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Inhibition of Proliferation by c-myb Antisense RNA and Oligodeoxynucleotides in Transformed Neuroectodermal Cell Lines

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ABSTRACT

Transfection of a neuroblastoma cell line with expression vectors containing two different segments of human c-myb complementary DNA in antisense orientation yielded far fewer transfected clones than did the transfection with the identical segments in sense orientation. In cell clones expressing c-myb antisense RNA, levels of the c-myb protein were down-regulated and the proliferation rate was slower than that of cells transfected with sense constructs or the untransfected parental cell line. Treatment of neuroblastomas and neuroepithelioma cell lines with a c-myb antisense oligodeoxynucleotide strongly inhibited cell growth. These data indicate a definite involvement of c-myb in the proliferation of neuroectodermal tumor cells extending the role of this protooncogene beyond the hematopoietic system. The availability of cell clones that transcribe c-myb antisense RNA provides a useful tool to study the involvement of other genes in the proliferation and differentiation of neuroblastoma cells.

INTRODUCTION

The c-myb protooncogene is the cellular homologue of the viral v-myb carried by avian myeloblastosis virus and E26 (1, 2), both of which transform hematopoietic cells with a distinct myeloid phenotype (1–3). The protein encoded by v-myb is localized in the nucleus (4, 5) and binds to DNA in vitro (6). The DNA-binding domain of the v-myb protein is composed of two imperfectly conserved 52-amino acid direct repeats located near the amino terminus (7) and corresponds to a truncated version of that found in chicken and mammalian c-myb proteins (8–10). The v-myb protein synthesized in bacteria binds specifically to the nucleotide sequence pyAAGC/TG (11), and concatamers of this consensus sequence can confer v-myb-dependent inducibility to otherwise unresponsive promoters, suggesting that the v-myb protein acts as sequence-specific DNA binding transcription factor (12). Also, v-myb directly regulates the expression of a cellular gene, MIM-1, in chicken myeloblasts infected with an avian myeloblastosis virus temperature-sensitive mutant (13). Nuclear localization and DNA binding activity are necessary but not sufficient for the oncogenic potential of myb (14). The c-myb protooncogene and v-myb share several biochemical and functional properties, including nuclear localization, DNA binding, and transcriptional regulator activity (15). These properties appear to be important in transformation of chicken myeloid cells (16).

It has long been suggested that c-myb expression is linked to proliferative and differentiative processes in the hematopoietic system (17, 18). Antisense c-myb oligodeoxynucleotides block normal hematopoiesis in vitro (19). More recently, the need for c-myb expression on fetal liver hematopoiesis has been demonstrated in transgenic animals in which the c-myb gene has been inactivated by homologous recombination (20).

Elevated c-myb expression has been demonstrated in human leukemia cells (21) the in vitro proliferation of which has been shown to be myb-dependent (22). Several solid tumors of different embryonic origin such as colon carcinoma (23), small cell lung carcinoma (24), teratocarcinoma (25), and neuroblastoma (26) also demonstrate c-myb expression.

NB* is a malignant childhood tumor thought to arise in migratory cells of the embryonal neural crest (26). NB is histopathologically indistinguishable from NE and the two malignancies are often considered as one entity. Nevertheless, Thiele et al. (26) have reported that the pattern of protooncogene expression differs in these tumors. In fact, N-my expression is generally high in NB and its amplification correlates with tumor progression and aggressiveness (27), whereas NE generally does not express N-my (27). On the contrary, c-my expression is high in NE but low in NB. However, both NE and NB express c-myb. NB cell lines induced to differentiate by retinoic acid demonstrate a rapid and sharp decrease in c-myb expression due to a decreased transcription rate rather than instability of c-myb mRNA (28). The temporal relationship between the levels of c-myb mRNA and the differentiative and proliferative processes occurring in NB makes c-myb a possible candidate for a key role in the proliferation and/or differentiation of neuroectodermal tumors.

In the present study we evaluated the effects of c-myb down-regulation in NB and NE cell lines by transfection of expression vectors carrying different domains of the c-myb cDNA in antisense orientation and by exposing cell cultures to antisense oligodeoxynucleotides.

MATERIALS AND METHODS

Cloning of Antisense and Sense c-myb Vectors. SstI-EcoRI (DNA-binding domain) and BamHI-BamHI (3' untranslated region) fragments were obtained from clone pMB1 dihydrofolate reductase (18) containing a full-length human c-myb cDNA. After end-repair with Klenow enzyme (Promega, Madison, WI), the fragments were cloned in the polylinker region of pRC/CMV vector (Invitrogen, San Diego, CA) as described (29). Sense and antisense orientation of the cloned fragments was determined by restriction analysis.

Cell Lines and Transfection. Neuroblastoma cell line LAN-5 (30) was grown in RPMI 1640 (Sigma, St. Louis, MO) supplemented with fetal bovine serum (Sigma). Cell lines SK-N-SH and SK-N-MC (31) were grown in minimal essential medium (GIBCO, Grand Island, NY) with 10% fetal bovine serum.

DNA transfections in LAN-5 cells were performed by the calcium phosphate precipitation technique according to standard procedures (29). Briefly, cells were seeded at a density of 10^6/dish and 48 h later were exposed to plasmid DNA (pRC/CMV) at 20 µg/plate for 6 h. After

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4 The abbreviations used are: NB, neuroblastoma; NE, neuroepithelioma; cDNA, complementary DNA; PBS, phosphate-buffered saline; CMV, cytomegalovirus; RT, reverse transcriptase; PCR, polymerase chain reaction.

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2 days cells were placed in medium containing the antibiotic G418 (Sigma; 400 μg/ml) and transfectant clones were isolated 3 weeks later.

Oligodeoxynucleotides. Unmodified deoxynucleotides were synthesized on an Applied Biosystem 380B DNA synthesizer by means of β-cyanoethylphosphoramidite chemistry. Oligodeoxynucleotides were purified by ethanol precipitation and multiple washes in 70% ethanol. Nucleotide (nt) numbers and codon positions for each c-myb oligodeoxynucleotide refer to the published human c-myb cDNA (10).

The primers for the PCR to control integration of the antisense inserts in the 5' and 3' antisense clones and to detect antisense RNA transcription are

CMV1: 5' AATGGAGATTGTGTGTTTTCACCAAC3' nt 699-722 of hRcCMV-myb1: 5' TGCCAAGCACTTAAAGGGGAGAATG3' nt 467-490
myb2-5': 5' ACCCTTGGGAGACCTCTGATT3' nt 2959-2983
myb3-5': 5' GCTGGCACTGCACATCTGTT3' complementary to nt 333-352
myb-4: 5' GCTGGCACTGCACATCTGTT3' nt 128-147
myb-5: 5' CTGAAGAAGCTGGTGGAACAGAATG3' nt 264-289
myb-6: 5' CTAGCAGCATGTCTACAGGC3' complementary to nt 2708-2729
myb-7: 5' CCATGGCACATTAAATCGA3' nt 2496-2515
myb-8: 5' GCTCATTATGTGTTAATGC3' nt 2525-2544

The c-myb sense oligodeoxynucleotide used in cell cultures was 5'GCCCGAAGACCChGGCCAC3' corresponding to codons 2-7. The c-myb antisense was 5'GCCCGGGGTCTTCGGGC3', complementary to the codons 2-7.

RNA Extraction and RT-PCR Analyses. Total RNA was prepared by acid guanidinium thiocyanate-phenol-chloroform extraction (32). RNA for RT-PCR analysis to detect c-myb antisense RNA in transfected clones was treated with RNase-free DNase I (Promega). Reverse transcription, PCR analysis, and hybridization to the specific probes were carried out as described (33). Plasmid integration was determined by PCR after isolating genomic DNA as described (29).

Immunocytochemical Analysis. LAN-5 cells were seeded in Labtek chamber slides (NUNC, Naperville, IL) at a density of 5 x 10^5 cells/cm². After 48 h, cells were rinsed in PBS, fixed in 4% paraformaldehyde in PBS for 10 min at 4°C, permeabilized in PBS containing 0.01% Triton X-100 for 5 min at 4°C, and treated with 1% bovine serum albumin (Sigma) for 30 min at room temperature. Incubation with sheep polyclonal antibody to c-myb protein (Cambridge Research Biochemicals, Valley Stream, NY) (34) at 1:100 dilution and anti-sheep IgG antibody (KPL, Gaithersburg, MD) and stained with an immunocytochemical staining kit (KPL).

RESULTS

Cloning Efficiency and Proliferation of a Human Neuroblastoma Cell Line Expressing c-myb Antisense Transcripts. Two different regions of the human c-myb cDNA were cloned in antisense orientation in the expression vector pRc/CMV in which the CMV promoter-enhancer (35) drives the transcription of the cloned genes and the SV40 promoter drives transcription of the gene encoding the G-418 resistance used to select transfected cells. Transient transfection assays to compare the efficiency of the Rous sarcoma virus, SV40, and CMV promoters in LAN-5 neuroblastoma cells showed that the CMV had highest activity in driving the transcription of a reporter gene.

Neuroblastoma cell line LAN-5 which expresses c-myb mRNA constitutively (26, 28) was transfected with plasmids expressing antisense and sense c-myb transcripts. Both size and number of G418 resistant clones were reduced in the transfections with constructs expressing the 5' or 3' antisense myb sequences as compared to those expressing the sense transcripts (Fig. 1). On average, the cloning efficiency of 5' antisense-myb transfectants was reduced 66% (from 62 to 70%) as compared to 5' sense-myb; for the 3' antisense-myb transfections, the average reduction was 91% (from 84 to 97%) as compared to 3' sense-myb (mean of 3 separate experiments).

Twelve single clones were isolated from the 5' and 3' antisense myb transfectants and the integration of each construct was determined by PCR. Primers for PCR were chosen in order to amplify 267 base pairs upstream of the NotI cloning site in addition to 208 base pairs of 5' antisense-wy¿> and 219 bp of 3' sense RNA transcripts in 5' antisense clones. In B, CMV-1 and myb-2 are the PCR primers to control the integration of the 3' antisense clones; myb-6 and myb-7 are the RT-PCR primers; and myb-8 is the probe for antisense RNA detection in 3' antisense clones.
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possibly because of rearrangement or deletion involving the antisense insert preventing the transcription of the antisense RNA. Accordingly, the inhibition of LAN-5 cell proliferation resulting from transfection with the 5' antisense myb construct might be underestimated. Clones positive for integration of the 5' antisense construct showed the expected amplified 475-base pair fragment.

Transcription of antisense c-myb RNA was confirmed in RT-PCR experiments (Fig. 3C). Total RNA from transfectant clones was extracted and treated with RNase-free DNase I before the RT-PCR reaction. Primers were designed to amplify a 224-base pair product for 5' antisense myb clones and a 231-base pair product for 3' antisense myb clones (Fig. 2). After size fractionation on an agarose gel and transfer to a nylon membrane, the amplified products were hybridized to specific probes for unambiguous identification; antisense c-myb RNA transcripts were clearly detected in both 5' and 3' antisense-myb transfectants (Fig. 3C). Most of the antisense transfectants do not show evident morphological alterations. All tested antisense clones retain the capability to differentiate toward a neural phenotype under the effect of retinoic acid (not shown). A more detailed phenotypic characterization of the transfectant clones is now in progress.

Comparison of growth curves for 3' and 5' antisense-myb clones which were found positive for integration of the constructs (Fig. 4) revealed consistently slower proliferation rates and generally lower growth plateaus than those of the LAN-5 parental cells and sense controls.

Immunocytochemical analysis using an anti-myb polyclonal antibody indicated the clear presence of myb protein in the nucleus of the LAN-5 parental cells and the 5' and 3' sense controls (Fig. 5, A–C), whereas myb protein was barely detectable in the 5' and 3' antisense transfectants (Fig. 5, D and E).

Effect of c-myb Antisense Oligodeoxynucleotides on Growth of Neuroblastoma and Neuroepithelioma Cell Lines. To further examine the role of c-myb in the proliferation of neuroectodermal tumors, we inhibited c-myb expression using antisense oligodeoxynucleotides and analyzed the effects of this
Fig. 5. Expression of c-myb protein in antisense clones. Cells were treated with an anti-myb specific polyclonal antibody (A-E) or nonspecific preimmune serum (F) as described in "Materials and Methods." A, LAN-5; B, 5' sense myb; C, 3' sense myb; D, 5' antisense myb; E, 3' antisense myb; and F, 3' sense myb treated with immune serum. × 200.

Fig. 6. Effect of c-myb antisense oligodeoxynucleotide on cell growth of neuroblastoma and neuroepithelioma cell lines. SK-N-SH (A), SK-N-MC (B), and LAN-5 (C) cells were untreated (C), sense-treated (S) and antisense-treated (AS). Cells were counted after 7 days for SK-N-SH (A) and after 9 days for SK-N-MC (B). LAN-5 (C) cells were counted at days 0, 5, 9, and 13 of culture. Cell count values, mean ± SD (bars) of experiment performed in triplicate.

inhibition on the growth of neuroblastoma cell lines LAN-5 and SK-N-SH and neuroepithelioma cell line SK-N-MC (31). In a typical experiment, 1 × 10^4 cells were seeded in the presence of antisense or sense oligodeoxynucleotides (80 µg/ml at 0 h, 40 µg/ml after 18 h, and 40 µg/ml after 36 h). Cells were counted after 7 or 9 days. As shown in Fig. 6, antisense c-myb oligodeoxynucleotide treatment resulted in almost complete growth inhibition in all three cell lines. To determine whether this inhibition correlated with c-myb transcript levels, total RNA was extracted from each tumor cell line 24 h after exposure to 120 µg of c-myb oligodeoxynucleotides and c-myb expression was measured by RT-PCR; c-myb mRNA was barely detectable in antisense-treated cells, but abundantly expressed in sense-treated and untreated cells (Fig. 7).

DISCUSSION

We have shown that down-regulation of c-myb expression exerts a strong inhibitory effect on the proliferation of neuroectodermal tumor cells. Two different strategies were used in our work. Neuroblastoma cell line LAN-5 was transfected with vectors carrying two different segments of the human c-myb cDNA in the antisense orientation and transfection efficiency was assayed; the yield of transfectants was dramatically reduced.
when either antisense vector was used as compared to their sense controls. Although the isolated antisense transfectants are heterogeneous in their growth rate, they show consistently a slower proliferation as compared to sense controls. Furthermore, they transcribe antisense c-myb mRNA and have a marked reduction of myb protein synthesis as indicated by immunocytochemistry. Together these data indicate that the slower proliferation of the isolated antisense clones is due to down-regulation of c-myb expression caused by antisense RNA production.

The second strategy involved exposure of neuroblastoma and neuroepithelioma cell cultures to a c-myb antisense oligodeoxynucleotide in order to suppress c-myb mRNA expression. Again, the same inhibitory effect on the proliferation rate of these cells was observed. The rescue of antisense stable transfectants is in apparent contrast with the nearly complete growth inhibition resulting from the exposure of neuroectodermal tumor lines to c-myb antisense oligodeoxynucleotides. Most likely these findings rest in the low level of antisense c-myb RNA transcribed by the transfectant clones and detectable by the sensitive RT-PCR technique but insufficient to completely block the function of c-myb mRNA. In this regard, Cotten et al. (36) have demonstrated the requirement for an antisense RNA:target mRNA ratio of 6:1 to completely abolish the function of protein U7. Nevertheless, the slow proliferation rate of the c-myb antisense transfectants indicates that even incomplete down-modulation of c-myb has a readily detectable effect on cell growth.

In neuroblastoma several structural abnormalities such as the deletion of the short arm of chromosome 1 (del1p32-pter), double-minute chromosomes and homogeneously staining regions are frequent findings (37–39) and have been associated with the development and the progression of this neoplasia (40). Furthermore, N-myc gene amplification has been correlated with advanced clinical stages and poor clinical outcome (27, 41). However, the proliferative activity of neuroblastoma has not been clearly associated with a distinct pattern of altered gene expression.

Two recent reports describe the block of N-myc expression by means of antisense RNA and oligodeoxynucleotides (42, 43). In both cases neuroblastoma proliferation was only partially affected by N-myc down-regulation and was probably secondary to the induction of differentiative processes. In addition, a large percentage of terminal neuroblastomas do not show amplification or detectable expression of N-myc. Together, these findings suggest that other gene activities besides N-myc are involved in the proliferation of neuroblastoma cells. The inhibitory effect on cell growth obtained by abolishing the expression of c-myb strongly suggests the involvement of this protooncogene in the regulation of neuroblastoma cell proliferation. Moreover, the findings reported here, provide direct evidence for the essential role of c-myb in nonhematopoietic tissues, perhaps through its effects on the expression of genes directly involved in DNA synthesis and cell cycle progression. The stable transfectant cell lines that express antisense c-myb RNA should prove useful in evaluating the possible cooperation of c-myb and other genes in regulating proliferative and differentiative processes in neuroblastoma, which, in turn, may lead to the development of an antisense-based therapy of these neoplastic disorders.

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