Binding of a 40-kDa Protein to the N-myc 3'-Untranslated Region Correlates with Enhanced N-myc Expression in Human Neuroblastoma*

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Subclones of neuronal (N) and non-neuronal (S) cells established from neuroblastoma tumors cultured in vitro differ in their growth characteristics and N-myc expression. N (W-N) cells derived from the NBL-W cell line express 5-fold higher levels of N-myc mRNA and 10–12-fold higher levels of protein than S cells (W-S), despite having the same N-myc copy number. This study demonstrates that the steady-state levels of N-myc are largely determined by differences in N-myc mRNA stability. The half-life of N-myc mRNA in the W-N cells is ~35 min compared with ~6 min in the W-S cells. Turnover of labile mRNAs is thought to be mediated in part by the interactions of trans-acting factors with elements within the 3'-untranslated region. RNA UV cross-linking assays using W-N cell lysate demonstrate abundant quantities of a protein complex that is 40 kDa in size (p40) that binds to the N-myc 3'-untranslated region. p40 is barely detectable in W-S cells. We have mapped two distinct regions within the 3'-UTR that specifically bind p40 (base pairs 5694–5715 and 6465–6482). Analysis of nine additional neuroblastoma cell lines shows that p40 activity correlates with enhanced expression of N-myc. p40 activity is also detected in 5 of 19 primary neuroblastomas, and activity is associated with clinically aggressive disease. In the accompanying study, we identify p40 as a member of the embryonic lethal abnormal vision (ELAV)-like family of RNA-binding proteins. Our studies suggest that ELAV-like proteins may play a role in the regulation of N-myc mRNA turnover and thereby modulate the steady-state levels of N-myc expression and tumor cell phenotype.

NB, a common neoplasm in children, arises from migrating neural crest cells (1). Reflecting the multipotent potential of neural crest tissues, these tumors are composed of a variety of cell types including neuroblasts, ganglion cells, Schwann cells, and rarely melanocytes (2, 3). Heterogeneous cellular subpopulations are also observed when human NB cells are cultured in vitro, and subclones consisting of neuroblastic cells (N) and non-neuronal cells that grow tightly adherent to the substratum (S) have been established (4–7). In addition to the unique morphological features of these two populations of cells, N and S cells also have distinct immunophenotypic, biochemical, and growth characteristics (5–7). Most S cells lack neuronal markers, fail to grow in soft agar, and are not tumorigenic in nude mice, whereas N cells express genes associated with neuronal differentiation, exhibit anchorage-independent growth and readily form tumors in nude mice (7–10). Subclones of N and S cells can spontaneously interconvert from one cell type to the other (6, 7, 10).

S cells derived from NB cell lines express lower levels of steady-state N-myc mRNA and protein than N cells (7–9, 11). The 5-fold disparity in steady-state N-myc mRNA expression in the N (W-N) and S (W-S) cells subcloned from the NBL-W NB cell line is not due to alterations in N-myc gene copy number, since both cell types contain ~100 copies of the gene (7). In this study we show that while N-myc is regulated at both transcriptional and post-transcriptional levels in both subclones, the lower steady-state level of N-myc in the W-S cells is largely determined by an increase in turnover of the N-myc mRNA.

Altering mRNA stability provides a powerful means for controlling the steady-state levels of gene expression, and others have shown that the metabolic lifetime of mRNA can be specified by cis-acting elements. Many labile mRNAs including N-myc contain AREs within their 3'-UTRs (for a review see Ref. 12). The AREs from the 3'-UTRs of c-fos, c-myc, granulocyte-macrophage colony-stimulating factor, and β-interferon function as RNA-destabilizing elements (13–16). Recently, several ARE-binding proteins have been identified, and it is thought that these proteins may influence the degradation of mRNA (16–23). Using RNA UV cross-linking assays we show that W-N cells contain abundant quantities of a protein complex 40 kDa in size (p40) that interacts with at least two distinct AU-rich sequences within the 3'-UTR of N-myc with high specificity, whereas p40 is barely detected in W-S cells. We also demonstrate that p40 activity correlates with enhanced steady-state levels of N-myc expression in NB.

MATERIALS AND METHODS

Cell Lines and NB Tumors—The NB cell lines NBL-W-N (7), NBL-W-S (7), LA-N-5 (24), IMR-5 (24), LA1–55a (9), LA1–5s (9), SK-N-SH (5), SH-SY5Y (5), SH-EP (5), GI-ME-N (25), and NBL-S (26) have been described previously. NB cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, glutamine and antibiotics at 37°C, 5% CO₂. At every passage and before each experiment, the morphology of the cultured cells was examined.

Primary tumors obtained from Chicago area hospitals between February 1986 and October 1994 were dissociated with collagenase and

(Received for publication, August 6, 1996)
DNase. Single cell suspensions were then placed over a Ficoll-Hypaque gradient (Pharmacia Biotech Inc.), and viable tue cells were counted. Approximately 1–5 × 10^5 tumor cells were viable frozen in (CH_3)_2SO and stored in liquid nitrogen until further analysis.

Soft Agar Colony Assay—W-N and W-S cells (2 × 10^2) were plated as described previously (27), and colonies were counted on day 28 with a Plaque Viewer (Bellco, Vineland, NJ).

Nude Mice Studies—Three- to four-week-old female athymic nude mice (BALB/c nu/nu) were given subcutaneous injections of 5 × 10^6 cultured W-N and W-S cells suspended in 0.2 ml of phosphate-buffered saline into the left and right flanks, respectively. The mice were housed in a laminar flow caging system and examined weekly. The time to detectable 5-mm diameter tumors was measured.

CAT Assays—W-N and W-S cells were co-transfected with 10 μg of N-myc CAT 2.1 RI-P CAT and 0.2 μg of pCH110, an SV40-β-galactosidase fusion construct (Pharmacia), using calcium phosphate precipitation as described previously (27). N-myc 2.1 CAT consists of N-myc sequence extending from base −1877 to +151; N-myc 2.1 RI-P CAT consists of N-myc sequence −1877 to −887; and N-myc 2.1 P-HI CAT consists of N-myc sequence −887 to +151. The construction of the CAT reporter gene fusion plasmids has previously been described (28). After 48 h, the cells were harvested, and extracts were prepared by freeze-thaw lysis. Transfection efficiency was determined by measuring β-galactosidase activity. Adjusted amounts of protein were assayed for CAT activity using standard methods (29). CAT assays were performed in the linear range for quantitation. More than three independent experiments were performed with separate extracts.

Nuclear Run-Off Assays—Nuclei were isolated from 1 × 10^6 cells, and nuclear run-off assays were performed as described previously (30). Approximately 1 μg of double-stranded DNA inserts corresponding to the coding region of N-myc, N-myc exons 1, 2, and 3, and β-actin were slot-blotted onto nitrocellulose and hybridized to 1 × 10^6 cpm of labeled run-off RNA as described. Signals were quantitated by laser scanning densitometry and standardized to β-actin. Each experiment was analyzed in duplicate, and three separate experiments were performed.

Messenger RNA Half-Life Studies—Cells were plated and grown to 75–80% confluence and then refed with fresh media containing 5 μg/ml actinomycin D (Sigma) and incubated at 37 °C. Total cellular RNA was isolated by standard methods (31), and Northern blotting was performed with 40 μg of RNA as described previously (32). N-myc mRNA levels were quantitated using a Fuji PhosphorImager, and the N-myc signals were standardized to β-actin. Mean half-life and standard deviations were calculated from four independent experiments.

Preparation of Cellular Extracts—Crude cytoplasmic extracts were prepared using the freeze-thaw method as described (18).

Preparation of DNA Templates for in Vitro Transcription—Plasmid pMC10 and pMC10-2.1RII were kindly provided by James Malter (University of Wisconsin, Madison, WI) and described elsewhere (18). Constructs containing the N-myc 3′-UTR were generated by polymerase chain reaction using the following primers: primer 1, NM3UTR-5′ (5631–5649), 5′-GGCTCTAGACGTCGGCTGCTAG-3′; primer 2, NMUT262 (5928–5945), 5′-CCCAAGTCTACCTGTTGTCGATCC-3′; primer 3, NMUT290 (5962–5980), 5′-GGATCTACGGCAACAGAG-3′; primer 4, NMUT569 (6215–6236), 5′-CCCAAG-ATCTCTGTTAATCTTTACAGTCGCC-3′; primer 5, NMUT590 (6236–6251), 5′-GGCGACCCTCGCAAGTCTACAGGCAACAGAG-3′.

All oligonucleotides were synthesized and provided by standard methods (33). All oligonucleotides were synthesized and provided by standard methods (33). An unique restriction site, which is underlined, and a G or C clamp at the 5′-end. Polymerase chain reaction products were cloned into pCRII vector (Invitrogen, La Jolla, CA) according to the manufacturer’s instructions. NU1, which corresponds to N-myc base pairs 5631–6962, was generated using primers 1 and 3; NU2 (5928–6236) was generated using primers 2 and 5, and NU3 (6215–6607) was generated using primers 4 and 6. All constructs were sequenced to verify the sequence and orientation. 3′-deletions of NU1 were generated by linearizing the NU1 template with HindIII, AvaI, and BstE II restriction enzymes, resulting in templates corresponding to base pairs 5631–5720 (NU1-H), 5631–5752 (NU1-A), and 5631–5864 (NU1-B). Similarly, 3′-deletions of NU3 were generated by linearizing the NU3 template with BstI, Msel, or BstEII, resulting in templates corresponding to base pairs 6215–6345 (NU3-D), 6215–6411 (NU3-M), and 6215–6482 (NU3-B).

Templates corresponding to the p40 wild-type and mutant binding sites were constructed as follows. Sense and antisense oligonucleotides corresponding to the sites were synthesized so that when annealed overhangs would be generated corresponding to a 5′ HindIII site and a 3′ BamHI site. pGE6MZ (Promega) was digested with HindIII and BamHI, and the oligonucleotides were ligated in the sense orientation. All constructs were digested in both directions to verify the orientation and sequence of the insert.

In Vitro Transcription—Radiolabeled RNA was in vitro transcribed from linearized templates by standard procedures (34). Template DNA was removed by digestion with RNase-free DNase I for 1 h at 37 °C. Probes were purified using G25-micro spin columns (5 Prime → 3 Prime, Inc., Boulder, CO). Typically, a specific activity of 10^7 to 10^8 cpm/μg RNA was obtained.

RNA UV Cross-linking Assays—Twenty-five μg of cytoplasmic extract and 50,000 cpm radiolabeled RNA (~5 pmol) were incubated in a final volume of 20 μl in 15 mM HEPES, pH 7.8, 500 μM yeast tRNA, 1 mM dithiothreitol, 1 μM ATP, 10 mM KCl, and 10% glycerol at 30 °C for 15 min. Reactions were then exposed to 0.5 J of 254-nm UV light at a distance of 3 cm in a Stratalinker (Stratagene, La Jolla, CA). 20 units of RNase T1 and 1 μg of RNaseA were added to the cross-linked products and incubated 30 min at 37 °C. SDS loading buffer (6 μl) was added to each tube, the samples were boiled for 5 min, and the complexes were resolved by 10% SDS-polyacrylamide gel electrophoresis. Following electrophoresis, gels were stained with Coomassie Blue to verify protein loading, dried, and exposed to x-ray film for 18–100 h with intensifying screens. For competition assays, competitor RNA was added to the extract 10 min before the addition of labeled RNA; otherwise, the reactions were processed as above. Each RNA UV cross-linking assay was repeated at least three times using fresh lysates.

RESULTS

N-myc Steady-state Levels Correlate with the Growth Characteristics of W-N and W-S NB Cells—To investigate if the steady-state levels of N-myc mRNA and protein in the NBL-W N and S clones correlate with growth characteristics, assays for soft agar colony formation and tumor growth in nude mice were performed. Four weeks after plating W-N and W-S cells an average of 26.5 colonies/dish (range 20–33) were formed in dishes with the W-N cells. However, W-S cells failed to form colonies. Tumors grew in the left flank of all six mice inoculated with W-N cells within 17–90 days (mean 40 days ± 29 days). W-S cells were injected into the right flank of these animals, and only one right-sided tumor was detected after 39 days of observation. The tumors were dissected from the animals, and the cells were mechanically dissociated and seeded into tissue culture flasks. In each case, the cultured cells exhibited the morphologic features of N-type cells within 24 h. Thus, it is likely that the solitary right-sided tumor resulted from an interconversion from S to N cells.

Transcriptional Control of N-myc Expression in W-N and W-S NB Cells—N-myc promoter activity was determined by assaying levels of CAT activity in W-N and W-S cells transfected with N-myc-CAT fusion constructs. Surprisingly, although W-S cells express lower levels of steady-state N-myc mRNA than W-N cells, 15-fold higher levels of CAT activity were detected in the W-S cells transfected with N-myc 2.1 CAT than N cells (Fig. 1). Similar to previous studies (28), maximal activity was observed in experiments performed with 2.1 CAT P-HI. The assay was repeated several times using fresh lysate, and each experiment yielded very similar results.

To further investigate the rate of N-myc transcription and to verify that attenuation of the N-myc transcript was not present, nuclear run-off experiments were performed. After correcting the signal intensity for target insert size, these studies demonstrated that the labeled RNA hybridized equally to the three N-myc exon probes, indicating that there was no block to elongation of the N-myc transcript in either the W-N or W-S cells (Fig. 2). Furthermore, approximately 10-fold higher levels of N-myc transcription were observed in the W-S cells compared with the W-N cells at each run-off experiment performed. The disparity in steady-state levels of N-myc mRNA in W-N and W-S cells does not appear to result from alterations in initiation of transcription or attenuation of the N-myc transcript. In the HL-60 promyelocytic cell line only β-actin tran
cells co-transfected with N-myc-CAT fusion constructs. N-myc promoter sequences are designated by the open box. N-myc exon 1 (bases +1 to +151) sequences are represented by the hatched box. Restriction sites within the N-myc promoter utilized for plasmid construction are indicated: EcoRI (RI), PstI (P), and BamHI (HI). Lower panel, CAT assays performed as described under “Materials and Methods” with W-N (N) and W-S (S) NB cells co-transfected with N-myc-CAT fusion plasmids and the β-galactosidase-expressing plasmid pH110.

**Fig. 2.** Nuclear run-off transcription assay. Labeled runoff transcripts from W-N (N), W-S (S), and HL-60 cells were hybridized to double-stranded DNA inserts corresponding to the entire N-myc coding sequence (indicated as cDNA), each of the N-myc exons, and β-actin as described under “Materials and Methods.” β-actin was present on the same filters, but the intervening lanes have been removed. N-myc signals were standardized to the β-actin signal.

Stability of N-myc Transcripts in W-N and W-S NB Cells—To determine whether the differential steady-state levels of N-myc in the W-N and W-S cells result from differences in mRNA degradation, the half-life of N-myc mRNA was measured using actinomycin D. After the addition of the RNA synthesis block, the time-dependent decay of the mRNAs was analyzed on Northern blots. β-Actin mRNA half-life was determined in all experiments as an internal long half-life control. The half-life of the 3.2-kilobase N-myc transcript in the W-N cells was approximately 35 min (± 10.7 min). However, the half-life of the N-myc mRNA in the W-S cells was extremely short, approximately 6 min (± 3.2 min) (Fig. 3). This experiment was repeated more than three times with fresh purified mRNA, and similar results were observed in each assay. Thus, a direct correlation exists between N-myc mRNA half-life and the steady-state levels of N-myc transcripts in the W-N and W-S cells, suggesting that the differences in N-myc mRNA stability are responsible, at least in part, for the disparities in the steady-state levels. Little β-actin mRNA degradation was observed during the 90 min of the study, indicating that message instability is not global in the W-S cells.

**W-N Cells Contain Abundant Quantities of a Protein Complex of 40 kDa**—As a first step toward determining whether the disparity of N-myc mRNA decay in the W-N and W-S cells is due to differential expression of trans-acting factors that specifically interact with N-myc AREs, RNA UV cross-linking assays were performed using AU-rich RNA probes and cytoplasmic extracts prepared from the subclones. The extracts were incubated with labeled AU4 (5′-AUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAU-3′) and AUG4 (5′-AUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGUAUGU...
the AU4 probe and protein extracts from the W-N and W-S nude mice tumors demonstrated the 40-kDa complex in all cases (data not shown). These observations further suggest that the solitary tumor that grew after inoculation of W-S cells resulted from a phenotypic switch.

All six cell lines with p40 activity express relatively high levels of N-myc. Three of the six cell lines are N-myc-amplified (LA1–55n, IMR-5, and LA-N-5), and Western blot analysis demonstrates readily detectable levels of N-Myc protein in all three (data not shown and Refs. 35 and 36). However, high levels of N-Myc protein are also seen in the N-myc-unamplified cell line NBL-S (32). We previously reported that the high levels of N-myc expression in this cell line is due to a decrease in turnover of the N-Myc protein (32). Furthermore, SK-N-SH and SH-SY5Y cells have been shown to express higher levels of both N-myc and c-myc mRNA than the SH-EP cells (11). Conversely, all three cell lines that lack p40 activity (SH-EP, LA1–5s, and GI-ME-N) express very low to undetectable levels of N-myc (data not shown and Refs. 9, 11, and 25).

p40 Activity Is Present in Primary NBs—To determine if p40 RNA binding activity is an artifact of tissue culture, binding assays were performed with 19 primary NBs and the probe AU4. RNA UV cross-linking assays demonstrated that 5 of the 19 primary NBs had active p40 (Fig. 4, Table I). N-myc amplification was detected by Southern blot analysis in 4 of the 5 tumors that exhibited active p40 (32). Furthermore, low levels of complexes 42 and 38 kDa in size were identified with these probes, indicating that three proteins may be binding to these N-myc sequences. Using probes NU1 and NU3, barely detectable levels of p40 were seen in the lanes containing W-S cell extracts. p40 was not detected in extracts from either subclone with the NU2 probe (N-myc sequences 5928–6236). Thus, N-myc sequences 5631–5962 and 6215–6607 are sufficient for p40 binding, while sequences 5928–6236 are not.

Identification of Two Binding Sites within the N-myc 3′-UTR That Interacts with p40—In an effort to map the NU1 binding site, three probes corresponding to various regions within the N-myc 3′-UTR sequence 5631–5962 (NU1) were transcribed (NU1-H, 5631–5720; NU1-A, 5631–5752; and NU1-B, 5631–5864) and used in RNA UV cross-linking assays (Fig. 5, A and B). Binding was seen with all three probes. Review of the NU1 sequence revealed an AU-rich region with 10 sequential U nucleotides in region 5694–5715. An RNA oligomer was synthesized corresponding to this region (5′-AUUUUUUUUU-AACAAAAUAU-3′), and binding assays demonstrated that this 22-base sequence (NMBS1) was sufficient to support binding (Fig. 9, A and B). Again, high levels of p40 binding were seen with W-N cell lysates, while barely detectable levels of binding were observed with lysates from W-S cell extracts. Disruption of the poly-U sequence by substitution of G (NMBS1U→G) abolishes binding (5′-AUGUGGUUUGAAACAAACAUU-3′).

Three additional probes were generated consisting of sequences 6215–6345 (NU3-D), 6215–6411 (NU3-M), and 6215–6482 (NU3-B). Only the full-length NU3 and NU3-B were sufficient to support p40 binding, suggesting that the binding site was located between 6411 and 6482 (Fig. 8C). Cross-linking experiments were then performed with W-N and W-S cell extracts and RNase T1 pre digested NU3-B probe, and p40 binding was again seen with W-N cell extracts (data not shown). Because RNase T1 specifically cleaves 3′ of G nucleotides, predigestion of the NU3-B probe should result in only one fragment of sufficient length to provide a suitable substrate for protein binding (5′-UUAUUUUCUUCAAACUAU-3′). To verify that this 18-base sequence was a binding site for p40, RNA cross-linking assays were performed using both in vitro transcribed probe and RNA oligomers corresponding to this fragment (NMBS2) (Fig. 9B). In addition, probe NMBS2U→G was generated from the sequence 5′-UGUGGUUGAAACAAUUG-3′ in which 4 G nucleotides were substituted for U nucleotides. p40 binding to NMBS2 was seen with W-N cell, but not W-S cell lysates. No detectable protein binding was seen to NMBS2U→G with either extract. Thus, at least two distinct
binding sites are present within the N-myc 3′-UTR that interact with p40.

To verify that p40 interacts with the N-myc binding sites with high specificity, RNA cross-linking experiments were performed in the presence of cold competitor RNAs (Fig. 10A). Both NMBS1 and NMBS2 competitor RNAs could compete for NMBS1 probe binding, with NMBS1 competing slightly more efficiently. Similarly, the NMBS2 probe was also competed efficiently by both NMBS1 and NMBS2 competitor. Once again, NMBS1 demonstrated slightly better competition. Since NMBS1 and NMBS2 both efficiently compete for p40 binding, it is likely that the same protein binds to both sites.

To further characterize the p40 binding activity, homopolymeric RNAs were assayed for their ability to compete with the NMBS1 and NMBS2 for binding of p40 (Fig. 10B). Binding of p40 was completely abolished in the presence of poly(rU), but not in the presence of poly(rA), poly(rG), or poly(rC). Together with the mutant binding site studies, these data suggest that p40 requires a U-rich element for efficient binding, possibly explaining why p40 has a lower affinity for NMBS2, which has two A residues in the middle of its U-rich element, than NMBS1.

**DISCUSSION**

N cells derived from the human NB cell line NBL-W express 5-fold higher levels of steady-state N-myc mRNA and protein than S cells although both subclones contain ~100 copies of the N-myc gene (7). No change in either the N-myc copy number or the level of N-myc expression has been detected in the NBL-W subclones after more than 5 years in culture. Because enhanced levels of N-myc expression have been shown to confer growth potential to NB cells both in vitro and in vivo (37), we analyzed the growth characteristics of the NBL-W subclones and found that N-myc mRNA and protein levels in the NBL-W subclones correlate with tumorigenicity. Others have similarly reported that N-myc expression closely parallels the growth characteristics of N and S subclones from other NB cells (8, 11). In most studies, S cells fail to form colonies in soft agar assays and are not tumorigenic in nude mice. However, Sleight and colleagues (38) have reported that a subclone of S cells from the NBL-W cell line are capable of forming tumors in nude mice, albeit with a longer lag phase and less efficiency than N cells.

To investigate the molecular mechanisms responsible for regulating the differential steady-state levels of N-myc expression in the W-N and W-S cells, N-myc promoter activity was analyzed using CAT reporter gene fusion plasmids containing
human N-myc promoter sequences. Surprisingly, although CAT activity was detected in both N- and S-cell types, N-myc promoter activity was higher in the W-S cells, which express lower levels of N-myc. Nuclear run-off experiments similarly demonstrated that the rate of N-myc transcription was actually higher in the W-S cells. Although a disparity between the CAT and nuclear run-off values exists (15-versus 10-fold difference), both assays indicate that the higher levels of steady-state N-myc mRNA in the W-N cells are not due to enhanced transcription of the mRNA. At present, it is unclear why the rate of N-myc transcription is lower in the W-N than the W-S cells, but others have shown that primary cells as well as nontransformed, established cell lines possess a Myc-negative feedback mechanism, whereby Myc protein suppresses transcription initiation from the c-myc promoter (39–41). It is possible that a similar negative feedback mechanism exists for N-myc as well.

Regulation at the level of transcriptional elongation of N-myc has been shown to be an important determinant of N-myc expression in normal developing tissues (42, 43). It has also been demonstrated that transcriptional attenuation plays a role in N-myc gene regulation in transformed rat embryo fibroblasts where loss of attenuation was associated with a progression to a more malignant phenotype (44). In contrast, our results show no evidence of transcriptional attenuation in either the W-N or W-S cells. It is possible that an early step in the development of NB is the loss of regulated transcriptional elongation, thus reconciling our findings with those in transformed rat embryo fibroblasts.

As shown in Fig. 3, the half-life of the N-myc mRNA in the W-N and W-S cells is markedly disparate. Because mRNA turnover is a geometric progression, modest changes in transcript half-life can result in great differences of steady-state mRNA levels. Thus, the disparity in the steady-state levels of N-myc in the NB subclones appears to be due (at least in part) to the differential regulation of N-myc mRNA turnover in the W-N and W-S cells. Others have shown that the half-life of N-myc mRNA in the NB cell line LA-N-5 and the retinoblastoma cell line Y79 is 30–40 min (45) and approximately 15 min in the NB cell line NGP (46). Thus, while the half-life of N-myc mRNA in the W-N cells is similar to the degradation rate seen in other cell lines, the N-myc mRNA in the W-S cell line is extremely unstable. It is possible that the labile nature of the N-myc transcript in the W-S cells makes it a poor template for translation, thereby leading to the relatively low levels of N-myc protein expression.

The molecular mechanisms controlling mRNA stability remain largely unknown. However, cytosolic proteins capable of binding AREs within the 3′-UTR or other cis-acting elements within the coding regions of labile mRNAs have recently been identified, and the interactions between these trans- and cis-acting elements appear to be important in the regulation of mRNA degradation (16–18, 20–23, 47). The mouse N-myc 3′-UTR increases the cytoplasmic instability of chimeric mRNAs containing the adenovirus E1A coding region and portions of the N-myc 3′-UTR (48). Also, it is interesting to note that proviral insertion of the Moloney murine leukemia virus is commonly found within first 100 bases of the N-myc 3′-UTR, resulting in increased expression of the truncated mRNA and T cell lymphoma (49). Taken together, these data suggest that the N-myc 3′-UTR plays an important role in the regulation of N-myc mRNA stability and expression. Thus, the 40-kDa protein complex that we identified in the RNA UV cross-linking assays that specifically interacts with at least two AU-rich

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**Fig. 9.** p40 binds to the N-myc binding sites. A, sequence of the p40 binding sites within the N-myc 3′-UTR and of the mutant probes. In the mutant probes, lowercase g represents a U → G substitution. B, RNA cross-linkings were performed with the wild type (WT) or mutant (U → G) binding sites (5 pmol) and 25 μg of W-N (W) and W-S (S) extract.

**Fig. 10.** Competition assays. A, unlabeled wild type NMBS1 or NMBS2 (WT) or mutant probe (U → G) (10- and 100-fold molar excess) was added to the W-N and W-S cell extracts 10 min prior to the addition of labeled NMBS1 or NMBS2 (5 pmol). RNA UV cross-linking assays were performed as described under "Materials and Methods." B, a 500-fold molar excess of unlabeled homopolymeric RNAs was incubated with extracts 10 min prior to the addition of labeled NMBS1 and NMBS2 (5 pmol).
Interestingly, these elements are highly conserved in the regulation of turnover of these short-lived mRNAs (50–52). Earlier studies have suggested that these elements play a role in granulocyte-macrophage colony-stimulating factor, and recently providing that p40 is a U-rich RNA-binding protein. Similar to NMBS2 contains a UUUAAUUUCUU motif (bases 6465–6475). Indeed, competition with homopolymeric RNAs indicated that poly(rU) was most efficient at binding p40, demonstrating that p40 is a U-rich RNA-binding protein. Similar sequences are also present in the 3'UTRs of c-fos, c-myc, and granulocyte-macrophage colony-stimulating factor, and previous studies have suggested that these elements play a role in the regulation of turnover of these short-lived mRNAs (50–52). Interestingly, these elements are highly conserved in the chicken, mouse, and woodchuck N-myc 3'-UTRs, further suggesting that they are functionally important.

Although there is no direct evidence as yet that any of the reported ARE-binding proteins function as an mRNA degradation or stabilization factor in vitro, other RNA processing events are regulated by trans-acting factors. It is therefore, quite likely that the ARE may trigger mRNA decay through formation of a complex of proteins on the ARE. Recently, the genes of several mammalian proteins having ARE- or U-rich sequence binding activity have been cloned. Using antibodies directed against several of these proteins, we report in the accompanying paper (54) that p40 appears to be a member of the ELAV-like family of RNA-binding proteins (53). Further studies investigating the interactions of ELAV-like proteins and N-myc mRNA should lead to a better understanding of the regulation of N-myc turnover and provide insight regarding the role these proteins play in determining NB phenotype.

Acknowledgments—We thank Dr. Randy Wada for the N-myc-CAT fusion constructs; Dr. Geoffrey Krystal for the N-myc cDNA probes that were used in the nuclear run-off assays; James Malter for the pAIUUA and pAUGUA plasmids; Helen Salwen and Dr. Mary Lou Schmidt for performing the soft agar assays; and Drs. Barbara Fays, Chitra Manohar, and Jeff Ross for helpful discussions and critical review of this manuscript.

sequences within the N-myc 3'-UTR may play a role in the regulation of N-myc mRNA metabolism.

RNA UV cross-linking assays performed with extracts from nine additional NB cell lines demonstrated p40 binding in six of the NB cell lines. Interestingly, all six of the NB cell lines with high levels of p40 activity contain N-type cells, express relatively high levels of N-myc mRNA, and exhibit anchorage-independent growth, while the three cell lines that lacked p40 are composed of S-type cells and express low levels of N-myc. p40 activity was also detected in primary NBs, demonstrating that activity is not an artifact of tissue culture. Further, in our small series of NB patients, p40 activity correlated with aggressive clinical behavior. The in vitro counterparts of N and S cells are not known, but NBs that lack p40 activity and are associated with clinically favorable outcomes may be composed of cells with an S-like phenotype.

Sequence analysis of the N-myc 3' binding sites does not display any shared sequence motifs between NMBS1 and NMBS2, although both regions are U-rich. NMBS1 does contain a stretch of 10 uridine residues (bases 5695–5704), and NMBS2 contains a UUUAAUUUCUU motif (bases 6465–6475). Indeed, competition with homopolymeric RNAs indicated that poly(rU) was most efficient at binding p40, demonstrating that p40 is a U-rich RNA-binding protein. Similar sequences are also present in the 3'UTRs of c-fos, c-myc, and granulocyte-macrophage colony-stimulating factor, and previous studies have suggested that these elements play a role in the regulation of turnover of these short-lived mRNAs (50–52).

Interestingly, these elements are highly conserved in the chicken, mouse, and woodchuck N-myc 3'-UTRs, further suggesting that they are functionally important.

Although there is no direct evidence as yet that any of the reported ARE-binding proteins function as an mRNA degradation or stabilization factor in vitro, other RNA processing events are regulated by trans-acting factors. It is therefore, quite likely that the ARE may trigger mRNA decay through formation of a complex of proteins on the ARE. Recently, the genes of several mammalian proteins having ARE- or U-rich sequence binding activity have been cloned. Using antibodies directed against several of these proteins, we report in the accompanying paper (54) that p40 appears to be a member of the ELAV-like family of RNA-binding proteins (53). Further studies investigating the interactions of ELAV-like proteins and N-myc mRNA should lead to a better understanding of the regulation of N-myc turnover and provide insight regarding the role these proteins play in determining NB phenotype.