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Original Paper

Activity of a 40 kDa RNA-Binding Protein Correlates with *MYCN* and *c-fos* mRNA Stability in Human Neuroblastoma

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Subclones of neuroblastic (N) and substrate adherent (S) cells have been established from neuroblastoma tumours cultured *in vitro* which differ in growth characteristics and *MYCN* expression. N cells derived from the NBL-W cell line (W-N) express 5-fold higher levels of *MYCN* mRNA and 10-fold higher levels of *MYCN* protein than S cells (W-S), despite having the same *MYCN* copy number. In an effort to identify the molecular mechanisms responsible for the disparity in steady-state *MYCN* levels, the rate of *MYCN* mRNA degradation was measured in the two subclones. The half-life of *MYCN* mRNA in the W-N cells was approximately 45 min compared to approximately 6 min in the W-S cells. Similarly, the half-life of another labile mRNA, *c-fos*, differed in W-N and W-S cells (30 min versus 15 min, respectively). The turnover of labile mRNAs is thought to be mediated by the interactions of trans-acting factors with AU-rich elements within the 3′ untranslated region. RNA UV cross-linking assays using W-N cell lysate demonstrated abundant quantities of a protein, 40 kDa in size (p40), that bound specifically to AU-rich elements within the *MYCN* and *c-fos* 3′ untranslated region. However, p40 was barely detectable in W-S cells. Our studies suggest that p40 may play a role in determining neuroblastoma phenotype by regulating *MYCN* and *c-fos* mRNA turnover. © **1997 Published by Elsevier Science Ltd.**

Key words: neuroblastoma, *MYCN*, *c-fos*, mRNA stability, RNA-binding protein
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INTRODUCTION

NEUROBLASTOMA, A common neoplasm in children, arises from migrating neural crest cells [1]. Reflecting the multipotent potential of neural crest tissues, these tumours are composed of a variety of cell types. In addition, heterogeneous cellular subpopulations are frequently observed when human neuroblastoma cells are cultured *in vitro* and subclones consisting of small, round neuroblastic cells (N) and large, flat substrate adherent (S or F) cells have been established [2–5]. 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 [3–5].

We and others have previously shown that S cells derived from neuroblastoma cell lines express lower levels of steady-state *MYCN* mRNA and protein than N cells [5–8]. The 5-fold disparity in steady-state *MYCN* mRNA expression in the N and S cells subcloned from the NBL-W NB cell line (W-N and W-S) is not due to alterations in *MYCN* copy number as both cell types contain approximately 100 copies of the gene [5]. In this study we show that the disparity in steady-state levels of *MYCN* in these cells results, at least in part, from differences in the rate of *MYCN* mRNA degradation. Turnover of *MYCN* mRNA is more rapid in the W-S cells than the W-N cells. In addition, another labile mRNA, *c-fos*, is degraded more rapidly in W-S cells than in W-N cells. The regulation of degradation of short-lived mRNAs is thought to be mediated by the interactions of trans-acting factors with AU-rich elements (AREs) within the 3′ untranslated region (3′UTR) of the mRNA [9–16]. Using RNA UV cross-linking assays, we show that W-N cells contain abundant quantities of a protein 40 kDa in size (p40) that specifically interacts with AREs within the 3′UTR of both *MYCN* and *c-fos*. However, p40 is barely detectable in W-S cells.

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MATERIALS AND METHODS

Messenger RNA half-life studies

To determine *MYCN* mRNA stability, neuroblastoma cells were plated and grown to 75–80% confluence, then re-fed with fresh media containing 5 μg/ml actinomycin D (Sigma Chemicals, St. Louis, Missouri, U.S.A.) and incubated at 37°C. Total cellular RNA was isolated by standard methods [17], and Northern blotting was performed as previously described [18]. *MYCN* mRNA levels were quantified using a Fuji Phosphoimager and the *MYCN* signals were standardised to β-actin. Serum chase experiments were performed to determine *c-fos* mRNA stability as previously described [19]. At various times postserum induction, cytoplasmic RNA was isolated from harvested cells and analysed by Northern blotting using a probe corresponding to exon 4 of the human *c-fos* gene.

RNA UV cross-linking assays

RNA UV cross-linking assays were performed using crude cytoplasmic extracts prepared by the freeze–thaw method as described [10]. Radiolabelled probes were *in vitro* transcribed from linearised templates by standard procedures [20]. Probes AU4 and AUG were prepared from the plasmids pAUUUA and pAUGUA, kindly provided by James Malter, University of Wisconsin–Madison, Madison, Wisconsin, U.S.A. *MYCN* probes were prepared from constructs containing portions of the *MYCN* 3′UTR which were cloned into pCRII vector (Invitrogen, La Jolla, California, U.S.A.). The *MYCN* templates were generated by PCR (polymerase chain reaction), using the following primers:

1: NM3UTR-5′ (5631–5649):
5′-GGGTCTAGACACGCTCGGACTTGCTAG-3′;
2: NMUT282+ (5928–5945):
5′-CCCAGATCTCACCTTGTGTGTTCCAAG-3′;
3: NMUT299−(5962–5945):
5′-GGGAAGCTTGGAACACACAAGGTG-3′;
4: NMUT569+ (6215–6236):
5′-CCCAGATCTCTGTACTAATTCTTACACTGCC3′;
5: NMUT590−(6236–6215):
5′-GGGAAGCTTGGCAGTGTAAGAATTAGTACAG-3′;
6: NM3UTR-3 (6607–6590):
5′-GGGGGGCCCGCTCCTTAAGGGACAGAG-3′.

All oligonucleotides are numbered as previously published [21], and contain a unique restriction site which is underlined and a G or C clamp at the 5′ end.

Sense and anti-sense oligonucleotides corresponding to the p40 wild-type and mutant binding sites (NMBS1: WT, T-G; and NMBS2: WT, T-G) were synthesised so that when annealed, overhangs would be generated corresponding to a 5′ HindIII site and a 3′ BamHI site. pGEM4Z (Promega, Madison, Wisconsin, U.S.A.) was digested with HindIII and BamHI, and the oligonucleotides were ligated in the sense orientation. The *c-fos* ARE transcription vectors pT3-AUFL, pT3-ARE, pT3-AU12 and pT3-AU2 were generously provided by Dr A. B. Shyu, University of Texas Health Science Center, Houston, Texas, U.S.A. Probes corresponding to full-length *c-fos* 3′UTR (AUFL) and portions of the *c-fos* 3′UTR (AU1, ARE, AU2, AU12) were prepared from these constructs as described [22]. The ARE probe contains the domains previously shown to function as destabilising elements [19]. AU1 and AU2 contain sequences that flank the ARE, and AU12 joins AU1 and AU2 splicing out the ARE. All constructs were sequenced in both directions to verify orientation and sequence of the insert.

RESULTS

Stability of MYC*N and* c-fos *transcripts in NBL-W-N and NBL-W-S neuroblastoma cells*

As shown in Figure 1a, the half-life of *MYCN* mRNA was approximately 45 min in the W-N cells, but only approximately 6 min in the W-S cells. Thus, a direct correlation exists between *MYCN* mRNA half-life and the steady-state levels of *MYCN* mRNA in the W-N and W-S cells. To determine if the rate of degradation of other short-lived mRNAs also differed in the W-N and W-S cells, *c-fos* mRNA turnover was examined. Serum chase experiments demonstrated that the pattern of degradation of *c-fos* mRNA in the W-N and W-S cells was similar to that of *MYCN*. The half-life of *c-fos* in the W-N cells was approximately 30 min compared to a half-life of only approximately 15 min in the W-S cells (Figure 1b), as calculated from the slope of each curve.

RNA UV cross-linking studies in NBL-W-N cells

As a first step toward determining whether the disparity of *MYCN* and *c-fos* mRNA decay in the W-N and W-S cells is due to differential expression of trans-acting factors that specifically interact with AREs within the 3′UTR of each mRNA, RNA UV cross-linking assays were performed using cytoplasmic extracts prepared from the W-N and W-S cells

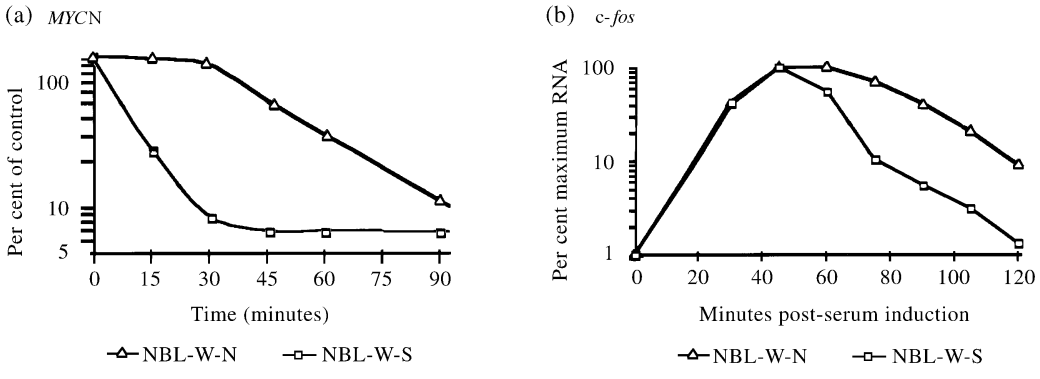

Figure 1. *MYCN* (a) and *c-fos* (b) half-life studies. Total cellular RNA was isolated from W-N and W-S cells for indicated times after either treatment with actinomycin D (*MYCN*) or serum pulse (*c-fos*), and Northern blot analysis was performed. A graphical representation of the rate of *MYCN* and *c-fos* transcript decay in W-N and W-S cells is shown.

and AU-rich RNA probes. Using the AU4 (5′AUUUAUUUAUUUAUUUA3′) probe, a 40 kDa complex (p40) was consistently identified in assays performed with W-N cell extracts while p40 was not detected in the W-S cells (data not shown). Protein binding was abolished using the AUG probe (5′AUGUAUGUAUGUAUUGA3′) in which 4 U's are replaced by 4 G's, indicating that AU-rich elements are necessary for protein binding. Furthermore, p40 binding was specific as competition assays using cold AU4 probe abolished binding (data not shown).

High levels of p40 activity were also seen with W-N cell extracts and the *MYCN* probes NU1 and NU3 (*MYCN* sequences 5631–5962 and 6215–6607, respectively), as well as the full-length *MYCN* 3′UTR (data not shown). 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 (*MYCN* sequences 5928–6236). Thus, *MYCN* sequences 5631–5962 and 6215–6607 are sufficient for p40 binding, while sequences 5928–6236 are not.

Identification of two binding sites within the MYC*N* 3′*UTR that specifically interact with p*40

In an effort to map the binding site in NU3, 3 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. Cross-linking experiments were then performed with W-N and W-S cell extracts and RNase T1 predigested NU3-B probe, and p40 binding was again seen only with W-N cell extracts. 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′UUUAAUUUCUUCAAAAUG-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). Once again, p40 binding was seen with W-N but not W-S cell lysates (Figure 2). Probe BS2T → G was generated from the sequence 5′-TGTAATGTCTGCAAAAGG-3′ in which 4 G's were substituted for T's and no detectable protein binding was seen with either extract.

Three probes corresponding to various regions within the *MYCN* 3′UTR sequence 5631–5962 (NU1) were also constructed (NU1-H, 5631–5720; NU1-A, 5631–5752 and NU1-B, 5631–5864) and used in RNA UV cross-linking assays. Binding was seen with all three probes. Review of the NU1 sequence revealed an AU-rich region with 10 sequential U's in region 5694–5715. An RNA oligomer was synthesised corresponding to this sequence (5′-AUUUUUUUUUUAA-ACAAACAUU-3′) and binding assays demonstrated that this 22 base sequence (NMBS1) was sufficient to support binding (Figure 2(a)). 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 cells. Disruption of the poly-U sequence by substitution of G's (BS1T → G) abolished binding (5′-AUGUGUUGUUGAA-ACAAACAUU-3′).

*Interaction of p*40 *with the* c-fos *ARE*

To determine whether p40 also interacts with the *c-fos* ARE, RNA UV cross-linking assays were performed using cytoplasmic extracts from W-N and W-S cells and *in vitro* transcribed RNAs corresponding to various portions of the *c-fos* ARE (Figure 2b). Similar to the pattern of binding seen with the *MYCN* probes, abundant quantities of an RNA-binding protein 40 kDa in size was detected in W-N cell lysates with an RNA probe corresponding to the full-length *c-fos* 3′UTR (AUFL) as well as a probe that contains two *c-fos* AU-rich RNA instability elements (ARE). Barely detectable levels of p40 were seen in W-S cell extracts. Binding was not detected with *c-fos* probes AU1, AU2 or AU12.

DISCUSSION

We have previously reported that W-N cells derived from the human NB cell line NBL-W express higher levels of steady-state *MYCN* mRNA and protein than W-S cells, even though both subclones contain approximately 100 copies of the *MYCN* gene [5]. To investigate the molecular mechanisms responsible for regulating the differential steady-state levels of *MYCN* expression in the W-N and W-S cells, the half-life of *MYCN* mRNA was measured in the neuroblastoma subclones and found to be markedly disparate.

The molecular mechanisms controlling mRNA stability remain largely unknown. However, cytosolic proteins capable of binding AREs within the 3′UTR or other *cis*-acting

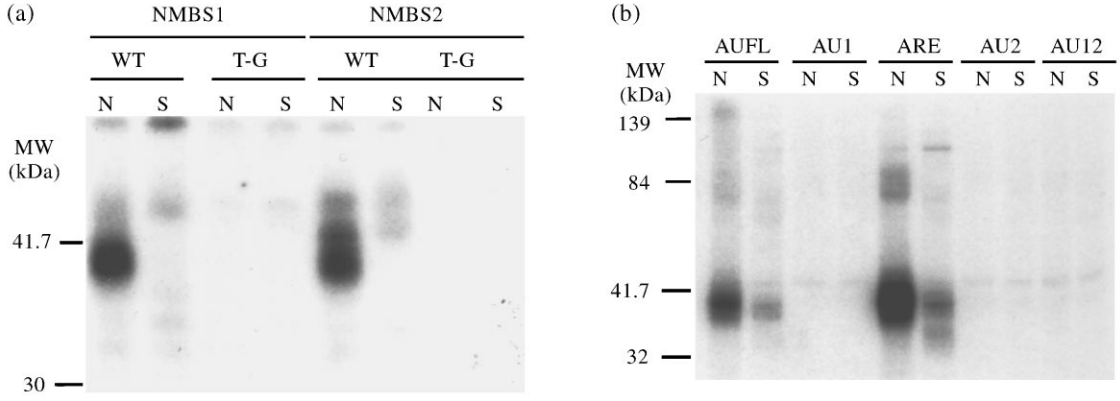

Figure 2. RNA UV cross-linking assays. Assays were performed with W-N (N) and W-S (S) protein lysates and *MYCN* probes corresponding to (a) two AU-rich binding sites in the 3′UTR (wild-type [WT] and mutated [T-G] NMBS1 and NMBS2) and (b) corresponding to regions of the *c-fos* 3′UTR (AUFL, AU1, ARE, AU2 and AU12).

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 [9, 10, 12–16, 23]. Thus, it is possible that the 40 kDa protein we identified that specifically interacts with *MYCN* and *c-fos* 3′UTR AU-rich *cis*-acting elements (NMBS1, NMBS2 and the *c-fos* ARE) plays a role in the regulation of *MYCN* and *c-fos* mRNA metabolism.

Sequence analysis of the *MYCN* 3′UTR does not display any shared sequence motifs between NMBS1 and NMBS2, although both regions are AU-rich. NMBS1 does contain a stretch of 10 uridine residues (bases 5695–5704), and NMBS2 contains a UUUAAUUUCUU motif (bases 6465–6475). Sequences similar to NMBS1 and NMBS2 are present in the 3′UTR of *c-fos*, *MYC* and GM-CSF and others have shown that ligation of these AU-rich elements to stable mRNAs results in enhanced degradation of the chimeric mRNAs [19, 24, 25]. *c-fos* mRNA turnover appears to be regulated by two separate AU-rich domains located within the 3′UTR [19]. Domain I of the *c-fos* mRNA has been shown to be required for rapid degradation of the message body. Domain II cooperates with domain I to accelerate decay, but does not confer instability alone. Thus, while the function of the *MYCN* binding sites is not yet known, p40 does interact with *c-fos* elements that are known to regulate mRNA turnover.

There is no direct evidence as yet that any of the reported ARE-binding proteins function as mRNA degradation or stabilisation factors *in vivo*. However, other RNA processing events are regulated by *trans*-acting factors. It is therefore tempting to speculate that p40 plays a role in determining neuroblastoma phenotype by regulating *MYCN* and *c-fos* mRNA decay. Further studies examining the function of p40 are ongoing in our laboratory.

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