Amplification and Rearrangement of DNA Sequences from the Chromosomal Region 2p24 in Human Neuroblastomas

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ABSTRACT

Seven DNA fragments which map to or very near human chromosome band 2p24 are shown to be differentially amplified in DNA from specific subsets of an enlarged series of human neuroblastoma cell lines and primary neuroblastomas. Of these DNA fragments, the probe NB-19-21 for the oncogene N-myc is the most frequently amplified, with a second expressed sequence (pG21) amplified in 9 of those 11 cell lines and 16 of those 25 tumors exhibiting amplification of N-myc. The remaining probes are in turn each amplified in progressively smaller, nested subsets of the cell lines and tumors in which both N-myc and pG21 are amplified. These data permit construction of models for the organization of a "neuroblastoma amplicon," i.e., an originally amplified DNA domain, with N-myc positioned most central and the other DNA fragments increasingly peripheral; comparable models result for the cell lines and the tumors. Five of the seven probes examined detect novel DNA fragments in these specimens, reinforcing previous observations that extensive DNA rearrangement can occur during DNA amplification in neuroblastoma cell lines and in primary neuroblastomas. Such rearrangements could contribute significantly to the evolution of the neuroblastoma amplicon in different specimens to progressively smaller units, preserving, in the limit, amplification of N-myc.

INTRODUCTION

Many of the cell lines derived from the childhood tumor neuroblastoma bear the cytological hallmarks of DNA amplification: chromosomal HSRs, or double minute bodies (1, 2). The mechanism of DNA amplification and the relevance of this phenomenon to neoplasia can be investigated by analysis of the structure and function of the amplified DNA sequences in normal and cancer cells. Different approaches have been successfully used to isolate such sequences from neuroblastoma resulting in a variety of molecular probes which were found to be amplified in different cell lines (3–7).

An insight into the complexity of the amplification process has been obtained in our laboratory by showing that in the neuroblastoma cell line IMR-32 (8), DNA sequences from different parts of 2p were spliced together into a novel unit which was amplified and relocated to form an HSR on chromosome 1 (3, 6, 9). It was also shown by us and by others that an oncogene of the myc family (N-myc), which also maps to 2p, was amplified and highly expressed in many neuroblastomas as well as in several retinoblastomas and small cell lung carcinomas (5, 6, 10–18). Amplification of N-myc in multiple HSRs on different chromosomes demonstrated in several neuroblastomas has supplied additional evidence for interchromosomal transposition during amplification (3, 10, 15, 16).

More recently, we have shown that N-myc as well as 4 other fragments from 2p were differentially amplified in nested subsets of advanced stage primary neuroblastomas (9, 17). This observation has given further support to the notion that amplification of specific 2p sequences in neuroblastoma is not merely an in vitro phenomenon, characteristic of cultured cells, but is part of the malignant process in advanced stage tumors at diagnosis. All of these 5 DNA fragments were mapped to a specific region, in or near the chromosomal band 2p24 (9), thus adding special interest to this genomic domain.

We now show that two additional DNA fragments from the same chromosomal region, both of which have some unique features, are amplified in neuroblastomas, adding new subsets to the molecular subdivision of these tumors. We also show that several probes including N-myc detect multiple "novel joints" in cell lines and in primary tumors, adding further evidence for the involvement of DNA rearrangements in the amplification process.

MATERIALS AND METHODS

Cell Lines and Tumors. A total of 11 cell lines and 25 primary neuroblastoma DNA samples with extensive (>10-fold) amplification of N-myc were examined. Cell lines NB-9, NB-16, NB-19 (19), CHP-126, and CHP-134 (20) were gifts from Dr. Fred Gilbert. IMR-32 (8) was obtained from the American Type Culture Collection, LA-N-1 (21), and LA-N-5 were from Dr. R. Seeger, and MCNB-1 was from Dr. J. Casper (22). DNA from cell line NLF (5, 10) was obtained from Dr. G. Brodeur. TNB-1 was established as a xenograft in nude mice from neuroblastoma tissue of a Japanese patient (23). Most of the primary tumors were obtained from the Children's Cancer Study Group, and the rest were collected at the Children's Hospital, Boston, MA. The tumors had been classified according to the clinical staging system of Evans et al. (24).

Probes. The N-myc probe, NB-19-21, probes 1, 3, and 8 cloned from the HSR of IMR-32 cells, and the probe NB-9-A cloned from NB-9 cells have been described before (3, 6, 9, 15). All of them map to the chromosomal vicinity of 2p24 and are amplified in different cell lines and primary neuroblastomas. In all of these tumors, amplification of N-myc was demonstrated, and smaller subsets of this group show amplification of various combinations of the other probes (6, 9, 15).

NB-915 is a 0.24-kilobase genomic fragment subcloned from NB-9 DNA at the EcoRV-BamHI sites of plasmid pAT153. This probe, together with probes 8 and NB-9-A are involved in a complex DNA rearrangement in this cell line (25).

Probe G21 is a 1.5-kilobase complementary DNA fragment subcloned in the EcoRI site of pBR322, which was isolated from an IMR-32 complementary DNA library. The genomic sequences corresponding to G21 were shown to be amplified in five neuroblastoma cell lines which also amplify N-myc (7). Using somatic cell hybrids, quantitative

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4 The abbreviations used are: HSR, homogeneously staining region; 2p, short arm of human chromosome 2.

5 K. Sakai, unpublished observations.
Novel Joints Detected by Amplified Probes. Novel joints, the result of recombinational DNA rearrangements, are detected by Southern blot analysis as extra bands, different in size from normal bands. Examples of such joints are demonstrated in Figs. 2–5.

The N-myc probe, NB-19-21 normally detects a 2.0–2.1-kilobase band on an EcoRI genomic blot and a band of approximately 24 kilobases when HindIII is used (Fig. 2). In one of the primary tumors (86) NB-19-21 detects an additional, amplified 5.0-kilobase EcoRI fragment (Fig. 2A). Whereas the 2.1-kilobase fragment is amplified 75-fold in this DNA, the additional band shows amplification of only 10-fold as determined by scanning densitometry. This new band is probably a result of a rearrangement which occurred after this region had already been amplified and was then followed by several additional cycles of amplification.

In CHP-126 DNA, NB-19-21 does not hybridize to any extra band detectable on a Southern blot when the DNA is cut by EcoRI (data not shown); however, when a HindIII genomic blot is used, an additional band of 13.5 kilobases appears, with intensity almost equal to that of the normal 24-kilobase band (Fig. 2B). This intensity corresponds to an approximately 100-fold amplification (6). This rearrangement therefore involves the large HindIII fragment, leaving the smaller EcoRI fragment intact.

The complementary DNA probe pG21, which hybridizes to multiple HindIII bands on genomic blots (Fig. 3) reveals new bands, reflecting DNA rearrangement in both tumors 83 and TNB-1; only some of the genomic sequences corresponding to this complementary DNA clone are amplified, whereas others appear in a single copy. There is also an extra band of 2.0 kilobases in tumor 83 (Fig. 3A). In tumor 86, probe G21 hybridizes to 2 extra bands of 9.0 and 2.9 kilobases with sequential decrease in the number of specimens exhibiting amplification of additional probes, always including those probes amplified in the previous, larger number of specimens, is similar in the primary tumors and the cell lines with only one exception, which has a simple interpretation (see below). In the cell lines, probes 1 and 3 are amplified in IMR-32 DNA only, whereas NB-9-A is amplified exclusively in NB-9.

Hierarchial Amplification of 2p24 Probes in Neuroblastomas. We have shown already that 5 DNA fragments from the chromosomal region 2p24 are differentially amplified in primary neuroblastomas, thus defining a molecular subdivision of these tumors into progressively smaller nested subsets (9) in which progressively greater numbers of the DNA fragments are amplified. Using a larger series of tumors as well as a series of neuroblastoma cell lines we have now carried out a similar analysis with these 5 probes plus 2 others: probe G21, a complementary DNA clone isolated from IMR-32 cells (7), and a small genomic fragment, NB-9J5, cloned from a DNA rearrangement in NB-9 DNA (25). Fig. 1 summarizes the current status of the molecular classification of neuroblastomas and neuroblastoma cell lines as defined by the seven probes. The

* H. Stroh, T. Donlan, Y. Shiloh, and J. Kang, unpublished observations.

Fig. 1. Current status of the partition of neuroblastoma cell lines (A) and primary tumors (B) into subsets characterized by different amplification patterns. In each section, the number of cell lines or tumors in each group is indicated on the right and the probes amplified in that group on the left. Each group is a subset of the one above.

RESULTS

Molecular Techniques. Extraction of genomic DNA, gel electrophoresis, Southern blotting, radiolabeling, hybridization, and scanning densitometry were performed as previously described in detail (15, 26). Typical blot washing stringency was 0.1 x 0.015 M NaCl-0.005 M sodium citrate, pH 7-0.1% sodium dodecyl sulfate, at 55°C.

DNA AMPLIFICATION IN NEUROBLASTOMA

Fig. 2. Southern blots revealing novel joints detected by the N-myc probe NB-19-21 in tumor 86 and CHP-126. A, Lane 1: 46.XX DNA; Lanes 2 and 3: tumors 84 and 86 DNA (enzyme: EcoRI); B, Lane 4: CHP-126 DNA; Lane 5: TNB-1 DNA (enzyme: HindIII). The normal EcoRI band detected by this probe is 2.1 kilobases (KB) and the size of the normal HindIII band is 24 kilobases.
DNA AMPLIFICATION IN NEOBLASTOMA

Fig. 3. Southern blots revealing novel joints detected by probe G21 in tumors 83 and 86 and in TNB-1. A, Lane 1: 46,XY DNA; Lane 2: IMR-32 DNA; Lane 3: NB-9 DNA; Lanes 4 and 5, tumors 81 and 83 DNA (enzyme: HindIII); B, Lanes 6 and 7: tumors 84 and 86 DNA (enzyme: HindIII); C, Lane 8: CHP-126 DNA; Lane 9: TNB-1 DNA (enzyme: HindIII). Lane 7 reveals 2 extra bands of 9.0 and 2.9 kilobases (KB) in tumor 86. Note the missing bands in tumor 83 (Lane 5) and TNB-1 (Lane 9).

Table 1 Summary status of probe rearrangement in neuroblastoma tissue

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<tr>
<th>DNA source</th>
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* The highest number of novel joints (R) in the tumors and cell lines studied per single probe (four) was detected by probe 8.

amplification of 50-fold, whereas the normal bands show 150-fold amplification (Fig. 3B).

Probe 8 detects an extra amplified band in the DNA of tumor 86 (Fig. 4A). It is of great interest that the amplification ratio of the normal and the novel bands is approximately similar to the 150:50 ratio detected by probe G21 in the same tumor but is different from the 75:10 amplification ratio of the bands detected by probe NB-19-21. Fig. 4A also shows once again the interesting novel joint detected by probe 8 in the NB-9 DNA.

Fig. 4. Novel joints detected by probe 8 in NB-9, tumor 86, CHP-126, and TNB-1 using Southern blot analysis and HindIII DNA digests. Normally this probe hybridizes to a single 1.75-kilobase (KB) HindIII band. A, Lane 1: 46,XY DNA; Lane 2: IMR-32 DNA; Lane 3: NB-9 DNA; Lanes 4-7: tumors 81, 83, 84, and 86 DNA; B, Lane 8: CHP-126 DNA; Lane 9: TNB-1 DNA.

Fig. 5. Detection of novel joints in NB-9 and tumor 26 using NB-9J5 as a probe and HindIII digestion of DNA. The band which normally appears with this probe is 6.7 kilobases (KB). A, Lane 1: NB-9 DNA; Lane 2: NB-19 DNA; A, Lanes 3-6: tumors 8, 26, 43, and 45 DNA.

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(6). In this case, the normal 1.75-kilobase band appears in a single copy, and the 100-fold amplification is confined exclusively to the rearranged segment. In CHP-126 DNA probe 8 hybridizes to a rearranged band of 1.45 kilobase, which is shown in Fig. 4B, together with a 1.65 kilobase rearranged band in TNB-1 DNA.

The NB-9J5 fragment was subcloned from a part of the 3.2-kilobase rearranged fragment detected by probe 8 in NB-9 DNA (Figs. 4A and 5A) with no probe 8 homology. It hybridizes in most samples (at relatively high hybridization stringency, i.e.,

7 P. Harris and Y. Shiloh, unpublished observations.
The 7 DNA fragments isolated so far from the chromosomal region 2p24 define different parts of this domain, which are differentially amplified in neuroblastomas. It is highly probable that N-myc is at or very near the core of any observed “neuroblastoma amplicon,” perhaps because this gene confers a selective growth advantage to cells, in this sense supplying the driving force for the amplification of the whole region. One indication for this is that there is no amplification of any other probe without concomitant amplification of N-myc (Fig. 1). Recently we have isolated several new amplified probes from CHP-126 DNA by using a novel phenol-enhanced DNA reassociation method of Kunkel et al. (27) for rapid cloning of multiple fragments from the amplified region.9 In this case too, all the probes detected amplification in various tumor-derived cell lines only when N-myc was also amplified. There is also evidence for a functional role of N-myc expression in the malignant process (11–14, 17, 19, 28–30).

The frequency with which each probe is amplified (Fig. 1) may be taken as an indication of its physical distance from the core of the amplicon: the less frequently amplified probes may be the more peripheral. Schematic diagrams, based on this model are shown in Fig. 6; notably, a similar probe order appears to exist in both neuroblastoma cell lines and in primary neuroblastomas. Federspiel et al. (31) have described an analogous situation when amplification of the dihydrofolate reductase gene was selected for in mouse cells by exposure to methotrexate: the same chromosomal region was amplified in different cell lines, but the amplified segments were extended to different lengths.

It is not possible at this point to estimate the actual distance between the different fragments in the various tumors. Amplification of several hundred kilobases of DNA around the dihydrofolate reductase gene is common in methotrexate resistant cells (31–35). The size of the amplicon in the neuroblastoma IMR-32 has been estimated to be 3000 kilobases (3); therefore, it is reasonable to assume that the probes used in this study define a chromosomal region of several hundred kilobases. Additional evidence supports this assumption that pG21 and probe 8 are probably close to each other (Fig. 6); however, following a “gene walking” experiment in which 30 kilobases of genomic DNA spanning probe 8 were cloned using a phage recombinant library, no homology to probe G21 was found in this segment (data not shown). If the distance between probe 8 and pG21 is at least several tens of kilobases, the distance between NB-19-21 and NB-9-A may well be on the order of several hundred kilobases.

The novel joints shown here indicate extensive and variable rearrangement of the amplified segments. It should be noted here that Nau et al. (14) also showed a novel joint detected by an N-myc probe in a small cell lung carcinoma cell line. Rearrangement thus appears to be almost typical when there is experimental selection for amplification of specific genes in vitro (31–35). Federspiel et al. (31) followed the formation of novel joints in the amplified region flanking the dihydrofolate reductase gene over long periods of continuous selection with methotrexate and could show an intensive dynamics of repeated rearrangements accompanying the amplification process. Ardesir et al. (36) made similar observations regarding amplification of the gene for the multifunctional protein CAD, consisting of carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase in Syrian hamster cells and noted the possibility that certain sequences are “hot spots” for recombinational events. It is, however, of interest that Montoya-Zavala and Hamlin (37) did not notice any rearrangements in contiguous 150 kilobases of amplified DNA spanning the dihydrofolate reductase gene in four Chinese hamster ovary cell lines.

Multiple novel joints might in fact be expected since phenomena such as relocation of the amplified sequences between different chromosomes (3, 5, 6, 10, 15, 16) and the splicing together of distant DNA fragments to form a novel amplified unit (9) have already been demonstrated in neuroblastoma cells. The novel joints can therefore be a result of internal rearrangement of the primary amplified DNA domain or recombination between remote sequences from the same chromosome or from different chromosomes. Additional evidence for the variability of these joints is supplied by the different patterns observed on the Southern blots; extra bands amplified concomitantly with the normal bands (see probe 8 and N-myc in tumor 86), or the appearance of an extra amplified band while the original one remains in single copy (see probe 8 in NB-9).

Since most of the probes used in this study detected novel joints, it is not clear whether any of these probes are especially prone to rearrangements (hot spots) (25) or whether the phenomenon is characteristic of any fragment cloned from the amplified region. We are now isolating and have started to sequence the recombinational joints detected by probe 8 in an attempt to reveal possible characteristics of the DNA sequences taking part in these events. Such studies as well as the present work are intended to provide a clearer picture of the molecular mechanisms involved in DNA rearrangements and amplification.

REFERENCES


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