A targeted mutation reveals a role for N-*myc* in branching morphogenesis in the embryonic mouse lung

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The N-*myc* proto-oncogene encodes a putative transcription factor that has been postulated to be involved in the control of differentiation in a number of lineages at various stages during mammalian embryogenesis. We have generated a leaky mutation in N-*myc* by gene targeting in embryonic stem cells. In this allele, the *neo^r* gene was inserted into the first intron of N-*myc*, in such a way that alternative splicing around this insertion could result in the generation of a normal N-*myc* transcript in addition to a mutant transcript. Mice homozygous for this mutation died immediately after birth owing to an inability to oxygenate their blood. Histological examination revealed a marked underdevelopment in the lung airway epithelium, resulting in a decreased respiratory surface area. Analysis of N-*myc* expression in wild-type and homozygous mutant embryonic lungs suggests that N-*myc* is required for the proliferation of the lung epithelium in response to local inductive signals emanating from the lung mesenchyme. Homozygous mutant embryos were slightly smaller than normal and also had a marked reduction in spleen size, whereas other tissues that normally express N-*myc* appeared to be unaffected by the mutation. Molecular analysis revealed that normal N-*myc* transcripts were found in tissues from homozygous mutant embryos. Different tissues expressed the normal N-*myc* transcript at different levels relative to those observed in wild-type embryos, with the lowest levels being observed in the lungs. These results illustrate one way in which gene targeting can be used to generate partial loss-of-function mutations and support the importance of generating a series of alleles at a given locus to elucidate the various different functions of a gene during development.

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N-*myc* is a proto-oncogene that is expressed during the differentiation of several lineages during mammalian embryogenesis and, when deregulated, participates in neoplastic transformation. The *myc* family of proto-oncogenes, which includes N-*myc*, c-*myc*, and L-*myc*, encodes nuclear proteins that share structural motifs with a number of transcription factors that control cell determination (for review, see Lüscher and Eisenman 1990). Myc proteins share a basic region/helix-loop-helix (b-HLH) domain with the products of a number of the myogenic genes (for review, see Olson 1990), as well as with products of genes that function in the specification of the *Drosophila* central nervous system, such as *hairy* (Rushlow et al. 1989), *daughterless* (Caudy et al. 1989), and the genes of the *achaete-scute* complex (Villares and Cabrera 1987). The Myc proteins also possess a leucine zipper dimerization motif, which is found in transcrip-

tion factors such as those encoded by CCAAT/enhancer-binding protein (C/EBP) and the *jun* proto-oncogene, and in Fos, which associates with Jun by this motif (Kouzarides and Ziff 1988; Landschulz et al. 1988). A number of transcription factors possess both a b-HLH domain and a leucine zipper domain, including TFE3 (Beckmann et al. 1990), AP4 (Hu et al. 1990), and USF (Gregor et al. 1990).

The c-Myc protein possesses sequence-specific DNA-binding activity (Blackwell et al. 1990), which is enhanced by association, by complementary HLH-leucine zipper domains, with the Max and Myn proteins in humans and mice, respectively (Blackwood and Eisenman 1991; Prendergast et al. 1991). Although sequence-specific DNA binding has not yet been shown for the N-Myc protein, it has been shown to associate with Max with the same affinity as does the c-Myc protein (Blackwood and Eisenman 1991). Thus, N-*myc* is also likely to encode a sequence-specific DNA-binding protein. These results, taken together with the similarity N-*myc* has

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with genes encoding known transcription factors, suggest that *N-myc* also encodes a transcription factor.

The expression pattern of *N-myc* during mouse development suggests a role in the differentiation of certain embryonic lineages. Unlike *c-myc*, which is generally expressed in proliferating cells during embryogenesis (Zimmerman et al. 1986; Schmid et al. 1989), and continues to be expressed in some tissues in the adult mouse (Zimmerman et al. 1986; Semsei et al. 1989), *N-myc* expression is confined to specific lineages during embryonic development and is expressed in the adult only at low levels in the heart (Zimmerman et al. 1986). *N-myc* is expressed in undifferentiated embryonic stem (ES) and embryonal carcinoma (EC) cells and is down-regulated upon EC cell differentiation in vitro (Jakobovits et al. 1985). Within the embryo, *N-myc* is expressed in primitive mesoderm cells arising from the primitive streak, and its down-regulation correlates with differentiation into somites (Downs et al. 1989). During organogenesis, *N-myc* is expressed in the brain, eye, kidney, lung, heart, and intestine (Zimmerman et al. 1986; Hirning et al. 1991). Detailed in situ analyses of *N-myc* transcripts during organogenesis (Mugrauer et al. 1988; Hirning et al. 1991) have shown that expression is restricted to particular undifferentiated populations of cells within these organs, regardless of their proliferative state. Expression of *N-myc* within the lung, kidney, and intestine is localized to epithelial cells and is not observed in the surrounding mesenchyme (Mugrauer et al. 1988; Hirning et al. 1991). *N-myc* is also expressed at higher levels in pre-B-cell lines than in mature B-cell lines (Zimmerman et al. 1986). Thus, in general, *N-myc* is expressed in subsets of cells that are in an early state of differentiation, and the further differentiation of these cells correlates with *N-myc* down-regulation. This suggests that *N-myc* plays a role in the completion of the differentiation pathway in a number of lineages by maintaining cells in an undifferentiated state.

N-myc was originally identified as an amplified gene in neuroblastoma (Kohl et al. 1983, 1984; Schwab et al. 1983), and has also been found often to be amplified or overexpressed in Wilms' tumor (Nisen et al. 1986), small-cell lung cancer (Nau et al. 1986; Wong et al. 1986), and retinoblastoma (RB) (Lee et al. 1984). This series of tumors correlates with tissue types that normally express *N-myc* during development: the brain, kidney, lung, and eye, respectively (Zimmerman et al. 1986; Hirning et al. 1991). All of these tumors are thought to arise from embryonic cells or, in the case of small-cell lung cancer, from undifferentiated progenitor cells. These observations support the hypothesis that *N-myc* normally plays a role in controlling the differentiation of these tissues, and that continued *N-myc* expression prevents differentiation and thus promotes uncontrolled growth.

To address directly the function of *N-myc* during development, we attempted to mutate it by homologous recombination in ES cells and to introduce the mutation into the mouse germ line (Capecchi 1989). A replacement-type targeting vector was used to eliminate gene

function. However, during our studies we identified a line in which the targeting vector had integrated into the first intron by an insertion-type event. Our analysis has shown this to be a leaky mutation that reduces but does not eliminate normal *N-myc* transcription. Mice homozygous for this mutation die at birth owing to an inability to oxygenate their blood, a phenotype that apparently arises from a defect in branching morphogenesis in the lung. This mutant phenotype suggests that one role for *N-myc* is in the epithelial–mesenchymal interactions that are the basis of lung morphogenesis.

Results

Gene targeting

N-myc was mutated in ES cells using a replacement-type targeting vector (Fig. 1b), which included a promoterless *neo* gene flanked by 0.8 kb of 5' homology and 3.4 kb of 3' homology to the endogenous *N-myc* gene (Fig. 1a; DePinho et al. 1986). Homologous recombination or gene conversion between the vector and the endogenous *N-myc* gene was expected to result in the replacement of the coding region of the second *N-myc* exon with the *neo* gene and its accompanying SV40 polyadenylation signal. Because *N-myc* is expressed at high levels in ES cells (Jakobovits et al. 1985), this type of vector was designed to enrich for the detection of homologous recombination events relative to random integration events. A similar targeting strategy for the *N-myc* gene has been described by others (Charron et al. 1990; Stanton et al. 1990; Sawai et al. 1991).

Eleven targeted G418-resistant colonies were identified by detection of a novel polymerase chain reaction (PCR) fragment at a frequency of 1 targeted colony per 65 G418-resistant colonies, or 1 targeted clone per 1.6×10^7 cells electroporated (Table 1). All five of the PCR-positive colonies that were established as ES cell lines were confirmed as targeted lines by Southern blot analysis (Fig. 2). The level of enrichment for *N-myc* homologous recombinants among G418-resistant colonies was lower than that reported by others (Charron et al. 1990; Stanton et al. 1990; Sawai et al. 1991). This discrepancy may be the result of differences in the targeting vectors used, as our vector did not include a splice donor in the 5' region of homology upstream of the *neo*-containing exon.

Four of the targeted ES cell lines had integrated the targeting vector by homologous recombination in the predicted manner (Fig. 1c), as determined by Southern blot analysis (Fig. 2). The remaining line, line 9a, had integrated the targeting vector into *N-myc* in an unexpected manner. Line 9a was initially detected as a homologous recombinant both by PCR and by Southern blot analysis using an upstream probe on DNA digested with an enzyme that cuts within *neo* (Fig. 2a). However, Southern blot analysis using an exon 2 probe demonstrated that exon 2 had not been replaced in this targeted cell line (Fig. 2b). Extensive Southern blot analysis of line

f) 5'-AGCTGAGGACACTGAAGAGTT GGCAGTGCCTTGTGTGAATGA-3'

Figure 1. N-myc targeting scheme. (a) The endogenous N-myc locus, contained within a 7.4-kb *Eco*RI fragment (DePinho et al. 1986). (b) Targeting vector. A 0.9-kb *Pvu*II–*Sma*I fragment of the N-myc gene, including all but three codons of the coding region of the second exon, was replaced with the promoterless *neo* gene, which also had an SV40 polyadenylation signal. The *neo* gene was flanked upstream and downstream by 0.8 and 3.4 kb of N-myc sequences, respectively. (c) The predicted structure of the N-myc gene after gene replacement via homologous recombination. Primer annealing sites for PCR detection of homologous recombinants are shown (arrows a and b). (d) The structure of the targeted N-myc locus in line 9a, N-myc^{9a}. A probable single homologous recombination event between the endogenous gene and the 5' region of homology of the targeting vector has resulted in the insertion of the majority of the targeting vector into intron I of the endogenous gene. The annealing sites for primers that amplify the junction fragment at the site of integration of the 3' end of the targeting vector are shown (arrows x and y). (e) Possible transcripts encoded by the N-myc^{9a} allele. Alternative splicing to the inserted *neo*-containing exon or to the endogenous exon 2 downstream of it results, respectively, in a truncated transcript that includes only three N-myc codons or a normal N-myc transcript. (f) Sequence of the PCR-amplified junction fragment between the 3' end of the targeting vector and the endogenous gene. Nucleotides in boldface type are derived from intron II of the targeting vector; underlined nucleotides are from intron I of the endogenous gene. (Solid bars) N-myc-coding regions; (open bars) N-myc-noncoding exon sequences; (lines) intron and flanking regions; (SA) splice acceptor; (SD) splice donor; (B) *Bam*HI; (E) *Eco*RI; (SII) *Sst*II; (S) *Sal*I; (Pv) *Pvu*II; (Sm) *Sma*I.

9a (not shown) demonstrated that most of the targeting vector was inserted within the first intron of the endogenous N-myc gene. The deduced structure of the targeted N-myc gene in line 9a is shown in Figure 1d.

To determine precisely the site of integration of the targeting vector in line 9a, the new junction created between intron II of the inserted targeting vector and intron I of the endogenous N-myc gene was amplified by PCR using primers x and y (Fig. 1d) and then sequenced. The sequence of the junction (Fig. 1f) shows a clear transition from targeting vector to endogenous gene sequences, and the joining occurred without the genera-

tion of repeats or deletions. These results suggest the possibility that the electroporated linear targeting vector was partially degraded, recircularized within the cell, and integrated by a single insertion-type recombination event in the 5' region of homology.

The structure of the N-myc allele in line 9a, which will henceforth be referred to as N-myc^{9a}, was such that it would be a null mutation if all transcripts terminated in the *neo* poly(A) addition site. However, the N-myc^{9a} mutation would be leaky if some primary transcripts were alternatively spliced to the normal second exon, such that the *neo*-containing exon was removed (Fig. 1e).

Table 1. Frequency of gene targeting

Experiment	Number of cells electroporated	Number of G418 ^r colonies	Number of homologous recombinants		
			assayed by PCR	assayed by Southern blotting	
				targeted by replacement	targeted by insertion
1	5×10^7	100	2	2	0
2	2.5×10^7	180	5	1	0
3	7.5×10^7	300	3	1	1
4	3×10^7	130	1	0	0
Total	1.8×10^8	710	11	4	1

Germ-line transmission of the targeted ES cell line

Cells from three of the targeted lines, including line 9a, were injected into blastocysts from C57BL/6J females. All were able to contribute extensively to the resulting chimeric pups. However only line 9a, which contains *N-myc*^{9a}, was transmitted through the germ line (Table 2). The four male germ-line chimeras produced by blastocyst injection of mutant line 9a were bred to C57BL/6J and CD1 females to generate stocks of *N-myc*^{9a/+} mice that were used for subsequent experiments. These mice had no apparent abnormal phenotype.

Mice homozygous for the N-myc^{9a} mutation die at birth

To investigate the effect of the *N-myc*^{9a} allele in the homozygous condition, *N-myc*^{9a/+} mice were intercrossed, and DNA isolated from their offspring was tested by Southern blot analysis for the presence of the mutation. A total of 171 pups from such matings were analyzed at 3–5 weeks of age, and none were *N-myc*^{9a/9a}. To delineate when *N-myc*^{9a/9a} embryos died, DNA from embryos at various stages of gestation from heterozygote intercrosses was analyzed by Southern blotting or PCR.

Figure 2. Southern blot analysis of homologous recombinants. DNA isolated from 5 of the 12 PCR-positive ES cell lines was digested as shown. (a) *Bam*HI digest, probed with probe 1 (Fig. 1a). The lower band in this digest represents the targeted allele (for predicted fragment sizes, see Fig. 1). (b) *Eco*RI + *Sal*I digest, probed with an exon 2 probe (probe 2, Fig. 1a). The band present in all lanes represents the normal allele. Note that only line 9a has a new exon 2-containing band, indicating that *neo* has not replaced the coding region of exon 2 in this line.

Table 2. Frequency of germ-line transmission of targeted ES cell lines

Cell line	Number of blastocysts injected	Number of live progeny	Number of chimeras			Number of males test bred	Number of germ line
			females	males	total		
D3-N-myc ^{9a}	119	34	3	6	9	5	4
D3-N-myc ³⁷	280	48	8	9	17	5	0
D3-N-myc ³⁸	182	28	1	3	4	2	0

Of a total of 372 embryos dissected between 10.5 and 18.5 days of gestation, there were 93 wild-type, 179 N-myc^{9a/+} and 100 N-myc^{9a/9a} embryos. Thus, the absence of N-myc^{9a/9a} mice observed at weaning was not the result of fetal death but of early postpartum death. Occasionally, dead pups were found the first morning after delivery from a heterozygote mating. All but one of these pups were subsequently found to be N-myc^{9a/9a}. Thus, N-myc^{9a/9a} pups died near birth and were generally being cannibalized by the mother before they could be analyzed. On two occasions, a pup that was later found to be N-myc^{9a/9a} survived as long as 12 hr after birth; these pups were small, did not feed, and had erratic breathing.

To rescue N-myc^{9a/9a} embryos for further analysis, pups from heterozygote intercrosses were delivered by Caesarian section at 18.5 days postcoitum (p.c.). Of 68 pups, 52 lived, but the remaining 16 attempted to breathe and failed to oxygenate their blood sufficiently. In the 30 min following delivery, while littermates turned pink and began to move and breathe regularly, these 16 pups remained gray, immobile, and only gasped for air sporadically. These pups were always the smallest ones in a litter. Subsequent analysis of DNA isolated from all pups showed that these 16 and no others were N-myc^{9a/9a}.

Germ-line chimeras were also crossed to 129Sv/J females to place the mutation on an inbred background. The phenotype of N-myc^{9a/9a} embryos produced by heterozygote intercrosses was identical on inbred or hybrid genetic backgrounds (data not shown).

N-myc^{9a/9a} pups die because of a defect in lung morphogenesis

The abrupt death and the inability of N-myc^{9a/9a} pups to oxygenate their blood suggested a defect either in their ability to switch blood flow from the placenta to the lungs, or in the lungs themselves. Histological analysis of the lungs showed the latter to be true. We observed that immediately after birth, the air spaces in the lungs of N-myc^{9a/9a} pups were reduced in number and were individually much larger than the air spaces of their littermates (Fig. 3a,b). Furthermore, in the N-myc^{9a/9a} pups, the septa that separate the air spaces were thinner and often appeared to have been broken by the pressure of the first breaths of the pups. The outer surface of the lung was very thin and fragile in the homozygotes,

whereas in wild-type littermates it was strengthened by progressively smaller air spaces.

To determine the developmental progression of this phenotype, we analyzed the lungs of 12.5- to 16.5-day embryos. Between 15.5 and 16.5 days p.c. the lung mesenchyme of wild-type embryos is regularly interrupted by the organized epithelium that forms the developing airways and air spaces. The lungs of N-myc^{9a/9a} embryos were smaller and the epithelium was not as extensively branched, giving the mesenchyme a smooth, uninterrupted appearance (cf. Fig. 3c and d). We quantitated this effect by counting the number of developing air spaces per unit area in matched sections of the same lobe of lungs dissected from N-myc^{+/+} and N-myc^{9a/9a} 16.5-day sibling embryos (Table 3), and found that N-myc^{9a/9a} lungs contained approximately two-thirds the number of developing airways per unit area when compared with wild-type lungs ($P < 0.001$). This number is an underestimate of the reduction in the total number of developing airways because N-myc^{9a/9a} lungs were roughly half the size of wild-type lungs. It further underestimates the phenotypic difference between mutant and wild-type lungs because the developing airways in wild-type lungs were individually larger than in N-myc^{9a/9a} lungs.

The number and distribution of bronchi and bronchioles, from which the developing airways branch and which are histologically distinct from the more distal airways, appeared to be normal in N-myc^{9a/9a} embryos at 15.5 and 16.5 days p.c. Thus, the effect of the N-myc^{9a} mutation in homozygotes was restricted to the more distal airways of the developing lungs. A change in the overall size and morphology of the lungs in N-myc^{9a/9a} embryos was detectable as early as 12.5 days p.c., when N-myc^{9a/9a} lungs were dramatically reduced in size compared to normal lungs, with considerably less epithelial branching (Fig. 6a,b, below). At 13.5 days p.c., when the right lung normally is subdivided into its four constituent lobes, it was often observed that in the N-myc^{9a/9a} embryo these lobes were fused together, making the single right lung larger than the left lung (not shown).

Analysis of hematoxylin- and -eosin (H&E)-stained sections of embryonic lungs throughout their development did not indicate that any cell types were absent from N-myc^{9a/9a} lungs. Furthermore, we performed immunohistochemistry on lungs at 14.5 and 18.5 days p.c. using an antibody against the endothelial cell-specific Von Willebrand's factor and did not observe any abnormalities in the vascularization of N-myc^{9a/9a} lungs (not shown).

Figure 3. Histological analysis of lungs of *N-myc*^{+/+} and *N-myc*^{9a/9a} embryos. (a,b) Matched sections through the left lung of newborn littermates delivered by caesarian section at 18.5 days p.c. Bar, 250 μ m. (a) *N-myc*^{+/+}; (b) *N-myc*^{9a/9a}; (sep) septum; (tas) terminal air sac. (c,d) Matched sagittal sections of 15.5-day sibling embryos showing the large left lung and the small postcaval lobe of the right lung where it crosses to the left side of the embryo. Bar, 500 μ m. (c) *N-myc*^{+/+}; (d) *N-myc*^{9a/9a}; (aw) airway; (br) bronchus; (rl) right lung (postcaval lobe); (liv) liver.

The effect of the N-myc^{9a} mutation on the development of other lineages

The most direct explanation for the abrupt postnatal death of *N-myc*^{9a/9a} pups was that reduced branching and/or proliferation of the lung epithelium during lung morphogenesis led to a severely reduced lung surface area, which did not permit full oxygenation of the blood of the pups after birth. However, it was important to examine fetuses for other possible morphological alterations caused by the mutation. The only other obvious morphological defect in *N-myc*^{9a/9a} embryos was that the spleens of 18.5-day p.c. embryos were reduced to approximately one-fifth of their normal size (not shown). Nevertheless, there were no clear histological abnormalities in the cellular composition of the mutant spleens (not shown), as determined by examination of H&E-stained sectioned spleens. *N-myc*^{9a/9a} embryos were slightly smaller than their littermates from 12.5 days p.c. on, without appearing to be developmentally retarded. This reduction in overall size was not as considerable as the specific reduction observed in the sizes of the lungs and spleen. Surprisingly, we did not observe any obvious defect in the morphology or cytodifferentiation of any other organs in *N-myc*^{9a/9a} embryos as determined by

examination of H&E-stained, sectioned embryos and dissected tissues. Unaffected tissues include the brain and kidney (not shown), which normally express *N-myc* at high levels during their development, but we cannot preclude the possibility that subtle defects exist in these and other tissues that would be manifested postnatally were the lung defect not lethal.

Expression of the normal N-myc transcript in N-myc^{9a/9a} embryos

Because many of the tissues that express *N-myc* were not affected by the *N-myc*^{9a} mutation, it seemed likely that the phenotype observed in *N-myc*^{9a/9a} embryos was the result of a leaky mutation generating some normal *N-myc* transcripts because of alternative splicing around the *neo*-containing exon in the targeted locus. Northern blot analysis of RNA isolated from 18.5-day embryonic brain and kidney using an *N-myc* exon 1 probe showed that normal-length *N-myc* transcript was indeed present in *N-myc*^{9a/9a} embryos, but at considerably reduced levels relative to those observed in *N-myc*^{+/+} or *N-myc*^{9a/+} embryos (Fig. 4). In *N-myc*^{9a/+} and *N-myc*^{9a/9a} embryos, an additional truncated transcript of

Table 3. Quantitation of the lung defect in *N-myc*^{9a/9a} 16.5-day embryos

Litter	Section	N-myc ^{+/+} or N-myc ^{9a/+}		N-myc ^{9a/9a}		Chi-square	Degree of freedom
		developing airways (no.)	mean	developing airways (no.)	mean		
38	1	68	55	25	24	12.2	1
	2	62		23			
	3	40		25			
57	1	46	44	15	27	4.1	1
	2	48		32			
	3	39		35			
58	1	37	38.5	23	25	2.9	1
	2	40		29			
				24			
57, 59	1	40	45	31	34	1.5	1
	2	47		40			
	3	47		31			
Total of chi-squares						20.7	4
Chi-square of totals			182.5		110	18.0	1
Heterogeneity chi-square						2.7	3

Population is homogeneous: $0.25 < P < 0.5$; therefore, data may be pooled.

Total of chi-squares = 18.0; thus, $P < 0.001$ that difference between *N-myc*^{9a/9a} and *N-myc*^{+/+} or *N-myc*^{9a/+} is due to chance alone. Chi-square analysis was performed on these data to determine whether the difference in the number of developing airways in the lungs of *N-myc*^{+/+} and *N-myc*^{9a/9a} embryos was significant or whether it could be due to chance alone.

the size expected for an exon 1-*neo* fusion transcript was also detected with an exon 1 probe. These results indicate that the primary transcript encoded by the *N-myc*^{9a} gene could produce two alternatively spliced secondary transcripts, joining exon 1 either with *neo* or with the intact exon 2, which is 3' of *neo* (see Fig. 1c). Thus, the *N-myc*^{9a} mutation is leaky, and the phenotype observed in *N-myc*^{9a/9a} embryos is unlikely to represent the null phenotype.

The exon 1-*neo* fusion transcript predominated over the normal transcript in *N-myc*^{9a/9a} embryos (Fig. 4), but this does not indicate the extent to which splicing to the *neo*-containing exon was favored over splicing to the downstream exon, because the mutant transcript also predominated over the normal *N-myc* message in *N-myc*^{9a/+} embryos (Fig. 4). Rather, it suggests that the *neo*-containing transcript was more stable than the normal *N-myc* transcript.

We wished to determine whether different tissues of *N-myc*^{9a/9a} embryos expressed different relative levels of the normal *N-myc* transcript compared with levels observed in those tissues in wild-type embryos, and whether there was any correlation between lower relative levels of the normal transcript and mutant phenotype. To this end, RNase protections were performed using an *N-myc* exon 2 probe that detects the normal *N-myc* transcript but not the *N-myc-neo* fusion transcript. Levels of *N-myc* message in *N-myc*^{9a/9a}, *N-myc*^{9a/+}, and wild-type kidney, brain, and lung were quantitated by scanning densitometry, and the ratio of transcript levels within each tissue in *N-myc*^{9a/9a} compared with wild type were determined. We observed that *N-myc*^{9a/9a} 18.5-day kidney expressed at 35% of normal

levels, 18.5-day brain expressed at 50% of normal levels, and 15.5-day lung expressed at 25% of normal levels (Fig. 5). Thus, this analysis, which was repeated three times, showed that splicing around the *neo*-containing exon occurred in all tissue types examined, and that the efficiency with which this splice was made varied from tissue to tissue. We note that splicing around the *neo*-containing exon was consistently least efficient in the lungs, which, of the tissues examined, were the most severely affected in *N-myc*^{9a/9a} mice.

Analysis of N-myc expression in normal and N-myc^{9a/9a} lungs by in situ hybridization

To examine the normal *N-myc* expression pattern and the effect of the *N-myc*^{9a} mutation on the expression of *N-myc* in earlier embryos, whole-mount RNA in situ hybridization was performed on 12.5-day p.c. lungs dissected from wild-type and *N-myc*^{9a/9a} littermates using an *N-myc* exon 2 probe (Fig. 6). In situ analysis of wild-type lungs showed that *N-myc* is normally expressed exclusively in the lung epithelium, with particularly high levels in the distal tips of the developing airways. The primary and secondary bronchi express *N-myc* at lower levels (Fig. 6a). This was also observed in whole-mount RNA in situ hybridizations of 13.5-day lungs (not shown) and is consistent with the *N-myc* expression pattern observed in the lung epithelium by RNA in situ hybridization to sectioned 14.5-day lungs (Hirning et al. 1991). In contrast to the expression pattern observed in 12.5-day wild-type lungs, the normal *N-myc* transcript was barely detected in the developing epithelium of 12.5-day *N-myc*^{9a/9a} lungs (Fig. 6b). The normal *N-myc* transcript appears to be present at even lower levels relative

to wild type than was determined by RNase protection analysis of 15.5-day lungs (Fig. 5), suggesting that the lung phenotype is the result of the particularly low levels of normal *N-myc* transcripts present during its morphogenesis.

Discussion

We have generated mice carrying a leaky mutation in the *N-myc* proto-oncogene by gene targeting in ES cells. The phenotype of mice homozygous for this *N-myc* allele, *N-myc*^{9a}, has indicated an important role for *N-myc* during organogenesis in the lung. This does not imply that *N-myc* does not function in controlling the proliferation and/or the differentiation of other lineages in which it is expressed. The nature of the *N-myc*^{9a} mutation is such that the lack of any apparent phenotypic defects in other lineages in *N-myc*^{9a/9a} embryos probably results from expression of sufficient levels of the normal gene product in these lineages.

The *N-myc*^{9a} allele reveals a function in lung morphogenesis

Mice homozygous for the *N-myc*^{9a} mutation died at birth owing to a defect in lung morphogenesis. Specifi-

Figure 5. RNase protection analysis of *N-myc*^{+/+}, *N-myc*^{9a/+}, and *N-myc*^{9a/9a} embryos. Five micrograms of total RNA from 18.5-day embryonic brain (lanes 1–3; 3-day exposure), 2 μ g from 18.5-day kidney (lanes 4–6; 7-day exposure), and 10 μ g from 15.5-day embryonic lung (lanes 7–9; 24-hr exposure) from *N-myc*^{+/+} (lanes 1,4,7), *N-myc*^{9a/+} (lanes 2,5,8), and *N-myc*^{9a/9a} (lanes 3,6,9) embryos were hybridized to an antisense probe transcribed from an *N-myc* exon II *Pvu*II–*Asu*II fragment, which only hybridizes to the normal *N-myc* transcript. The densitometric intensities of *N-myc* bands within one tissue type were corrected for differences in loading by use of a β -actin control (bottom) and were normalized to the wild type. The ratios of band intensities are shown below each lane. (Lane 10) *N-myc* sense probe hybridized to 5 μ g of wild-type 18.5-day brain RNA; (lane 11) *N-myc* antisense probes hybridized to 10 μ g of tRNA; (lane 12) undigested *N-myc* antisense probe.

Figure 4. Northern analysis of *N-myc*^{+/+}, *N-myc*^{9a/+}, and *N-myc*^{9a/9a} embryos. Ten micrograms of total RNA from 18.5-day embryonic brain and kidney was run and probed with a 300-nucleotide *Sma*I fragment of *N-myc* intron I (probe 3, Fig. 1). This probe detects both an *N-myc*–*neo* fusion transcript of 1.8 kb in *N-myc*^{9a/+} and *N-myc*^{9a/9a} tissues, and a normal *N-myc* transcript of 2.9 kb in wild type, *N-myc*^{9a/+} and, at low levels, in *N-myc*^{9a/9a} tissues.

cally, we observed that the pulmonary epithelium, which lines the airways and air spaces of the developing lung and which normally expresses *N-myc*, failed to proliferate and branch normally in *N-myc*^{9a/9a} embryos. This phenotype was evident as early as 12.5 days of embryogenesis, when *N-myc* expression in normal lungs is at its highest level as determined by Northern analysis (Mugrauer et al. 1988), and appeared ultimately to result in small lungs with an insufficient surface area to oxygenate the blood of the newborn mouse. Morphologically, the lung phenotype was restricted to the developing airways while the number and distribution of the more proximal bronchi appeared to be normal. This is consistent with the normal expression pattern of *N-myc* observed in the lung, where there were very low levels of transcripts in the trachea and primary and secondary bronchi but higher levels in the tips of the developing airways.

Many studies have addressed the mechanism of morphogenesis in epitheliomesenchymal organs in general and in the lung in particular. In the lungs there is a mesodermally derived mesenchymal component and an epithelial component that is endodermally derived. The murine lung begins to form at the 25–27 somite stage as two endodermal evaginations from the pharyngeal region of the gut that extend into mesenchyme derived from splanchnic mesoderm (Weston 1984). These endodermally derived buds develop into the bronchial tree and the respiratory surface of the lung while the mesenchyme eventually forms the connective tissue, pulmo-

Figure 6. Whole-mount RNA in situ analysis of N-myc expression in N-myc^{+/+} and N-myc^{9a/9a} 12.5-day p.c. embryonic lungs showing expression (black staining) in the wild-type epithelium, but not in the mesenchyme, and very little expression in the N-myc^{9a/9a} epithelium. (a) N-myc^{+/+}; (b) N-myc^{9a/9a}. The probe used was the antisense transcript of an N-myc PvuII–AsuII exon 2 fragment. (br) Primary bronchus; (mes) mesenchyme; (ep) epithelium. Bar, 200 μ m.

nary smooth muscle, and vasculature of the lung. The lung grows by progressive and stereotyped branching of the proliferating epithelium into the mesenchyme.

Grafting experiments involving explanted, cultured embryonic lung in vitro have shown that the bronchial mesenchyme induces budding of the epithelium and controls its specific pattern of branching. This occurs during early lung morphogenesis [Alescio and Cassini 1962; Alescio and Colombo Piperno 1967] at a time when a defect was first observed in N-myc^{9a/9a} lungs. The mesenchyme appears to control epithelial branching morphogenesis by inducing local, differential mitotic activity in the lung epithelium with which it is in contact [Goldin and Wessells 1979; Goldin and Opperman 1980; Goldin et al. 1984]. The phenotype of N-myc^{9a/9a} embryos, combined with the normal localization of N-myc expression to the lung epithelium, suggests that N-myc is required for the epithelium to receive or respond to the inducing signals from the mesenchyme. Because N-myc is a nuclear protein and a putative transcription factor, it presumably acts downstream of receptor mechanisms in this signal transduction pathway and may be involved in locally activating genes required for the proliferation of the lung epithelium. The identification of a role for

N-myc in branching morphogenesis may help in uncovering other genes that function upstream of N-myc in this epithelial–mesenchymal interaction.

N-myc has been shown to be amplified or otherwise deregulated in small-cell lung cancer (SCLC; Nau et al. 1986; Wong et al. 1986). The role for N-myc in lung morphogenesis revealed by the N-myc^{9a} mutation is consistent with N-myc deregulation contributing to the transformation of adult lung cells and to the progression of lung cancer, particularly because SCLC has its origin in the lung epithelium [Andrew et al. 1982, and references therein]. Specifically, SCLC is derived from bronchial epithelial stem cells that are committed to differentiate toward neuroendocrine cells of the lung [Gazdar et al. 1981; Gazdar 1984]. N-myc amplification is not a first step in the progression of SCLC [Bergh 1990; Noguchi et al. 1990], and the inactivation of tumor suppressor genes, such as p53 or RB—events that are common in lung cancer [Bergh 1990; Kaye et al. 1990; Takahashi et al. 1991]—may initiate the process of tumorigenesis in bronchial epithelial stem cells. Amplification of N-myc may then be one factor that provides a selective growth advantage to transformed cells and that promotes tumor progression.

Phenotypic effects of the *N-myc*^{9a} mutation on other lineages

A number of tissues other than the lung express *N-myc* at particular stages during their development. RNA in situ hybridization analysis has shown that subsets of cells within the developing kidney, lung, brain, and intestine express *N-myc* (Mugrauer et al. 1988; Hirning et al. 1991), and Northern blot analysis has shown that low levels of *N-myc* transcripts are also detectable in the embryonic spleen and heart (Zimmerman et al. 1986; Semsei et al. 1989). Despite expression at these sites, the only tissue other than the lung that showed a clear defect in *N-myc*^{9a/9a} embryos was the spleen. The effect of the *N-myc*^{9a} mutation on the spleen was a reduction in the size of the spleen without an alteration of its morphology. The exact nature of this defect is not yet clear, as little is known about the mechanism of spleen morphogenesis or about the expression pattern of *N-myc* within the developing spleen. The primary spleen phenotype could not have been the result of an absence of hematopoietic cells, because the phenotype could be observed before hematopoiesis occurs in the spleen. Furthermore, lethally irradiated mice reconstituted with fetal liver cells from *N-myc*^{9a/9a} embryos were healthy and expressed terminally differentiated B-cell antigens, suggesting that later stages of hematopoiesis and lymphopoiesis were not affected by the *N-myc*^{9a} mutation (C.B. Moens et al., unpubl.).

The *N-myc*^{9a/9a} phenotype provides no information about the function of *N-myc* in epithelial–mesenchymal interactions other than in the lung. However, it is interesting to note that within the developing kidney and intestine, *N-myc* is expressed exclusively in epithelial cells (Mugrauer et al. 1988; Hirning et al. 1991). Other *N-myc* alleles or combinations of alleles may make it possible to study whether *N-myc* functions more generally in epithelial–mesenchymal interactions during development.

Because *N-myc*^{9a/9a} mice die at birth, we could not determine whether other tissues suffered subtle defects that would manifest themselves postnatally. We note, however, that *N-myc*^{9a/9a} embryos were slightly smaller than their wild-type or *N-myc*^{9a/+} littermates, suggesting that there were subtle effects in some lineages other than the lungs and the spleen. This observation is unlikely to indicate a role for *N-myc* in general cell proliferation, because *N-myc* is expressed in a restricted manner during development.

Molecular basis of the *N-myc*^{9a/9a} phenotype

The phenotypic effects of the *N-myc*^{9a} mutation are very specific to the lungs and the spleen. Recent results from studies of mice bearing a null mutation at the *N-myc* locus indicate that *N-myc* must play a vital role earlier in development, as homozygous embryos die before birth (Sawai et al. 1991). Embryos homozygous for a *N-myc* null allele generated elsewhere have been found to die prior to organogenesis when neither the lungs nor the spleen are required to function (B. Stanton and L. Parada,

pers. comm.). Presumably the difference in phenotypes between homozygotes of *N-myc*^{9a} and of the null mutation is the result of the leaky nature of the *N-myc*^{9a} mutation.

The *N-myc*^{9a} allele contains a splice donor in the endogenous first exon, followed by two identical exon 2 splice acceptors, the first preceding *neo* and the second in the normal exon 2, which is downstream of the inserted targeting vector (Fig. 1). The *neo*-containing exon has no splice donor but does contain an SV40 polyadenylation signal. We have observed that both splice acceptors are used, generating two alternate transcripts: a mutant transcript that terminates after *neo*, and a normal *N-myc* transcript. It has been shown that the presence of a splice donor is required for exon definition and efficient splicing in vivo, whereas a mutation of the splice donor of an internal exon often results in the skipping of that exon in vitro and in vivo (Robberson et al. 1990; Talerico and Berget 1990). However, the polyadenylation machinery likely functions to define an exon that lacks a splice donor but includes a polyadenylation site (Robberson et al. 1990). Thus, in cells containing the *N-myc*^{9a} allele, there may be a competition between the RNA-processing machinery: If *neo* is polyadenylated first, a mutant transcript is generated; and if the flanking exons are defined prior to *neo* polyadenylation, the *neo*-containing exon is skipped and a wild-type *N-myc* transcript is generated.

All tissues tested were able to splice around the *neo*-containing exon to make a normal *N-myc* transcript, but with different efficiencies. The lungs, which were severely affected in *N-myc*^{9a/9a} embryos, appeared to splice around *neo* with the lowest efficiency as judged by RNase protection analysis. Furthermore, RNA in situ hybridizations showed the levels of normal *N-myc* transcripts in very early *N-myc*^{9a/9a} lungs to be extremely low compared with wild-type lungs. This suggests that the lung phenotype was caused by these low levels of normal transcript, and the absence of a phenotype in other tissues that normally express *N-myc* was attributable to sufficient levels of the normal transcript. However, the differences in levels of the normal transcript between different tissues in *N-myc*^{9a/9a} embryos was not so significant as to preclude the possibility that the lungs are particularly sensitive to reduced levels of the *N-myc* transcript. Unfortunately, the small size of the *N-myc*^{9a/9a} spleen and the low levels of *N-myc* expression in the spleen prevented a similar analysis of *N-myc* expression in this tissue.

The *N-myc*^{9a} mutation: mechanism of targeting vector integration

N-myc^{9a} was an unexpected mutation in which the targeting vector was inserted into the first *N-myc* intron. A likely mechanism for this type of recombination event is that the linear replacement-type targeting vector, upon entering the cell, circularized and underwent a single homologous recombination event between the endogenous gene and one of the regions of homology within the

targeting vector. This type of insertion event would only be detected as a homologous recombinant by our PCR screening approach if the short arm of homology were involved in the crossover, as was the case in *N-myc*^{9a}. Another possible mechanism by which this type of insertion could occur is that the targeting vector concatenated prior to integration and then underwent a double crossover or gene conversion event involving the two 5' regions of homology. Regardless of the mechanism, the insertion of replacement-type vectors resulting in this type of targeted locus is not uncommon (Hasty et al. 1991).

Given that the integration of the targeting vector results in a duplication of *N-myc* sequences, the *N-myc*^{9a} mutation might be expected to be somewhat unstable, with intrachromosomal homologous recombination resulting in either the regeneration of the wild-type *N-myc* allele or in the generation of an *N-myc*^{null} allele. If this occurred regularly during the development of *N-myc*^{9a/9a} embryos, the ratio of *N-myc*^{9a/+} to *N-myc*^{9a/9a} embryos detected by PCR in litters from heterozygote intercrosses would be expected to exceed 2:1, because *N-myc*^{9a/9a} embryos in which this had occurred would be scored as *N-myc*^{9a/+}. This was not observed: among 372 embryos dissected during gestation, there were 93 *N-myc*^{+/+}, 179 *N-myc*^{9a/+} and 100 *N-myc*^{9a/9a} embryos. Thus, within the limits of detection, the insertion mutation appears to be stable, and mosaicism need not be a concern in interpreting the phenotype.

Among the embryos that were dissected during gestation, the gross phenotypes of very few were inconsistent with their genotype. Three embryos appeared to have a mutant phenotype (small lungs and spleen) upon dissection but were *N-myc*^{9a/+}, and one embryo that did not have a mutant phenotype was *N-myc*^{9a/9a}. These anomalous embryos may have been mosaics, or more likely are indicative of variable penetrance of the *N-myc*^{9a} mutation, whereby in some embryos the *N-myc*^{9a} allele makes slightly more or less of the normal *N-myc* transcript than in others. Variable penetrance would not be entirely unexpected because the mutation is on an outbred genetic background. An alternative possibility is that the phenotype we have associated with the *N-myc*^{9a} mutation is a result of an independent, closely linked mutation, which, in anomalous embryos, has segregated from the *N-myc*^{9a} locus. Because the *N-myc*^{9a} mutation is unique to the cell line we generated, and it would be very difficult to generate precisely the same mutation again, we cannot rule out this possibility by testing other cell lines. However when *N-myc*^{9a/+} mice are crossed to mice heterozygous for a null mutation in *N-myc*, the resulting compound heterozygotes have a related embryonic lethal phenotype. This demonstrates that the two mutations are clearly allelic (C. Moens and B. Stanton, unpubl.).

Conclusions

We have described the phenotype of mice that are homozygous for a leaky mutation in the *N-myc* proto-on-

cogene. This phenotype has allowed us to propose a function for *N-myc* in the epithelial-mesenchymal interaction that is the basis of lung morphogenesis. Such a function is consistent with the known expression pattern of *N-myc* in the lung and with the involvement of *N-myc* in the progression of lung cancer.

If patterns of expression are a guide, many developmentally important genes play multiple roles at different stages and in a number of different lineages during embryogenesis. A phenotypic series generated by different mutant alleles or combinations of alleles is therefore useful in determining the functions of a gene during development. These alleles would include null mutations, leaky mutations, and more subtle site-directed mutations either in tissue- and stage-specific *cis*-acting regulatory elements or in the gene itself, which would lead to tissue- or stage-specific defects.

N-myc is one such gene, whose wide expression pattern during embryogenesis indicates a multiplicity of roles. Embryos homozygous for a null mutation in *N-myc* die at mid-gestation (B. Stanton and L. Parada, pers. comm.), and clearly this mutation is central to defining the earliest essential role for the gene. Two later roles in lung and spleen morphogenesis are revealed by the *N-myc*^{9a/9a} mice. By crossing *N-myc*^{null/+} mice with *N-myc*^{9a/+} mice, we can generate *N-myc*^{9a/null} embryos that should have further reduced levels of normal *N-myc* transcript and may reveal other sensitive developmental stages. Thus, this kind of leaky mutation can help to achieve the goal of producing an allelic series of mutations for a developmentally interesting gene while strategies for directing mutations to particular stages of embryogenesis are being developed.

Materials and methods

Targeting vector construction

The *N-myc* genomic clone (DePinho et al. 1986) was the gift of R. DePinho and F. Alt (Columbia University, NY). The targeting vector extends from an *Sst*II site in intron 1 to an *Eco*RI site downstream of exon 3 but is deleted for a *Pvu*II-*Sma*I fragment, which includes the coding region of exon 2 and 70 bp of intron 2 and is replaced with a 1-kb promoterless *neo* (*Bgl*II-*Sma*I) with a 250-bp SV40 polyadenylation signal. There are two in-frame stop codons between the *N-myc* ATG and the *neo* ATG, the 5'-most being 15 codons upstream of the *neo* ATG.

Electroporation and selection of ES cells

D3 ES cells (5×10^6) (Joyner et al. 1989) per 0.8 ml of PBS were electroporated with 40 μ g of *Eco*RI-digested targeting vector DNA using a Bio-Rad Gene Pulser (500 μ F, 250 V). Treated cells were allowed to recover for 20 min at room temperature before being seeded onto gelatinized 90-mm tissue culture dishes (Nunculon) at a density of 5×10^5 cells per plate in Buffalo rat liver cell (BRL)-conditioned medium (Smith and Hooper 1987). BRL-conditioned medium was replaced 48 hr after electroporation with BRL-conditioned medium containing 100 μ g of active G418 (GIBCO). Cells were maintained under selection for ~10 days, with selection medium being changed every other day.

Screening of G418-resistant colonies

After 10 days of selection, one-half of each resistant colony was picked and analyzed by PCR as described (Joyner et al. 1989) to distinguish targeted from random integrations. The remaining half-colony was left under selection until the PCR result was obtained. The 3' and 5' oligonucleotide primers for PCR anneal in the 5' end of the *neo* gene and in the first N-*myc* intron outside of the sequences included in the targeting vector (primers a and b, Fig. 1c). PCR was run for 40 cycles of 2 min denaturation (94°C), 2 min annealing (55°C), and 10 min extension (70°C) in a Perkin-Elmer thermal cycler. The remaining halves of those colonies that gave positive signals by PCR were subsequently picked onto dishes containing a primary embryonic fibroblast layer and were expanded in 15% FCS in the absence of G418 and later used for Southern analysis and blastocyst injection.

Southern blot analysis of ES cell lines and mice

Ten micrograms of DNA purified from ES cells or tails of mutant mice was digested, either with *Bam*HI or *Eco*RI plus *Sal*I. Digested DNA was transferred onto GeneScreen nylon membrane and was probed either with probe 1 [Fig. 1a (for *Bam*HI digests)] or with probe 2 [Fig. 1a (for *Eco*RI plus *Sal*I digests)]. This latter probe is deleted in a predicted homologous recombinant but is not deleted in N-*myc*^{9a}. Blots were hybridized in 50% sodium phosphate, 15% formamide, 7% SDS, 100 mg/ml of fatty acid-free BSA, and 1 mM EDTA at 63°C. Blots were washed in 30 mM sodium phosphate at 60°C.

Blastocyst injection

Groups of 10–20 cells from the three mutant cell lines indicated were injected in separate experiments into blastocysts flushed from the uteruses and oviducts of 3.5-day p.c. C57BL/6J (Jackson Lab) mice. Injected blastocysts were transferred into the uterus of 2.5-day p.c. pseudopregnant CD1 (Charles River) recipients.

Breeding of chimeric animals

Because the parental D3 ES cell line is derived from 129/J agouti mice, its descendants in the skin of the chimera will produce agouti hair on an otherwise black background. Male chimeras with extensive ES contribution in the coat were bred with C57BL/6J females to test for germ-line transmission of the dominant Agouti coat color marker. Agouti offspring of chimeric males were then tested for the presence of the N-*myc*^{9a} mutation by Southern blot analysis of *Bam*HI-digested tail DNA using probe 1 (Fig. 1a).

Typing and fixation of embryos for histology

Dissected embryos were typed by Southern blot analysis or by PCR. For PCR, three 21-mer primers were used to distinguish between N-*myc*^{+/+}, N-*myc*^{9a/+}, and N-*myc*^{9a/9a} embryos. Primer 1 annealed to the antisense strand of N-*myc* intron 1, upstream of the site of integration of the targeting vector; primer 2, which is the same as primer b in Figure 1, annealed to the sense strand in the 5' end of *neo*; and primer 3 annealed to the sense strand in the 5' end of N-*myc* exon 2. Primers 1 and 2 amplified a 475-nucleotide fragment from the N-*myc*^{9a} allele, and primers 1 and 3 amplified a 583-nucleotide fragment from the wild-type allele. Tissues from N-*myc*^{+/+}, N-*myc*^{9a/+}, and N-*myc*^{9a/9a} embryos were either fixed for histology or pooled according to genotype for extraction of total RNA. Dissected

embryos and organs were fixed overnight in 30% ethanol, 10% acetic acid, and 10% formaldehyde and were cleared and embedded in paraffin wax (Tissue Prep). Blocks were sectioned at 5–7 μ m and stained with H & E.

RNA preparation, Northern and RNase protection analysis

Fetal brain, kidney, lung, and liver were dissected from embryos at the stages stated in the text. Tissues were frozen immediately in liquid nitrogen until embryos were genotyped. RNA from genotyped embryos was prepared using an acid guanidinium thiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi 1987). For Northern analysis, 10 μ g of total RNA was run on a denaturing formaldehyde gel and transferred onto a GeneScreen nylon membrane. Blots were probed under the conditions described for Southern blots with an exon 1 probe (0.3-kb *Sma*I fragment of the N-*myc* genomic clone) and were washed in 30 mM sodium phosphate at 62°C. For RNase protection analysis, probes were transcribed in vitro using Promega Riboprobe System II. For N-*myc* probes, a 215-nucleotide *Pvu*II–*Asu*II fragment from exon 2 was cloned into pGEM7, and sense and antisense probes of 290 nucleotides were transcribed with T7 and SP6 polymerases, respectively. For β -actin probes, a 245-nucleotide *Kpn*I–*Xba*I fragment of the mouse β -actin cDNA was cloned into pGem3Z, which was cut with *Hinc*II to make a 150-nucleotide antisense transcript when transcribed with SP6 polymerase. RNase protections were performed as described (Sambrook et al. 1989). A phosphor screen was exposed to the dried gel and scanned using the Molecular Dynamics PhosphorImager. Band intensities were quantitated using the ImageQuant software (Molecular Dynamics). Levels of N-*myc* transcripts were corrected for uneven loading by the use of a β -actin control.

*Sequence analysis of N-*myc*^{9a} mutation*

Southern analysis of DNA from cell line 9a and from embryos homozygous for N-*myc*^{9a} localized the point at which targeting vector sequences rejoin endogenous N-*myc* sequences to be within 200 bp, 5' of the *Xho*I site in intron 2 of the targeting vector, and within 200 bp, 3' of the *Bgl*II site in intron 1 of the endogenous gene. Primers capable of amplifying a junction fragment in this region were designed (primers x and y in Fig. 1d) and were used to amplify a 170-nucleotide fragment. This fragment was blunt ended and cloned into *Sma*I-digested pGem7. Sequencing was carried out using the universal primer and Sequenase Sequencing Kit (U.S. Biochemical).

Whole-mount in situ hybridization

The in situ hybridization procedure was adapted from Tautz and Pfeifle (1989) and from Hemmati-Brivanlou et al. (1990) as described (R. Conlon and J. Rossant, in prep.). The probe used was transcribed from a 215-bp *Pvu*II–*Asu*II fragment of exon 2.

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