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Characterization of DNA rearrangements of N-myc gene amplification in three neuroblastoma cell lines by pulsed-field gel electrophoresis

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We characterized N-myc gene amplification in three human neuroblastoma cell lines (IMR-32, TGW, GOTO). Rearrangements in long-range regions surrounding amplified N-myc genes were examined by pulsed-field gel electrophoresis. Since rare-cutting enzymes completely digested DNA at the middle of the N-myc gene, we were able to construct a physical map upstream and downstream of the germline N-myc gene, and to obtain information on restriction sites surrounding amplified N-myc genes. This method enables us to envisage the organization of amplified units over a long range. Digestion patterns differed considerably among the germline and the three cell lines, but were simple in each case. We estimated that the minimal distance between neighboring N-myc genes is at least several hundred kilobases. Our data suggest that amplification units contain several DNA fragments derived from different loci, but that they are homogeneous.

Alu sequence; Gene amplification; Neuroblastoma; Gene, N-myc; Pulsed-field gel electrophoresis

1. INTRODUCTION

Many genes, possibly any gene, can be amplified in various organisms and, if a suitable selective agent or marker exists, amplification of a specific DNA can be identified (reviews [1–3]). Typical examples are amplification of drug resistance genes in the presence of cytotoxic agents [4] and of oncogenes in neoplasms [5]. To gain a better understanding of the mechanism of gene amplification, the structure of amplified DNA sequences has been characterized. The size of amplification units has been estimated to be in the range 150–3000 kb (review [6]). At first, these values were calculated based on the DNA content in the homogeneously staining region (HSR) and number of repetitions [7]. Recently, Looney and Hamlin [8] isolated a complete 220-kb-long amplification unit containing the dihydrofolate reductase (DHFR) gene by chromosome walking. Randomly selected DNA fragments in amplified regions have also been cloned by enrichment of DNA present in HSR by chromosome sorting [7], Roninson's in situ gel denaturation and renaturation technique [6,9], and direct microdissection [10]. Using these DNAs as probes, the organization of amplified DNA regions has been examined. They appear to be of two types [6]: one contains many DNA rearrangements and its amplified units are quite heterogeneous; the other contains few rearrangements and its amplification units are more homogeneous. Here, we adopted pulsed-field gel electrophoresis (PFG) [11] to examine rear-

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Abbreviations: DHFR, dihydrofolate reductase; HSR, homogeneously staining region; PFG, pulsed-field gel electrophoresis

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rangements surrounding N-myc genes amplified in neuroblastoma cell lines. This method enables us to envisage the organization of amplified units over a long range.

2. MATERIALS AND METHODS

2.1. *Cell lines*

IMR-32 [12] was obtained from the American Type Culture Collection. TGW [13] and GOTO [14] were kindly provided by Dr T. Kato (Nagoya City University).

2.2. *Probes, cloning and sequencing*

Probes for the N-myc gene were kindly supplied by Dr Y. Taya (National Cancer Center). Three probes were prepared as shown in fig.1. Clone TGW-1 was isolated by the ordinary cloning procedure described by Sakano et al. [15]. Probe A (0.4 kb) was prepared from TGW-1 and is connected to the N-myc gene in TGW. Clone 804 was isolated by screening the human genomic library of Maniatis et al. [16] with probe A, using the method of Benton and Davis [17]. The nucleotide sequence was determined by the chain termination technique of Sanger et al. [18] using Bluescript M13 vectors (Stratagene).

2.3. *Pulsed-field gel electrophoresis*

For PFG analysis, agarose blocks containing high molecular mass cellular DNA were prepared as described by Schwartz and Cantor [11]. A DNA sample embedded in 100 μl of agarose block was digested with 40 units of restriction enzyme as described by Smith and Cantor [19]. Digested samples were loaded onto a 1% agarose gel in TBE buffer (0.1 M Tris, 0.1 M boric acid, 2 mM EDTA, pH 8.5) with dimensions of 20 × 20 × 0.5 cm formed in a pulsed-field gel electrophoresis apparatus (Pharmacia LKB Biotechnology). Gels were run for 42 h at 330 V at 7°C and pulsed usually for 90 s. When a hexagonal electrode was used, agarose gels (15 × 15 × 0.5 cm) were run at 170 V. Southern hybridization was performed according to [20] using a nylon membrane (Hybond N, Amersham).

3. RESULTS

3.1. *Identification of a DNA rearrangement in the vicinity of the N-myc gene in human neuroblastoma cell line, TGW*

Southern blot analysis was carried out in order to examine DNA amplification and rearrangements at and around N-myc gene loci in the three human neuroblastoma cell lines, IMR-32, TGW and GOTO, using the three probes described in fig.1. The N-myc gene was amplified 20-, 40- and 25-fold in IMR-32, TGW and GOTO, respectively. In TGW DNA, a rearranged fragment was observed in addition to the germline band (not shown). One third of amplified fragments contained the rearranged configuration. We cloned it (named TGW-1) from TGW DNA, and the germline counterpart in the rearranged fragment (denoted clone 804). We determined the nucleotide sequences around the rearranged regions in TGW-1 and clone 804 (fig.2). The rearrangement occurred 11 nucleotides downstream of the additional poly(A) signal of the N-myc gene. The rearranged point in clone 804 is located in the Alu sequence and this region between two putative promoter elements, box A and B, for RNA polymerase III is a hot point for DNA rearrangements involved in Alu-Alu homologous recombination [21]. Probe A prepared from TGW was used as a probe for PFG analysis of TGW DNA.

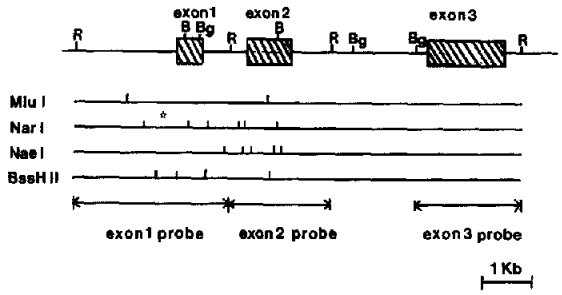

Fig.1. Restriction map of N-myc gene. Restriction sites of the four rare-cutting enzymes were mapped on the basis of the sequence data of Stanton et al. [25] and from results on digestion of cloned N-myc DNA. (☆) Additional *Nar*I site(s) present; DNA fragments used as probes are indicated. R, *Eco*RI; B, *Bam*HI; Bg, *Bgl*II.

3.2. *Long-range restriction map around germline N-myc gene*

We constructed long-range restriction maps of four rare-cutting enzymes, *Bss*HII, *Nae*I, *Nar*I and *Mlu*I, around the N-myc gene by using normal human peripheral lymphocyte DNA. Identification of their restriction sites has frequently been hindered by methylated CpG. However, there is a region of high CpG content in the middle of the N-myc gene (fig.1), and most CpG in such a region is reportedly unmethylated [22]. In fact, all four enzymes digested DNA completely at the middle of the N-myc gene (not shown). Therefore, we were able to construct a physical map upstream and downstream of the N-myc gene by using exon 1 and 3 probes, respectively. The lymphocyte DNA

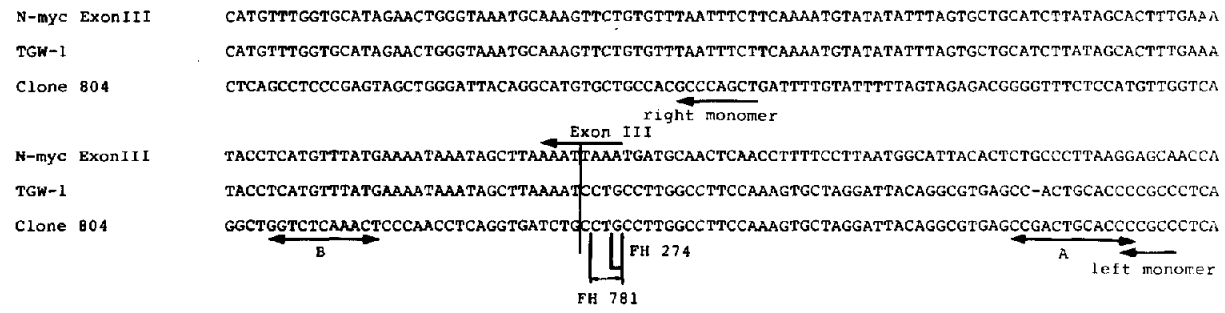

Fig.2. DNA sequence involved in a DNA rearrangement of TGW cells. Rearrangement occurred between the 3'-end of the N-myc gene and the Alu sequence. Sequence of the end of N-myc exon 3 referred from Stanton et al. [25]. Arrows: end of exon 3, and starting points of left and right monomers in the Alu sequence; A,B (delinated with double-headed arrows): regions of the putative promoters for RNA polymerase III [26]; vertical line: breaking point; FH 274, FH 781: regions of DNA rearrangement in LDL receptor gene [21].

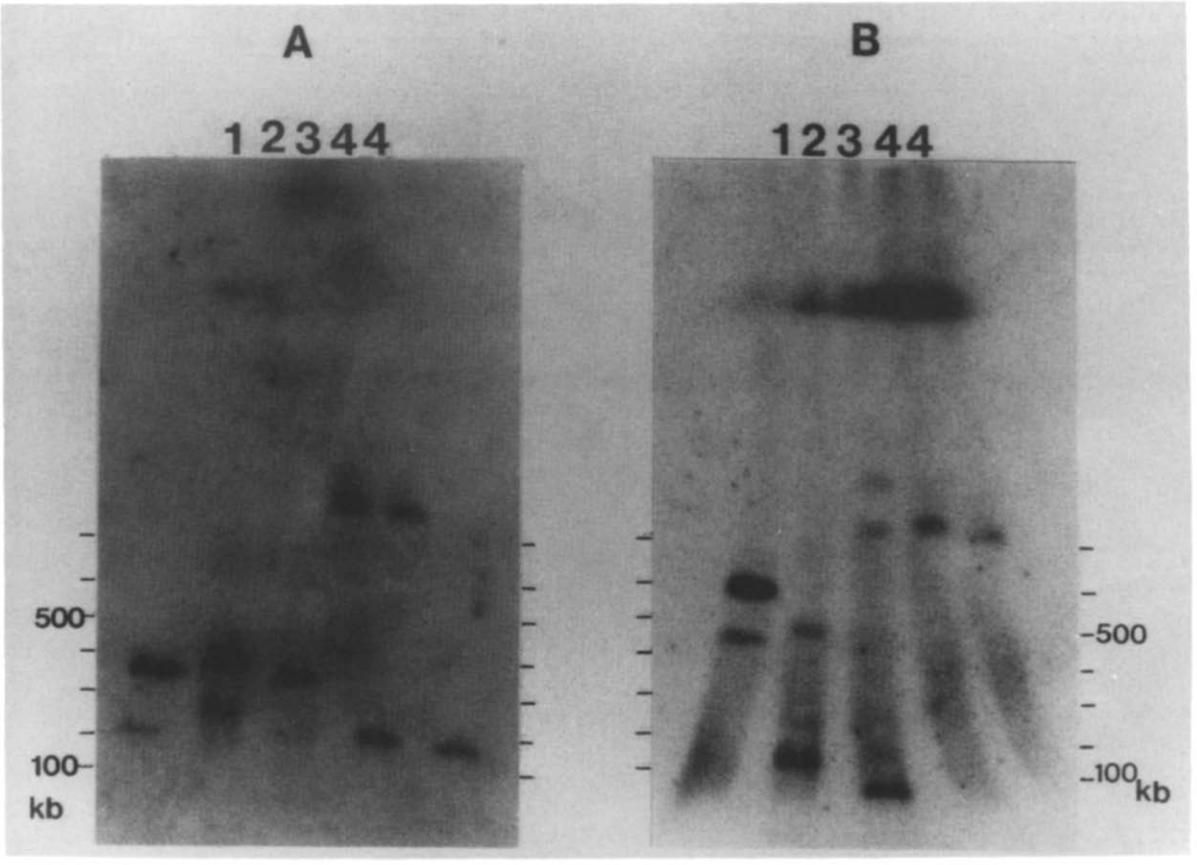

Fig.3. PFG analysis of germline DNA. DNA from human peripheral lymphocytes was digested with rare-cutting enzymes, separated by PFG and hybridized with exon 1 probe (A) and exon 3 probe (B). Lanes: 1, *Bss*HII; 2, *Nae*I; 3, *Nar*I; 4, *Mlu*I. Sizes were estimated using λ-phage concatemers. Exposure time, 1 week.

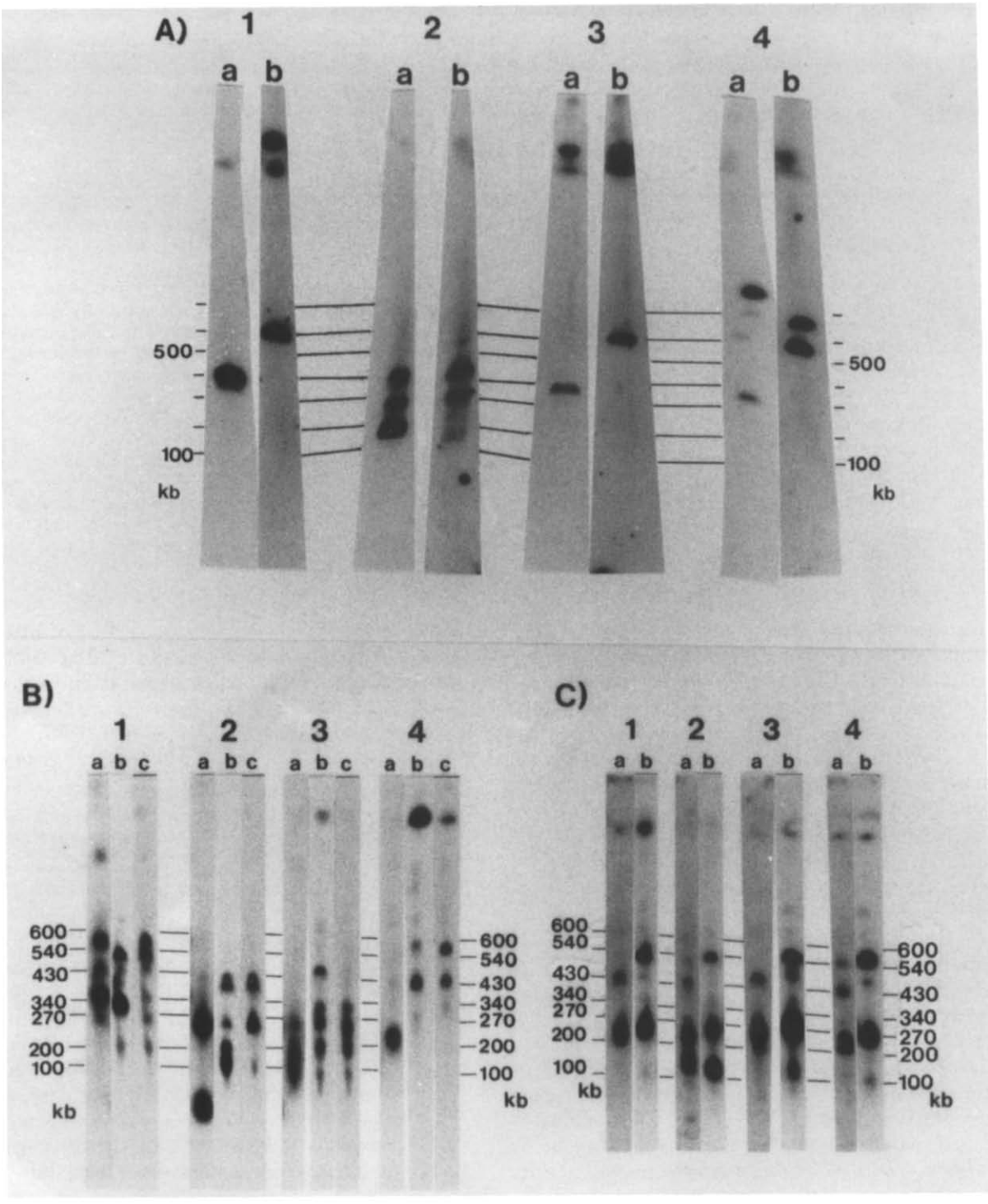

Fig.4. PFG analysis of three neuroblastoma cell line DNAs. DNAs from IMR-32 (A), TGW (B) and GOTO (C) cells were digested with rare-cutting enzymes, separated by PFG and hybridized with exon 1 probe (a), exon 3 probe (b) and probe A (c). Enzymes: 1, *Bss*HII; 2, *Nae*I; 3, *Nar*I; 4, *Mlu*I. A hexagonal electrode was used in the experiments of (B,C). Sizes of yeast chromosomes (strain 334, Beckman) were calibrated using λ-phage concatemers, and employed in experiments (B,C). Exposure time, 20 h.

was digested with the rare-cutting enzymes, separated by PFG, blotted and hybridized with exon 1 and 3 probes, with the results being depicted in fig.3. Exon 1 and 3 probes detected one or two discrete fragments in each case. The reason why more than one fragment was identified is because of the polymorphism of methylation, and/or partial digestion. DNA samples from three independent human peripheral lymphocytes gave identical digestion patterns. Fig.5 (upper lines) shows the restriction map of the four enzymes around the N-myc gene.

3.3. *Characterization of amplified N-myc genes in neuroblastoma cell lines by PFG*

DNAs from the IMR-32, TGW and GOTO cell lines were digested with the four enzymes described above, separated by PFG, blotted and hybridized with exon 1 and 3 probes. As shown in fig.4A, the exon 1 probe detected only one major band at 400 and 360 kb in *Bss*HII- and *Nar*I-digested IMR-32 DNA, respectively. The exon 3 probe also detected only one major band at 600 kb in both *Bss*HII- and *Nar*I-digested DNA. In contrast, several bands were observed for *Nae*I-digested DNA with both probes, with different sizes compared to those of germline DNA. The exon 1 probe detected one major band at 800 kb and three faint bands at 350, 610 and 700 kb in *Mlu*I digests. The exon 3 probe detected two bands at 580 and 680 kb. Since Southern hybridization of *Bss*HII and *Nar*I digests gave single bands on both sides of the N-myc gene, the reason why several bands were detected in *Nae*I and *Mlu*I digests may not be connected with the heterogeneity of DNA rearrangements but rather be the result of partial digestion.

In the case of TGW cells (fig.4B), the exon 1 probe detected one major band at 230 kb in digests with *Mlu*I, three bands at 30, 50 and 250 kb for *Nae*I, three bands at 100, 150 and 270 kb in the case of *Nar*I, and two intensely stained bands (360 and 560 kb) and two faint bands (270 and 440 kb) in *Bss*HII digests. As described above, one third of amplified fragments in TGW DNA contained rearranged points at the 3′-end of exon 3. Southern hybridization was carried out with probe A, and the results compared with those for exon 3 probe. In *Nae*I digests, the exon 3 probe identified five bands at 120, 200, 250, 390 and 420 kb, of which two (those at 390 and 420 kb) were identified with

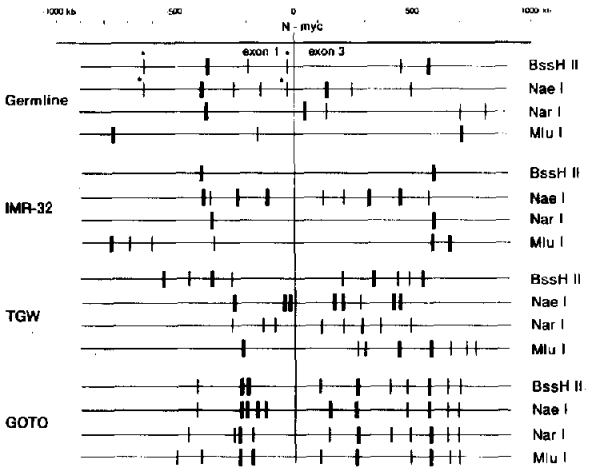

Fig.5. Positions of restriction sites around the N-myc gene in germline and three neuroblastoma cells. Restriction enzyme sites obtained from the results in figs 3,4 are summarized. Enzyme sites upstream and downstream of N-myc gene were identified with exon 1 and 3 probes, respectively. Thick and thin vertical bars represent the intensities of detected bands. Thin bars indicated with an asterisk were not detected in fig.3, but weakly detected in another experiment.

probe A with the same degree of intensity. Although one more band was observed at 250 kb with probe A, it was of much greater intensity than that with exon 3 probe, and ordinary Southern hybridization of *Hin*dIII-digested TGW DNA with probe A showed that the DNA fragments containing probe A were amplified not only in the N-myc gene-connected form but also in the germline configuration (not shown). The degree of amplification of the probe A-containing DNA in the germline configuration is greater compared to the N-myc gene-connected form. Thus, the probe A-containing DNA region was involved in this amplification in at least two forms. However, this region was amplified in neither IMR-32 nor GOTO cells (not shown). The sizes of two other bands, 120 and 200 kb, detected with the exon 3 probe, differ from those of the germline or the two other cells. Although the digestion patterns obtained for TGW with the exon 3 probe appear to be more heterogeneous vs those of IMR-32, the bands can be classified as either containing or lacking probe A, the patterns being simple in each case.

In GOTO cells (fig.4C), a few fragments were detected with either probe, and were similarly sized for all four enzyme digests.

4. DISCUSSION

4.1. *Heterogeneous rearrangements and homogeneous amplification around amplified N-myc genes in neuroblastoma cell lines*

The analyses with rare-cutting enzymes were sometimes affected by methylation polymorphism among different cell types and/or variation in culture conditions. Since the cells used here are all very similar and were cultivated under similar conditions, comparisons among these three cell lines were believed to be informative. Fig.5 summarizes the results we obtained thus. Although some bands may represent partially digested products, the digestion patterns surrounding N-myc genes vary considerably among the germline and three cell lines. We interpret heterogeneity in the digestion patterns among the three cell lines to reflect variation in the rearrangements in these cells, and conclude that DNA rearrangements occurred at both sides of the N-myc genes in all three neuroblastomas. Rearrangement of points and fragments connected to N-myc gene-containing DNA would result in differences in each case. However, the amplification units are homogeneous, or at least only very slightly heterogeneous over regions encompassing several hundred kilobases surrounding amplified N-myc genes.

Shiloh et al. [23] demonstrated that at least three DNA fragments derived from different chromosome loci were simultaneously amplified in HSR of IMR-32. Since they employed in situ hybridization, it was unclear whether these DNA fragments were connected to each other in the same amplification unit. Zehnbauer et al. [6] isolated 140 kb DNA surrounding the N-myc gene and showed that rearrangements in this region are rarely observed in NGP cells and 12 primary neuroblastoma cells. Our studies with PFG analysis provided information on the long-range organization of amplified units: DNA rearrangements had occurred relatively close to the N-myc gene, however amplification units were much longer and homogeneous. Based on our observations and the data of Shiloh et al. [23], the connection of several DNA fragments might be necessary in some cases for amplification of DNA fragments. Such rearrangements should have occurred before, not during amplification. If rearrangements did occur during amplification, digestion patterns with rare-cutting enzymes would have been more heterogeneous than in our results.

4.2. *Estimation of minimal distance between neighboring N-myc genes*

Based on the above observations, we estimated the minimal distance between neighboring N-myc genes in amplified regions. Since DNAs were completely digested at the middle of the N-myc gene under the conditions used, the distance between neighboring N-myc genes should be the same as or longer than the sizes of the fragments detected using the exon 1 and 3 probes. For example, in IMR-32 DNA, an 800 kb fragment was detected with the exon 1 probe in *Mlu*I digests, from which the minimal distance was evaluated as 800 kb. One should also take into consideration the orientations of neighboring N-myc genes. Looney and Hamlin [8] reported that some amplified DHFR genes have a head-to-tail configuration while others adopt the head-to-head type. In IMR-32, the longest fragments identified as single major bands with the exon 1 and 3 probes were 800 and 600 kb, respectively. If the orientation is head-to-tail, then the minimal distance should be longer than the 1400 kb (800 + 600) in IMR-32. However, if the orientation is head-to-head, the 800 kb DNA may contain two N-myc genes at both ends. This would be possible because the sizes of the DNA fragments detected in the other three enzyme digests are less than 400 kb. The size of fragments detected with the exon 3 probe is around 600 kb in *Bss*HII, *Nar*I and *Mlu*I digests. It is unlikely that these fragments of 600 kb contain two N-myc genes in tail-to-tail configuration because the DNA sizes determined in *Nae*I digests were 330 and 450 kb. If the orientation is indeed tail-to-tail, the distance between two neighboring N-myc genes should be longer than 1200 kb (600 × 2).

In TGW DNA, the longest fragments detected were 560 kb with the exon 1 probe and 520 kb with the exon 3 probe in *Bss*HII digests. Therefore, we can argue that the minimal distance must be at least several hundred kilobases.

In GOTO DNA sizes of the fragments detected with the exon 1 and 3 probes appear similar, not only among the four enzyme digests but also between the two probes. This suggests that fragments may occur that contain two N-myc genes in head-

to-tail configuration. If this is indeed the case, then the distance between neighboring N-myc genes should be of exactly the same size as the fragments.

Kanda et al. [7] estimated the size of N-myc gene amplification units in IMR-32 to be 3000 kb, based on the DNA content of the HSR and the number of repetitions. Kinzler et al. [24] reported that the amplification unit in eight neuroblastomas containing N-myc amplification was 290–430 kb long. They estimated the values from the sum of the sizes of bands detected using Roninson's technique [9]. Apparent differences in size between the amplification units would appear to be due to variations in the characteristics of the cells and/or differing methodology. Cloning of the entire amplicon will provide a direct demonstration of the organization of the amplified region.

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