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# Identification and characterization of a RING zinc finger gene (C-RZF) expressed in chicken embryo cells

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**ABSTRACT** **To identify changes in gene expression that occur in chicken embryo brain (CEB) cells as a consequence of their binding to the extracellular matrix molecule cytotactin/tenascin (CT/TN), a subtractive hybridization cloning strategy was employed. One of the cDNA clones identified was predicted to encode 381 amino acids and although it did not resemble any known sequences in the nucleic acid or protein data bases, it did contain the sequence motif for the cysteine-rich $C_3HC_4$ type of zinc finger, also known as a RING-finger. This sequence was therefore designated the chicken-RING zinc finger (C-RZF). In addition to the RING-finger, the C-RZF sequence also contained motifs for a leucine zipper, a nuclear localization signal, and a stretch of acidic amino acids similar to the activation domains of some transcription factors. Southern analysis suggested that C-RZF is encoded by a single gene. Northern and *in situ* hybridization analyses of E8 chicken embryo tissues indicated that expression of the C-RZF gene was restricted primarily to brain and heart. Western analysis of the nuclear and cytoplasmic fractions of chicken embryo heart cells and immunofluorescent staining of chicken embryo cardiocytes with anti-C-RZF antibodies demonstrated that the C-RZF protein was present in the nucleus. The data suggest that we have identified another member of the RING-finger family of proteins whose expression in CEB cells may be affected by CT/TN and whose nuclear localization and sequence motifs predict a DNA-binding function in the nucleus.**

An understanding of development requires knowledge of how genes control the patterned organization of tissues in the embryo and how gene networks operate in individual cells as a consequence of the feedback they receive from cellular interactions. Specific interactions of cells with other cells and with the extracellular matrix (ECM) molecules depend upon cell adhesion molecules (CAMs) and substrate adhesion molecules (SAMs) and their receptors. To explore the possibility that cellular interactions in turn lead to changes in gene expression, we have used the technique of subtractive hybridization to examine changes in gene expression in embryonic cells resulting from cellular aggregation mediated by neural CAM (N-CAM) (1). The main result of these studies was that the transcription rates of genes involved in cell adhesion, such as those for N-CAM, neuron-glia CAM (Ng-CAM), and the neural form of α-catenin (α-N-catenin), as well as in transcriptional regulation, such as *HoxA4* (*Hox1.4*), decreased after aggregation of chicken embryo brain (CEB) cells. It is now becoming apparent that adhesion molecules not only specify cellular interactions but also cause changes in the expression of developmentally significant genes that may be essential for the subsequent differentiation or development of those cells into tissues and organs (2, 3).

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In a subsequent study, we explored changes in gene expression that occur when the substrate adhesion molecule cytotactin/tenascin (CT/TN; reviewed in refs. 4–6) binds to neurons. The results of this study revealed alterations in the expression of a number of genes (7). One of these differentially expressed clones encodes a novel protein specifying a cysteine-rich sequence containing a "RING finger" or "$C_3HC_4$" consensus motif (8–11) and was enriched in the library prepared from CEB cells treated with CT/TN. The sequence of the cDNA clone that encodes the chicken-RING zinc finger (C-RZF) protein also contains motifs that predict a leucine zipper, a nuclear localization signal (NLS), and a transcription activation domain.‡ Immunofluorescence studies with anti-CRZF antibodies showed nuclear staining in chicken embryo heart cells. These properties raise the possibility that C-RZF may bind DNA and function as a transcriptional regulator.

## MATERIALS AND METHODS

**Subtractive Hybridization.** Cells were isolated from E8 CEBs (12) and allowed to attach to polylysine-coated tissue culture dishes at a density of $5 \times 10^7$ cells per dish for 20 min in Dulbecco's modified Eagle's medium without serum. The medium was changed, and the cells were incubated for 1 hr in the presence or absence of chicken CT/TN at 15 μg/ml, an amount previously shown to be maximal for the induction of several biological effects (13, 14). Under these conditions, the cells remained rounded throughout the incubation period thereby minimizing potential effects of CT/TN-induced morphological changes. A subtractive hybridization cloning strategy (see ref. 1) was used to generate two subtracted cDNA libraries called (+)CT/TN and (−)CT/TN that were enriched for sequences whose expression was either increased or decreased as a result of cell binding to CT/TN. These subtracted libraries were analyzed by hybridization and sequence analysis. Data base searches were performed with the FASTA program (Genetics Computer Group), version 7-UNIX to scan the GenBank (release 91), EMBL (release 44), and Swiss-Prot (release 31) data bases.

***In Situ* Hybridization.** *In situ* hybridization was performed with digoxygenin(DIG)-labeled RNA probes that were revealed with an alkaline phosphatase-conjugated anti-DIG antibody (Boehringer Mannheim). The specificity of the signal was verified by controls including hybridization with sense probes, hybridization in the absence of probe, pretreatment of the tissue sections with RNase, and omission of the anti-DIG antibody during detection.

**Immunoblotting and Immunofluorescence.** Rabbit polyclonal antibodies were generated against the C-RZF fusion protein representing amino acids 264–381 (Fig. 1) prepared in the pGEX-5X2 vector (Pharmacia). This construct was trans-

Abbreviations: CEB, chicken embryo brain; C-RZF, chicken RING zinc finger; CT/TN, cytotactin/tenascin; ECM, extracellular matrix; NLS, nuclear localization signal.

‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. X95455).

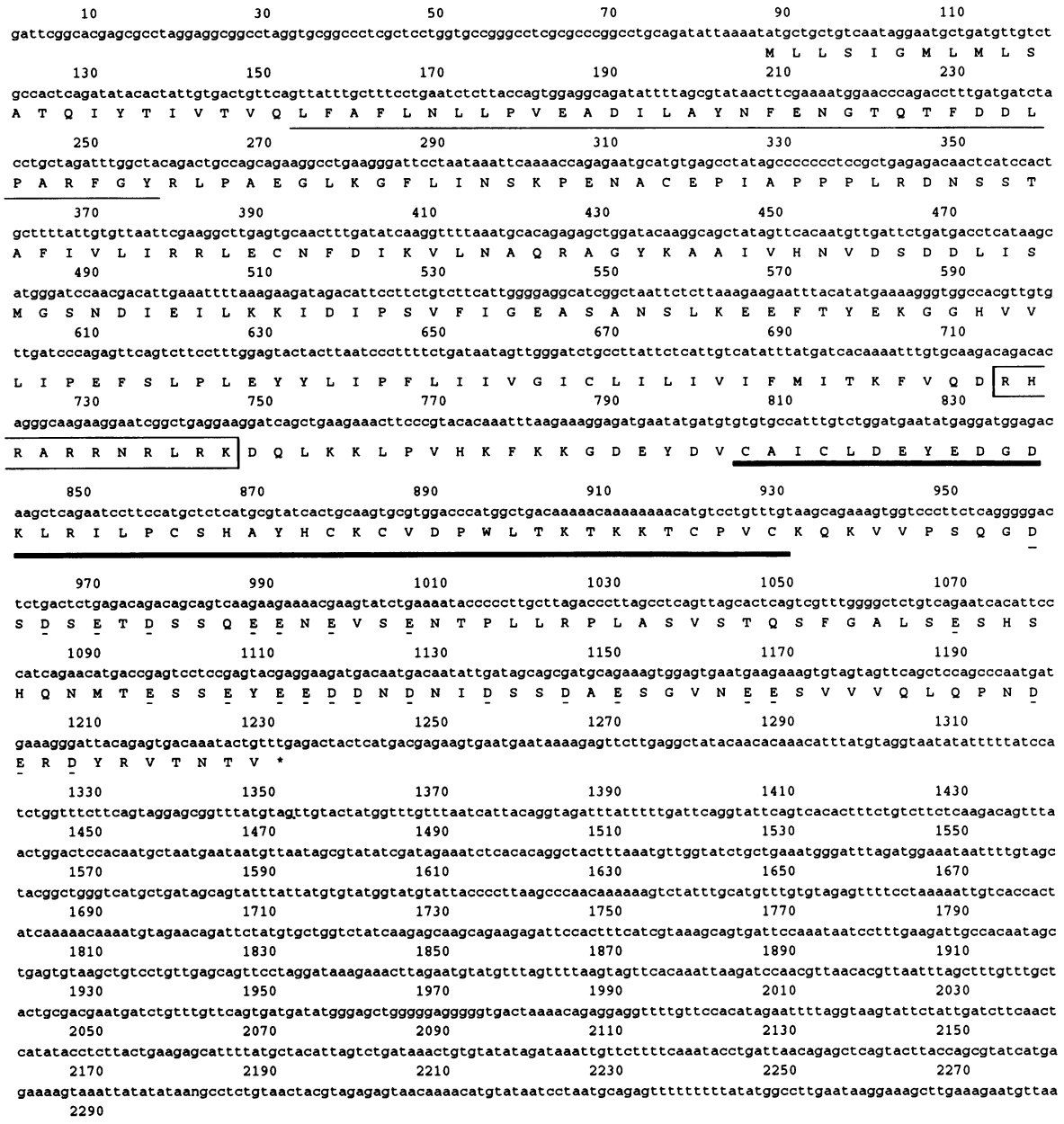

FIG. 1. C-RZF cDNA sequence. Nucleotide sequence and deduced amino acid sequence of C-RZF. The longest open reading frame contains 381 amino acids terminating at a TGA termination codon. The RING-finger motif is underlined with a thick line. The nuclear localization signal (NLS) motif is boxed. The leucine zipper motif is underlined with a thin line. The acidic region is indicated by underlined single letters.

formed into *Escherichia coli* BL21(NovaGen), and the fusion protein was purified (15). Nuclear and cytoplasmic protein extracts were isolated from the hearts of E9 chicken embryos (16) with a final step in which both fractions were clarified by centrifugation at 32,500 × *g*. Protein was determined by using a modified Lowry assay (Bio-Rad). After SDS/PAGE and transfer to nitrocellulose filters (17), immunoblots were performed with a polyclonal antibody to the C-RZF fusion protein. In addition, a monoclonal antibody to β-tubulin (Boehringer Mannheim) and a polyclonal antibody to lamin (provided by L. Gerace, ref. 18) were used as markers for cytoplasmic and nuclear compartments, respectively. Indirect immunofluorescence staining was done as described (19) using cultured chicken embryo cardiocytes provided by V. M. Fowler (20).

## RESULTS

**CT/TN-Binding Causes Changes in Gene Expression.** Subtractive hybridization was used to identify changes in gene expression that occur in CEB cells as a consequence of their binding to the ECM molecule CT/TN. One of the cDNA clones enriched in the (+)CT/TN subtracted library, the C-RZF cDNA clone, comprised 1.2% of the clones in that library as compared with fewer than 0.1% of the clones in the (−)CT/TN subtracted library. To confirm this change in gene expression, poly(A)$^+$ RNA from E8 CEB cells that were treated with CT/TN or untreated, as in the original experiment, were used to generate radiolabeled first-strand cDNA probes that were hybridized to duplicate slot blots containing C-RZF DNA. The hybridization signals were normalized against values for the small nuclear RNA, U6 (see ref. 1). Based on this analysis, the expression of the C-RZF gene, at the mRNA level, was 2.2-fold higher in cells treated with CT/TN compared with untreated cells.

The C-RZF cDNA clone was small (122 bp), and closer analysis of its sequence revealed that it was similar to a zinc-dependent RING-finger, or $C_3HC_4$ structure (reviewed in

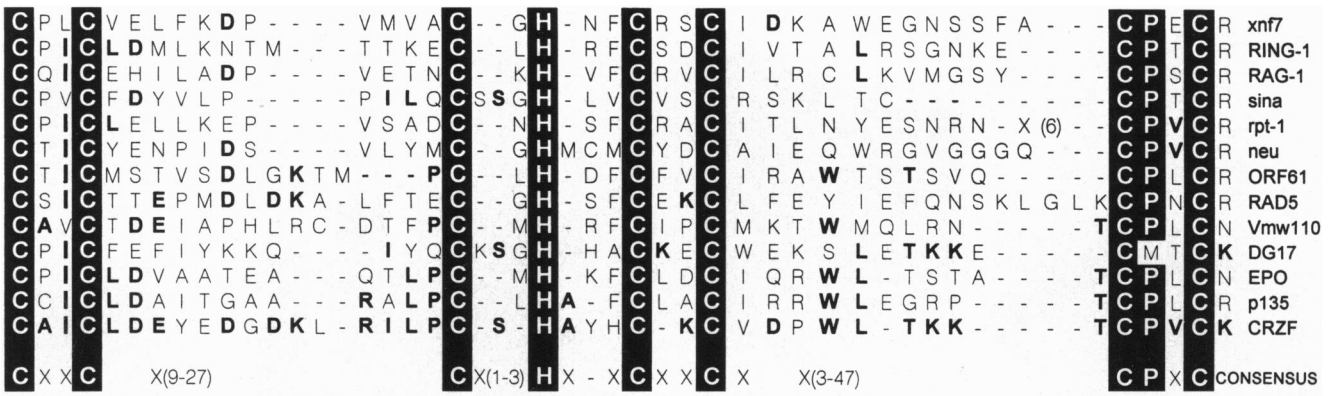


FIG. 2. Comparison of RING-finger motifs. The RING-finger consensus sequence is indicated in the bottom line, and the conserved amino acids are shown in white type within a black box. Other residues with identity to C-RZF are shown in boldface type. Amino acids X(9–27) and X(3–47) are variable regions. X(6) = (TDGKGN). Proteins: xnf7 = *Xenopus* nuclear factor 7 (22), RING1 (23), RAG-1 = recombination activating gene 1 (24), sina = seven in absentia (25), rpt-1 = regulatory protein, T-lymphocyte 1 (26), neu = neuralized (27), ORF61 = open reading frame 61 (28), RAD5 (29), Vmw110 (30), DG17 (31), EPO = early protein O (32), and p135 (33).

ref. 21). A CEB cDNA library was therefore screened to obtain larger cDNA clones. The largest of these cDNA clones (2.3 kb) was then sequenced on both strands (Fig. 1).

**Sequence Analysis of the C-RZF Clone.** The predicted protein sequence encodes 381 amino acids and contains a RING-finger motif (Fig. 1). The consensus RING-finger sequence and a comparison with other RING-finger proteins is shown in Fig. 2. The seven cysteine residues and one histidine residue are conserved in all members of this family. The sequences outside of the RING-finger had no significant homology with any sequences in the nucleic acid or protein data bases. However a visual examination of the predicted amino acid sequence revealed a potential NLS (Fig. 1) located at a site amino-terminal to the RING-finger motif. This sequence contains two overlapping hexapeptides that exactly meet the criteria for a core NLS (34). Both hexapeptides are flanked by acidic amino acids and a proline residue, contain four arginine or lysine residues, and do not contain aspartic or glutamic acids, phenylalanine, tyrosine, or tryptophan residues.

Preceding the NLS is a sequence that resembles a leucine zipper. Leucine zippers occur in $\alpha$-helical regions, and according to the Chou and Fasman predictive method (35), the structure of the putative C-RZF leucine zipper is likely to be $\alpha$-helical. The leucine zipper motif consists of repeats of seven amino acids in which leucine or some other hydrophobic amino acid occupies position 1 and an uncharged or hydrophobic amino acid occupies position 4 (reviewed in refs. 36 and 37). These repeats form two adjacent helical arms that can interact with the similar arms of another leucine zipper on another molecule. Within the C-RZF sequence are five such repeats, consistent with the four to five repeats normally found in a leucine zipper.

**Embryonic Expression of the C-RZF Gene.** Northern blot analysis of E8 chicken embryo tissues with a 1200-bp C-RZF radiolabeled insert as a probe indicated that the mRNA encoding this molecule is present as a single mRNA species of $\approx$3.2 kb in neural tissue and as a 3.0-kb mRNA in heart tissue (Fig. 3a). It was not detected in gizzard or liver.

The C-RZF protein appears to be encoded by a single gene in chicken, as revealed by Southern blots of genomic DNA digested with *Eco*RI, *Hin*dIII, and *Pst*I and probed with the same probe used for the Northern analysis. In each case, the probe hybridized to only one band (Fig. 3*b*).

*In situ* hybridization revealed expression of the C-RZF mRNA in some regions of the brain and spinal cord (Fig. 4). The optic lobe and forebrain had the highest levels of expression of the brain structures showing hybridization. The optic lobe from E8 chicken embryos is divided into neuroepithelium and four distinct layers containing postmitotic neurons. The highest level of expression was seen in the ventricular zones, a region of cell proliferation. High levels of staining were also seen in layer II but not in layers I or III–IV. In the retina, a moderate to high level of expression was seen only in the optic fiber layer; there was no expression in the inner or outer plexiform layers. In the rostral telencephalic region of the chicken forebrain, a moderate to high level of expression was homogeneously distributed in the basal (B1-4) and dorsal (D1-3) zones. Some basal zones had high levels of expression, as did the trigeminal and dorsal root ganglia. Moderate levels of expression were observed in the dorsal mesencephalon and rhombencephalon and in the spinal cord. Lower levels of

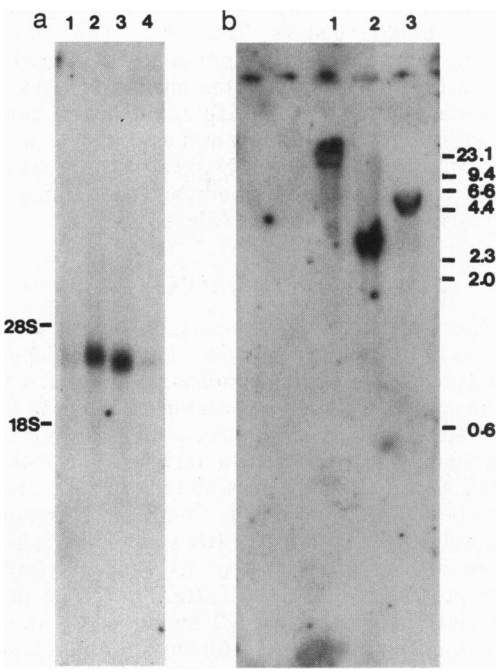


FIG. 3. Northern and Southern blots with a C-RZF cDNA probe. (*a*) Total chicken embryo RNA (10 $\mu$g) from day 8 gizzard (lane 1), brain (lane 2), heart (lane 3), or liver (lane 4) was electrophoresed on 0.8% formaldehyde gels, transferred to Immobilon-N$^+$ (Amersham), and probed with the C-RZF cDNA insert. Positions of 28S and 18S rRNA are indicated. (*b*) Recognition of genomic DNA by the C-RZF probe. Adult chicken genomic DNA (10 $\mu$g) was digested with *Eco*RI (lane 1), *Hin*dIII (lane 2), and *Pst* I (lane 3); electrophoresed on a 0.8% agarose gel; transferred to Immobilon N$^+$; and probed with the C-RZF cDNA insert. The relative migrations of the molecular weight standards are indicated at the right in kilobases.

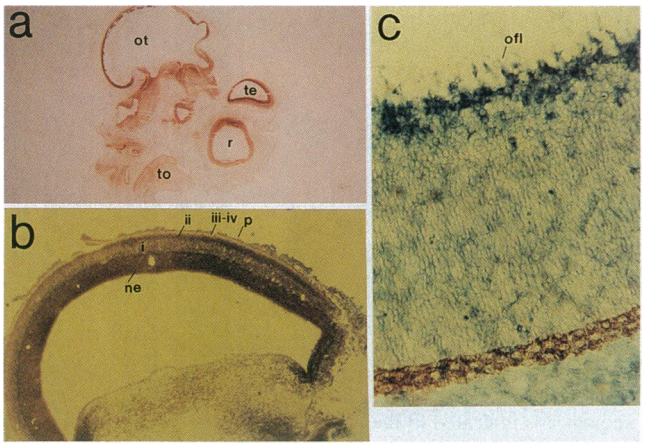


FIG. 4. *In situ* hybridization of day 8 CEB with a C-RZF probe. (*a*) Sagittal section of a day 8 chicken embryo head showing C-RZF mRNA expression in the retina and different brain regions. The highest levels of expression were in the retina (r), telencephalic region (te), optic tectum (ot), as well as the dorsal region of the brainstem. Expression was low or absent in the nonneural tissue encompassing the brain (to = tongue). (*b*) Sagittal section of the optic tectum at higher magnification. C-RZF mRNA expression was localized in the neural epithelium (ne) and layer ii, but not in layers i or iii–iv (p = pial surface). (*c*) Sagittal section of the retina at higher magnification. Cells containing C-RZF mRNA were found exclusively in the optic fiber layer (ofl).

expression were detected in the heart and blood vessels. No expression for C-RZF was observed in the liver or gut.

**Subcellular Localization of the C-RZF Protein.** To establish the subcellular localization of the C-RZF protein, embryonic heart tissue was fractionated into nuclear and cytoplasmic fractions, and proteins in each of these fractions were analyzed by a Western blot analysis. The results (Fig. 5*a*) clearly demonstrate that the C-RZF protein is restricted to the nuclear fraction. The purity of the nuclear and cytoplasmic fractions was determined by using antibodies to lamin as a nuclear antigen and to β-tubulin as a cytoplasmic marker. In addition, cultures of chicken embryo cardiocytes were immunostained by using the same antibody, and the staining was also found exclusively in the nucleus (Fig. 5*b*).

## DISCUSSION

In an effort to determine the extent to which cell–matrix interactions influence the expression of other developmentally significant genes, subtractive hybridization was used to identify changes in gene expression that occur in CEB cells in response to their binding to the ECM molecule CT/TN. In the course of these studies, we identified a number of cDNA clones encoding CAMs, ECM molecules, and ribosomal proteins that were enriched in the library made from CT/TN-treated cells. One of the cDNA clones, whose mRNA expression is increased by the binding of CT/TN to neurons, encodes a previously unknown protein, C-RZF. The C-RZF mRNA contains an open reading frame encoding 381 amino acids. Although it shows no significant homology with any known protein, it has sequence motifs that predict a number of protein structures, including a RING-finger, a leucine zipper, a nuclear localization signal, and an acidic activation domain.

Initial Northern blots and *in situ* hybridization analyses of E8 chicken embryonic tissues indicate that expression of the C-RZF gene is predominant in the brain and heart at this stage. In E8 chicken embryos, the C-RZF gene appears to be expressed in brain structures that also express CT/TN. A more thorough analysis of the C-RZF expression pattern will determine how extensively the expression pattern overlaps that of CT/TN.

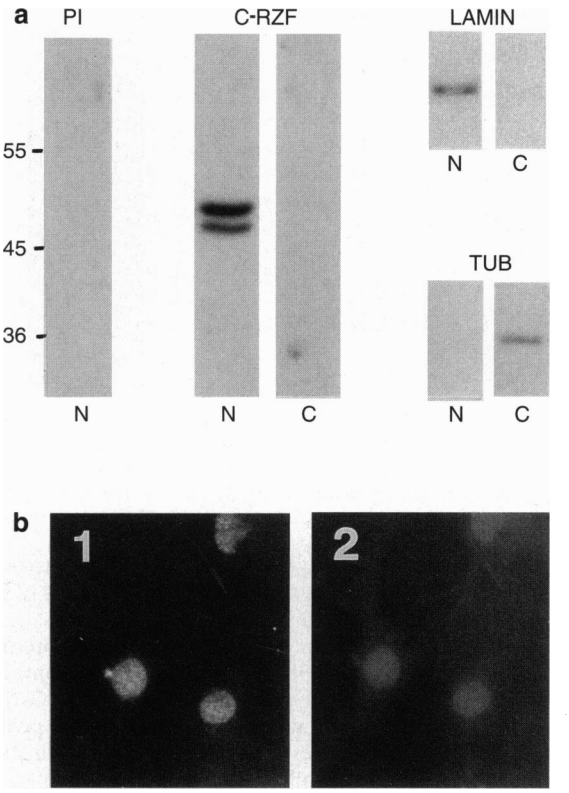


FIG. 5. Subcellular localization of the C-RZF protein. (*a*) Autoradiogram of immunoblots showing nuclear (lanes N) and cytoplasmic (lanes C) fractions of chicken embryo heart cells probed with preimmune serum (PI), antibodies to the C-RZF fusion protein, β-tubulin (TUB), and lamin. (*b*) Immunofluorescence staining of cultured chicken embryo heart cells. (*Left*) Staining with anti-C-RZF antibodies. (*Right*) Cells of the same field stained with the DNA-specific Hoechst 33258 (Molecular Probes).

The presence of a RING-finger motif in the C-RZF sequence suggests that this protein may bind to DNA. Most RING-finger proteins are localized in the nucleus and are involved in processes concerned with nucleic acid recognition (reviewed in ref. 21). More than 40 proteins containing this motif have been identified from a variety of organisms, although their sequences outside of the RING-finger are quite different. The structure of the RING-finger itself is similar to the other classes of zinc fingers in that they all use either cysteine or histidine residues as zinc ligands. The sequences within the C-RZF RING-finger (Fig. 2) are most similar to the bovine herpesvirus p135 protein (33) and also closely resemble the viral immediate early and early gene product EPO (32). The p135 protein is known to be a transcriptional regulator, and the EPO protein is postulated to function as one.

Although a very diverse group of proteins contain RING-finger motifs, many have been found to play significant roles during development. These include the *Drosophila* protein encoded by seven in absentia (*sina*; ref. 25), the *Drosophila* neuralized (*neu*) gene (27), and two related neighboring *Drosophila* genes, Posterior sex combs (*Psc*) and Suppressor two of zeste [*Su(z)2*] (8, 38). Other proteins containing a RING-finger motif include the products of several oncogenes (reviewed in ref. 21), RAG-1, a protein that activates immunoglobulin gene recombinations (24), and RAD5, a 134-kDa protein from yeast that functions in error-free postreplication DNA repair (29).

In addition to the RING-finger motif, the C-RZF protein contains a region with features of a leucine zipper that may function to homodimerize the C-RZF protein or allow it to interact with other proteins. The leucine zipper occurs in a

number of transcription factors such as Fos and Jun and is normally preceded by a region of basic amino acids that bind DNA (reviewed in ref. 37). While often located in the carboxyl-terminal region of proteins containing them, leucine zippers are not restricted to this region. They can occur at the amino terminus of a protein, both with and without a basic region [e.g., meq (39) and IFP (40, 41)]. In addition, leucine zippers have also been identified in proteins without a basic region but containing other DNA-binding domains, such as zinc fingers (e.g. cbl-b, ref. 40) or homeodomains (e.g., Athb-3, ref. 42). The leucine zipper in the C-RZF protein is located at the amino terminus and is not preceded by a basic region, suggesting that, in C-RZF this domain may be involved in protein–protein interactions.

It is noteworthy that the C-RZF protein contains a region of acidic amino acids near the carboxyl terminus, a feature seen in many transcription factors. Transcription factors normally contain two domains, one for binding to DNA and the other for activating transcription. The most common family of activation domains are the acidic activators, sometimes referred to as acid blobs. These domains consist of a sequence rich in acidic amino acids but lacking any obvious common motif or sequence constraints (43, 44).

Further characterization of C-RZF may help to clarify the function of this protein, which we have found to be located mainly in the cell nucleus. This result is consistent with the presence of two NLS motifs (34). This finding and the presence of an acidic stretch similar to those of transcriptional activators open the possibility that C-RZF may function in regulating gene expression.

We have shown that the expression of the C-RZF gene increased when embryonic brain cells were incubated with the ECM protein CT/TN and that the expression pattern of the C-RZF gene overlaps that of CT/TN in some regions of the E8 embryo. Recently we have found that the C-RZF protein binds to DNA (unpublished data). Given these results, the C-RZF protein may be a regulatory molecule that is part of a gene network related to CT/TN expression. Alternatively, it may link cell adhesion or binding events at the cell membrane to changes in the nucleus that subsequently affect cell shape, migration, or differentiation.

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