Many short-lived mRNAs contain AU-rich instability elements within their 3′-untranslated region. Cellular factors that bind to these elements are thought to play a role in the regulation of mRNA degradation. In the accompanying paper (Chagnovich, D., and Cohn, S. L. (1996) J. Biol. Chem. 271, 33580–33586) we characterized the binding activity of a 40-kDa protein (p40) that interacts with high specificity with at least two AU-rich elements located within the 3′-untranslated region of N-myc. p40 activity correlates with N-myc mRNA stability in subclones of the NB-L W neuroblastoma cells line (W-N and W-S). In an effort to determine the identity of p40 we performed immunoblotting studies, immunoprecipitation experiments, and RNA gel mobility shift assays using antibodies that are directed against known RNA-binding proteins. In this paper we demonstrate that in W-N and W-S cells, p40 activity parallels the expression of embryonic lethal abnormal vision (ELAV)-like proteins, and that antibodies directed against this family of RNA-binding proteins recognize p40. We also show that purified ELAV-like proteins (HuD and Hel-N1) bind with high specificity to the same N-myc 3′-untranslated region sequences as p40. Our data indicate that p40 is a member of the ELAV-like family, and suggest that this family of RNA-binding proteins may regulate N-myc mRNA turnover.

The RNA-binding protein embryonic lethal abnormal vision (ELAV)1 is essential for proper development of the Drosophila nervous system (1, 2). Mutations in the elav locus result in a dysfunctional nervous system in which neuroblasts continue to proliferate, fail to differentiate, and migrate inappropriately (1). Furthermore, studies of flies expressing temperature-sensitive elav demonstrate that at the restrictive temperature the flies lose the ability to hop and fly (3, 4). Recently, a number of proteins highly homologous to ELAV have been identified in various species including Xenopus, zebrafish, mouse, and man (5–9). ELAV-like proteins are first detectable in neurogenic precursor cells that have exited the ventricular zone of the spinal cord, and expression continues in mature neurons (10).

The human homologues HuD and HuC/pLE21 were independently discovered as target antigens in patients with small cell lung cancer-associated paraneoplastic neurologic syndrome (6, 7), Hel-N1 was cloned due to its homology with the elav RNA-binding domain (8), and HuR was cloned due to its homology with HuD (9). Together these data suggest that vertebrate homologues of ELAV may also play a role in the development and differentiation of neurons.

The molecular mechanisms underlying the function of ELAV-like proteins remain unknown. However, because all ELAV-like proteins contain three copies of the RNA recognition motif (5, 6, 11), it is thought that these proteins promote neuronal differentiation by regulating the expression of genes that control cell proliferation and differentiation at the post-transcriptional level. In support of this hypothesis, HuD, HuR, and Hel-N1 have recently been shown to bind with high affinity to AREs within the 3′-UTR of mRNAs for genes involved in cellular growth, such as c-myc and c-fos, and neuronal differentiation, such as Id (8, 9, 12–14). Because many immediate early mRNAs are targeted for rapid degradation by virtue of their AREs (15, 16), these observations suggest that this family of RNA-binding proteins may be important regulators of mRNA turnover.

Previously, we and others have demonstrated that many NB cell lines contain two phenotypically distinct cell types; neuroblastic cells (N) and substrate adherent cells (S) (17–20). N cells exhibit anchorage independent growth and readily form tumors in nude mice, whereas S cells are not tumorigenic (21–23). In addition, N cells express higher levels of the N-myc mRNA compared to S cells (20). In the accompanying paper (46), we demonstrate that mRNA stability is a major regulatory step governing the expression of N-myc in the N (W-N) and S (W-S) subclones of the human NB cell line NBL-W. Furthermore, we show that a 40-kDa protein (p40) binds with high specificity to at least two AU-rich sequences within the N-myc 3′-UTR, and that p40 binding correlates with N-myc mRNA stability and neuroblastic phenotype. In this paper we demonstrate that ELAV-like protein expression parallels the activity of p40 in NB cell lines, and that antibodies directed against human ELAV-like proteins recognize p40. In addition, we show that purified HuD and Hel-N1 proteins bind with high specificity to the same N-myc AU-rich sequences as p40. These studies indicate that p40 is a member of the ELAV-like protein family.

MATERIALS AND METHODS

NB Cell Lines—The NB cell lines NBL-W-N and NBL-W-S (20) and LA1–55n and LA1–5s (22) have been described previously. NB cell lines

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1 The abbreviations used are: ELAV, embryonic lethal abnormal vision; ARE, adenosine uridine-rich element, 3′-UTR, 3′-untranslated region; NB, neuroblastoma; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; hnRNP, heterogeneous ribonuclear protein; GST, glutathione S-transferase.
were cultured in RPMI-1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum, penicillin, and antibiotics at 37 °C, 5% CO2. At each passage and before every experiment, the morphology of the cultured cells was examined.

**Western Blot Analysis**—Twenty-five μg of cellular extract or cellular equivalents of cell fractions, or approximately 1 μg of purified GST-HuD (12) or partially purified g10-tagged Hel-N1 (14) protein was resolved by 12% SDS-PAGE. HuD protein was generously provided by Dr. Henry Furenaux (Memorial Sloan Kettering Cancer Center) and Jack Keene (Department of Microbiology, Duke University Medical Center) provided Hel-N1 protein. Proteins were transferred to nitrocellulose using standard methods (24). Blots were blocked in 10% non-fat dried milk in phosphate-buffered saline for greater than 2 h at room temperature with gentle rocking. The blots were then incubated at room temperature overnight with a 1:1000 dilution of monoclonal antibody 16A11 (4 µg/ml), also provided by Dr. Henry Furenaux (10), preimmune rabbit serum or anti-AUF1 polyclonal serum (1:000 dilution), a generous gift from Dr. Gary Brewer (Wake Forest University) (22) or 1.000 dilution of monoclonal antibodies 9H10 or 4F4 (ascites) (27–29), known to react with GST and huRNCP1C2, which were that graciously provided by Dr. Gideon Dreyfuss (Howard Hughes Medical Institute, University of Pennsylvania School of Medicine). After at least 2 h of incubation the blots were rinsed three times with phosphate-buffered saline and washed with phosphate-buffered saline three times for 10 min with gentle shaking. Secondary antibody labeled with horseradish peroxidase (KPL, Gaithersburg, MD) was diluted 1:2000 in blocking solution and incubated on the blot for 1 h at room temperature. Blots were washed as above and developed using the ECL chemiluminescence procedure as described by the manufacturer (Amersham). Results were visualized using Hyperfilm (Amersham).

**Immunoprecipitation of Bound Complexes**—Fifty μg of cytoplasmic extract from W-N cells were incubated with radiolabeled RNA (10 µg of NMBS1 or NMBS2 probe), and subjected to RNA UV cross-linking, as described below. The assay volumes were brought up to 600 µl in TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) plus 1% Nonidet P-40, antibody was added, and the mixture incubated at 4 °C overnight with gentle rocking. Twenty μl of packed volume of Protein A/G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) was added, and the reaction was incubated an additional 2 h at 4 °C. Complexes were collected by centrifugation and the supernatant was removed to a fresh tube for precipitation.

**Semi-quantitative Reverse Transcriptase-Polymerase Chain Reaction**—Fetal brain poly(A) mRNA was obtained from Clontech (Palo Alto, CA). Cytoplasmic RNA was extracted from cell lines using Nonidet P-40/urea method as described elsewhere (30). The amount of RNA used in the reactions was titered over a broad range, and 1 μg of cytoplasmic RNA or 10 ng of poly(A) mRNA was found to be suitable for these experiments. RNA was reverse transcribed with random hexamers using standard procedures (31). One-tenth of the reverse transcription reaction was used for amplification with [α-32P]dCTP (3000 Ci/mmol) and primer pairs specific for HuD (upstream 5′-CCAGGCCCCGTCCACG-3′; downstream 5′-GCTTCTCTTACCCATTAC-3′; pNMBS1-HuD) or Hel-N1 (upstream 5′-AACAACCAAGTTGAGACACAG-3′; downstream 5′-TTTGTCAGAAATGGACAC-3′), Hel-N1 (upstream 5′-GTATCAGGAGCCTCAGTAC-3′, upstream 5′-TTATATTCCTCAGCCAAACG-3′), and β2-microglobulin (upstream 5′-CTGAATTCACCCACCTGCTG-3′, downstream 5′-TTCTATCATATAAACCTTGG-3′) as described previously (6). One-tenth of the reaction was resolved by denaturing PAGE, stained with Coomassie Blue to verify protein loading, dried, and exposed to x-ray film for 1–7 days with intensifying screens.

**RESULTS**

**ELAV-like Protein Expression and Localization Correlates with p40 Binding in the W-N and W-S NB Cells**—As a first step toward identifying p40, immunoblotting experiments were performed using antibodies directed against known RNA-binding proteins that are approximately 40 kDa in size, to determine if the pattern of expression of any of these proteins in W-N and W-S cells correlates with the differential binding activity observed with p40. As shown in Fig. 1A, serum containing anti-Hu human autoantibody detects a strong 40–42-kDa doublet in whole cell extracts derived from the W-N cells, while a faint/undetectable signal is seen in extracts derived from W-S cells. Immunoblotting with anti-Hu monoclonal antibody 16A11 detects a single 40-kDa peptide which is W-N cell specific. In contrast, antibodies against the AUF1, hnRNPA, and hhnRNPC proteins detect equivalent levels of proteins of the predicted size in both the W-N and W-S cell extracts.

Since p40 binding activity is found predominantly in the cytoplasm, we next investigated if, in the W-N and W-S cell lines, ELAV-like protein expression and p40 binding activity co-localize to the same cellular fraction. Cellular fractions were subjected to Western blot analysis (Fig. 1B), and corresponding fractions were analyzed for p40 activity by RNA cross-linking assays using an RNA probe that corresponds to the p40-binding site located in the N-myc 3′-UTR (NMBS1) described in the accompanying paper (46) (Fig. 1C). ELAV-like protein expression and p40 activity were mainly detected in the postnuclear (S20) fraction of W-N cells, with smaller amounts found in the nucleus (N). Further fractionation of the cytoplasm demonstrates that both ELAV-like protein expression and p40 binding activity co-localize with the high speed pellet corresponding to the polysomal fraction (P), with only a small portion in the S20 fraction of W-N cells, with smaller amounts found in the nucleus (N).
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W-N and W-S cells and the N-myc NMBS1 RNA probe (Fig. 2). This complex (bracket) was super-shifted (arrow) in the presence of anti-HuD monoclonal antibody 16A11. No super-shift was seen in the W-S cells. The addition of HuD epitope-specific peptide HuDpep (10) blocks the super-shift of the W-N-specific complex seen with 16A11 antibody. As expected, the complex remains super-shifted in the presence of a nonspecific peptide, HuDmex (10).

We next sought to determine if the complex super-shifted by monoclonal antibody 16A11 is the p40 complex described in the accompanying paper (46). RNA cross-linking assays were performed with W-N cytoplasmic extracts and N-myc NMBS1 RNA. Following cross-linking, immunoprecipitation experiments were performed with antibodies directed against ELAV-like proteins and the RNA-binding proteins AUF1, hnRNPA, and hnRNPC C1/C2. As shown in Fig. 3, lane 1, the 40-kDa complex is specifically precipitated with monoclonal antibody 16A11, and completely removed from the supernatant. The other antibodies tested were unable to immunoprecipitate this complex. In experiments performed with the AUF1, hnRNPA, and hnRNPC antibodies, the 40-kDa complex is found exclusively in the post-precipitation supernatant.

**elav-like mRNA Expression in the W-N and W-S Cell Lines**—
Due to the high homology between the known ELAV-like proteins, none of the available antibodies demonstrate specificity for one specific family member. We, therefore, performed RT-PCR to investigate which members of the elav-like family are expressed in the W-N and W-S cell lines. Expression was also examined in two additional subcloned neuroblastoma cell lines (LA1–55n and LA1–5s) which also demonstrate differential p40 binding activity and expression (accompanying paper (46) and data not shown). A reverse transcriptase-dependent product was detected for all primer pairs on analysis of the infant brain poly(A) RNA. HuD-, Hel-N1, and Hel-N2-specific products can be detected in all the NB cell lines, although the quantities of products appear to differ relative to the β2-micro-
Interestingly, others have shown that in normal tissues, fractionation, both ELAV-like protein expression and p40 activity. In addition, when the W-N cells were subjected to cell fractionation, both ELAV-like protein expression and p40 activity were detected with the polysomal fraction of the cytosol. Interestingly, others have shown that in normal tissues, ELAV-like proteins are expressed in W-N cells but not W-S cells, paralleling the pattern of p40 activity. Using RNA binding assays several groups have identified cellular factors that bind to AREs located within the 3'-UTR of many immediate early mRNAs (8, 9, 12–14, 26, 32, 37, 38). Although the precise functions of these proteins remain unknown, it is believed that these factors regulate ARE-mediated mRNA degradation. In the accompanying paper (46), we characterized the binding activity of a 40-kDa protein (p40) that interacts with high specificity with at least two N-myc AU-rich sequences. In subclones of the NB cell line NBL-W (W-N and W-S) p40 binding correlates with enhanced N-myc mRNA stability, increased levels of steady-state N-myc expression, and a tumorigenic phenotype. The studies described in this paper indicate that p40 is a member of the ELAV-like RNA-binding protein family.

We have shown that ELAV-like proteins are expressed in W-N cells and are associated with polysomes. In their studies, significantly higher levels of Hel-N2, an alternatively spliced form of the Hel-N1, were detected in brain tumor cells compared to normal tissues, suggesting that alternate splicing may result in differential location and/or function within the cell. Alternately, we find low levels of Hel-N1 and Hel-N2 in the cytosol of W-N and W-S cell lines, while both cell lines express full-length HuD. In contrast, Hel-N2 is the predominant transcript in the polysomal cell fraction of LA1–55n and LA1–5s NB cell lines, indicating that alternate splicing may be a cell-specific phenomena.

To further determine the identity of p40 we performed gel mobility shift assays and immunoprecipitation experiments using antibodies directed against known RNA-binding proteins. Only the addition of antibody directed against ELAV-like proteins resulted in a super-shift of an RNA-protein complex unique to the W-N cells. Furthermore, monoclonal antibody 16A11 immunoprecipitated the p40 RNA-protein complex, indicating that p40 is a member of the ELAV-like RNA-binding protein family. Recently several studies have demonstrated that members of the ELAV-like protein family bind with high specificity to AU-rich instability determinants located within...
the 3′-UTR of rapidly degraded mRNAs. Hel-N1 can bind sequences within the 3′-UTR of c-myc, c-fos, and Id (8, 14); HuR has been shown to bind to the AREs of c-myc, N-myc, and interleukin-3 (9); and both HuR and HuD specifically bind the AU-rich sequences in the c-fos 3′-UTR known to function as destabilizing elements (9, 12). Our binding studies indicate that N-myc is another potential RNA ligand for HuD and Hel-N1.

Because regulated expression of N-myc is crucial for normal neural crest differentiation (43, 44), our studies support the idea that ELAV-like proteins play a role in neuronal differentiation. However, in contrast to our results, Levine and co-workers (14) reported that Hel-N1 did not bind to the N-myc 3′-UTR. The probes used by these investigators consisted of just 632 bases of the N-myc 3′-UTR, and the actual sequence was not published. Our binding sites are separated by over 700 bases. It is, therefore, possible that these investigators used the intervening N-myc sequences, which we have shown do not bind the ELAV-like proteins. Similar to what has been demonstrated with the c-fos ARE, our studies show that both HuD and Hel-N1 bind with comparable affinities, suggesting a redundant activity for these proteins (13).

In summary, the results presented here and in the accompanying paper (46) suggest that ELAV-like proteins may mediate the turnover of the N-myc message in human. At the present time, the molecular mechanism by which these RNA-binding proteins function remains to be determined. However, because the half-life of N-myc mRNA is prolonged in the NB cells that express abundant quantities of ELAV-like protein, we believe that binding of these factors to AREs within the 3′-UTR of N-myc may protect the mRNA from degradation enzymes. Prokipcak and colleagues (45) have proposed a similar function for a 70-kDa RNA-binding protein that interacts with a c-myc coding region instability element. Further study of the interactions of N-myc and ELAV-like proteins should lead to a better understanding of the function of these RNA-binding proteins and their role in NB.

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