Luciferase Expression Vectors For Analyzing RNA Interference within the *MYCN* 3´UTR

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Genen *MYCN* kodar för N-Myc, ett protein som fungerar som transkriptionsfaktor, det vill säga det kontrollerar vilka gener som transkriberas aktivt från DNA till RNA. Många neuroendokrina tumörer, speciellt neuroblastoma, uppvisar ofta onormalt höga nivåer av N-Myc, och eftersom N-Myc startar transkriptionen av många gener som är viktiga för cellens delning, tillväxt och mobilitet, skulle det kunna vara en bidragande orsak till cancerutveckling. Både genens och proteinets struktur är mycket lika de för det närbesläktade c-Myc, med undantag för den 3´icke-translaterade regionen (3´UnTranslated Region, 3´UTR). När messenger RNA (mRNA) translateras till protein är det inte allt som kodar för protein och den icke-kodande sekvensen som ligger efter mRNAts stopkodon kallas 3´UTR. Att just den sekvensen skiljer sig så mycket mellan N-Myc och c-Myc antyder att den skulle kunna vara viktig när det gäller bestämmandet av det vitt skillda uttrycket mellan dem, speciellt med tanke på att 3´UTR är den regionen som är utsatt för den största andelen av mRNA reglering. MicroRNA (miRNA) är små, icke-kodande RNA som binder till 3´UTR och förhindrar translation av mRNA. Datorbaserade algoritmer kan förutsäga vilket mRNA som de binder till, men dessa beräkningar tycks göra en grov överskattning av deras antal. Datorprogrammen är därför en bra indikation på vilka interaktioner som skulle kunna ske, men de kan inte ge någon säker information. Inbindningssekvenserna för flera intressanta miRNA är beräknade till att befinna sig i *MYCN*s 3´UTR och skulle kunna föklara dess reglering. Vissa av dessa miRNA är sedan tidigare kända för att reglera cellulära processer med motsatt utgång i förhållande till N-Myc. Vissa ligger i en kromosomal region som ofta har blivit förlorad i neuroblastoma celler. Ifall det skulle visa sig att de förhindrar N-Myc protein från att bildas skulle detta ge en klarare av hur N-Myc interagerar i cellen. För att undersöka detta sattes här olika delar av *MYCN*s 3´UTR in nedanför eldfluge-luciferasgenen på en plasmid. Plasmider är små cirkulära DNA-molekyler som kodar endast för ett fåtal protein. Luciferas är ett enzym som skapar luminiscens och dess aktivitet kan mätas med en luminometer. När *MYCN* 3´UTR sätts in efter dess stopkodon påverkar regleringen av denna (t.ex genom miRNA) hur mycket av luciferasets mRNA som hindras från att translateras. På detta sätt skapades en reporterplasmid som visar hur *MYCN* skulle kunna regleras av miRNA när den transfekteras in i humana celler.Transfektion med en miRNA prekursor eller inhibitor av samma cell som har plasmiden kan visa effekten av denna på mängden luciferasprotein. Renilla-luciferas, ett annat luciferas från sjöpennan *Renilla reniformis* används som en kontroll för transfektioneffektiviteten. Denna kodas antingen från samma plasmid eller från en helt annan. Tre olika plasmider konstrurerades i detta syfte och testades med siRNA, en motsvarigheten till miRNA som ofta används för att på konstgjord väg blockera translationen av det mRNA de binder till.

ABSTRACT

The gene *MYCN* encodes a transcription factor with a fundamental role in many neuroendocrine tumors, especially neuroblastoma. The gene and protein are very similar to the closely related c-Myc protein, except for the 3´untranslated region (3´UTR), suggesting that it may be an important factor in determining their very different expression pattern at the post-transcriptional level. MicroRNA (miRNA) are small non-coding RNA that bind to the 3´UTR and prevent translation of their target mRNA. Targets can be predicted with computational algorithms, but the number of targets seems to be greatly overestimated. The target sequence of several interesting miRNAs exist in the *MYCN* 3´UTR that may explain its regulation and deregulation. To test if these targets are true, an *in vitro* approach with a plasmid-encoded reporter gene can be used. Here, the *MYCN* 3´UTR has been inserted

downstream of the firefly luciferase gene. Renilla luciferase, on the same plasmid or separate, is used as a control. Three plasmids with different insert lengths were generated.

INTRODUCTION

MYC transcription factors (c-Myc, L-Myc and N-Myc) are critical regulators of the cell cycle by promoting proliferation. c-Myc was described first and is most deeply studied, but N-Myc seem to be able to replace c-Myc in almost all settings (Aubry et al. 2000, Malynn B.A. et al.), suggesting redundancy, and that they function differently only because of variations in their temporal and spatial expression. N-Myc is critical during development, but becomes restricted to specific tissues, such as the brain, testis and heart in adults (Xu et al. 1991). In addition to their proliferative capabilities, MYCs seem to equip cancer cells with all properties required; such as enhancing DNA replication (Dominguez-Sola et al. 2007), favoring glycolysis (Morish et al. 2008) and facilitating metastasis (reviewed by Eilers and Eisenmann 2008). Hence, MYCs are notorious oncogenes.

Although the functions of MYCs seem to be similar, their regulation must be different and studies have to be done on each MYC in this context. The malignancy most characteristic for N-Myc is neuroblastoma (Schwab 2004), a paediatric tumor believed to develop from migrating neural crest cells that fail to differentiate. Neuroblastoma is responsible for approx. 15% of childhood cancer deaths. The critical role of N-Myc is well established, high protein levels is a direct cause (Weiss et al. 1997) and MYCN amplification correlates with very poor prognosis. In this context, revealing the regulation mechanisms of N-Myc protein levels are of great value. In addition to transcription, control of MYC protein levels is likely very important also at post-transcriptional level. MYC transcripts are very similar, the only exception being the 3´untranslated region (3´UTR) that is much longer in *MYCN* (N-Myc) than *MYC* (c-Myc) (Stanton et al. 1986). Conceivably, the 3´UTR is the major contributor to differences in their post-transcriptional regulation.

MicroRNAs (miRNAs) are non-translated small RNAs (22 nucleotides) that regulate cellular protein levels by inhibiting translation or reducing the stability of mRNA by one or several mechanisms (reviewed by Kutter and Svoboda 2008, Eulalio et al. 2008). The miRNA seed region, generally consisting of nucleotides 2-8 from the 5´ end, is highly conserved and crucial for target recognition. Animal miRNAs bind only to the 3´UTR, perhaps because it lacks scanning or translating ribosomes that otherwise disrupt binding (Gu et al. 2009). Recent data however, suggests that there can be interactions in the coding sequence (CDS) as well (Elcheva et al. 2009), perhaps because of rare codons upstream that slow downs the ribosomes or alternatively by interactions with different proteins (Ago2 instead of Ago1). In any case, so far, this can be viewed as an exception rather than a rule and by restricting targeting to the 3´UTR the number of possible targets is greatly diminished. Furthermore, as mentioned above, the 3´UTR can be suspected to be participating profoundly in regulation of protein synthesis. Targets of a particular miRNA can be predicted using one of many internet based programs, but all have in common that they greatly overestimate the number of true targets (reviewed by Mendes et al. 2009). Factors that may contribute to *in vivo* and *in vitro* functions include mRNA structure (Long et al. 2007) and the presence of RNA binding proteins (Reviewed by Glisovic et al. 2008).

Reporter systems utilizing Firefly luciferase (Fluc) from *Photinus pyralis* are perhaps the most effective for measuring regulation of a nucleotide sequence *in vitro* (Fan and Wood 2006). Benefits of Fluc are

the high sensitivity and high dynamic range. Fluc is often used together with Renilla luciferase (Rluc) from *Renilla reniformis*. FLuc and Rluc can be used to validate a predicted miRNA target by inserting the sequence downstream of the luciferase stop codon. Cotransfecting synthetic miRNA precursors or inhibitors would then give the expected effect on luciferase protein levels and luminescence activity. Because Rluc uses a different substrate, coelenterazin instead of luciferin, it can be used together with Fluc as a measurement of transfection effiency. For convenience, Fluc and Rluc could be encoded on the same plasmid. In theory, this would also give a more reliable system. However, it is possible that it could be limiting when the amount of each enzyme cannot be manipulated with different amounts of transfected plasmid. Therefore, two vector backbones were utilized for creating *MYCN* 3´UTR constructs; one with Fluc, and one encoding both Fluc and Rluc.

To make sure that the silencing is not artificial, as much as possible of the sequences surrounding the predicted target site should be included in the vector. If cloning not much more than the target site, a downregulation is not much more validating than a computational target prediction. A natural choice is to insert the full 3´UTR. This will give the most native three-dimensional mRNA structure and target site availability. Although the predicted target site is included, and perhaps will be validated with site-directed mutagenesis, excluding surrounding sequences may disrupt binding sites for RNA-binding proteins. Many RNA binding proteins influence RNAi and their binding sites do not need to be directly linked to the miRNA target site (Elcheva et al. 2009). The correct distance between the end of ribosomal activity (the stop codon) and the target site is also important (Bartel 2009). Including upstream bases of the coding sequences in the insert will perhaps give a correct nucleotide arrangement, but if the luciferase stop codon is not removed, the 3´UTR will be an artificial elongated version that could harbour additional target sites. By using insets with different surrounding sequences, a hint of potential regulatory elements can be given and direct further investigations on the full 3´UTR.

METHODS

Polymerase Chain Reaction (PCR)
The *MYCN* 3´UTR (Figure 1) was amplified using three different sets of primers (1-3 Table 1). Two of the PCR products (inserts 1 and 2) were engineered with SacI and HindIII restriction sites for cloning into the pMIR-REPORT Expression Vector (Ambion). The size of these fragments differed greatly; inserts 1 included the full 3´UTR while insert 2 was only the middle part of the 3´UTR (nucleotides 276-650). Insert 1 had 7bp upstream and 13 bp downstream, giving a total size of 936 bp after restriction cutting). Approximately the same, full sequence but with one nucleotide missing upstream and 4 bp downstream was produced by the third primer pair (fragment size 918 bp), but with sites for NheI and SalI for cloning into the pmir Glo Dual Luciferase Expression Vector (Promega). Both plasmid backbones contain the β-galactosidase encoding gene (amp^r), making ampicillin selection possible.

A DNA template was extracted from cells of the human neuroblastoma cell line LAN-1, using a Genomic DNA Purification Kit (Fermentas) according to the manufactures instructions. *MYCN* is greatly amplified in this cell line, rendering it a promising template for primers targeting the *MYCN* gene.

For the PCR, Fermentas PCR Master Mix (2X) was used. To optimize the PCR different volumes of 25 mM $MgSO_4$

(Fermentas) was added. The PCR program for primer pairs 1 and 2 was as follows: initial denaturation, 94 ˚C for 2 min; denaturation 94 ˚C for 1 min; annealing, 55˚C for 1 min; elongation 72 ˚C for 1.5 min; final elongation, 72˚C for 10min. Primer pair 3 used the following program: initial denaturation, 94 ˚C for 2 min; denaturation 94 ˚C for 1 min; annealing, 50 ˚C for 1 min; elongation 72 ˚C for 1 min; final elongation, 72 ˚C for 10 min. The number of cycles of both programs was 25.

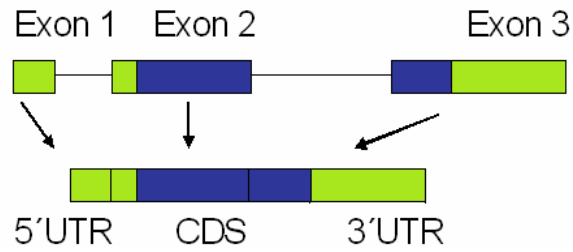
Samples were analyzed with agarose gel electrophoresis using Tris-Acetate EDTA (TAE) buffer (4.84 g tris base, 1.14 ml glacial acetic acid, 100 ml EDTA (pH = 8), and 1% agarose in the gel, and a final concentration of 10 μg ethidium bromide per ml TAE buffer. After optimizing the PCR, the products were purified directly using QiaQuick PCR Purification Kit (Qiagen) without prior gel purification. This gave greater yield compared to gel excision and purification using QiaQuick Gel Extraction Kit (Qiagen).

pMIR-REPORT vector and inserts 1 and 2 were double digested with FastDigest HindIII (Fermentas) and FastDigest SacI (Fermentas) according to the manufactures protocol but with slightly longer incubation of approx. 15 min, followed by inactivation for 5 min in 65 ˚C. Shorter digestion times (5min) were not tested however. The pmir Glo vector and insert 3 were double digested with FastDigest SalI (Fermentas) and FastDigest NheI (Fermentas) as above. In the case of pmir Glo, the cleaved plasmid was purified with agarose gel electrophoresis and extracted using QiaQuick Gel Extraction Kit (Qiagen).

Ligations, using T4 DNA ligase (Fermentas) were carried out overnight in the case of inserts 1 and 2. Insert 3 was incubated with linearized pmir Glo vector for 2 h. All reactions were carried out at room temperature and inactivated at 65 ˚C for 10 min. The mass ratio (insert:vector) was 1:1 in both cases. This gives a molar ratio of approx. 6:1 and 7:1, respectively.

Molecular Cloning

A)



B)

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5´-   1 GCTTCTCAAAACTGGACAGTCACTGCCACTTTGCACATTTTGATTTTTTTTTTAAACAAA
     61 CATTGTGTTGACATTAAGAATGTTGGTTTACTTTCAAATCGGTCCCCTGTCGAGTTCGGC
    121 TCTGGGTGGGCAGTAGGACCACCAGTGTGGGGTTCTGCTGGGACCTTGGAGAGCCTGCAT
    181 CCCAGGATGCTGGGTGGCCCTGCAGCCTCCTCCACCTCACCTCCATGACAGCGCTAAACG
    241 TTGGTGACGGTTGGGAGCCTCTGGGGCTGTTGAAGTCACCTTGTGTGTTCCAAGTTTCCA
    301 AACAACAGAAAGTCATTCCTTCTTTTTAAAATGGTGCTTAAGTTCCAGCAGATGCCACAT
    361 AAGGGGTTTGCCATTTGATACCCCTGGGGAACATTTCTGTAAATACCATTGACACATCCG
    421 CCTTTTGTATACATCCTGGGTAATGAGAGGTGGCTTTTGCGGCCAGTATTAGACTGGAAG
    481 TTCATACCTAAGTACTGTAATAATACCTCAATGTTTGAGGAGCATGTTTTGTATACAAAT
    541 ATATTGTTAATCTCTGTTATGTACTGTACTAATTCTTACACTGCCTGTATACTTTAGTAT
    601 GACGCTGATACATAACTAAATTTGATACTTATATTTTCGTATGAAAATGAGTTGTGAAAG
    661 TTTTGAGTAGATATTACTTTATCACTTTTTGAACTAAGAAACTTTTGTAAAGAAATTTAC
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721 TATATATATATGCCTTTTTCCTAGCCTGTTTCTTCCTGTTAATGTATTTGTTCATGTTTG
781 GTGCATAGAACTGGGTAAATGCAAAGTTCTGTGTTTAATTTCTTCAAAATGTATATATTT
841 AGTGCTGCATCTTATAGCACTTTGAAATACCTCATGTTTATGAAAATAAATAGCTTAAAA
901 TTAAATGA -3´
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Figure 1. A) The *MYCN* gene consists of three exons. Nothing of the first exon is translated, and the 5´UTR is unusually long. All MYCs share these gene arrangements. In contrast, the 3´UTR of *MYCN* in the last exon is much longer than that of *MYC*. B) The DNA sequence of the *MYCN* 3´UTR. Inserts 1 and 3 included the full 3´UTR plus some additional basepairs. Insert 1 had 7 bp extra upstream and 13 bp downstream, and insert 3 had one nucleotide missing upstream and 4 bp extra downstream. The sequence indicated in bold is insert 2.

Primer pairs

1.
SacI
Forward: GCTAAAGAAAATTGAACGAGCTCGGACTT
Reverse: CCATTAAGGAAAAGCTTGAGTTGCATCAT
HindIII

2.
SacI
Forward: AAAAGAGCTCTCACCTTGTGTGTTCCAAG
Reverse: AAAAAGCTTTACTCAAAACTTTCACAACTC
HindIII

3.
NheI
Forward: ACTTGCTAGCCTTCTCAAAACTGGAC
Reverse: CCATTAAGGAAAAGGTCGACTTGC
SalI

Table 1. Primer pairs. The numbers 1-3 corresponds to the numbers of the PCR products 1-3. Engineered restriction sites are underlined. Sequences are written in 5´ to 3´ direction.

Bacterial cell culture

Escherichia coli cells of the strain JM-106 were grown in a rotating incubator at 37 ˚C in Luria Bertani (LB) media composed of (per liter) 10 g Tryptone, 10 g NaCl and 5 g yeast extract. The media was also supplied with amplicillin to a final concentration of 100 μg/ml.

An overnight 5 mL culture of JM-106 was put into a baffled flash containing 1 litre LB media and grown to an OD of 5.6. At this point, cells were harvested by centrifugation and resuspended in a total volume of 600 ml 10 % cold glycerol. After a second centrifugation for 15 min, the pellet was resuspended in 14 ml 10 % glycerol and spun down for 20 min. 500 μl 10 % glycerol was used to resuspend the pellet and the cells were stored as aliquotes of 50 μl in -80 ˚C.

50 μl JM-106 was transformed with approx. 40 ng of plasmid directly from the ligation mixtures using electroporation. The transformation was followed by an hour of phenotyping in 1 mL LB media without ampicillin.150 μL of the transformation mix was plated on ampicillin-containing agar plates and grown overnight. Colonies were checked for insert with colony PCR using the same primer pair that yielded insert number 2 (giving a 374 bp fragment). Positive colonies were further analyzed with restriction cutting using the same enzymes that provided the sticky ends for ligation. The plasmids were confirmed by sequencing (Eurofin MWG Operon). Plasmids were propagated in JM-106 grown in LB media with ampicillin and purified using GeneElute HP Plasmid Midiprep (Sigma) and GeneJet Plasmid Miniprep Kit (Fermentas).

Human cell culture

Human non-cancerous breast epithelial HB-2 cells were grown in a humidified atmosphere with 5 %CO_2. The medium

was composed of 450 mL Dulbecco´s Modified Eagle Medium (DMEM) supplied with 10 % Fetal Bovine Serum (FBS), 1 % L-glutamine, 1 % penicillin and streptomycin, 0.1 % insulin and 0.1 % hydrocortisone.

Transfection
On day prior to transfection, HB-2 cells were seeded in 24-well plates. The cells were transfected with the empty vectors or with vectors containing the constructs using Lipofectamine 2000 (Invitrogen). siRNA targeting the *MYCN* 3´UTR was cotransfected. 40 h after transfection, cells were treated with Passive Lysis Buffer (Promega) according to the manufactures recommendations and were then stored at -20 ˚C until analysis. The cell lysates were measured for luciferase activity with a Bioorbit 1250 Luminometer and using Promegas Dual-Glo Luciferase Assay System. In brief, substrates for Fluc activity are first added and luminescence is measured. The reaction is then quenched at the same time as Rluc reactants are added, and luminescence is measured again. The ratio between Fluc and Rluc activity is used to eliminate variance in transfection effiency.

RESULTS

Generation of plasmids
The transformation yielded approx. 10-20 colonies at a time, of which almost all were sceened with colony PCR. Of these more than half (insert 3) or a third (insert 1 and 2) were positive. The colony PCR (Figure 2) results correlated well with subsequent restriction cutting (Figure 3).

Sequencing
Sequencing revealed that DualGlo-MYCN lacked an adenosine at position 730 and a thymine at 731 in the *MYCN* 3´UTR. This is likely a true mutation since it was identified by sequencing in both direction of the two different strands and there was nothing suspicious with the chromatogram. No predicted miRNA site lies within this region (MiRanda, miRBase).

A. B.

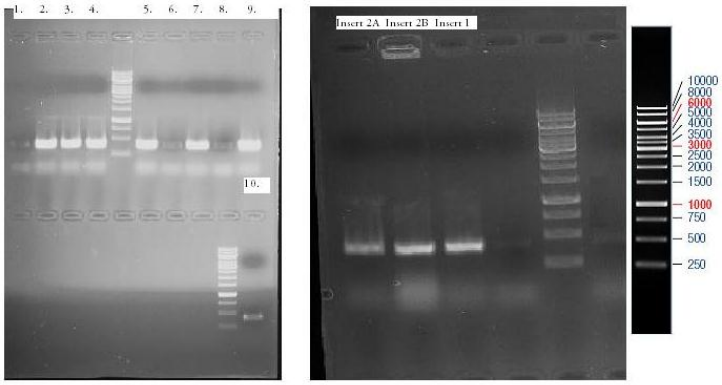


Figure 2. Agarose gels of colony PCRs. A) Colony PCR after electroporation with ligation mixture with pmir Glo and insert 3. The numbers represent the colonies, which were all that were analyzed at this step. Plasmid DNA from the colonies that gave the strongest bands was purified with miniprep. B) Part of a gel from colony PCR with transformants suspected to have insert 1 or 2. The DNA marker ladders are the same in all pictures (1kb DNA Gene Ruler (Fermentas)).

A. B. C.

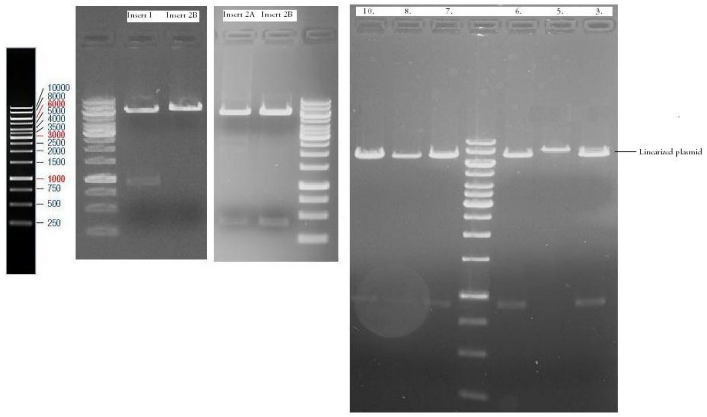
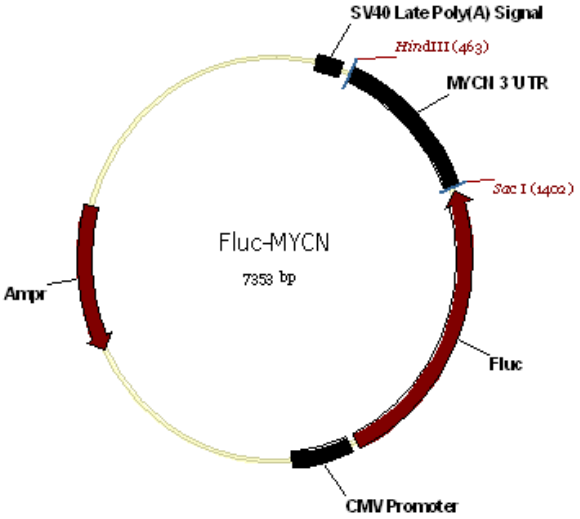
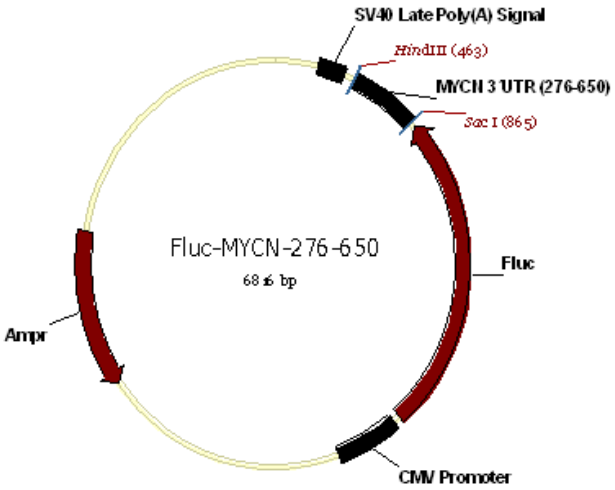


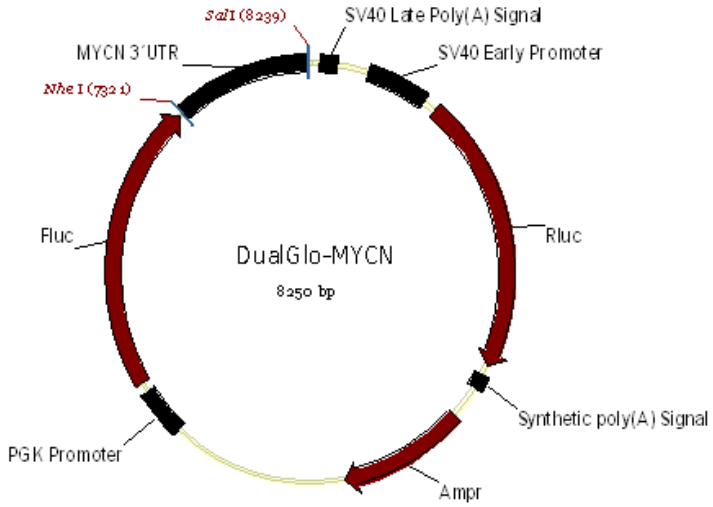
Figure 3. Restriction cleavages. The restriction sites engineered in the primers were used to excise the fragments. Insert 1 is 936 bp, insert 2 is 374 bp and insert 3 is 918 bp. A) Restriction cleavage of purified plasmid from colonies that were checked with PCR and labelled Insert 2B and Insert 1 in picture 2B. This gel showed insert 2B as a negative, but when it was checked again (3B) it became clear that this was due to improper cutting. B) Restriction cleavage of purified plasmid from colonies that were checked with PCR and labelled Insert 2A and Insert 2B in picture 2B. C) Restriction cleavage of the purified plasmids from colonies 1, 3, 5, 6, 7, 8 and 10 (upper) of the gel in 2A. All except number 5 were positive, but it looks like it was also positive and just poorly cleaved. The DNA marker ladders are the same in all pictures (1 kb DNA Gene Ruler (Fermentas)).

1.



2.





3.

Figure 4. The three plasmid constructs containing the *MYCN* 3´UTR or part of it. The two upper plasmids were created from a pMIR-REPORT backbone, while the third is derived from the pmir Glo vector.

siRNA knockdown of firefly luciferase

To test the function of the plasmids, three different siRNA (named scramble, siRNA1 and siRNA2 in Figure 4) were employed. siRNA 1 and siRNA 2 were constructed to target the *MYCN* 3´UTR in a region that was included in all inserts. Luciferase activity was also measured without siRNA. Firefly luciferase (Fluc) activity was normalized against Renilla luciferase (Rluc). Rluc was expressed from a cotransfected plasmid in the case of Fluc-MYCN and Fluc-MYCN-276-650. DualGlo-MYCN expresses both luciferases and cotransfection was not necessary. As shown in Figure 5, the scrambled control gave the same result with or without the insert, while the other siRNAs gave a strong downregulation, especially siRNA 2 against insert 2 (9-fold). On the other hand, siRNA 2 against insert 3 was not very efficient.

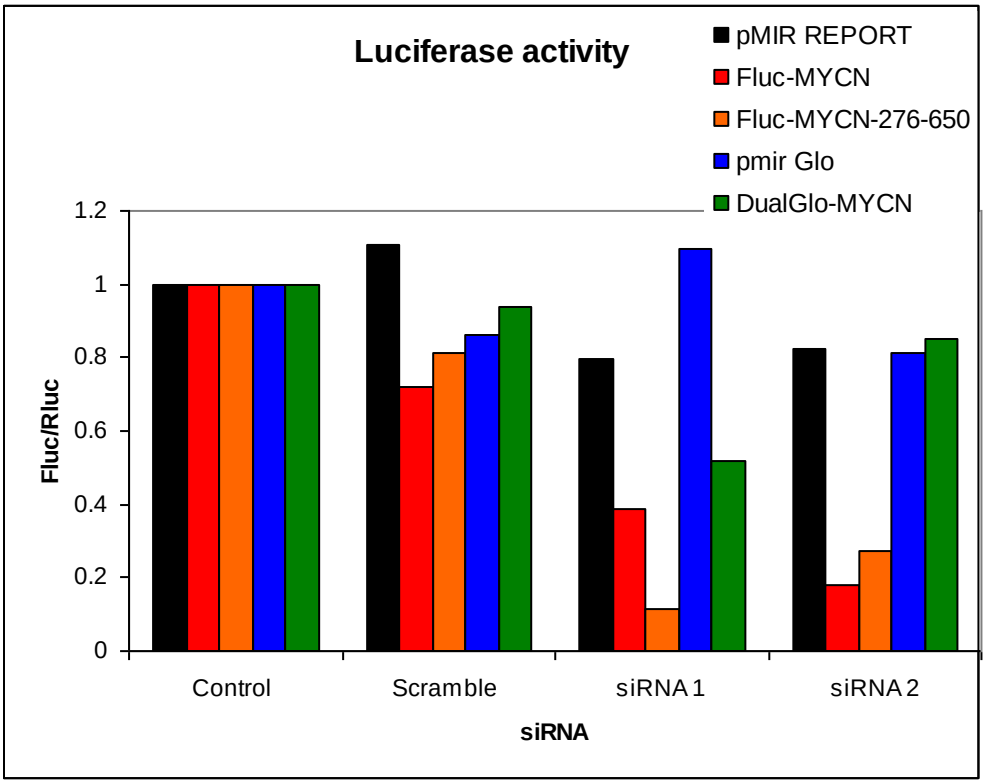


Figure 5. Luciferase activity of cell lysates after transfection of 5 different plasmids. The two empty vectors (blue and black) were also tested with siRNA. siRNA1 and siRNA2 targets the *MYCN* 3´UTR. Control is without siRNA, scramble is a siRNA targeting another region of *MYCN* that was not in the inserts.

DISCUSSION

Since the discovery of endogenous RNAi (i.e. miRNA) (Lee et al. 1993, Wightman et al. 1993), the 3´UTR has drawn much attention because of the extensive post-transcriptional control of mRNA and protein levels that is at work in this region. Here, luciferase constructs with parts of or the complete 3´UTR of the oncogene *MYCN* are presented (Figure 4). *MYCN* encodes a transcription factor with functions in neuronal development and great clinical importance in the treatment of some cancers, in particular neuroblastoma, but also other neuroendocrine tumors that include a large proportion of all lung cancers. The evolutional sequence divergence of the *MYCN* 3´UTR suggests that it has important functions that specifies N-Myc expression. However, little of this has been revealed so far.

The reporter vectors created here can be used in many ways to quickly validate a miRNA as a regulator of N-Myc. In a miRNA-gain-of-function approach, synthetic miRNA precursors can be cotransfected. In a loss-of-function approach, miRNA inhibitors (antagomirs) are used. Alternatively, the effect of the reduction of whole groups or the complete population of miRNA can be tested, as well as the modulation of cellular processes and interactions with the environment (an overview of miRNA techniques is given by Wang 2009). All such modifications can give information of what mechanisms could confer abnormal N-Myc levels through the 3´UTR.

To my knowledge, only one miRNA has been validated as a direct repressor of *MYCN*, i.e. miR-34a (Wei et al. 2008). Interestingly, miR-34a is located in a band

of the short arm of chromosome 1 (1p36.term-1p36.2) that is commonly deleted in neuroblastoma (Mosse et al. 2007). Thus, miR-34a is likely to be involved in the pathology. Furthermore, members of the miR-200 family are clustered in the same region and predicted to target *MYCN*. The same study (Wei et al. 2008) also investigated the effect of these miRNAs on growth, but due to their modest effect compared to miR-34a, the study went further only with miR-34a. It should be noted though that miR-34a also targets some other regulators of cell viability (Tazawa et al. 2007), and somehow enhances the activity of p53 (Yamakuchi and Lowenstein 2009). It is possible that p53 and others have a greater influence on cellular growth than N-Myc and was responsible for much of the effect of miR-34a. miR-200s are notorious regulators of epithelial-to-mesenchymal transition (EMT) (Park et al. 2008, Hurteau et al. 2007), a process that facilitate metastasis. Invasiveness is a characteristic of the most severe neuroblastomas (Killion and Fidler 1989). Both clinical data (Seeger et al. 1985) and molecular studies (Goodman et al. 1996, Janardhanan et al. 2009) suggest that N-Myc has a prominent role in mobility gain of neuroblastomas. It is possible that another specification of miR-200s control of EMT involves N-Myc-controlled cell functions, and that the loss of the short arm of chromosome 1 releases their regulation of *MYCN*. Hence, it would be interesting and more informative to test if miR-200 actually targets *MYCN in vitro*.

Neuronal precursors have stem cell properties, show glial characteristics and exist also the human brain (reviewed by Kriegstein et al. 2009). The glial precursors, radial glia, differentiate into various intermediate precursor cells depending on their final cell type. N-Myc, in contrast to its proliferative and otherwise differentiation-repressive features, promotes neuronal differentiation into intermediate precursors of neurons, neuroblasts (Wakamatsu et al. 1997). These are maintained in a proliferative state by N-Myc (Nagano et al. 2009). During final differentiation into quiescent neurons, N-Myc is downregulated. Likely, miRNAs are part of the process.

Some of the most well-understood miRNAs, members of the let-7 family, are among the most crucial factors modulating stem cell properties, and have been connected to c-Myc. Let-7 targets c-Myc (Sampson et al. 2007), but there is also a dual relationship, because c-Myc induces Lin28 that prevents let-7 processing. Let-7 is expressed in terminally differentiated neurons (Wulczyn et al. 2007). c-Myc, perhaps interchangeable with N-Myc, is one of the four factors needed to artificially produce induced pluripotent stem cells (iPSs) (reviewed by Konrad and Plath 2009). Lin-28, however, can substitute for c-Myc, and is necessary for this function, suggesting that it is the major downstream actuator for this function (Chang et al. 2009). c-Myc widely represses miRNA expression (Chang et al. 2008), giving a possible explanation for the induced pluripotency, but except for let-7, Lin-28B seems not to be much involved in this regulation. Hence, let-7 could be one of the most critical molecular regulators of stem cell maintenance. Recently, N-Myc was found to induce Lin-28B in neuroblastoma cells (Cotterman and Knoepfler 2009). A cell culture population of neuroblastoma cells contain approx. 10% multipotent cells, and they may be fundamental for invasive and proliferative potential of the tumor population. Although *MYC* (c-Myc) and *MYCN* 3´UTRs differ greatly, *MYCN* harbours two predicted target sites for let-7 (using Target-Scan), one of which shows additional 3´complimentarity to some let-7 members, something that is believed to strengthen repression. The 3´ part of the mature miRNA is also suggested to be the distinguishing factor of which different

mRNA targets are regulated by different let-7 members (Bartel 2009). Hence, this predicted target site is particulary interesting when it comes to potential regulation discrimination between *MYC* and *MYCN*. Future studies will reveal if a feedback loop between N-Myc and let-7 is present, and if it plays a role in the switch between neuronal precursors and differentiated cells during neurogenesis.

Another important interactor with the *MYCN* 3´UTR is the neuronal ELAV-like protein HuD. ELAV proteins stabilize mRNA by binding to AU-rich elements (AREs) and replacing proteins that promote mRNA degradation. Several binding sites for HuD exist in *MYCN* (Monohar et al. 2002). Although prevention of ARE-mediated decay seems to be a general function, there are some discrepancies about this binding. Since HuD was found to be highly expressed in some neuroendocrine tumors (Chagnovich et al. 1996), it was suggested that it stabilizes *MYCN* mRNA. HuD antisense treatment decreased HuD expression and increased N-Myc levels in these cells (Manohar et al. 2002). Recently, neuroendocrine lung cancers were associated with a HuD mutation that probably rendered it incapable of binding RNA (D'Alessandro et al. 2009). Thus, it can be speculated that the effect of HuD overexpression on *MYCN* stability is altered in these tumors. Another scenario was proposed reasoning that because the HuD gene (*ELAV4)* is often deleted in *MYCN* amplified neuroblastoma, this would be the reason for *MYCN* amplification (Grandinetti et. al. 2006). In this model, HuD loss causes all cells without *MYCN* amplification to die (because N-Myc is essential during neuronal development) and a rapid selection for cells with abnormal amounts of *MYCN* copy number (and hence sufficient amounts of mRNA) would occur. It should not be taken for certain that HuD has a positive role in *MYCN* mRNA stabilization, however. This was the case with c-Myc and the ubiquitous HuD homolog HuR, that had been identified to bind c-Myc mRNA. Only recently the importance of this interaction was revealed; HuR recruits let-7 and its associated Ago2, promoting c-Myc degradation instead of stabilization (Kim et al. 2009). Perhaps exists a similar relationship between *MYCN* and HuD/let-7. In support of this, HuD promotes terminal differentiation of neurons (reviewed by Deschenes-Furry et al. 2006). Furthermore, neurons are the only cells that express HuD, while they are completely devoid of N-Myc but a specific residence of mature let-7 in the nervous system cell population (Wulczyn et al. 2007). In contrast to HuB, neuronal progenitors in HuD double knockout mice showed enhanced self-renewal capacity and decreased ability to differentiate into mature neurons (Akamatsu et al. 2005). These results directly contradict a stabilizating role of HuD on N-Myc mRNA. Does overexpression of defective HuD in some tumors exhibit a competive inhibitory function and impede proper degradation of *MYCN*, and is a similar deregulation achieved by loss of the *ELAV4* locus? In any case, the significance of these interaction in determining *MYCN* mRNA fate needs to be elucidated.

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