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Megabase-scale analysis of the origin of N-myc amplicons in human neuroblastomas

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ABSTRACT

In order to elucidate the initiation of the N-myc gene amplification, we have analyzed the original structures of the N-myc amplicons among 38 human neuroblastomas. Nineteen DNAs isolated from the N-myc amplicons recognized a continuous stretch totally encompassing a 5.5 megabase region spanning the normal N-myc gene. The co-amplification profiles with these DNAs showed that two of them, which mapped into a 300 kb region flanking the N-myc gene, were commonly amplified in most specimens, while others were differentially amplified among various subsets. These profiles enabled us to divide the N-myc amplicons into several groups and outline their original domains as a continuous stretches, pointing to the existence of 'consensus sites' for the ends of the initial domains in the original region. In one cell line, the domain was found to be several times larger than that of the derivative amplicon; and the rearranged sites identified within the amplicons, which showed no site specificity, were consistent with those deduced from the domain structure. These results lead to a model in which N-myc gene amplification is initiated at some consensus sites by a preferential mechanism and followed by a random loss of the domain structures during subsequent stages.

INTRODUCTION

In human neuroblastomas, the N-myc gene is often rearranged and amplified, and it is manifested as double minute chromosomes (DM) and/or a homogeneously staining region (HSR) (1, 2, 3, 4). The amplification of the N-myc gene is correlated with the progressive stages and poor prognosis of the disease (5, 6). To gain a better understanding of the correlation between this phenomenon and the genesis and/or progression of neuroblastomas, cytogenetic and molecular analyses of the mechanism underlying N-myc amplification have been performed. Chromosomal analyses show that the amplified N-myc gene is found at sites other than the original locus (7, 8), and that one of the N-myc alleles is deleted at the original site (9). Analyses of the structural organization of the amplified DNA show that the amplified region is organized as a tandem repeat of amplicons, called 'N-myc amplicons', each of which contains the N-myc gene along with flanking sequences ranging up to several hundred kilobases in size. This is a consequence of complex rearrangements (8, 10, 11, 12, 13, 14, 15, 16, 17). These studies lead us to consider the N-myc gene amplification in two contexts: one is rearrangements in the initial stage, that is, either over-replication or excision, and the other is rearrangements during subsequent stages. In order to draw a complete picture of the whole process of N-myc amplification, more evidence needs to be accumulated, and special attention needs to be paid to the initial events. Even if the original locus is in fact excised from the chromosome, little is known about the initial site and size involved, in particular whether such a region is commonly conserved, or whether the site of initiation varies among different neuroblastomas. Recently, Schneider *et al.* (18) cloned a 1.2-megabase region surrounding the N-myc locus into yeast artificial chromosomes (YACs). Such an effort is of great use in elucidating events in the early stages of N-myc amplification.

Thus far, we have isolated a large number of DNAs that are amplified in the cell lines MC-NB-1 and/or IMR-32 (10, 15, 19). In the present study, we were able to map these DNAs into a continuous 5.5-megabase stretch spanning the N-myc locus and complete the co-amplification profiles of these DNAs in 38 different neuroblastoma specimens with N-myc amplification including primary tumors, xenografts, and cell lines. Our efforts to define the original site and size of the domains initially involved in the formation of N-myc amplicons around the normal N-myc region and to compare the domain structures with those of the derivative amplicons enabled us to divide N-myc amplification into two classes of rearrangements: one is rearrangements preferentially initiated around some 'consensus sites', and the other is those with a random loss at subsequent stages. This model fits our previous idea that the flanking segments are preferentially co-segregated with the N-myc gene by a non-random mechanism.

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MATERIALS AND METHODS

Cell sources

The human lymphoblastoid cell line GM3798, which shows a germline configuration at the N-myc locus, was used as a cell source. A total of 14 human neuroblastoma cell lines (MC-NB-1, NB9, NB16, NB19, LA-N-1, LA-N-2, LA-N-5, CHP126, CHP134, IMR-32, TNB-1. NB-I, GOTO, and cNBI), and 5 xenografts (TNB-4, TNB-6, TNB-9, TNB-11 and NMB22), and 19 tumors (#7, #8, #9, #11, #12, #861, #876, #1104, #1119, #1120, #1121, #1256, #1277, #1283, #1287, #1840, #1944, NB6S, and NB13RLN) were used for co-amplification studies. In all of the neuroblastoma cell lines, xenografts, and tumor specimens, N-myc was amplified 20 to a few hundred-fold (4, 7, 15, 20, 21, unpublished data). Detailed information on these specimens is given elsewhere (10, 11, 15).

Pulsed field gel electrophoresis (PFGE)

PFGE was performed using a CHEF-DRII Pulsed Field Electrophoresis System (Bio-Rad, CA USA). Gel blocks containing 2×10^6 cells in 100 μl of 0.6% low melting agarose (FMC BioProducts, Rockland, ME, USA) were lysed with 1% lauroyl sarcosine (Wako Pure Chemical Industries, Ltd, Tokyo, Japan), 1 mg/ml Proteinase K (Wako Pure Chemical Ind.) and 0.5 M EDTA (Sigma, St Louis, MO, USA) at 50°C for a day and two nights. Proteinase K was inactivated by 1 mM phenylmethylsulfonyl fluoride (Wako Pure Chemial Ind.). Each block was digested with 25−50 units of restriction enzymes. A total of 20 restriction enzymes, NotI, SfiI, NruI, NaeI, NarI, SacII, SalI, XbaI, EagI, FspI, HaeII, PmlI and BspEI from NEN Research Products (Boston, USA), MulI, BssHII, ApaI, and EheI from TOYOBO (Osaka, Japan), and SmaI, CpoI, and SplI from Takara Shuzo (Kyoto, Japan) were used in this experiment. Digestion was carried out for 15 hours at 50°C for BssHII and SplI, at 30°C for SmaI and at 37°C for the others. Pieces of agarose blocks were loaded and electrophoresed in 1% agarose gel in 0.5×modified TBE buffer (1×TBE: 0.45 M Boric acid, 0.45 M Tris−base, and 0.5 mM EDTA) at 150 V for 60 hours at 4°C with a pulsed time cramped from 100−200 sec. The DNAs were then transferred to nylon filters (GeneScreen *Plus:* NEN Research Products).

Co-amplification profiles on different neuroblastomas

Genomic DNAs were prepared as described elsewhere (22). They were completely digested with HindIII (Nippon Gene, Toyama, Japan). The concentration of DNA was determined fluorometrically using fluorodye 4′, 6-diamino-2-phenylindole dihydrochlorode hydrate (Sigma). Exactly 2 μg of each digested DNA were electrophoresed in 0.7% agarose gel, transferred to nylon membranes and hybridized with the DNA probes. Amplified fragments were determined by comparing the intensity of the hybridization signals of the neuroblastoma specimens with those of GM3798.

Probes, radiolabelling and hybridization

The probes, 60, 116, 118, 129, 176, 212, 229, 260, and 264 (formerly named #60, #116, #118, #129, #176, #212, #229, #260, and #264) were single-copy sequences randomly cloned from the amplified sequences from the cell line MC-NB-1 using the phenol emulsion reassociation technique (PERT) (15). 1G6-5 and 2B7-107 were single-copy sequences subcloned from the EMBL3 phages 1G6 and 2B7 (data not shown). The phage clones 1G6 and 2B7 were isolated from the genomic library derived from the cell line MC-NB-1 using the Probe 277, which was isolated from the cell line MC-NB-1 using the PERT (10). Probe 1, Probe 3, Clone 8 (formerly named Probe 8), Probe 9, and Probe 10 were single-copy sequences randomly cloned from the amplified sequences from the fluorescence-activated and flow sorted metaphase chromosomes from the cell line IMR-32 (19). Probe 1-2 was a single-copy sequences subcloned from Probe 1 (data not shown). G21 is a 1.5 kb cDNA fragment subcloned in the EcoRI site of pBR322, which was isolated as a 'neuroblastoma-specific' cDNA clone, by differential screening of a cDNA library prepared from poly(A)$^+$ RNA of the cell line IMR-32 (3). As there is a NotI site within exon II of the N-myc gene, the NotI-linking clones isolated were used. They were N-myc A, a 0.9 kb NotI−EcoRI fragment of the 3′ downstream of the NotI site, and N-myc B, 1.1 kb EcoRI−NotI fragment of the 5′ upstream of the NotI site of the EcoRI−EcoRI fragment that includes exon II of the N-myc gene (15).

Hybridization was performed in 6×SSC, 1×Denhardt's solution, 0.1% SDS, and 100 μg/ml sonicated salmon sperm DNA (Sigma). In using probes 129 and 118 and Probe 3, which contains repeat sequences, sonicated and denatured total human placental DNA at 250 μg/ml was added to the hybridization mixture.

RESULTS

Megabase-scale mapping of the region flanking the normal N-myc gene

The probes used in this experiment came from two different DNA sources; one is from the cell line MC-NB-1 (10, 15) and the other is from the cell line IMR-32 (3, 19), which showed differential amplification among the subsets of the various human neuroblastomas (3, 7, 8, 10, 11, 15, 16, 19). To generate a restriction map, we used the strategy of performing single digests with up to 20 different rare cutting enzymes and hybridizing the resulting fragments with a number of DNA probes (Fig. 1). Using a set of probes, we first identified band(s) of the same size resulting from digestion by each enzyme. Next, using the same set of probes, we identified other bands resulting from digestion by at least two different enzymes. Then, the bands were aligned based on the sizes and the probe positions were also determined.

Table 1 lists the informative probes hybridized with the same-sized bands digested with different enzymes. First, seven restriction enzymes, NotI, MulI, NruI, BssHII, NarI, SacII, and SalI, were used. For example, Probe 9 and Probe 10 both identified bands of the same size, namely 1080, 680, 1020, 900 and 900 kb upon digestion with NotI, MluI, NruI, BssHII and SacII, respectively (Table 1). So, we can conclude that Probes 9 and 10 are linked together within 680 kb. In this way, linking was demonstrated between Probe 1-2 and Probe 3 from the NotI, NruI, BssHII, NarI and SacII bands; among Clone 8, G21 and 129 from the MluI and SalI bands; among G21, 129, and N-myc B from the NotI and BssHII bands; among N-myc A, 229, 212 and 176 from the MluI and SacII bands; among 118, 60, 2B7-107 and 260 from the MluI, BssHII, SacII and SalI bands; between 116 and 150 from the MluI, NruI SacII and SalI bands; and between 264 and 260 from the NruI (3000 kb) and the BssHII bands, respectively. Based on these analyses, a physical map spanning a continuous stretch from Clone 8, positioned at around 500 kb upstream, to 260, positioned at around 1700 kb downstream, of N-myc was constructed (Table 1, Fig. 2).

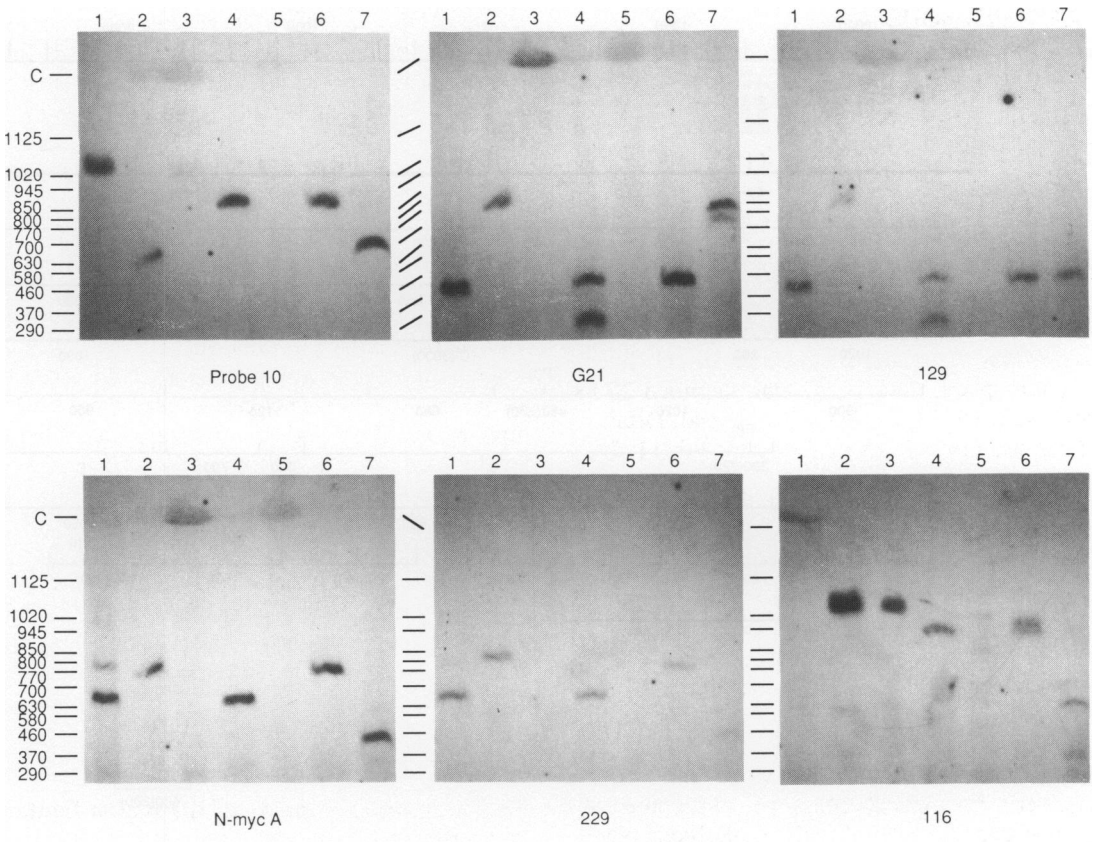

Figure 1. Examples of the Southern blot hybridization of the genomic DNA derived from the lymphoblastoid cell line GM3798 after PFGE. The genomic DNA was digested with NotI (lane 1), MulI (lane 2), NruI (lane 3), BssHII (lane 4), NarI (lane 5), SacII (lane 6), and SalI (lane 7). The probes used for hybridization are indicated below each panel. The chromosomal DNAs of *Saccharomyces cerevisiae* are indicated as a size marker.

However, such combinations of enzymes do not provide sufficient information on linking among three other regions containing Probes 9 and 10, Probe 1-2 and Probe 3, and 116 and 150. In order to gain further information, thirteen other enzymes, NaeI, SfiI, EagI, EheI, FspI, HaeII, SmaI, XbaI, CpoI, SplI, PmlI, BspEI, and ApaI were used. Among them, the bands identified only in digests with FspI, SmaI, PmlI and BspEI were informative (Table 1), while those identified in digests with other enzymes were not, since most of the sizes were smaller than 150 kb (data not shown). In these analyses, Probes 10, 1-2 and 3 identified the same 700 kb SalI and 1200 kb (and 850 kb) FspI bands. Therefore, the region containing Probes 1-2 and 3 must be adjacent to the 3′ end of the main region containing Clone 8 and some other probes, and the one containing Probes 9 and 10 must be linked with 3′ downstream from the region containing Probes 1-2 and 3. Probes 260 and 116 identified the same 250 kb SmaI, 170 kb PmlI and 200 kb (and 400 kb) BspEI bands. Thus, the region containing 116 and 150 is linked 5′ upstream with the main region containing 260. The results for these enzymes taken together enable the physical mapping of a continuous stretch spanning a 5.5-megabase region (Fig. 2).

As shown in Fig. 2, the probes were scattered throughout the region, although the exact positions couldn't be determined nor could the order of Probes 3 and 1-2; of 1G6-5, 264, 212 and 176; of 60, 2B7-107, and 260; and of 116 and 150 be uniquely determined. According to this map, Probe 9 is located at the 5′ end of the region, some 1900 kb upstream from the N-myc gene, and 150 (or 116) is at the 3′ end, some 1900 kb downstream from the N-myc gene. The other probes are located at distances ranging from some 10 kb (1G6-5 to 176) to 900 kb (Probe 1-2 and Clone 8). Most of the probes isolated from the cell line IMR-32 (Probes 9 to G21) are in the 5′ region of N-myc, and most of those isolated from the cell line MC-NB-1 (129 to 150 or 116) are in the 3′ region.

Analysis of amplified regions in 38 different neuroblastoma tumors, xenografts and cell lines

Some of the co-amplification profiles of the DNA probes isolated from the cell lines MC-NB-1 and IMR-32 have been previously described (7, 8, 10, 11, 15, 16, 19). In this study, we completed the co-amplification profiles of the probes used to construct the physical map for the neuroblastoma specimens, which include 14 cell lines, 5 xenografts and 19 tumors. The results combined with those of previous studies are shown in Fig. 3. Only two probes, 129 and 229, which were mapped into a 300 kb region spanning the N-myc gene, were commonly amplified in most of the specimens examined. Most of the other probes were scattered throughout the region and were co-amplified among subsets of the specimens. Based on similarities in the co-amplification profiles, amplicons were classified into five types. Type A includes seven specimens (MC-NB-1, #876, NB19, #1287, NB16, #1121, and #1944) nd shows amplification from 129 to those in the downstream region with the exception of #876, which showed amplification from N-myc. Type B includes six specimens (#1283, LA-N-5, #1840, NMB22, TNB-1 and CHP126) and shows amplification from Clone 8 to those in the

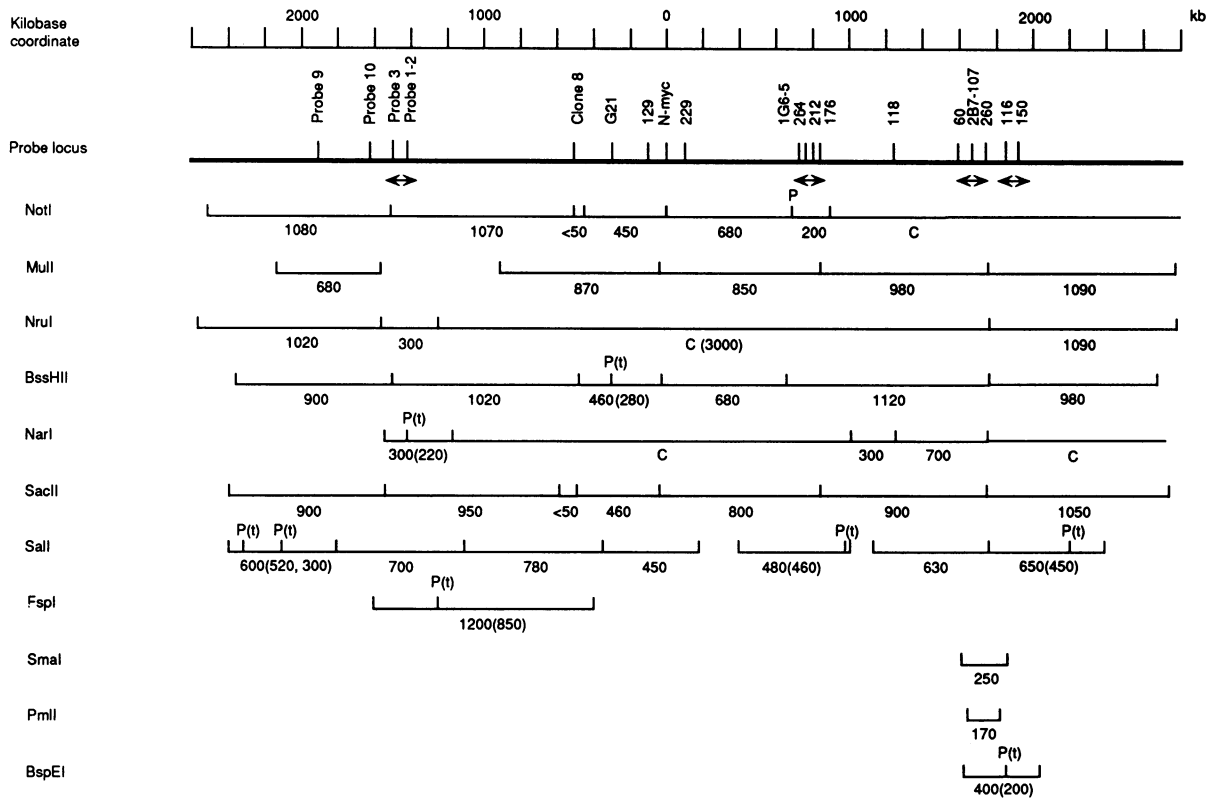

Figure 2. A physical map of the region spanning the N-myc locus. The positions of the probes used in the Southern blot hybridization are shown as Probe locus with scales expressed in kilobase coordinates in which the position of the N-myc gene is taken as 0. The sizes of bands identified by each of ten restriction enzymes are shown at the bottom in kb. The compressed bands, the sizes of which exceeded 2000 kb under our conditions, are indicated as C. C (3000) means that compressed bands digested with NruI were further separated by PFGE under different conditions (see legend in Table 1). The different sized bands appeared in the same digests are also indicated in parentheses. Taken together, the site identified at 680 kb downstream from the NotI site at exon II could be interpreted as partial digestion, thus it is designated as P and the other sites of the partial digestion tentatively determined are designated as P(t). There is possibility that the polymorphic sites are represented by these bands. The order of the probes from Probe 1-2 to Probe 3, from 1G6-5, to 176, from 60, to 260, and from 116 to 150 is tentative, as indicated by horizontal bars with arrows under the probe positions.

downstream region. Type C includes four specimens (IMR-32, NB9, #9, and CHP134) and shows amplification from the end of the upstream region to those of the downstream region. Type D includes eleven specimens (#1256, LA-N-1, cNBI, TNB-9, #861, #11, #8, #1277, #12, NB13RLN, and TNB-6) and shows amplification from Clone 8 to 229 with the exception of NB13RLN, which showed amplification from G21. Type E includes the remaining 10 specimens (NB6S, TNB-11, TNB-4, NB-I, GOTO, LA-N-2, #7, #1119, #1120, and #1104) and shows amplification from 129 to 229 with the exception of #1104, which shows amplification from N-myc.

Finally, the present results did not show any specific correlation of the sub-type of amplicon with the clinical data so far obtained, such as tumor stages and/or degree of N-myc amplification (data not shown), nor with cytogenetically defined markers, such as DM or HSR (Fig. 3).

DISCUSSION

In the present study, we were able to construct a physical map spanning a 5.5-megabase region around the normal N-myc region by examining digests of a human lymphoblastoid cell line obtained with twenty different rare cutting enzymes and hybridizing them with nineteen DNA probes, which were previously isolated from the N-myc amplicons of two different cell lines, MC-NB-1 and IMR-32 (10, 11, 15, 19). Our success in constructing the map surrounding the normal N-myc region is due to the use of a fairly large number of rare cutting enzymes to obtain single digests rather than performing multiple digestions with a small number of enzymes, even though only half of the enzymes used yielded informative bands. As yet, the order of some of the probes has not been determined because of a lack of appropriate enzymes spanning these probes.

The co-amplification profiles, when considered in relation to the map positions in the normal locus, indicate that the region commonly segregated with the N-myc gene seems to be limited to a 300 kb region surrounding the N-myc gene and that there is great heterogeneity in the flanking DNAs co-segregated with the N-myc gene, which are distributed broadly within the mapped region. The heterogeneity, however, seems to be non-random, since the amplicons can be grouped into several types (A to E) based on the co-amplification profiles. Such classification permits us to consider the original structure of the putative domains as a continuous stretch in the normal region that could be initially involved in the formation of N-myc amplicons. The majority of the domains (domains of Type D and E) are relatively small in size, their size ranging from some hundred to a thousand kb. The other classes (domains of Type A to C) are large enough

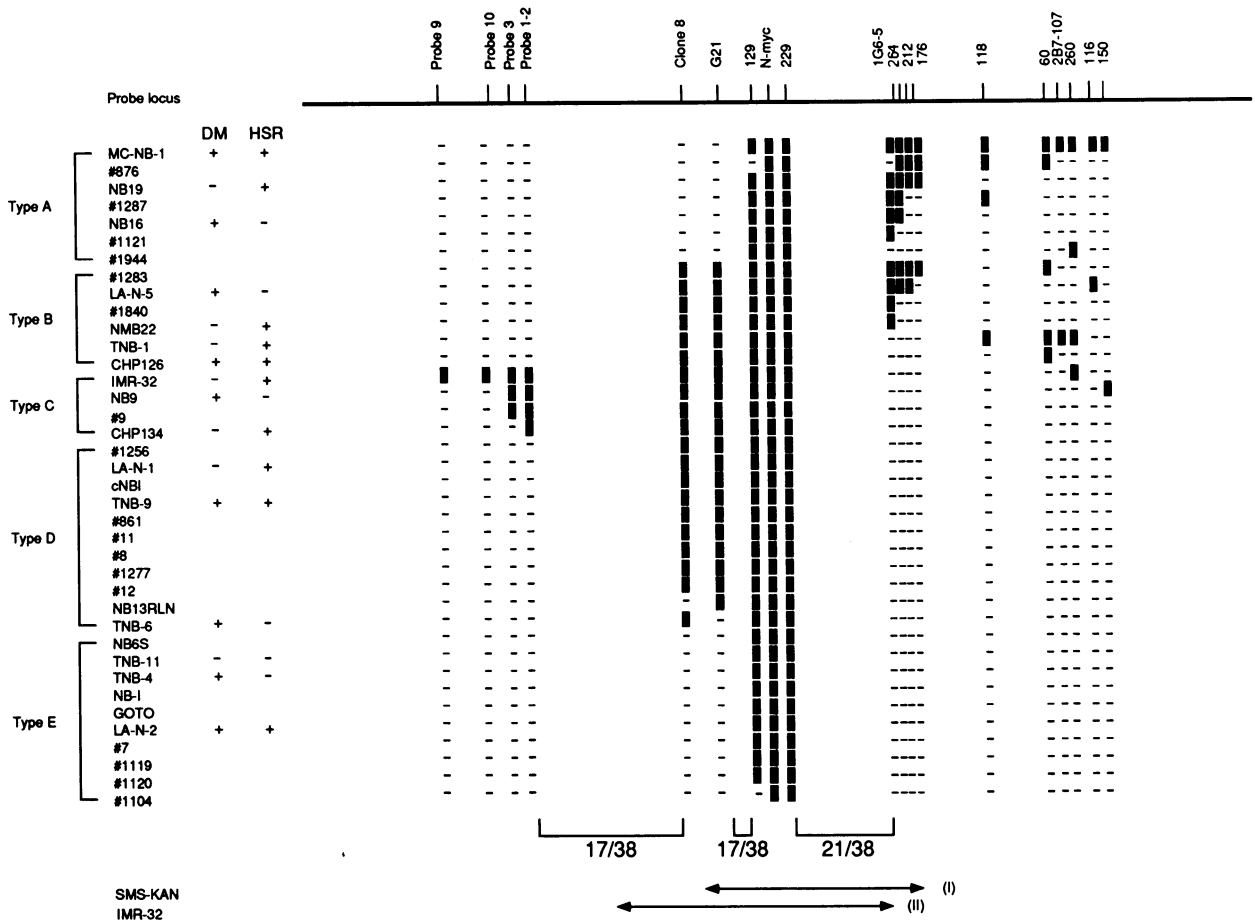

Figure 3. The amplification profiles of 19 probes isolated from the cell lines MC-NB-1 and IMR-32 for 38 neuroblastoma tumors, xenografts, and cell lines. The probe positions are the same as in Fig. 2. The amplification defined by the Southern blot hybridization is indicated by closed boxes (amplified) or bars (not amplified). The order of the specimens was determined on the basis of similarities in the amplification profiles. The presence or absence of DM and/or HSR available from the data (7, 20, 26, 27, 28, 29, 30, 31, 32) are indicated as + or − in the third and fourth columns. The columns are left open in the specimens without any cytogenetical information. The 'consensus sites (or regions)' are indicated as brackets with the number of neuroblastoma specimens whose putative domains ended up at the bracketed sites per total number of neuroblastomas examined. The arrows at the bottom indicated either the region identified by PFGE in the normal locus or the corresponding amplified domains in the cell line IMR-32 (I) (12), or the region cloned into the YACs from the normal locus or the corresponding amplified domains in the cell line SMS-KAN (II) (18). The boundaries of the data (I) are adjusted to the corresponding restriction sites of our data (a MluI site 870 kb upstream and a NotI site 680 kb downstream from the Not I site in the N-myc gene). The boundaries of the data (II) are unclear in relation to our restriction sites. There is no inconsistency between our and their amplification profiles of the cell line IMR-32. In the case of the cell line SMS-KAN, it is still unknown whether the probes contained in the cloned regions are amplified or not.

to cover half or most of the mapped region, their sizes being some thousand kb. The site distributions point to the existence of 'consensus sites' for the ends of the putative domains initially involved. For example, in 17 out of 38 neuroblastomas the domains ended up at a region between 129 and G21, a 5′ flanking region to the N-myc gene; and in 17 out of 38 they ended up at a region between Clone 8 and Probe 1-2, a further flanking region to the N-myc gene; and in 21 they ended up at a region between 229 and 1G6-5, a 3′ flanking region to the N-myc gene.

In our previous work (11), we surveyed the co-amplification of a 330 kb cloned region in a large number of neuroblastomas. A histogram of the frequency with which the flanking amplified sequences from one specimen matched those of the cell line MC-NB-1 showed a non-random distribution. From this observation, we speculated that the initiation of amplification is regulated by a preferential mechanism. The present results again suggest that there is some non-random mechanism at work during the formation of the initial domains of N-myc amplicons. The regions, defined to be areas within which a large proportion of the domains end, may be 'hot or warm regions' for the initiation of amplification.

It is interesting to compare our results with those of others. Amler and Schwab (12) analyzed the structure of the N-myc amplicons of several cell lines and the normal N-myc locus by using PFGE and two probes in the N-myc gene. They defined the size of the normal locus and the amplified regions in the cell line IMR-32 surrounding the N-myc gene as a 820 kb MluI band in the 5′ direction and a 600 kb NotI band in the 3′ direction of the N-myc gene (Fig. 3). Both bands correspond well to those we identified as a 850 kb MluI band in the 5′ direction and a 680 kb NotI band in the 3′ direction from the N-myc gene (Table 1). Based on these observations, they concluded that the amplicons of IMR-32 stretch over 1400 kb without rearrangements. Our findings may extend their results to a longer stretch, since the region initially involved in the formation of N-myc amplicons in the cell line IMR-32 exceeds 3500 kb in total.

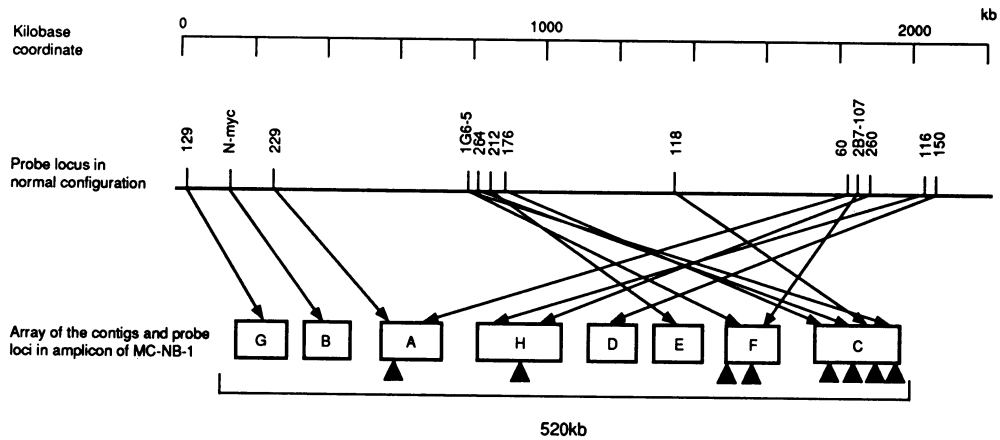

Figure 4. A comparison of structural organization of the normal locus spanning the N-myc gene and contigs identified in the cell line MC-NB-1. The upper scales are in kilobase coordinates in which the position of the probe 129 is taken as 0. The relative probe positions are the same as those in Figs 2 and 3. The contigs (Contigs A to H) were re-aligned to best fit the order of the probes. For help in understanding the sizes of the contigs and the inter-contig distances, the size of a representative amplicon identified in the cell line MC-NB-1 (520 kb) is indicated. Therefore, the scale of the amplicon does not correspond to the upper scale. Arrows with bars indicate the relation between the probe position in the normal locus and that in the contigs of the amplicons. The arrows without bars attached to the contigs indicate the rearranged sites previously determined (10).

Our results for IMR-32, however, further suggest that the large region from 1G6-5 to 116 in the 3′ direction after the 680 kb NotI band is deleted during the subsequent stages in the resulting amplicons. Very recently, Schneider *et al.* (18) cloned a 1.2-megabase region around the N-myc gene from a cell with a germline configuration into YAC vectors (Fig. 3). By comparing this structure with those of the N-myc amplicon of the cell line SMS-KAN, they concluded that the normal 1.2-megabase region was almost completely conserved in the amplicon of this cell line. The NotI/BssHII sites located around 600 kb, and the NotI site around 800 kb, in the 3′ direction of the N-myc that appeared in their data were also observed in our study as sites around 680 kb and 200 kb downstream from the NotI site of the N-myc gene (Table 1 and Fig. 3), while most of the other BssHII sites that they identified were not found in our study. Since their mapping data on NotI and BssHII were from the digests of YAC DNAs derived from a yeast background, in which the methylation of cytosines does not occur (23), all rare cutting restriction sites are available. Therefore, most cytosines within the BssHII sites surrounding the N-myc gene are methylated in GM3798. Following our typing, the amplicons identified in the cell line SMS-KAN seem to belong to Type B or D.

Analyses of N-myc amplicons performed in some ways suggest that amplification organization has a heterogeneous nature (8, 10, 11, 12, 13, 14, 15, 16, 17, 19) and those performed in other ways suggest that it has a homogeneous one (18, 24, 25). This discrepancy can be explained by noting that co-amplification profiles will be rather homogeneous if the DNAs are isolated from amplicons spanning a relatively small region; while the profiles will be heterogeneous if the DNAs are isolated from amplicons spanning relatively long stretches. In our collection, the majority of the amplicons were derived from rather small regions, so most regions in such amplicons will be well represented by the DNAs cloned from the amplicons from the small regions, such as SMS-KAN (18), while they will be less represented by those from large regions, such as MC-NB-1 and IMR-32. Again, the fact that the amplicons from the cell lines

Table 1. Results on the Southern blot hybridization of genomic DNA from GM3798 with DNA probes

Probes	NotI	MluI	NruI	BssHII	NarI	SacII	SalI	FspI	SmaI	PmlI	BspEI
Probe 9	1080	680	1020 [a]	900	C [b]	900	600 520 300	ND [c]	ND	ND	ND
Probe 10	1080	680	1020	900	C	900	700	1200 850	ND	ND	ND
Probe 1-2	1070	C	300	1020	300 220	950	700	1200 850	ND	ND	ND
Probe 3	1070	C	300	1020	300 220	950	700	1200 850	ND	ND	ND
Clone 8	<50	870	C	1020	C	<50	780 760	1200 850	ND	ND	ND
G21	450	870	C	460 280	C	460	780 760	ND	ND	ND	ND
129	450	870	C	460 280	C	460	450	ND	ND	ND	ND
N-myc B	450	850	C	460 280	C	800	450	ND	ND	ND	ND
N-myc A	680 800 [d]	850	C	680	C	800	450	ND	ND	ND	ND
229	680 800 [d]	850	C	680	C	800	450	ND	ND	ND	ND
1G6-5	200	850	C	1120	C	800	480 <50	ND	ND	ND	ND
264	200	850	C [e] (3000)	1120	C	800	480 <50	ND	ND	ND	ND
212	200	850	C	1120	C	800	480 <50	ND	ND	ND	ND
176	200	850	C	1120	C	800	480 460	ND	ND	ND	ND
118	C	980	C	1120	300	900	630	ND	ND	ND	ND
60	C	980	C	1120	700	900	630	ND	ND	ND	ND
2B7-107	C	980	C	1120	700	900	630	ND	ND	ND	ND
260	C	980	C [e] (3000)	1120	700	900	630	620	250	170	400 200
116	C	1090	1090	980	C	1050	650 400	ND	250	170	400 200
150 [f]	C 200	1090 850	1090 C	980 1120	C	1050 800	650 400 480	ND	ND	ND	ND

[a] As hybridization signals were positive but weak, we re-examined these probes using the digests of a male placental DNA.
[b] C, Compressed bands
[c] ND, Not determined
[d] The bands were weakly hybridized.
[e] Compressed bands were further separated by PFGE at 55 V for 140 hours at a pulse time of 60 minutes.
[f] The multiple bands identified by most of the enzymes indicate that 150 recognized two different loci, but the bands do not represent either polymorphism or partial digestion.

MC-NB-1 and IMR-32 do not overlap (10, 11, 15, 16) is because the domains involved in the formation of the amplicons are very different from each other, that is, the 3′ ends were mainly

represented in MC-NB-1, while the 5′ ends were mainly represented in IMR-32, since it lost the 3′ region.

Finally, we compared the normal map positions of the DNA probes obtained in the present study with those within the contigs localized in the amplicons in the cell line MC-NB-1 obtained in previous studies (10, 11). The sizes of the amplicons of MC-NB-1 identified by PFGE in terms of the distance between neighboring N-myc genes were 100 kb (truncated), 420, 480 and 520 kb (10, 11, 15). The total cloned regions forming contigs spanning 330 kb were included in these amplicons (10). Fig. 4 illustrates that the normal map positions of the DNAs included in the contigs are scattered throughout some 2000 kb, and that there are gross alterations in the alignment of the DNAs in the amplified domains in MC-NB-1 as compared with those mapped in the normal N-myc region. These results strongly suggest that rearrangements with large losses of DNA sequences are common in the formation of the amplicons in this cell line. In a previous study, we identified the eight rearranged sites within the contigs in the amplicons in this cell line occurring every 40 kb on average by identifying discontinuities in the amplification by at least two specimens in the same region (10). The rearranged sites deduced from the present mapping data are completely consistent with those identified previously (Fig. 4 and 10). As speculated before (10, 11), such rearrangements seem to be associated much more with the later stages of N-myc amplification in this cell line.

In conclusion, the present results lead to a model of N-myc amplification in human neuroblastomas in which amplification is preferentially initiated at some consensus sites flanking the N-myc gene, and is followed by subsequent rearrangements with a random loss of domain sequences, resulting in the formation of amplicons.

There have been no reports so far on the construction of a physical map encompassing such a large region surrounding the N-myc locus in the normal position to reveal the original sites and sizes of the N-myc amplicons involved at the initial event. We think that these probes and the mapping data with the co-amplification profiles provide a valuable means of investigating the mechanism involved in the initial events of N-myc amplification. Our next step is to examine more directly the recombinational events in the N-myc locus.

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