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Defects of embryonic organogenesis resulting from targeted disruption of the N-*myc* gene in the mouse

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SUMMARY

The highest expression of the N-*myc* gene occurs during embryonic organogenesis in the mouse ontogeny, with the peak of expression around embryonic day 9.5. Homozygous N-*myc*-deficient mice, produced by germline transmission of a disrupted allele in ES cells, developed normally to day 10.5, indicating dispensability of N-*myc* expression in the earlier period, but later accumulated organogenic abnormalities and died around day 11.5. The most notable abnormalities were found in the limb bud, visceral organs (lung, stomach, liver and heart) and the central/peripheral nervous systems, and were highly correlated with the site of N-myc expression. The limb buds and the lungs excised from N-*myc*-deficient mutant embryos were placed in culture to allow their development to stages beyond the point of death of the embryos. Analyses indicated that the mutant limbs failed to develop distal structures and the development of bronchi from the trachea was defective in the lungs. The latter defect was largely corrected by addition of fetal calf serum to the culture medium, suggesting that an activity missing in the mutant lung was replenished by a component of the serum. The phenotype of N-*myc*-deficient mutant embryos indicated requirement of the N-*myc* function in many instances of tissue interactions in organogenesis and also in cell-autonomous regulation of tissue maturation.

Key words: N-*myc*, targeted gene disruption, ES cells, organogenesis, limb buds, organ culture, mouse

INTRODUCTION

It is a widely accepted notion that proto-oncogenes have essential regulatory functions in normal ontogenesis. Many of them are expressed primarily in the embryos and are thought to regulate embryogenesis in direct or indirect ways. A straightforward approach to clarify the function of such regulatory genes is to analyze the consequence of mutational alteration of the gene in an animal's life. This can be accomplished by gene targeting technology using embryonic stem (ES) cells (Thomas and Capecchi, 1987).

The N-*myc* gene is one of the oncogenes whose normal function is implicated in the regulation of embryogenesis. Although recognized by its similarity with the c-*myc* gene and its amplification in malignant neuroblastomas (Kohl et al., 1983; Schwab et al., 1983), analysis of normal ontogeny indicated that N-*myc* gene is primarily expressed in the early part of embryonic development, especially during organogenesis (Jakobovitz et al., 1985; Kato et al., 1991). This is in contrast to the expression of c-*myc*, which appears to persist throughout an animal's life. N-*myc* also appears to differ from L-*myc*, another member of the myc family, in its spatiotemporal regulation of expression (Zimmerman et al., 1986; Sawai and Kondoh, unpublished observation).

The N-*myc* protein is usually found in the nucleus (Ikegaki et al., 1986; Ramsay et al., 1986; Slamon et al., 1986; Ueno et al., 1988; Kato et al., 1991), and is considered to bind to DNA in a sequence-specific manner upon hetero-dimerization with a partner protein (Max/Myn), as demonstrated for the c-*myc* protein (Blackwood and Eisenman, 1991; Prendergast et al., 1991). There is evidence that c-*myc* protein has the capacity to regulate transcription (Kato et al., 1990). Since there is a considerable conservation of amino acid sequence motifs among the myc family proteins, especially between N-myc and c-myc, similarity of their molecular actions as transcriptional regulators has been strongly suggested. However, there are N-myc-specific domains in the sequence that are conserved among the animal species (Sawai et al., 1990), indicating activities that are unique to N-myc. Thus, it is a likely hypothesis that N-*myc* protein regulates transcription of a particular group of genes that participate directly in the organogenic process.

Involvement of the N-*myc* activity in the process of organogenesis was even more strongly suggested by histological analyses of N-*myc* expression utilizing in situ hybridization. During organogenesis of the mouse, N-*myc* expression was initially localized in the primitive streak (day 7.5), then in the neural fold and presomitic mesoderm (day 8.5; Downs et al., 1989). At day 9.5, N-*myc* expression was high in the central nervous system, neural crest derivatives, limb bud mesenchyme, myocardium, and a few additional condensed mesoderm derivatives (Kato et al., 1991). The distribution of N-*myc* transcripts in mouse embryos at day 9.5 was highly correlated with that of N-*myc* protein detected by N-myc-specific antibodies (Kato et al., 1991), and almost identical to that in 3.5 day chicken embryos (Sawai et al., 1990). During the later period of organogenesis, the expression of N-myc in previously positive tissues gradually diminished (Wakamatsu and Kondoh, unpublished observations), and in turn the expression commenced in endodermal epithelia of the lung (Hirning et al., 1991) and the digestive tract as will be described below.

Previously, we reported disruption of N-*myc* alleles, singly or doubly, in ES cells by insertion of neomycin/hygromycin- resistance sequences into the N-*myc* coding region by utilizing homologous recombination (Sawai et al., 1991). Unexpectedly, totally N-*myc*-deficient ES lines made by consecutive gene disruption were normal in cell morphology, growth properties and differentiation repertoire under culture conditions, indicating the non-essentiality of N-*myc* activity in the ES cells in spite of the high N-*myc* expression in normal ES lines (Sawai et al., 1991), a situation analogous to the one observed in *pim* double knockout ES lines (Riele et al., 1990).

However, when disrupted N-*myc* alleles were transmitted to descendants from the primary mouse chimeras made between normal blastocysts and the ES cells, and resulting heterozygotes were intercrossed, the embryonic death of homozygous N-*myc*-deficient mice became apparent (Sawai et al., 1991). We describe here the defects of organogenesis observed in N-*myc*-deficient homozygous mutants (hereafter simply called 'mutants') which are highly correlated with the tissue distribution of N-*myc* expression during the period from day 9.5 to day 12, and indicate involvement of N-*myc* in tissue interactions required for normal organogenesis. The mutant phenotype described here is very different from that described for very leaky N-*myc* mutants generated by non-replacement type mutagenesis (Moens et.al., 1992), where the lung was the only organ affected during embryogenesis.

MATERIALS AND METHODS

Staging and genotyping of embryos and mice

Embryos were staged according to their development as described by Theiler (1989) and the stages expressed as days post coitum. To detemine genotype of the N-*myc* locus, DNAs were extracted from yolk sacs or adult tails after digestion with Proteinase K (Sawai et al., 1990), and were analyzed by Southern blotting (yolk sacs of 10.5 day and later embryos) done according to Sawai et al. (1990) or by use of polymerase chain reaction (PCR) for other cases (Fig. 1). The primers used were:

a, CGGACGAAGATGACTTCTAC;
b, ACATGCAGTCCTGAAGGATG;
c, GCTTGCCGAATATCATGGTG.

Histology

Histological sections stained with hematoxylin and eosine were prepared from Bouin-fixed and paraffin-embedded specimens according to the conventional method. In situ hybridization of N-*myc* transcripts on paraffin sections (Wakamatsu and Kondoh, 1990; Kato et al., 1991) and immunofluorescent staining of N-*myc* and neurofilaments on cryosections (Kato et al., 1991) were done as described.

Organ cultures

Organ cultures of embryonic lungs and limbs were carrried out according to Hirai et al. (1989), except that the culture medium was L-15, the gas phase was the air and fetal calf serum was omitted where indicated.

RESULTS

Apparently normal development of N-*myc*-deficient mutant embryos up to day 10.5

The previous observations that N-*myc* deficiency resulted in embryonic lethality but that N-*myc* activity is dispensable in ES cells (Sawai et al., 1991) suggested the requirement of N-*myc* in the postblastocyst periods. Thus, we analyzed the implanted embryos produced from crosses between the N-*myc* heterozygous mice. In assessing the phenotype, the genotype of the embryo was determined using yolk sac DNAs, either by Southern blotting or PCR (Fig. 1), and the embryo proper was analyzed for the phenotype.

Since the phenotype of N-*myc*-deficient homozygous embryos might differ depending on the genetic background of the mouse, we crossed the N-*myc*-heterozygous mice for several generations with Balb/c or ICR and made two isolated pedigrees. Then, sibling heterozygotes were mated to obtain homozygous embryos. However, since the results on homozygous embryos were, in fact, the same between Balb/c and ICR pedigrees, the data were combined without specifying the genetic background.

Among the 9.5 and 10.5 day embryos collected from several litters, wild-type, heterozygous and homozygous embryos of the N-*myc* locus were found in the ratio approximated by the Mendelian 1:2:1, demonstrating the viability of N-*myc*-deficient embryos up to these stages (Table 1).

At day 9.5, homozygous mutants were normal in size, external morphology and development of internal organs (data not shown). N-*myc*-deficient mutant embryos were also recovered with normal morphology at day 10.5. Overall histogenesis of the limb buds, visceral arches and the central nervous system was not significantly affected by the lack of N-*myc* activity (data not shown). N-*myc* expression is prominent in normal embryos during the period 9.5 to 10.5 days (Kato et al., 1991; and described below). Thus, although the N-*myc* expression is high in the embryonic period up to day 10.5, embryogenesis did proceed even in the absence of N-*myc* expression as far as this stage.

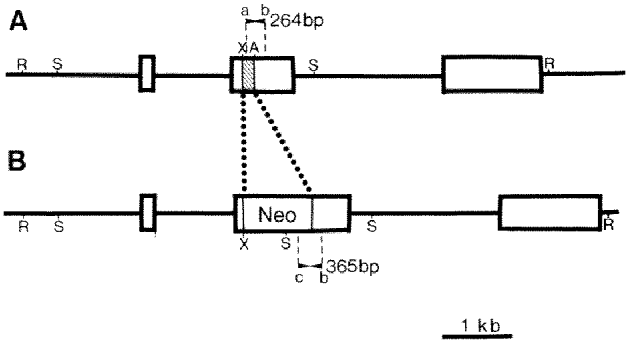

Fig. 1. Structure of normal (A) and mutant (B) alleles of N-*myc*. The exons are in boxes. The restriction sites: A, *Asu*II; R, *Eco*RI, S, *Sph*I; X, *Xho*I. The coding sequence starts immediately to the 5′ (left) of the *Xho*I site. The part of the coding sequence between the *Xho*I and *Asu*II sites in the second exon (hatched) is replaced by a neo sequence in the mutant allele. As a result, translation of the rest of the N-*myc* sequence (to the right) is inhibited in the mutant allele (Sawai et al., 1991). The locations of PCR primers (A-C) and the size of the amplified sequence for each allele are also indicated.

Table 1. Effect of N-myc deficiency on embryonic development

Day	(Litters examined)	Embryos recovered* +/+	+/−	−/−	Resorbed embryos
9.5	(8)	20	34	19	
10.5	(4)	6	14	7	
11.5	(12)	29	47	18†	4
12.5	(2)	6	6	4§	5
14.5	(2)	3	12	0	5
15.5	(1)	2	4	1¶	3
16.5	(1)	2	7	0	3
Total	(30)	68	124	49	20

*+/+, wild type; +/−, heterozygous; −/−, homozygous mutant at the N-*myc* locus.

†Six of the embryos were dead when examined. In these cases, the dead embryos had developed to the stage of day 11.5 and normal live siblings had developed to the stages slightly more advanced.

§All were dead and in the process of being resorbed.

¶The embryo shown in Fig. 2C, dead but not resorbed.

Morphological abnormalities and lethality which developed on day 11

In contrast to the earlier periods, N-*myc*-deficient mutant embryos became clearly distinguishable at day 11.5 because of the development of morphological abnormalities, most notably in limb buds. Fig. 2A,B compare wild-type and homozygous mutant embryos of the same litter which were clearly recovered alive, i.e. with active heartbeats and blood circulation visible under a microscope. The photographs were taken after fixation with Bouin's fixative. In normal embryos, limb buds not only protruded outward but widened in the rostrocaudal direction, and the distal half of the limbs spread in the form of a disk, a structure called the limb plate. However, mutant limbs did not significantly show a gain in width and the limb plates were formed very poorly or not at all. In addition, the trunk of homozygous embryos was appreciably smaller than normal (i.e., wild-type and heterozygous) embryos, and there was slightly less expansion of the brain.

Beyond day 11.5, a substantial fraction of the embryos were found dead or were resorbed (Table 1). Analysis of DNA recovered from any remnant tissues indicated that all those that had died, but none still living were N-*myc*-deficient homozygotes (Table 1). Inclusion of resorbed embryos in the statistics, on the assumption that the majority of these were N-*myc*-homozygous mutants, satisfied Mendelian distribution of the three genotypes.

In one exceptional case, a homozygous embryo appeared to have survived to a stage later than day 11.5 and escaped resorption. The embryo shown in Fig. 2C was recovered without resorption, though it was already dead, when normal littermates had grown to the stage of 15.5 days. This embryo displayed a morphology with exaggerated abnormalities of the homozygotes observed at day 11.5, namely, limbs lacking obvious distal structures, poorly expanded brain, and, in addition, underdeveloped maxilla and mandible. The last defect may be correlated with the high N-*myc* expression in the facial primordium and the visceral arches in normal embryos of around day 9.5 (Kato et al., 1991).

Defects of limb morphogenesis

The limb bud is one of the prominent sites of N-*myc* expression (Sawai et al., 1990; Kato et al., 1991), and the defective morphogenesis of mutant limbs was of particular interest in understanding how N-*myc* activity is linked to embryonic morphogenesis. In the limb buds of embryos around day 10, N-*myc* is expressed primarily in the mesenchyme, rather than in the ectodermal cells, making an increasing proximodistal gradient of the expression level (Kato et al., 1991). At day 11.5, however, N-*myc* expression was reduced throughout the limb buds (data not shown). Histological sections of 11.5 day mutant embryos indicated that apical ridges were formed but the mutant ridges had a slightly rounder external contour in cross sections than normal (data not shown).

To extend the analysis of limb development in the absence of N-*myc* expression beyond the point of death of the mutant embryos, we cultured the forelimb buds excised from 11.5 day embryos on a membrane floated on a culture medium for three days (Fig. 2D,E). With the culture methods employed, external morphology of the limbs did not always give a decisive indication of development of the cartilagenous structures, but staining with alcian green clearly showed the formation of zeugopodial and autopodial cartilages in the normal (wild-type and heterozygous) limbs (Fig. 2D); cultured mutant limb buds, in contrast, had not formed these distally located cartilages (Fig. 2E). Thus, in the absence of N-*myc* expression, the elaboration of distal structures of limbs was seriously inhibited. These formation defects were consistent with the phenotype of an exceptionally long-developed mutant embryo (Fig. 2C).

Developmental abnormalities of mutant internal organs and correlations with the site of N-*myc* expression in normal embryos

Sagittal sections showing internal organs of normal (wild-type) and homozygous mutant embryos of day 11.5 are

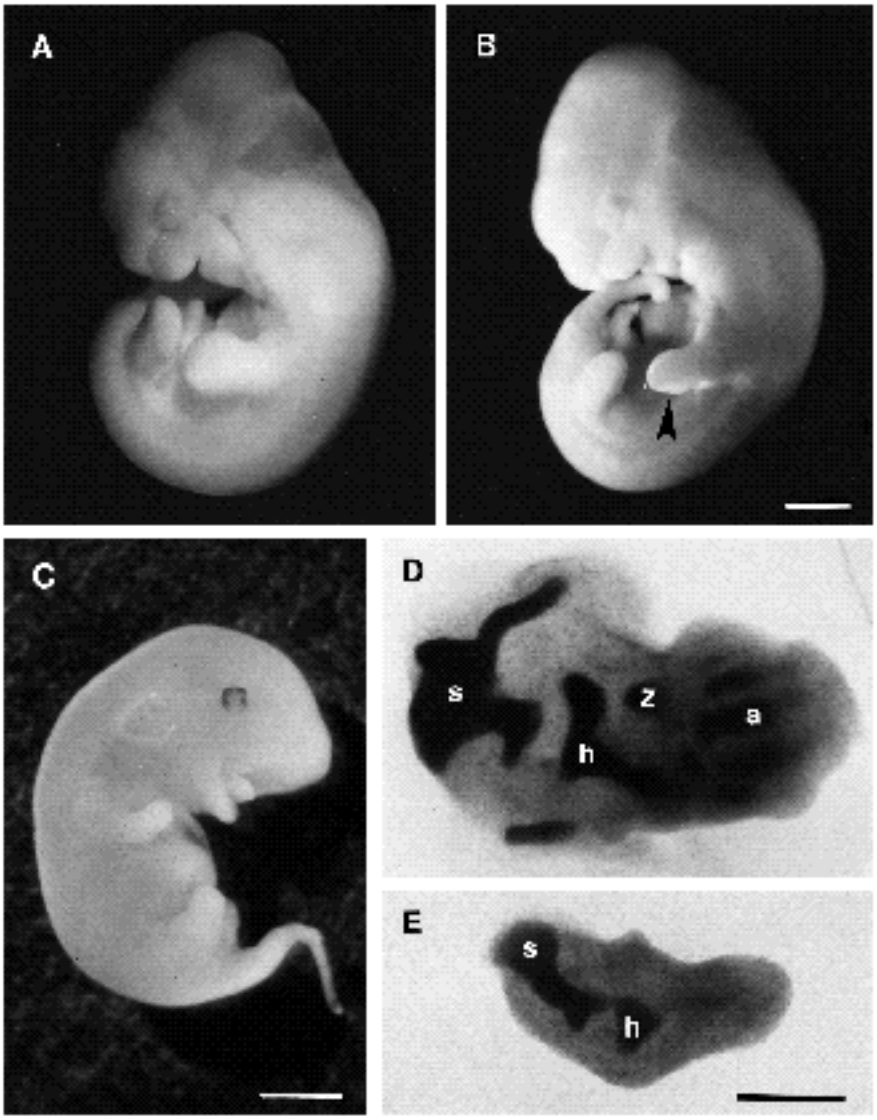

Fig. 2. Developmental abnormalities of N-*myc* mutant embryos. (A) 11.5 day wild-type embryo fixed with Bouin's fixative. (B) 11.5 day mutant embryo of the same litter as A recovered alive and fixed with Bouin's. The arrowhead points to a mutant forelimb bud where the development of the limb plate was very poor. (C) An exceptional mutant embryo which developed to a stage later than day 11.5 and escaped from resorption. When isolated, this embryo was dead, while normal siblings had developed to the stage of 15.5 days. (D) Normal forelimb bud excised from an 11.5 day embryo, cultured on a filter for 3 days, fixed and stained for cartilage with alcian green. s, scapula; h, humerus; z, zeugopodial components; a, autopodial components. (E) Mutant forelimb bud cultured in the same way as D. Note poor development of the distal structure. The bars indicate 1 mm (A,B); 2 mm f(C); 500 μm (D,F).

compared in Fig. 3. This kind of histological analysis as well as dissection of the internal organs of the mutant embryos revealed various defects characteristic to each organ. As is generally true of the mutant embryos, visceral organs were smaller than normal and apparently underdeveloped, features which were reflected by the smaller trunks.

Stomach

Along the digestive tract of the mutant embryos, the most conspicuous abnormality was the absence of a bulging stomach structure (Fig. 3B). In situ hybridization of N-*myc* transcripts in the stomach of a normal 10.5 day embryo indicated high N-*myc* expression in the endodermal epithelium as well as a low but significant transcript level in the surrounding mesenchyme (Fig. 4A,B).

Lung

A remarkable difference was also found in the lung. In the normal embryos, the pair of trachea showed growth at their ends and branching had already begun at least on the right (Fig. 5A), indicating the beginning of formation of bronchial trees. By contrast, mutant trachea remained Y-shaped and showed no sign of branching (Fig. 5D). In situ hybridization analysis of developing lung at day 12 indicated that bronchial epithelia were the major sites of N-*myc* expression (Hirning et al., 1991; Fig. 4C-E).

Lung development in organ culture

To follow mutant lung development beyond the stage of embryonic death, we made organ cultures of lung buds excised at day 11.5 from normal and mutant embryos. Cultures were initially done using media without supplement of fetal calf serum. Within 24 hours of culturing, normal lung developed a number of bronchial branches, and later developed further to form numerous bronchioles (in all three cases examined; Fig. 5B). Homozygous lung, on the other hand, increased in size but remained almost the same morphologically (Y-shaped). After 48 hours, homozygous lung exhibited a very rudimentary branching reaction only

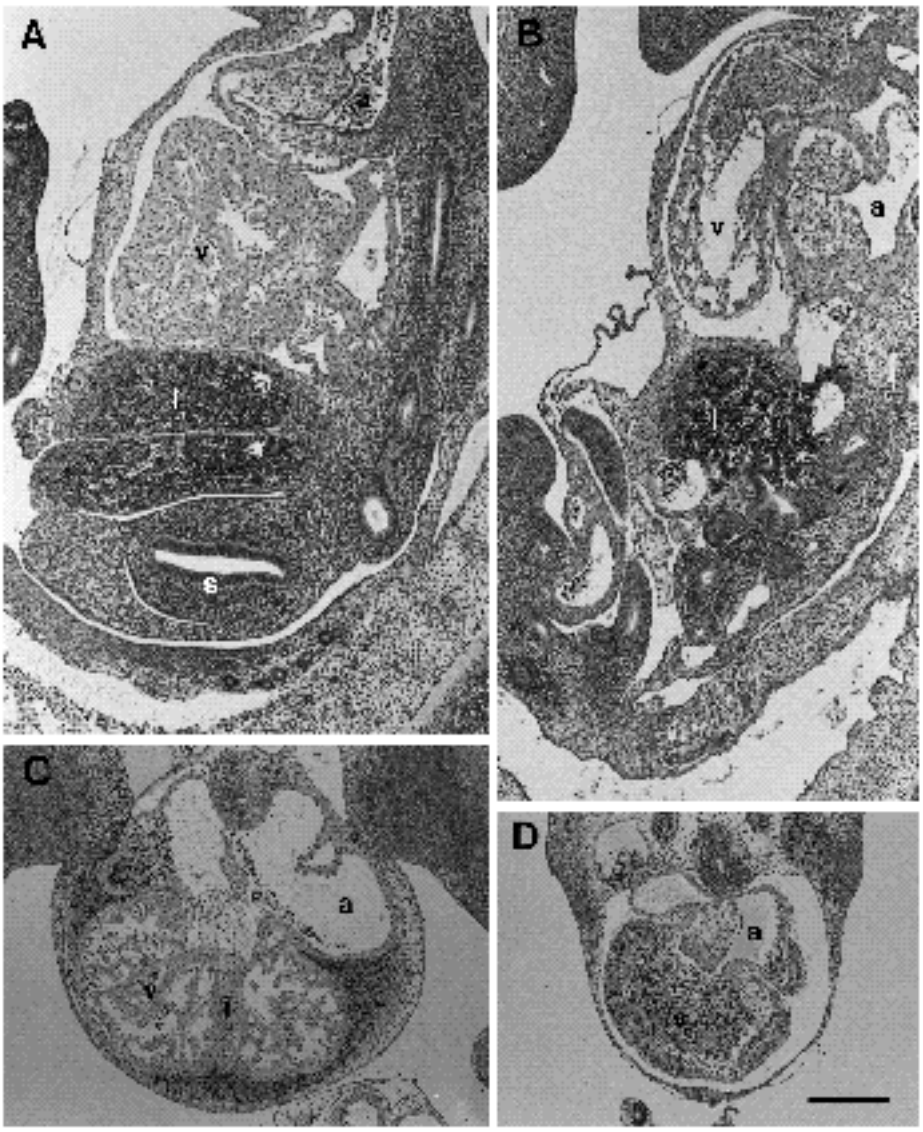

Fig. 3. Internal organs of normal and N-*myc* mutant embryos at day 11.5. Sagittal sections of normal (A) and mutant (B) embryos, as well as cross sections of normal (C) and mutant (D) embryos at the level of the heart are shown. The sections were stained with hematoxylin and eosin. a, atrium; i, interventricular septum; l, liver; s, stomach; v, cardiac ventricle. Note in the mutant section that an obvious stomach structure is lacking, that the liver is small and not divided into lobes, that the cardiac musculature is very thin and that the interventricular septum is absent. Blood cells remained in the ventricle in D, but were displaced into the thoracic cavity during sample preparation of C. The bar indicates 200 μm (A,B); 300 μm (C,D).

at the tip (in all 3 cases examined; Fig. 5E arrowhead). Thus, mutant lung buds by themselves were very defective in producing bronchial branches.

Addition of fetal calf serum to the medium from the beginning of the culture had a dramatic effect on branching of the mutant lung buds: they initiated significant branching after 24 hours and, by 72 hours, had produced numerous branches not only from the tip but from the side of the trachea, though much smaller than normal (three examined cases; Fig. 5F). Normal lung buds also developed better in the presence of the serum (three examined cases; Fig. 5C), but this was attributable to the general effect of the serum on the cultures. Thus, it seems that the added serum contained one or more substances that were missing from mutant lung buds and which were essential for the budding of bronchi from the trachea.

Liver

The liver of homozygous embryos was not only smaller than normal but composed of a single lobe (Fig. 3B) as opposed to four major lobes in the normal embryos due to dextrosinistral and dorsoventral divisions (Fig. 3A, for dorsoventral division). In the developing liver, there was no appreciable N-*myc* expression in the endodermal hepatic cells (Kato et al., 1991; Fig. 4B), but it occurred at a significant level in the surrounding mesenchymes at day 9.5 (Kato et al., 1991).

Heart

When homozygous embryos were recovered alive, hearts were beating at the frequency of 40-50 per minute at room temperature, which is normal for embryos of this stage where heart beats were autonomously paced by the ventricle. However, the hearts of mutant embryos (Fig. 3B,D) were significantly smaller than normal (Fig. 3A,C), and had no interventricular septum (Fig. 3D). In addition to these gross morphological features, it was also found that individual cardiac muscle cells and, accordingly, myocardium and trabeculae carneae were significantly thinner in the mutant embryos (Fig. 3).

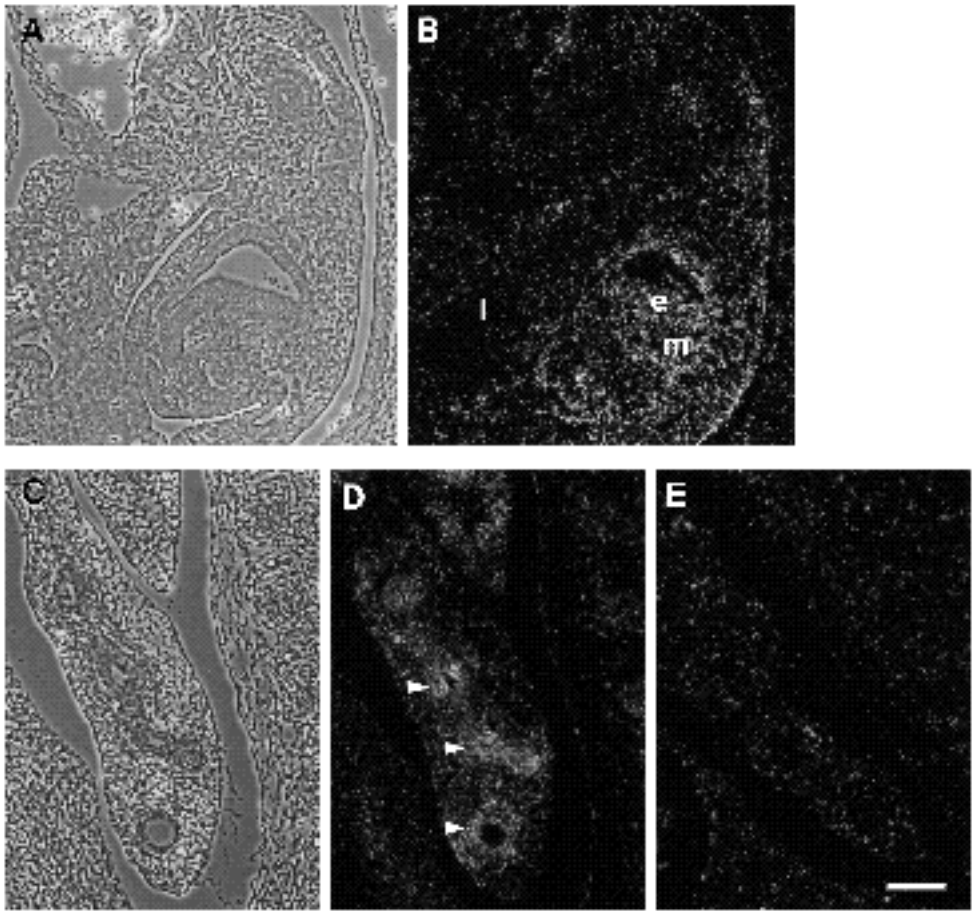

Fig. 4. Distribution of N-*myc* expression in developing stomach, liver and lung, as shown by in situ hybridization. (A,B) A section through the liver and stomach of a normal 10.5 day embryo, hybridized with antisense N-myc probe. (A) Phase-contrast and (B) dark-field photographs. l, liver, e, endodermal epithelium of the stomach and m, stomach mesenchyme. Note absence of hybridization signal on the liver cells. (C,D) A section through the lung of a normal 12.0 day embryo hybridized with antisense probe as in A and B. Note high hybridization signals on the bronchial epithelia (arrowheads). (E) Sense probe hybridization control of a nearby section. The bar indicates 10 μm.

Development of the central and peripheral nervous systems

The cephalic region

The basic organization of the central nervous system was normal in the N-*myc*-deficient embryos; brain components, ventricles, hypophysis and retina were correctly positioned. However, the thickness of the encephalic walls was consistently thinner than in the normal embryos. These are shown in Fig. 6A,B.

It is interesting to note that development of the ocular tissues was scarcely affected in the mutant embryos (Fig. 6A,B), since lens and neural retina are the representative sites of high N-*myc* expression, and expression continues from the stages of lens induction and lens vesicle formation (Sawai et al., 1990; our unpublished results) to later stages of maturation (Hirning et al., 1991; Yamada et al., 1992).

The trunk neural tube and sensory and autonomic nervous systems

The trunk neural tube and sensory spinal ganglia were compared in the wild-type and N-*myc*-deficient mutants in cross sections (Fig. 6C-F). The neural tubes of the homozygotes (Fig. 6E,F) were not as thick as those of normal (Fig. 6C,D), as was also true of the cephalic neural tube (Fig. 6A,B). There were mitotic figures observed in the most ventricular zone of the mutant neural tube at a frequency not far below that in the normal embryos. We estimated that on average mutant neural tubes had 20% less cells per section and two thirds of the wall thickness compared with normal embryos at the same section level. Thus, in addition to the reduced cell number, tight packing of the cells equally contributed to thinning of the mutant tube wall.

The spinal ganglia were more conspicuously different. Those of the mutant embryos were generally thinner and smaller. Comparison of the spinal ganglia located in the rostral (Fig. 6E) and caudal portions (Fig. 6F) of the same mutant embryos with those of a normal embryo (Fig. 6C,D) showed that ganglia were more severely affected in the more rostral portions. The parasagittal sections of the mid trunk region showed that the mutant ganglia contained considerably fewer mature neurons than normal, as indicated by fewer cells having enlarged nuclei, but the number of putative supporting cells with small nuclei appeared less affected (Fig. 7A,B).

Staining with anti-neurofilament antibodies and comparison with normal embryos (Fig. 7C,D) not only confirmed the above features of the spinal cord and spinal ganglia, but revealed more details of the nervous systems. First, the basic architecture of these systems had developed in the absence of N-*myc* activity. The autonomic nervous systems derived from the neural crest were formed and located normally in the trunk: the sympathetic ganglia were found in ventrolateral locations of the notochord, and parasympathetic neurons were distributed along the digestive tract. Both spinal ganglia and ventral horns were sending axons

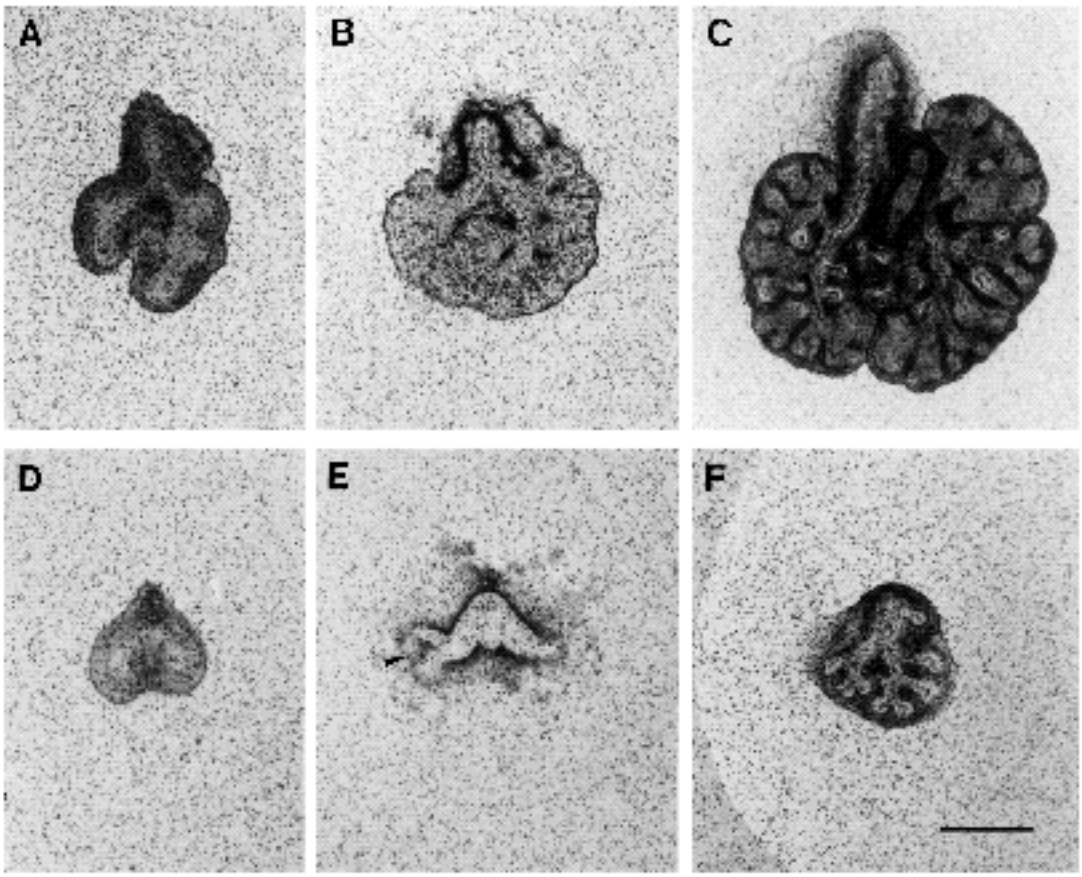

Fig. 5. Isolated and cultured lungs from normal and mutant embryos, and the effect of fetal calf serum on bronchial development. (A) A lung taken from an 11.5 day normal embryo and placed on a filter. (B) The same lung as in A cultured for 3 days in a medium without fetal calf serum. Note the numerous branchings. (C) Normal lung cultured in the same way but in the presence of 10% fetal calf serum. (D) A lung taken from an 11.5 day mutant embryo. (E) The same mutant lung as in E cultured for 3 days in the absence of serum. The arrowhead points to the rudimentary branches at the tip of a trachea. (F) Mutant lung cultured for three days in the presence of 10% fetal calf serum. Note budding and branching of bronchi. The bar indicates 500 μm.

to form the spinal nerves. Second, even without N-*myc* expression, the neurons had developed to the maturity of the normal developmental stage, as revealed by the synthesis of neurofilaments and by having sent axons along appropriate tracts. In other words, although the number of neurons was generally less in the mutants and the extent of this decrease was dependent on the particular nervous system, the individual neurons, once formed, appeared to develop in a more-or-less normal fashion.

DISCUSSION

N-*myc* is required for embryonic organogenesis

Histological analysis of N-*myc* expression at various stages in developing embryos has revealed that the major sites of N-*myc* expression do not correlate with cell multiplication but with the process of cell differentiation (Mugrauer et al., 1988; Downs et al., 1989; Hirning et al., 1991; Kato et al., 1991). To address the question of what kind of regulation the N-*myc* activity is involved in, we generated N-*myc*-deficient mice by targeted mutagenesis utilizing homologous recombination in ES cells (Sawai et al., 1991). The homologous recombinant ES lines were obtained at a relatively high frequency (Charron et al., 1990; Stanton et al., 1990; Sawai et al., 1991), transmitted through mouse germ lines (Stanton et al., 1990, Sawai et al., 1991), and homozygous mutations were embryonic lethal (Sawai et al., 1991) because of abnormalities that developed in the embryos, most notably in the limb buds and internal organs, by day 11.5.

The abnormalities of organogenesis in the mutant embryos were tightly correlated with the site of high N-*myc* expression during days 9.5-11.5. The tissues expressing N-*myc* per se were affected in certain instances and those interacting with the tissues expressing N-*myc* in others, although there were more complicated situations and a few exceptional cases that were unaffected by N-*myc* deficiency. From a chronological point of view, it seems that the time of onset of abnormal development was delayed from the peak of expression (day 9.5) in normal embryos. It is also noted that the phenotype expression was very consistent among the mutants, and that most embryos died after day 11.5 (Table 1).

The abnormalities were not correlated with the sites of expression earlier than day 9, however, and N-*myc* expres-

sion was not essential for development up to day 10.5. Blastocysts, both inner cell mass and trophoblasts (Wakamatsu and Kondoh, unpublished observation), as well as the ES cells derived therefrom (Stanton et al., 1990; Sawai et al., 1991), express abundant N-*myc* RNA and protein. After implantation, the primitive streak and presomitic mesoderm become the major sites of N-*myc* expression (Downs et al., 1989). However, homozygous mutant embryos did implant or go through gastrulation, and normally form axial as well as para-axial structures. This fact and the observation of non-essentiality of N-*myc* activity in the differentiation of ES cells (Sawai et al., 1991) argue for the dispensability of embryonic N-*myc* expression up to day 10.5. Activation of other genes that compensate the loss of N-*myc* function during this period has not been totally ruled out, but no evidence for it has been obtained among the myc and related genes (S. Sawai and H. Kondoh, unpublished observation).

A schematic presentation of N-*myc* expression during organogenesis of normal embryos is shown in Table 2 and compared with the mutant phenotype of the organs. In no instance did we observe excessive morphogenesis, indicating that the N-*myc* gene generally functions to promote rather than subdue the organogenesis process, although the actual mechanisms of regulation may vary among the tissues or organs.

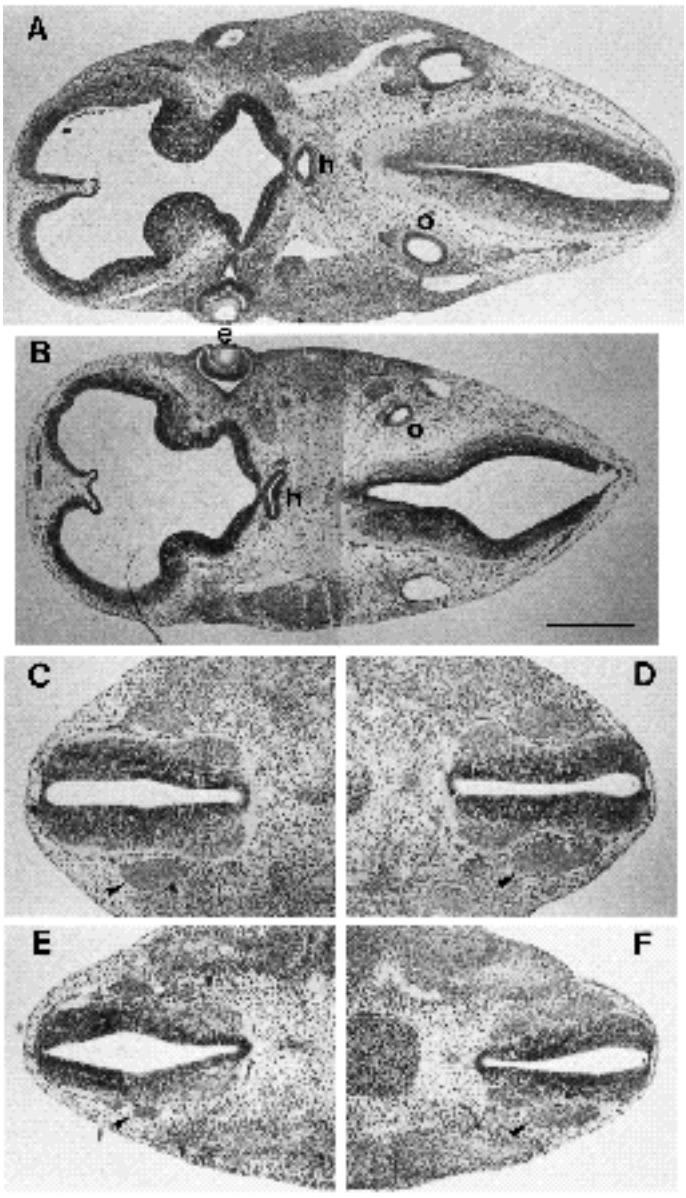

Fig. 6. Comparison of the central nervous system and the spinal ganglia of normal and N-*myc* mutant embryos at day 11.5. Rostral side is to the left. (A,B) A pair of matched sections through the head of normal and mutant embryos, respectively. e, eyes; h, hypophysis; o, otic vesicle. (C-F) Sections through the spinal cord and spinal ganglia (arrowheads) of the same normal (C,D) and mutant (E,F) embryos near the forelimb bud (C,E) and hindlimb bud (D,F) levels. Note that the spinal ganglia are greatly inferior in the rostral side of the mutant embryo. The bar indicates 500 μm (A,B); 250 μm (C-F).

How N-*myc* is involved in organogenesis: examples of tissue autonomy

There are cases where the tissues expressing N-*myc* were themselves seriously affected, e.g. heart, where the myocardium was the normal site of N-*myc* expression. The myocardium was consistently thinner and development of the trabeculae was inferior in the mutants, also the heart overall was smaller and devoid of an interventricular septum.

The mutant neural tube, from the cephalic region through the trunk, was characterized by thin tube walls. At day 11.5 when the mutant phenotype became evident, there were still mitoses occurring with significant frequency along the ventricle, indicating that the mutant neural tube was still growing. There was a slightly smaller number of cells per unit length of a mutant tube than normal, but equally or even more significant was the tight packing of the cells. It is not clear whether this was due to smaller cell size, possibly reflecting poor maturation of the cells, to the increased pro-

Table 2. The summary of the sites of N-myc expression during organogenesis and the abnormalities of the mutant embryos

Organ	Site of expression on day 9.5-10.5	Site of expression on day 11.5	The mutant phenotype at day 11.5
Heart	Myocardium	Myocardium	Small Thin musculature No interventricular septum
Neural tube	Whole	Dorsal	Thin walls
Spinal ganglia	Whole	Neurons	Fewer neurons
Visceral arches	Mesenchyme		Abnormal mandible*
Liver	Mesenchyme		Small No division into lobes
Lung	Mesenchyme	Bronchial tubes	No bronchial branches
Stomach	Mesenchyme	Mesenchyme Epithelium	No bulging
Limb buds	Mesenchyme		Poor development of distal structures
Eyes	Neural retina Lens	Neural retina Lens	Little affected

*The case of an embryo which developed to a stage beyond day 11.5 and is shown in Fig. 2C.

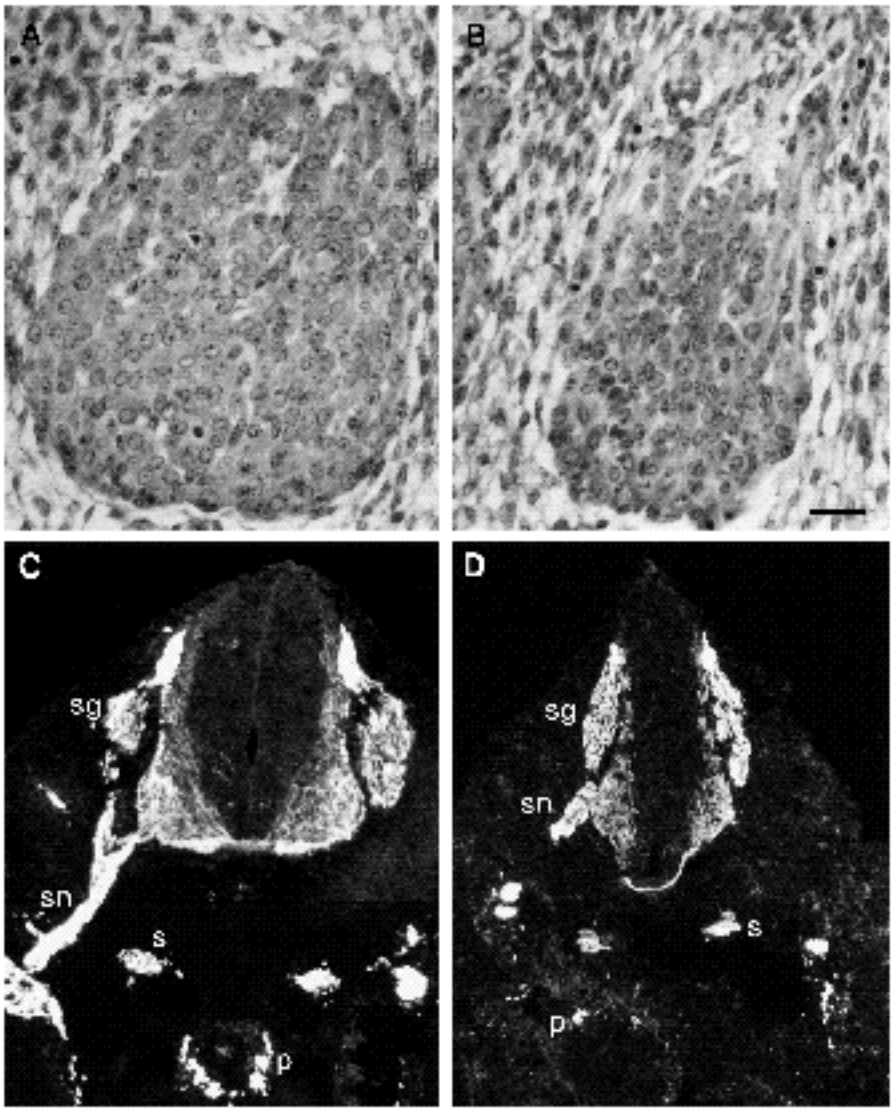

Fig. 7. Development of the peripheral nervous systems in normal and N-*myc* mutant embryos at day 11.5. (A,B) Parasagittal sections of normal (A) and mutant (B) embryos through the spinal ganglia stained with hematoxylin and eosin. (C,D) Cross sections of normal (C) and mutant (D) embryo stained with anti-neurofilament antibodies and fluorescein. sg, spinal ganglia,; sn, spinal nerve; s, sympathetic ganglia; p, parasympathetic neurons. The bar indicates 25 μm (A, B); 100 μm (C, D).

portion of smaller cells in the heterogeneous neural tube cell population, or to the tight associations among the cells.

In the mutant embryos, the spinal ganglia were increasingly inferior toward the rostral end of the trunk. The major symptom of these mutant ganglia was a significant decrease in neurons, a cell type that was recognized by its large nucleus and strong staining of the cytoplasm with anti-neurofilament antibodies. The mutant ganglia were smaller and contained fewer neuronal cells than normal. In spite of these lower neuronal cell populations, the neurons, once produced, appeared normal as judged by their extension of axons to the neural tube and along the normal pathway of the spinal nerves.

Several models may explain the reduction of ganglion sizes in the absence of N-*myc* expression: fewer neural crest cells colonizing in the ganglia; limited multiplication of the cells in the spinal ganglia; biased differentiation to non-neuronal cells among the population of the ganglion cells; and/or regression of the ganglia once formed by extensive cell death. These models are not mutually exclusive and await rigorous tests. However, the cells that were in apoptosis as characterized by condensed nuclei were not significantly more numerous than in a normal ganglion, probably ruling out the last model. The mechanism of the greater disparity than normal in the rostrally located mutant ganglia is not clear.

Very exceptionally among the tissues with high N-*myc* expression, development of ocular tissues, neural retina and lens (Sawai et al., 1990; Hirning, 1991; Yamada et al., 1992) were least affected in the mutants. Comparing the extent of ocular histogenesis within a single litter confirmed that mutant and normal embryos had undergone the same chronological development.

Cases where tissue interaction was implicated: internal organs

The effect of N-*myc* deficiency on liver development was characterized by smaller mass and absence of division of the organ into lobes. Since hepatocytes never express significant levels of N-*myc* transcript, the hepatic phenotype is likely ascribable to the surrounding mesenchymes. In contrast to the case of the myocardium, individual hepato-

cytes in mutant embryos were indistinguishable from those of normal embryos.

In the mutant embryos at day 11.5, the lung had a feature resembling primitive lung buds: a blunt-ended Y shape without any sign of branches. To follow lung development beyond the stage of death of the mutant embryos, lungs were excised from the embryos and placed in organ culture, with or without a supplement of fetal calf serum. Mutant lungs formed numerous bronchial branches from the trachea, only when fetal calf serum was added to the medium. This branch-promoting activity of the serum in the medium strongly suggested that N-*myc* deficiency resulted in a lack of the extracellular factors required for bronchial morphogenesis. Lung is one of the well-characterized organs where tissue morphogenesis depends on an intimate interaction between the epithelium and the mesenchyme (Wessels, 1977). Whether the primary cause of the defect in the mutant lung lies in the epithelial, the mesenchymal or in both components awaits rigorous testing, and the organ culture system should provide a way for this to be done.

Moens et al. (1992) recently reported the phenotype of extremely leaky N-*myc* mutants in which the targeting vector did not replace but was inserted into the normal N-*myc* alleles. The only significant effect of the N-*myc* mutation in that case was a moderate underdevelopment of the lung epithelium, a phenotype that can be extrapolated from that of the stringent N-*myc* deficiency found in the present investigation.

The case of the stomach may be analogous to that of the lung discussed above, in that moderate N-*myc* expression in the mesenchyme preceded very high expression in the endodermal epithelium, and morphogenic development of the epithelium was seriously hindered by the lack of N-*myc* activity.

Limb morphogenesis: analogy to limb deformity mutants

In the early period of normal limb morphogenesis, N-*myc* expression takes place in the mesenchyme with increasing gradients toward the distal ends (Sawai et al., 1990; Kato et al., 1991). In the total absence of N-*myc* expression, limb morphogenesis proceeded without gaining rostrocaudal width and without spreading of the distal part. In later development of mutant limbs, as demonstrated by the culture of limbs excised from embryos, distal structures were more severely affected than proximal ones, indicating a larger N-*myc* activity in the more distal part of the limb.

It is tempting to point out that the limb phenotype of N-*myc*-deficient embryos strongly resembles that of limb deformity (*ld*) mutants (Zeller et al., 1989; Jackson-Grusby et al., 1992; and Trumpp et al., 1992), in the absence of increase in width of limb buds, in slightly altered apical ridge morphology (Zeller et al., 1989) and in more serious malformation of the distal than of the proximal structures in later stages. The morphology of the limb buds in *ld* (Fig. 1E of Zeller et al. (1989)] and N-*myc* (Fig. 2B) mutants is very similar. The *ld* gene encodes a group of nuclear proteins (Trumpp et al., 1992), and is expressed in both ectoderm and mesenchyme with a specifically spliced form of transcript in the apical ectodermal ridge (Jackson-Gruby et al., 1992), while N-*myc* expression is confined to the mesenchyme and the distribution of the expression in the mesenchyme does not coincide with that of *ld* (Sawai et al., 1990; Kato et al., 1991). Nevertheless, the resemblance of the mutant phenotypes suggests that these two genes are involved in the same regulatory cascade of limb morphogenesis where multiple and tight interactions between the mesenchymal and ectodermal components take place (Wessels, 1977).

Genetic basis of the mutant phenotype

The N-*myc* gene codes for a sequence-specific DNA-binding protein, which is thought to regulate transcription of a group of genes, either positively or negatively (Kato et al., 1990; Blackwood and Eisenman, 1991). N-*myc*-deficient mutants were defective in the organogenic processes, some of which are dependent on tissue interactions and others are apparently tissue autonomous. This strongly argues that the genes regulated by N-*myc* are those that directly participate in tissue interactions and tissue maturation. The N-*myc*-deficient condition, either in embryos or in ES cells, will provide invaluable materials for biochemically identifying, e.g. by subtraction screening, the candidate genes that are regulated by N-*myc* and whose actions are central to the process of organogenesis. The candidate genes will then be tested to see whether their forced expression rescues mutant organogenesis in embryos or in organ cultures.

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