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Excision of N-*myc* from Chromosome 2 in Human Neuroblastoma Cells Containing Amplified N-*myc* Sequences

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Amplification of one of three growth-stimulating *myc* genes is a common method by which many tumor types gain a proliferative advantage. In metastatic human neuroblastoma, the amplification of the N-*myc* locus, located on chromosome 2, is a dominant feature of this usually fatal pediatric cancer. Of the many models proposed to explain this amplification, all incorporate as the initial step either disproportionate overreplication of the chromosomal site or recombination across a loop structure. The original locus is retained within the chromosome in the overreplication models but is excised in the recombination models. To test these models, we have used somatic cell hybrids to separate and analyze the chromosomes 2 from a neuroblastoma cell line containing in vivo amplified N-*myc*. Our results demonstrate that N-*myc* is excised from one of the chromosomes, suggesting that deletion is a requisite part of gene amplification in a naturally occurring system.

Amplification of genes in eucaryotic cells is a common mechanism associated with normal cellular development and has been observed in organisms as diverse as plants and animals. This increase in gene copy number, as observed in the amplification of the dihydrofolate reductase gene in the presence of the drug methotrexate, is frequently used by tumor cells as a defense against toxic molecules (7, 15). Amplification of genes coding for regulatory proteins also appears to give tumor cells a proliferative advantage, as demonstrated by the amplification of one of three growth-stimulating *myc* genes in a variety of tumor types (6, 21, 28, 29). One such neoplasm, neuroblastoma, is the most common noncranial solid tumor of young children. Advanced-stage neuroblastoma cells frequently possess multiple copies of the proto-oncogene N-*myc* found on double minutes (DMs) (2, 5, 16, 28).

Although detailed analyses of the amplified genes found on episomes, DMs, and homogeneously staining regions have been performed, data on the original chromosomal site following amplification is lacking in any naturally occurring higher eucaryotic system. Of several models proposed to explain DNA amplification in eucaryotic cells (13, 15, 20, 27, 35, 36), research has focused on those models that explain observed head-to-head repeats in amplified extrachromosomal DNA segments (22, 25, 38). These models include the onionskin or disproportionate replication model (for reviews, see references 27 and 36), the double rolling circle model proposed by Futcher (10) and modified by Passananti et al. (22) and Ruiz and Wahl (25), and recombination across a replication loop (39). On the basis of the observation that amplified human genes are localized to replicating submicroscopic circular DNA (38), Wahl (39), Passananti et al. (22), and Carroll et al. (3) have proposed models for gene amplification based on the premise that recombination occurs within the replication bubble leading to excision of the chromosomal region to be amplified.

Excision has been observed in chemically stressed, methotrexate-resistant *Leishmania* spp. cell lines (1) and in Chinese hamster cell lines transfected with the multifunctional Syrian hamster CAD genes (3). However, there has been no direct evidence for this excision event occurring during the de novo amplification of an endogenous gene in higher eucaryotes. This lack of data can be attributed to two problems associated with observing the original chromosomal locus. First, there are two alleles of the target gene present in the genome, and excision of one homolog would be masked by the gene on the other homolog. Second, because the gene is amplified, there are from dozens to hundreds of copies of the gene present in the cells.

To determine whether excision is an aspect of the amplification mechanism in a naturally occurring human tumor, we have examined the amplification of N-*myc* in neuroblastoma cells. Chromosomes 2, which contain N-*myc* at position p24 (31) in normal cells, were isolated from a neuroblastoma cell line by using the technique of somatic cell hybridization, thereby placing them in an environment free of amplified N-*myc*-containing DMs and other human chromosomes 2. DNA from the hybrid cells that contained these chromosomes 2 were probed with an N-*myc*-specific sequence to determine if one homolog had been excised from the chromosome during the amplification process.

MATERIALS AND METHODS

Cell lines. The neuroblastoma cell line N0315L was derived from a patient enrolled in the Pediatric Oncology Group (study 8105 on the biology of neuroblastoma). N0315L is a typical neuroblastoma cell line which has a deletion in the short arm of chromosome 1 (the result of a nonreciprocal translocation [24]) and cytogenetically visible DMs, which contain amplified copies of the gene N-*myc*. No homogeneously staining region is visible, indicating that reinsertion of the amplified sequences into a chromosome has not occurred. The hypoxanthine-guanine phosphoribosyltransferase-deficient Chinese hamster cell line E36, isolated from male lung tissue, has been described elsewhere (11). Both cell lines were maintained as adherent cultures in Ham F10 medium (Flow Laboratories, Inc., McLean, Va.) supplemented with 10% fetal bovine serum (Flow), 10% tryptose phosphate (Oxoid Ltd., Basingstoke, Hamshire, England), 50 μg of gentamicin sulfate (SoloPak Laborato-

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ries, Franklin Park, Ill.) per ml, and 1 μg of Fungizone (E. R. Squibb & Sons, Princeton, N.J.) per ml at 37°C in a humidified 5% CO_2 atmosphere.

Hamster-human somatic cell hybrids. Somatic cell hybrids were formed by fusion of E36 hamster cells to the human neuroblastoma cell line N0315L. Neuroblastoma and E36 cells (5×10^5 of each) were mixed and cultured overnight at 37°C. The mixed monolayer was washed with Tris-buffered saline (0.139 M NaCl, 5.1 mM KCl, 0.7 mM Na_2HPO_4, 0.1% glucose, 25 mM Tris hydrochloride, 67 μg of penicillin per ml, 55 μg of streptomycin per ml [pH 7.4]) and incubated for 20 min in serum-free medium (Ham F10). After removal of the serum-free medium, fusion was induced by a 2-min incubation with 45% polyethylene glycol 1450 (J. T. Baker Chemical Co., Phillipsburg, N.J.) in 0.15 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (Sigma Chemical Co., St. Louis, Mo.) adjusted to pH 8.0. Polyethylene glycol 1450 was removed by four washes with serum-free growth medium, and the cells were then incubated overnight in serum-containing growth medium. Hybrid cells were selected for and then propagated in growth medium containing hypoxanthine-aminopterin-thymidine (13.6 μg of hypoxanthine per ml, 0.17 μg of aminopterin per ml, 3.9 μg of thymidine per ml) and ouabain (10^{-6} M).

Isozyme analysis. Somatic cell hybrids containing human chromosome 2 were identified by analysis of cell lysates for isocitrate dehydrogenase 1 (IDH1) activity (13a). The gene coding for IDH1 has previously been assigned to chromosome 2q32-qter (12). Cell lysates were prepared from 10^7 cells suspended in 30 μl of water by freeze-thawing three times. Lysates were clarified by centrifugation for 2 min at 6,700 × *g* and were stored at −70°C. Cell lysates (1 μl) were electrophoresed for 60 min at 150 V on 1% agarose gels (1 mm thick) containing 5% sucrose and 0.05 M sodium phosphate, pH 6, at 4°C in 0.05 M sodium phosphate, pH 6, running buffer. After electrophoresis, the gels were incubated for 15 min in 0.05 M Tris hydrochloride, pH 8.8, at room temperature to increase the pH of the gel so that the enzymatic staining reaction could take place. The gels were stained for 30 min at 37°C for IDH1 activity (AuthentiKit ICD1 staining system; Innovative Chemistry, Marshfield, Mass.).

Chromosome spreads. Somatic cell hybrid clones were incubated in standard growth media supplemented with 0.1 μg of colcemid per ml for 5 h. Chromosome spreads were then prepared by using the standard method of Wray and Stubblefield (40) and were stained by GTG banding. DNA for Southern and polymerase chain reaction (PCR) analyses were isolated from somatic cell hybrids at the time chromosome spreads were made to allow comparison of data obtained from the different technical approaches.

DNA probes. The cloned sequence L2.30 was obtained from the American Type Culture Collection (Rockville, Md.). This plasmid contains unique human sequences located at 2p25 (8, 23, 32) that detect a restriction fragment length polymorphism (RFLP) consisting of two alleles in *Bgl*II restriction endonuclease-digested human DNA. The plasmid pNB-1 contains a 1-kilobase (kb) cloned sequence from the first intron and second exon of N-*myc* (28) and was obtained from Garret Brodeur. The N-*myc*-specific sequence in this plasmid was derived from the *Eco*RI-*Bam*HI fragment shown in Fig. 1A and B.

An oligonucleotide 40-mer was synthesized that corresponds to a region within the second exon of N-*myc* (Fig. 1C [represented by P]). The sequence was 5'-GAGCGAGAAG CTGCAGCACGGCCGCGGGCCGCCAACCGCC-3'.

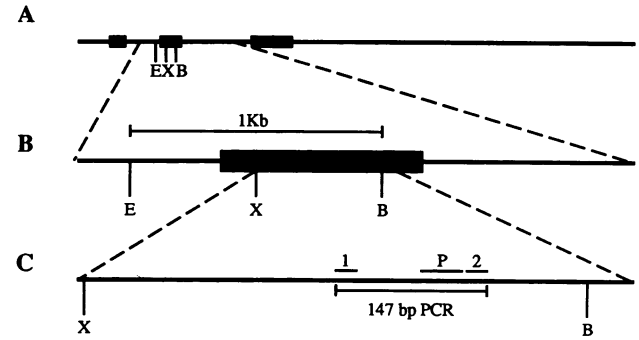

FIG. 1. Partial restriction map of the N-*myc* locus. (A) Entire N-*myc* gene containing 3 exons (heavy bars) and 2 introns. (B) Restriction sites are *Eco*RI (E), *Xho*I (X) and *Bam*HI. The 1-kb *Eco*RI-*Bam*HI fragment is contained in the plasmid pNB-1. (C) The region within the second exon that was amplified by PCR analyses. The 147-bp region is shown. Primer 1 encompasses N-*myc* bases 1101 to 1121; primer 2 encompasses bases 1247 to 1228. An oligonucleotide 40-mer (P) was used to detect the PCR-amplified sequences.

DNA preparations. Eucaryotic DNA was isolated by suspension of 10^7 cells in 1 ml of reticulocyte standard buffer (10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris hydrochloride [pH 7.4]) and 1 ml of 2× DNA buffer (80 mM Tris hydrochloride [pH 7.4], 40 mM EDTA, 0.2 M NaCl). Cells were lysed by the addition of sodium dodecyl sulfate to a final concentration of 0.25%. Proteins were removed by an overnight digestion with 100 μg of proteinase K per ml at 47°C and by subsequent phenol-chloroform (1:1) extractions. The DNA was ethanol precipitated and dissolved in TE (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA). Bacterial plasmid DNA was isolated by using the alkali lysis method (18).

Electrophoresis of DNA and Southern blots. Whole cellular DNA was digested with the appropriate restriction endonuclease and was electrophoresed overnight at 25 mA in a horizontal 1% agarose gel (25 by 20 by 0.5 cm) in TBE running buffer (89 mM Tris hydrochloride [pH 8.0], 89 mM boric acid, 2 mM EDTA). The DNA was transferred to a GeneScreenPlus hybridization transfer membrane (Dupont, NEN Research Products, Boston, Mass.) by the method of Southern (19, 34) as modified by Chomczynski and Qasba (4).

Radiolabeling of DNA probes and hybridization. DNA probes were labeled with [α^{32}P]dCTP (Amersham Corp., Arlington Heights, Ill.; 3,000 Ci/mmol) by using random primer initiation and the Klenow 1 fragment of DNA polymerase I (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) (9). Hybridization was performed for 16 h at 47°C in 30% formamide, 10% dextran sulfate, 1 M NaCl, 50 mM Tris hydrochloride (pH 7.5), 1% sodium dodecyl sulfate, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, and 100 μg of sonicated, denatured salmon sperm DNA per ml. After hybridization, excess probe was removed by two 15-min washes at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) followed by successive 90-min washes at 65°C with constant gentle rocking in 2× SSC and 1% sodium dodecyl sulfate.

The oligonucleotide 40-mer was labeled with ^{125}I by using a modification of the iodination procedure of Tereba and McCarthy (37). To keep the DNA single stranded during the iodination reaction, sodium perchlorate was added to the reaction mixture to a final concentration of 11.5 M. The reaction was performed at pH 4.5 for 30 min at 80°C, and the

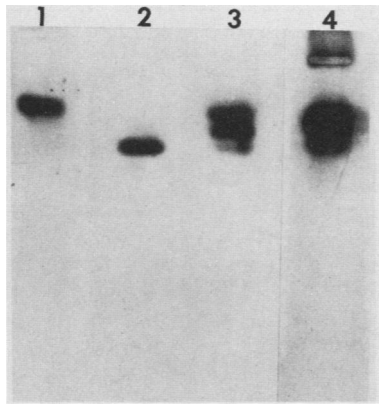

FIG. 2. Detection of human chromosome 2 in somatic cell hybrids by isozyme analysis of isocitrate dehydrogenase 1 in agarose thin gels. Lane 1, Hamster cell lysate; lane 2, human cell lysate; lanes 3 and 4, lysates from somatic cell hybrids 9C6 and 6D6, respectively.

unstable intermediate was destroyed by incubating the mixture in 0.5 M ammonium acetate, pH 9.0, for 60 min at 60°C. The iodinated DNA was separated from unincorporated ^{125}I by using a Sephadex G-50 fine column. The iodinated DNA was ethanol precipitated before use.

PCR analysis. PCR analyses (26) were performed by using synthetic oligonucleotides 20 base pairs (bp) in length. Primer 1 was homologous to the region starting at N-*myc* nucleotide 1101 (5′-TGGGTGGCCTCACCCCCAAC-3′) (17). Primer 2 was the inverse complement to the region between nucleotides 1247 and 1228 (5′-CCGGGGACTGGG CGGTGGAA-3′). The location of these primers is shown in Fig. 1C. Sample DNA (1 μg) was cycled through 29 rounds of 55°C for 2 min (anneal), 72°C for 2 min (extension), and 94°C for 1 min (denature) and one final cycle of 55°C for 2 min, 72°C for 10 min, and 94°C for 1 min. The amplified DNA was electrophoresed through a 1% low-melting-point agarose (Bethesda Research Laboratories) and 3% NuSieve GTG agarose (FMC Corp., Marine Colloids Div., Rockland, Maine) gel in TAE (0.04 M Tris, 0.02 M acetic acid, 0.001 M EDTA) running buffer and blotted onto a membrane, as described above. Iodinated oligonucleotide DNA was used to detect the amplified sequences.

RESULTS

Isolation of chromosome 2 from neuroblastoma cells. Normal human cells contain an N-*myc* gene on each chromosome 2. In addition, neuroblastoma cells frequently contain multiple copies of N-*myc* located on DMs. To independently examine each original N-*myc* chromosomal locus following amplification, the technique of somatic cell hybridization was used to isolate chromosomes from the neuroblastoma cell line N0315L in a Chinese hamster background. Following fusion of N0315L and hamster E36 cells, the resulting hybrids were cloned and screened for the presence of human chromosome 2 by isozyme analysis in thin agarose gels. Cell lysates from several cloned somatic cell hybrids were positive for human IDH1 (located at 2q32-2qter [12]) activity, indicating the presence of human chromosome 2. Analyses of two of these hybrids, E36 × N0315L-9C6 and E36 × N0315L-6D6, are shown in Fig. 2. A single band was observed in the hamster lysate (lane 1), and a faster-migrating single band was observed in the human lysate (lane 2). Hybrids 9C6 and 6D6 each contained human chromosome 2, as demonstrated by the presence of both the human and hamster bands in lanes 3 and 4, respectively. The third (middle) band seen in these lanes represents a hybrid isozyme produced by the association of hamster and human monomers.

Cytogenic confirmation of the presence of chromosome 2. To confirm that a human chromosome 2 was present in the hybrid cells 9C6 and 6D6, chromosome spreads were prepared (Fig. 3). In both 9C6 (Fig. 3A) and 6D6 (Fig. 3B), 17 of 20 spreads contained one normal human chromosome 2. The chromosomes were cytogenetically intact (Fig. 3C [c and d]) and were identical to the chromosomes 2 taken from the original tumor tissue used to develop the cell line N0315L (Fig. 3C [b]). These chromosomes were compared with chromosomes 2 taken from normal human blood (Fig. 3C [a]). Upon heavy staining of several spreads, no DMs were observed. These results indicated that individual chromosomes 2 were separated from each other and from DMs (which contain amplified N-*myc*) in independent somatic cell clones.

Molecular differentiation of the chromosomes 2. It was important to distinguish each locus of N-*myc* to ensure that we were examining the locus that had been amplified. In order to accomplish this, the probe L2.30 that detects RFLPs at the adjacent 2p25 chromosome band was used to distinguish the chromosomes 2 from N0315L contained in hybrids 6D6 and 9C6. Southern blot analysis (Fig. 4) demonstrated that the parental neuroblastoma line N0315L is heterozygous for this marker (lane 1), as demonstrated by the presence of both the A1 and A2 alleles at 9.0 and 6.3 kilobases (kb), respectively. This probe does not detect sequences in hamster DNA (lane 4) with the hybridization conditions used. DNAs from the two somatic cell hybrids that tested positive for the presence of chromosome 2 (6D6 and 9C6) are shown in lanes 2 and 3. The hybrid 6D6 has one chromosome 2 (allele A2 of L2.30) from N0315L, and the hybrid 9C6 possesses the other chromosome 2 (allele A1 of L2.30). These results indicated that we had isolated each of the chromosomes necessary to examine the original N-*myc* chromosomal locus following gene amplification.

Molecular analysis of the N-*myc* loci. To determine if the N-*myc* gene had been excised from one of the chromosomes 2, *Eco*RI-digested DNA from the chromosome 2-containing clones was probed with the radiolabeled N-*myc* sequence pNB-1. The results of this experiment are shown in Fig. 5A. The amplification of N-*myc* in the parental neuroblastoma cell line N0315L (lane 1) is evident when the band located at 2.0 kb is compared with that obtained from a neuroblastoma, N1003L, that contains two copies of N-*myc* per cell (lane 2). No band was detected in hamster DNA (lane 3) with the hybridization conditions used. DNA from the hybrid 6D6 (lane 4) contains the N-*myc* gene and this indicates that this locus is not excised from the chromosome 2 that contains the A2 allele of L2.30. However, DNA from the hybrid 9C6 (lane 6), which possesses the other chromosome 2 from N0315L, lacked detectable amounts of N-*myc*, suggesting that the gene is absent from this chromosome. DNA from a hybrid, 10D5, that contains a relatively small number of both chromosomes 2 (lane 5), appeared to contain a small but reproducible amount of N-*myc*. The presence of N-*myc* sequences in 6D6 and their absence in 9C6 DNA was reproducible on several Southern blot analyses.

To confirm the presence of human chromosome 2 DNA in each lane that contained somatic hybrid cell DNA, the blot in Fig. 5A was reprobed with the L2.30 sequence. The blot

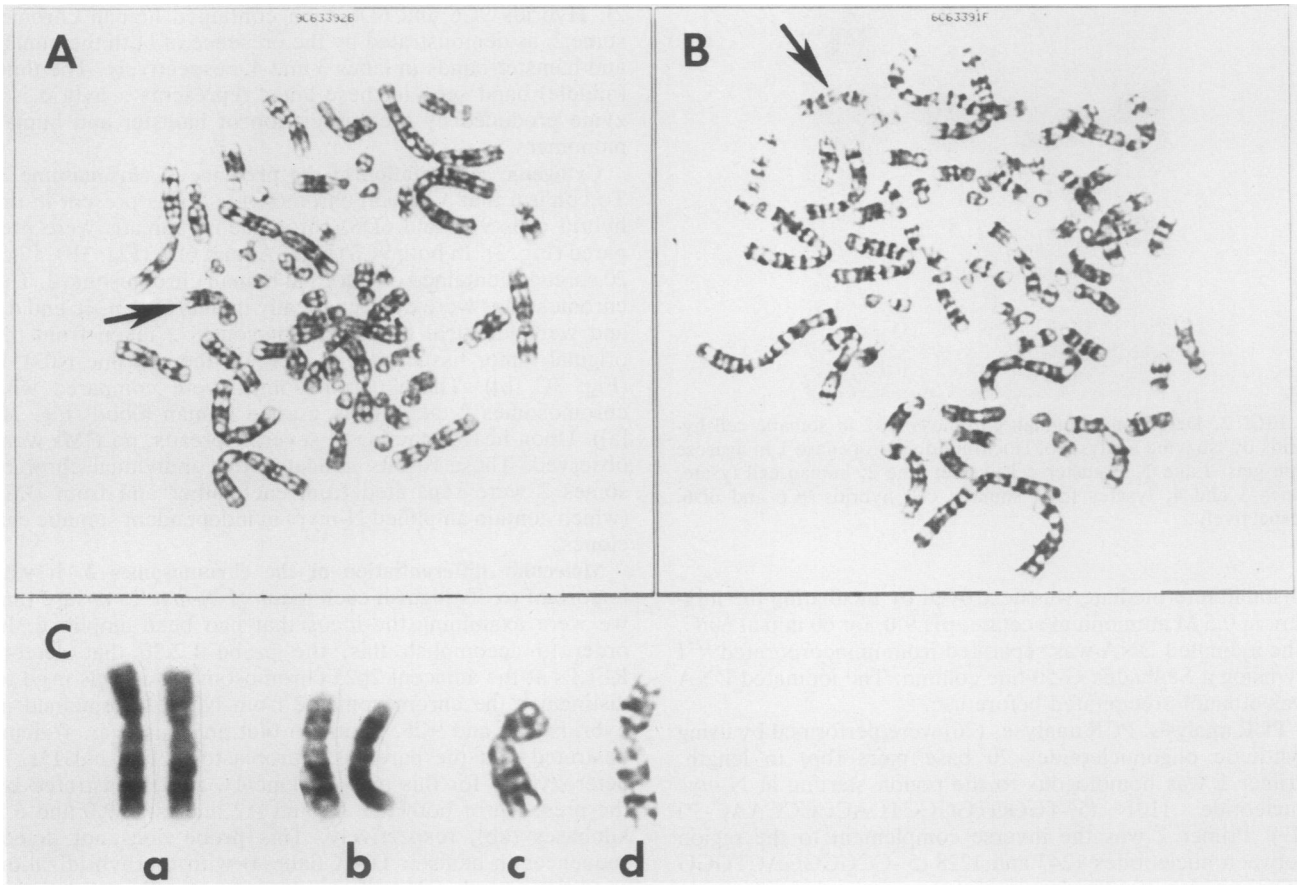

FIG. 3. Chromosome spreads of somatic cell hybrids. Chromosome spreads from hamster-human somatic cell hybrids 9C6 (A) and 6D6 (B) demonstrate that one normal human chromosome 2 (arrow) is present in each hybrid. These chromosomes (C [c and d]) are compared with chromosomes 2 derived from normal human blood (a) and the tumor tissue used to derive the parental cell line N0315L (b).

was not stripped of the N-*myc* probe before being reprobed (to eliminate the unlikely possibility that DNA would be lost from the membrane during the stripping process). The results of this rehybridization are shown in Fig. 5B. A distinct

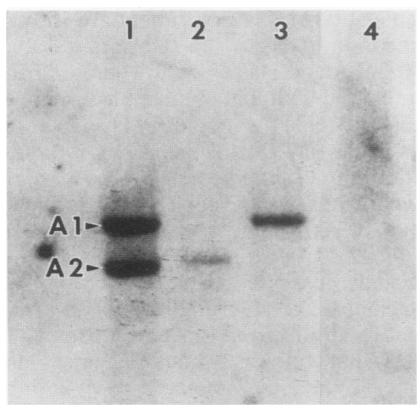

FIG. 4. Identification of individual human chromosomes 2. *Bgl*II-digested DNA from neuroblastoma N0315L (lane 1), somatic cell hybrid 6D6 (lane 2), somatic cell hybrid 9C6 (lane 3), and hamster cell line E36 (lane 4) were electrophoresed, transferred onto a membrane by the method of Southern, and hybridized to the probe L2.30 (specific for an RFLP at 2p25). Allele A1 is 9.0 kb, and A2 is 6.3 kb.

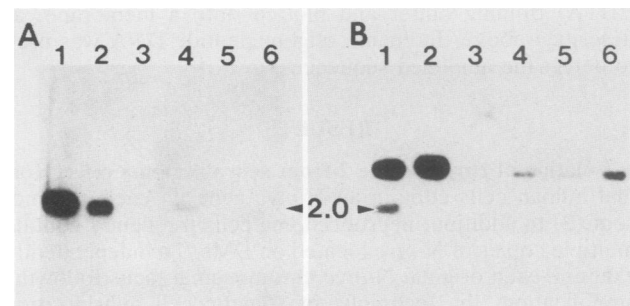

FIG. 5. Genotypic analysis of somatic cell hybrids. (A) Analysis of the N-*myc* locus in somatic cell hybrids by hybridization with N-*myc* probe pNB-1. *Eco*RI-digested DNA from neuroblastomas N0315L (lane 1) and N1003L (lane 2), hamster cell line E36 (lane 3), somatic cell hybrid 6D6, which contains one chromosome 2 (with the A2 allele) from N0315L (lane 4), somatic cell hybrid 10D5, which contains both chromosomes 2 from N0315L in relatively small amounts (lane 5), and somatic cell hybrid 9C6, which contains the other chromosome 2 from N0315L (with the A1 allele) (lane 6). The *N-myc* band is at 2.0 kb. (B) Detection of human chromosome 2 DNA. The filter used to create panel A was rehybridized with L2.30. Lanes 1 to 6 are the same as in panel A. The L2.30 band is at 2.4 kb. The filter was autoradiographed for 14 days after each hybridization.

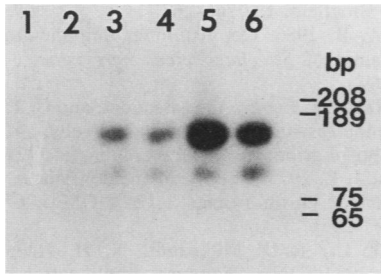

FIG. 6. PCR analysis of hybrids 6D6 and 9C6. DNAs (1 μg) from hamster cell line E36 (lane 1), somatic cell hybrid 9C6 (lane 2), somatic cell hybrid 6D6 (lane 3), 9C6 and 6D6 (1 μg from each) (lane 4), parental neuroblastoma cell line N0315L (lane 5), and plasmid pNB-1 (10 pg) (lane 6) were subjected to PCRs using *Taq* DNA polymerase. The major band corresponds to a 147-bp fragment, and the minor band corresponds to a 90-bp sequence. The blot was autoradiographed for 2 h after hybridization with an iodinated N-*myc*-specific oligonucleotide 40-mer, which does not detect primer DNA.

band can be seen in all three lanes containing the hybrid cell DNA (lanes 4 to 6). Note that L2.30 does not detect an RFLP in *Eco*RI-digested DNA; thus only one size of band is observed in the hybrids. The band in lane 6 of Fig. 5B is significantly more intense than in either lane 4 or 5, indicating that there was more human chromosome 2 DNA from 9C6 present on the filter than from either 6D6 or 10D5. If 9C6 DNA contained N-*myc* sequences, it should have been easily detected in the experiment presented in Fig. 5A.

Confirmation of N-*myc* deletion by PCR analysis. Southern blot analyses demonstrated that the hybrids contained chromosome 2 DNA and suggested that hybrid 6D6, but not 9C6, contained N-*myc* sequences. Because of the presence of less than one human chromosome 2 per cell, a more sensitive technique was needed to confirm the absence of N-*myc* sequences from hybrid 9C6. This was accomplished by amplifying the DNA from hybrids 6D6 and 9C6 using the *Taq* PCR technique (26). The same batch of DNA used in the Southern analyses was amplified in the PCR experiments, ensuring that human chromosome 2 DNA would be present. Two 20-mer oligonucleotide primers were selected that corresponded to N-*myc* sequences within the second exon that were nonhomologous with c-*myc* (Fig. 1C). Amplification of this region would generate a 147-bp sequence.

The results of the PCR analysis are shown in Fig. 6. No amplified sequence was observed in E36 DNA (lane 1), indicating that the hamster N-*myc* sequences are not amplified with the chosen primers. Likewise, no band was detected for 9C6 DNA (lane 2), confirming the results obtained from the Southern blot in Fig. 5A. As expected, a major band at 147 bp was observed for 6D6 DNA (lane 3). To eliminate the possibility that the 9C6 DNA contained an inhibitor of *Taq* polymerase, a 1:1 mix of 9C6 and 6D6 DNA was analyzed (lane 4). The amount of N-*myc* DNA amplified was the same as for the hybrid 6D6, indicating that no inhibitor was present. DNA extracted from the parental cell line N0315L (lane 5) and 10 pg of pNB-1 plasmid DNA (lane 6) served as positive controls for the PCR reactions. The minor band detected at 90 bp may be the result of mismatched alignment of the primers with the N-*myc* sequence. Computer analyses indicated that primer 1 could align with the N-*myc* sequence starting at nucleotide 1146 and primer 2 could align with the sequence at nucleotide 1235. The resulting 90-bp fragment would be detected with the oligonucleotide probe. Alternatively, the secondary structure of the high GC sequence may account for this second minor band. From the results of the isozyme, karyotype, Southern, and PCR analyses, it is evident that the N-*myc* gene is not present on one of the chromosomes 2 from N0315L, which indicates that this locus may be excised during the amplification process.

DISCUSSION

Amplification of specific human genes is a relatively common event, especially in tumor cells. Using the naturally occurring amplification of N-*myc* in human neuroblastoma cells as a representative system, we have tested the validity of models of amplification that propose as the initial step either disproportional DNA replication, which leaves the original locus intact on the chromosome, or rare recombination, which results in excision of the locus that is amplified. By isolating the individual chromosomes in somatic cell hybrids, we were able to independently analyze each homolog of chromosome 2 and demonstrate that the N-*myc* locus was excised from one of these chromosomes. Although it is difficult to prove the loss of a gene, our multipart approach using isozymes, karyology, and RFLP analyses demonstrated that our hybrids contained, in a significant percentage of cells, either one or the other chromosome 2 homolog in approximately equal amounts. By using both Southern blot and PCR analyses on the same material, it is clear that the N-*myc* gene has been lost from at least a vast majority of one of the chromosomes 2.

These results are consistent with and most easily explained by the rare recombination models, which propose that the deletion of the N-*myc* gene is part of the amplification process. However, because we examined the end results of an event and not the actual occurrence, more than one interpretation concerning the mechanism of deletion is possible. The deletion may be independent of the amplification process, occurring randomly during cell growth or as a result of an unstable complex formed by disproportionate replication. While this alternative mechanism cannot be excluded, karyotypic analysis has demonstrated that the chromosomes 2 in the hybrids are identical to the chromosomes from the patient material, and Southern blot analysis has demonstrated the continued presence of a locus one band distal to N-*myc* on both chromosomes 2. Thus, the deletion would have to be submicroscopic. In addition, the parental cells were not cloned before formation of the hybrids or after the initial fusion. If the loss of an N-*myc* locus were to occur during the passage of the parental line, that cell would have to have a growth advantage to predominate. This is counter to the observed correlation of increased proliferation with increased N-*myc* copy number (14, 30, 33, 41). If the deletion occurred after hybrid formation, it would have had to occur in the heterokaryon or we would have detected at least some N-*myc* sequences in the 9C6 hybrid.

Our results are in agreement with those obtained using a transfected CAD gene as an amplification system (3). In this system, a foreign gene was transfected into a cell line and induced to amplify, and then, after a period of time, cells were selected that had lost the amplified sequences. The original locus was also lost, which provided reasonable evidence for a recombination event, but the data were obtained from an artificially derived system that relied on observations of only a few clones. The possibility of nondisjunction and subsequent loss of the chromosome containing

the transfected gene or of the innate instability of a transfected gene in a small population from the cell line could not be eliminated.

Ascertaining whether excision is an integral part of the amplification process is not only important in determining the mechanism of amplification but also may lead to an understanding of other chromosomal abnormalities. With excision as an obligate step in amplification, a subsequent loss of the DMs would lead to a gene deficiency at that locus. Thus, the overexpression or underexpression of a gene may be initiated through similar mechanisms.

Finally, in *Leishmania* spp., excision has been observed in some studies (1) and not in others (27). This creates the impression that more than one mechanism may be used for amplification in lower eucaryotes. Our results provide strong evidence that excision is an integral part of amplification in at least some naturally occurring higher eucaryotic systems, but they do not exclude alternate mechanisms as important elements in other amplification systems. To determine whether additional mechanisms also exist in higher eucaryotes, the technical approach presented here could be easily adapted to other systems.

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