Identification of N-myc Regulatory Regions Involved in Embryonic Expression

JEAN CHARRON, JEAN-FRANÇOIS GAGNON, AND JEAN-FRANÇOIS CADRIN-GIRARD

Center of Research on Cancer, University Laval, CHUQ, L'Hôtel-Dieu de Québec,
Québec, Québec G1R 2J6, Canada

ABSTRACT

Our knowledge on the regulation of the N-myc proto-oncogene expression comes mostly from *in vitro* studies. Very few *in vivo* analyses have been performed to identify the regulatory elements involved in N-myc developmental expression. In the present study, we defined DNA regions required for the regulated expression of N-myc during early embryogenesis. We showed that the expression of N-myc driven by the human N-myc sequences previously described to control N-myc expression in appropriate cell types *in vitro* cannot rescue the mouse N-myc mutant phenotype, suggesting that regulatory elements necessary for N-myc embryonic expression were missing. To identify the regulatory DNA regions involved in N-myc expression, transgenic mouse lines carrying N-myc/lacZ reporter constructs were generated. β -Galactosidase staining analysis at different stages of gestation revealed that >16 kb of mouse N-myc genomic sequences are required to recapitulate the entire spatiotemporal

expression pattern of the endogenous N-myc gene between embryonic d 8.5 and 11.5. This observation supported the notion that the sequences previously identified by *in vitro* assays were not sufficient to reproduce the N-myc embryonic expression pattern. However, regulatory elements that can direct specific expression in the visceral arches, the limb buds, the CNS, and the dorsal root ganglia are included into the mouse N-myc genomic sequences tested. Altogether, these findings indicated that the regulation of the spatiotemporal expression pattern of N-myc during development necessitates multiple regulatory DNA elements. (*Pediatr Res* 51: 48–56, 2002)

Abbreviations

E, embryonic day
DRG, dorsal root ganglia

N-myc gene is a member of the myc gene family, which encodes highly related transcription factors that share many structural and biochemical functions (1–5). c-myc, N-myc, and L-myc genes are the best-characterized members of this family. These three myc members are involved in the initiation and progression of naturally occurring neoplasms. Their tumorigenic potential has also been demonstrated by transformation assays both *in vitro* and *in vivo* (6). Most likely the tumorigenic capacities of both c-Myc and N-Myc reside in part in their ability to drive cells into the cell cycle (7, 8).

The N-myc gene is associated with specific tumors, such as neuroblastoma, retinoblastoma, and small cell lung carcinoma. The activation of N-myc in these various neoplasms always leads to inappropriate and elevated expression of a gene encoding an apparently normal Myc protein (9). Gene amplification is the main mechanism that leads to N-myc overexpression in tumor cells (10–12). However, among the most

aggressive neuroblastomas, a class exists in which no gene amplification is detected. Thus, gene amplification cannot account for the high level of N-myc mRNA in this subset of tumors (13, 14). Therefore, other mechanisms might be involved in the deregulation of N-myc expression, such as modification of N-myc regulatory sequences or deregulation of N-myc transcriptional regulators.

N-myc is involved in organogenesis. Homozygous null N-myc mutant mice die at E11.5. In mutant embryos, defects such as reduced size and decreased cellularity are present primarily in tissues and organs that express high levels of N-myc transcripts, including the cranial and spinal ganglia, mesonephros, lung, liver, and gut (15–21). In a similar way, c-myc homozygous mutant embryos die before E10.5, and they also exhibit a marked embryo size reduction and a generalized delay in the development of many organs (22, 23). Overlapping expression pattern and redundant functional properties among myc family members was proposed to explain survival of individual myc family gene mutant mice through early embryogenesis (15–17, 22). More recently, Malynn *et al.* (3) have shown, by replacing the endogenous c-myc coding sequences by the N-myc ones, that N-Myc can complement c-myc functions in cell growth and differentiation during mouse development, suggesting that

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Correspondence and reprint requests: Jean Charron, Ph.D., Centre de recherche en oncologie de l'Université Laval, CHUQ, L'Hôtel-Dieu de Québec, 9, rue McMahon, Québec, QC, G1R 2J6 Canada; e-mail: jean.charron@crhdq.ulaval.ca

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the distinct functions of both genes reside in a large extent in their specific expression pattern.

The expression patterns of the *c-myc* and *N-myc* genes are distinct, albeit overlapping, during early embryogenesis. Both genes are expressed at preimplantation stages, and their expression profiles diverge with advancing development. *c-myc* gene is widely expressed in tissues and organs in proliferation, whereas *N-myc* expression is restricted to a limited number of tissues and organs and it is not necessarily associated with cells in mitosis (24–30). At E9.5, *N-myc* is highly expressed in the CNS including the mesencephalon, prosencephalon, and neural tube (28). At this stage, *N-myc* expression is also detected in neural crest–derived tissues such as the facial primordia, the visceral arches, and the DRG, as well as in mesodermal derivatives including the caudal part of the somites and the sclerotomes derived from it, the mesonephric tubules, and the limb buds. *N-myc* is also expressed in a complementary fashion with *c-myc* in some tissues, such as the early gut, brain, lung, and kidney (17–19, 26, 31). In tissues in which *N-myc* and *c-myc* are coexpressed, *c-myc* is often associated with rapidly proliferating cells, whereas *N-myc* persists through cellular differentiation. These different expression patterns suggest specific roles for *c-myc* and *N-myc*. *Cis*-acting sequences involved in the regulation of *N-myc* gene expression have been identified mainly from *in vitro* assays using *N-myc* promoter-reporter constructs introduced into cells from different species (32–39). Major regulatory elements are located within sequences 2 kb upstream of the transcription initiation site and in the first exon and intron of the gene. Transgenic mice analyses have suggested that genomic fragments containing these regulatory elements are able to direct *N-myc* expression in newborn and adult tissues in a manner that parallels the endogenous *N-myc* gene (34, 38, 40). However, these studies have not shown whether these sequences are sufficient to recapitulate the embryonic spatiotemporal expression profile of the gene.

To tackle this problem, we have used two approaches. First, we attempted to complement the embryonic lethal phenotype of the *N-myc* mutant mice with a human transgene containing previously described regulatory elements (15, 40). The complete absence of rescue suggested that the transgene was not adequately expressed during mouse development. Second, we initiated the identification of regulatory regions implicated in mouse embryonic expression of the *N-myc* gene. Transgenic mice carrying the bacterial *lacZ* gene under the control of mouse *N-myc* genomic fragments were generated. Although none of the transgenes recapitulated the entire expression pattern of *N-myc* during early development, some of the constructs directed the expression to a subset of *N-myc* expression sites. Our findings indicated that a combination of distal and proximal regulatory elements is required for the proper spatial and temporal expression of *N-myc* during embryogenesis.

METHODS

Construction of *N-myc/lacZ* transgenes. The mouse *N-myc* genomic sequences contained in the *N-myc* 40, *N-myc* 41X, and *N-myc* 41S transgenes were derived from a normal mouse DNA genomic library. All the constructs contain the 7.7-kb

EcoRI fragment described in DePinho *et al.* (41). This fragment was previously modified by the insertion of the *neo* gene into the second exon of *N-myc*, which generated the vector pJC7/*N-myc*-P (42). To construct the plasmid *N-myc* 40, the *neo* sequences of pJC7/*N-myc*-P were replaced by *lacZ* sequences. The *Bam*HI-*Hind*III *lacZ* fragment obtained from pCH110 (Pharmacia, Baie d'Urfé, Québec, Canada) was blunted using the T₄ DNA polymerase and cloned into the *Kpn*I-*Xba*I/T₄ DNA polymerase–blunted sites of pJC7/*N-myc*-P to produce pJC40. The *N-myc* 40 fragment was derived from the pJC40 construct by a *Tth*III1-*Sal*I digestion.

The transgenes *N-myc* 41X and *N-myc* 41S were obtained from pJC21, a pUC18 plasmid containing a 16-kb *Sal*I *N-myc* genomic fragment that extends from –4.4 kb to +11.6 kb, the *Sal*I sites deriving from the phage clone. pJC41 was obtained by introducing the *Rsr*II-*Nru*I *N-myc/lacZ* fragment of pJC40 into the *Rsr*II-*Nru*I unique *N-myc* genomic sites of pJC21. The pJC41 construct was digested with either *Xba*I or *Sal*I to generate the *N-myc* 41X and *N-myc* 41S transgenes, respectively.

Production and identification of transgenic mice. All transgenes were separated from the vector sequences after migration on agarose gel before being microinjected. *N-myc* 40 and *N-myc* 41X fragments were isolated from the agarose using the glass powder–NaI DNA isolation procedure (43). *N-myc* 41S fragment was isolated from the agarose by electro-elution and phenol extraction as described in Hogan *et al.* (44). Transgenic lines were obtained by microinjection of the purified fragments into the pronuclei of fertilized eggs derived from (C57Bl/6 × CBA) F1 hybrid intercrosses as described in Larochelle *et al.* (45). All animal experiments were performed according to guidelines established by the Canadian Council of Animal Care, and protocols were approved by the Animal Care Committee of Université Laval.

Transgenic mice were identified by Southern blot analysis of DNA extracted from mouse tail biopsy at 2 to 3 wk of age. Purified DNA was digested with *Eco*RI, fractionated by electrophoresis through 0.8% agarose gels, and blotted onto Qiabran membrane (QIAGEN, Mississauga, Ontario, Canada). The filters were hybridized with a random-labeled *Cla*I-*Eco*RI third exon *N-myc* probe (42). The DNA of each transgenic founder was analyzed to verify the copy number and the integrity of the inserted construct. Southern blot analysis was performed using appropriate restriction sites and probes. None of the constructs in the transgenic lines that show X-gal staining was rearranged. The copy number of the transgene sequences varied from 2 to >100 copies, organized in a head-to-tail configuration at a single integration site (data not shown).

Whole-mount *in situ* hybridization analysis. The whole-mount *in situ* hybridization protocol was based essentially on that of Larochelle *et al.* (45). The 817-bp *Pvu*II-*Cla*I murine cDNA fragment of *N-myc*–containing sequences from the second exon was used as a template for synthesizing the digoxigenin UTP-labeled riboprobe.

β-Galactosidase staining. Transgenic males were bred with nontransgenic (C57Bl/6 × CBA) F1 hybrid females to generate timed matings. The day of the vaginal plug was considered

as E0.5. Embryos were collected at various stages between E8.5 and E11.5, and yolk sacs were used to determine the genotype of the embryos. Embryos were fixed in ice-cold 0.25% glutaraldehyde prepared in 0.1 M phosphate buffer, pH 7.0, 2 mM MgCl_2 , and 5 mM EGTA for 12 min for E8.5, 15 min for E9.5 and E10.5, and 30 min for E11.5. Fixation was followed by three 20-min rinses at room temperature in 0.1 M phosphate sodium buffer, pH 7.0, 2 mM MgCl_2 , 5 mM EGTA, 0.01% deoxycholic acid (sodium salt), and 0.02% Nonidet P-40, Sigma, Oakville, Ontario, Canada. Embryos were stained overnight at room temperature in 1 mg/mL X-gal (dissolved at 20 mg/mL in dimethyl formamide), 5 mM $\text{K}_3\text{Fe}(\text{CN})_6$, 5 mM $\text{K}_4\text{Fe}(\text{CN})_6$, 1 mM spermidine, and 0.2 mM NaCl prepared in the above rinse solution. After staining, tissues were briefly rinsed in 0.1 M phosphate sodium buffer, pH 7.0, and then progressively dehydrated by successive transfers in graded alcohol. For photography, whole embryos were clarified with benzyl benzoate–benzyl alcohol (2:1) for 30 min to 2 h depending on the size of the specimen.

Histologic analysis. Embryos were dissected from the visceral yolk sac, fixed for 16 h in ice-cold 4% paraformaldehyde, dehydrated by successive transfers in graded alcohol and xylene, and embedded in paraffin as previously described (15). Six-micrometer sections were stained with Harris hematoxylin and eosin following standard procedures.

RESULTS

hN1 transgene cannot rescue the N-myc mutant phenotype. Zimmerman *et al.* (40) have described a mouse transgenic line containing regulatory elements identified by *in vitro* assays that apparently direct normal expression of a human transgene in newborn and adult tissues. This transgenic line was termed TgN(hN1). To determine whether this transgene can reproduce N-myc early embryonic expression, the TgN(hN1) mouse line was bred with the N-myc mutant mouse line (N-myc^{+/-}) to rescue the N-myc mutant phenotype (15). N-myc^{-/-} TgN(hN1)^{+/-} mice were generated by breeding N-myc^{+/-} females to N-myc^{+/-} TgN(hN1)^{+/+} males, with an expected ratio of embryos or mice being 1 N-myc^{+/+} TgN(hN1)^{+/-}:2 N-myc^{+/-} TgN(hN1)^{+/-}:1 N-myc^{-/-} TgN(hN1)^{+/-}.

The genotype of the resulting progeny was determined by Southern blot analysis using specific human and mouse N-myc probes as previously described (15, 40). Of 59 live offspring obtained between 1 d and 3 wk after birth, none was found homozygous for the N-myc mutation, indicating that the lethal phenotype associated with the N-myc mutation was not rescued by the TgN(hN1) transgene (Table 1). To determine whether the TgN(hN1) transgene can partially rescue the N-myc muta-

tion, N-myc^{+/-} TgN(hN1)^{+/+} males were mated with N-myc^{+/-} females, and the females were killed at different times of gestation. The embryos were dissected from the visceral yolk sac, and their genotype was determined. At E10.5, the ratio of N-myc^{+/+} TgN(hN1)^{+/-}:N-myc^{+/-} TgN(hN1)^{+/-}:N-myc^{-/-} TgN(hN1)^{+/-} embryos was 1:2:1 as expected (Table 1). However, at E12.5, all the N-myc^{-/-} TgN(hN1)^{+/-} embryos as determined by genotype were found dead or in the process of resorption. Histopathologic analysis of E10.5 N-myc^{-/-} TgN(hN1)^{+/-} embryos revealed that they present the same defects as the ones previously described for N-myc^{-/-} mutant embryos (15). For instance, the heart of N-myc^{-/-} TgN(hN1)^{+/-} embryos was underdeveloped, and analysis of successive sections indicated that it retained the S-shape more typical of an earlier developmental stage (Fig. 1A). The pericardial cavity was empty owing to the underdevelopment of the heart. Observation of transverse sections at the level of the brain also showed the size reduction of the cranial and spinal ganglia, as well as irregularities and decreased cellularity in the neural ectoderm of the telencephalon and myelencephalon (Fig. 1B; data not shown). Therefore, the presence of the TgN(hN1) transgene could not complement the N-myc phenotype, suggesting either that the TgN(hN1) transgene was unable to recapitulate the endogenous expression pattern or reach the mRNA levels of the endogenous N-myc gene during early embryogenesis. Although the possibility exists that the human N-Myc protein may not have the same properties as the mouse one, we hypothesized that the human N-myc transgene tested lacks essential regulatory elements for appropriate N-myc expression during early development. To identify the *cis*-acting sequences required to reproduce the endogenous embryonic expression pattern of N-myc, transgenic lines containing mouse N-myc genomic sequences linked to a *lacZ* reporter gene were produced.

Expression of N-myc gene at mid-gestation. To determine whether the N-myc/*lacZ* transgenes analyzed in the present study can recapitulate the N-myc endogenous expression pattern, we performed whole-mount *in situ* hybridization using a probe derived from the second exon of the murine N-myc cDNA. This probe was shown to specifically reveal N-myc transcripts in E12.5 lung epithelium as expected (20) (data not shown). In E9.5 embryos, N-myc expression was detected in the CNS, the facial primordia, the branchial arches, the limb buds, and the somites as previously described by *in situ* hybridization on section (Fig. 2A) (28). A faint staining of the myocardium of the cardiac ventricle could be observed. In contrast, N-myc expression in the mandibular and the hyoid arches was clearly defined. At E11.5, signal was still

Table 1. Viability of N-myc^{-/-}/TgN(hN1)^{+/-} embryos

Age*	Number of litters	Number of pups	Genotype of alive embryos or mice TgN(hN1) ^{+/-}		
			N-myc ^{+/+}	N-myc ^{+/-}	N-myc ^{-/-}
E10.5	2	17	5	8	4
E12.5	2	15	4	6	0†
D1–21	11	59	19	40	0

* D1–21 represents a range between 1 and 21 d of age in the pups.

† Four dead embryos in the process of being resorbed were genotyped N-myc^{-/-}. One embryo was not genotyped.

Figure 1. Comparison of heart and cranial development between wild-type and mutant embryos in presence or absence of the TgN(hN1) transgene. *A*, transverse sections through the heart of E10.5 wild-type and homozygous N-myc mutant embryos complemented with the TgN(hN1) transgene are shown. Similar transverse sections of wild-type and homozygous mutant N-myc embryos lacking the transgene are presented for comparison. In contrast to wild-type specimens, N-myc^{-/-} hearts were not divided into atrial (*a*) and ventricular (*v*) chambers, and the trabeculation of the ventricles was greatly reduced (*arrows*). *B*, comparative morphology of the ganglion of trigeminal nerve V from transverse brain sections of wild-type and homozygous mutant carrying or not the TgN(hN1) transgene. Reduction in size or cellularity, and disorganization of the structure were observed in both mutant specimens (*arrows*). Original magnification *A* and *B*, $\times 20$ and $\times 50$, respectively.

observed in the CNS, the branchial arches, and the somites located in the caudal region of the embryo. Expression in the DRG as well as in the cranial ganglia was detected, but the

highest level of expression was in the proliferating zone of the limb bud with a proximal–distal gradient of labeling (Fig. 2*B*).

Figure 2. Expression pattern of the endogenous *N-myc* gene. Whole-mount *in situ* hybridization was performed on embryos at 9.5 (A) and at 11.5 (B) day of gestation. At E9.5, expression was detected in the CNS, the somites (S), the forelimb buds (Flb), the heart (H) and the branchial arches. The first branchial arches are indicated mandible (Mb), second and third branchial arches (2, 3). At E11.5, *N-myc* expression remained in the CNS and in the somites. *N-myc* transcripts were also detected in the dorsal root ganglia (DRG), the progressive zone of the limbs (Pz), and in the maxillary (Mx).

Partial reconstitution of *N-myc* embryonic expression pattern in transgenic mice. Zimmerman *et al.* (40) have shown that a transgene containing a 7.7-kb genomic fragment of the mouse *N-myc* gene, extending from -1.7 kb to $+6.0$ kb, was able to direct expression in mouse newborn brain and kidney, albeit at a lower expression levels than the endogenous *N-myc* gene. This fragment contains the regions described to exert positive and negative effects on the mouse *N-myc* promoter by *in vitro* assays (32–36, 38, 46). Our rescue experiments with the human *N-myc* transgene, which contains more upstream and downstream sequences than the 7.7-kb mouse genomic fragment, suggested the need for more *cis*-acting sequences to correctly direct the spatiotemporal expression profile of *N-myc* gene during mouse early embryogenesis. We first tested whether the 7.7-kb mouse genomic fragment can reproduce the *N-myc* developmental expression pattern by producing transgenic animals carrying a chimeric construct that contains this 7.7-kb mouse genomic fragment fused in frame to *lacZ* sequences. The *lacZ* sequences were inserted immediately downstream of the initiation codon of the N-Myc protein in the *N-myc* second exon, replacing 300 bp of *N-myc* coding sequences (construct *N-myc* 40; Fig. 3). The deletion of the *N-myc* coding sequences, which are not expected to contain any regulatory element, should not affect *N-myc* gene regulation. Of seven independent transgenic mouse lines established, three displayed embryonic transgene expression, including two with identical X-gal staining pattern (Fig. 4, A–D).

β -Galactosidase activity of the *N-myc* 40 transgene was monitored in embryos from E8.5 to E11.5, as the expression domain of *N-myc* is clearly defined at these stages. Moreover, these embryonic ages include the time of death of the *N-myc* homozygous mutant embryos (15, 28–30). At E8.5, expression was detected in the first branchial arch, and it remained restricted to these structures at E9.5 and E10.5 (Fig. 4, A–C). At E11.5, β -galactosidase expression was observed along the CNS with the loss of the specific staining in branchial arches (Fig. 4D). Additional *cis*-acting sequences were therefore required to recapitulate the appropriate spatiotemporal regulation of *N-myc* during embryogenesis.

To test this hypothesis, we generated transgenic animals carrying an *N-myc/lacZ* transgene containing 9.2 kb of genomic sequences and encompassing the *N-myc* gene from position -2.2 kb to position $+7.0$ kb (construct *N-myc* 41X; Fig. 3). Five transgenic lines were generated, among which three expressed the transgene and showed similar X-gal staining patterns. As for the *N-myc* 40 transgene, staining was localized in the first branchial arch at E8.5 (Fig. 4E). One day later, transgene expression was detected in branchial arches as well as in limb buds (Fig. 4F). At E10.5 and E11.5, X-gal staining was located in the same structures, but the expression was more restricted. Expression was localized in the apical ectodermal ridge of the limb and it was limited to the anterior region of the mandibular arch (Fig. 4, G and H). One transgenic line did not show expression in the limb buds, but

instead, expression in the CNS and DRG was observed at E11.5 (data not shown). Thus, additional 5' and 3' *N-myc* genomic sequences to the *N-myc* 40 transgene revealed the presence of regulatory elements specific to the limb buds.

A longer construct was tested, *N-myc* 41S, that contained a 16-kb *N-myc* genomic fragment including sequences from position -4.4 kb to position $+11.6$ kb (Fig. 3). Four *N-myc* 41S transgenic lines were established, and they all expressed the transgene with an identical pattern of X-gal staining in the branchial arches and limb buds. At E8.5 and E9.5, these lines presented the same expression pattern as the one observed with the *N-myc* 41X construct, with expression in the mesodermal tissues of the visceral arches and in the limb buds (Fig. 4, E, F, I, and J). At E10.5, the *N-myc* 41S transgenic lines reproduced the expression pattern observed in the *N-myc* 41X lines, but additional expression was seen in the CNS and DRG for two lines (Fig. 4K). However, staining intensity in the CNS was lower than in the visceral arches and limb buds. One day later, the same pattern was observed with a stronger signal in the CNS and DRG (Fig. 4L). Staining in the DRG was confirmed by sectioning the embryos (data not shown). A construct including *N-myc* genomic sequences from -8.7 kb to $+7.0$ kb was also tested in transient transgenics. This construct contained 4.3-kb more 5' sequences than the longest transgene *N-myc* 41. At E10.5, a similar expression pattern to the *N-myc* 41 construct ones was observed with staining in the branchial arches and the limbs, indicating that sequences required for the entire *N-myc* embryonic expression are located in even more 5' or 3' DNA regions (data not shown).

DISCUSSION

Regulation of gene expression implies a considerable number of molecular elements, all necessary to obtain a precise modulation of expression. *In vitro* assays are often used to

identify regions implicated in regulation. However, these assays may reproduce partial aspects of the regulation that takes place *in vivo*. Such *in vitro* experiments have revealed that correct *N-myc* expression can be supported by 1 kb of 5' flanking sequences and 6 kb of 3' flanking sequences of the human *N-myc* gene. These experiments demonstrated correct expression in human and murine cell lines as well as in mouse newborn and adult tissues (34). In contrast, our analysis using a transgenic approach has revealed that *N-myc* embryonic expression pattern cannot be entirely reconstituted with a similar mouse genomic fragment (*N-myc* 40). Moreover, 16 kb of mouse *N-myc* genomic sequences appears to be insufficient to reproduce the endogenous *N-myc* expression profile during embryogenesis.

Even if the pattern is not entirely reconstituted with the constructs we tested, sequences included in the genomic fragment extending from -4.4 kb to $+11.6$ kb contain regulatory elements able to direct expression in the branchial arches, the limb buds, the CNS, and the DRG. The *lacZ* expression detected in the branchial arches of the *N-myc* 40 transgenic mice indicated that branchial arch-specific control elements are included in the shortest construct. However, the higher levels of X-gal staining in branchial arches of specimens generated with the *N-myc* 41X transgene suggest that additional elements might be involved in *N-myc* branchial arch regulation. Furthermore, CNS expression was observed for all transgenes tested, but because expression in this structure was delayed when compared with endogenous *N-myc* gene expression, more regulatory sequences are most probably required.

The differences observed in the X-gal staining pattern between transgenes *N-myc* 40 and *N-myc* 41X suggest that regulatory elements directing *N-myc* expression in the limb buds must be present either in the 5' region located between positions -2.2 kb and -1.7 kb, or in the 3' region in the

Figure 3. Characterization of the regulatory regions of the mouse *N-myc* gene in transgenic mouse embryos. Schematic representation of the mouse *N-myc/lacZ* constructs and their sites of expression from E8.5 to E11.5. *A*, structure of mouse *N-myc* gene, from which the various constructs were derived. Open boxes indicate transcribed regions and black boxes correspond to translated regions. *B*, diagram of the *N-myc/lacZ* constructs used to generate the transgenic mice. The production of these constructs is described in the "Methods" section. *C*, summary of transgenic expression analysis. The first column represents the number of *lacZ*-expressing founders from the total number of transgenic founders obtained. The number of positively stained lines for each structure listed is indicated. BA, branchial arches; DRG, dorsal root ganglia; LB, limb buds; SOM, somites; B, *Bam*HI; Bs, *Bss*HI; N, *Nru*I; P, *Pvu*I; R, *Rsr*II, RI, *Eco*RI, S, *Sal*I, X, *Xba*I.

Figure 4. Developmental time course of β -galactosidase expression obtained with different *N-myc/lacZ* constructs in transgenic embryos. A–D, construct *N-myc* 40; E–H, construct *N-myc* 41 X; I–L, construct *N-myc* 41 S. Expression was analyzed at E8.5 (A, E, I), E9.5 (B, F, J), E10.5 (C, G, K), and E11.5 (D, H, L). Expression in branchial arches (arrowheads) and limb buds (arrows) is shown for E9.5 stage. Similar pattern of staining in branchial arches was observed for all three constructs, whereas expression in limb buds was restricted to longer constructs *N-myc* 41 X and *N-myc* 41 S (F–H, J–L).

+6.0-kb to +7.0-kb region (Fig. 3). The temporal expression of the *N-myc* 41X transgene in the limb buds mimicked the endogenous one (28). However, the tissue distribution did not correspond to the normal *N-myc* expression; *N-myc* transcripts are detected in the progression zone of the limb whereas transgene expression was restricted to the apical ectodermal region. Therefore, more regulatory sequences are needed to achieve cell-specific expression in the limb.

β -Galactosidase activity was never observed in the heart, and in only one instance in the somites. In the latter case, the expression was detected in the rostral part of the somites although normally *N-myc* expression is confined to the caudal moiety (28). Because a unique transgenic line (1 of 16) presented ectopic X-gal staining in the somites, this may reflect the influence of the site of integration on the expression of the transgene.

N-myc mutant embryos die because of heart defects (15–17, 20). Therefore, absence of the ability of the N-myc regulatory regions to direct expression in the heart might explain the lack of rescue of the N-myc mutant phenotype when a human N-myc transgene was used. However, we cannot rule out the possibility that the appropriate control sequences were present in the human transgene but that the human N-Myc protein differs in its properties from the mouse protein. This latter possibility appears unlikely owing to the high levels of identity between both proteins (41). In addition, recent data have shown that despite a lesser degree of homology, the N-Myc protein can replace the c-Myc one in its function (3). Accumulating experimental evidence demonstrates that regulatory elements of important developmental genes are conserved during evolution. In that regard, we can hypothesize that the mouse N-myc regulatory regions that will be identified from transgenic analyses will be similarly organized at the human N-myc locus (47).

Characterization of murine N-myc regulation has revealed several control elements, including two promoting regions located between positions –980 bp and –797 bp, and positions –279 bp and +108 bp, as well as an inhibiting sequence localized in the –860-bp to –797-bp region (32, 33). In addition, an orphan nuclear receptor element involved in the activation of N-myc transcription by ROR α 1 has been identified in the first intron of human and mouse N-myc genes (37). Moreover, in the human gene, sequences located in the exon 1–intron 1 region were shown to be implicated in the regulation of tissue-specific expression of N-myc (38, 39, 48).

Our findings provide evidence that these *cis*-acting elements known to regulate N-myc gene expression *in vitro* are insufficient to drive its proper expression *in vivo*. They strongly suggest that important distal regulatory elements exist in the N-myc gene, although definitive proof awaits their identification.

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