (14). Our laboratory has isolated cDNA clones encoding the entire amino acid sequence of a chicken intestinal epithelial cell MHC (32). The 0.9-kb clone published by Katsuragawa et al. (14) was included in this sequence, whereas the 2.8-kb cDNA clone showed differences throughout the sequence with 73% nucleotide identity and 79% amino acid identity.

1. *Abbreviation used in this paper*: MHC, myosin heavy chain.

To understand the biological significance of MHC isoforms in vertebrate nonmuscle cells, we studied the expression of two types of MHCs in a number of avian tissues at different stages of development and quantitated the relative content of mRNA encoding the MHC isoforms in a number of tissues. We also studied the effect of serum in altering the distribution of mRNA encoding the two different MHCs in cultured fibroblasts. In addition, we provide preliminary evidence that the two genes encoding the two nonmuscle MHC isoforms encode peptides that can be separated by SDS-PAGE.

Materials and Methods

DNA Probes

Two 45-mer single-stranded DNA oligonucleotides, 5'-GGC CTG GAA AGT CTC CAT CTC ACT CAG TCT ATT TTC TGC AGC TAG-3' (oligo A) and 5'-TTG ATG TTT CCT TTC CAT TTC CTC AAG TTC TGC TTC CAC TTT TGT-3' (oligo B) were synthesized on a DNA synthesizer (Biosearch Model 8700; Millipore Corp., Burlington, MA). Oligo A and B are complementary to the mRNA sequence for MHC-A and B, respectively. The location of the oligonucleotides in the cDNA clones is shown in Fig. 1. The oligonucleotides were labeled using T4 polynucleotide kinase (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) and [gamma-^{32}P]ATP (7,000 Ci/mmol; ICN Biomedicals, Inc., Irvine, CA).

RNA and DNA Blot Analyses

Chickens were killed in the laboratory and tissues were used immediately for RNA extraction. Intestinal epithelial cells were prepared as described previously (3). Total RNA was isolated from tissues or cultured cells by the acid guanidinium thiocyanate-phenol-chloroform extraction method (5). The concentration of RNA was determined by reading the A_{260} for each sample before gel electrophoresis. RNA was size-fractionated on a 0.9% agarose gel containing formaldehyde by electrophoresis, and capillary-transferred from the gel to Nytran membranes (Schleicher & Schuell, Inc., Keene, NH) (24) and cross-linked by UV irradiation using a UV Stratalinker 2400 (Stratagene Corp., La Jolla, CA). Gels were stained with ethidium bromide and photographed before transfer to ascertain that equal amounts of intact RNA was loaded.

To quantitate the amounts of mRNA encoding MHC-A and B, two 45-mer DNA oligonucleotides a and b, which are complementary to oligo A and B, respectively, were synthesized and used as standards. A series of known amounts of oligo a and b were applied to a Nytran membrane using a slot blot apparatus and crosslinked by UV irradiation. To calibrate individual autoradiograms of RNA blots probed with the two different oligonucleotides (oligo A and B), RNA blots and the standard oligonucleotide blots (a and b) were processed together. Blots were prehybridized in a solution containing 5× SSPE (1× SSPE = 0.149 M NaCl, 0.01 M $NaH_2PO_4 \cdot H_2O$, 1 mM EDTA, pH 7.4), 5× Denhardt's solution, 2% SDS, 100 μg/ml sonicated and heat-denatured herring sperm DNA for 2 h at 37°C and hybridized in the solution containing 40% formamide, 5× SSPE, 2.5× Denhardt's solution, 0.5% SDS, 10% dextran sulfate, 100 μg/ml sonicated and heat-denatured herring sperm DNA and [^{32}P]5'-end labeled oligonucleotides (5–10 × 10^8 cpm/μg, 2–4 × 10^7 cpm/ml) at 37°C. The final wash contained 0.3× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) plus 0.5% SDS at 57°C. The blots were exposed to Kodak XAR x-ray film for a period of 4–24 h at −70°C using intensifying screens and developed with an automatic processor (Kodak X-Omat M20 Processor, Eastman Kodak Company, Rochester, NY). Autoradiograms were scanned using a laser densitometer (LKB Utroscan XL) to quantitate the relative amounts of radioactivity. Only those bands that were in the linear response range of the film were used in quantitation.

Chicken genomic DNA was obtained from Clontech Lab., Inc. (Palo Alto, CA). 30 μg of genomic DNA was digested using the indicated restriction enzymes and the digests were size-fractionated by field inversion gel electrophoresis in a 0.7% agarose gel. DNA was transferred to a Nytran membrane (24) and probed as described above.

Cell Culture

Chicken embryonic fibroblasts were obtained from American Type Culture Collection (Rockville, MD) and maintained in Eagle's minimum essential medium with Earle's balanced salt solution and nonessential amino acids supplemented with 5% tryptose phosphate broth (basal medium) and 5% FBS (GIBCO BRL Life Technologies, Inc., Gaithersburg, MD). The cells were grown in 75-cm^2 flasks to confluence in the above medium. The medium was switched to the basal medium plus 0.5% FBS for 24 h and then the basal medium plus 1 mg/ml bovine albumin (Pentex-fatty acid-free fraction V; Miles Laboratories Inc., Naperville, IL) for another 24 h. The cells were then stimulated with 10% FBS. For RNA extraction, the cells were washed in PBS and a solution containing guanidinium thiocyanate (5) was added directly to the monolayer culture. Various inhibitors, such as cycloheximide (30 μg/ml), actinomycin D (3 μg/ml), and mitomycin C (0.2 μg/ml) were added to the medium 15 min before serum stimulation.

For [^{3}H]thymidine incorporation, cells were plated on 24-well plates and the medium changed as for RNA extraction. The cells were pulse-labeled for the last 1 h of the incubation period with [^{3}H]thymidine (0.4 μCi/ml, 6.7 Ci/mmol; New England Nuclear, Boston, MA). The cells were then washed with ice-cold PBS and [^{3}H]thymidine that was incorporated into DNA was precipitated by addition of 10% TCA and assayed for radioactivity using an aqueous counting scintillant in a scintillation counter.

Preparation of Tissue Extracts (Crude Actomyosin)

Tissue or isolated intestinal epithelial cells were homogenized in a buffer containing 40 mM MOPS-NaOH (pH 7.6), 0.6 M NaCl, 5 mM EDTA, 5 mM EGTA, 1 mM DTT, and 0.1 mM PMSF and centrifuged at 7,000 *g* for 10 min. ATP was added to the supernatant to a final concentration of 10 mM and centrifuged at 3,000 *g* for 20 min. The resulting supernatant (high salt-ATP extract) was fractionated by ammonium sulfate (30–60% saturation) in the presence of 10 mM $MgSO_4$ and 5 mM ATP and dialyzed against a buffer containing 40 mM MOPS-NaOH (pH 7.6), 0.6 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 0.1 mM PMSF. The high salt-ATP extracts or the 30–60% ammonium sulfate fraction were used for SDS-PAGE.

SDS-PAGE

Gel electrophoresis was performed in a 5% acrylamide gel with 0.065% bisacrylamide, using the buffer system of Laemmli (20). To resolve the nonmuscle MHCs, electrophoresis was carried out until bromophenol blue dye, loaded with the samples, reached the bottom of the gel. At this time another aliquot of dye was applied into the wells and electrophoresis was continued until the second dye front reached the bottom of the gel. Under these conditions, MHCs are resolved approximately one-third to one-fourth of the way from the bottom of the gel.

Two-dimensional Peptide Mapping of Radioiodinated Proteins

Radioiodination and the mapping of chymotryptic peptides of proteins recovered from polyacrylamide gels were carried out as described previously (15), except chymotrypsin (final concentration 100 μg/ml; Worthington Biochemical Corp., Freehold, NJ) was used in this study instead of trypsin.

Results

Specificity of the Probes

To study the expression of mRNA encoding MHC-A and MHC-B we synthesized two oligonucleotides (45 mers), oligo A and oligo B, which are complementary to the two different mRNAs in an area of relative sequence dissimilarity. Fig. 1 shows the location of the two probes with respect to the MHC. In the area selected, only 40% of the nucleotide sequence is identical as compared with 73% nucleotide identity when the published nucleotide sequence for MHC-B (14) is compared with MHC-A (32). The specificity of these oligonucleotides was tested using chicken genomic Southern blot analysis. As shown in Fig. 1 (*left*), oligo A hybridized to a single DNA fragment using three different restriction digests of genomic DNA. When the same blot was stripped and reprobed with oligo B, it also hybridized to a single, but different DNA fragment in each of the three lanes (Fig. 1,

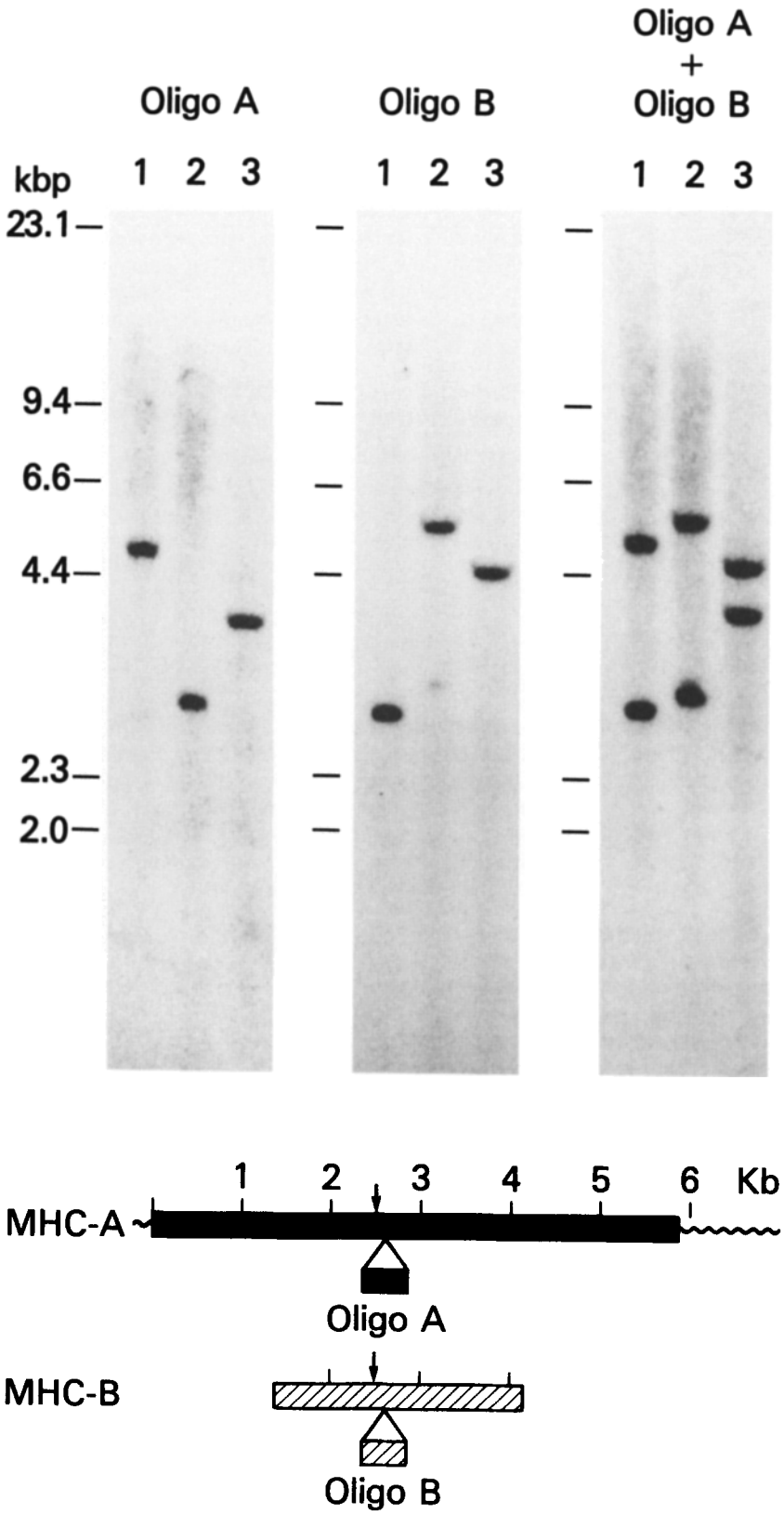

Figure 1. Southern blot analysis of chicken genomic DNA. Chicken genomic DNA (30 μg) was digested with Xba I (lanes *1*), Eco RI (lanes *2*) or Hind III (lanes *3*), electrophoresed in a 0.7% agarose gel and transferred to a nylon membrane. The same blot was hybridized in all three panels with oligo A (*left*), oligo B (*middle*), or both oligo A and B (*right*). The diagram below the autoradiograms depicts two different cDNAs for chicken nonmuscle MHCs and the location of the two oligonucleotide probes. MHC-A: cDNA isolated from intestinal epithelium library (32). Solid box indicates the coding region (5,877 nucleotides) and wavy line the untranslated regions. An arrow indicates the codon for the proline residue just before the helical rod region. Oligo A (45 mer) is complementary to the mRNA sequence from nucleotide 2,584 to 2,628 (where nucleotide 1 is A of the initiating methionine codon for MHC-A). MHC-B: cDNA isolated from a cultured fibroblast library (14). This clone corresponds to the sequence from nucleotide 1,384 to 4,156 of the MHC-A coding region. An arrow indicates the codon for the proline residue, as described above. Oligo B (45 mer) complements the mRNA encoding the same portion of the MHC-B molecule that Oligo A encodes in MHC-A.

middle). Fig. 1 (*right*) shows the same blot hybridized with both oligo A and oligo B to confirm that the two probes hybridize to distinct DNA fragments. These results show that oligo A and oligo B can be used to distinguish the two different genes and also confirm that the two MHCs are encoded by different loci in the chicken genome (14).

Cell Type- and Tissue-dependent Expression of Two MHC mRNAs

Expression of the two MHC mRNAs in cultured chicken fibroblasts and isolated intestinal epithelial cells was analyzed by Northern blots. Oligo A hybridized to a 7.3-kb mes-

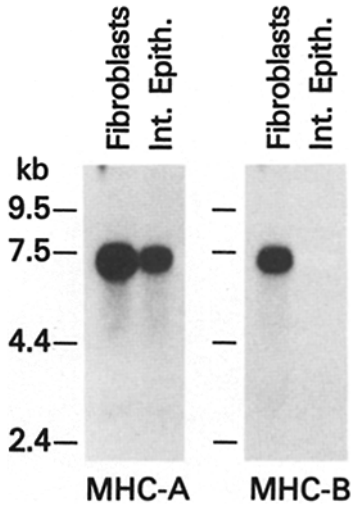

Figure 2. Differential expression of two nonmuscle MHC mRNAs. Each lane contains 30 μg of total RNA. The same blot was hybridized in both panels with oligo A (*left*) and oligo B (*right*). The blot hybridized with oligo B was exposed to x-ray film for approximately twice as long as that hybridized with oligo A. (*Int. Epith.*) Intestinal epithelial cells.

sage in both fibroblasts and epithelial cells (Fig. 2). In contrast, oligo B hybridized to mRNA isolated from fibroblasts, but not from epithelial cells (Fig. 2). The size of the message encoding MHC-B is also 7.3 kb. Thus, the mRNAs encoding MHC-A and MHC-B are the same size, but the expression of the two MHC mRNAs differs, depending on cell type.

Fig. 3 shows the expression of nonmuscle MHC mRNAs in a variety of chicken tissues and developmental stages (adult, newborn and 15 d-old embryo). The MHC-A mRNA is detected in all tissues examined, including muscle tissues. Fibroblasts, spleen and intestinal epithelium (lanes *5*, *8*, and *10*) contained relatively more of the mRNA encoding MHC-A. On the other hand, relatively more mRNA was detected with oligo B in aorta and brain (lanes *4* and *6*). The bands detected by the two oligos in muscle tissues are not due to cross-hybridization with skeletal, cardiac, or smooth muscle MHC mRNA since cDNA probes for the respective muscle MHCs hybridize to distinct bands of the expected smaller size (23, 38) on the same blots (data not shown). The relative distribution of mRNA encoding MHC-A and MHC-B in different nonmuscle tissues is similar throughout the developmental stages we analyzed, although more mRNA encoding MHC-B is detected in embryonic skeletal and gizzard smooth muscle compared to adult chicken muscle. Because of the relatively small amounts of mRNA encoding nonmuscle MHCs in skeletal and gizzard cells, and the possible contamination of these cells with nonmuscle cells, these experiments must be interpreted with caution.

Differential Effects of Serum on Expression of Two MHC mRNAs

The above data show that both MHC-A and B mRNAs are present in most tissues, including cultured fibroblasts. Next we studied whether the level of the two MHC mRNAs can be altered in cultured fibroblasts by extracellular signals. Chicken embryo fibroblasts were grown to confluence and starved in a serum-free medium. The quiescent cells were then stimulated with 10% FBS and RNA was extracted at various times for RNA blot analysis. The same blots or blots treated in an identical manner using the same preparation of mRNA were hybridized with oligo A and oligo B. Representative data are shown in Fig. 4 *A* and data from a number of sets of experiments are summarized as a graph in Fig. 4 *B* (*top two panels*). The expression of MHC-A mRNA began to increase one hour following serum stimulation and reached a peak at 6 h. Thereafter, the level declined, and by 26 h, it returned to the control value. In contrast, the level of MHC-B mRNA decreased 6–10 h after serum stimulation and, thereafter, returned to the control level. The MHC-A mRNA level was increased approximately threefold in stimulated cells as compared with resting cells, and the MHC-B mRNA was decreased by about one-third at 6 h. Chicken embryo fibroblasts enter the G_0/G_1 phase when they are deprived of serum. Addition of serum to such quiescent cells triggers an increase in the rate of initiation of DNA synthesis

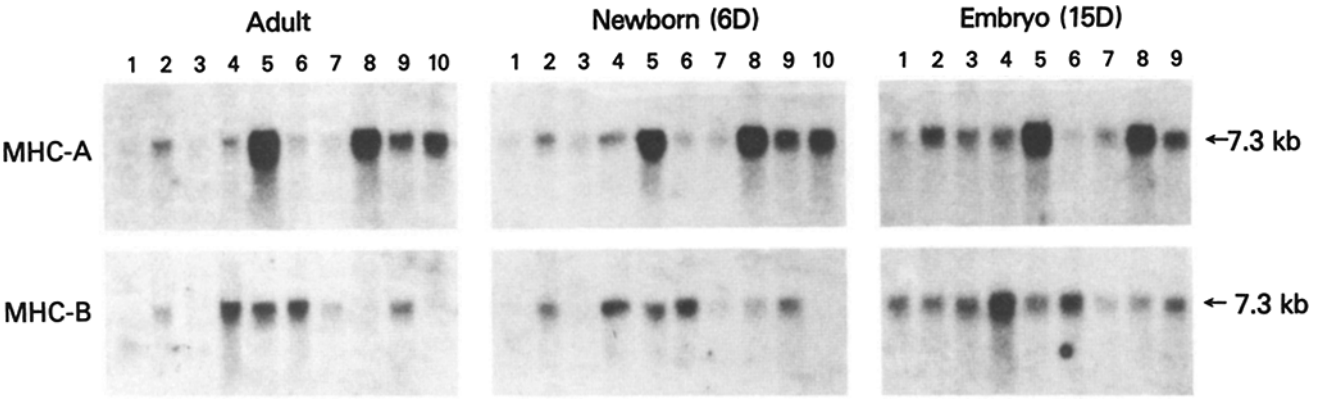

Figure 3. Tissue-dependent expression of two nonmuscle MHC mRNAs. Each lane contains 30 μg of total RNA from skeletal muscle (lanes *1*), cardiac ventricle (lanes *2*), gizzard smooth muscle (lanes *3*), aorta (lanes *4*), brain (lanes *6*), liver (lanes *7*), spleen (lanes *8*), kidney (lanes *9*), and intestinal epithelial cells (lanes *10*) from adult (*left*), 6-d-old newborn (*middle*), and 15-d-old embryo chickens (lane *10* is absent in the embryo panel). Lanes *5* of all three panels contains 30 μg of the same preparation of RNA from cultured embryo fibroblasts as a standard in order to allow a comparison of the three panels. The same RNA blots were probed with oligo A (*top*) or oligo B (*bottom*). Exposure time to x-ray film was about the same for all blots.

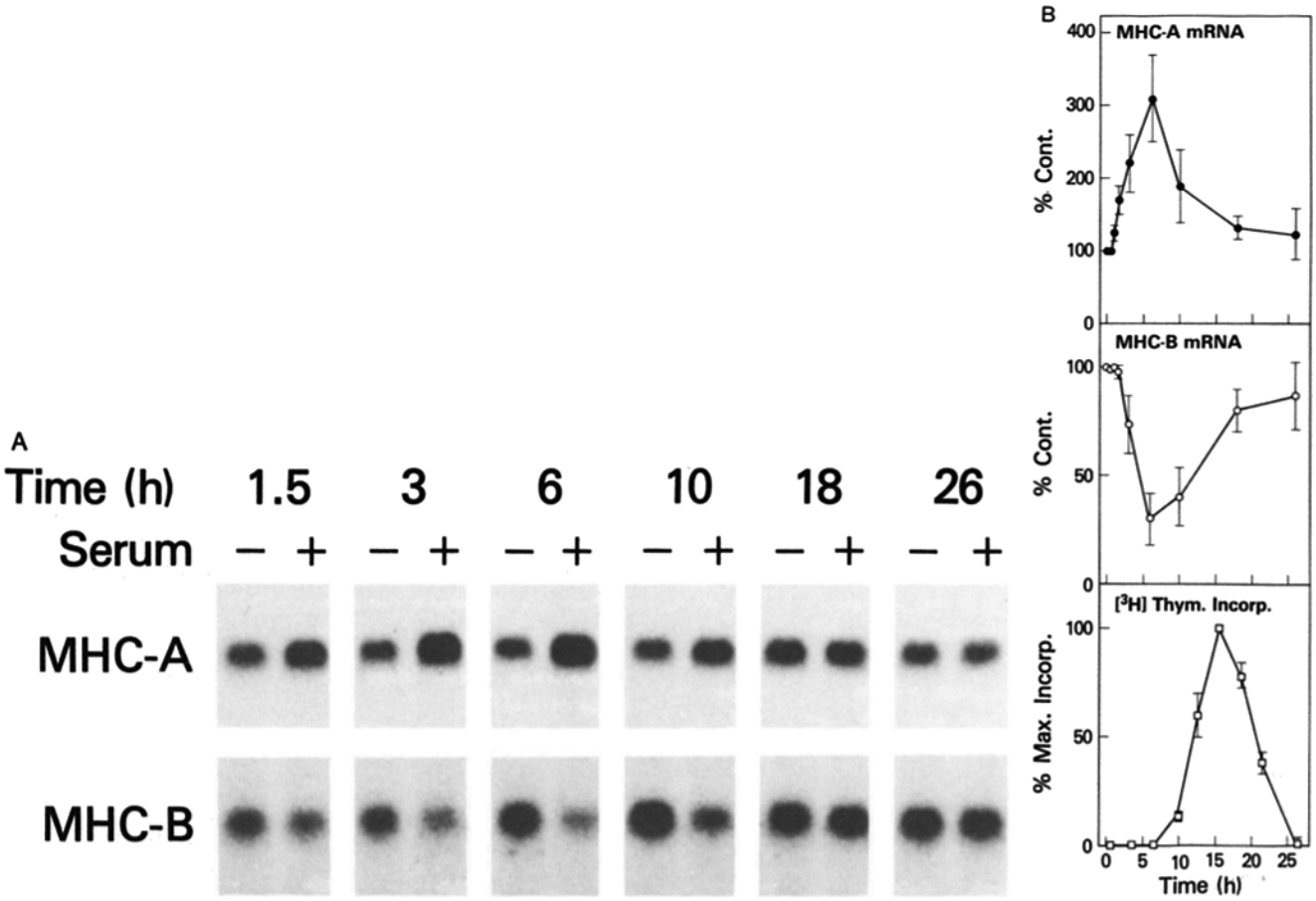

Figure 4. Effect of serum on nonmuscle MHC mRNA expression. (*A*) Chicken embryo fibroblasts were grown to confluence and starved in serum-free medium as described in Materials and Methods. The quiescent cells were then stimulated with 10% FBS (*serum +*) or not stimulated (*serum −*). RNA was extracted at each of the indicated time points. Each lane contains 30 μg of total RNA. The RNA blots were probed with oligo A (*top*) or oligo B (*bottom*). To obtain a signal for quantitative analysis by densitometry, the blots probed with oligo B were exposed for a longer time. (*B*) Expression of MHC-A mRNA (*top*) and MHC-B mRNA (*middle*). The autoradiograms of the RNA blots were analyzed densitometrically and the results are expressed as percent of the signal intensity measured in parallel cultures not stimulated with serum. The data shown are the mean ± SD, $n = 3$ except for the 6-h time point ($n = 6$). Incorporation of [^{3}H]thymidine (*bottom*). The cells were pulse-labeled for the last 1 h at each of the indicated time points. Results are expressed as percent of the maximum [^{3}H]thymidine incorporation obtained at the 15–16-h time period. The radioactivity detected in parallel cultures not stimulated with serum were subtracted from those in serum-stimulated cultures. The data shown are the mean ± SD, $n = 3$.

after a lag phase. As shown in Fig. 4 *B* (*bottom*), [^{3}H]thymidine incorporation starts after a 10-h lag period (this period represents the G_1 phase of the cell cycle) and reaches a maximum at 15 h after serum stimulation, under the given conditions. Thus, the maximum changes in both MHC-A and B mRNA expression occur during the G_1 phase.

Changes in mRNA expression can be affected by increased or decreased transcription or posttranscriptional changes affecting mRNA stability. Inhibitors of mRNA transcription or translation were used to determine the level of control. All inhibitors were added to the quiescent cultures 15 min before serum stimulation. The cultures were harvested 6 h later, and the purified RNA was analyzed by Northern blots. The serum-induced changes in the MHC-A and B mRNA levels were still seen in the presence of cycloheximide, an inhibitor of protein synthesis (Fig. 5). In contrast, actinomycin D, an inhibitor of transcription, abolished the serum-induced changes in both MHC-A and B mRNAs (Fig. 5). These results suggest that the changes in nonmuscle MHC mRNAs seen after serum stimulation is regulated, at least to some extent, at the transcriptional level. Additionally, we tested the effect of an inhibitor of DNA synthesis, mitomycin C (Fig. 5). Expression of neither gene was affected by mitomycin C, a finding consistent with the notion that changes in the expression of both genes is a prereplicative rather than a postreplicative event.

Quantitation of MHC mRNAs in Four Tissues

To quantitate the relative amounts of MHC-A and B mRNAs in four different tissues, we used oligonucleotides which are complementary to oligo A and B (i.e., the same strand as mRNA), as standards for MHC-A and B mRNAs (see Fig. 6 *A*). Known amounts of oligonucleotides were applied to a Nytran membrane using a slot blot apparatus, UV cross-linked and hybridized in the same hybridization solution used for RNA blots. Oligo A and B were labeled at the 5′ end phosphate using [gamma-^{32}P]ATP, so that both probes had

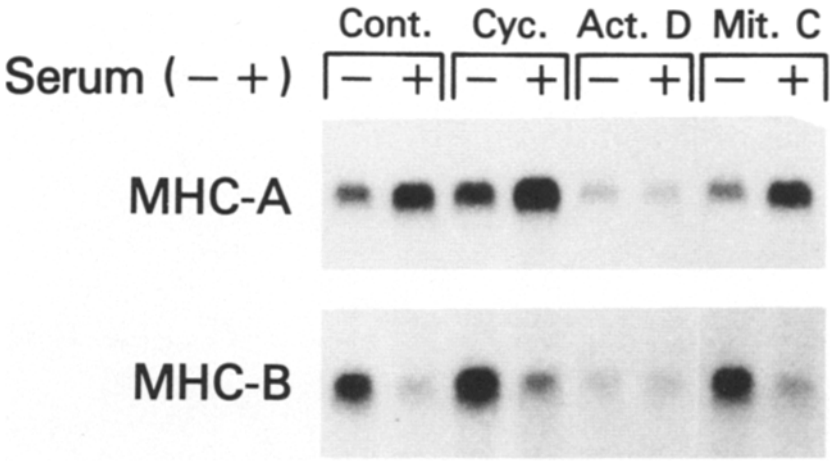

Figure 5. Effect of inhibitors on nonmuscle MHC mRNA expression. The quiescent chicken embryo fibroblasts were stimulated with 10% FBS (*serum +*) or unstimulated (*serum −*) in the presence of cycloheximide (30 μg/ml, *Cyc.*), actinomycin D (3 μg/ml, *Act. D*), mitomycin C (0.2 μg/ml, *Mit. C*) or no inhibitors (*Cont.*). RNA was extracted 6 h after serum stimulation. Each lane contains 20 μg of total RNA for probing with oligo A (*top*), and 40 μg (the same RNA preparation used for oligo A) for oligo B. The blot probed with oligo B was exposed for a longer time to obtain a signal of comparable intensity.

the same specific activity and RNA blots were then exposed along with the standard oligonucleotide blots, and individual autoradiograms were scanned. The intensity of the signals were calibrated, based on that of the standard blots (Fig. 6 *A*). Fig. 6 *B* shows the distribution of mRNA encoding MHC-A and B for four different tissues. The relative amounts of MHC-A and B mRNA are quite different, depending on the tissue. In intestinal epithelial cells and spleen, >95% of the mRNA is the A-form, whereas in brain >80% is the B form. Kidney contains roughly equal amounts of A and B mRNA. Fig. 6 *B* also indicates that the content of both MHC-A and B mRNAs is similar comparing intestinal epithelium, spleen and kidney, but the MHC mRNA content in brain is approximately one-third of the above tissues.

Separation of Two MHCs by SDS-PAGE in Four Tissues

In an attempt to distinguish the two nonmuscle MHC iso-

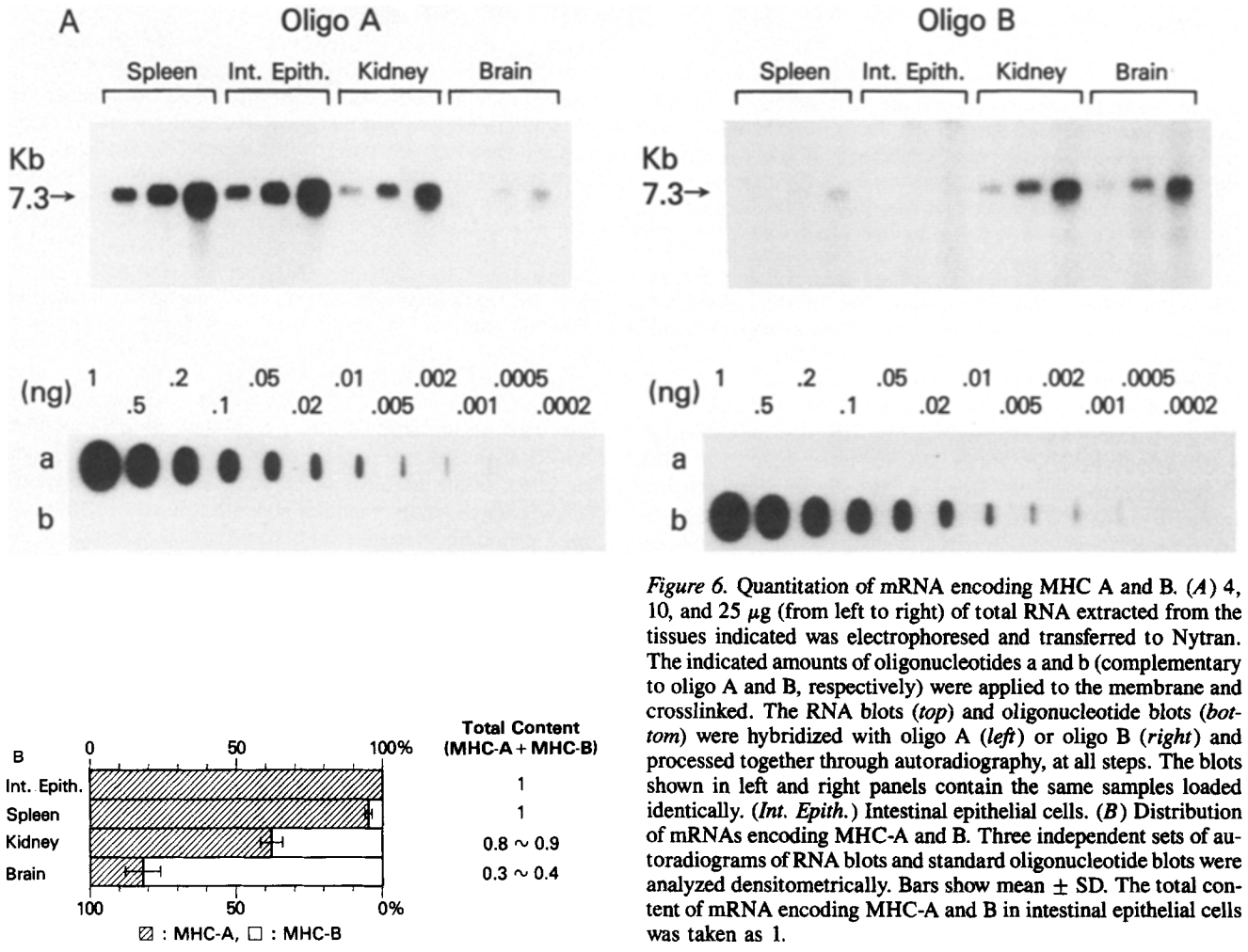

Figure 6. Quantitation of mRNA encoding MHC A and B. (*A*) 4, 10, and 25 μg (from left to right) of total RNA extracted from the tissues indicated was electrophoresed and transferred to Nytran. The indicated amounts of oligonucleotides a and b (complementary to oligo A and B, respectively) were applied to the membrane and crosslinked. The RNA blots (*top*) and oligonucleotide blots (*bottom*) were hybridized with oligo A (*left*) or oligo B (*right*) and processed together through autoradiography, at all steps. The blots shown in left and right panels contain the same samples loaded identically. (*Int. Epith.*) Intestinal epithelial cells. (*B*) Distribution of mRNAs encoding MHC-A and B. Three independent sets of autoradiograms of RNA blots and standard oligonucleotide blots were analyzed densitometrically. Bars show mean ± SD. The total content of mRNA encoding MHC-A and B in intestinal epithelial cells was taken as 1.

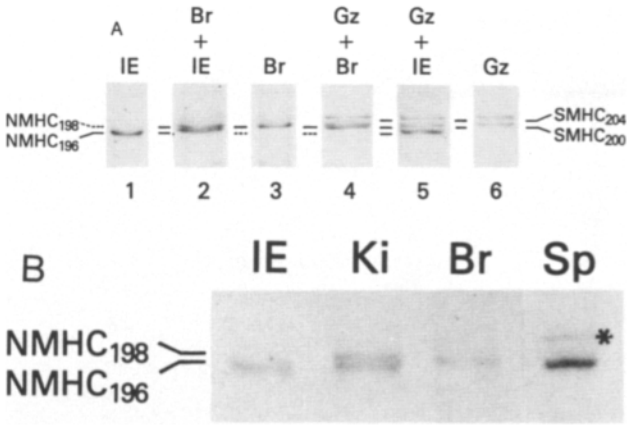

Figure 7. Separation of two nonmuscle MHCs by SDS-PAGE. (*A*) MHCs in tissue extracts from intestinal epithelial cells (*IE*, lane *1*), brain (*Br*, lane *3*) and gizzard smooth muscle (*Gz*, lane *6*) were separated by SDS-PAGE and the proteins were stained with Coomassie blue. Lane *2* contains a mixture of the extracts from intestinal epithelial cells and brain; lane *4*, brain and gizzard and lane *5*, intestinal epithelial cells and gizzard. Only the part of the gel that includes MHCs is shown. $NMHC_{196}$: 196-kD nonmuscle MHC; $NMHC_{198}$. 198-kD nonmuscle MHC; $SMHC_{200}$: 200-kD smooth muscle MHC; $SMHC_{204}$: 204-kD smooth muscle MHC. The broken line indicates that the MHC is absent from the panel to the right of the line. (*B*) MHCs in tissue extracts from intestinal epithelial cells (*IE*), kidney (*Ki*), brain (*Br*), and spleen (*Sp*) were separated by SDS-PAGE. The band labeled with an asterisk in the spleen lane is the 204-kD smooth muscle MHC.

forms at the protein level, we made use of low concentrations of acrylamide and bisacrylamide in SDS-PAGE. This gel system was originally used to separate the two smooth muscle MHC (204 and 200 kD) isoforms from a nonmuscle MHC isoform (196 kD) (15, 29), but we modified the running condition as described in Materials and Methods. In this system, the MHC extracted from intestinal epithelial cells migrates as a single band, as seen in Fig. 7 *A*, lane *1*. The MHC from chicken brain also migrates predominantly as a single band as detected by Coomassie blue, but slightly slower than the intestinal epithelial MHC (Fig. 7 *A*, lane *3*). The different migration of the epithelial cell and brain MHCs is confirmed by running them together (Fig. 7 *A*, lane *2*). We estimated the relative molecular mass of the intestinal epithelium and brain MHCs as 196 and 198 kD, respectively. Using the same gel system, the two MHCs of gizzard smooth muscle myosin also can be separated (M_r 204 and 200 kD) and both smooth muscle MHCs are seen to migrate slower than the two nonmuscle MHCs (Fig. 7 *A*, lane *6*). Coelectrophoresis of brain MHC or intestinal epithelium MHC with smooth muscle MHCs shows that brain and intestinal epithelium MHCs migrate faster than the two smooth muscle MHCs and clearly indicates that the distance between the intestinal epithelium MHC and the 200-kD smooth muscle MHC is greater than that between brain MHC and the 200-kD smooth muscle MHC (Fig. 7 *A*, lanes *4* and *5*).

Since intestinal epithelial cells contain mRNA encoding essentially only MHC-A and >80% of the brain mRNA encodes MHC-B, it is possible that the two polypeptides (196 and 198 kD) represent products of the two different mRNAs. Fig. 6 shows that kidney contains ~60% MHC-A mRNA and spleen contains >95% MHC-A mRNA. Therefore, we also analyzed the MHC peptides extracted from these tissues using the porous polyacrylamide gel. As expected, the MHCs from kidney resolved as a doublet which comigrates with both intestinal epithelium and brain MHCs, whereas the MHC from the spleen migrates with the same relative molecular mass as intestinal epithelium MHC (Fig. 7 *B*). The asterisk in the spleen lane indicates a band that comigrates with the 204-kD smooth muscle MHC. We confirmed the identity of this polypeptide by immunoblots, using an antibody that is specific for smooth muscle MHCs (15). Our results above suggest that MHC-A mRNA encodes the 196-kD polypeptide and that MHC-B mRNA encodes for the 198-kD polypeptide.

In the course of examining the migration of the MHCs from a number of different tissues and cell lines (including human cell lines), we were able to confirm that those cells and tissues expressing predominantly MHC-A mRNA encoded a polypeptide chain of 196 kD. We did, however, find one exception. Chicken embryonic fibroblasts expressed predominantly MHC-A mRNA (see Fig. 3), yet it encoded a polypeptide migrating more slowly than chicken intestinal epithelial cells (data not shown). We were able to confirm that the polypeptide chain was, in fact, MHC-A by using two-dimensional peptide mapping of iodinated MHCs which, as demonstrated below, can be used to distinguish between MHC-A and MHC-B.

To examine the differences in protein structure between the MHCs obtained from intestinal epithelial cells and brain, we prepared chymotryptic peptide maps of iodinated MHCs following separation of MHCs by SDS-PAGE. The peptide maps prepared from the 196- (intestinal epithelium) and 198-kD (brain) MHCs show a large number of radioactive spots in common, but they also demonstrate that there is a well-defined and reproducible difference as noted by the arrows (Fig. 8, *A* and *B*). Fig. 8 *C* shows a map of an equal mixture of intestinal epithelium and brain chymotryptic peptides, and confirms the differences indicated in Fig. 8, *A* and *B*. Fig. 8 *D* is a map of the chymotryptic peptides obtained from the digestion of the kidney MHCs. As shown in Fig. 7 *B*, kidney contains approximately equal amounts of both the 198- and 196-kD MHC. As expected, the map of the iodinated kidney MHC peptides resembles the map obtained by mixing equal amounts of the intestinal epithelium and brain peptides (compare Fig. 8, *C* and *D*). These results show that the two nonmuscle MHCs are similar, but not identical, and support the idea that intestinal epithelium and brain MHCs, which can be separated by SDS-PAGE, differ in their primary structure.

Discussion

In this study, we demonstrate the tissue- and cell type-specific expression of two nonmuscle MHC mRNAs and the effect of serum stimulation on the expression of the two MHC mRNAs in cultured fibroblasts. Although some of our results agree with those of Katsuragawa et al. (14), there are important differences between the two studies. First of all, to quantitate the distribution of mRNA encoding the two MHC isoforms, we used oligonucleotide probes that were labeled to the same specific activities. Each oligonucleotide probe was shown to be specific for a particular MHC since it hybridized to a single band in a genomic Southern blot

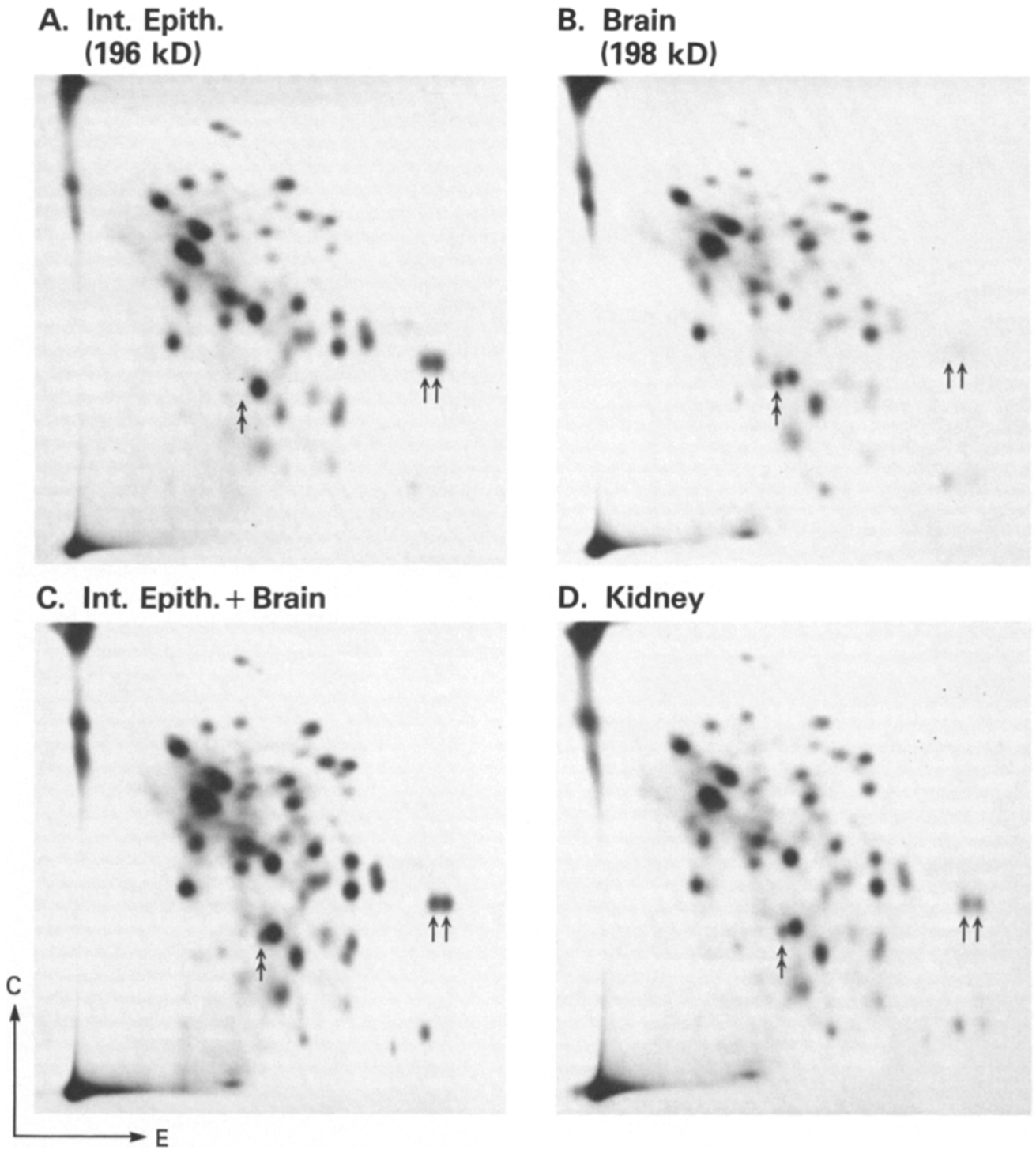

Figure 8. Comparison of two-dimensional chymotryptic peptide maps of iodinated MHCs. (*A*) 196-kD nonmuscle MHC obtained from intestinal epithelial cells. (*B*) 198-kD nonmuscle MHC obtained from brain. (*C*) A mixture of equal amounts (based on measurement of ^{125}I radioactivity) of intestinal epithelial cell and brain nonmuscle MHCs. (*D*) Kidney MHCs which contain both 196- and 198-kD MHCs (see Fig. 7 *B*). The appropriate MHC(s) was cut from an SDS-5% PAGE, labeled with ^{125}I, and digested with chymotrypsin. Samples were applied to silica gel plates and subjected to electrophoresis (*E*) and ascending chromatography (*C*) and autoradiography as previously described (15). Single head arrows point to peptides unique to intestinal epithelial cell MHCs. Double headed arrow points to a peptide unique to the brain MHC. (*Int. Epith.*) Intestinal epithelial.

(Fig. 1) and also hybridized to its complement of nucleotides by a factor of $>10^4$ than it did to a complement of the other oligonucleotide probe (Fig. 6 *A*). In contrast, Katsuragawa et al. employed cDNA probes from the coding region that were labeled by random priming. Quantitative analysis of our Northern blots showed that spleen and intestinal epithelial cells are particularly enriched for mRNA encoding MHC-A whereas brain is enriched in mRNA encoding

MHC-B. Kidney, on the other hand, contains approximately equal amounts of both mRNAs (Fig. 6).

Our results extend those reported by Katsuragawa et al. (14) by including newborn (6 d) as well as adult and embryo tissues. In addition, we included a number of different nonmuscle tissues in our analysis of embryonic tissues, whereas they only analyzed muscle and brain. They found that embryonic tissue contains significantly more mRNA encoding MHC-B than adult, whereas our studies show that, with the exception of skeletal and gizzard smooth muscle cells, for any given tissue there is no major change in this distribution comparing embryonic, newborn and adult. Rather, the expression of the two nonmuscle MHC mRNAs is dependent on the tissue or cell type. Thus, although the sarcomeric forms of the contractile proteins are expressed in a developmental and tissue-specific manner (18, 23, 34, 36, 37), we find that the two nonmuscle MHC genes are expressed at similar levels throughout different developmental stages.

The presence of two different MHC isoforms in vertebrate nonmuscle cells raises questions about whether these myosins serve specific functions. Although we cannot answer this question directly, the ability of serum to alter MHC mRNA expression is of note (Fig. 4). Previous work has demonstrated that serum contains a number of factors capable of altering the expression of mRNA encoding a number of cytoskeletal proteins (9, 11, 25). In this paper, we show that stimulation of quiescent fibroblasts with serum results in an increase in mRNA encoding MHC-A and a decrease in mRNA encoding MHC-B. It is well established that the activation of certain genes is required for quiescent cells to respond to mitogens and proliferate. These genes include the proto-oncogene c-*fos* and c-*myc* (11, 21), and genes encoding cytoskeletal proteins such as nonmuscle actins (11, 25) and vimentin (9) and others, as yet unidentified (21). The rapid accumulation of MHC-A mRNA after serum stimulation, and the fact that this effect does not require de novo protein synthesis (Fig. 5), suggests that the increased expression of MHC-A mRNA and the concomitant decreased expressed of MHC-B mRNA is due to growth factor(s) present in the serum.

Our working hypothesis is that this alteration in MHC mRNA expression might also be necessary for cell proliferation. In addition to the experiments cited above (see Fig. 4), we have also observed a similar phenomenon with smooth muscle cells that are placed in culture. Previous experiments by us (15), as well as others (29), demonstrated that when smooth muscle cells are placed into primary culture, the smooth muscle MHC isoforms (204 and 200 kD) are replaced by nonmuscle isoforms, particularly during log-phase growth. Experiments similar to those shown here for chicken embryonic fibroblasts show that proliferating smooth muscle cells contain a high proportion of the mRNA encoding MHC-A (Kawamoto, S., unpublished observation). This is in contrast to the mRNA content of intact vascular tissue that is enriched for mRNA encoding MHC-B (see Fig. 3). Although it is tempting to postulate, on the basis of these experiments, a specific function for MHC-A in cellular proliferation, for example, a role in cytokinesis (7, 22), it must be pointed out that the serum-induced changes in MHC mRNA described here appear to be transient and occur before DNA replication. Thus, the exact function of the myosin isoforms remains to be determined.

The presence of two different mRNAs encoding nonmuscle MHCs suggests the existence of two different protein isoforms. Using SDS-PAGE, we were able to separate two MHC species with different M_rs (196 and 198 kD). Our data suggest that MHC-B mRNA encodes the 198-kD MHC and MHC-A mRNA encodes the 196-kD MHC, but we have not yet demonstrated this directly. Moreover, we cannot rule out the presence of additional MHC isoforms, although we have failed to detect their presence to date screening multiple libraries and probing genomic Southern blots at low stringency. The existence of additional, smaller forms of the MHC (e.g., 110 kD) similar to those found in intestinal brush border cells (6, 12), is also not precluded by these studies.

Two-dimensional chymotryptic peptide maps prepared from 196 kD and 198 kD MHCs show a number of distinct radioactive peptides with many common peptides (Fig. 8). In the procedure used for this study, mostly tyrosine residues are iodinated, and other amino acid residues, such as histidine, cysteine, tryptophan, and methionine are labeled to a lesser extent (39). Although cDNA cloning allows for direct comparison of the amino acid sequence between the chicken smooth muscle (38) and nonmuscle MHC-A (32), only part of the MHC-B sequence (14) has been published to date. Both the chicken intestinal epithelial cell MHC (32) and the human macrophage MHC cDNA-derived sequences (30) are for MHC-A. The amino acid sequence identity between chicken MHC-A and the chicken 204-kD smooth muscle MHC is 84% in the head region and 69% in the rod region with 80–85% of the tyrosine residues located in the head region. Similar values are found in comparing MHC-A and B (unpublished observation, this laboratory). Nevertheless, at least three of the chymotryptic peptides generated from the 196-kD intestinal epithelium MHC and the 198-kD brain MHC migrate to unique positions using peptide mapping, confirming that the two MHCs are related, but distinct proteins.

Burridge and Bray have demonstrated that platelet and brain myosins are distinct by analysis of fragments of myosin produced by chemical cleavage at cysteine residues (4), although they were not able to separate the MHCs in their SDS-PAGE system. It is of note that they found that kidney and fibroblast myosin generated a pattern consistent with their being a simple mixture of brain and platelet myosins. This agrees with our observation that kidney contains roughly equal amounts of both the 196- and 198-kD MHCs. Together, the results presented in this paper suggest that at least two types of nonmuscle MHC exist both at the peptide and mRNA levels. These results should help in defining the functional differences between the two MHC isoforms.

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