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Characterization of N-*myc* Amplification Units in Human Neuroblastoma Cells

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A set of DNA clones comprising 48 independent *Hin*dIII fragments (215 kilobases of sequence) was derived from the N-*myc* amplification unit of the neuroblastoma cell line NGP. These clones were used to investigate N-*myc* amplification units in NGP cells and 12 primary neuroblastoma tumors. Three parameters were evaluated: (i) the number of rearrangements from germ line configuration that had occurred during the amplification process; (ii) the homogeneity of amplification units within individual tumors; and (iii) the conservation of amplified sequences among different tumors. The results indicated that remarkably few rearrangements had occurred during amplification, that the amplification units within any one tumor were quite homogeneous, and that although each tumor contained a unique pattern of amplified DNA fragments, there was considerable similarity between the amplification units of different tumors. In particular, the amplification units were strikingly similar over a contiguous domain of at least 140 kilobases surrounding the N-*myc* structural gene.

Gene amplification is a potent and common mechanism for increasing the amount of a gene product in a mammalian cell. Such amplification has been shown to occur in mammals in two different contexts. The first, and most well studied, is the amplification of drug resistance genes that occurs in cultured cells in the presence of a variety of cytotoxic agents (12, 28, 34). The second involves amplification of an oncogene in neoplasms in vivo (1). Gene amplification is particularly prevalent in tumors of neuroectodermal origin, occurring in neuroblastomas (4), retinoblastomas (18), glioblastomas (18a, 36b), and small cell carcinomas of the lung (23, 37). In neuroblastomas, amplification of the N-*myc* gene has been shown to correlate with advanced disease stage and poor prognosis (4, 31). Thus, gene amplification occurs in a variety of important biological contexts. Elucidation of the structure and organization of amplification units should provide essential information for eventual understanding of the mechanisms underlying this genetic alteration.

Many studies of cell lines containing amplified drug resistance genes have shown that the amplification unit (amplicon) consists of a 200- to 3,000-kilobase (kb) domain containing the target gene (i.e., the gene whose increased expression confers the resistant phenotype) (12, 28, 34). In such drug-resistant cells, two types of amplicon organization have been observed. One type, described most commonly, consists of amplification units that contain numerous DNA rearrangements compared with the germ line organization (2, 5, 7, 12, 28, 34). Such amplification units are extremely heterogeneous when different cell lines resistant to the same drug are compared; moreover, the amplicons within single cells are often variable, making it difficult to define an amplification unit characteristic of a given cell line. These amplification units are usually associated with cell lines which have been passaged for long periods in the presence of selective agents. They may represent an advanced stage of amplification, and the numerous rearrangements and heterogeneity may be a result of multiple rounds of sister chromatid exchange or recombination or both (12, 28, 34). Conversely, some drug-resistant cell lines contain relatively few rearrangements and are much more homogeneous. This second type of amplification unit organization has been observed during the early stages of amplification (10), but in at least one case persists through later stages (19, 21).

In contrast to the large amount of information available on amplicon organization in drug-resistant cell lines of rodents, there have been relatively few studies on the structural organization of amplicons containing oncogenes (3, 16, 32, 33). In the study reported here, we established numerous clones from the N-*myc* amplification unit of a neuroblastoma tumor to determine several basic structural features of the amplification units in these cells. Our results indicate that N-*myc* amplification units are organized in a way similar to the second type described above, characteristic of early stages of the amplification process. In particular, we found that there were relatively few rearrangements within the N-*myc* amplification unit, that there was significant homogeneity between the amplification units contained in any one tumor, and that there was great similarity between the amplification units in different tumors. The results leading to these conclusions are described below.

MATERIALS AND METHODS

Cosmids, bacteriophage, plasmids, bacterial strains, and genomic DNA. Cosmid pJB8 (13) arms were purchased from Amersham Corp. (Arlington Heights, Ill.). Cosmid pTL5 (35) was obtained from Leroy Hood. Bacteriophage EMBL3 (9) arms were purchased from Vector Cloning Systems (San Diego, Calif.). Plasmid pKB358 (25) was obtained from Keith Backman. *Escherichia coli* K-12 MC1061, KH802 *recA*, CES200, and DB1316 were obtained from Malcolm Casadaban, Fred Blattner, Helmut Sauer, and David Botstein, respectively. High-molecular-weight DNA was prepared from the NGP cell line and primary neuroblastoma tumors by sodium dodecyl sulfate-proteinase K digestion

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and phenol extraction (4, 11). Recombinant cosmid and plasmid DNAs were isolated from minicultures by the boiling method, and recombinant phage DNA was isolated by the plate lysate method (20).

Enzymes and electrophoretic analysis. Restriction endonucleases and DNA ligase were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.) and were used according to the instructions of the manufacturer except that 10 units of restriction endonuclease per μg of DNA was added. Agarose gel electrophoresis was performed on 1% gels with Tris-acetate-EDTA buffer (20) in a vertical apparatus (Hoefer Scientific Instruments, San Francisco, Calif.). Southern transfer to nylon membranes (Nylon Plus; Hoefer) was done by the alkaline technique described by Reed and Mann (24).

Library construction and cloning. Partial *Mbo*I digests of NGP genomic DNA were size selected on 10 to 40% sucrose gradients centrifuged at 25,000 rpm for 20 h at 25°C in an SW27 rotor (Beckman Instruments, Inc., Fullerton, Calif.). Fractions containing 35- to 45-kb fragments were pooled for cosmid libraries, and 18- to 23-kb fragments were pooled for phage libraries. Target DNA fragments were treated with calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) before ligation. In vitro packaging was done with the Gigapack system from Vector Cloning Systems.

Hybridization. Radiolabeled probes were prepared by nick translation or oligo labeling (8). Repeated sequences were removed from the probe by the preannealing method described by Sealey et al. (30). Hybridization was performed at 60°C in 0.5% nonfat dried milk (14)–10% formamide–1% sodium dodecyl sulfate–0.5 mg of heparin per ml–0.2 mg of salmon sperm DNA per ml–0.9 M NaCl–2 mM EDTA–50 mM sodium phosphate, pH 7.0. Posthybridization washing, autoradiography, and densitometric analysis of autoradiographs were performed as described previously (36a).

Cloned sequences surrounding the N-*myc* gene. The cosmid library from NGP cell DNA was first screened with nick-translated NGP genomic DNA to identify a sublibrary of clones which contained amplified DNA sequences. This sublibrary was subsequently screened with pNB-1, a plasmid clone containing N-*myc* sequences (29), to obtain cosmid clones of the N-*myc* gene. Subsequent clones were selected by walking with end fragments, isolated from agarose gels (36) in both the 5′ and 3′ directions. We initially performed the walking experiments with a cosmid library, but were unable to extend the walk beyond 30 to 50 kb from the N-*myc* gene in either the 5′ or 3′ direction. Therefore, subsequent walking experiments employed an EMBL3 phage library of NGP DNA sequences which allowed us to overcome these blocks to cloning. CES200 or DB1316 cells were used to facilitate bacteriophage cloning of sequences which are difficult to clone in standard bacteria such as KH802 *recA*, the strain used as the host for the cosmid library (22).

Fragments were prepared from digests of phage or cosmid clones and used for hybridization to Southern transfers of cloned and genomic NGP DNA. The arrangement of the fragments shown in Fig. 1 was determined by repeated hybridization with individual DNA fragments and overlapping, nonidentical phage and cosmid clones not shown in the map. The DNA fragments shown in Fig. 1 were also detected as fragments of the same molecular weight in *Hin*dIII-digested normal human DNA hybridized with individual fragments, confirming that variations did not occur during cloning.

Randomly selected clones from the NGP amplification unit. Amplified NGP restriction fragments were enriched by subjecting them to in situ gel denaturation and renaturation without S1 nuclease treatment (26, 27). S1 nuclease was eliminated to preserve the sticky ends produced by *Hin*dIII digestion and thus to facilitate subsequent cloning. Fragments of 2.2 to 4.5 kb were electrophoretically eluted from agarose gel slices and subcloned in the positive-selection vector pKB358. This size range was chosen because of the high concentration of amplified fragments (see Fig. 5) and the ease of subcloning fragments of this size. These plasmids were then screened to select those that were amplified in the NGP cell line. Redundant clones were eliminated by restriction mapping and cross-hybridization to each other and to the sequences previously cloned in cosmid and phage vectors.

Denaturation-renaturation gels. Amplified sequences which were conserved among NGP and the other 12 neuroblastomas tested were identified by mixing 0.2 μg of end-labeled *Hin*dIII-digested NGP DNA (tracer) with 9.8 μg of nonradiolabeled *Hin*dIII-digested genomic DNA (driver) from each of the other neuroblastomas before electrophoresis. The DNA fragments were then separated by electrophoresis through a 1% agarose gel (26 cm long) and subjected to denaturation-renaturation treatment as previously described (16, 26).

RESULTS

Two sets of clones were used in this study. Both were obtained from DNA of the neuroblastoma cell line NGP,

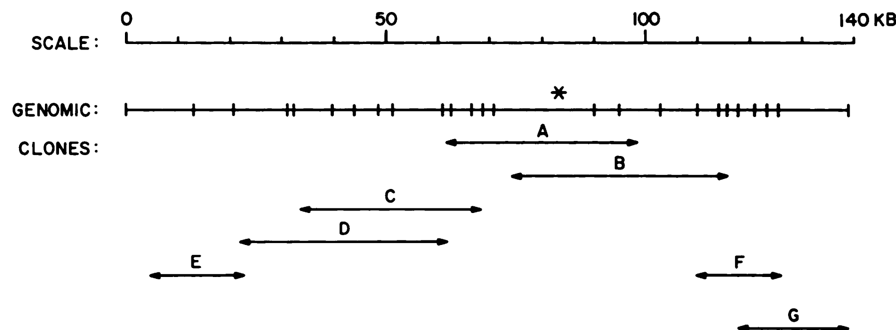

FIG. 1. Sequences surrounding the N-*myc* gene. The map was assembled by aligning genomic *Hin*dIII fragments identified from digests of NGP DNA and cloned in cosmid (A to D) and phage (E to G) clones. The genomic *Hin*dIII fragments detected by each clone are drawn to scale. Regions spanned by each clone are indicated at the bottom. The asterisk notes the fragment which contains the entire N-*myc* structural gene indicated by hybridization to the p9D cDNA probe (17).

which had previously been shown to contain an amplification unit similar to that of many primary neuroblastoma tumors (16). The first set of clones encompassed 140 kb surrounding the N-*myc* structural gene (Fig. 1). These were obtained by walking from the N-*myc* gene in both directions with cosmid and phage vectors. These clones contained 24 contiguous *Hin*dIII fragments. The second set of cloned fragments consisted of 24 plasmid clones containing individual *Hin*dIII inserts ranging from 2.2 to 4.5 kb. These inserts were randomly cloned from the N-*myc* amplicon, using denaturation-renaturation gels to enrich for amplified fragments as described in Materials and Methods. Through hybridization experiments, 24 plasmids from this random set were selected which did not contain significant amounts of repeated sequences and did not contain sequences from within the 140 kb surrounding the N-*myc* gene. Thus, the two sets of clones represented 48 different *Hin*dIII fragments encompassing a total of 215 kb of DNA.

The clones were used as probes in hybridization experiments of genomic DNA from the NGP cell line and 12 primary neuroblastoma tumors containing amplification of N-*myc* genes. The analysis of the amplification units was examined in terms of (i) the number of rearrangements that had occurred during the amplification process, (ii) comparison of amplification units within an individual tumor, and (iii) comparison of amplification units between different tumors.

Number of rearrangements that occurred during amplification. Studies of the amplicons of drug-resistant cell lines have often noted the presence of novel joint fragments (summarized in references 12, 28, and 34). These joint fragments represent rearrangements of germ line sequences which had occurred during amplification. In the N-*myc* amplification units studied here, however, such novel joint fragments were quite uncommon. The *Hin*dIII fragments identified in hybridization experiments to DNA from neuroblastomas (Fig. 2 and 3) were the same sizes as those identified in normal placenta DNA with the following two exceptions. (i) Three probes (cosmids C and D and plasmid 4) detected restriction fragment length polymorphisms (RFLPs), so that different *Hin*dIII fragments were detected in different patients with the same probe. For example, cosmid clone C detected an RFLP with two alleles of either 9.6 or 5.1 and 4.5 kb; cosmid D detected the same polymorphism (Fig. 2). Plasmid clone 4 detected an RFLP with two alleles of 3.4 or 4.7 kb (Fig. 3). These polymorphisms were detected in 65 normal and tumor DNA samples with frequencies of occurrence of 12 to 1 for the 9.6-kb allele compared with the 5.1-kb allele (cosmid C) and 2 to 1 for the 3.4-kb allele compared with the 4.7-kb allele (plasmid 4). Only one polymorphic allele was amplified in any given tumor. These fragments therefore did not represent rearrangements within amplification units but simply represented amplification of sequences normally variable in the population. (ii) A single case (tumor 60) showed an apparent deletion within a 15-kb fragment detected by cosmid clone F resulting in a new fragment of 1.7 kb (Fig. 2). We could not be certain that this deletion did not also represent a polymorphism of the deletion type (6). However, this variation was not found in DNA samples from 44 other individuals (normal DNA from patient 60 was not available for comparison).

Comparison of amplification units within individual tumors. Studies on the amplification units containing drug resistance genes have often indicated that a complicated pattern of amplification units existed within any individual cloned cell line. Heterogeneity of the amplification units within a cell line was indicated by three findings. (i) During walking experiments, clones were found which were identical on one end but diverged at the opposite end, thus defining two separate amplification units (7). (ii) Individual probes which detected one fragment in normal genomic DNA detected more than one amplified fragment in drug-resistant cell lines (2, 5, 7). This also indicated heterogeneity within the amplification units, with the germ line fragment contained in some amplification units and rearranged fragments contained in other amplification units within the same cell line. (iii) Fragments from the amplified region were amplified to different extents in the same cell line (2, 5, 7, 12, 28, 34). This resulted from the fact that some fragments (e.g., the target gene) were present in all the amplification units, while other fragments were present in only a portion of the amplification units.

The results obtained with probes from the N-*myc* amplification unit provided little evidence for heterogeneity. First, more than 100 phage and cosmid clones were obtained from the NGP cell line by the walking procedures outlined in Materials and Methods; all clones could be mapped linearly with respect to the map illustrated in Fig. 1. These clones encompassed over 140 kb of DNA flanking the N-*myc* structural gene.

Second, each of the 48 unique *Hin*dIII fragments identified at most one major amplified fragment in digests of genomic DNA from each of the neuroblastomas and the NGP cell line; thus, there was no indication that any tumor contained two major classes of amplicons (one amplicon containing the germ line fragment and the other containing a rearranged fragment). Even for tumor 60, in which an unusual 1.7-kb fragment was identified with cosmid clone F, the amplification units were homogeneous in that all contained the amplified 1.7-kb fragment and none contained the more common 15-kb fragment (Fig. 2). By overexposing autoradiographs such as those shown in Fig. 2 and 3, it was estimated that we could detect minor fragments present at a level of 15% or more of the fragments actually detected. Thus, if there were amplification units present in the tumors which involved rearrangements within any of the 48 tested *Hin*dIII fragments, these amplification units must have been relatively minor, representing less than 15% of the total number of amplicons in any individual tumor.

Third, all the amplified fragments detected in a given tumor were amplified to the same extent as N-*myc* in that tumor. The extent of amplification of each individual fragment was estimated by comparing the relative intensity of the hybridization signal from tumor DNA with that of the signal obtained with DNA of normal lymphoid cells with the same probe. For example, the N-*myc* gene was amplified 100-fold in the NGP cell line and all 48 *Hin*dIII fragments were amplified to similar extents in NGP DNA (i.e., between 70- and 130-fold, the limits of quantitation obtained with Southern blotting; blots used for quantitation were rehybridized to the N-*myc* probe to control for variation in DNA loading). Similar results were obtained for all the fragments amplified in the other tumors, although the extent of N-*myc* amplification varied from tumor to tumor. The similarity in amplification level was found not only with probes close to the N-*myc* target gene (such as those represented by the cosmid and phage clones) but also in all the random clones, some of which were probably distant from the target gene. Assuming that the N-*myc* gene was contained in every amplification unit in every tumor, these results suggest that all amplified fragments detected with these clones were amplified in the majority of the amplification units within each individual tumor.

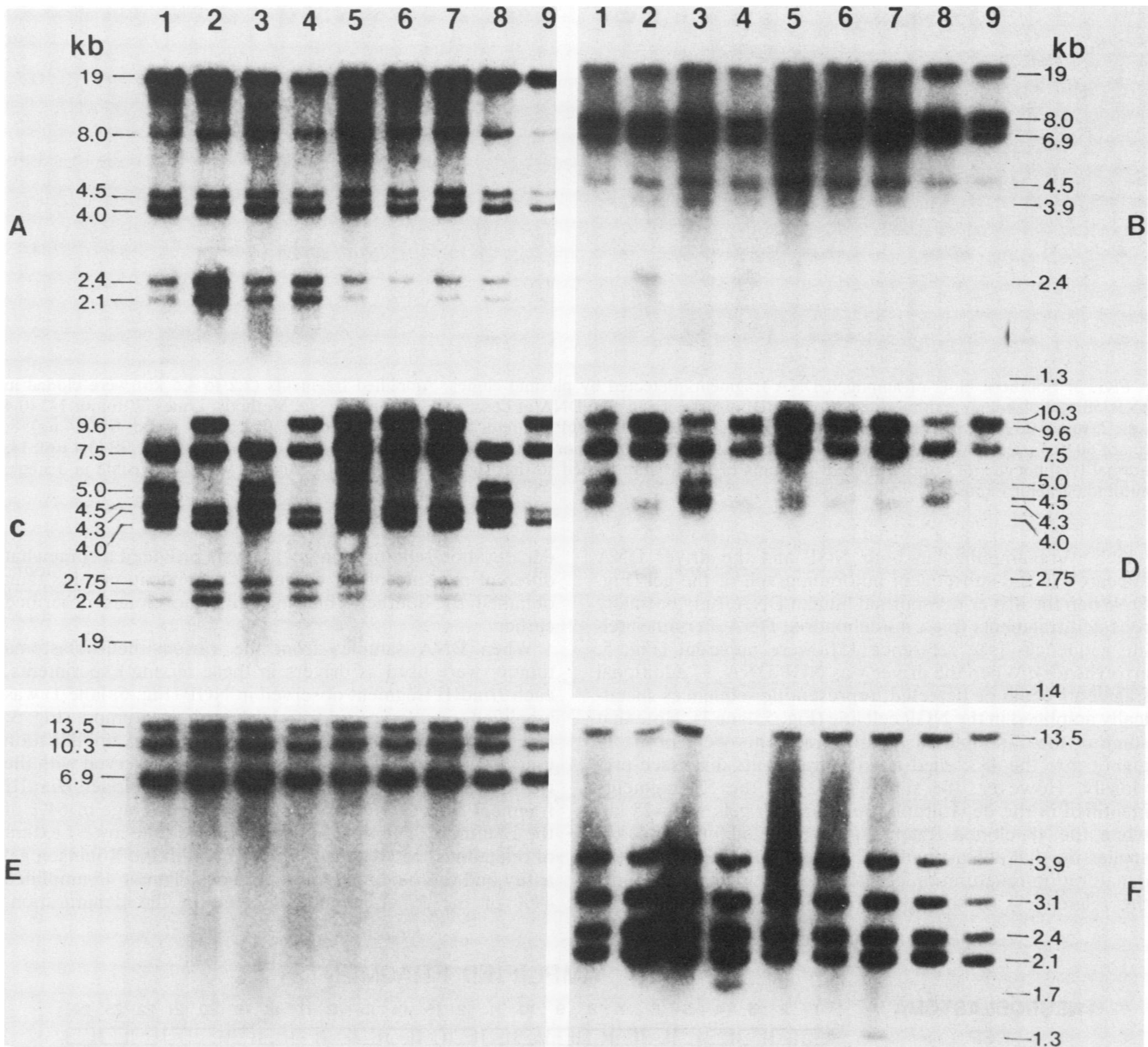

FIG. 2. Southern blots of amplified DNA fragments detected by cosmid and phage clones. Recombinant DNA from each clone (A to F) was radiolabeled and hybridized to *Hin*dIII digests of NGP or neuroblastoma tumor DNA. The clones are mapped in Fig. 1. Lanes: 1, tumor 86 (1.2 μg); 2, tumor 78 (0.8 μg); 3, tumor 64 (1.6 μg); 4, tumor 60 (2 μg); 5, tumor 53 (4 μg); 6, tumor 51 (2.8 μg); 7, tumor 45 (3.2 μg); 8, tumor P-34 (1.2 μg); 9, cell line NGP (2 μg). Different amounts of DNA were loaded so that signals from all DNA samples would be comparable in a single autoradiographic exposure. The size of each fragment is noted to the right or left in kilobase scale. Some smaller fragments (e.g., the 1.9-kb fragment in clone C, the 1.4-kb fragment in clone D, and the 1.3-kb fragment in clone F) cannot be seen in the exposures illustrated. However, all fragments illustrated in the map in Fig. 1 could be visualized on longer exposures.

Comparison of amplification units in different tumors. The fact that each tumor was characterized by a single major amplification unit, as described above, made it possible to compare the units in different tumors. A total of 24 *Hin*dIII fragments within the 140-kb area surrounding the N-*myc* gene were each amplified in all the 12 primary tumors. Thus, this identical region was amplified in all tumors, with the possible exception of tumor 60 as noted above. There was more variation in the randomly cloned fragments outside the 140-kb domain (Fig. 4). Some fragments were amplified in all the tumors (e.g., fragments 1 to 5) and thus showed as much conservation as those fragments within the 140-kb domain surrounding the N-*myc* gene. The least conserved fragments (fragments 16, 18, 19, and 24) were amplified in only 3 of the 12 primary tumors tested. The other 16 fragments were amplified in 4 to 9 of the 12 tumors (Fig. 4).

Another way to assess the conservation of amplified fragments was through mixing experiments, using the denaturation-renaturation gel system developed by Roninson (26). In these experiments, end-labeled *Hin*dIII-cleaved NGP DNA (the tracer) was mixed with DNA from individual neuroblastomas or normal cells (the driver). After electrophoresis, the gel was subjected to denaturation-renaturation and subsequent S1 nuclease treatment; only fragments

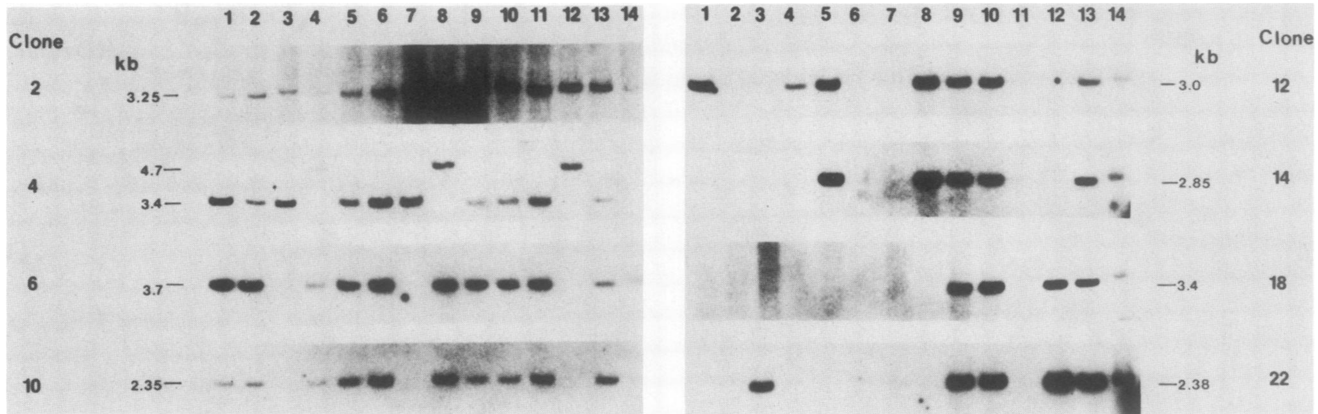

FIG. 3. Southern blots of amplified DNA fragments detected by plasmid clones. Amplified fragments (2.2 to 4.5 kb) were cloned in pKB358, radiolabeled, and hybridized to *Hin*dIII digests of genomic DNA as described in Materials and Methods. Lanes: 1, tumor 142 (0.4 μg); 2, tumor 139 (0.7 μg); 3, tumor 138 (1.8 μg); 4, tumor 119 (0.1 μg); 5, tumor 86 (0.3 μg); 6, tumor 78 (0.2 μg); 7, tumor 64 (0.4 μg); 8, tumor 60 (0.4 μg); 9, tumor 53 (0.9 μg); 10, tumor 51 (0.6 μg); 11, tumor 45 (0.7 μg); 12, tumor P-34 (0.3 μg); 13, cell line NGP (0.4 μg); 14, normal lymphocytes (4.4 μg). Different amounts of DNA were loaded so that signals from all DNA samples would be visible in a single autoradiographic exposure.

which were amplified in both NGP and the driver DNA appeared in the subsequent autoradiograph of the gel (Fig. 5). When the driver was normal human DNA, only normally repeated fragments (e.g., mitochondrial DNA, certain satellite sequences [see reference 15]) were apparent (Fig. 5, arrowheads). When the driver was NGP DNA, 57 additional fragments could be detected, representing sequences specifically amplified in the NGP cell line (Fig. 5, lane 1). Note that some of the 57 amplified *Hin*dIII fragments were probably identical to the 48 cloned *Hin*dIII fragments discussed previously. However, the summed size of the 57 fragments identified in the denaturation-renaturation gels was 425 kb, while the 48 cloned fragments represented only 215 kb, suggesting that at least half of the fragments identified in the denaturation-renaturation gels had not been cloned. Hence, the relatedness of the amplification units as assessed by denaturation-renaturation gel analysis provided a somewhat different measure of relatedness of amplification units than obtained by Southern blotting with the clones described earlier.

When DNA samples from the various neuroblastoma tumors were used as drivers in these mixing experiments, each *Hin*dIII fragment observed in NGP (Fig. 5, lane 1) was amplified, on average, in 66% of the primary tumors (Fig. 5, lanes 2 to 9). The degree of relatedness of these amplification units in the gel analysis was similar to that observed with the random clones depicted in Fig. 3 and 4; each cloned *Hin*dIII fragment from NGP was amplified in an average of 64% of the 12 tumors. Tumors 45 and 64 exhibited the lowest extent of relatedness to NGP as assessed by both the Roninson gel assay and the randomly cloned probes. Tumor 45 amplified 49% of the 57 fragments detected in the denaturation-

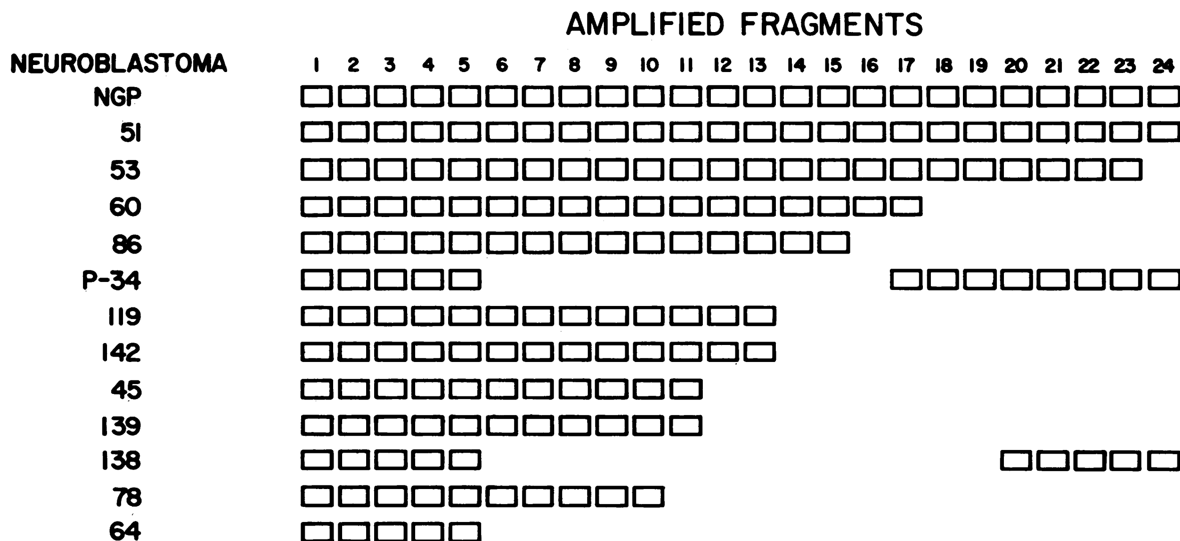

FIG. 4. Relatedness of amplified fragments in different neuroblastomas. The bar graph summarizes the hybridization patterns observed for each of the 24 randomly cloned *Hin*dIII fragments hybridized to neuroblastoma tumor DNA digests as illustrated in Fig. 3. Each box represents the amplification of one unique cloned fragment. This graph does not reflect position within the N-*myc* amplicon or relative size of an amplified fragment. None of the 24 fragments were contained within the region mapped in Fig. 1.

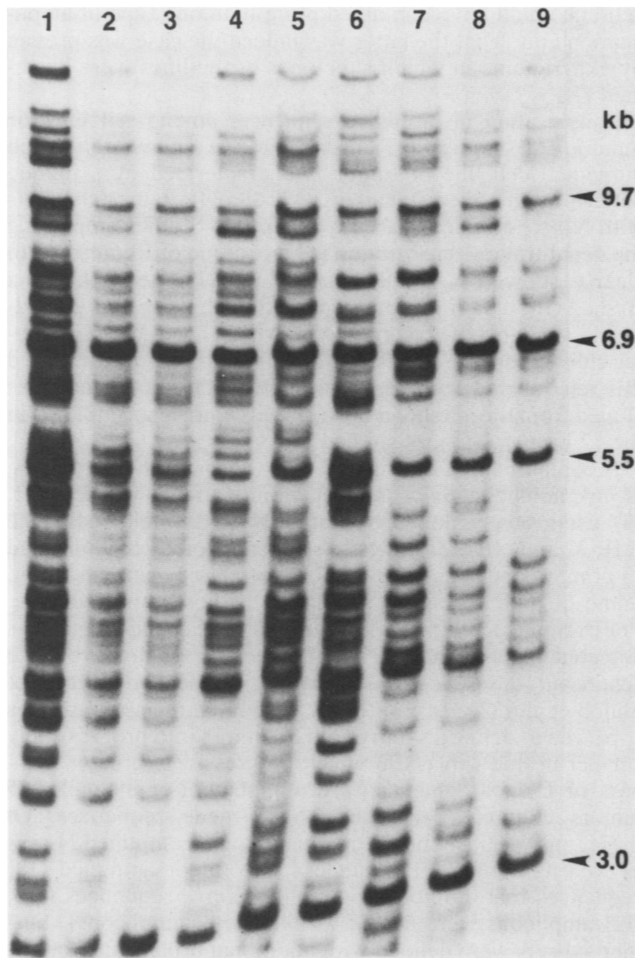

FIG. 5. Conservation of N-*myc* amplification units. Labeled NGP *Hin*dIII DNA fragments (tracer) were mixed with *Hin*dIII DNA fragments from NGP (lane 1) or each of eight neuroblastomas (driver) (lanes 2 to 9). Amplified restriction fragments contained in both NGP and the driver DNA formed hybrid molecules which appear as bands in the autoradiograph. The driver DNAs were as follows: lane 1, NGP; lane 2, tumor 51; lane 3, tumor 53; lane 4, tumor 60; lane 5, tumor 86; lane 6, tumor P-34; lane 7, tumor 78; lane 8, tumor 45, lane 9, tumor 64. The four fragments identified in normal human DNA (i.e., normally repeated sequences) are indicated to the right.

renaturation gels and amplified 46% of the 24 randomly cloned fragments. Tumor 64 amplified 35 and 21% of the fragments, respectively, in these assays. Similarly, the amplicons from tumors 60, 53, and 51 were the most related to NGP as estimated by both hybridization to the cloned fragments and conservation of the 57 amplified fragments observed with denaturation-renaturation gels. The correlation coefficient between the percentage of NGP fragments which appeared to be amplified in individual tumors by using either the gel assay of Roninson (26) (Fig. 5) or the cloned *Hin*dIII fragments as probes (Fig. 4) was statistically significant ($r = 0.84$, $P < 0.01$).

DISCUSSION

Our primary interest in this investigation was to gain information about the basic organization of amplified oncogenes in human tumor cells. Using numerous cloned, amplified sequences as DNA probes in hybridization experiments, we obtained data regarding the rearrangement, heterogeneity, and conservation of N-*myc* amplicons in several primary neuroblastomas.

To facilitate comparison between our results and those obtained by other workers, we converted the data from published reports to a form consistent with our present study (Table 1). To compare these results directly and quantitatively, we first determined the number of different cell lines or tumors examined for amplified DNA sequences for each of the amplicons studied (Table 1, columns A and B). Next, the total size in kilobases of cloned, amplified DNA sequences examined in each study was defined (column C) as the sum of nonoverlapping DNA fragments cloned in plasmid, phage, or cosmid vectors from the amplicon of interest. The total number of kilobases of amplified genomic DNA examined (column D) was calculated as the sum of amplified sequences detected in the genome of the various cell lines with the cloned probes. Thus, if 100 kb of cloned, amplified sequences was amplified in all 10 cell lines, the entry in column D would read "1,000 kb." If one of the cell lines had only amplified half of the cloned 100 kb, the entry in column D would read "950 kb." When the restriction fragment detected by hybridization of a cloned probe to the DNA of a cell line or tumor differed in size from the restriction fragment detected by hybridization to germ line DNA from the parental cell without amplification, this event was scored

TABLE 1. Amplification unit comparisons[a]

Target gene (A)	No. of cell lines or tumors examined (B)	No. of kilobases cloned (C)	Total kilobases amplified with cloned fragments as probes[b] (D)	No. of rearrangements observed[c] (E)	Rearrangements per 1,000 kb amplified[d] (F)	Reference(s)	Notes
dhfr	6	37.2	169	10	59	5	Highly resistant
CAD	12	236.5	1,629	19	11.7	2	Highly resistant
CAD	33	380	12,540	3	0.24	10	First step, low resistance
dhfr	4	150	600	2	3.3	19, 21	Highly resistant
N-*myc*	11	14.2	494	7	14.1	32	11 cell lines
	25	14.2	976	5	5.1	32	25 tumors
N-*myc*	13	215	2,405	1	0.43	Present work	1 cell line, 12 tumors

[a] The table includes only studies in which it was possible to derive the information listed from published values.
[b] Sum of sizes of germ line fragments detected by cloned probes in all cell lines and tumors examined; $D = B \times C$ after correction for germ line fragments not amplified in particular cell lines or tumors.
[c] Restriction fragments not detected in germ line DNA were counted as rearrangements.
[d] $F = (E/D) \times 1{,}000$.

as a DNA rearrangement (column E). For example, the deletion we detected in the present study in tumor 60 using our cosmid clone F (Fig. 2) was scored as a DNA rearrangement. Definite DNA polymorphisms were not considered to be DNA rearrangements. Finally, the frequency of rearrangements was estimated as the number of total rearrangements (column E) per total amplified DNA sequences examined in all cell lines and tumors of each study (column D) normalized to 1,000 kb (column F). These comparisons led to the following conclusions.

Rearrangements. The number of rearrangements detected in the various studies is only meaningful when normalized to the amount of total amplified sequences examined, as rearrangements will be more frequently identified as either the amount of cloned sequences or the number of cell lines examined increases. When normalized in this manner, there are large differences between the frequencies of rearrangements detected in the various studies. Table 1 shows the number of rearrangements per 1,000 kb (Mb) of amplified sequence examined (column F). *dhfr* amplicons in mouse cells (5) and CAD amplicons in highly resistant Chinese hamster cells (2) exhibit a high frequency of rearrangements (59 and 12 per Mb, respectively). *dhfr* amplicons in hamster cells contain fewer rearrangements than those seen in other highly resistant cell lines, for reasons which are not clear (19, 21). The N-*myc* amplicons from primary tumors contain a relatively low incidence of rearrangements (0.4 per Mb in our study and 5.1 per Mb in reference 32). The frequency of rearrangements in N-*myc* amplicons that we observed is similar to that seen in CAD amplicons during the early steps of the amplification process (0.24 per Mb [Table 1] from reference 10). It is possible that N-*myc* amplicons in primary tumors represent an earlier stage in the amplification process than that seen in established cell lines which have been selected through sequential increases in toxic drug concentrations. Neuroblastoma cell lines appeared to have higher frequencies of rearrangements than seen in primary neuroblastoma tumors (14.1 versus 5.1 per Mb [Table 1] from reference 32).

Heterogeneity of amplicons within an individual cell line or tumor. In addition to the relative absence of rearrangements in the N-*myc* amplicons, two other observations suggested homogeneity of amplicons within individual cell lines. First, in walking experiments, all of more than 100 clones we obtained could be mapped linearly without any branch points (joint fragments). In the experiments of Ardeshir et al. (2), Federspiel et al. (7), and others (12, 28, 34), divergent clones were often obtained at sites closely surrounding the target gene, sometimes even within the target gene. We detected no divergent clones within 140 kb of the target gene (N-*myc*) in NGP cells.

Second, we found that all amplified fragments in a given tumor were amplified to the same extent in that tumor. This observation included randomly cloned fragments which were presumably distant from the target gene (as evidenced by their amplification in only a fraction of the tumors). Similarity in the amplification levels of different amplified sequences was also found in the relatively homogeneous *dhfr* amplicons of CHO cells (19) and in early stage CAD amplicons (10). In contrast, individual cell lines with heterogeneous amplicons exhibited "vastly different degrees of amplification of flanking sequences relative to the DHFR gene" (7). There were two possible explanations for this latter finding: there may be subpopulations of cells, each of which has a homogeneous amplicon that differs to some degree from that of other subpopulations; alternatively, each cell line could have contained more than one type of amplification unit. That the latter was indeed the case was proven by experiments in which subclones of the lines were examined (7).

Conservation of amplified sequences among cell lines or tumors. All studies of amplicons have shown that each individual cell line or tumor is characterized by a unique pattern of amplified fragments (12, 28, 34); neuroblastomas with N-*myc* amplicons are no exception. Studies employing the denaturation-renaturation gel technique of Roninson (26) clearly showed a unique pattern in each neuroblastoma studied (16; data not shown), even though there was considerable similarity between the patterns. The studies employing clones from the N-*myc* amplicon reported here confirmed this relationship; an average of 81% of the total sequences cloned from the amplicon of NGP cells were also amplified in the 12 primary tumors studied.

A comparison of our results with those of others studying N-*myc* amplicons is of interest. Shiloh and co-workers (32, 33) used seven clones from the N-*myc* amplicon of the IMR-32 and NB-9 cell lines to study nine other cell lines and 25 primary neuroblastoma tumors with N-*myc* amplification. Some of their results were similar to those reported here. For example, a relatively low number of rearrangements was detected in both studies (Table 1). There were, however, two significant differences between our studies and those of Shiloh et al. (32, 33). First, they noted that the seven probes appeared to define a subdivision of tumors into progressively smaller nested subsets in which progressively greater numbers of DNA fragments were amplified. For example, 25 tumors contained amplified N-*myc* gene sequences. Of these, only a subset of 16 tumors contained amplified N-*myc* as well as probe G21 sequences. No tumor amplified G21 sequences that did not also amplify N-*myc* sequences (i.e., G21 amplicons were nested within N-*myc* amplicons). Further subsets were defined with additional probes until only 1 of the original 25 tumors contained amplified sequences for all seven probes. This is the progressively smaller nested subset phenomenon described by Shiloh et al. (32, 33). Our results showed that as more probes are examined, exceptions to such nesting are obvious (Fig. 4), thus precluding many simple models for the structure of N-*myc* amplicons. Second, the conservation of amplicons that we measured among tumors was significantly greater than that noted by Shiloh et al. (32, 33). In our study, 48 independent restriction fragments cloned from the NGP amplicon were also amplified in an average of 81% (range, 25 to 100%) of 12 tumors; in the study of Shiloh et al., seven independent restriction fragments cloned from the IMR-32 or NB-9 amplicons were amplified in an average of 37% (range, 4 to 100%) of 25 tumors. This difference may be due to the fact that the probes used by Shiloh et al. (33) were largely derived from the IMR-32 cell line, which has an unusual amplification unit composed of sequences joined from widely scattered regions of chromosome 2. In contrast, our cloned probes were derived from the NGP cell line, which was chosen specifically because its amplicon was quite similar to those found in primary tumors (16). Another difference is that our probes were biased toward those that detected sequences surrounding the N-*myc* gene, while those of Shiloh et al. (32, 33) represented random clones that may not have been near the N-*myc* gene. When we consider only the randomly cloned probes that we used (the 24 *Hin*dIII fragments cloned in plasmid vectors, displayed in Fig. 4) the conservation that we found was 64%, still considerably higher than that found by Shiloh et al. Considering the number of probes which we

used and the fact that the results with these cloned probes agreed with those measured with the Roninson (26) gel assay (66%), the higher conservation values observed in the present study are probably more representative of the true level of conservation of N-*myc* domains.

On a practical note, the probes detecting RFLPs within the N-*myc* amplicon may prove useful. The 3.4-kb *Hin*dIII fragment cloned in plasmid clone 4 (Fig. 3 and 4), for example, detects a frequent *Hin*dIII polymorphism. In heterozygotes, DNA from normal tissue exhibits two fragments of exactly equal intensity with this probe. In DNA from tumor tissue, one of the fragments is increased in intensity to a degree reflecting the level of amplification. Hybridizations with this probe to DNA from heterozygotes would present two advantages for quantifying the amount of amplification. First, the hybridization is internally controlled by the signal from the nonamplified allele. Second, the signal-to-noise ratio is increased by a factor of 2, because the amplification is measured against one unamplified fragment per cell instead of two unamplified copies per cell identified when other probes are used in control hybridizations to the same filter. These advantages might prove useful when attempting to detect low levels of amplification in remission bone marrow samples. The sequences detected by clone 4 are amplified in all 12 tumors we have studied so far. Development of additional polymorphisms in the N-*myc* domain is in progress.

In summary, each neuroblastoma tumor contains a unique pattern of amplified fragments. These amplicons appear quite homogeneous within any single tumor, with significant overlap among the amplified sequences in different tumors. The amplicons in neuroblastoma tumors were more like those found in the early steps of CAD gene amplification or in methotrexate-resistant hamster cells than in those found in other established cell lines containing drug resistance genes. These data provide a working model of the N-*myc* amplicon. In particular, it appears that a single major amplicon can be defined for each neuroblastoma tumor, unlike the situation in many drug-resistant cell lines. Cloning of the complete amplicon from one or more of these tumors should further clarify the nature of this important genetic alteration.

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