Nucleotide Sequence and Expression of a cDNA Encoding Chick Brain Actin Depolymerizing Factor†,‡

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ABSTRACT: Chick brain actin depolymerizing factor (ADF) is a 19-kDa protein that severs actin filaments and binds actin monomers. We have obtained a cDNA encoding ADF by screening a chick embryo λgt11 cDNA library with both a rabbit anti-ADF antiserum and two oligonucleotide probes. Several non-full-length clones of 636 bases and one full-length clone of 1886 bases were isolated and sequenced. The full-length cDNA encodes a protein of 165 amino acids with a calculated molecular weight of 18 520. The deduced amino acid sequence shows 73% identity with the porcine brain actin binding protein cofilin. The coding region of the ADF cDNA has been placed in an expression vector, and the resulting protein shows immunoreactivity with an anti-ADF antiserum but not with an anti-cofilin antibody. The expressed ADF has been purified and has an actin depolymerizing activity identical with that of brain ADF. Like cofilin, ADF contains a sequence similar to the nuclear transport signal sequence of the SV40 large T antigen and a calcium/calmodulin-dependent protein kinase II phosphorylation consensus sequence. Northern blots of both embryonic chick brain and muscle RNA revealed two ADF mRNAs of length 2.1 and 0.9 kilobases. Southern blots suggest that the ADF gene is present in a single copy within the chicken genome. ADF contains regions of homology with other actin binding proteins including tropomyosin, gelsolin, and depactin.

Chick brain actin depolymerizing factor (ADF)[1] was first identified by its ability to rapidly depolymerize filamentous actin (F-actin) (Bamburg et al., 1980). It has been purified and characterized as a single polypeptide with apparent molecular mass of 19 kDa on SDS–polyacrylamide gels (Giuliano et al., 1988). ADF is an actin binding protein capable of severing F-actin and binding actin monomers (G-actin) (Nishida et al., 1984; Hayden & Bamburg, 1987), but it does not cosediment with F-actin (Koffer et al., 1988; Abe & Obinata, 1989). These activities are pH and calcium independent in vitro. ADF is present in most embryonic and many adult chicken tissues, but it occurs in highest amounts, up to 0.5% of the total protein, in embryonic chick brain (Bamburg & Bray, 1987). A protein of similar size that is recognized by ADF antiserum occurs in mammalian brain (Bamburg & Bray, 1987; Nishida et al., 1984).

Although many studies have focused on characterizing the biochemical activity and cellular distribution of ADF, the amino acid sequence has not been reported. This paper reports the primary structure of ADF derived from an ADF cDNA. The primary structure shows 73% identity between the sequence of chick brain ADF and porcine brain cofilin (Nishida, 1985; Matsuzaki et al., 1988). Cofilin has an actin depolymerizing activity similar to ADF at pHs above 7.5, but at lower pH, it binds stoichiometrically to actin subunits in F-actin and cosediments with the actin (Yonezawa et al., 1985). A cofilin-like protein occurs along with ADF in embryonic chick muscle and chick brain (Abe & Obinata, 1989). Therefore, to be certain that the cDNA we have isolated codes for ADF and not cofilin, we have expressed the cDNA clone in a bacterial system and characterized the protein product as ADF.

Materials and Methods

Materials. A cDNA library in λgt11 prepared from whole 10-day embryonic chick mRNA was obtained from Clontech Laboratories, Inc., Palo Alto, CA. Nitrocellulose filters (Ba45) were obtained from Schleicher & Schuell, Keene, NH. Nylon membranes (Hybond-N) and all radiolabeled nucleotides were from Amersham Corp., Arlington Heights, IL. Oligonucleotides were synthesized in the Macromolecular Resource Facility, Colorado State University, or by Operon Technologies, Inc., San Pablo, CA. pET vector and *Escherichia coli* BL21(DE3) were kindly provided by Drs. Alan Weeds and Michael Way (Medical Research Council Laboratory of Molecular Biology, Cambridge, England). Monoclonal antibody raised against chick muscle cofilin (MAB-22) was provided by Dr. Takashi Obinata (Abe et al., 1989). All immunochemicals, restriction enzymes, and DNA modifying enzymes were obtained from GIBCO-BRL, Gaithersburg, MD. Low gelling temperature aragose was obtained from FMC Corp., Rockland, ME. Isopropyl β-D-thiogalactopyranoside (IPTG) and bovine pancreatic DNase I were obtained from Sigma Chemical Co., St. Louis, MO. All other chemicals were reagent grade.

Peptide Isolation and Sequence Analysis. ADF was isolated from 19-day embryonic chick brains as described by Giuliano et al. (1988). Purified ADF (1 mg) was reduced and carboxamidomethylated by the method of Mayes (1984). Cyanogen bromide peptides of ADF were prepared by using the method of Gross (1967) as modified by Phillips and Azari (1971). Peptides were filtered through 0.45-μm filters and separated by HPLC on a reverse-phase C-8 column. Peptides

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‡ The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J02912.

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[1] Abbreviations: ADF, actin depolymerizing factor; F-actin, filamentous actin; G-actin, monomeric actin; IPTG, isopropyl β-D-thiogalactopyranoside; TFA, trifluoroacetic acid; SSPE, saline–sodium phosphate–EDTA buffer; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; SSC, saline–sodium citrate buffer; PAGE, polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; bp, base pair(s); DNase I, deoxyribonuclease I; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); pET, plasmid for expression by T7 RNA polymerase.

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(0.1 mg) were injected onto the column (0.46 × 5 cm) equilibrated in 0.1% trifluoroacetic acid (TFA) and eluted over 30 min with a 0–75% linear gradient of 80% acetonitrile/0.1% TFA. Fractions containing each 214-nm absorbance peak were collected, lyophilized and subjected to a second round of HPLC to enhance purity. Selected peptides were lyophilized in preparation for sequence analysis. Peptide sequence analysis was performed at the University Of Wyoming regional facility.

Oligonucleotide Synthesis. Two degenerate, 17-base, oligonucleotide probes, ADFO-1 (128-fold degenerate) and ADFO-2 (64-fold degenerate), were made by using minimally degenerate nucleotide sequences derived from the amino acid sequence of the CNBr peptides. These synthetic oligonucleotides were made corresponding to the noncoding DNA strand and were purified by electroelution from gel slices after separation by gel electrophoresis on 50% urea-containing 20% polyacrylamide gels.

Isolation of cDNA Clones. The chick embryo cDNA library in λgt11 was plated at a density of 5000 plaques/100-mm dish and screened with both rabbit antiserum to chick brain ADF (Bamburg & Bray, 1987) and the two oligonucleotide mixtures described above. Expression screening was based on the method of Young and Davis (1983). Primary antibody was detected with horseradish peroxidase conjugated goat anti-rabbit IgG and peroxidase substrate (peroxide and diaminobenzidine). Hybridization screening used oligonucleotides labeled at their 5′ end with [γ-^{32}P]ATP by T4 polynucleotide kinase. Duplicate filters were hybridized at 37 °C overnight with ADFO-1 and ADFO-2. Hybridization was carried out in 6 × SSPE (1 × SSPE contains 0.18 M NaCl, 10 mM sodium phosphate, pH 7.4, and 1 mM EDTA), 1 × Denhardt's solution [0.2 mg/mL each of bovine serum albumin, Ficoll (type 400, Sigma Chemical Co.), and poly(vinylpyrrolidone)], 0.05% tetrasodium pyrophosphate, 0.1% SDS, 100 μg/mL herring sperm DNA, and labeled probe. Filters were washed in 6 × SSPE/0.05% tetrasodium pyrophosphate at 47 °C for 10 min and subjected to autoradiography. Only clones hybridizing to both probes were isolated for further analysis.

DNA Sequence Analysis. Restriction fragments from three clones, CBA-2, CBA-6, and CBA-4, were subcloned into M13mp18 or M13mp19 for single-strand sequencing or into pUC 19 for double-strand sequencing by the dideoxynucleotide chain termination method of Sanger et al. (1977).

RNA Blot Analysis. RNA was isolated from fresh or liquid nitrogen frozen chick brain and chick breast muscle using the guanidinium/CsCl method (Chirgwin et al., 1979). RNA was separated by agarose–formaldehyde electrophoresis, transferred to a Hybond-N membrane, and hybridized with an 807 bp fragment of clone CBA-4 (1-807) which had been labeled with ^{32}P by the random primer method (Feinberg & Vogelstein, 1983). Hybridization was carried out in 50% formamide, 5 × Denhardt's solution, 5 × SSPE, 0.1% SDS, 0.05% tetrasodium pyrophosphate, 10 μg/mL poly(A) DNA, and 100 μg/mL herring sperm DNA at 42 °C overnight. Filters were washed for 1 h at 65 °C in 1 × SSPE/0.05% tetrasodium pyrophosphate.

Southern Blot Analysis. Genomic DNA was isolated from chick brain by the method of Gross-Bellard et al. (1972), cleaved with restriction enzymes, separated on 0.8% agarose, and transferred to a Hybond-N membrane. The filters were hybridized with a 1687 base pair fragment (1-1687) of clone CBA-4 (labeled with ^{32}P by the random primer method) in 6 × SSC (1 × SSC contains 0.15 M NaCl and 15 mM sodium citrate, pH 7.0), 5 × Denhardt's solution, and 0.5% SDS at 65 °C overnight. Filters were washed repeatedly with 2 × SSC and with a final 1-h wash in 0.1 × SSC at 65 °C.

Expression of ADF in E. coli. A 1377 bp *Eco*RI/*Sst*I DNA fragment containing the complete ADF coding sequence was first subcloned into the *Eco*RI/*Sst*I sites of pUC 19 for convenience. Standard methods for subcloning, bacterial culture, and bacterial transformations were performed as described by Maniatis et al. (1982). The ADF coding sequence was then excised from pUC 19 with either *Nco*I and *Sca*I (718 bp fragment) or *Nco*I and *Hin*dIII (1330 bp fragment). The *Nco*I cleaves at the initiating methionine of ADF, Sc*a*I cleaves 122 bp downstream of the termination codon, and the *Hin*dIII cleaves in the polylinker of pUC 19. The resulting fragments were purified by agarose electrophoresis using low gelling temperature agarose and ligated into a modified pBR 322 vector (pET) containing a bacteriophage T7 RNA polymerase promoter and termination signal surrounding unique cloning sites (Studier et al., 1990). The pET vector containing the ADF cDNA coding fragment was used to transform *E. coli* JM101 (ampicillin selection). Plasmids containing an ADF cDNA insert were identified by restriction enzyme analysis, and the pET/ADF plasmid DNA was used to transform *E. coli* BL21(DE3) (ampicillin selection). Selected clones were grown to OD_{600nm} = 1.0 in liquid medium, and IPTG (0.6 mM final concentration) was added. The cultures were grown for 3 h and the bacteria harvested by centrifugation at 1200*g*.

SDS–PAGE. SDS–polyacrylamide gel electrophoresis was performed by the method of Laemmli (1970) in isocratic acrylamide gels (15 T, 2.7% C) prepared as mini-slab gels. Samples were prepared in 0.25 M Tris, pH 6.8, 20% glycerol, 2% SDS, and 20% 2-mercaptoethanol.

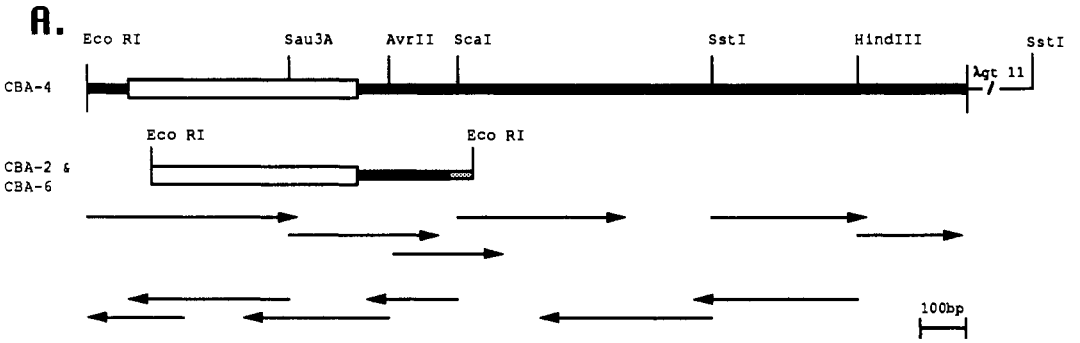
Immunoblotting. Immunoblots of proteins separated by SDS–PAGE were performed by the method of Towbin et al. (1979). Following transfer to nitrocellulose, blots were incubated in 5% powdered milk, and then in primary antibody. Rabbit antiserum to chick brain ADF (Bamburg & Bray, 1987) or MAB-22 to chick muscle cofilin (Abe et al., 1989) was used as primary antibody. Rabbit immunoglobin was detected by treatment with biotinylated goat anti-rabbit IgG, streptavidin–alkaline phosphatase, and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt; mouse monoclonal antibody was detected with peroxidase-conjugated goat anti-mouse IgG, peroxide, and diaminobenzidine.

DNase I Inhibition Assay for ADF Activity. ADF activity was measured by the DNase I inhibition assay developed by Blikstad et al. (1978) as modified by Harris et al. (1982) for quantitating the depolymerization of F-actin by other proteins. Monomeric actin and ADF–actin complex inhibit DNase I to the same extent (Daoud et al., 1988).

Results and Discussion

Isolation of ADF cDNA. Two peptides separated from the CNBr digest of purified brain ADF were selected for sequence analysis on the basis of their 280-nm absorbance. The amino acid sequence of each of these peptides was determined by automated Edman degradation. Nineteen amino acids were identified in peptide CBD-1 (IYASSKDAIKKKFQGIKHE) and 11 amino acids in peptide CBD-2 (FFLWAPEQAPL). Degenerate oligonucleotide probes were constructed from the sequence information of the underlined regions in each peptide.

Expression screening of a chick embryo cDNA library produced three positive clones, CBA-1, CBA-2, and CBA-3, each with an insert length of ~700 base pairs. Western blot analysis of a lysate of each of the clones indicated that the antibody reacted with a protein of about 135 kDa which is the size expected for an ADF/β-galactosidase fusion protein (data not shown). Screening of the library with oligonucleotides



B.

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                Met Lys Val Arg Lys Cys Ser
CBA-4  140- C ATG AAA GTG CGG AAA TGC TCT -161
CBA-2&6  1- G TGC TGG GCG CGG CGC GGC TCT - 22
                Cys Trp Ala Arg Arg Gly Ser
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C

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GCAACGCCCTCGCCGCTTCCCTTCCTGCTGCCGGAGCCCGCGCCGGCCCAGCACCCGAGGGAAGCAGCAGCCGCCGCTCCGCCGACACC      89

ATGGCATCTGGAGTACAAGTTGCCGATGAGGTATGCCGTATCTTCTACGACATGAAAGTGCGGAAATGCTCTACGCCTGAGGAAGTGAAG     179
 M  A  S  G  V  Q  V  A  D  E  V  C  R  I  F  Y  D  M  K  V  R  K  C  S  T  P  E  E  V  K
                                                          1

AAGAGGAAGAAGGCTGTCATCTTCTGCCTCAGTCCAGACAAAAAGTGCATTATCGTGGAAGAAGGCAAAGAGATTCTAGTGGGAGATGTT     269
 K  R  K  K  A  V  I  F  C  L  S  P  D  K  K  C  I  I  V  E  E  G  K  E  I  L  V  G  D  V
 2

GGTGTGACGGTTACTGACCCTTTCAAGCACTTTGTCGAGATGCTTCCCGAGAAGGATTGCCGTTATGCCTTGTATGATGCAAGTTTTGAG     359
 G  V  T  V  T  D  P  F  K  H  F  V  E  M  L  P  E  K  D  C  R  Y  A  L  Y  D  A  S  F  E

ACCAAGGAATCCAAAAAAAGAAGAGCTGATGTTCTTCTTGTGGGCACCAGAACAAGCACCTCTCAAAAGTAAGATGATCTATGCAAGCTCC     449
 T  K  E  S  K  K  E  E  L  M  F  F  L  W  A  P  E  Q  A  P  L  K  S  K  M  I  Y  A  S  S
                                                          3

AAGGATGCAATCAAAAAAGAAGTTCCAAGGCATAAAGCATGAATGCCAAGCAAATGGGCCAGAGGACCTCAATCGAGCTTGTATTGCTGAG     539
 K  D  A  I  K  K  K  F  Q  G  I  K  H  E  C  Q  A  N  G  P  E  D  L  N  R  A  C  I  A  E
 4

AAGCTAGGAGGCTCCCTAGTCGTAGCTTTTGAAGGAAGTCCTGTGTAGATGTCATACAGTGCCACAACTCTACAAGCATTCCATGTTTTA     629
 K  L  G  G  S  L  V  V  A  F  E  G  S  P  V

ATATATCAGTTATGTAAAGCAACCATCCTAGGCAATGGGTTCACTAACTGTAGGGAAGTTGTCTTATAGAATAAAGTAGATTTTGGAGAT     719

GTAGCTCTATGTATAAATGACCTTAAATAAATCAAATTAAGGATTTATCTTGGTGTATTAATATTTGCATTGGTTGTCTATCCCAGTACT     809

ATCCCCGATGAATTAACACTAAAGGTCTCCAAAAATTCTGATTCAGCAGTGTATCGTGGGATGAAGGGGGGGGTTGATGAGAACAATGTG     899

GTTCCTGTTCTCTTCTAAATTAAAATGAGCAAAATCATCAGTCTTTCTGAGGCTACTGGCCAAATTGCCAAACTCAAAACTTTCCCCTCG     989

AACCGTTGAATGCAAGACTTAAAACCTGTAGTTCAAGTTAGGCACCTAAACAGAGCTAAATTCTTAGTGCTTGCAGTTGCCCGGCGTTAC    1079

TAATAAGGGTTAATCAGGAGGTTAAAATAGAGATTAAAGTAGGTACGCTTTCCTGGAGAAAGTCCTAATCATCCTTGTAAACTCAGCGTG    1169

CAGTAATCTTTAAAGGTTTGCTTGTTCCTACAGGTTAACATTGGTTATGAAGTGTCTCGAGGTCTCCTCCAAGGCTGAACGATTCTATTA    1259

AAATTGCTTTTCTTTTGGCATAGGTTAACTTAGGACCACCTTATTTCCACTAACACACTTAGTAGCGGATCAGACGCTTCTGAGGCTTTG    1349

ACAAGTGTAGATCCTTCCGTTGGAGCTCTCCAGTTCTGGGCAGTACCATGAGAAAGAACCACTTGGAAAAACAGCAAAAGATCACAAGTG    1439

GTGTTCTCAGCCCCACTCTTTGGTCACTTGGTCCATTTGTCCGTAATTATTATTGATTTAACTTAGTAGAACCAATCAGCCTGACTAATA    1529

TGGCTTGATCTGGGCTTTTTGTCTGTTAGCTTCTTTAATTCTGTAGAAATGCATGGAAATATCACCTACTAGAAAGTTTGACCTGAAATG    1619

CCTTTTGTATGGGGGGGGGGGGGAGGGGGGTAGAGAACATTCCTGTGCCAGCTACAGAGATCTTAAAAGCTTCCATGTTTCACAGTGGTT    1709

GATTAAAGCTGTTTCCTTTGTGTAATGTGTACATGGAAGTTGCATAGAGTTTTCCTGTATGTCTGTAGAGAAAAAAAAAAAAAGAAAAAAA    1799

AACAAAACCCTTAGCCTCCAGTGCGTTACTGTGCTATGTTCTGTGTGTGCCAAACTTGAATAGCTTAAATAAATGGGGTTTCCTTC(A)19  1905
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FIGURE 1: (A) Restriction map of the ADF cDNAs and sequencing strategy. Arrows indicate the direction and extent of DNA sequence determination. The open box indicates the coding region of the cDNA for each clone. Clones CBA-2 and CBA-6 are identical and are contained entirely within clone CBA-4 except for the poly(A) tail indicated by the shaded region and the extreme 5′ end shown in detail in (B). (B) Sequence comparison of the 5′ end of CBA-2 and -6 with CBA-4. Nucleotide differences are shown in bold print. (C) Complete nucleotide sequence and the amino acid sequence of the open reading frame of the cDNA encoding ADF. Nucleotides are numbered on the right. (1) The consensus phosphorylation sequence recognized by calcium/calmodulin-dependent protein kinase II. (2) The region similar to the nuclear translocation signal sequence of the SV40 large T antigen. (3) Region homologous to a similar region of depactin. (4) The sequence similar to the amino terminus of tropomyosin. Polyadenylation signal sequences in the 3′ region are in bold print. The arrow indicates the position of polyadenylation in clones CBA-2 and CBA-6.

yielded eight positive clones, seven (CBA-5 to -11) of which contained an insert similar in size to the inserts in clones obtained by expression screening and one (CBA-4) which contained a much larger insert of ~2000 base pairs. Inserts from one expression positive clone (CBA-2) and from two hybridization positive clones (CBA-6 and CBA-4) were subcloned and sequenced by using the strategy outlined in Figure 1A. The 3′ *Eco*RI site that should have been present in clone CBA-4 had been lost, presumably during library construction and amplification. The 3′ end of the clone was obtained by using the *Sst*I site 1060 base pairs 3′ of the *Eco*RI site in λgt11 (Young & Davis, 1983).

Sequence of the cDNAs and the Encoded Protein. The complete DNA sequence of CBA-4 is shown in Figure 1C. It contains 1886 nucleotides and 1 long open reading frame of 495 bases which codes for a protein of 165 amino acids and includes both ADF peptide sequences in unique CNBr peptides. Both DNA strands were completely sequenced in the coding region, and 67% of the entire sequence data was confirmed by sequencing both strands (Figure 1A). This cDNA appears to contain the full-length coding region because (1) the derived protein has a molecular weight of 18 520 which agrees with the apparent molecular mass of 19 kDa estimated by mobility on SDS–polyacrylamide gels and (2) the sequence surrounding the initiation AUG codon contains the consensus translation initiation sequence described by Kozak (1986). The 3′ end of the cDNA sequence contains three separate polyadenylation signals at positions 699, 745, and 1867 (Figure 1C).

Clones CBA-2 and CBA-6 contain 626 bp and have identical nucleotide sequences. They differ in sequence from CBA-4 in that (1) they are incomplete, lacking 53 base pairs at the 5′ end of the ADF coding region, (2) clone CBA-4 uses the polyadenylation signal sequence at position 1867 whereas clones CBA-2 and CBA-6 use the polyadenylation signal sequence at position 745 and begin polyadenylation at the point indicated by the arrow in Figure 1C, and (3) 12 out of the first 17 bases at the 5′ end of clones CBA-2 and CBA-6 differ from those in the corresponding region (139–155) of clone CBA-4 (Figure 1B). This sequence difference may represent a cDNA coding for a different isoform of ADF which has been observed by 2D PAGE of brain ADF (Giuliano et al., 1988), or it may simply be an artifact introduced during library construction and amplification. Efforts are currently underway to resolve this question.

Confirmation That the Cloned cDNAs Code for ADF. As shown below, ADF shares over 73% identity at the amino acid level and 68% identity in the coding region of its cDNA with cofilin, an actin binding protein isolated from porcine brain. Given the high degree of sequence homology between these proteins and the finding that both proteins are present in chick tissues (Abe & Obinata, 1989), the following experiments were done to demonstrate that the cDNAs characterized here code for ADF and not cofilin.

SDS–PAGE of total protein from control and vector-containing *E. coli* BL21(DE3) cultures before and after induction with IPTG shows that only bacteria containing the pET/ADF vector produce a 19-kDa protein that increases upon induction (Figure 2A). *E. coli* BL21(DE3) is somewhat leaky for T7 RNA polymerase so low levels of 19-kDa protein are made prior to induction. An immunoblot of a similar gel, but one containing less protein (Figure 2B), shows that the 19-kDa protein is immunoreactive with the antiserum to brain ADF. The induced protein does not react with the cofilin antibody MAB-22 specific for chick cofilin (Figure 2C, upper panel).

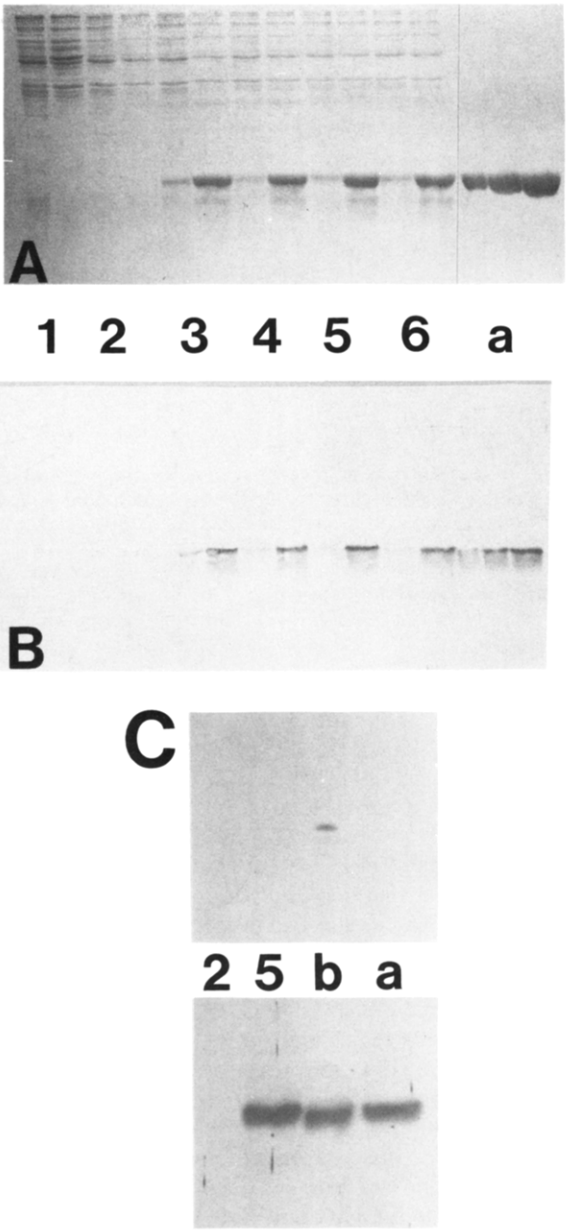

FIGURE 2: SDS–PAGE and immunoblots of proteins extracted from control and pET/ADF transformed cultures of *E. coli* BL21(DE3). Pairs of samples are presented in (A) and (B) with those on the left obtained before induction and those on the right obtained 3 h after IPTG addition. (A) Proteins stained with Coomassie Brilliant Blue R-250. (B) Immunoblot of a similar gel containing $^1/_{40}$th the amount of protein loaded on gel A developed with ADF antiserum. (C) Immunoblot using monoclonal antibody specific for cofilin (upper panel) and an ADF antiserum (lower panel). Samples are from cultures containing (1) no vector, (2) pET vector without insert, (3 and 4) pET vector with a 718 bp ADF cDNA insert, and (5 and 6) pET vector with a 1330 bp insert. a = ADF standards [1, 2, and 3 μg on gel in (A); 10, 20, and 30 ng on gel B; 15 ng on gel C]; b = chick brain homogenate; total protein loaded, 30 μg on upper gel, 5 μg on lower gel.

The cofilin antibody does, however, react with a 20-kDa protein in chick brain extract. The λgt11 fusion protein between β-galactosidase and the product of the CBA-2 also reacted with antiserum to chick brain ADF but not with MAB-22 (data not shown). Thus, it is likely that the shorter cDNAs also code for ADF.

Treatment of postinduction bacteria containing the pET/ADF vector with lysozyme (4.5 mg/mL in 50 mM Tris, pH 8.0, and 2 mM EDTA) for 15 min at 30 °C and centrifugation at 13400*g* resulted in liberation of most of the ADF in the

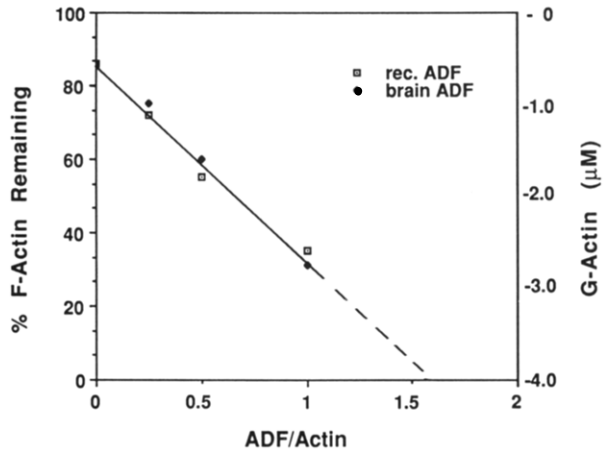

FIGURE 3: Comparative actin depolymerizing activity of brain ADF and recombinant ADF measured by DNase I inhibition. F-actin (4 μM) was incubated with different amounts of ADF (to give the molar ratios shown) at pH 7.0 (50 mM PIPES, 70 mM KCl, and 2 mM $MgCl_2$) for 15 min at room temperature. Aliquots were assayed for DNase I inhibition, and the amount of G-actin (actually total ADF–actin plus free G–actin) in each aliquot was obtained from a standard curve of DNase I inhibition by G-actin alone.

soluble fraction. The amount of ADF produced per milliliter of bacterial culture following a 3-h induction period is approximately 100 μg, or about 35% of the total protein present. The ADF released following the lysozyme treatment has biological activity comparable to purified brain ADF at pH 7.0 (Figure 3), a pH at which cofilin binds and cosediments with F-actin (Yonezawa et al., 1985).

Northern Blot Analysis. Northern blots of total chick brain and muscle RNA contain two distinct classes of mRNA of 2100 and 900 bases that hybridize with an 807 base pair fragment of clone CBA-4 containing the entire coding region of ADF (Figure 4A). These two mRNAs occur in a similar ratio in both embryonic chick brain and muscle. It is likely that CBA-4 and CBA-2 represent the cDNAs produced by reverse transcription of the 2100 and 900 base pair mRNAs, respectively.

Southern Blot Analysis. Southern blots of total chicken genomic DNA probed with a 1687 base pair fragment from CBA-4 showed strong hybridization to a single fragment of about 13 kb in *Kpn*I and *Bam*HI digests, and slightly smaller fragments in *Xba*I and *Eco*RI digests (Figure 4B). A major single band of about 2 kb was obtained in *Pst*I digests. Multiple enzyme digest showed that the smallest single band of about 4 kb is obtained with *Xba*I and *Kpn*I (Figure 4C). These results suggest that the gene for ADF is present as a single copy within the chicken genome.

Analysis of the Deduced Primary Sequence of ADF. The deduced sequence of chick ADF is one amino acid shorter than that of porcine cofilin (Figure 5A). Alignment of the two cDNA nucleic acid sequences indicates that it is the Thr-148 of cofilin that is missing in the ADF sequence. A consensus phosphorylation sequence recognized by calcium/calmodulin-dependent protein kinase II (Czernik et al., 1987; Blackshear et al., 1988) is found at residues 21–24 of both ADF and cofilin. ADF can be phosphorylated in vivo (Morgan & Bamburg, 1988) and is capable of being phosphorylated by calcium/calmodulin-dependent protein kinase II in vitro (T. E. Morgan, M. Browning, and J. R. Bamburg, unpublished results). Cofilin also occurs in a phosphorylated state in the cell (Ohta et al., 1989).

Cofilin has been shown to be translocated to the cell nucleus following dimethyl sulfoxide treatment or heat shock (Matsuzaki et al., 1988: Ohta et al., 1989). Matsuzaki et al. (1988) identified a sequence in cofilin similar to the nuclear translocation signal sequence of the SV40 large T antigen (Kalderon et al., 1984), and Ohta et al. (1989) have hypothesized that this sequence does not function as a nuclear translocation signal in cofilin when the protein is in the phosphorylated state. ADF contains the identical sequence in the same position (Figure 1C). At present, it is not known if ADF can be translocated to the nucleus.

ADF primary structure also contains regions that are homologous to regions of other actin binding proteins (Figure 5), several of which have been previously identified in cofilin (Matsuzaki et al., 1988). The DAIKKK sequence, which is in common among tropomyosin, cofilin, and ADF, has been shown to be important in the binding of cofilin and tropomyosin to F-actin since synthetic peptides containing this sequence block the cosedimentation of cofilin or tropomyosin with F-actin (Yonezawa et al., 1989; Cho et al., 1990). However, ADF does not cosediment with F-actin (Koffer et

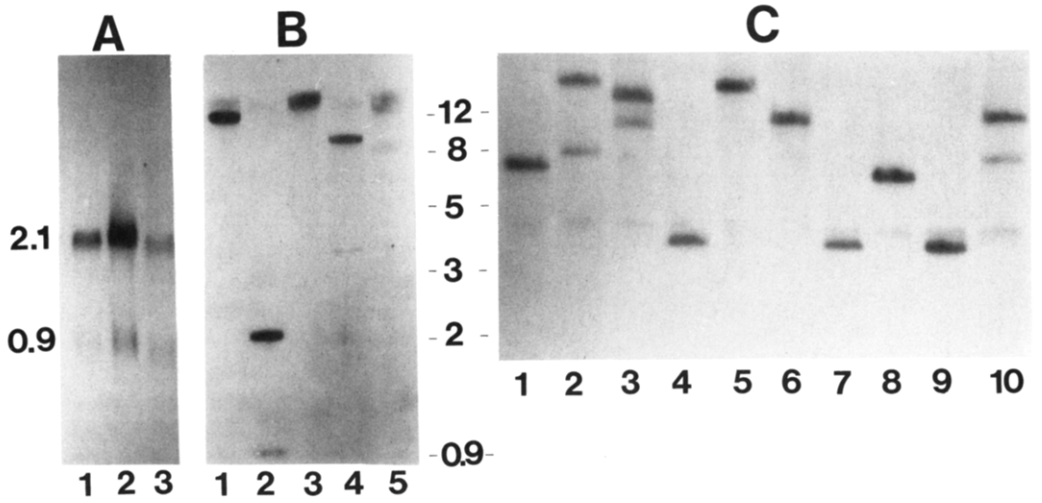

FIGURE 4: (A) Northern blot of total RNA from brain and muscle of embryonic chick. The probe for hybridization was an 807 bp fragment obtained from CBA-4 containing the complete coding region. (1) 15-day embryonic brain RNA (10 μg); (2) 19-day embryonic brain RNA (20 μg); (3) 10-day embryonic muscle RNA (20 μg). The size of the of the two mRNA classes was determined from RNA standards. (B and C) Southern blot of total chicken genomic DNA probed with an 1687 bp fragment of CBA-4. Restriction enzymes used: (B-1) *Xba*I; (2) *Ps*'I; (3) *Kpn*I; (4) *Eco*RI; (5) *Bam*HI; (C-1) *Bam*HI and *Xba*I; (2) *Bam*HI and *Kpn*I; (3) *Bam*HI and *Sma*I; (4) *Kpn*I and *Xba*I; (5) *Sma*I and *Xba*I; (6) *Kpn*I and *Sma*I; (7) *Bam*HI, *Kpn*I, and *Xba*I; (8) *Bam*HI, *Sma*I, and *Xba*I; (9) *Kpn*I, *Sma*I, and *Xba*I; (10) *Bam*HI, *Kpn*I, and *Sma*I. Fragment sizes were determined from DNA standards.

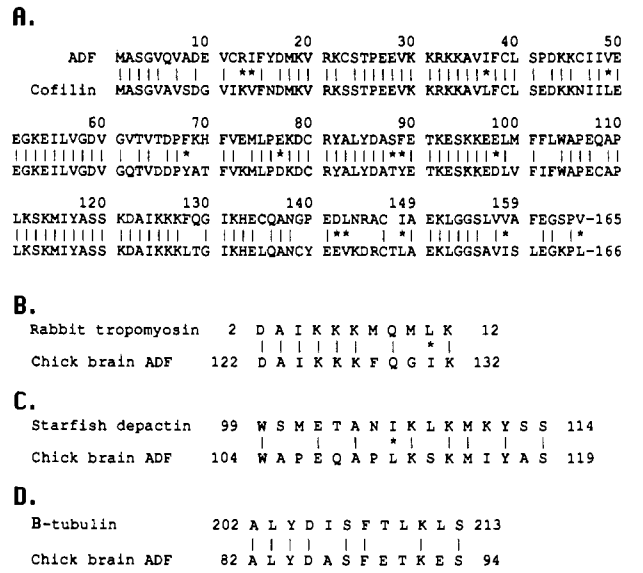

FIGURE 5: Sequence comparison of ADF with other proteins. Vertical lines indicate identical amino acids. An asterisk indicates a conservative substitution. Chick brain ADF is compared with (A) porcine brain cofilin (Matsuzaki et al., 1988), (B) the amino terminus of rabbit tropomyosin (Mak et al., 1980), (C) a homologous region of starfish depactin (Takagi et al., 1988), and (D) a homologous region of β-tubulin (Singhofer-Wowra et al., 1986).

al., 1988; Abe & Obinata, 1989). Thus, either additional sites of interaction are required for cofilin binding to F-actin or the topography surrounding the DAIKKK sequence in ADF and cofilin must be different.

ADF contains a region of homology which occurs in cofilin, profilin, fragmin, and gelsolin (Matsuzaki et al., 1988). In addition, ADF has a region of homology with depactin, a protein from starfish oocytes with similar actin binding properties (Mabuchi, 1983; Nishida et al., 1984; Takagi et al., 1988). Also, ADF contains a region of sequence that is homologous to an interior segment of β-tubulin, although the importance of this homology is unknown.

It is not yet clear which, if any, of these homologous regions defines the ADF actin binding domain(s). The ability to express a single isoform of ADF in bacteria and obtain it in a fully active form and in high amounts should now permit us to crystallize this protein and examine its three-dimensional structure, both alone and in a complex with actin. These studies should allow us to define more clearly the interaction between actin and chick brain actin depolymerizing factor.

Added in Proof

After we submitted this paper, the cDNA sequence of a similar actin depolymerizing factor from porcine brain was published (Moriyama et al., 1990). This protein differs from chick brain ADF by eight amino acid substitutions.

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Sequence of cDNAs Encoding Actin Depolymerizing Factor and Cofilin of Embryonic Chicken Skeletal Muscle: Two Functionally Distinct Actin-Regulatory Proteins Exhibit High Structural Homology[†,‡]

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ABSTRACT: Two actin-regulatory proteins of 19 and 20 kDa are involved in the regulation of actin assembly in developing chicken skeletal muscle. They are homologous with actin depolymerizing factor (ADF) and cofilin, a pH-dependent actin-modulating protein, which were originally discovered in chicken and mammalian brain, respectively. In this study, full-length cDNA clones were isolated by screening a λgt11 cDNA library constructed from poly(A+) RNA of embryonic chicken skeletal muscle with the antibodies specific for each protein, and their complete sequences were determined. The chicken cofilin cDNA encoded a protein of 166 amino acids, the sequence of which had over 80% identity with that of porcine brain cofilin. The amino acid sequence of the ADF was 165 amino acids and showed about 70% identity with either chicken or mammalian cofilin, in spite of the fact that ADF and cofilin are functionally distinct. Like chicken and mammalian cofilin, ADF contained a sequence similar to the nuclear transport signal sequence of SV40 large T antigen. ADF and cofilin shared a hexapeptide identical with the amino-terminal sequence of tropomyosin as well as the regions homologous to other actin-regulatory proteins, including depactin, gelsolin, and profilin. The overall nucleotide sequences and Southern blot analysis of genomic DNA, however, indicated that the two proteins were derived from different genes.

Actin is a major constituent of thin filaments of cross-striated myofibrils in skeletal muscle. In the developing skeletal muscle, it is synthesized, polymerized, and organized into sarcomeric structures. Little is known as to how the polymerization of actin and the organization of actin filaments into the sarcomeric structures are regulated in developing muscle cells. Purified actin is known to be polymerized spontaneously and form actin filaments in vitro under physiological salt and pH conditions. Most actin in adult muscle is filamentous, and monomeric actin is present only at the level of critical concentration. However, in young embryonic muscle, actin is largely present in unpolymerized forms in the cytoplasm (Shimizu & Obinata, 1986). Since purified embryonic muscle actin is polymerizable to the same degree as adult muscle actin, regardless of the difference in major actin isoforms between embryonic and adult muscles (Hayward & Schwartz, 1986; Shimizu & Obinata, 1980), it was postulated that actin polymerization in developing muscle is controlled or suppressed by some regulatory proteins (Shimizu & Obinata, 1986). Recently, three actin regulatory proteins of 16, 19, and 20 kDa, respectively, have been isolated from chicken embryonic skeletal muscle. The first one, the 16K protein, was identified with profilin (Carlsson et al., 1977) from its functional characteristics and size (Ohshima et al., 1989). The second one, the 19K protein, was regarded as being the same as actin depolymerizing factor (ADF) of chicken brain (Bamburg et al., 1980) or its variant, because, like ADF, the 19K protein forms a 1:1 complex with G-actin to inhibit its polymerization, and quickly depolymerizes F-actin, and the antigenicity and peptide maps of this protein were similar to those of ADF (Abe & Obinata, 1989). The third one, the 20K protein, binds to both G- and F-actin, inhibits actin polymerization in a pH-dependent manner, and inhibits binding of tropomyosin to F-actin (Abe et al., 1989a,b). Since such characteristics have been detected in porcine brain cofilin (Nishida et al., 1984; Yonezawa et al., 1985), the 20K protein appears to be homologous to porcine cofilin. However, it has been reported that cofilin is absent from muscle cells (Yonezawa et al., 1987). To better understand the structures and functions of these actin binding proteins in embryonic muscle, we have isolated cDNA clones for their respective proteins from a cDNA library constructed from poly(A+) RNA of

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