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Extrachromosomal amplification mechanisms in a glioma with amplified sequences from multiple chromosome loci

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Accumulation of extrachromosomal DNA molecules (double minute) is often responsible for gene amplification in cancers, but the mechanisms leading to their formation are still largely unknown. By using quantitative PCR, chromosome walking, *in situ* hybridization on metaphase chromosomes and whole genome analysis, we studied a glioma containing four extrachromosomally amplified loci (7p11, 1q32.1, 5p15 and 9p2). Complex extrachromosomal DNA molecules were formed by the fusion of several syntenic or non-syntenic DNA fragments from 7p11, 5p15 to 9p2. Fragments ranged from a few base pairs to megabase pairs. Scars of the amplification process remained at the original locus in the form of deletions or chromosome rearrangements. Chromosome fragmentation, due to replication stress, could explain this complex situation. In contrast, at 1q32.1, the initial extrachromosomal DNA molecule resulted from the circularization of a single fragment associated with an intrachromosomal deletion including, but larger than, the amplified sequence. The nature of the sequences involved in these rearrangements suggests that a V(D)J-like illegitimate recombination contributes to its formation.

INTRODUCTION

Gene amplification is one of the genomic alterations found in cancer cells by which proto-oncogenes may be activated. Amplified sequences may be found within homogeneously staining regions of the chromosomes (hsr) or on autonomously replicating circular and acentric extrachromosomal DNA molecules, named double minutes (dmins). The size of dmins ranges from a few hundred kilobases to megabases.

Currently, there are few data available on the precise molecular structure of dmins. Data were obtained by studying amplified mutants selected *in vitro* for their resistance to various cytotoxic drugs. In some cases, the amplified fragment was apparently extruded from the chromosome and circularized without further rearrangements (1). In other cases, each circular element comprises two copies of the amplified sequence linked in inversed orientation (2,3). However, more complex structures were also found, for example dmins may contain multiple copies of the same sequence or several sequences originating from various loci (4–8). Several mechanisms have been proposed to explain the formation of dmins: (i) circularization of a DNA fragment after chromosome breakage across replication bubbles at stalled folks (9); (ii) looping out of chromosome fragments in G1 or G2 phase with (10) or without (11,12) deletion of the corresponding sequence from the chromosome; (iii) circularization of the products of chromosome fragmentation process (13,14). These studies suggested that different mechanisms may drive extrachromosomal amplification.

Molecular analyses were also performed to determine the structure of dmins found in cancers. For example, dmins are present in up to 40% of glioblastomas, and most often bear

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the epidermal growth factor receptor (EGFR) gene. By studying a series of seven gliomas in which EGFR was amplified, we have previously shown that all the amplicons of a given tumor derive from a single founding extrachromosomal DNA molecule (15). In each tumor, the founding molecule was generated by a simple event leading to circularize a segment from band 7p11.2, overlapping the EGFR gene. The signature of microhomology-based non-homologous end joining (NHEJ) was observed at all junctions. In this series of glioma, the intrachromosomal EGFR locus was never deleted or rearranged, suggesting that a post-replicative event was responsible for the formation of each initial amplicon (15). In hematological malignancies, dmins are infrequently observed. In a follicular lymphoma in which the REL gene was amplified on dmins, the intrachromosomal copies of the gene were not deleted (16). In contrast, in acute myeloid leukemia and myelodysplastic syndrome, a deletion of the same size or larger than the amplicon was often observed at the MYC locus on one chromosome 8, suggesting that the corresponding sequences popped out of the chromosome and formed the initial extrachromosomal molecule (17–22). The same mechanism was proposed for amplification of the NUP214-ABL1 gene fusion in T-cell acute lymphoblastic leukemia (23). In the few analyzed cases, the microhomology-based NHEJ was involved in the formation of dmins (19). Thus, data are now available suggesting that, in human cancer cells, different mechanisms lead to the formation of extrachromosomal elements comprising a single segment. In contrast, very little is known about the structure and mechanisms of formation of complex extrachromosomal amplifications involving multiple loci.

We analyze here a glioma containing four chromosome loci (7p11, 1q32.1, 5p15 and 9p2) amplified in dmins. In 7p11, 5p15 and 9p2, a replication stress may have led to chromosome fragmentation and the formation of complexes extrachromosomal DNA molecules by the fusion of several syntenic or non-syntenic DNA fragments. In contrast, at 1q32.1, a V(D)J-like illegitimate recombination could result in an intrachromosomal deletion associated with the circularization of the initial extrachromosomal DNA molecule.

RESULTS

Structure of the amplicons

Previous studies using chromosomal comparative genomic hybridization (CGH) have shown that four loci (1q32.1, 5p15, 7p11 and 9p2) are amplified in glioblastoma 26 (24). FISH with BACs overlapping each amplified region established that the amplified sequences are present on dmins (Fig. 1A and B and not shown). The structure of the amplicons was determined by quantitative PCR. The first primer pairs used covered the chromosome regions identified by CGH. Once the regions of switch in DNA copy number were roughly identified, the transition points were more precisely mapped by scanning each region with a series of closed primer pairs. Chromosome walking from both extremities of these amplified segments was then performed to identify the junctions. We characterized five types of circular DNA molecules, hereafter named amplicons (Fig. 2 and Table 1).

Three levels of amplification were observed at 7p11.2 corresponding to partially overlapping segments of about 400, 180 and 50 kb, respectively (Supplementary Material, Fig. S1). Three amplicons contain fragments from this locus. Amplicon 1, amplified about 8-fold, corresponds to the circularization of a 406 kb-long DNA fragment from 7p11.2. Amplicon 2, amplified about 8-fold, contains a 182 kb-long DNA fragment from 7p11.2 associated with two small fragments of 84 and 292 bp, originating, respectively, from 5p15.1 and 7p11.2. The 292 bp fragment normally lies 500 kb centromeric to the other 7p11.2 fragment of the amplicon. Amplicon 3, amplified some 250-fold, contains a 52 kb-long fragment from 7p11.2 associated with a fragment of 375 kb from 5p15.2. Amplicon 4, is formed by circularization of a 1 Mb-long fragment from 1q32.1, and is amplified about 10-fold. Amplicon 5 is 4.6 Mb-long and amplified some 15 times. It results from the association of six fragments, ranging from 0.14 to 2.28 Mb. Four of them come from different regions of chromosome 5 (5q11.2, 5p15.1, 5p15.2 and 5p15.33), and the last two from chromosome 9 (9p22.3 and 9p24.2).

Thus, 13 DNA fragments from four chromosomes underwent amplification in this tumor, giving rise to five types of amplicons. The amplification factor is constant along each type of amplicon, independently of the origin of the sequence. Two-color FISH experiments with BAC covering various amplified fragments have established that all types of amplicons coexist in each cell of the tumor (Fig. 1B and not shown).

Characterization of the junctions on extrachromosomal molecules

We identified 13 junctions and determined their nucleotide sequence, which allowed us to analyze the sequence of 26 breakpoints (Supplementary Material, Fig. S2). Ten (40%) involved interspersed repeated elements. The same interspersed repeated element was found on both sides of two junctions (amplicon 1 and amplicon 5, junction g–e) but the fusion took place in non-homologous sequences. In eight cases interspersed repeated elements of the same family were not found on both sides of a junction. Low-copy repeats or segmental duplications were not present in the involved regions (not shown). Thus, the rearrangements identified here were not generated by illegitimate homologous recombination events. In addition, we searched for, but failed to identify, consensus matches to several sequence motifs previously associated with chromosome rearrangements (Supplementary Material, Supporting Methods S1). However, in amplicon 4, a variant of the recombination signal sequence (RSS) involved in the V(D)J recombination process was found at the junction (see below). In six cases, microhomologies of 1–3 bp in length were found in the normal counterparts of the fusion and maintained as a single copy in the junction (Supplementary Material, Fig. S3). Three insertions of 1 bp were also observed. These data suggest that, as previously found in other glioblastomas (15), microhomology-based mechanisms are involved in the formation of the junctions.

Chromosomal status of the amplified loci

We analyzed the status of the four chromosomes involved in the amplification process by FISH with probes specific to

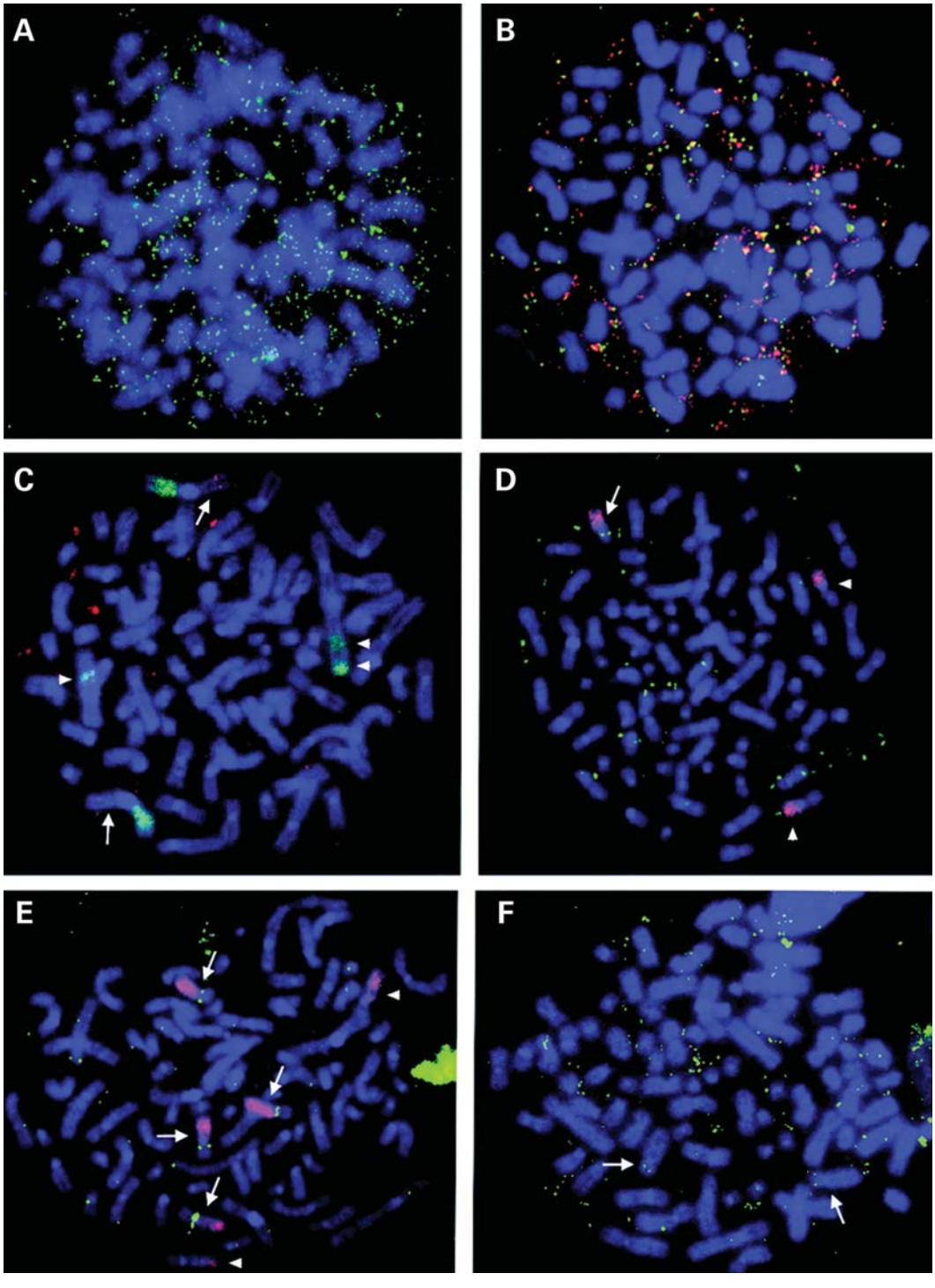

Figure 1. FISH chromosome analysis. (**A**) Hybridization of RP11-433C10 (green) localized at 7p11.2; a high number of double minutes (dmins) are labeled. (**B**) Co-hybridization of RP5-1091E12 (green), localized at 7p11.2 and RP11-803F2 (red), localized at 5p15.2; the probes reveal different sets of dmins, however, dmins bearing the two sequences could be observed (yellow labeling). (**C**–**F**) Co-hybridizations using a chromosome arm specific painting and a probe corresponding to an amplified segment. (C) Chromosome 1 short arm painting (green) and RP11-832D8 localized at 1q32.1 (red); the BAC hybridized on the two normal chromosomes (arrow) and not on the chromosome rearranged in 1p (arrow head); the deleted 1p fragment of the rearranged chromosome was translocated on another chromosome (double arrow heads). (D) Chromosome 9 long arm painting (red) and RP11-265C24 (green) localized at 9p22.3; the BAC hybridized on the single normal chromosome present in the cells (arrow) and not on the two rearranged chromosomes (arrow head). (E) The RP11-23F4 probe (green), localized in the amplified region of the 7p11.2 locus present only in amplicon 1, labeled only a few dmins and allowed characterization of the intrachromosomal locus; four of the six chromosomes 7, identified by their chromosome long arm painting (red), were labeled by the probe (arrow), the two others were not labeled (arrow head). (F) Hybridization of RP11-803F2 localized at 5p15.2, FISH signals (green) were visible on the two chromosomes 5 at the expected position. Chromosomes were stained in blue with DAPI. See supporting information Supplementary Material, Figure S3 for complementary data.

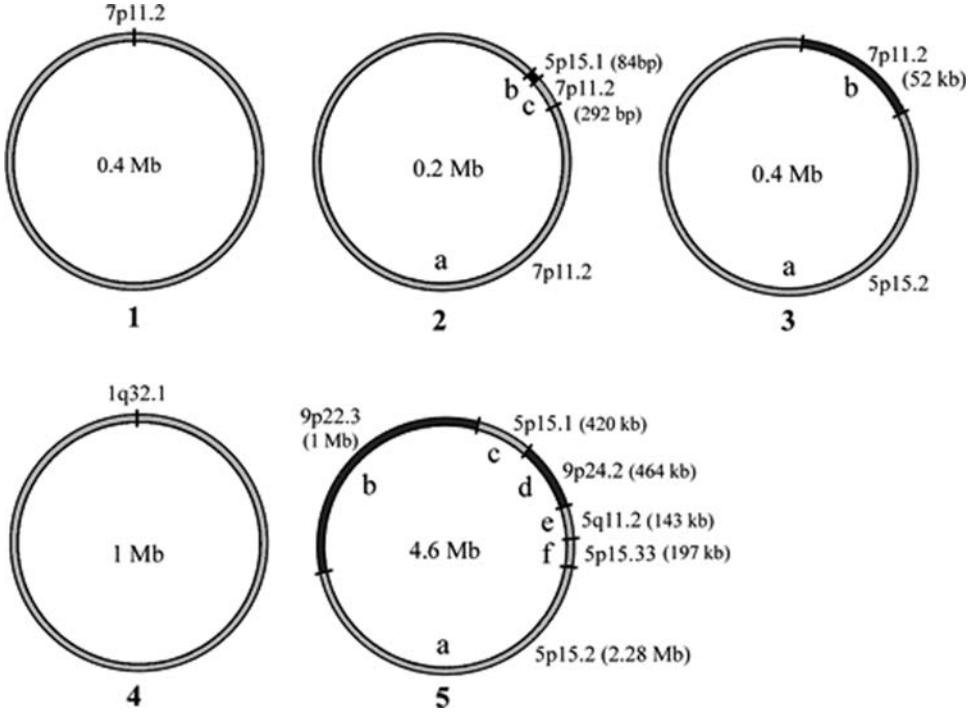

Figure 2. Structure of the amplicons. The five extrachromosomal circular DNA molecules present in tumor 26 are presented. The total length of the amplicons is indicated in the circle with the position of the constituting fragments. The chromosome origin and the length of the fragments are listed outside the circles. Fragment lengths are not to scale.

Table 1. Copy number, localization and size of the amplicons

Amplicon[a]	Fragment[a]	Band[b]	5′ Breakpoint[c]	3′ Breakpoint[c]	Length[d]	Strand[e]
1 (×8)		7p11.2	54 871 384	55 277 220	405 837	+
2 (×8)	a	7p11.2	55 109 202	55 291 006	181 805	+
	b	5p15.1	16 111 003	16 111 085	83	+
	c	7p11.2	55 695 353	55 695 641	292	−
3 (×250)	a	5p15.2	11 481 177	11 855 754	374 578	−
	b	7p11.2	55 192 562	55 245 040	52 479	+
4 (×10)		1q32.1	201 939 768	202 925 623	985 856	+
5 (×15)	a	5p15.2	11 206 687	13 486 241	2 279 555	+
	b	9p22.3	14 429 810	15 500 315	1 070 506	+
	c	5p15.1	15 773 175	16 193 433	420 259	+
	d	9p24.2	2 551 839	2 976 369	424 531	−
	e	5q11.2	56 390 561	56 533 655	143 095	+
	f	5p15.33	1 157 330	1 353 928	196 599	+

[a]Amplicons are extrachromosomal circular DNA molecules comprising a single fragment or several fragments from various regions of the genome; the level of amplification is given in brackets.
[b]Position of the fragment on normal chromosomes.
[c]5′ And 3′ positions of the ends of the amplified fragments.
[d]Length of the amplified fragments in base pairs.
[e]Orientation of the fragment in the amplicon as compared with it position in the normal chromosome.

chromosome arms (Supplementary Material, Fig. S4). Our results confirm previous data showing that cells of tumor 26 contain highly rearranged chromosomes and are near tetraploid (24). The presence of two copies of several marker chromosomes indicates that passage to tetraploidy took place after the formation of at least some of the rearrangements.

We then attempted to determine the structure of the chromosomes in the regions from which the amplified segments originate, by performing FISH experiments with BACs specific to these sequences. We found that the 1q32 segment of amplicon 4 is maintained on the two normal chromosomes 1 present in these cells, but is deleted from a third chromosome 1 with a rearranged short arm (Fig. 1C). The amplified 7p11.2 sequences were found to be deleted from two of the six copies of chromosome 7 present in these cells (Fig. 1E). We also showed that the 5p15.2 locus of amplicon 5 is maintained on the two normal copies of this chromosome (Fig. 1F) and that the two regions of chromosome 9

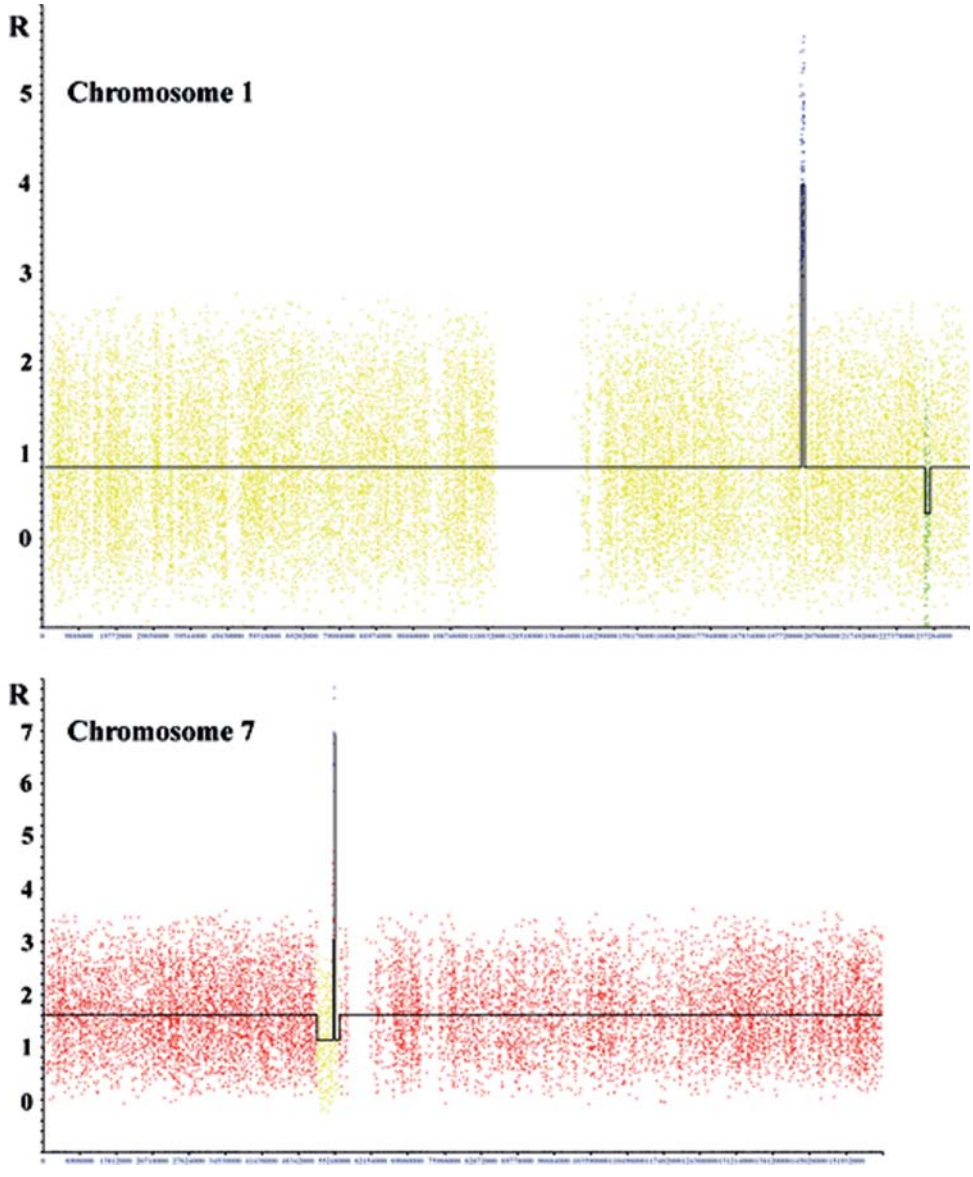

Figure 3. Genomic profiles of chromosomes 1 and 7 of tumor 26 normalized using the ITALICS algorithm. R is the ratio, at each SNP position, between the copy number measured in the near tetraploid tumor cells and in normal diploid cells. Regions with no or low DNA copy number alteration are in yellow, with gains in red, with losses in green and with amplifications in blue. The black line represents the smoothing line. Chromosome 1: three chromosomes 1 are present in the tumor cells, corresponding to the loss of one chromosome in the tetraploid cells and to an *R* value of 0.8 for normal chromosome regions, the 232–233 Mb region is deleted in one chromosome and the 201–202 Mb region is amplified. Chromosome 7: six chromosomes 7 are present in the tumor cells, i.e. a gain of two chromosomes in the tetraploid cells, thus $R = 1.6$ in the normal chromosome regions, the amplified region (54.8–55.3 Mb) is localized in a region (52.1–57.1 Mb) deleted in two chromosomes.

found in amplicon 5 are maintained on the single normal chromosome 9 present in these cells (Fig. 1D and not shown). The DNA copy number variations were also measured using microarrays. No DNA copy number variations could be observed in the sequences either side of the amplified region in 1q, but a deletion of 1.44 Mb was found in one chromosome, 31 Mb telomeric to the amplified region (Fig. 3). A deletion of about 5 Mb surrounded the amplified region in one-third of the chromosome 7 (Fig. 3). Chromosomes 5 and 9 displayed complex situations with numerous DNA copy number changes in the chromosome regions from which the amplified sequences originate (Supplementary Material, Fig. S5). Thus, in this glioma, we detected chromosomal scars of amplification, a situation that differs notably from that previously found in a series of glioma with a simple amplification of the EGFR locus, in which no deletion or rearrangement was observed ((15) and unpublished data). This suggests that different mechanisms of amplification drive the formation of simple and complex amplicons.

Characterization of the chromosome deletion associated with the amplification at 1q32.1

Amplicon 4 was formed by circularization of a 1 Mb-long fragment from 1q32.1. By chromosome walking from the chromosome sequence centromeric to the amplified segment,

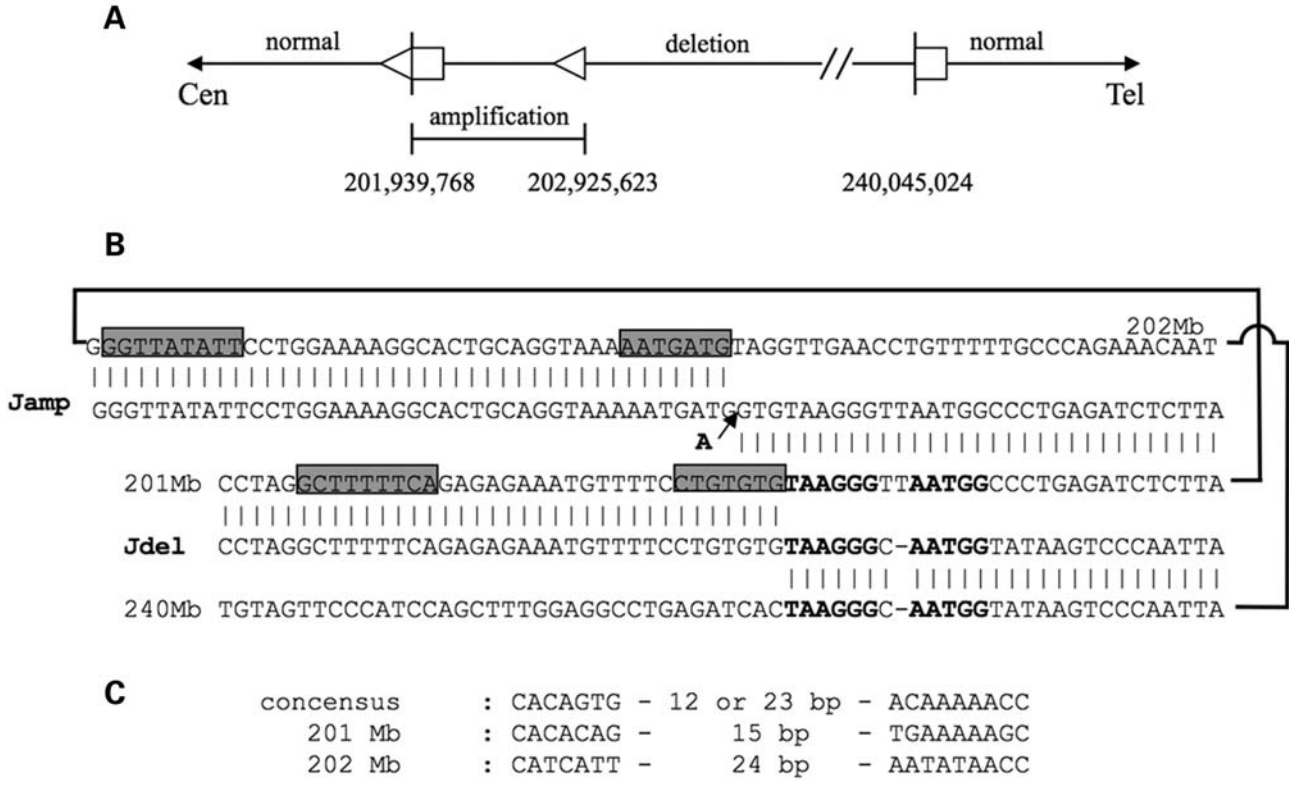

Figure 4. Sequences of chromosome 1 involved in the formation of the extrachromosomal DNA molecule and of the intrachromosomal deletion. (**A**) Summary of the structure of the locus; the positions on the chromosome sequence of the ends of the amplified and deleted regions are indicated, triangles: RSS variants, squares: short homologous sequences. (**B**) The sequences of the junction of the deletion (Jdel) and of the amplicon 4 (Jamp) are aligned with respect to the sequences of the normal counterparts. Positions on chromosome 1 of the normal sequences are given in megabase. The insertion of an A was observed at Jamp (arrow, see Supplementary Material, Fig. S2). Microhomologies are shown in bold and RSS sequences are boxed. (**C**) Comparison between the consensus RSS sequence and the variants found at positions 201.9 and 202.9 Mb on chromosome 1.

we characterized a deletion associated with the amplification. This deletion extends about 38 Mb beyond the amplified sequence toward the telomere (Fig. 4A). The sequence centromeric to the break, mapping at 1q32 (position 201 939 768), is fused to a sequence localized at 1q43 (position 240 045 024; Fig. 4A and Supplementary Material, Fig. S3). The fusion took place 3 bp telomeric to the 5' breakpoint of amplicon 4. A microhomology of 6 bp (possibly 11 bp if two mismatches are accepted) was present at the junction (Fig. 4B). This deletion was not detected upon analysis of copy number variations by SNP microarray (Fig. 3) suggesting that the deleted segment was retained in the genome. Indeed, using chromosome 1q specific painting, we detected a partially labeled marker chromosome in addition to the three copies of chromosomes 1 (Supplementary Material, Fig. S4). This translocated fragment is likely to be the fragment deleted from chromosome 1. Examination of the sequences in the vicinity of the junctions of the amplified and deleted segments (Fig. 4B and C) revealed variants of RSS involved in the V(D)J recombination process (25). A typical RSS contains moderately well conserved heptamer (consensus 5′-CACAGTG-3′) and nonamer (consensus 5′-ACAAAAACC-3′) sequences separated by about 12 or 23 bp of not conserved spacer (26). At position 201.9 Mb, a heptamer 5′-CACACAG-3′ is associated to an 5′-TGAA AAAGC-3′ nonamer by 15 bp spacer whereas at position 202.9 Mb, 24 bp separate the 5′-CATCATT-3′ and the 5′-AATATAACC-3′ sequences (Fig. 4). Since highly divergent RSS variants are active in the formation of the variable region of the immunoglobulin (IG) and T-cell receptor (TCR) as well as in illegitimate chromosome rearrangements (27), a V(D)J-like-mediated translocation mechanism could have been involved in the complex amplification/deletion observed at this locus. However, no significant expression of the RAG1 and RAG2 genes was found in the tumor cells (not shown).

DISCUSSION

We have analyzed a glioma containing five distinct types of extrachromosomal circular DNA molecules (amplicons) resulting from the fusion of several DNA fragments from various chromosome localizations or resulting from the circularization of a single fragment. The lack of homologies between fused chromosome regions exclude homologous recombination mechanisms for amplicon formation. The presence of microhomologies at some junctions show that microhomology-driven non-homologous end-joining is involved as previously found for a series of gliomas in

which only the EGFR locus was amplified after circularization of a single fragment. In this series, no deletion or chromosome rearrangement was observed at 7p11.2, suggesting that a post-replicative event was responsible for the formation of the amplicons ((15) and unpublished data). In striking contrast, in tumor 26, scars of the amplification process, such as deletion or chromosome rearrangements, were found at the original intrachromosomal position (Figs 1 and 3 and Supplementary Material, Figs S4 and S5).

Our finding that non-contiguous fragments of different size, originating from different chromosomes joined on the same amplicon (Table 1 and Fig. 2), suggests that the initial step of amplification was the simultaneous fragmentation of large chromosome regions. This type of chromosome destabilization could be induced by replication stress. In the apoptotic independent mechanism analyzed by Stevens *et al*. (28), fragmented chromosomes are condensed and have a grouping and localization similar to what would be expected if that chromosome was intact. Such chromosome fragmentation occurs spontaneously at a low level in numerous cell lines, at frequencies that correlate with the level of genomic instability. It is also induced by treatment with methotrexate, which inhibits DNA synthesis by depletion of the nucleotide precursor pool, or with doxorubicin, an inhibitor of topoisomerase II, an enzyme involved in replication fork progression (28). Moreover, spontaneous chromosome fragmentation is high in $ATR^{-/-}$ knockout mouse embryonic cells, likely due to cells entering mitosis with DNA damage and ineffective G2 checkpoint inactivation (29). The consequences of the chromosome fragmentation depend on its extent. When numerous or all chromosomes are involved, cell death is induced. In contrast, when only one or a few chromosomes are fragmented, the cells are expected to survive, and it has been proposed that incomplete chromosome fragmentation can potentially lead to chromosome rearrangements or dmins formation (28). Thus, we propose that a replication stress induced the partial fragmentation of chromosomes 5, 7 and 9 in the tumor 26. Some of these fragments fused by NHEJ and generated the initial extrachromosomal circular DNA molecules. The associations between fragments could be driven by chromosome proximity in the nucleus (30). The remaining chromosome fragments could engage in the formation of rearranged chromosomes or could be lost.

The extrachromosomal DNA molecule corresponding to amplicon 4 resulted from the circularization of a single fragment from 1q32.1, associated with a chromosomal deletion including, but larger than, the amplified sequence. Three chromosome sites were involved, localized at positions 202, 203 and 240 Mb (Fig. 4A). We identified two RSS variants at positions 202 and 203 Mb and two short homologous sequences at positions 202 and 240 Mb (Figs 4B, C and 5A). The presence of the two RSS variants suggests that a V(D)J-like-mediated translocation mechanism was involved. Beside the well-regulated mechanism of formation of the complete VDJ exon coding for the variable region of the IG and TCR (25,31), it has been shown, in hematological malignancies, that the high flexibility of the V(D)J recombination system may drive illegitimate chromosome rearrangements (27,32–34). The RAG1 and RAG2 proteins are able to recognize a large panel of variant RSS and it has been estimated that several millions of cryptic sites dispersed in the genome could potentially mis-target the V(D)J recombination process (35). Moreover, the characterization of chromosome translocations leading to ontogeny activation in lymphoid malignancies demonstrated that broken ends fortuitously introduced in a chromosome in a random sequence may infiltrate or be captured by the post-cleavage RAG complex generated as an intermediate during V(D)J recombination, and that three joining products may be produced (27). Thus, the V(D)J-mediated translocation mechanisms are very flexible and able to manage a large panel of situations during DNA end processing. Up to now, such mechanisms were observed only during lymphoid differentiation and in hematologic malignancies. Nevertheless, the structure of the junctions argues for a V(D)J-like-mediated translocation mechanism in the deletion/amplification observed at 1q32.1. Indeed, DNA double-strand breaks were introduced at the 5′ limits of the heptamer of the two RSS variants as found in the legitimate V(D)J-mediated translocation and strand exchanges as well as final joining products are strongly reminiscent of those found in some illegitimate recombinations observed in lymphoid malignancies (27). It should be noted that a V(D)J-like-mediated translocation mechanism requires the RAG1 and RAG2 proteins. These proteins are thought to be expressed exclusively in B and T cell precursors, nevertheless transcripts have been found in the mouse central nervous system or derived cell lines (36,37). We found that RAG1 and RAG2 genes are not expressed at a detectable level in the cells of tumor 26. However, they may have been transiently expressed during tumor development and then switched off, as observed in lymphocyte differentiation (25). The formation of the extrachromosomal DNA molecule and of the intrachromosomal counterpart requires a coordinated succession of events suggesting the formation of a DNA/protein stable complex during the rearrangement process (Fig. 5B). A series of steps of DNA strand breaks and repairs may be proposed for the formation of the observed final products (Fig. 5C and D). In this mechanism, a new role for microhomologies is suggested in the DNA repair process. When microhomologies are present, it is assumed that illegitimate pairing of distant sequences occurs after double-strand breaks, followed by excision of the intervening regions and ligation, a single copy of the microhomologous sequence remaining at the junction. In the present case, each copy of the microhomologous sequences remains in distinct DNA molecules. The presence of the short homologous sequence at positions 202 and 240 Mb may have favored the strand exchange between these chromosome segments rather than between the segments bearing the RSS variants as generally observed in the V(D)J recombination.

In conclusion, we have shown that during tumor formation several mechanisms of extrachromosomal amplification such as chromosome fragmentation, V(D)J-like-mediated translocation or post-replicative circularization of a single fragment (15) could take place in glial cells. A definitive answer concerning the actual recurrence of the proposed mechanisms will be possible only after characterization at the nucleotide level of series of paired extrachromosomal circular DNA molecules and intrachromosomal scars in various tumor types.

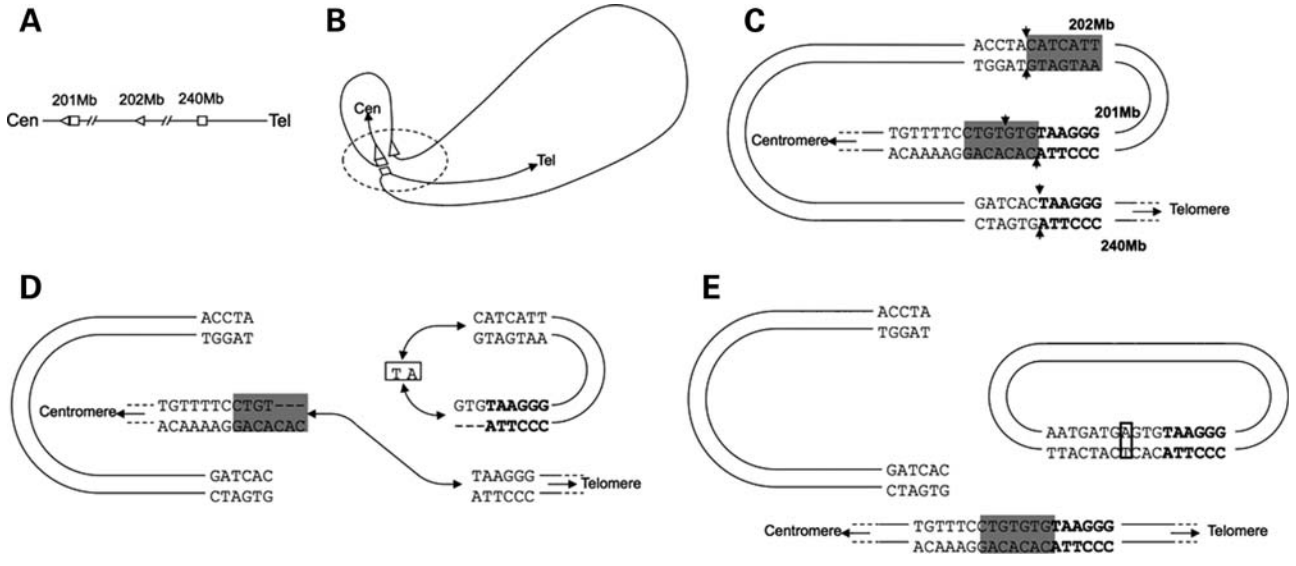

Figure 5. Model for the formation of the deletion-amplification at chromosome 1. (**A**) Positions of the three involved chromosome sites localized at positions 202, 203 and 240 Mb; 2 RSS variants (triangles) and two short homologous sequences (squares) are present. (**B**) Loop formation: the setting side by side of these sequences in a DNA/protein complex (dotted cycle) induced the formation of two DNA loops. (**C**) Relative position of the sequences in the complex; heptamers of the RSS are highlighted, microhomologies are in bold; arrow heads: position of the DNA strands breaks; two nicks are introduced at position 202 Mb, 3 bp apart, with the formation of 5′ protruding ends corresponding to the GTG sequence present in the normal chromosome between the positions of the junctions in the extrachromosomal DNA molecule and in the intrachromosomal deletion (Fig. 4B); two blunt-end breaks are also formed, at positions 203 and 240 Mb. (**D** and **E**). Fusions between broken fragments and final products; double-headed arrows link fragments to be fused, box: A:T base pair insert; after strand exchange between ends at positions 202 and 240 Mb, filling of the 5′ overhang and ligation, the deleted chromosome may be formed; the ligation, after insertion of an A/T bp, of the other 202 Mb filled end with one of the broken ends at position 203 Mb generate the extrachromosomal DNA molecule; the unamplified third fragment, of about 37 Mb, is mainly maintained in the cells since only a 1.44 Mb segment was partially lost in the tumor (Fig. 3). The remaining part is likely included in the marker chromosome labeled by FISH with the 1q chromosome painting (Supplementary Material, Fig. S3B).

MATERIALS AND METHODS

Biological material

The glioblastoma multiforme (tumor 26) was collected at the Hôpital de la Salpêtrière (Paris). Informed consent was obtained from the patient. The tumor was grown as xenografts in athymic mice and recovered for analysis at passage 2 or 3. Cytogenetic analysis has been previously published (24).

Fluorescent *in situ* hybridization

Cell preparations were obtained after short-term culture (1–2 days) of tumor fragments. Metaphase spreads were hybridized with BAC (BACPAC Resources, Children's Hospital Oakland Research Institute, Oakland, CA, USA) or chromosome-specific paintings (MetaSystem, Altlussheim, Germany) as previously described (15).

Amplicon analysis

The level of amplification was measured by real-time quantitative PCR using the 7500 Real-Time PCR System and SYBR Green PCR kits (Applied-Biosystems), as previously described (15). The nucleotide sequences of primers are available upon request. Amplification levels were calculated using a standard curve constructed with serial dilutions of control DNA amplified in a parallel experiment (DNA from normal lymphocytes). The ends of each amplicon were localized by chromosome walking using the Universal Genome Walker kit (BD Biosciences). PCR fragments corresponding to the junctions were directly sequenced using Big Dye Terminator Sequencing kits (Applied Biosystems). Sequence data used in this work refer to the human genome sequence (released March 2006) available at the UCSC Genome Bioinformatics site (http://genome.ucsc.edu/) (38).

DNA copy number determination

The Affymetrix GeneChip Human Mapping 500K set (StyI array) was used to detect copy number alterations. Genomic DNA was prepared for microarray hybridization as previously described (39). Washing, staining and scanning of chips were performed using materials and methods provided by the manufacturer. Labeling and hybridization were performed in the Affymetrix Core Lab facility, (Translational Research Department, Institut Curie, Paris, France). DNA samples were processed following the instructions of the GeneChip Human Mapping 500K Set manual (Affymetrix). The preprocessing of microarray data was done using the iterative and alternative normalization of copy number SNP array (ITALICS) algorithm (40) with default parameters. Briefly, ITALICS alternatively estimates the biological signal (i.e., the DNA copy number at each SNP locus) with the Gain and Loss Analysis of DNA algorithm (41) and normalizes the data to correct the non-relevant effects (CG content and fragment length of PCR products, oligonucleotide CG content and SNP effect). These two steps are repeated iteratively to improve the biologic signal estimation until no more improvement is seen.

The result of this process is a segmented genomic profile that consists of regions of constant DNA copy number. Each region is given a smoothing value (i.e., the median of the SNP copy numbers within the region) and a status (i.e., amplification, gain, normal or loss).The profiles were visualized and analyzed with the VAMP software (42). Microarray data were recorded to the ArrayExpress database (accession: E-MEXP-2423).

RAG1 and RAG2 expression

RAG1 and RAG2 expression was measured by real-time fluorescent quantitative RT–PCR. cDNAs were prepared using the SuperScript II system according to the manufacturer's protocol (Invitrogen) and amplified using the GeneAmp 7500 sequence detection system and SYBR Green PCR Kits (Applied Biosystems).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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