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Characterization of a β-Actin mRNA Zipcode-Binding Protein

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Localization of β-actin mRNA to the leading edge of fibroblasts requires the presence of conserved elements in the 3′ untranslated region of the mRNA, including a 54-nucleotide element which has been termed the "zipcode" (E. Kislauskis, X. Zhu, and R. H. Singer, J. Cell Biol. 127:441–451, 1994). In order to identify proteins which bind to the zipcode and possibly play a role in localization, we performed band-shift mobility assays, UV cross-linking, and affinity purification experiments. A protein of 68 kDa was identified which binds to the proximal (to the coding region) half of the zipcode with high specificity (ZBP-1). Microsequencing provided unique peptide sequences of approximately 15 residues each. Degenerate primers corresponding to the codons derived from the peptides were synthesized and used for PCR amplification. Screening of a chicken cDNA library resulted in isolation of several clones providing a DNA sequence encoding a 67.7-kDa protein with regions homologous to several RNA-binding proteins, such as hnRNP E1 and E2, and with consensus mRNA recognition motif with RNP1 and 2 motifs and a putative REV-like nuclear export signal. Antipeptide antibodies were raised in rabbits which bound to ZBP-1 and coimmunoprecipitated proteins of 120 and 25 kDa. The 120-kDa protein was also obtained by affinity purification with the RNA zipcode sequence, along with a 53-kDa protein, but the 25-kDa protein appeared only in immunoprecipitations. Mutation of one of the conserved sequences within the zipcode, an ACACCC element in its proximal half, greatly reduced its protein binding and localization properties. These data suggest that the 68-kDa ZBP-1 we have isolated and cloned is an RNA-binding protein that functions within a complex to localize β-actin mRNA.

It is now evident that one mechanism used by cells to establish polarity is to restrict the synthesis of certain proteins to certain regions of the cell. This is observed in oocytes, where segregation of mRNAs such as Vg1, Xcat-2 in *Xenopus laevis*, and *bicoid*, *oskar*, and *nanos* in *Drosophila melanogaster* has been described in detail (for reviews see references 12 and 26). In several asymmetric cell types, β-actin mRNA is localized near the leading edge of the cell in a region referred to as the lamella. These cell types include chicken embryo fibroblasts (CEFs) (18), 3T3 fibroblasts (8), endothelial cells (10), and C2 myoblasts (9). Since the leading edge of the lamella, the lamellipodium, contains actively polymerizing actin filaments (25), the sorting of this mRNA provides a congruence of the sites of synthesis with the utilization of the cognate protein.

It has been suggested that this asymmetric distribution of β-actin mRNA functions to support the polarity of the cell, through restricted spatial distribution of actin protein synthesis, which is necessary for directional movement (18). Recently, we have obtained evidence that directly implicates peripheral β-actin localization in cellular polarity and motility. In these studies, β-actin mRNA was delocalized by treatment with antisense oligonucleotides directed against the *cis*-acting localization element (see below). In these "delocalized" cells, polarity (14), and also cellular motility (14a), was severely reduced. Thus, the establishment of a polar phenotype, i.e., where a cell has a clear leading edge and a trailing edge, depends on positional β-actin protein synthesis. This may be necessary for the long-term directional movement observed when cells migrate in a developmental pattern or in response to chemotactic agents.

The sequence elements required for β-actin mRNA sorting have recently been identified. In a series of experiments using a reporter gene linked to mutated segments of the actin gene (13, 14), it was shown that several sequence elements in the 3′ untranslated region (UTR) of β-actin were necessary and sufficient to localize mRNA in the periphery. Fine analysis of the region showed that a 54-nucleotide (nt) segment could direct the localization of the entire transcript. This segment was termed the "zipcode." Sequence analysis showed several regions in the zipcode which are conserved among β-actins of several species but which were absent in other mRNAs and other actin isoforms. Among these are several AC-rich regions comprising the sequence ACACCC. While the significance of these elements is not clear, their conserved nature in β-actins from several species (27) suggested that they played a role in the peripheral distribution of the mRNA, possibly by binding proteins which mediate localization.

The mechanism by which β-actin mRNA sequence information is transduced into peripheral localization remains to be elucidated, although some facts have emerged. First, localization is energy dependent, since cordycepin, an inhibitor of ATP production, prevented this process (17). Second, localization does not require ongoing protein synthesis, since it occurred in the presence of puromycin or cycloheximide (22). Third, localization is inhibited by disruptors of the actin cytoskeleton, and not by disruptors of the microtubule system, indicating that the transport and/or anchoring steps require the actin cytoskeleton (17a, 23). The involvement of the microfilament system for β-actin mRNA localization in fibroblasts differs from localization of other mRNAs in other systems. In oocytes (5) and neurons (2), similar studies suggested that a microtubule system was used in the transport and/or the anchoring stages of mRNA localization. Fourth, serum-induced signal transduc-

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tion mechanisms were involved in the regulation of β-actin mRNA localization (17).

The leading lamellae of the cell contain a variety of cytoskeletal elements, including a network of actin filaments and actin binding proteins which function to maintain the structural integrity of this region of the cell. Sundell and Singer have previously reported that actin mRNA in the lamellae appears in the light microscope to be in "granules" (23), suggesting that the RNA is in a rather large complex, presumably with proteins and possibly other RNAs. Electron microscopic examination of poly(A) RNA showed that the majority of mRNAs are present at the intersection of actin filaments (1a), often at intersections containing the actin binding protein ABP-280 (filamin), which is known to form actin networks. These intersections frequently contain large electron-dense masses, presumably consisting of proteins or protein-RNA complexes (1a). Others have reported granules of myelin basic protein mRNA in oligodendrocytes (1), of *bicoid* mRNA in *Drosophila* (5), possibly involved with Exu protein (24), and in *Xenopus* (6, 15, 21). It is likely, then, that mRNA is either transported to or anchored at the lamella in a complex with a number of proteins.

In this study we employ band-shift, UV cross-linking, and affinity purification methods to isolate proteins binding to the localization zipcode of β-actin mRNA. This approach has yielded several candidate proteins, primarily the 68-kDa protein which we have termed ZBP-1. It has been purified and cloned, and the sequence indicates that it is an RNA-binding protein with several regions of homology to hnRNP proteins and putative REV-like nuclear export signal (NES). Mutational analysis of the zipcode indicates that binding of this protein to the zipcode in vitro correlates strongly with its localization in vivo, suggesting a direct role of ZBP-1 in this process. In addition, several other proteins either copurify or coimmunoprecipitate with ZBP-1. Our data are consistent with the existence of a complex of proteins binding both to the zipcode and to the actin network, suggesting a mechanism for mRNA transport and/or anchoring within the cell periphery.

MATERIALS AND METHODS

Tissue culture and metabolic labeling. Fibroblast cells were isolated from breast muscle tissue of 12-day chick embryos as described previously (22). Cells were plated onto 10-cm culture dishes at a density of 6×10^5 cells/ml and grown at 37°C in minimal essential medium supplemented with 10% fetal calf serum in an atmosphere of 95% air/5% CO_2. Cultures were then passaged into 15-cm plates to remove residual myotubes and were grown to approximately 90% confluence before harvesting. For metabolic labeling, cultures were incubated in methionine-free media (Gibco, Inc.) containing 100 μCi of [^{35}S]methionine (Amersham) per ml for 4 h at 37°C. Cells were rinsed and scraped in cold phosphate-buffered saline (PBS), pelleted in a tabletop centrifuge, and resuspended in the appropriate buffer supplemented with 1% Triton X-100. Cells were extracted for 30 min on ice and were clarified at 1,500 × *g* for 10 min at 4°C. Supernatants were collected and used in further procedures.

UV cross-linking. Oligoribonucleotide probes corresponding to the zipcode sequences were constructed on an oligonucleotide synthesizer. Probes were end-labeled with ^{32}P by T4 polynucleotide kinase and isolated by G-50 gel filtration chromatography. For binding, 10 ng of probe was combined with 10 μl of cell extract and the sample was exposed to UV light (Bio-Rad GS Genelinker) at a distance of 3 to 5 cm for 5 min (total power, 125 mJ). Sodium dodecyl sulfate (SDS) sample buffer was added and the sample was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) autoradiography.

Preparation of oligoribonucleotide probes. Oligoribonucleotide probes corresponding to regions of the zipcode were constructed with deoxyribonucleotides at the ends. Probes were as follows (RNA inserts are shown by boldface characters): proximal zipcode, 5′-TT-**CCGGACUGUUACCAACACCCACACCC**-TT-3′; distal zipcode, 5′-TT-**CUGUGAUGAAACAAAACCCAUAAAUGCGCA**-TT-3′; proximal antisense, 5′-TT-**GGGUGUGGGUGUUGGUAACAGUCCGG**-TT-3′; distal antisense, 5′-TT-**UGCGCAUUUAUGGGUUUUGUUUCAUCACAG**-TT-3′; and poly(A), 5′-TT-**AAAAAAAAAAAAAAAAAAAAAAAAAAA**-TT-3′.

For affinity purification, the 3′ end TT is replaced by the following motif: -TT(biotin)dATT(biotin)T-3′. Biotin-modified T (Glen Research, Sterling, Va.) was incorporated during synthesis on an ABI synthesizer.

Mutant RNAs. The wild-type RNA used had the sequence 5′-TT-**CCGGACUGUUACCAACACCCACACCC**-TT(biotin)dATT(biotin)T-3′ (boldface characters show RNA insert). Mutant RNAs, shown with RNA inserts in boldface and mutated bases underlined, were as follows: mutant 1, **TT-taaACCGGACUGUUACCAUGUGUGACACCC**-TT(biotin)dATT(biotin)T-3′; mutant 2, TT-**taaACCGGACUGUUACCACACCCUGUGUG**-TT(biotin)dATT(biotin)T-3′; mutant 3, **-TT-taaACCGGACUGUUACCAUGUGUGUGUGUG**-TT(biotin)dATT(biotin)T-3′; and mutant 4, -TT-**taaACCCCUGAGTTACCAACACCCACACCC**-TT(biotin)dATT(biotin)T-3′.

Mutant RNA localization assay. Mutations parallel to those described above were synthesized as pairs of complementary deoxyoligonucleotides that were ligated into a 3′ polylinker of the expression plasmid RSVβgal (13). Mutants were tested for their ability to localize a *lacZ* reporter (14).

Affinity purification. The probe was immobilized by overnight incubation with either streptavidin-agarose (binding capacity, 3 μg of probe/25 μl of beads; Sigma) or streptavidin magnetic beads (binding capacity, 300 ng of probe/25 μl of beads; Dynal, Lake Success, N.Y.) in a buffer of 1 M NaCl–50 mM Tris 7.4–5 mM EDTA–0.1% Triton X-100 (coupling buffer). Unbound probe was removed by rinsing three times with fresh coupling buffer. Cell extracts were incubated with the appropriate affinity resin overnight at 4°C on a circular rotator. Nonspecifically bound proteins were removed with five rinses with binding buffer (100 mM NaCl, 50 mM Tris-HCl [pH 7.4], 10 mM $MgCl_2$, 2% Triton X-100), and specific proteins were eluted in Laemmli SDS sample buffer (16) and analyzed by SDS-PAGE.

Protein sequencing. In five separate experiments, 10 mg of probe was immobilized and combined with 500 μl of unlabeled extract from 50 10-cm plates prepared as described above. After affinity purification and SDS-PAGE, proteins were electroblotted to polyvinylidene difluoride (PVDF) membranes overnight at 4°C. Blots were stained with 0.1% Ponceau red in 1% acetic acid, and the blots were destained in 1% acetic acid to reveal bands. Bands corresponding to proteins specifically purifying with the zipcode probes were cut out and eluted by digestion with either thermolysin K or trypsin, and the digested peptides were analyzed by high-pressure liquid chromatography. Peaks containing peptides in sufficient quantity were sequenced with an Applied Biosystems Procise protein sequencer at the W. M. Keck Foundation Protein Chemistry facility at the Worcester Foundation for Experimental Biology (Shrewsbury, Mass.).

Peptide synthesis and polyclonal antibody production. Peptide sequences used for antipeptide antibody synthesis were NH2-KITTILAQVRRQQXK-COOH (sequence 629) and NH2-KVRMVVIGPPEAQFK-COOH (sequence 627). Peptide sequences used for partial cloning were sequence 627 and NH2-LKEENFFGPK-3′ (sequence 133). Peptides were synthesized in milligram quantities by the peptide synthesis facility at the University of Massachusetts—Worcester. Approximately 10 mg each of peptides 627 and 629 were combined with adjuvant and injected into rabbits (East Acres Biologics, Southbridge, Mass.). Antisera were tested by Western blotting and immunoprecipitation.

Cloning of ZBP-1 (Yuri Oleynikov). Two of the peptide sequences obtained by Edman degradation were reverse translated into nucleic acid sequences, and degenerate inosine-containing 30-base oligonucleotides were synthesized on a DNA synthesizer (Applied Biosystems). The primers were used for PCR amplification from CEF cDNA. The PCRs were optimized for correct $MgCl_2$ concentration and annealing temperature, and a 97-nt product was obtained. A cDNA library was constructed from CEF poly(A) plus RNA isolated at 90% confluency. The mRNA was reverse transcribed with Moloney murine leukemia virus RNase H^- reverse transcriptase (cDNA synthesis kit; Promega, Madison, Wis.) and primed with a mixture of oligo(dT) and random hexamers (Sigma). The cDNA was ligated with *Eco*RI adaptors and inserted into LambdaZapII phage vector (Stratagene, La Jolla, Calif.). The 97-nt PCR fragment of ZBP-1 was used to make a single-stranded [^{32}P]dCTP-labeled probe in an asymmetric PCR. Approximately 500,000 clones were screened, and 6 clones were isolated and sequenced. The biggest contiguous sequence was 2,023 nt long, and it encoded an open reading frame of 67.7 kDa, which contained the three peptide sequences obtained earlier.

Sequence analysis. Homology searches were performed with BLAST and FASTA algorithms on NCBI and EMBL servers. The EMBL server has also been used for protein sequence analysis through Worldwide Web access. The sequence was analyzed and manipulated with various commercial and shareware packages available for Apple Macintosh computers.

Immunoblotting. After SDS-PAGE, gels were electroblotted onto either PVDF or nitrocellulose membranes by using a Bio-Rad electrotransfer chamber. Transfer was carried out at 50 mA overnight at 4°C in a buffer of Trizma base (3 g/liter)–glycine (14.4 g/liter)–methanol (10%). Blots were blocked with 1% I-block (Tropix, Inc., Bedford, Mass.) supplemented with 0.1% Tween 20 (block buffer) for 3 h at room temperature. Primary antibodies were diluted 1:100 in block buffer, and blots were incubated overnight at room temperature. Blots were rinsed three times with block buffer and then incubated in a 1:10,000 dilution of goat anti-rabbit immunoglobulin G-alkaline phosphatase-conjugated antibody (Tropix, Inc.) in block buffer. After 30 min at room temperature, blots were rinsed twice with block buffer, once with PBS–0.1% Tween 20, then twice in assay buffer (1% diethanolamine, adjusted to pH 10). Blots were incubated for 5 min in assay buffer supplemented with CSPD, a light-emitting substrate of alkaline phosphatase, and exposed to film for 1 to 10 min.

Immunoprecipitation. Cell extracts (in bind buffer) were incubated with a 1:100 dilution of antipeptide antisera for 3 h at 4°C. Fifty microliters of protein

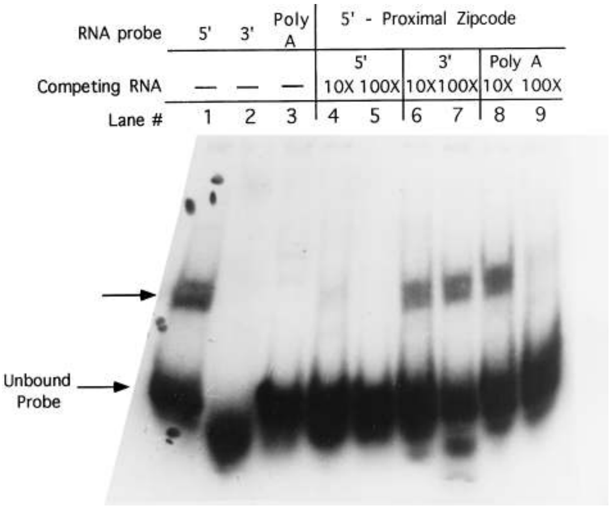

FIG. 1. The proximal zipcode forms specific complexes with cellular proteins in band shift. ^{32}P-labeled RNA probes (constructed with DNA bases on the ends as described in Materials and Methods) were incubated for 10 min at room temperature in 20 ml of CEF extract in a buffer of 10 mM MgCl$_2$–100 mM NaCl–50 mM Tris (pH 7.4)–1% Triton X-100. Twenty microliters of 50% glycerol was added, and the samples were separated on a 4% nondenaturing polyacrylamide gel. Lane 1, proximal zipcode; lane 2, distal zipcode; lane 3, poly(A) sequence; lanes 4 and 5, proximal zipcode with a 10-fold (lane 4) or 100-fold (lane 5) excess of unlabeled proximal zipcode; lanes 6 and 7, proximal zipcode with 10-fold (lane 6) or 100-fold (lane 7) unlabeled distal zipcode; lanes 8 and 9, proximal zipcode with 10-fold (lane 8) or 100-fold (lane 9) unlabeled poly(A). Note the specific complex formation with the proximal zipcode (arrow), which is competed effectively with the specific probe (lanes 4 and 5) but not with nonspecific probes (lanes 6, 7, and 8), although a 100-fold excess of poly(A) resulted in significant competition (lane 9).

G-Sepharose (Sigma, St. Louis, Mo.) was added for 1 h, and beads were rinsed five times with bind buffer. Forty microliters of Laemmli sample buffer supplemented with 10 mM dithiothreitol was added, and samples were heated to 90°C for 1 min. Beads were pelleted in a tabletop centrifuge, and the supernatants were analyzed by SDS-PAGE.

RESULTS

Identification of proteins binding to the zipcode. To identify the proteins binding to the localization sequence, band-shift, UV cross-linking, and affinity purification procedures were employed using cell extracts prepared from CEFs mixed with various oligoribonucleotide probes (see Materials and Methods). The 54-base zipcode was synthesized as two separate 27-base sequences, corresponding to proximal (to the coding region) and distal halves. Several deoxybases were put on both ends of the probe for the purpose of protection against RNase activity; these did not affect protein binding. For band-shift and UV cross-linking experiments, probes were 5′ labeled with ^{32}P by T4 polynucleotide kinase.

In Fig. 1 we show that the proximal zipcode forms a stable and specific complex with proteins in CEF extract. A strong complex (lane 1) is specifically competed by the unlabeled proximal zipcode (lanes 4 and 5). It is not competed by a nonspecific RNA (antisense to distal zipcode) even at high concentrations (lanes 6 and 7). The poly(A) probe competed the proximal zipcode only, but at high concentrations (lanes 8 and 9), which may reflect the relatively A-rich nature of the zipcode (42.5%). The distal zipcode formed only weak complexes that are competed off with specific and nonspecific probes (data not shown). In addition, the complexes formed with proximal zipcode are stable when exposed to heparin sulfate in concentrations up to 25 mg/ml (data not shown).

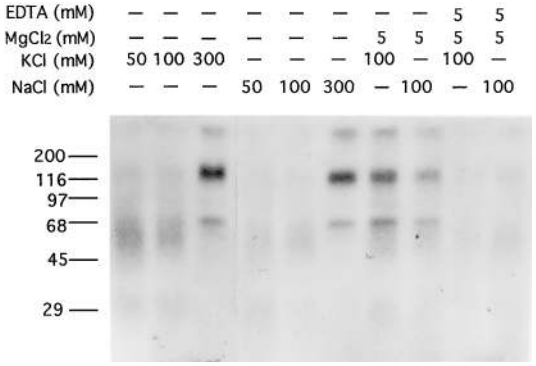

FIG. 2. UV cross-linking of proximal zipcode to cellular proteins reveals salt-dependent binding of proteins of 68 and 120 kDa. Cell extracts were prepared as described in Materials and Methods by using the given buffer supplemented with 1% Triton X-100. Ten nanograms of ^{32}P-labeled oligoribonucleotide probe corresponding to the proximal zipcode was cross-linked to cellular proteins by exposure to UV light (125 mJ) at a distance of 1 cm. Cross-linked proteins were visualized by SDS-PAGE. Note the presence of three specific bands at 68, 120, and >200 kDa, which appear with either 300 mM NaCl or KCl or 5 mM MgCl$_2$.

To identify the size of this protein-RNA complex, UV cross-linking experiments were performed with ^{32}P-labeled proximal zipcode RNA. When the protein-RNA complex was stabilized by UV light and separated by SDS-PAGE, specific bands were seen at 68 and 120 kDa, and a band was seen at a molecular size greater than 200 kDa (Fig. 2). The same bands were seen when cross-linking was done on the gel-shifted band in Fig. 1 (data not shown). This binding pattern was affected by salt concentrations and was enhanced by either MgCl$_2$ (at 5 mM) or high (300 mM) monovalent cations of either NaCl or KCl (the complex was stable in up to 1.5 M NaCl). Formation of a complex of these sizes was sequence specific, since neither antisense nor other sequences used exhibited complex formation (data not shown). These data supported the results from band-shift experiments indicating that the proximal zipcode sequence had the capacity to form specific complexes with one or more proteins and indicated the sizes of the prospective binding proteins.

A method was then developed to obtain quantities of these proteins sufficient for analysis by sequencing (2 to 5 mg). Oligoribonucleotide probes corresponding to the zipcode sequences were constructed with a 3′ end spacer labeled with biotin (see Materials and Methods). Probes were immobilized on streptavidin-coated beads (Dynal) and used as affinity resins in batchwise purification of binding proteins. Cell extracts were incubated with these resins overnight and then washed in buffer, and bound proteins were eluted in SDS sample buffer and analyzed by SDS-PAGE. Initially, extracts from [^{35}S]methionine-labeled cells were used to screen different RNA oligonucleotides for protein binding activity. Consistent with the results obtained by UV cross-linking, the proximal localization element probe bound a 68-kDa protein specifically, while other sequences showed no complex formation (Fig. 3). Also, as was observed in the UV cross-linking, binding of the 68-kDa protein was affected by either 300 mM monovalent salts (NaCl or KCl) or 5 mM MgCl$_2$ (Fig. 3B). The 68-kDa protein had the highest affinity and specificity and was designated the zipcode-binding protein (ZBP-1). In addition to ZBP-1, other proteins were specifically selected by this sequence, including proteins of 120, 95, 53, and 35 kDa.

Microsequencing the ZBP and antipeptide antibody production. Proteins were transferred to PVDF membranes, digested,

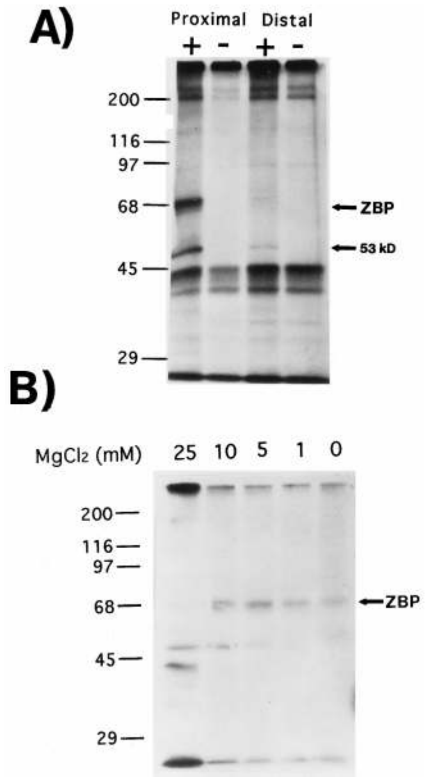

FIG. 3. Affinity purification of a major protein of 68 kDa with a proximal zipcode probe. CEF cultures were labeled with [^{35}S]methionine for 4 h, rinsed, and extracted with bind buffer (100 mM NaCl, 10 mM $MgCl_2$, 50 mM Tris [pH 7.4], 1% Triton X-100). Unlabeled RNA probes were immobilized on magnetic beads, and clarified supernatants were incubated in batches overnight with the given affinity resins. The beads were washed three times with bind buffer, and bound proteins were eluted and analyzed by SDS-PAGE. (A) The two halves of the zipcode (proximal, 5′; distal, 3′), both sense (+) and antisense (−). Note the presence of a 68-kDa band (ZBP-1) specifically in the proximal sense lane. Also, the 53-kDa protein is evident in this lane only. (B) ZBP-1 binding to the proximal zipcode (sense probe) is affected by a high $MgCl_2$ concentration. Note that maximal binding appears at 5 to 10 mM.

and sequenced by conventional methods (see Materials and Methods). Five peptides of approximately 15 residues each were obtained for the 68-kDa protein. A search of databases using these sequences did not reveal proteins with similar sequences; therefore, this protein appears to be novel. The other binding proteins were identified by their peptide sequences: the 95-kDa protein had sequences identical to those of gelsolin; the 35-kDa protein had sequences identical to those of fibroblast tropomyosin. Actin was also common in the preparations (Fig. 3A).

Peptides corresponding to the amino acid sequences obtained were synthesized and injected into rabbits to generate antipeptide polyclonal antibodies. The development of immunogenicity was monitored by Western blotting against either total cellular protein or affinity-purified ZBP (Fig. 4). As shown, a light band in the 68-kDa region was seen in proteins purified from a cell extract. If this band was indeed ZBP-1, it would be expected that affinity purification with the zipcode would constitute an enrichment of this band. After isolation by using the zipcode sequences, the cell extract was assayed for antibody binding, and a severalfold enrichment for the 68-kDa protein was seen (right lane). This confirmed that the antibodies were directed against a 68-kDa protein which was purified by the zipcode affinity resin.

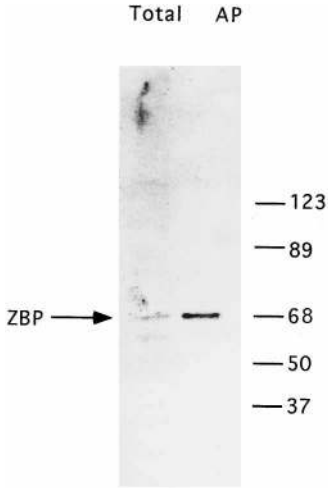

FIG. 4. Western blot of antipeptide antibodies to the ZBP-1. Affinity-purified ZBP-1 was microsequenced, and two peptides were synthesized (as described in Materials and Methods) and injected into rabbits for antibody production. Left lane, binding of rabbit antibodies to total cell extract; right lane, binding to affinity-purified ZBP-1.

To test the ability of the antipeptide antibodies to interact with the ZBP-1 in solution, immunoprecipitations were performed. When [^{35}S]methionine-labeled cell extracts were immunoprecipitated with the antibody, a band corresponding to the ZBP was specifically precipitated (Fig. 5). The identity of this band as the ZBP was confirmed by Western blot analysis of immunoprecipitated material (data not shown). This indicated that this antipeptide antibody interacted with ZBP-1 in its native state in solution. To test whether this antibody inter-

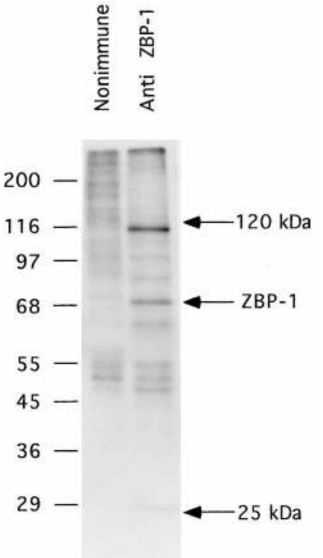

FIG. 5. Immunoprecipitation with antipeptide antibodies shows coprecipitation of proteins of 120 and 25 kDa. Cultures of CEF cells were labeled with [^{35}S]methionine and extracted in bind buffer supplemented with 1% Triton X-100. Clarified extracts were immunoprecipitated with 10 ml of the antipeptide antibody or preimmune serum for 3 h at 4°C on Sepharose-protein G beads. Precipitated material was centrifuged, and proteins were separated by SDS-PAGE and visualized by autoradiography. Note the specific immunoprecipitation of the ZBP-1 and the specific coprecipitation of proteins of 120 and 25 kDa.

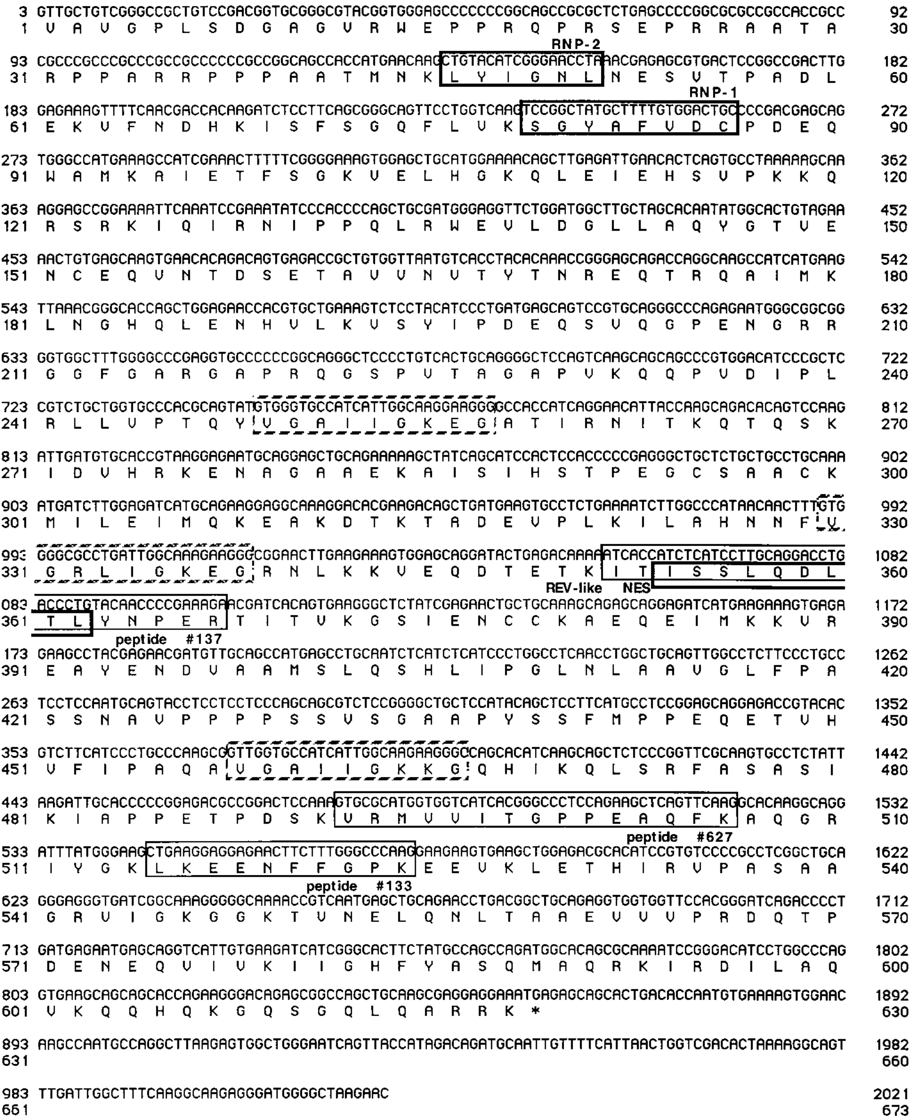

FIG. 6. The sequence of a 3′ fragment of the ZBP-1 mRNA reveals RNA-binding domains. Sequences were initially obtained by PCR amplification of ZBP-1 cDNA with inosine-containing degenerate primers from a chicken fibroblast cDNA pool. Subsequently, sequences were used to screen a chicken cDNA library. Sequencing of isolated clones indicates the presence of an RRM domain, with two highly conserved RNP regions, termed RNP-1 and RNP-2 (boxes at top of figure), and a REV-like NES (in peptide 137). A 9-amino-acid sequence (VGAIIGKE/KG) of unknown function which repeats three times is also shown in dashed boxes. Small boxes indicate potential stop codons.

acted with ZBP-1 when the protein was stoichiometrically associated with RNA, ^{32}P-labeled probe to the proximal zipcode was incubated in cell extracts under conditions identical to those used to purify ZBP-1, and the ability of the antibody to coimmunoprecipitate the RNA was tested. In this experiment none of the antibodies were able to precipitate labeled probe (data not shown). Thus, it appears that the antibody can interact with ZBP only when the protein is not bound to its RNA target.

In addition to ZBP, proteins of 120 and 25 kDa were coprecipitated with the antibody (Fig. 5). This indicated that these proteins were physically associated with ZBP when the ZBP was not associated with the RNA. The presence of the 120-kDa protein was of interest, since a protein of this size is cross-linked to the zipcode (Fig. 2) and is seen in affinity purification to exhibit the same salt dependence as the ZBP (Fig. 3). The 53-kDa protein was not coimmunoprecipitated; thus, it did not appear to associate with ZBP-1 when the RNA was not present.

Cloning and sequence analysis of ZBP-1 (Yuri Oleynikov). By using the obtained peptide sequences, a 97-nt fragment corresponding to the zipcode-binding protein was obtained by PCR. The fragment was used to screen a chicken cDNA library constructed from fibroblast poly(A) RNA. A clone of 2,023 nt was isolated which had an open reading frame encoding 67.7 kDa of polypeptide (Fig. 6). The 5′ end of the mRNA is not yet identified. The peptide sequence contained RNP-1 and RNP-2 consensus sequences and regions homologous to the hnRNP family of proteins as well as other known RNA-binding proteins. In particular, hnRNP E1 and E2 proteins contain a sequence of unknown function that is almost perfectly repeated in ZBP-1 three times. The sequence is present also in

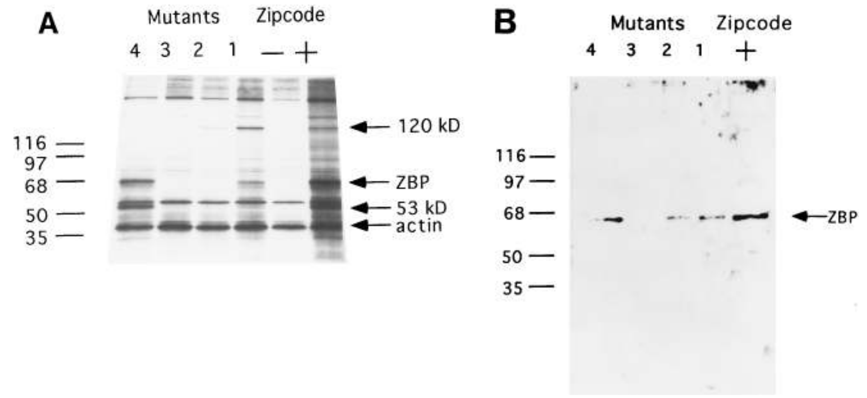

FIG. 7. Mutation of the zipcode reveals an element in the proximal zipcode necessary for binding ZBP-1. Oligoribonucleotides were constructed as described in Materials and Methods and were used in affinity assays with CEF extracts. (A) [^{35}S]methionine-labeled cell extracts were affinity purified with the given probes, and bound proteins were visualized by SDS-PAGE. (B) Western blot with anti-ZBP-1 peptide antibodies of proteins affinity selected by using the mutated zipcode sequences. Note that ZBP-1 requires at least one of the ACACCC domains in the distal portion of the proximal zipcode. Note that the mutants 1 to 4, listed in Materials and Methods, have mutations in motifs B, C, B and C, and A, respectively.

hnRNP K, transformation-upregulated nuclear protein, and onconeural ventral antigen with less strict homology, where it also is repeated. A potential REV-like NES was also found in amino acid positions 354 to 362. There are also several potential phosphorylation sites in this sequence, as determined with PROSITE and BLOCKS databases. The sequence appears to be novel, as no protein or nucleic acid in the current database shows high extensive homology to ZBP-1.

Correlation of ZBP binding with localization activity. To establish the sequence requirements for binding of the ZBP to the zipcode, the ability of mutated zipcode sequences to bind to the protein was analyzed by the affinity purification method. Sequence comparison of human and chicken zipcode revealed several regions of homology (27). First, there are several AC-rich regions in the zipcode, including a set of tandem ACA CCC repeats at positions 16 to 27 (termed motifs B and C, respectively), the second of which is conserved in human β-actin (27). In addition, there is a conserved sequence, GGACU (termed motif A), at positions 4 to 8 past the stop codon. It was of interest to determine if the ZBP binding relied on any of these sequences. To test this hypothesis, oligoribonucleotides which were mutated in these regions were constructed and immobilized on streptavidin-agarose. Affinity purifications were carried out, and protein binding was monitored either by visualizing proteins from [^{35}S]methionine-labeled cell extracts (Fig. 7A) or by Western blotting using the antipeptide antibody (Fig. 7B).

As shown, mutation of motif A (designated mutant 4; GGACU, positions 4 to 8) had little effect on binding of the 68-kDa protein in both assays, although the 120-kDa protein was eliminated. Mutation of the first ACACCC element (motif B), at positions 16 to 21 (designated mutant 1), reduced binding to less than 50% of control levels (see also Fig. 8), without affecting the 120-kDa protein. Mutation of the second tandem ACACCC element (motif C), at positions 22 to 27 (designated mutant 2), reduced binding of both the 68- and the 120-kDa proteins to less than 5% in the [^{35}S]methionine assay and to less than 40% in the Western blot assay. Mutation of both ACACCC motifs (designated mutant 3) reduced protein binding to background by [^{35}S]methionine labeling and to undetectable levels in the Western blot assay. These results indicated that protein binding was significantly reduced upon mutation of the ACACCC motifs. The binding of the 120- and 53-kDa proteins, exhibiting a similar, but not identical, dependence on the motifs, further supported the hypothesis that these two proteins are involved in a complex with the ZBP and the RNA.

To establish whether localization activity correlated with binding of the ZBP to the zipcode, sequences corresponding to the mutants used in the protein binding assays were inserted into the zipcode trap assay (14). As summarized in Fig. 8, mutations which affected the binding of the proteins also af-

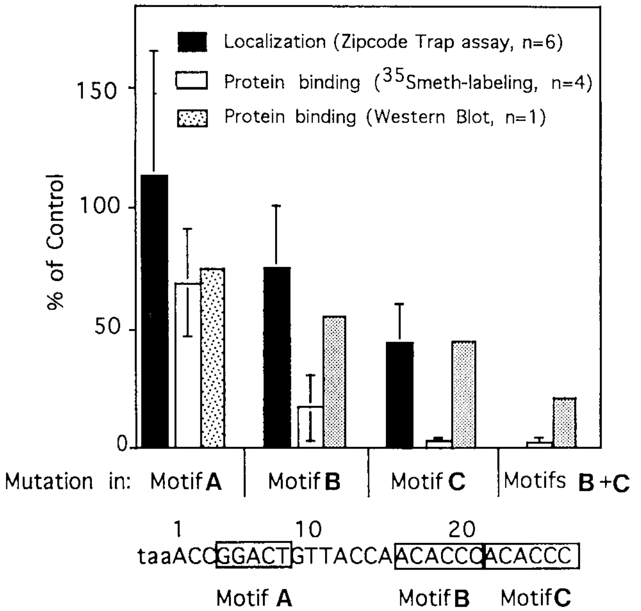

FIG. 8. Correlation of the binding of ZBP-1 to mutated zipcode sequences with localization. Mutations in the positions shown in Fig. 7 were constructed in the zipcode and inserted into the zipcode trap as described previously (14). CEFs were transfected, and the ability of the transfected constructs to localize peripherally was monitored by X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) production. Zipcode activity was expressed relative to the unmutated sequence and was not tested for mutations of both motifs B and C (right). Binding of ZBP-1 was determined by affinity binding as described in the legend to Fig. 7 and was quantitated by either [^{35}S]methionine labeling or Western blot analysis with the antipeptide antibody. Note that mutation of the ACACCC domains severely impairs the ability of the zipcode to localize peripherally and to bind ZBP-1.

fected the localization ability of the zipcode. Mutation of the GGACU element had essentially no effect on the ability of the zipcode to localize the chimeric zipcode peripherally. In contrast, mutation of either the proximal or distal ACACCC element significantly reduced the ability of the insert to direct peripheral localization, with the distal element having the greatest effect, as it did with the protein binding. The localization assay was less sensitive than the protein binding assay to mutation of the domains, possibly because the entire 54-nt zipcode was used in the localization assay, in contrast to the protein binding assay, in which the 54-mer was split into two 27-mers. It is possible that AC-rich elements in the distal half of the 54-nt sequence partially compensated for loss of the ACACCC motifs. Taken together, these data indicate a strong positive correlation pari passu between the ability of the zipcode to localize and the binding of the ZBP.

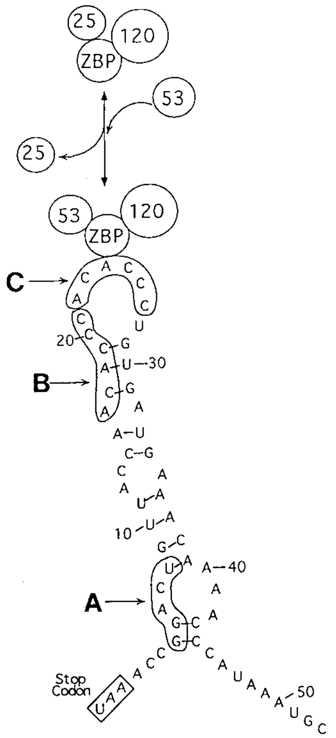

FIG. 9. Hypothetical model of the binding of the ZBP and associated proteins with the zipcode. A proposed secondary structure model of the β-actin localization zipcode region shows the sites of mutated motifs A, B, and C and indicates the proposed site of ZBP-1 binding.

DISCUSSION

These results indicated that a protein of 68 kDa specifically interacted with the proximal 27 nt of the β-actin mRNA zipcode, and evidence discussed below implicates it in the localization process. First, the zipcode formed a specific complex with CEF proteins in band-shift experiments: complex formation can be competed with excess specific probe, but not with nonspecific probes, and was stable in heparin sulfate. Second, the zipcode could be cross-linked by UV light to several proteins, including proteins of 68 and 120 kDa, and these interactions required either 300 mM NaCl or KCl or 5 mM $MgCl_2$. Third, this fragment purified a 68-kDa band by affinity from labeled cell extracts and required a similar salt dependency, evident by UV cross-linking. This protein binding pattern was not evident with either the distal half of the zipcode, the 43-nt element which had been shown to exhibit localization activity in the zipcode trap assay, or a variety of other nonspecific sequences. Fourth, this protein did not bind with high affinity to a mutated zipcode which could not localize. Fifth, the sequence of this protein contained an RNA binding domain. The RNA binding domain has strong homology to the RNP1 and 2 motifs of the RNA recognition motif (RRM) (see reference 3). A putative REV-like NES was also found in the sequence. It will be interesting to determine if ZBP-1 shuttles between the nucleus and cytoplasm, as has been seen with the hnRNP A1 (3), raising the possibility that the zipcode is recognized in the nucleus by ZBP-1 and translocates with it to the cytoplasm. The protein also seems to have other elements that require further analysis, such the 9-amino-acid sequence repeated thrice that is homologous to, for example, hnRNP E1 and E2 proteins.

By comparing the proteins selected by affinity to the zipcode with those selected by immunoprecipitation using the antibodies to the synthetic peptides, a hypothetical picture of the RNA-protein localization complex could be presented (Fig. 9). First, the antipeptide antibodies failed to immunoprecipitate added labeled RNA probe; thus, it is likely that the epitope is blocked when the ZBP is bound to the RNA, and any precipitation with this antibody may represent proteins not bound to the RNA target. Since this anti-ZBP antibody coimmunoprecipitates the 120- and the 25-kDa proteins, but not the 53-kDa protein, it is likely that the 120- and the 25-kDa proteins are associated with the ZBP when free in solution. Both the 120- and the 53-kDa proteins may be associated with the RNA (data not shown in the model), since they are also selected by affinity, whereas the 25-kDa protein is not. Thus, we hypothesize that the 25-kDa protein may cycle off the ZBP, and the 53-kDa may cycle on, when the ZBP is induced to bind the RNA target. This model, therefore, is supported by current evidence and provides a working hypothesis of the protein-protein and protein-RNA interactions occurring during localization. The RNA binding site of the protein corresponds to a hypothetical stem-loop structure which can be obtained with a best-fit algorithm. In this model, the preferred binding site of the protein as determined from the mutation analysis corresponds exactly with the sequences at the end of the loop.

The process of mRNA sorting has been suggested to involve the following steps: assembly of an RNP particle, translocation of this RNP particle to the proper cellular location, and anchoring of the RNP to the cytoskeleton (26). In the case of β-actin mRNA, the transport and the anchoring steps have been suggested to involve the actin cytoskeleton (23). It might be expected, then, that purification of proteins binding to the actin mRNA zipcode would yield known actin binding proteins. Actin was often nonspecifically copurified due to its high abundance in the cell. However, our results indicate that the affinity approach enriched for at least two actin binding proteins, gelsolin and tropomyosin, which appeared to vary with the degree of stringency of the binding. This could result if these actin binding proteins are bound, either directly or indirectly, to the ZBP. Conceivably, the ZBP could be recruited to the actin cytoskeleton when bound to the mRNA.

The identification of mutations in the ACACCC motif that affect localization constitutes a further definition of the sequence requirement for β-actin mRNA localization. Tandemly repeated ACACCC sequences occurred only in chicken β-actin; however, a single ACACCC sequence, in a position homologous to the essential distal one in the chicken required for efficient localization of β-actin mRNA, was conserved in humans.

Finally, the question of regulation of the localization complex remains to be addressed. The process of β-actin mRNA localization is under the control of the intracellular signalling systems which are activated by cell surface receptors for chemotactic factors, including platelet-derived growth factor and lysophosphatidic acid (17). These signaling systems may regulate the localization complex. Preliminary experiments showed that the ZBP-1 is not phosphorylated, but that the 120-kDa protein is a phosphoprotein (21a). Possibly, the formation and/or function of the localization complex was regulated by a signal transduction event, triggered by the fibroblast response to chemoattractants. This would result in actin mRNA localization toward the signaling event, and hence actin protein would be provided for motility (14a).

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