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Genetics

Gene amplification accompanied by the loss of a chromosome containing the native allele and the appearance of the amplified DNA at a new chromosomal location

(oncogene amplification/chromosome loss/DNA transposition/fluorescence *in situ* hybridization/inverted repeats)

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ABSTRACT **The organization of amplified DNA in mammalian cells in the form of inverted repeats rather than tandem repeats was first observed and studied in the 3B rat cell line. The structure and chromosomal location of the amplified inverted duplications in this cell line have been further analyzed by cloning, long-range mapping, and fluorescence *in situ* hybridization. The amplification unit is at least 450 kilobases in size and all of the amplicons are located in a single chromosomal location of approximately 10 or 11 megabases. No heterogeneity in either size or molecular structure is detected between the 3B amplicons, indicating that the 20- to 40-fold amplification occurred in a *single event* and not through a series of events, which would result in heterogeneity among the amplicons. Thus the amplification in 3B cells may reflect more closely the situation seen in tumors containing amplified oncogenes/protooncogenes than the amplifications present in cell lines after multiple selections with cytotoxic drugs. The progenitor Rat-2 cell line contains three alleles of the region of DNA that is amplified in 3B cells; two are located on the two normal homologues of rat chromosome 2 and the third is at the equivalent position on a marker chromosome, der(3)t(2;3). 3B cells contain only *one* of the two normal homologues of chromosome 2 in addition to chromosome der(3)t(2;3). All of the amplified DNA is located on a new marker chromosome, M2, whose amplified DNA region does not resemble chromosome 2. These results are consistent with the amplification model proposed by Passananti *et al.* [Passananti, C., Davies, B., Ford, M. & Fried, M. (1987) *EMBO J.* 6, 1697–1703], in which the excision from a chromosome of the DNA to be amplified results in the loss or rearrangement of that chromosome. In this model the excised DNA can be amplified extrachromosomally *during a single S phase* before becoming stabilized by integration into a chromosome, probably at a different location to that of its unamplified allele.**

Gene amplification results in the increased expression of a particular gene through an increase in its copy number rather than through any alteration in its transcriptional or translational regulation. In some lower eukaryotes, gene amplification can occur as part of an organism's developmental program (1). However, in mammals gene amplification is often observed in transformed or neoplastic cells and its study in such systems is of great importance as amplification of various different protooncogenes (e.g., c-*myc*, N-*myc*, *ras*, *erbB*) has been implicated in carcinogenesis (2). Gene amplification has been studied as a model system *in vitro* after multiple rounds of selection of cultured mammalian cells to resistance to cytotoxic drugs that affect DNA and/or chromosome replication.

It was initially demonstrated that inverted repeats, as opposed to direct repeats, are a common feature of gene amplification in mammalian cells for an amplified polyoma virus oncogene in the transformed 3B rat cell line (3, 4) and subsequently for the amplified c-*myc* protooncogene in various human tumor cell lines and for the amplified *CAD* gene in baby hamster kidney cells selected with the drug *N*-(phosphonacetyl)-L-aspartate (5). Inverted duplications were later detected associated with amplification of *AMPD* (6), *APRT* (7), *CAD* (8, 9), and *DHFR* (10–12). Such findings indicate that the formation of inverted duplications may be a primary event in mammalian gene amplification and consequently new models were postulated to explain the generation of amplified DNA (4, 6).

Regions of amplified inverted duplications containing the polyoma virus (Py) oncogene in the transformed 3B rat cell line have previously been cloned and characterized. The inverted duplication was found to be amplified by 10- to 20-fold (3) and the inversion joint was found to have been created by a simple illegitimate recombination event (4). In this work we have further analyzed the size and structure of the 3B amplicon using pulsed-field gel electrophoresis (PFGE) and have used fluorescence *in situ* hybridization (FISH) to determine the chromosomal location of the amplified DNA in 3B cells as well as the original location, in the progenitor Rat-2 cells, of the DNA involved in the amplification.

MATERIALS AND METHODS

A library of size-selected *Bam*HI fragments was made using λ vector EMBL4 (13). A library of size-selected, partially cut *Sau*3A fragments [average size > 20 kilobases (kb)] was constructed in λ DASH and a library of size-unselected, partially cut *Sau*3A fragments was constructed in λ FIX (Stratagene). The Rat-2 and 3B cell lines and their culture conditions and treatment with 5-azacytidine (5-azaC) have been described (3, 14). High molecular weight DNA from rat cells was prepared in low-melting-temperature agarose plugs and digested with restriction enzymes before being fractionated by PFGE using the LKB Pulsaphor system with either point electrodes or the hexagonal electrode kit (15). Positive bands were detected by conventional Southern blotting with random-primed ^{32}P-labeled probes. Metaphase chromosome spreads from rat cells were prebanded by incubation in 2× SSC (0.3 M NaCl/30 mM sodium citrate) followed by Wright's stain. FISH was performed essentially as described by Pinkel *et al.* (16), except that biotinylated probes were preannealed with sonicated rat DNA.

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Abbreviations: PFG, pulsed-field gel; PFGE, PFG electrophoresis; Py, polyoma virus; 5-azaC, 5-azacytidine; FISH, fluorescence *in situ* hybridization; G, Giemsa.

RESULTS

Short- and Long-Range Mapping and Cloning of the 3B Amplified DNA. Approximately 16.5 kb of the 3B amplicon has been cloned in two overlapping λ clones, λ3B (3) and λBcl (4) (Fig. 1). A 15.0-kb *Bam*HI fragment, which partially overlaps the λBcl insert, was cloned into the *Bam*HI site of EMBL4, in a clone called λBam (Fig. 1). To define the 3B amplicon further, a library of *Sau*3A partially cleaved 3B cellular DNA was cloned in the λ vectors DASH and FIX. The DNA inserts from a number of positive clones, detected by a unique 3.9-kb *Eco*RI–*Bam*HI probe fragment derived from the insert of the λBam, were mapped along the length of the inverted duplication (Fig. 1). Only two of these, λF8 and λF9, extended the cloned region by a significant amount. This region was found to be rich in highly repetitive sequences that inhibited the isolation of useful probes for further cloning of the amplicon using λ vectors. When the cloned unique 3.9-kb *Bam*HI–*Eco*RI fragment was used as a probe no differences could be detected in the bands generated from 3B DNA or the parental Rat-2 DNA with various restriction enzymes (data not shown), indicating that over this region the DNA from 3B and Rat-2 cells is identical.

PFGE of 3B cellular DNA cleaved with rare cutter restriction enzymes was used to investigate the size, structure, and homogeneity of the amplified DNA. A blot of a pulsed field gel (PFG) of 3B cellular DNA cleaved with 10 restriction enzymes and hybridized with an amplification-specific Py DNA probe (only present in the amplified DNA of 3B cells) is shown in Fig. 2. Seven of these enzymes (*Eag* I, *Nae* I, *Nar* I, *Nru* I, *Pvu* I, *Sac* II, and *Sfi* I) did not cleave the 3B amplified DNA into hybridizing fragments of <600 kb (the limit of gel resolution). In addition, it was found that the restriction enzymes *Not* I, *Nru* I, *Sfi* I, and *Sma* I did not cleave the 3B amplified DNA into fragments of <3000 kb (Fig. 3*C*). The discrete *Cla* I band of 240 kb (Fig. 2) is most probably generated by cleavage of two identical *Cla* I sites, each 120 kb away from the center of the inversion joint.

The Py probe detects a series of hybridizing fragments (major ones of 180, 320, and 460 kb) after *Xho* I cleavage of the amplified DNA from 3B cells (Fig. 2). Cleavage with *Sal* I generates faint hybridizing fragments just below 600 kb (Fig. 2), which are more clearly resolved in the PFG blot shown in Fig. 3*A* (gel resolution is 700–750 kb) where a major band of 600 kb and a fainter fragment of 550 kb are seen. We have previously shown, through the use of 5-azaC, that such multiple bands correspond to differential methylation of restriction enzyme cleavage sites present at the same positions within identical 3B amplicons as opposed to heterogeneity in the structure of the 3B amplicons (14). Incorporation of 5-azaC into 3B cellular DNA leads to hypomethylation and

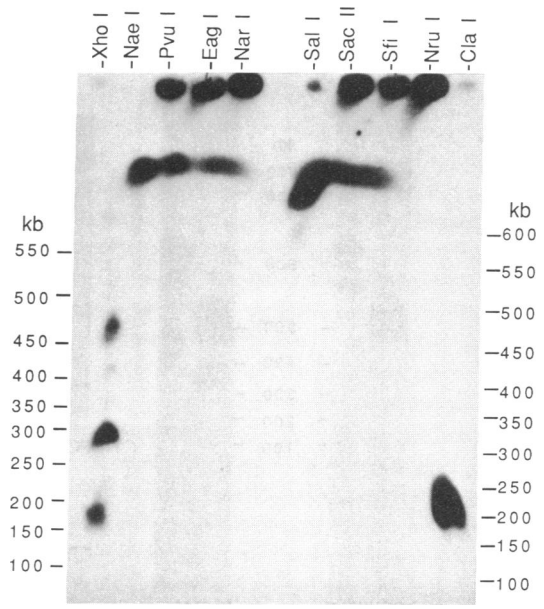

FIG. 2. Southern blot of a PFG of 3B DNA restricted with various enzymes and probed with the Py probe, which only detects amplified sequences. Each track contains 10 μg of 3B DNA digested with the enzyme indicated. The LKB point electrode system was used with a pulse interval of 30 sec at 350 V for 16 hr. Note that DNA fragment sizes are not parallel across the width of the gel owing to the curvature of the electric field.

relieves the inhibition of cleavage by *Xho* I and *Sal* I of sites within the 3B amplified DNA. *Sal* I cleavage of hypomethylated 3B amplified DNA, when compared to methylated 3B amplified DNA, results in a striking increase in the proportion of lower molecular weight fragments (500 and 550 kb) (Fig. 3). A similar Southern analysis of *Xho* I-cleaved, amplified DNA from 3B cells grown in 5-azaC also showed a striking increase in the intensity of lower molecular weight fragments with accumulation of most of the hybridizable DNA in the *Xho* I 180-kb fragment (14). Based on the complete and partial digestion products of 3B DNA cleaved with *Sal* I, *Xho* I, and *Cla* I in the absence and presence of 5-azaC (Figs. 2 and 3) (14, 17), the amplicon is estimated to be at least 450 kb in length and the complete inverted duplication is estimated to be at least 900 kb in length.

In addition to the Py probe, which detects only the amplified DNA in 3B cells (see above), the 3.9-kb *Eco*RI–*Bam*HI unique rat DNA fragment (RI-Bam), located 20.0 kb from the Py sequences in the 3B amplicon (Fig. 1), was also used as a probe for Southern blot analyses (Fig. 3 *B* and *D*). The

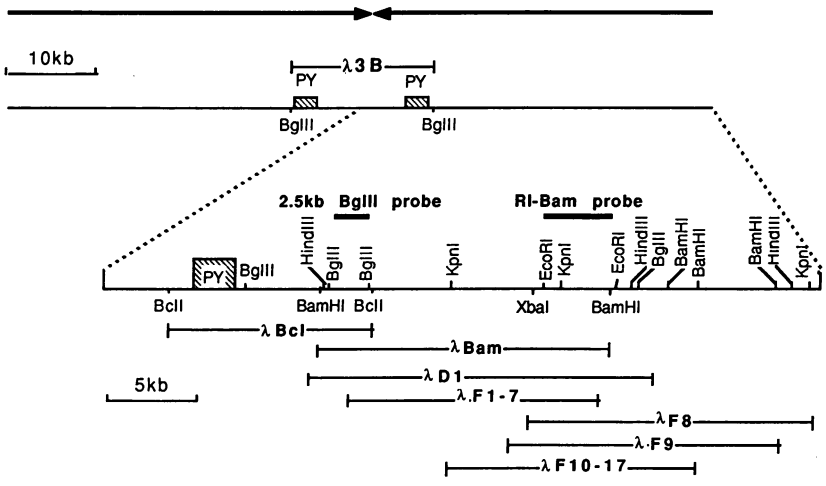

FIG. 1. The upper map shows the cloned region of the inverted duplication amplified in the genomic DNA of 3B cells. The arrows above the map represent the palindromic arms of the inverted duplication. Hatched boxes represent the 2.8-kb Py DNA inserts. The lower map is a detailed restriction map of one arm (40 kb) of the inverted duplication. The two unique DNA probes are shown as solid bars. Inserts of the various λ clones isolated using these probes are presented below the map.

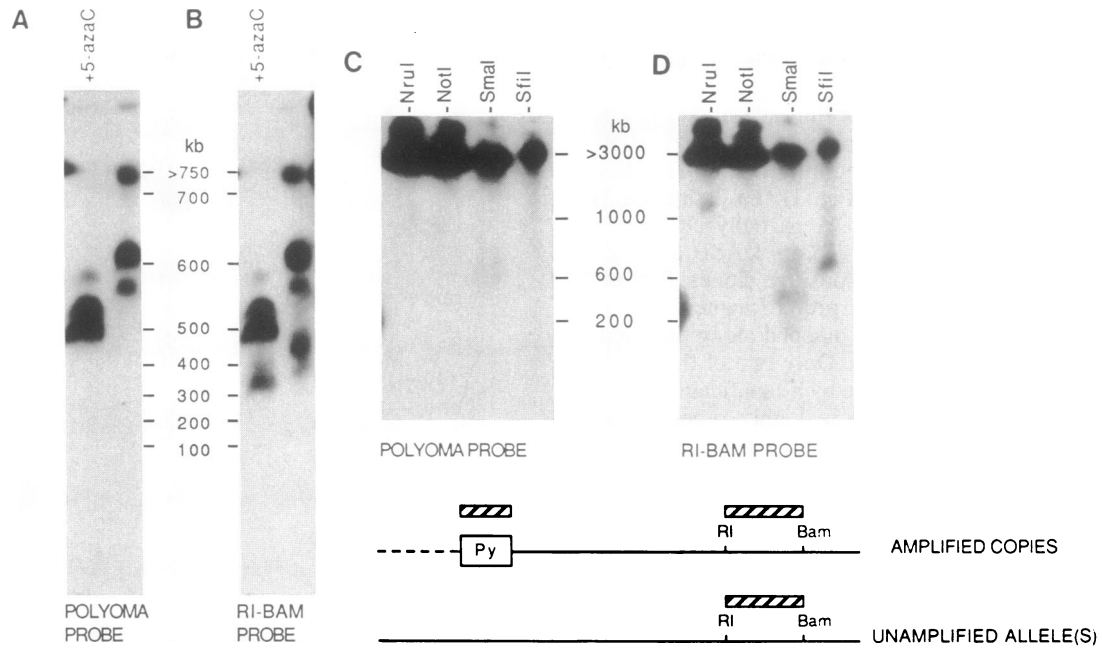

FIG. 3. (*A* and *B*) Southern blot of a PFG of 3B DNA restricted with *Sal* I and hybridized with the Py probe (*A*) and the RI-Bam rat probe (*B*). The DNA in the right-hand track of the gel was derived from 3B cells grown in the absence of 5-azaC, whereas the DNA in the left-hand track (+5-azaC) was derived from 3B cells grown in the presence of 5 μM 5-azaC. The mobilities of the bands in the tracks without 5-azaC are slightly impeded relative to the same bands in the tracks with 5-azaC, with the amplified 600- and 550-kb bands detected with both probes and the 400- and 350-kb unamplified bands detected with the RI-Bam probe being positioned slightly above the corresponding bands in the tracks with 5-azaC. For a more detailed analysis, see ref. 14. The gel was run at 170 V for 18 hr with a pulse interval of 60 sec (size resolution of up to 750 kb). (*C* and *D*) Southern blot of 3B DNA digested with the restriction enzymes indicated above, run on a PFG that resolved fragments up to 3000 kb in size (60 V for 5 days with a pulse interval of 20 min), and hybridized with the Py probe (*C*) and with the RI-Bam rat probe (*D*). The two maps at the bottom illustrate that the Py probe will only detect restriction fragments from the amplified DNA in 3B cells (*A* and *C*), whereas the RI-Bam rat probe will detect the amplified DNA and the unamplified allele(s) of this region of rat DNA (*B* and *D*). Thus the extra bands present in *B* but not in *A*, and in *D* but not in *C*, represent the unamplified rat allele(s).

RI-Bam probe will detect not only the amplified DNA but also the unamplified alleles in 3B and the parental Rat-2 cells. Thus the 350-kb and 400-kb *Sal* I fragments detected by the RI-Bam probe (Fig. 3*B*) but not by the Py probe (Fig. 3*A*) must be derived from the unamplified allele(s) of the rat DNA that has been amplified in 3B cells. These two fragments are also detected in the Rat-2 DNA (14). In Rat-2 and 3B the 400-kb fragment predominates in DNA that has not incorporated 5-azaC, whereas the 350-kb fragment predominates in 3B or Rat-2 DNA that has incorporated 5-azaC, suggesting that the *Sal* I sites in the native locus and the amplified DNA are methylated in a similar fashion (compare Fig. 3 *A* and *B*) (14). No differences could be detected between the amplified 3B DNA and the unamplified rat allele(s) DNA over the 450 kb analyzed, indicating there are no gross rearrangements in the rat sequences that lie within the amplicon in 3B cells.

The above results illustrate the sensitivity of the RI-Bam probe to detect low- or single-copy sequence (the unamplified rat alleles) in the presence of the 20–40 amplified copies (compare Fig. 3 *A* and *B*). Thus the RI-Bam probe could be used in conjunction with the Py probe to identify any heterogeneity between amplicons in DNA from 3B cells. All of the fragments detected using various different restriction enzymes can thus be attributed to either the amplified DNA (detected by the Py probe in 3B genomic DNA) or the unamplified allele(s) (detected by the RI-Bam probe in Rat-2 and 3B genomic DNA). It is therefore concluded that there is no gross heterogeneity between the 3B amplicons within the 450 kb of amplified DNA analyzed.

The previous results showed that the 3B amplicon is large and homogeneous. To determine whether the 3B amplicons are distributed in multiple locations or clustered at a single site in the 3B genome, 3B cellular DNA restricted with either *Not* I, *Nru* I, *Sfi* I, or *Sma* I was fractionated using PFGE under conditions that resolved fragments of up to 3000 kb. Hybridization of a blot of this gel with the amplification-specific Py probe revealed no bands of <3000 kb (Fig. 3*C*). All of the restriction enzyme digestions must have been complete, as hybridization of the same blot with the RI-Bam probe revealed the presence of the native (unamplified) restriction fragments in each case (see fragments present in Fig. 3*D* that are not present in Fig. 3*C*). These results suggest that the 3B amplicons are clustered in a single or small number of arrays that are >3000 kb in size and not dispersed in multiple locations in the 3B genome.

Location of the Amplified DNA in 3B Cells and the Unamplified Alleles in 3B and Parental Rat-2 Cells by FISH. FISH on 3B and Rat-2 metaphase spreads was performed using biotinylated probes. The 15.5-kb λBam probe is composed entirely of rat DNA (Fig. 1) and will detect the amplified DNA as well as the chromosomal regions containing the unamplified alleles in 3B and the parental Rat-2 chromosomes. The λBam probe detects a single-copy signal on three chromosomes in Rat-2 cells. By Giemsa banding (G-banding) of Rat-2 chromosome spreads that were subsequently fluorescence hybridized with the λBam probe, two of the chromosomes with a positive signal were found to be identical and were identified as the two homologues of rat chromosome 2 (Fig. 4*A*). The third Rat-2 chromosome with a positive signal is a marker chromosome, M1, which was identified by G-banding/fluorescence hybridization to be derived from a translocation between a proximally broken rat chromosome 2 and most of rat chromosome 3, including its centromere (Fig. 4*A*), and is termed der(3)t(2;3). Nearly all of the metaphase spreads of the Rat-2 cells examined were observed to be effectively trisomic for most of chromosome 2 owing to the presence of chromosome der(3)t(2;3). The position of the single-copy signals, detected with the λBam probe, on the

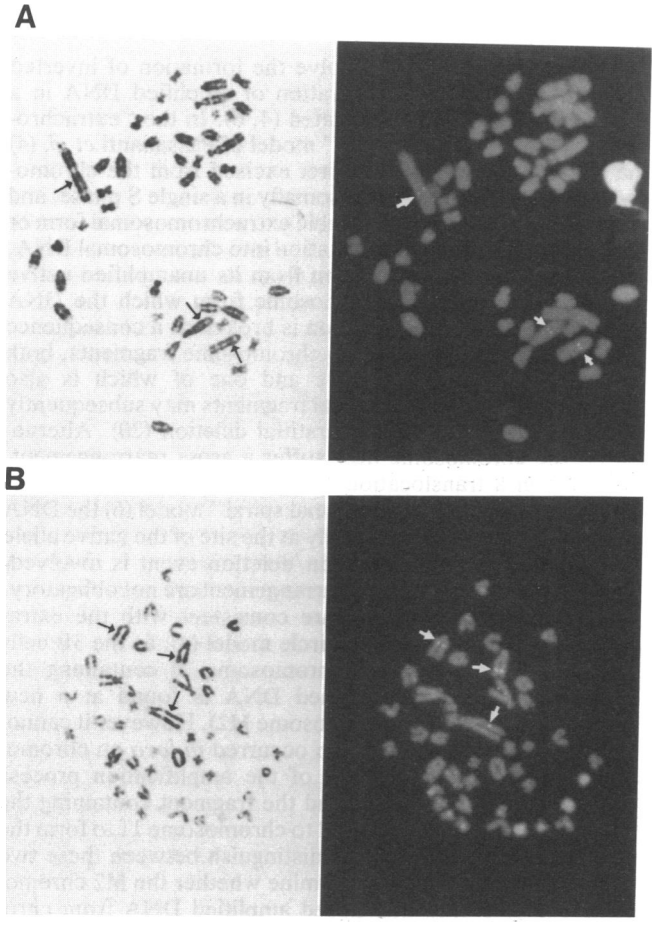

FIG. 4. (*A*) A Rat-2 metaphase spread, first G-banded (left) and then fluorescence-hybridized (right) with biotinylated λBam rat DNA probe. Arrows indicate the positions of fluorescein signal (right) and the corresponding positions on the G-banded chromosomes (left). The three chromosomes with a positive signal have been identified as the two homologues of rat chromosome 2 and a long marker chromosome M1, which is the product of a translocation between a complete chromosome 3 and most of chromosome 2 and is termed der(3)t(2;3). Rat-2 cells are essentially triploid for rat chromosome 2. (*B*) A 3B metaphase spread, first G-banded (left) and then fluorescence-hybridized (right) with biotinylated λBam rat DNA probe. The single-copy, native alleles are seen on only *one* chromosome 2 (compare with Rat-2 spread above in *A*) and on the marker chromosome der(3)t(2;3). The amplified DNA lies on the upper arrowed chromosome whose G-banding pattern bears no obvious resemblance to chromosome 2, or other rat chromosomes, which is denoted as marker chromosome M2. See Fig. 5 for G-band pattern comparisons of chromosomes 2, M1/der(3)t(2;3), and M2.

three Rat-2 chromosomes was located by G-banding to band 2q23 [using the banding nomenclature of Levan (18)].

The λBam probe detects a fluorescein signal on three chromosomes in 3B cells. Single-copy sequence is detected on two of these chromosomes, whereas on the third the signal corresponds to amplified multicopy sequences (Fig. 4*B*). FISH using the λBam probe on G-banded 3B metaphase spreads (Fig. 4*B*) shows that the single-copy signal, representing the unamplified alleles, is located on one normal homologue of rat chromosome 2 and on the der(3)t(2;3) marker chromosome as previously seen with Rat-2 (Figs. 4*A* and 5). The second normal copy of rat chromosome 2, found in Rat-2 cells, is absent in 3B cells (Figs. 4*B* and 5). However the amplified copies are located on a new marker chromosome (present only in 3B) that has a very different banding pattern to chromosome 2 (Figs. 4*B* and 5) and has been termed marker chromosome M2. The localization of the

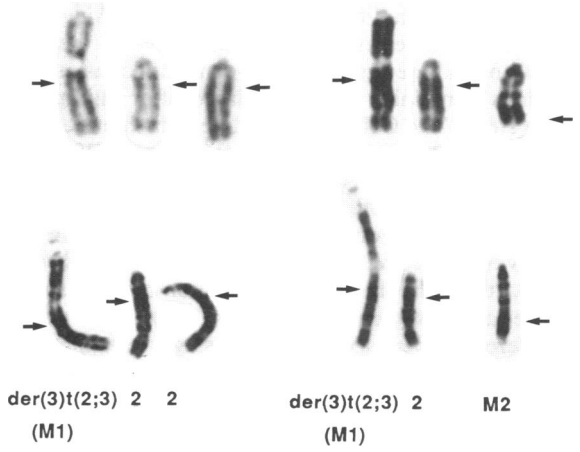

FIG. 5. G-banded chromosomes to which the λBam probe hybridizes from two Rat-2 metaphase spreads (*Left*) and two 3B metaphase spreads (*Right*). The arrows indicate the position of the signal detected by the λBam probe on each chromosome (see Fig. 4). The amplified DNA is all located on chromosome M2 in 3B cells. Unamplified alleles are located on chromosomes 2 and M1/der(3)t(2;3) in Rat-2 and 3B cells.

amplified DNA to a single site was confirmed by the use of the amplification-specific λ3B probe (Fig. 1), which detected a single amplified signal in the same position on the M2 marker chromosome in 3B cells (data not shown) as the λBam probe (Fig. 4*B*). In all of the 3B metaphases analyzed, whether from early passage (about 25 cell divisions) or late passage (40–50 cell divisions), the signal representing the amplified DNA was always detected at the same location on the M2 marker chromosome (Figs. 4*B* and 5). The G-banding pattern of the proximal part of the M2 chromosome in 3B cells appears to be that of chromosome 11. This is supported by the presence of only one normal homologue of chromosome 11 in 3B cells as opposed to the two chromosome 11 homologues present in the parental Rat-2 cells. In all of the 3B spreads examined only one copy of the M2 chromosome that contains the amplified DNA was ever seen to be present. Finally, the cytogenetic analysis revealed that all of the amplified DNA in 3B cells appears to be chromosomally located, as no hybridization to extrachromosomal elements or acentric fragments could be detected.

DISCUSSION

The discovery that arrays of inverted repeats, as opposed to direct repeats, was a common feature of gene amplification in mammalian cells (3) led to the postulation of new models to explain the generation of amplified DNA (4, 6). Small regions of the inverted duplicated 3B amplicon containing the Py oncogene and adjacent mouse and rat DNA sequences had previously been cloned and characterized (3, 4). In this work we have further analyzed the 3B amplicon by cloning, long-range mapping, and FISH.

Long-range mapping revealed that the 3B amplicon is at least 450 kb in size and that all of the 3B amplicons are clustered in a single or a few sites in the 3B genome. One end of the 3B inverted duplication has previously been shown to be the same for all of the 3B amplicons (3) and our present results indicate that the other end of the inverted duplication is probably the same for all of the 3B amplicons. Our PFGE analysis, which made use of different probes within the amplicon, was sensitive enough to detect the presence of the two unamplified alleles in the presence of the 20–40 copies of amplified DNA [Figs. 2 and 3 (14, 17)] and indicates that we could have detected heterogeneity in the size or structure of even a single copy of the amplified DNA. The homogeneity

between the amplicons in the 3B cells strongly suggests that the 20- to 40-fold amplification took place as a *single event* and not through a series of amplification events that would have resulted in heterogeneity between amplicons as is seen in cell lines containing DNA amplified after multiple drug selection steps. The CHOC400 cell line, which has amplified inverted duplications containing the *DHFR* gene (10), and the $HC_{50}474$ cell line, which has amplified inverted duplications containing the adenylate deaminase gene (6), show heterogeneity among amplicons resulting from the series of amplification events that occurred from the multiple rounds of drug selection. It may be that the amplification mechanism(s) for oncogenes and selection for resistance to cytotoxic drugs are quite different, especially at the early stages of amplification. Thus the 3B cell line may reflect the situation seen in tumors containing amplified oncogenes/protooncogenes more closely than the amplifications in drug-resistant cell lines. It is also possible that transfected DNA is integrated with a high frequency into the cellular genome at chromosomal sites that are more susceptible to amplification.

The cytogenetic analysis of the 3B amplified DNA by FISH confirmed the molecular analysis. The amplified DNA was all localized to a single chromosomal region that was estimated to be about 10 or 11 megabases in size, which is consistent with a 20-fold amplification of units of 500–550 kb. By G-banding and FISH the parental Rat-2 cells were found to contain three unamplified alleles of the DNA region that is amplified in 3B cells. Two of these alleles were located in band 2q23 on the two normal homologues of chromosome 2 and the third was at the equivalent position (band 2q23) on the marker der(3)t(2;3) chromosome (Figs. 4*A* and 5). In the 3B cells one of the normal homologues of chromosome 2 was absent and only two unamplified alleles, one on the single copy of chromosome 2 and the other on der(3)t(2;3), were detected. The amplified DNA in the 3B cells was found on a new marker chromosome, M2. The proximal part of M2 chromosome appears to contain a large region of chromosome 11; however the part of chromosome M2 in which the amplified DNA resides does not appear to be derived from chromosome 11. Neither the G-banding pattern nor the chromosomal location of the amplified DNA indicated that this region of M2 was derived from chromosome 2. Apart from the disappearance of a single homologue of each of the chromosomes, 2 and 11, and the appearance of the marker chromosome M2, no other consistent chromosomal differences between the 3B and Rat-2 karyotypes could be associated with the amplification event in 3B cells. The results show that the 2q23 DNA region was the target site of the integration of transfected Py and mouse DNA and its subsequent amplification in 3B cells (3). The 2q23 region also contains a fragile site (17) and has been implicated in tumor development in rats (19).

No differences (rearrangements) were detected in structure and location of the amplified DNA or in the karyotypes of early-passage (about 25 cell doublings) or late-passage (40–50 cell doublings) 3B cells, indicating that the amplified DNA is stable. Furthermore, the consistent karyotypic differences between the parental Rat-2 cells and the 3B cells only involved those chromosomes that contained either the original or the final location of the DNA that was amplified. This suggests that after the amplification event took place the resulting 3B cell line was karyotypically stable. The parental Rat-2 cell line is effectively trisomic for most of chromosome 2, so that the loss of one copy of chromosome 2, as the result of the amplification event, only reduces the chromosome 2 complement to near-diploid state in 3B cells, which should not result in severe chromosome unbalance. If, on the other hand, the amplification had resulted in the loss of one of a pair of diploid chromosomes, extensive chromosomal rearrangement might have subsequently occurred to compensate for gene dosage differences.

Two main models that involve the formation of inverted duplications during the generation of amplified DNA in a *single event* have been postulated (4, 6). In the "extrachromosomal double rolling circle" model of Passananti *et al.* (4) the DNA to be amplified is first excised from the chromosome, amplified extrachromosomally in a single S phase, and then either remains as an unstable extrachromosomal form or becomes stabilized by reintegration into chromosomal DNA, probably at a different location from its unamplified native site. In this model the chromosome from which the DNA excises prior to its amplification is broken as a consequence of the excision event into two chromosome fragments, both containing only one telomere and one of which is also acentric. The two chromosomal fragments may subsequently fuse together to form an interstitial deletion (20). Alternatively, the chromosome may suffer a gross rearrangement, take part in a translocation event, or be partially or completely lost. In the "chromosomal spiral" model (6) the DNA is amplified intrachromosomally at the site of the native allele (*in loco*) and since no excision/deletion event is involved, chromosome breakage and rearrangement are not obligatory. The results presented here are consistent with the extrachromosomal double rolling circle model (4), as the 3B cells have lost a chromosome (chromosome 2) containing the native allele and the amplified DNA is found at a new chromosomal location (chromosome M2). However it cannot be ruled out that amplification occurred *in loco* on chromosome 2 and that as a result of the amplification process chromosome 2 was broken and the fragment containing the amplified DNA was then fused to chromosome 11 to form the marker chromosome M2. To distinguish between these two models it is important to determine whether the M2 chromosome contains only transposed amplified DNA from chromosome 2 or a translocated fragment of chromosome 2 containing amplified DNA as well as unamplified flanking DNA sequences.

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