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Isolation and Structural Analysis of a 1.2-Megabase N-*myc* Amplicon from a Human Neuroblastoma

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Oncogene amplification is observed frequently in human cancers, but little is known about the mechanism of gene amplification or the structure of amplified DNA in tumor cells. We have studied the N-*myc* amplified domain from a representative neuroblastoma cell line, SMS-KAN, and compared the map of the amplicon in this cell line with that seen in normal DNA. The SMS-KAN cell line DNA was cloned into yeast artificial chromosomes (YACs), and clones were identified by screening the YAC library with amplified DNA probes that were obtained previously (B. Zehnbauer, D. Small, G. M. Brodeur, R. Seeger, and B. Vogelstein, Mol. Cell. Biol. 8:522–530, 1988). In addition, YAC clones corresponding to the normal N-*myc* locus on chromosome 2 were obtained by screening two normal human YAC libraries with these probes, and the restriction maps of the two sets of overlapping YACs were compared. Our results suggest that the amplified domain in this cell line is a ~1.2-Mb circular molecule with a head-to-tail configuration, and the physical map of the normal N-*myc* locus generally is conserved in the amplicon. These results provide a physical map of the amplified domain of a neuroblastoma cell line that has de novo amplification of an oncogene. The head-to-tail organization, the general conservation of the normal physical map in the amplicon, and the extrachromosomal location of the amplified DNA are most consistent with the episome formation-plus-segregation mechanism of gene amplification in these tumors.

Gene amplification is one of the mechanisms by which proto-oncogenes may be activated in malignant cells (3, 12, 13, 44). The amplified DNA generally is detectable by cytogenetic analysis either as extrachromosomal double minutes (DMs) or as a homogeneously staining region that is chromosomally integrated (7, 22). The most characteristic examples of oncogene amplification in human tumors include (i) N-*myc* amplification in neuroblastomas (9, 14, 46), (ii) amplification of *HER2/neu*, *Myc*, and fibroblast growth factor-related genes in breast cancer (8, 23, 51, 52, 57), and (iii) *Myc* family gene amplification in small-cell lung cancer (32, 34, 35, 62).

To gain a better understanding of this mechanism of oncogene activation, we have studied the amplified DNA containing the N-*myc* proto-oncogene in human neuroblastomas. This example is particularly important because the N-*myc* oncogene is amplified in 25 to 30% of primary neuroblastomas and 90% of tumor-derived cell lines (9, 10). In addition, N-*myc* appears to be the only oncogene that is amplified in neuroblastomas (9, 10). Finally, N-*myc* amplification is associated strongly with advanced stages of the disease and a poor prognosis (9, 14, 46).

To determine the size and structure of the amplified domain around the N-*myc* oncogene, we constructed a yeast artificial chromosome (YAC) library from a representative neuroblastoma cell line. We have used a series of probes obtained previously from an N-*myc* amplified domain (63) to screen this library and construct a physical map of the region. We also screened two normal YAC libraries to construct a map of the region corresponding to the normal locus on chromosome 2 (band 2p24). Comparison of the two maps indicates that the normal N-*myc* locus generally is preserved in the same linear array in the amplicon, but the amplicon appears to be arranged in a head-to-tail organization and contains at least one region of instability.

MATERIALS AND METHODS

Probes and reagents. Amplicon probes 1 to 24, phage E, and phage F were cloned from the NGP cell line in an earlier study (63). However, probes 5, 10, and 23 could not be revived for this study, and probes 16 and 20 were not used in this analysis because they contained highly repetitive sequences. Probe F1 is a 1.3-kb *Hin*dIII fragment subcloned from phage F, and phage E was used as a probe directly (63). Probes for N-*myc* included pNB-1 (43) and a polymerase chain reaction (PCR) product specific for exon 3 (54). The YAC cloning vector pYAC4 and yeast strain AB1380 (both provided by Maynard V. Olson) were used as the transformation system for these studies (18). The neuroblastoma cell line SMS-KAN (39), which has 150 copies of N-*myc* per haploid genome, was provided by C. Patrick Reynolds. This line was chosen for analysis by YAC cloning because it contained DMs, which are representative of primary tumors (6, 10, 11) and less likely than a homogeneously staining region-bearing cell line to have undergone subsequent rearrangements (60).

Construction and screening of the neuroblastoma YAC library. The neuroblastoma YAC library was constructed as described previously (18). Briefly, high-molecular-weight DNA was prepared from the SMS-KAN neuroblastoma cell

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line. DNA with an average size greater than 900 kb was digested partially with *Eco*RI to produce DNA fragments that averaged 500 kb in size as the insert DNA for YAC cloning. This partially digested DNA was ligated into the pYAC4 vector and transfected into spheroplasted AB1380 yeast cells, which were allowed to grow in a layer of agar under selective conditions as described previously (17, 18, 48). Replicate filters were screened by hybridization with the amplicon probes (63) that were labeled by the random primer labeling method (24).

Isolation of normal YAC clones. YAC clones corresponding to the normal N-*myc* locus were obtained from the Center for Genetics in Medicine (CGM) and the Centre d'Etude du Polymorphisme Humain (CEPH) libraries (2, 17). The latter also is known as the CEPH Mark I YAC library (30). At the time of this study, both centers employed a PCR screening approach coupled with one filter hybridization step (25) to isolate YACs from their libraries. Oligonucleotides for the PCR assays were derived from sequence analysis of various amplicon probes (63).

The oligonucleotides for the locus-specific PCR assays were as follows: F1 (120 bp), 5'ATGGATAACATAATGA CATTATGCC3' and 5'TCCTTAAAAGTTTGCTGGAACT CAC3'; 1 (218 bp), 5'TGTTGGAGGATTACAGTTAAGG AAG3' and 5'TGTTTTGCTCCCACAAGAATGTAAG3'; 2 (236 bp), 5'TACCATCTGCAGTGTACCGTGTTGC3' and 5'GAGGGAAGCCTCACTGGACTTTAGC3'; 3 (322 bp), 5'GTAGTTTGTTTAATAGAGTCTCCCC3' and 5'AAAAA TGTGAGCAACCTTTGTGGCC3'; 4 (140 bp), 5'AAGAAG GACAGCTCAGAAAGACTTG3' and 5'TGCTATTCTGTA GGATGCCTATGTG3'; 7 (231 bp), 5'GAGTAACGCCTTC CCACCTTCAAAG3' and 5'TACTAGCTTATAGTGTTG GAGTCGG3'; 8 (167 bp), 5'AGCTCATGAAGCAGTATC TGTGGCC3' and 5'GTGCTTTCCCCAAATGACCAAGC AG3'; 11 (111 bp), 5'TGAGAATCATTAAGGCCATAGC CAC3' and 5'TCAAACTCGAAGGAACCTTGATCTC3'; 13 (187 bp), 5'ATTCCATTACCCACCTTACAAAGAC3' and 5'TGCAGTGAATGTCATCTATCAACAG3'; NB-1L (150 bp), 5'CAAGATGATTTTTGGGTGAGGACAC3' and 5'AACTATTGTAGATTATGGTGCACTC3'; NB-4L (99 bp), 5'TAACTAATAGATGGTTCCATGACAG3' and 5'CT TCATGGCTAAAAACCATTAAGAC3'; and N-*myc*/exon 3 (1,135 bp), 5'GTATCAGCGTCATACTAAAG3' and 5'GCC GGAAGAGACAGATAAGC3'.

The following protocol was used for essentially all of the PCR assays specified above: heating to 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, renaturation at 55°C for 2 min, and extension at 72°C for 2 min; and extension at 72°C for 4 min. The 10× buffer was used contained 100 mM Tris (pH 8.3), 500 mM KCl, and 15 mM $MgCl_2$.

Characterization of YAC clones. YAC clones were confirmed by colony screening (17) with the respective amplicon probes. DNA was prepared in solution by a crude sucrose gradient procedure (19, 37), while high-molecular-weight DNA was embedded in agarose (20, 45). *Hin*dIII genomic digests were performed on DNA in solution, and Southern blots were hybridized with all of the probes amplified in SMS-KAN. Contour-clamped homogeneous electric field (CHEF) gel electrophoresis (21) was used to separate the yeast chromosomes embedded in agarose plugs to determine the size of the YAC. HY1150 is a YAC clone (obtained from David D. Chaplin) that contains a 160-kb insert from the human HLA locus, and this YAC was used as control for nonspecific human DNA.

YAC end isolation. YAC end clones were isolated by using a modification of the bubble-vectorette, PCR-based approach (40). The YAC clone was digested with either *Rsa*I or *Hin*fI and ligated to a vectorette containing an extensive internal region of noncomplementary DNA. This fragment was amplified by PCR with a pYAC4-specific primer and a vectorette-specific primer, and the PCR product was subcloned and sequenced. PCR primers used to screen the YAC libraries were chosen from the insert-specific sequence, using the PRIMER program (31).

Rare-cutting restriction analysis and CHEF gel electrophoresis. DNA in agarose plugs was digested overnight in 500 μl of 1× restriction buffer plus 50 U of *Bss*HII or *Not*I (New England BioLabs) at the recommended temperature. The plugs were loaded onto a 0.8% pulsed-field gel electrophoresis agarose gel (Boehringer Mannheim) and run on the CHEF gel apparatus at 6 V/cm for 20 h. The ramped switching time for the *Bss*HII digests started at 6 s and proceeded to 12 s, while the time for *Not*I digests began at 15 s and ended at 30 s. The DNA was transferred to ONCOR filters and hybridized with the various amplicon probes (63) as described above.

RESULTS

YAC clones from the N-*myc* amplicon. Twenty YACs were identified by using either N-*myc* or the amplicon probes described above to screen the neuroblastoma YAC library. In essentially all cases, the size of the band identified in these YACs was the same as the size of the *Hin*dIII fragment used as a probe (data not shown). On the basis of this pattern of probe hybridization, as well as the pattern of digestion with the rare-cutting restriction enzymes *Bss*HII (Fig. 1) and *Not*I (data not shown), the YACs could be arranged into two subsets of contiguous, overlapping clones (contigs) (Fig. 2A).

The first contig spans ~550 kb and contains the N-*myc* oncogene as well as a substantial amount of flanking DNA. The loci included in this contig are F1, N-*myc*, E, 2, and 3. The second contig spans ~700 kb and is located 3' of the N-*myc* gene relative to its transcriptional orientation. The probes found within this contig include (in order) 1, 4, 7, 9, 6, 8, 11, 13, and 12 (Fig. 2A). The YAC clone yNB-8 spans the gap between the two contigs, but it appears to contain an internal deletion (see below), which has been documented previously in YACs (2, 15, 16). Alternatively, yNB-8 may be representative of a subset of amplicons in this cell line that contain this deletion, or this region may be unstable in a yeast genetic background.

In general, the internal consistency of the maps of the overlapping contigs is quite striking. However, yNB-2 contains a *Bss*HII site not found in three overlapping clones (yNB-1, yNB-3, and yNB-4). Similarly, yNB-6 lacked a *Bss*HII site found in several other YACs in the region. Therefore, these YACs may be chimeric, or they may be representative of a subset of amplicons that differ from the majority of the YACs isolated. No other obvious chimeric neuroblastoma YAC clones were identified, but it is possible that additional chimeric YACs with small amounts of noncontiguous DNA are present. YAC yNB-3 was found to be circular, as judged from its aberrant electrophoretic mobility as well as the pattern of restriction digestion and probe hybridization (to be described elsewhere).

YAC clones from the normal 2p24 locus flanking the N-*myc* proto-oncogene. Of the 19 YACs isolated, 11 were isolated from the CGM library (yWNL YACs) and 8 were isolated from the CEPH library (yCNL YACs). The map of the combined clones is shown in Fig. 2B. As in the neuroblas-

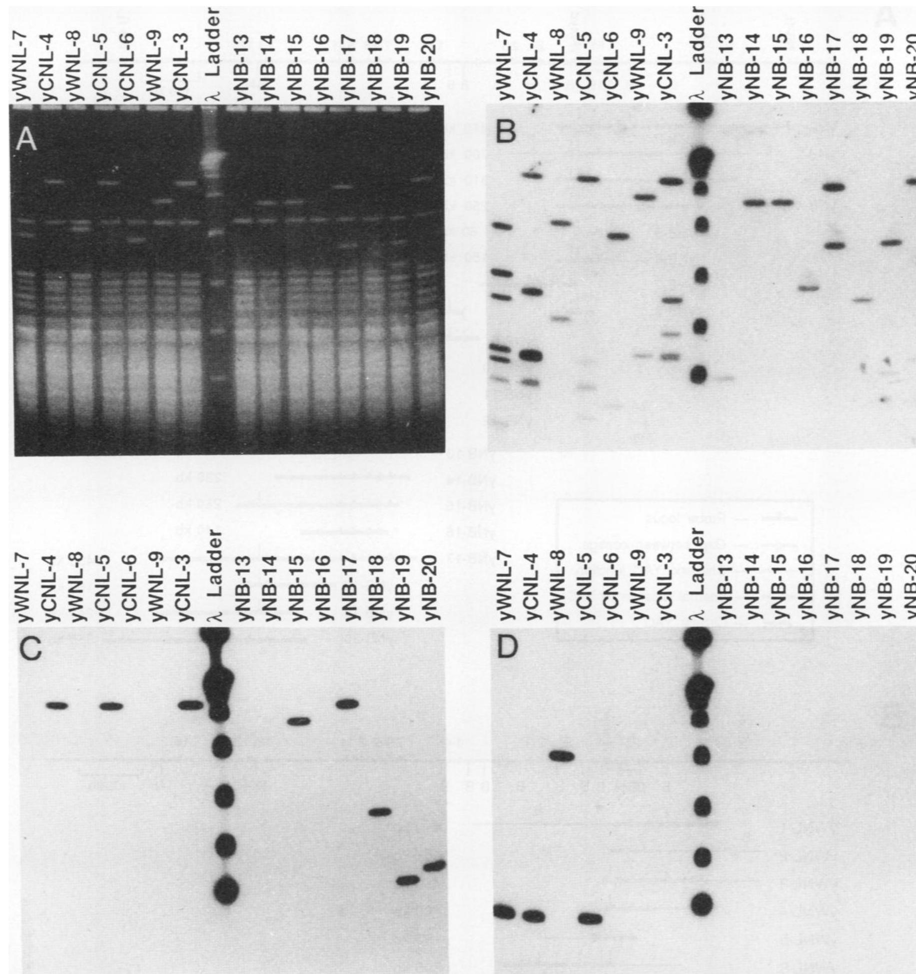

FIG. 1. *Bss*HII digests of normal and neuroblastoma YACs. (A) Ethidium bromide-stained CHEF gel of various YAC DNAs digested with *Bss*HII. (B through D) Hybridizations to the digested YAC DNAs with the following probes: SMS-KAN genomic DNA (yWNL-7 was cotransformed, accounting for extra bands) (B), probe 13 (C), and probe 4 (D). The center lane is a ladder of lambda DNA concatemers in integer multiples of 48.5 kb.

toma map, two YAC contigs were isolated in this region. The first contig spans ~400 kb and contains the N-*myc* gene as well as probes F1, E, 2, and 3. The extent of the map represented in the contig does not include the entire length of the clones isolated, but only the DNA that appears to be specific for the 2p24 locus. All of the eight YACs isolated in this contig were thought to be chimeric because their *Bss*HII restriction maps and/or their patterns of probe hybridization were inconsistent with two or more overlapping YACs. In addition, some isolates of yCNL-1 were unstable (see below). The second contig in the normal YAC map covers ~650 kb of DNA and includes probes 1, 4, 7, 9, 6, 8, 11, 13, 12, 14, and 15, in that order (Fig. 2B). Seven of the eleven YACs isolated in this region were presumed to be chimeric for the same reasons as cited above.

Isolation and characterization of the 5′ ends of yNB-1 and yNB-4. The 5′ end of yNB-1, a neuroblastoma-derived YAC, was isolated by using a modification of the bubble-PCR approach (40). The PCR primers derived from the insert-specific sequence of this end clone detected the same-size PCR product in YAC yNB-1 and in SMS-KAN DNA (Fig. 3A) as well as in chromosome 2-specific hybrid DNA and normal human DNA (data not shown). PCR assays then were performed on a representative panel of YAC DNAs from both the neuroblastoma and normal YAC libraries. Only yNB-1 and yNB-20 gave the appropriate PCR product (Fig. 3A). No PCR product was seen with this set of primers in any of the normal YACs. These data suggested that the 5′ end of yNB-1 resides 5′ to the most distal region of the normal map and that yNB-20 contains sequences derived from this region. This finding was confirmed by hybridization of the PCR product from the 5′ end of yNB-1 (NB-1L) to the same set of representative YACs spanning both the normal and neuroblastoma maps (Fig. 3B). This probe hybridized to a 4.6-kb *Hin*dIII fragment that was amplified in SMS-KAN. As expected, a unique 1.8-kb fragment hybridized in yNB-1. yNB-20 showed hybridization to a 4.6-kb fragment as well as to a 3.2-kb fragment, suggesting that a rearrangement had occurred in yNB-20 involving this locus. No other YACs hybridized with the NB-1L probe.

The 5′ end of neuroblastoma YAC yNB-4 was isolated by using an approach identical to that described above, and the PCR product obtained from the sequence of this end was used as a probe (NB-4L) against blots of the normal and

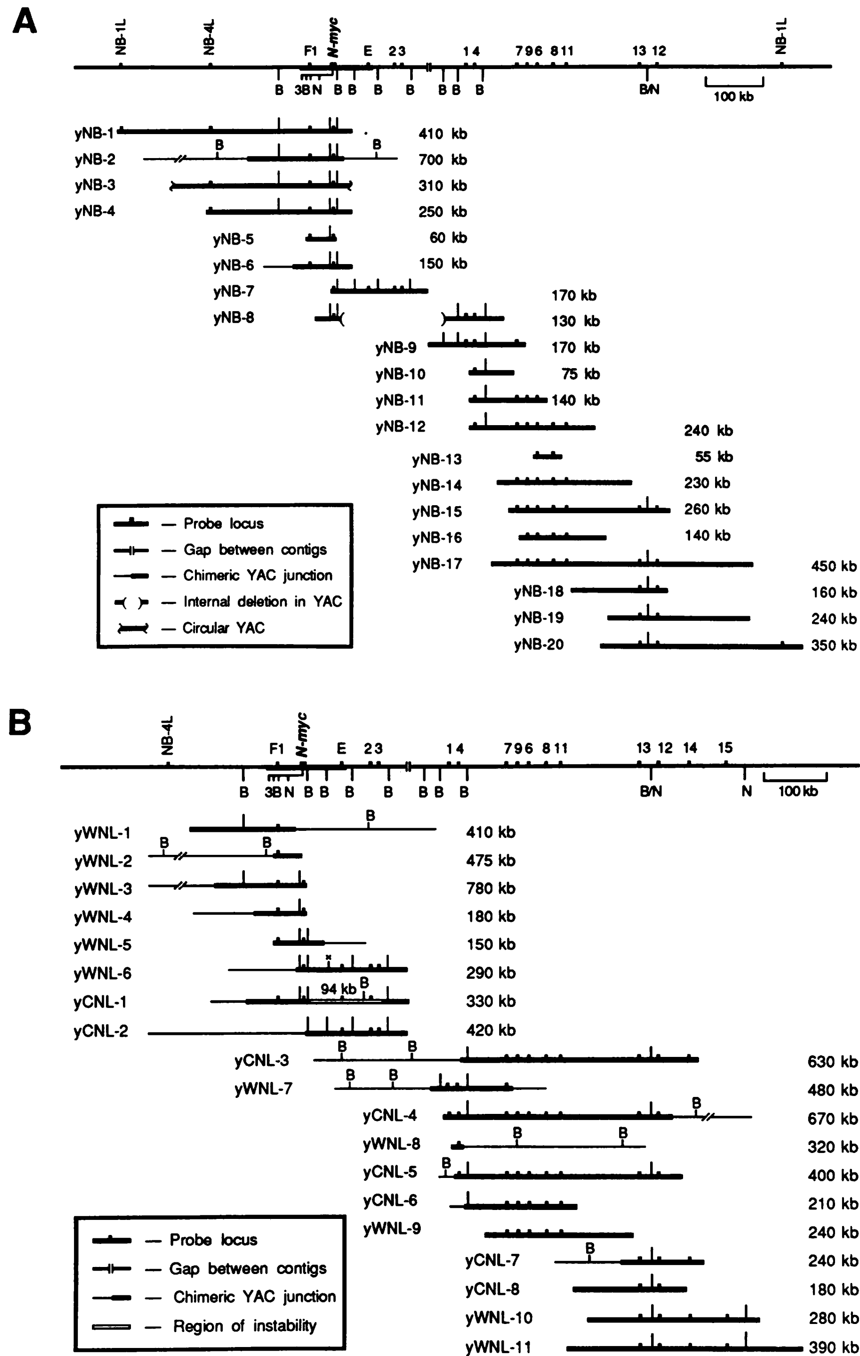

FIG. 2. (A) YAC map of the N-*myc* amplicon. Twenty YAC clones that span the N-*myc* amplicon in the neuroblastoma cell line SMS-KAN are depicted. Amplicon probes are shown above the top horizontal line. The rare-cutting restriction enzyme sites indicated below the map line by vertical lines are *Bss*HII (B) and *Not*I (N). The cluster of rare-cutting restriction sites in and immediately adjacent to the N-*myc* gene is shown below the gene locus. The 140-kb region around the N-*myc* gene mapped previously (63) is shown with a thicker line. *Bss*HII sites that are unique to individual YACs (presumably in chimeric regions) are indicated on the respective region of each YAC. All other symbols are indicated in the box. (B) YAC map of the normal N-*myc* locus. Nineteen YACs that span the normal N-*myc* locus at 2p24 are depicted. All symbols are the same as in the YAC map of the neuroblastoma amplicon except for yWNL-6, which has a missing *Bss*HII site in an otherwise conserved region shown with an asterisk. The following are official designations for the CEPH YACS: yCNL-1 = 223D12; yCNL-2 = 252E4; yCNL-3 = 305B2; yCNL-4 = 217D9; yCNL-5 = 27F6; yCNL-6 = 388F3; yCNL-7 = 303B11; and yCNL-8 = 83H11.

neuroblastoma YACs (data not shown). NB-4L hybridized to yNB-1, yNB-3, and yNB-4 but not to yNB-2, which is thought to be chimeric (see above). None of the normal YACs hybridized with this probe, confirming that this locus is 5′ of the region represented in the normal YACs.

Comparison of the two YAC maps suggests a head-to-tail junction in the amplicon. Comparison of the *Bss*HII and *Not*I restriction maps and patterns of probe hybridization between the amplicon-containing YACs and the normal YACs reveals striking similarity. All of the *Bss*HII and *Not*I sites

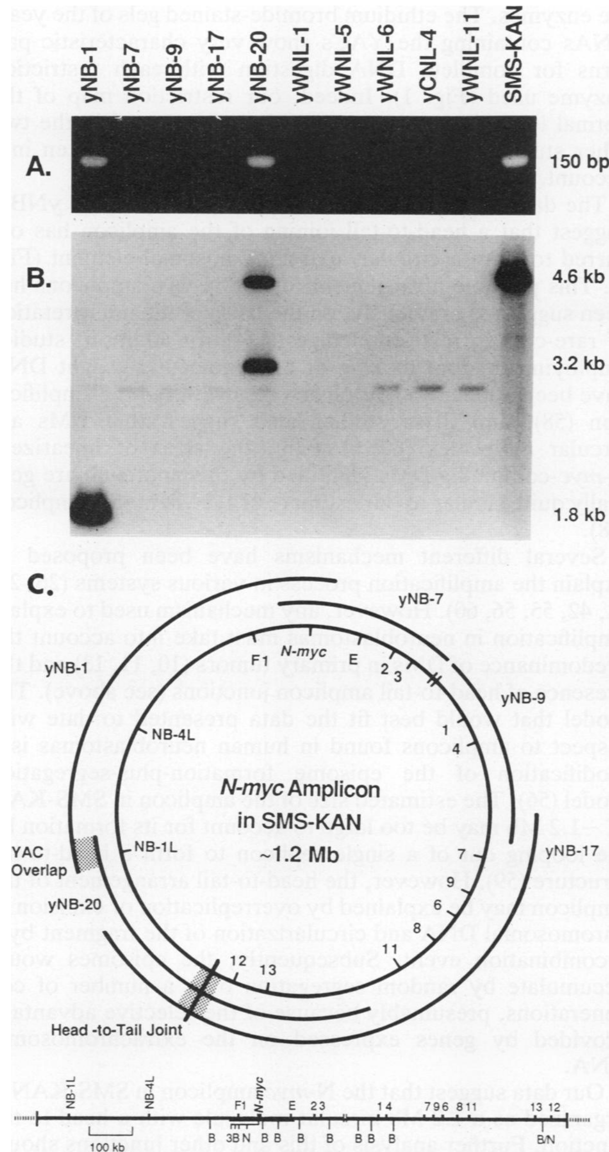

FIG. 3. Evidence for head-to-tail circular structure of the SMS-KAN amplicon. (A) PCR assay with NB-1L primers. Primers derived from the 5′ end of yNB-1 were used to assay neuroblastoma and normal YAC DNAs as well as SMS-KAN DNA. (B) Pattern of hybridization of the NB-1L PCR product to a set of representative neuroblastoma and normal YACs digested with *Hin*dIII, confirming the pattern seen by PCR analysis. An extra band was seen in yNB-20 DNA, presumably representing a rearrangement involving this locus. (C) Structure of the N-*myc* amplicon. The region of overlap between yNB-1 and yNB-20 is represented by the shaded area. The location of the head-to-tail joint is indicated by a bar, although the precise location of the joint could be within 25 kb on either side of this bar (shaded). The linear map of the region (from Fig. 2A) is shown below.

contained consistently in the normal map were found also in the neuroblastoma amplicon map. However, the normal map contained probes 14 and 15, which were not amplified in SMS-KAN. In addition, the normal map contained one *Not*I site 3′ of probes 14 and 15 that was not found in the map of SMS-KAN. Thus, the region between probes 12 and 14 in the normal map probably contained the 3′ end of the amplicon in this cell line. The neuroblastoma YAC yNB-20, as well as yNB-17 and yNB-19, extended beyond this 3′ junction identified in the normal map, but they did not contain probe 14 or 15. In addition, yNB-20 contained DNA derived from the 5′ end of yNB-1 (see above). Furthermore, the 5′ end of yNB-1 hybridized to a 275-kb *Bss*HII fragment in yNB-1 (which contains the 5′ end) and a 270-kb *Bss*HII fragment of yNB-20 (which contains the 3′ end). These data suggested that YACs yNB-1 and yNB-20 overlapped and that YACs yNB-17, -19, and -20 contained a head-to-tail junction fragment between the 5′ end and the 3′ end of an N-*myc* amplicon (Fig. 3C) or two adjacent amplicons in a head-to-tail orientation.

Region of instability 3′ of N-*myc*. Another similarity between the two maps was the region of apparent instability between N-*myc* and probe 1 (Fig. 2 and 3). One YAC colony (yCNL-1) with four different subclones was isolated from the normal library, and the region between N-*myc* and probe 2 was unstable (Fig. 2B). Furthermore, one YAC from the neuroblastoma library, yNB-8, appeared to contain a stable internal deletion in the same region, and this was the only YAC isolated from all three libraries that spanned the region from N-*myc* to probe 1. Thus, this region appeared to be difficult to clone in the YAC cloning system, and it was inherently unstable when cloned. Two stable clones from this region, one from the neuroblastoma library and one from the normal library (yNB-7 and yCNL-2, respectively), had the same *Bss*HII restriction map, and YAC yWNL-6 from the normal library shared two of the three internal *Bss*HII sites with these two YACs. Thus, it is likely that yNB-7 and yCNL-2 accurately represent the map of N-*myc*-flanking DNA in this region.

Defining the SMS-KAN amplicon. The consensus amplicon isolated from SMS-KAN spanned about 1.2 Mb. The 3′ end of the amplicon appeared to reside within a 50-kb region of DNA between probes 12 and 14 in the normal map, which is about 700 kb downstream from the N-*myc* gene. The 5′ end of the amplicon has not been identified in the corresponding region of the normal DNA, but it appeared to be about 500 kb upstream of the N-*myc* gene. This region included all of YAC yNB-1 that is 5′ of N-*myc* as well as the 3′ end of YAC yNB-20 distal to probe 12 (as it is oriented in the linear array of Fig. 2A). The pattern of hybridization with various amplicon probes (63) indicated that this 1.2-Mb amplicon from SMS-KAN was of intermediate size and appeared to include the core region amplified in most neuroblastomas.

DISCUSSION

We have used YAC cloning technology to isolate the amplicon containing N-*myc* from a representative, DM-containing neuroblastoma cell line. Similarly, we have cloned the corresponding normal locus of chromosome 2 (band 2p24) from two normal YAC libraries. Our structural analysis suggests that the amplicon is ~1.2 Mb in size and is organized as a circular molecule with a head-to-tail joint. We presume that each DM contains a single amplicon, although our data are equally consistent with two or more direct repeats of the amplified domain joined in a head-to-tail fashion. We found no evidence for head-to-head or tail-to-tail junctions, but the possibility exists that such junctions occur in this or other neuroblastomas. The internal consistency of the neuroblastoma YAC map and the general correspondence of the amplicon map with the map from the normal locus suggest that few gross rearrangements are present.

These results extend those of two previous studies (29, 63), which have shown that the amplification units containing N-*myc* are conserved among different neuroblastomas, that relatively few rearrangements had occurred during amplification, and that the amplification units within any one tumor were quite homogeneous. The amplification units in one of the studies (63) were found to be strikingly similar over a contiguous region of at least 140 kb surrounding the N-*myc* structural gene. We have reached similar conclusions in our analysis of a 1.2-Mb amplicon. However, our preliminary, fine-structure analysis of this region in SMS-KAN indicates that there are areas of apparent instability or rearrangement (data not shown), and there may be subsets of amplicons that differ from the consensus amplicon shown in this study.

Other groups have analyzed the amplified domain containing N-*myc* in neuroblastomas by using probes derived from the neuroblastoma cell line IMR-32 (27, 49, 50). They showed that the probes detected different patterns of DNA amplification that could be arranged into nested subsets. In addition, they detected rearrangements of the amplified DNA rather frequently and suggested that extensive rearrangements take place during the amplification process. In many cases, the rearranged bands were of lesser intensity than the amplified band commonly seen, suggesting that rearrangements may have occurred in minor subsets of amplicons. Nevertheless, their data suggest that a substantial region of DNA is amplified in many neuroblastomas and that the process of amplification may involve, or lead to, frequent rearrangement.

Another group has cloned regions of the N-*myc* amplicon from a neuroblastoma cell line in lambda phage; they arranged contigs of 25 to 60 kb each, which together comprise about 330 kb of the amplified domain (1). They concluded that rearrangements occurred, leading to subsets of amplicons that have sequence homogeneity. Other groups have physically mapped the DNA surrounding N-*myc* in normal lymphocytes and in neuroblastoma cell lines, using various rare-cutting restriction enzymes and pulsed-field gel electrophoresis (4, 5, 28, 61). They concluded that rearrangements were common in the amplified DNA from these neuroblastoma cell lines.

Clearly, some degree of rearrangement of the N-*myc* amplicon has been identified in all of the studies mentioned above as well as in our own. However, it is less clear whether the extent of rearrangements identified in some of the studies of neuroblastoma cell lines is representative of what occurs de novo in primary tumors with N-*myc* amplification. Indeed, some differences in the rare-cutting restriction patterns of different tissues seen in these studies could be due to differences in tissue-specific methylation patterns or to technical problems in digesting DNA embedded in agarose. In addition, homogeneously staining regions, which are the predominant form of amplified DNA in neuroblastoma cell lines (9, 10, 13), are more likely to have undergone secondary rearrangements (as a result of chromosomal integration) that are not representative of the original amplification event. Finally, secondary rearrangements are more likely to have occurred in continuously growing cell lines than in primary tumors. Thus, these possible confounding factors need to be taken into consideration in the interpretation of the data described above.

The rare-cutting restriction data presented here were based on digests of YAC DNAs derived from a yeast background, in which methylation of cytosines does not occur (38), so all rare-cutting restriction sites are available to the enzymes. The ethidium bromide-stained gels of the yeast DNAs containing the YACs show very characteristic patterns for complete DNA digestion with each restriction enzyme used (Fig. 1). Indeed, our restriction map of the normal locus is consistent with the maps found in the two other studies (4, 28), if incomplete digestion is taken into account.

The data obtained with use of the 5′ end of YAC yNB-1 suggest that a head-to-tail joining of the amplicon has occurred to form a circular, extrachromosomal element (Fig. 3). This possible arrangement for the N-*myc* amplicons has been suggested previously, on the basis of the interpretation of rare-cutting restriction digests (4). In addition, studies employing random nicking of high-molecular-weight DNA have been applied to neuroblastomas with N-*myc* amplification (58), and these studies also suggest that DMs are circular molecules (60). Finally, the sizes of linearized, N-*myc*-containing DMs identified by this approach are generally quite similar to our estimate of 1.2 Mb in this amplicon (58).

Several different mechanisms have been proposed to explain the amplification process in various systems (26, 27, 41, 42, 55, 56, 60). However, any mechanism used to explain amplification in neuroblastomas must take into account the predominance of DMs in primary tumors (10, 11, 13) and the presence of head-to-tail amplicon junctions (see above). The model that would best fit the data presented to date with respect to amplicons found in human neuroblastomas is a modification of the episome formation-plus-segregation model (56). The estimated size of the amplicon in SMS-KAN of ~1.2 Mb may be too large to account for its formation by the looping out of a single replicon to form a head-to-tail structure (59). However, the head-to-tail arrangement of the amplicon may be explained by overreplication or excision of chromosomal DNA and circularization of the fragment by a recombination event. Subsequently, the episomes would accumulate by random segregation over a number of cell generations, presumably because of the selective advantage provided by genes expressed on the extrachromosomal DNA.

Our data suggest that the N-*myc* amplicon in SMS-KAN is organized as a 1.2-Mb circular molecule with a head-to-tail junction. Further analysis of this and other junctions should allow us to determine whether there are characteristic features of the sequences that are involved in these recombination events, which may provide insight into the mechanism of amplification in these tumors. Given the large size of this and other N-*myc* amplicons (29, 63), it is likely that other expressed sequences are coamplified in neuroblastomas. Indeed, increased N-*myc* expression levels in tumors without N-*myc* amplification does not correlate with aggressive clinical behavior (33, 36, 47, 53). Thus, it is possible that other expressed sequences in the region flanking the N-*myc* proto-oncogene contribute to the aggressive phenotype associated with amplified tumors.

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