The EMBO Journal Vol.16 No.11 pp.2985–2995, 1997

Targeted expression of *MYCN* causes neuroblastoma in transgenic mice

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The proto-oncogene *MYCN* is often amplified in human neuroblastomas. The assumption that the amplification contributes to tumorigenesis has never been tested directly. We have created transgenic mice that overexpress *MYCN* in neuroectodermal cells and develop neuroblastoma. Analysis of tumors by comparative genomic hybridization revealed gains and losses of at least seven chromosomal regions, all of which are syntenic with comparable abnormalities detected in human neuroblastomas. In addition, we have shown that increases in *MYCN* dosage or deficiencies in either of the tumor suppressor genes *NF1* or *RB1* can augment tumorigenesis by the transgene. Our results provide direct evidence that *MYCN* can contribute to the genesis of neuroblastoma, suggest that the genetic events involved in the genesis of neuroblastoma can be tumorigenic in more than one chronological sequence, and offer a model for further study of the pathogenesis and therapy of neuroblastoma.

Keywords: comparative genomic hybridization/*MYCN*/neuroblastoma/N-*myc* /transgenic

Introduction

Neuroblastoma is a tumor of neural crest origin that comprises 8–10% of all childhood malignancies and causes 15% of cancer-related deaths in children. Ninety percent of cases strike children <10 years old (Matthay, 1995). The tumor arises anywhere in the sympathetic nervous system, most frequently in the adrenal medulla and in paraspinal ganglia (Brodeur and Castleberry, 1993). The best characterized genetic abnormality in neuroblastoma is amplification of *MYCN*, a gene originally isolated from neuroblastoma cells (Kohl *et al.*, 1983; Schwab *et al.*, 1983). Amplification of *MYCN* occurs in approximately one-third of neuroblastomas and correlates with advanced disease, suggesting that amplification of *MYCN* is a late event in tumorigenesis (Brodeur *et al.*, 1984; Kohl *et al.*, 1984). An alternative interpretation of these data is that *MYCN*-dependent and *MYCN*-independent pathways exist early in neuronal transformation, and that the *MYCN*-dependent transformation leads to more aggressive tumors with a worse prognosis. In either event, it is not known whether the genetic events that give rise to neuroblastoma must occur in a particular chronological sequence.

The evidence that *MYCN* participates in the genesis of neuroblastoma has until now been entirely circumstantial. However, there are experimental demonstrations that the gene does have oncogenic potential. For example, overexpression of *MYCN* can cooperate with a mutationally activated H-*RAS* gene to transform rat embryo cells (Schwab *et al.*, 1985) and can convert established rat cell lines to tumorigenicity (Small *et al.*, 1987). Transgenic mice that overexpress *MYCN* under control of the immunoglobulin heavy chain enhancer develop lymphoid tumors (Dildrop *et al.*, 1989; Rosenbaum *et al.*, 1989). These data suggest that *MYCN* has a role in modulating cellular proliferation, but the evidence that *MYCN* actually contributes to neuroblast transformation remains inferential.

In an effort to explore the role of *MYCN* in neuroblastoma, we created transgenic mice that overexpress *MYCN* in cells derived from the neural crest. Such animals develop neuroblastoma several months after birth, demonstrating that overexpression of *MYCN* can initiate tumorigenesis. The latent period prior to neuroblastoma development suggests that additional genetic events contribute to tumor formation. By comparative genomic hybridization (CGH), neuroblastomas from the transgenic mice demonstrate a number of consistent chromosomal gains and losses in regions syntenic with those observed in human neuroblastoma. These mice represent a model for neuroblastoma that may be useful both to identify additional genes involved in tumorigenesis, and to test new therapies in preclinical trials.

Results

Targeting MYCN overexpression to the neural crest

During development, neuroblasts appear as a normal constituent of the neural crest. To target expression of human *MYCN* to these cells, we used the tyrosine hydroxylase promotor (Figure 1). This promotor is active in migrating cells of the neural crest early in development (Banerjee *et al.*, 1992), and has been shown to direct expression of chloramphenicol acetyl transferase to sympathetic ganglia and the adrenal, in which neuroblastomas often arise (Banerjee *et al.*, 1992). As hoped, mice carrying the transgenic construct showed tissue-specific expression of the *MYCN* protein product (Mycn). By Western analysis, Mycn was expressed at high levels in the adrenal gland, which is derived from the neural crest (Figure 2a). There was marginal expression in brain, heart, testes and spleen (data not shown).

Neuroblastomas in mice overexpressing the MYCN transgene

Three independent *MYCN* transgenic lines developed thoracic paraspinous masses (Table I). The histology of

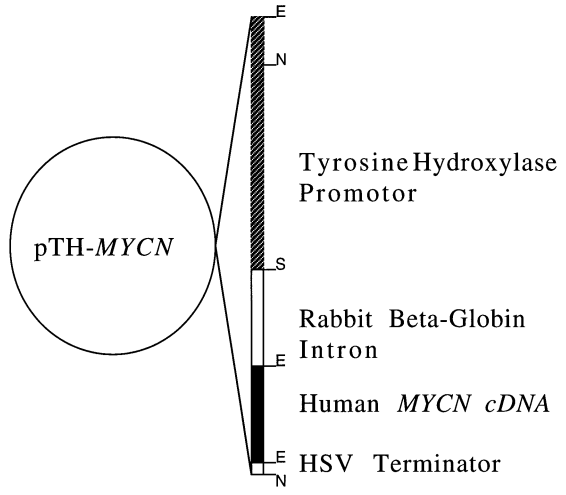

Fig. 1. Construct used for generating tissue-specific expression of the *MYCN* transgene. A cDNA for human *MYCN* was ligated downstream of the rat tyrosine hydroxylase promotor. The rabbit β-globin enhancer was used to enhance expression, and a herpes simplex virus thymidine kinase gene sequence was used as a transcription terminator. The transgene was cleaved from vector sequences using *Nsi*I. E, *Eco*RI; S, *Sal*I; N, *Nsi*I.

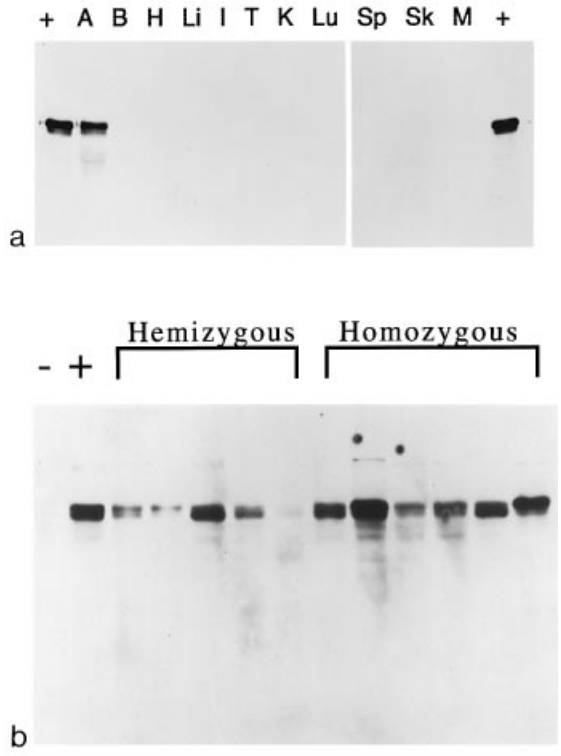

Fig. 2. Expression of the *MYCN* transgene. (**a**) Western blot analysis of total protein from various transgenic organs. Protein extracts prepared from equal amounts of organ tissues were immunoprecipitated, fractionated by PAGE and hybridized with an anti-Mycn monoclonal antibody (Boehringer Mannheim, Indianapolis, IN). A, adrenal; B, brain; H, heart; I, intestine; K, kidney; Li, liver; Lu, lung; M, muscle; Sk, skin; Sp, spleen; T, testes. The positive control was from Kelly cells (Schwab *et al.*, 1983) and represented approximately one-quarter of the protein loaded in other lanes. The level of Mycn expressed in the adrenal gland varied among mice in the same line. (**b**) Mycn protein levels in tumor samples. Equal quantities of protein from tumor and control tissues were subjected to SDS–PAGE, transferred to nitrocellulose membranes and probed with a Mycn monoclonal antibody. The positive control was from Kelly cells. The negative control was kidney tissue from a transgenic mouse. Hemizygous and homozygous refer to mice with one or two alleles of the *MYCN* transgene insertion.

these tumors was consistent with neuroblastoma. Tumors showed varying degrees of neuronal differentiation, as evidenced by ganglionic cells and neuropile (Figure 3). We do not believe that the presence of ganglionic cells can be explained by the adventitious inclusion of normal tissue in the tumors: first, ganglionic tumors were often found in regions where ganglia are not known to reside; and second, many ganglionic tumors showed signs of proliferation, such as mitotic figures and double nuclei (data not shown). Figure 3a shows a typical small round blue cell tumor adjacent to a paraspinous ganglion, Figure 3b a tumor with both small round blue cells and more differentiated elements.

Tumors stained positively for the neuronal markers synaptophysin and neuron-specific enolase (Figure 3c and d). Tumor cells with ganglionic differentiation were positive for the marker S100 (Nakagawara *et al.*, 1986), while anaplastic tumor cells remained negative. Tumor cells did not react with antibodies for chromogranin A and O13 (data not shown).

Electron microscopy of tumor tissue demonstrated synapse formation (Figure 3e) and neurosecretory granules (Figure 3f). Western blotting showed that in some tumors, the levels of the Mycn were equivalent to those found in the human neuroblastoma cell line Kelly (Figure 2b) which has high levels of *MYCN* amplification (Schwab *et al.*, 1983). The relative amounts of Mycn varied from one tumor to another, but in every instance they far exceeded those found in normal tissue(s).

One line of mice has developed tumors in multiple animals. This line had ~4 copies of the *MYCN* transgene by Southern analysis (data not shown). Mice from this line were back-crossed twice to C57B6/J, and 50 offspring were followed for tumor development. Fourteen of these animals developed tumors, with an incidence of ~5% at 3 months and 20% at 6 months (Figure 4a). Six animals had large abdominal tumors incorporating the adrenal glands, kidney, intestine and other organs. Six animals

Table I. Transgenic lines produced with the tyrosine hydroxylase–*MYCN* transgene

Mouse strain	No. of founders	Lines established	Lines with tumors
C57B6/J×Balb/c	8	3[a]	2[b]
FVB/N	9	6[c]	1[d]
C57B6/J	7	3[e]	1[f]

[a]All of these lines overexpressed *MYCN* in adrenal tissue.
[b]These mice developed thoracic paraspinous masses with the histology of neuroblastoma. One died as a sterile founder, and the other occurred in a line that expresses *MYCN*, and transmits both the transgene and tumor susceptibility.
[c]Three of these six lines overexpressed *MYCN*.
[d]This mouse died of a thoracic paraspinous mass with the histology of neuroblastoma. No additional tumors have been seen in this line.
[e]One of these three lines overexpressed *MYCN*.
[f]This sterile founder died of a brain tumor with neuronal elements as evidenced by ganglionic differentiation and positive cell staining for synaptophysin and neuron-specific enolase. The histology was consistent with that of a primitive neuro-ectodermal tumor with neuronal differentiation (cerebral neuroblastoma).

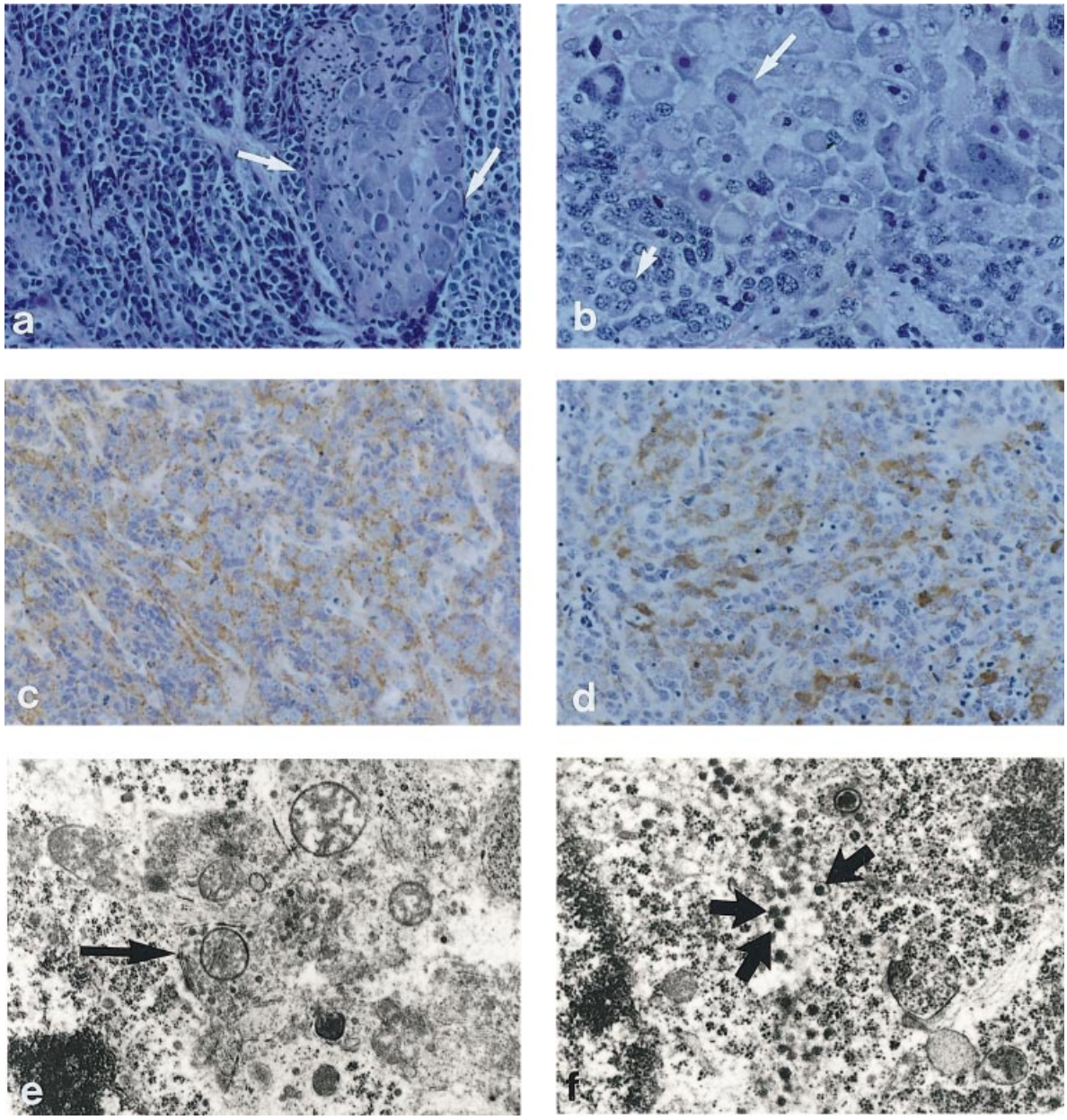

Fig. 3. Histopathology of tumors in *MYCN* transgenic mice. Tissues were processed for staining and electron microscopy as detailed in Materials and methods. Sections are shown at high (×250) power. (**a**) Anaplastic tumor. Arrows denote the normal paraspinous ganglion in a section of the tumor. (**b**) Tumor showing a broader spectrum of differentiation. The short arrow denotes more primitive small round blue cells, while the longer arrow denotes malignant cells with a more ganglionic appearance. (**c**) Synaptophysin staining of a tumor section. (**d**) Neuron-specific enolase staining of a tumor section. (**e** and **f**) Neuronal elements seen on electron microscopy of tumor sections (×25 000). The arrow in (e) denotes synapse formation in a tumor section. The arrows in (f) denote neurosecretory granules.

had thoracic paraspinous tumors and two had both abdominal and thoracic tumors.

Clinical presentations varied among animals. Some animals became emaciated. These had either thoracic tumors (Figure 5a) or small abdominal masses. Others developed very large palpable abdominal masses (Figure 5b) or a progressive paralysis of the lower extremities. The paralyzed mice had either abdominal or thoracic paraspinous masses, with tumor tracking along the peripheral nerve and encasing the spinal cord (Figure 6).

Metastatic spread of mouse neuroblastoma

Occasional animals had gross metastases to liver, lung or ovary. This observation prompted us to analyze organ histology from eight animals that died of neuroblastoma, but in which no gross metastases were seen. Five such animals showed microscopic metastases to liver, lung, lymphatics, kidney, ovary, testes, brain and muscle. Selected examples are shown in Figure 7a and b. No mice showed metastases to cortical bone, and a single mouse had bone marrow involvement.

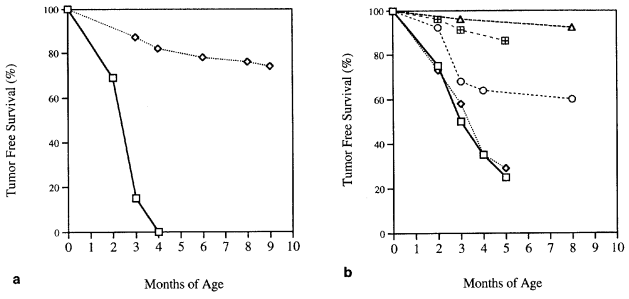

Fig. 4. Survival of mice carrying the *MYCN* transgene. Wild-type mice had 100% tumor-free survival in these experiments (data not shown). (**a**) Survival of mice hemizygous and homozygous for the *MYCN* transgene. The outbred founder line (C57BL6/J×Balb/c) was back-crossed twice to C57BL6/J. Fifty such mice were followed (◇). F1 offspring from the above cross were interbred and 13 offspring homozygous for the *MYCN* transgene were followed (□). (**b**) Survival of *MYCN* transgenic mice carrying hemizygous inactivating mutations in *p53*, *NF1* and *RB1*. The outbred founder line was back-crossed once to C57BL6/J, and then to a *p53*+/– mouse in an FVB/N strain background. Twenty three mice hemizygous for the *MYCN* transgene and heterozygous for the *p53* inactivating mutation were followed (⊞). Twenty seven littermates transgenic for *MYCN* but wild-type at *p53* were followed to control fo strain effects (△). The outbred founder line was back-crossed once to C57BL6/J, then crossed to a 129S/v mouse heterozygous for *NF1* or *RB1* inactivating mutations. Fifteen *MYCN* transgenics heterozygous for the *NF1* mutation (◇), 20 *MYCN* transgenic mice heterozygous for the *RB1* mutation (□) and 25 *MYCN* transgenic littermates wild-type at these loci (○) were followed for 1 year.

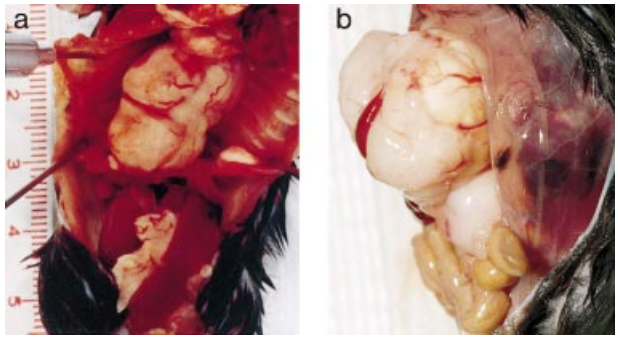

Fig. 5. Tumors induced by the *MYCN* transgene. (**a**) Thoracic paraspinous mass. Mice with thoracic paraspinous masses became emaciated, dyspneic and hunched. (**b**) Abdominal mass. Mice with abdominal masses appeared otherwise normal.

Tumor formation is dependent on MYCN gene dosage

To determine whether transgene dosage is related to tumor incidence, transgenic mice were crossed to produce homozygosity for the *MYCN* transgene. Homozygotes displayed both increased incidence and decreased latency of tumor formation, approaching 100% at 4 months (Figure 4a).

To test whether γ irradiation could increase tumor penetrance, mice hemizygous for the *MYCN* transgene were irradiated at 3–4 Gy before day 3 of life. No increase in tumor incidence was seen in these animals.

Additional genetic lesions contribute to tumorigenesis

In the limited studies performed to date, loss of *NF1* occurs commonly, and loss of *RB1* rarely in human neuroblastoma cell lines or tumor tissue (Nakamura *et al.*, 1991; Johnson *et al.*, 1993; The *et al.*, 1993). To test whether loss of these tumor suppressor genes could increase tumor penetrance, mice lacking *NF1* and *RB1* were crossed to mice that overexpressed *MYCN*. Mice homozygous for *NF1* and *RB1* deletions die at midgestation (Jacks *et al.*, 1992, 1994). In order to avoid this complication, mice were generated that were hemizygous for the *MYCN* transgene and heterozygous for deletions of *NF1* or *RB1*. Such mice had a decreased latency, and an increased incidence of tumors, to ~75% by 10 months (Figure 4b). The *NF1* and *RB1* mutant mice were in a 129S/v strain background, so littermates wild-type at the tumor suppressor loci and carrying the *MYCN* transgene were also followed to control for strain effects. Littermates had a tumor penetrance of 40% at 10 months (Figure 4b). No neuroblastomas were seen in mice heterozygous for deletions of *RB1* or *NF1*, but not carrying the *MYCN* transgene. These data suggest that loss of either *NF1* or *RB1* can contribute to tumorigenesis in mice overexpressing *MYCN*.

Structural mutations in *p53* are rare in human neuroblastoma (Imamura *et al.*, 1993; Hosoi *et al.*, 1994). To test the role of *p53* in mouse neuroblastoma, we crossed the *MYCN* transgenic to an FVB/N mouse heterozygous for a *p53* deletion. Mice hemizygous for the *MYCN* transgene and heterozygous for deletion of *p53* showed a tumor incidence similar to that of littermates hemizygous for the *MYCN* transgene and wild-type at the *p53* locus (Figure 4b). The tumor penetrance in mice hemizygous for the *MYCN* transgene and wild-type at the *p53* locus was less than that seen in similar mice back-crossed to a C57BL6/J background (Figure 4), even though these mice were back-crossed to FVB/N only once. *MYCN* transgenic mice lacking both *p53* alleles were also generated, and did not show increased tumor penetrance (data not shown).

Analysis of tumors by comparative genomic hybridization

To examine genetic aberrations at the chromosomal level, we used CGH to analyze 21 tumors from hemizygous and homozygous mice. This technique is based on two-color fluorescence *in situ* hybridization and scans the entire genome for gains and losses of chromosomal material. Tumors from five homozygous mice and three hemizygous mouse showed no chromosomal changes. Thirteen tumors (all from hemizygous mice) showed gains and losses of chromosomal regions (Figure 8, Table II). In mice hemizygous for the *MYCN* transgene, chromosomal regions were most commonly gained on chromosomes 11 (5/16) and 17 (6/16). These regions are syntenic with human chromosomes 6 and 17 respectively (Copeland *et al*., 1993), both of which commonly are gained in human neuroblastoma (Plantaz *et al.*, 1997). Chromosomal loss was most often detected on chromosomes 5 (4/16), 9 (3/16), 16 (4/16) and X (4/16). These chromosomes are syntenic with human chromosomes 4, 11, 3 and X respectively (Copeland *et al*., 1993), all of which commonly are lost in human neuroblastoma (Plantaz *et al.*, 1997).

Gain of chromosome 17 also correlated with lack of ganglionic differentiation. Five tumors showed predomin-

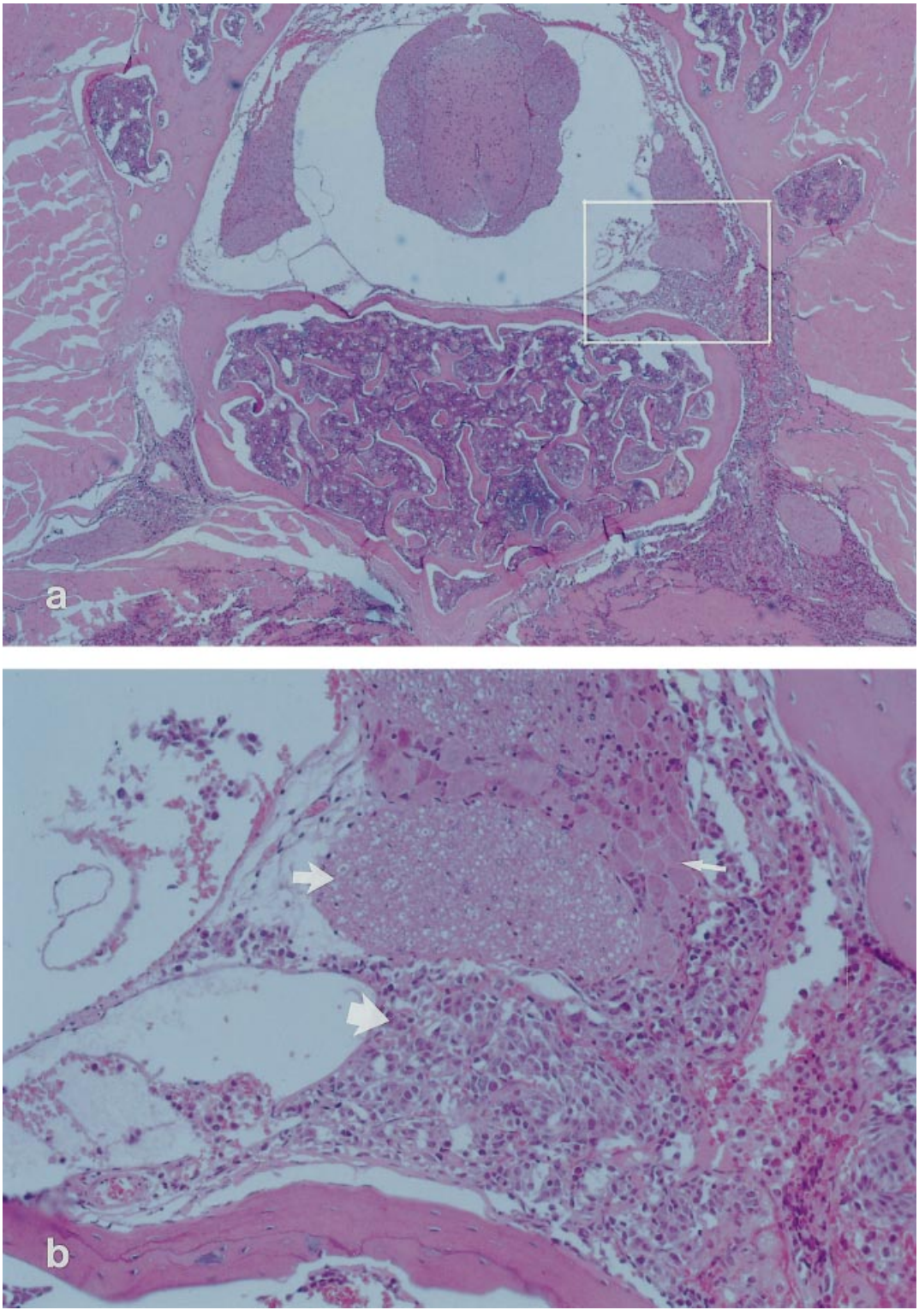

Fig. 6. Tumor tracking along radicular nerves and encasing the spinal cord. A number of mice developed progressive paraparasis of the lower extremities. These animals had paraspinous masses that infiltrated neural foramina and encased the spinal cord. The tumor and spinal column were fixed, decalcified, sectioned and stained with hematoxylin and eosin. (**a**) Low power micrograph of the spinal column and tumor in a decalcified axial photograph. Note the tracking of the tumor around the spinal cord in the center of the bony canal. (**b**) High power view (×250) of the boxed region in (a). The thin arrow denotes the peripheral nerve. The medium arrow denotes the dorsal root ganglion. The thick arrow shows the tumor.

antly ganglionic differentiation, and all showed gain of chromosome 17. Six tumors were predominantly poorly differentiated, and only one of these showed gain on chromosome 17. Two tumors were intermediate in differentiation, and showed no abnormalities of chromosome 17.

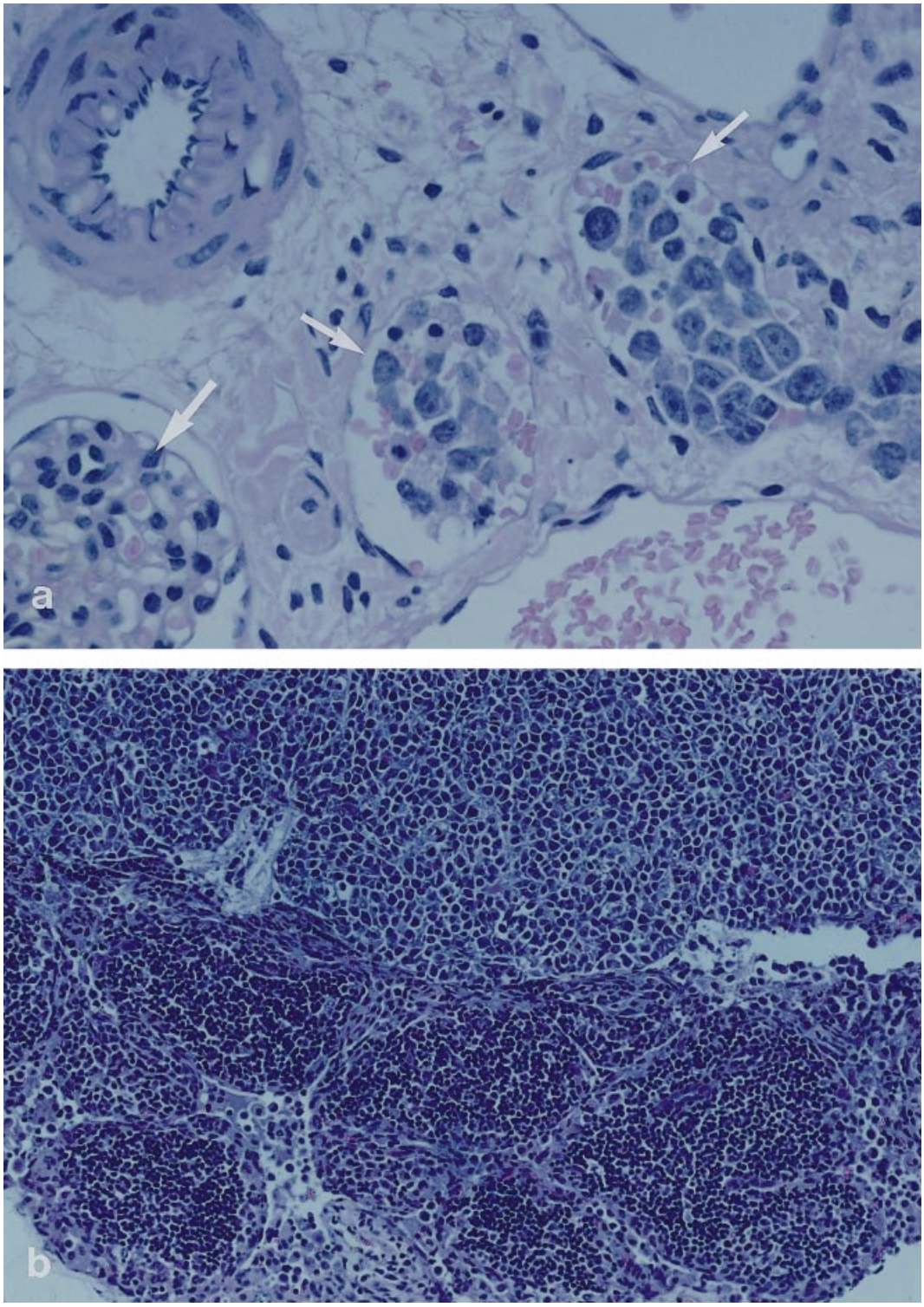

Fig. 7. Metastatic spread of tumor. (**a**) Hematogenous spread to the kidney. Thin arrows denote the tumor in renal venules. The thick arrow shows the renal glomerulus. The structure at the upper left is a renal arteriole. (**b**) Lymphatic spread to regional lymph nodes. The top of the structure shows the tumor effacing the normal lymph node architecture. The lower area of the photograph shows the normal lymphatic ultrastructure.

Discussion

Overexpression of MYCN in the neuroectoderm predisposes mice to neuroblastoma

Our data demonstrate that targeted expression of *MYCN* causes neuroblastoma in transgenic mice and represent the first demonstration that *MYCN* can contribute to the transformation of neuroblasts *in vivo*. This murine model mirrors human neuroblastoma in that: (i) tumors arise in appropriate locations (abdomen and thorax); (ii) mice may present with spinal cord involvement; (iii) tumorigenesis is affected by *MYCN* gene dosage; (iv) tumors have the characteristic histology of human neuroblastoma (small round blue cells, ganglionic differentiation and neuropile);

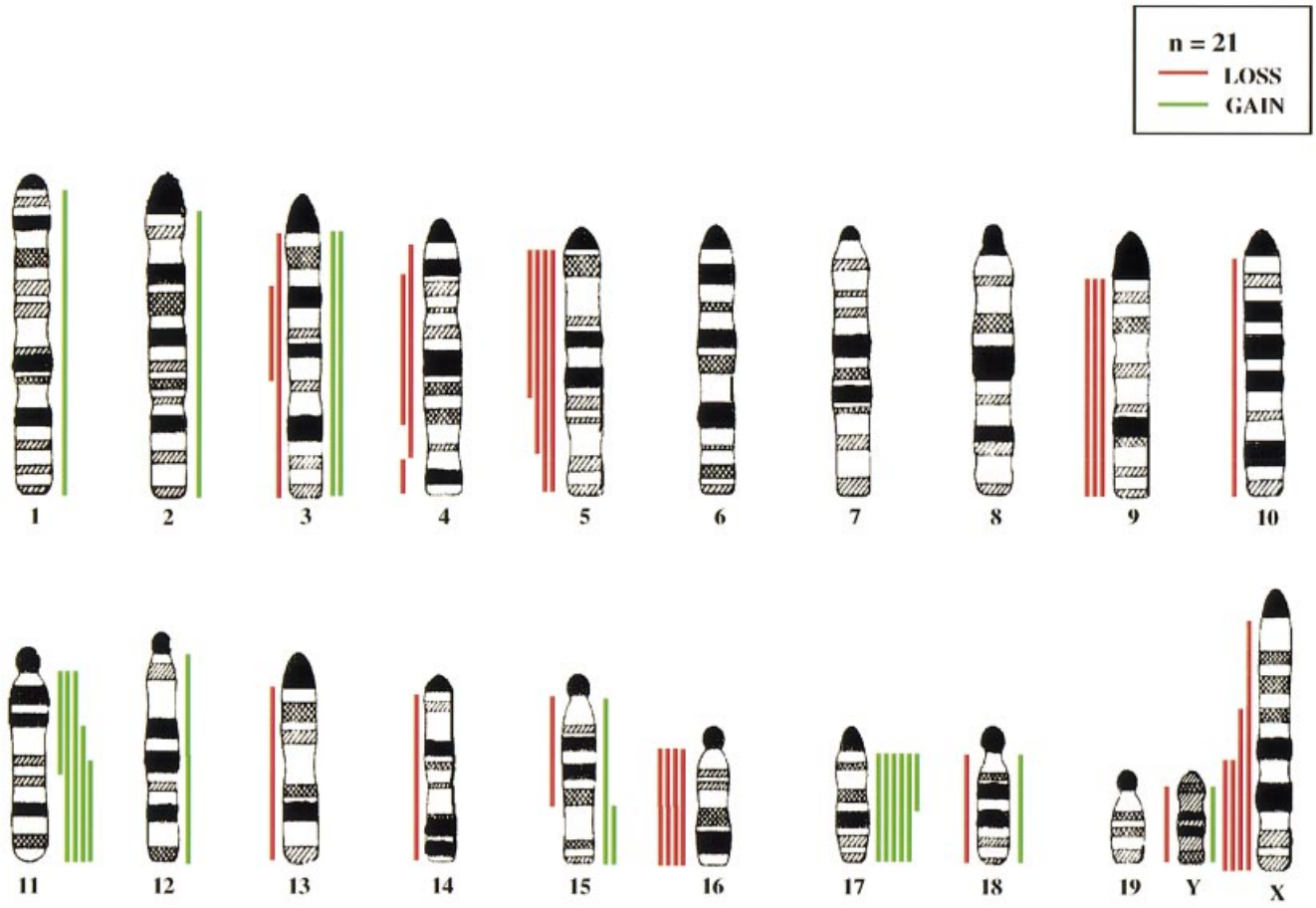

Fig. 8. Chromosomal changes in tumors analyzed by comparative genomic hybridization. Tumors were analyzed as in Materials and methods. Five tumors showed no chromosomal changes.

(v) tumor cells stain positively for synaptophysin and neuron-specific enolase; (vi) tumors form synapses and contain neurosecretory granules; and (vii) tumors show gains and losses of chromosomes in regions syntenic with those observed in human neuroblastoma.

MYCN is amplified in one-third of human neuroblastomas and is associated with an unfavorable prognosis. These observations suggest that *MYCN* acts late in tumor progression. Alternatively, *MYCN* amplification may occur early in a subset of neuroblastomas, generating more aggressive tumors than those transformed through *MYCN*-independent pathways. It is unlikely, however, that amplification of *MYCN* is the initial event in tumorigenesis, since amplification is alleged not to occur in normal mammalian cells (Tlsty, 1990; Wright *et al.*, 1990). Thus, it is probable that early events in the genesis of neuroblastoma promote genetic instability and allow cells to tolerate such instability. Genomically unstable cells can then be transformed by lesions such as *MYCN* amplification in conjunction with other genetic events. By overexpressing *MYCN* in a transgenic mouse, we bypass the need for destabilization of the genome which is a likely prerequisite for *MYCN* amplification, while maintaining a requirement for other genetic events that contribute to neuroblastic transformation.

For some human tumors, one or more genetic lesions typically occur early during the course of tumorigenesis, whereas others occur later (Kinzler and Vogelstein, 1996). Must the multiple mutations culminating in malignancy occur in a particular order? The data presented here demonstrate that overexpression of *MYCN*, although

Table II. Comparative genomic hybridization results from individual tumors

Tumor[a]	Copy number change by CGH
1[b]	+11(A2–B3), +15(E–F3)
2[b]	+3, +11, +Y
3[b]	–5, –9, –16
4[b]	–4(A2–D3), –5, –9, –16
5[c]	–5(A2–E5), +11(B1–E2), –16
6[b]	–3(C–F1)
7[d]	+17, –X
8[b]	+15, +17, +18, –X(D–F3)
9[c]	+11 (B2–E), –X(D–F3)
10[d]	+1, +2, +3, –4(A4–C7), –4(E), –5(A2–E2), –9, –10, +11, –13, –14, –15(A2–D3), –16, +17, –18, –X (A7–F)
11[d]	+17(A–D)
12[d]	–3, +17, +18
13[d]	+12, +17

[a]Eight tumors, including all five tested from mice homozygous for the *MYCN* transgene, had no changes by CGH.
[b]Undifferentiated tumors.
[c]Tumors showing mixed differentiation.
[d]Tumors with predominantly ganglionic histology.

unlikely to be the initial event in the genesis of human neuroblastoma, can in fact initiate the sequence of events that lead to neuroblastoma in mice.

Additional genetic lesions contribute to tumorigenesis

The prolonged latent period for tumor formation and the additional chromosomal lesions seen by CGH both argue that tumor formation in the mouse, as in humans, requires genetic lesions in addition to overexpression of *MYCN*. Five mice homozygous for the *MYCN* transgene had no chromosomal gains or losses seen by CGH, suggesting that neuroblasts with increased *MYCN* gene dosage may require fewer additional genetic events for transformation than those with lower levels of *MYCN*. Three of 16 mice hemizygous for the *MYCN* transgene also showed no chromosomal copy number abnormalities. This number is comparable with 4/29 human neuroblastomas examined by CGH which also showed no abnormalities (Plantaz *et al.*, 1997). It has been suggested that the amplicon carrying *MCYN* in neuroblastomas may contain other genes that also contribute to tumorigenesis (Manohar *et al.*, 1995). On the other hand, a survey of amplified DNA in human neuroblastomas found that *MYCN* may be the only genetic element held in common among the amplicons in the various tumors (Reiter and Brodeur, 1996). In the mouse model presented here, we encountered one tumor that was trisomic for chromosome 12 (which carries *mycn*). Otherwise, we found no evidence for amplification of endogenous *mycn* (which could serve as an indicator for amplification of linked genes as well).

In mice hemizygous for the *MYCN* transgene, the genetic losses that occurred most often were of chromosomes 5 (4/16 mice), 9 (3/16 mice), 16 (4/16 mice) and X (4/16 mice). The involved chromosomes are syntenic with human chromosomes 4 (lost in 7/29 human neuroblastoma samples), 11 (lost in 11/29 human tumors), 3 (lost in 8/29 human tumors) and X (lost in 10/29 human tumors) respectively (Plantaz *et al.*, 1997). The chromosomal region 1p36.1–36.2 may carry a tumor suppressor gene involved in the genesis of human neuroblastoma (White *et al.*, 1995). Loss of this region may correlate with amplification of *MYCN* (Fong *et al.*, 1992; Caron *et al.*, 1993), although this view is controversial (Weith *et al.*, 1989). In contrast, CGH detected loss of the syntenic region of chromosome 4 in only two of the 16 mouse tumors studied here. We cannot presently account for this apparent discrepancy. It is possible that lesions will be found when the syntenic region is studied at higher resolution. Alternatively, providing an excess of Mycn early in tumor progression may alter the requirements for subsequent genetic events.

The genetic gains that occurred most often were of chromosome 17 (in 6/16 mice) and chromosome 11 (in 5/16 mice). The chromosomes involved are syntenic with human chromosomes 6 (gained in 10/29 human neuroblastoma samples) and 17 (gained in 21/29 human tumors) respectively (Plantaz *et al.*, 1997). Gain of mouse chromosome 17 also correlated with ganglionic differentiation in 5/6 tumors showing predominantly ganglionic differentiation by histology.

In human studies, gain of chromosome 17 was the most commonly found genetic abnormality, and the minimal region of chromosome 17 which showed gain was 17q21.3-qter (Plantaz *et al.*, 1997). In the data presented here, the five tumors showing gain of mouse chromosome 11 shared an area defined by the much smaller region between bands B2 and B3. Much of this mouse interval is syntenic with the short arm of human chromosome 17, and only a very limited region is syntenic with 17q21.3-ter (Mouse Genome Database, 1996). The region of chromosome 11 gained in mouse tumors potentially provides a better definition of the interval gained in human neuroblastoma. Experiments to better characterize this region by fluorescence *in situ* hybridization are in progress.

It is intriguing that the chromosomal regions most commonly affected in this mouse tumor sample are all syntenic to those affected in human tumors. These data suggest that the genetic pathways contributing to mouse neuroblastoma are similar to those observed in the human disease.

In an effort to identify specific genetic lesions that might contribute to the genesis of neuroblastoma in the transgenic mice, we explored the effects of deficiencies in the tumor suppressor genes *NF1*, *RB1* and *p53*. We found that heterozygous deficiencies in either *NF1* or *RB1* augmented tumorigenesis. While we cannot exclude the possibility that increased tumorigenesis results from haploinsufficiency for *NF1* and *RB1*, we presume that spontaneous damage inactivated or eliminated the remaining alleles of the genes. In contrast, even homozygous defects in *p53* had no apparent effect on tumorigenesis. These results are in at least partial accord with the findings to date in human neuroblastoma cell lines, which show relatively frequent defects only in *NF1* (Johnson *et al.*, 1993; The *et al.*, 1993) Our results with the transgenic model encourage a more diligent examination of *RB1* in human neuroblastoma.

Twenty percent of the *NF1* hemizygous knock-out mice developed phaeochromocytomas of the adrenal gland (Jacks *et al.*, 1994). We cannot exclude the possibility that some of the increased tumor penetrance seen in our *NF1* cross relates to an increase in phaeochromocytomas. This possibility is unlikely, however, as, in contrast to the study of Jacks *et al.* (1994), nearly all of the tumors in our cross were extra-adrenal. In addition, whereas phaeochromocytoma is a relatively well differentiated neoplasm, the tumors in our study showed a range of neuronal differentiation from predominantly ganglionic elements to undifferentiated small round blue cell tumors.

Different mouse strains showed differing penetrance for neuroblastoma. Mice back-crossed once to a 129S/v strain had increased penetrance over mice back-crossed to C57B6/J, whereas mice back-crossed to an FVB/N strain had a lower penetrance of tumors. In all of these experiments, mice were not back-crossed extensively, and therefore the contribution from genetic background was presumably limited. Nevertheless, the data with FVB/N are particularly striking and suggest that a tumor suppressor in this strain may protect mice from *MYCN*-induced neuroblastoma. These mice are being back-crossed repeatedly to achieve homogeneous genetic backgrounds.

Increased tumor incidence in mice homozygous for the MYCN transgene

The amounts of Mycn varied from one tumor to another. In some of the present examples, the level of expression

was low, but it inevitably exceeded that found in normal tissues. The variable levels of expression may in part reflect variations among tumors in the ratio of tumor cells to stroma. Previous experience has indicated that transgenes can be tumorigenic even when expressed in relatively small amounts, so long as the expression is abnormal to the tissue in question (Nusse *et al.*, 1984). It is possible that the impact of transgene expression may be enhanced by the additional genetic lesions that accumulate during progression.

By inter-breeding transgenic mice that overexpress *MYCN*, we generated animals with increased dosage of the transgene. Surprisingly, this small increase in gene dosage led to a substantial shortening of and an increased penetrance of tumors. This result suggests that a small change in protein levels may have a dramatic impact on signaling pathways. The mechanism by which doubling the gene dosage leads to increased tumorigenesis remains uncertain. It is possible that increased levels of *MYCN* lead to further genomic instability. However, if this were true, we would expect that tumors from such mice would display a large number of chromosomal abnormalities by CGH. In fact, all of the five tumors analyzed displayed no additional gains or losses of chromosomal regions. This result suggests that increased gene dosage of *MYCN* does not promote further genomic instability. By analogy with *MYCC*, perhaps *MYCN* overexpression leads to transformation by up-regulating *CDC25* or other signaling molecules and pushing quiescent cells into the cell cycle (Galaktionov *et al.*, 1996)

MYCN and neuroblast transformation

MYCN frequently is amplified in neuroblastoma, but rarely has been implicated in other human malignancies. This observation raises the question of whether *MYCN* is particularly limited in the types of cells it can transform. Certainly *MYCN* can transform other cell types, as evidenced by B- and T-cell malignancies in transgenic mice which overexpress *MYCN* under control of the immunoglobulin heavy chain enhancer (Dildrop *et al.*, 1989; Rosenbaum *et al.*, 1989). Similarly, *MYCN* is the only proto-oncogene that has to date been implicated in neuroblastoma. This observation suggests that neuroblasts may be especially susceptible to the effects of *MYCN*. It is notable that overexpression of viral transforming genes from polyomavirus, papovavirus and adenovirus all cause neuroblastic tumors in transgenic mice (Small *et al.*, 1986; Aguzzi *et al.*, 1990; Koike *et al.*, 1990; Skalnik *et al.*, 1991). In contrast, few human proto-oncogenes other than *MYCN* have been implicated in neuronal transformation (Iwamoto *et al.*, 1993).

We have shown that overexpression of *MYCN* in the developing neuroectoderm of transgenic mice leads to neuroblastoma, and that chromosomal gains and losses in these tumors occur in regions syntenic to those in human neuroblastoma. These data suggest that the pathogenesis of neuroblastoma in the transgenic model resembles that of human tumors. Transgenic mice overexpressing *MYCN* may therefore be useful for finding additional genes involved in tumorigenesis, and for developing better therapies for children with this malignancy.

Materials and methods

Plasmid construction

A pGEM7Zf(+)-based plasmid (Promega, Madison, WI) was constructed containing a 4.8 kb fragment of the tyrosine hydroxylase promotor (Banerjee *et al.*, 1992), the rabbit β-globin intron (O'Hare *et al.*, 1981), a human *MYCN* cDNA (Ramsay *et al.*, 1986) and the herpes simplex virus thymidine kinase gene terminator/poly(A) sequence (Cole and Stacy, 1985). This construct was cut with *Nsi*I (Figure 1), purified from low melting agarose gels and diluted to 5 μg/ml for injections.

Transgenic mice

Mice were created by standard methods (Hogan *et al.*, 1994). Founders were derived from Balb/c×C57B6/J, FVB/N or C57B6/J (Jackson Labs, Bar Harbor, ME). Tail DNA was analyzed by Southern analysis using the *MYCN* probe or by PCR using 5′ primer CTTCATCATCTGAATCGCTCAGG and 3′ primer TCATCCTCCAGGACTGCATGTG within *MYCN*.

Brain, tumor and grossly abnormal organs were submitted for pathology in all cases. Eight mice had full microscopic analysis of adrenal, brain, heart, lung, cortical bone, bone marrow, liver, kidney, pancreas, intestine, skin, spleen, skeletal muscle and gonads.

Mice homozygous for the *MYCN* transgene were identified by Southern blotting of genomic DNA using a human *MYCN* probe. The cross-hybridizing mouse genomic *MYCN* band served as internal standard. Tail DNA was cut with *Eco*RI, which cleaves human transgenic *MYCN* as a 1.8 kb band, and the genomic mouse *mycn* as a 7 kb band. Band intensities were determined by phosphorimager analysis.

NF1 and *RB1* knock-out mice (Jacks *et al.*, 1992, 1994) were from Jackson Labs (Bar Harbor, ME). Mice deleted for *p53* (Donehower *et al.*, 1992) were from Jeff Arbeit. These mice were screened by PCR using primers for the neomycin resistance gene which marks the disrupted allele (5′-GATGGATTGCACGCAGGTTC, 3′-CTTGAGTGACAACGTCGAGC).

Histology, immunohistochemistry and electron microscopy

Tissues and tumors were fixed in 10% formalin, paraffin embedded, sectioned and stained with hematoxylin and eosin. For immunohistochemistry, 7 μm sections were deparaffinized, hydrated and treated with the appropriate mouse monoclonal antibodies directed against synaptophysin (Boehringer Mannheim, Indianapolis, IN, 1 μg/ml), neuron-specific enolase (Dako, Carpinteria, CA, 3 μg/ml), O13 (Signet, Dedham MA, 1 μg/ml), S100 (Dako, 1 μg/ml) or chromogranin A (Biogenex, San Ramon, CA, 1 μg/ml). Detection was accomplished using the Vectastain kit (Vector labs, Burlingame, CA). For ultrastructural analysis, tumors were fixed with glutaraldehyde, and imaged with a Jeol electron microscope (Peabody, MA).

Immunoblotting

For Western analyses, tissues and tumors were homogenized in Tris-buffered saline (TBS). SDS and β-mercaptoethanol were added to 2 and 5% respectively. Lysates were sonicated, boiled, microcentrifuged, and supernatants loaded on 10% SDS–polyacrylamide gels. Proteins transferred electrophoretically to nitrocellulose membranes were blocked with TBS containing 0.5% NP-40, 0.1% Tween-20, 5% bovine calf serum and 5% non-fat dry milk (TBSTN). Membranes were incubated with Mycn antisera (Boehringer Mannheim, Indianapolis, IN, 1 μg/ml) and treated with a horseradish peroxidase-linked sheep anti-mouse secondary antibody (Amersham, Arlington Heights IL, 1 ng/ml), Bands were visualized by ECL (Amersham, Arlington Heights, IL). Equal amounts of protein were added as measured by Coomassie staining, and by re-probing with an antibody for phosphofructokinase.

For immunoprecipitations, equal weights of tissues were homogenized in TBS, and SDS was added to 0.5%. Homogenates were boiled, and 4 volumes of 1.25× RIPA without SDS were added prior to the centrifugation. To obtain adequate amounts of tissue, adrenals were harvested from six littermates and pooled. Other tissues were taken from a single mouse. Immunoprecipitation was performed using rabbit polyclonal anti-Mycn antibody Y2 (Ramsay *et al.*, 1986) followed by Western blotting as above.

Radiation

Newborn mice were irradiated before 3 days of age with 3 Gy from a cesium source. Transgenic mice and non-transgenic littermates were followed for 10 months for development of tumors.

Comparative genomic hybridization

CGH was performed as described previously (Mohapatra *et al.*, 1995). Tumor DNA was labeled by nick translation with fluorescein isothiocyanate (FITC)–dUTP. Normal spleen DNA from C57BL6/J mice was labeled with Texas red–dUTP (Du Pont Inc., Wilmington, DE). The probe mixture [200 ng each of labeled tumor and normal DNAs, 20 μg of unlabeled cot-1 DNA (Gibco BRL, Gaithersburg, MD) in 10 μl of 50% formamide, 10% dextran sulfate and 2× SSC, pH 7.0] was denatured at 75°C for 5 min and hybridized to normal mouse fibroblast metaphase chromosomes for 48 h at 37°C. After hybridization, slides were counterstained with 0.2 mM 4′,6'-diamidino-2-phenylindole (DAPI) in anti-fade solution.

Digital images of red, green and blue fluorochromes were acquired using a Quantitative Image Processing System, and ratio profiles of fluorescence intensity along chromosomes were quantitated (Piper *et al.*, 1995). At least six metaphase chromosomes were analyzed and averaged to obtain CGH ratio profiles. Profiles were normalized to an average value of 1.0 for the entire genome, and regions where the green:red ratio deviated significantly from the average were scored as increases or decreases in DNA sequence copy number.

Acknowledgements

We are grateful to Jeff Arbeit, Dona Chikaraishi, William C.Mobley, Nadya Milshteyn, Eric Radany, Gary Ramsay, Werner Roseneau and members of the Bishop laboratory for their help. This work was supported by grant CA44338 from the National Institutes of Health, and by funds from the G.W.Hooper Research Foundation to J.M.B. W.A.W. is a recipient of the Burroughs Wellcome Fund Career Award in the Biomedical Sciences, and was supported by an National Institutes of Health Neurological Sciences Academic Development Award (K12NS01692), and by a grant from the American Philosophical Society. G.M. and B.G.F. were supported from the National Cancer Institute (CA13525, CA61147 and CA64898) and from Vysis, Inc.

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Received on December 20, 1996; revised on February 24, 1997