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Co-amplified markers alternate in megabase long chromosomal inverted repeats and cluster independently in interphase nuclei at early steps of mammalian gene amplification

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Two-colour *in situ* hybridization with probes for two co-amplified markers located several megabases apart on chromosome 1 has been used to analyse early stages of adenylate deaminase 2 (AMPD2) gene amplification in Chinese hamster cells. In the amplified chromosomal structures, the distribution of hybridization spots identifies megabase-long inverted repeats. Their organization is remarkably well accounted for if breakage-fusion-bridge cycles involving sister chromatids drive the amplification process at these early stages. During interphase the markers often segregate into distinct nuclear domains. Many nuclei have bulges or release micronuclei, carrying several copies of one or both markers. These observations indicate that the amplified units destabilize the nuclear organization and eventually lead to DNA breakage during interphase. We propose a model in which interphase breakage has a role in the progression of gene amplification.

Key words: chromosomal instability/gene amplification/nuclear organization

Introduction

Genomic instability, a typical property of cancer cells, probably helps such cells to acquire new phenotypes and to escape normal growth regulation. One type of genomic rearrangement, gene amplification, leads to increased gene expression through alteration of gene copy number. Amplification of oncogenes has been observed in many tumours (Bishop, 1991) and an understanding of the mechanisms underlying gene amplification will be of major clinical importance. However, the early events of amplification that occur *in vivo* are not easy to study. On the contrary, model systems of cells grown *in vitro* that acquire resistance to cytotoxic drugs by gene amplification can be studied from early stages. The results obtained by single-colour fluorescence *in situ* hybridization (FISH) techniques show that the first events of mammalian DNA amplification involve unequal segregation of gene copies rather than local overreplication (Trask and Hamlin, 1989; Smith *et al.*, 1990; Windle *et al.*, 1991; Toledo *et al.*, 1992).

We study the amplification of adenylate deaminase 2 (AMPD2) genes in Chinese hamster fibroblasts. Mutants with amplified AMPD2 genes are recovered from the GMA32 Chinese hamster cell line selected with coformycin, an inhibitor of the AMPD enzymes, when adenine is supplied as a purine source (Debatisse *et al.*, 1982, 1988). Recently, we have performed FISH analysis of several such clones ~20 cell divisions after the initial event of amplification (Toledo *et al.*, 1992). The extra copies were found on a variant chromosome 1, together with the original copy. There was also a striking heterogeneity both in the copy number and in the size of the amplified units from cell to cell within each of several clones. In such clonal populations, the various structures are necessarily related to one another, indicating that cells engaged in an early amplification process are, at least transiently, undergoing extensive genomic rearrangements, leading to a rapid increase in copy number and to the shortening of amplified units. Ten generations later the populations were more homogeneous because cells with smaller amplified units tend to overgrow the previously heterogeneous clonal populations.

To gain further insight into the mechanisms underlying the transient genomic instability of amplified cells, we isolated from a chromosome 1-specific library a second probe (P3C4) that lies several megabases (Mb) away from the AMPD2 gene but can still be co-amplified with it. This permitted a two-colour FISH study of newly amplified mutants with P3C4 and AMPD2 probes labelled, respectively, with biotin or digoxygenin. The analysis of doubly marked metaphase chromosomes implies that the chromatid type of breakage-fusion-bridge (BFB) cycles (McClintock, 1951) is involved during early stages of the amplification process. Moreover, the study of interphase nuclei reveals that P3C4 and AMPD2 sequences often segregate into distinct nuclear domains. Nuclear 'blebs' (Ruddle, 1962) are frequently observed, generally labelled by one or both markers. Labelled 'micronuclei' are also observed, indicating that breakage occurs during interphase. We propose a model that implies interphase breakage in the progression and/or initiation of BFB cycles, and possibly in the formation of double-minute chromosomes (DMs).

Results

Single-colour in situ analyses of cell populations amplified for AMPD2

We previously reported (Toledo *et al.*, 1992) the analysis of 13 drug-resistant clones ~20 cell cycles after the initial event of gene amplification. This analysis revealed the striking heterogeneity of these small clonal cell populations but may have described only a fraction of the possible early events; some resistant colonies were picked but died rapidly after the first transfer. Moreover, for each surviving colony, only 5-18 metaphases were informative.

To observe a more complete spectrum of genetic structures present during the early phase of amplification, we adopted a different strategy that avoids the critical step of plating at a very low cell density. Several cell populations, small enough to be statistically devoid of pre-existing mutants, were isolated and expanded independently for 12 generations

in non-selective medium. Ten days after plating in selective medium (~20 cell cycles after the initial event(s) of gene amplification), the resistant cells (20–40 colonies) derived from a single initial population were pooled. Spreads from each independent pool of mutant cells were prepared. With this protocol, an average of two initial events of gene amplification have given rise to the resistant cells of each pool (see Materials and methods). Lineage studies of the amplified structures are not possible, but each pool yielded several tens or hundreds of metaphases. Eight such pools, analysed with an AMPD2 probe alone, displayed the various chromosomally amplified structures previously observed in the study of clones: (i) 'duplications' and 'large ladders': structures for which two to four copies of the AMPD2 genes are regularly spaced every 47 Mb along the amplified chromosome; (ii) 'mixed ladders': structures containing 5–15 amplified units of various size, generally 1–10 Mb long and (iii) 'clusters': structures with much shorter units, which are found alone or associated with ladders. Moreover, in two of the eight pools, ~10% of the metaphases contained DMs, variable in size within a single cell but <25 Mb (Figure 1A). Each DM contains only one or a few AMPD2 genes since the intensity of the fluorescent AMPD2 spots is roughly similar for DMs and a chromosomal single copy (Figure 1B and C). DMs and chromosomally amplified units were generally not present in the same cell (Figure 1B); two exceptions were encountered among the 16 metaphases observed (Figure 1C).

In situ analysis of metaphases with P3C4 and AMPD2 probes

In unamplified cells, a P3C4 probe gives a large hybridization spot ~5–10 Mb away from the site of the AMPD2 gene (Figure 2A). Two pools have been studied in detail using biotinylated P3C4 and digoxygenin-labelled AMPD2 probes: pool A contains only chromosomally amplified cells and pool B contains both chromosomally amplified cells and cells with DMs.

In pool A, out of some 250 observed metaphases, 62 metaphases chromosomally amplified for P3C4 were identified: 60 displayed AMPD2 genes organized in mixed ladders. Remarkably, in 57 out of 60 mixed ladders, the hybridization spots were distributed according to perfectly symmetrical patterns of variable complexity (Figure 2B–D). The mixed ladder of Figure 2B comprises two domains, each containing two inverted units. Symmetry of two different orders can be recognized in this structure. More generally the AMPD2 copy number was high whereas the P3C4 sequences were only slightly amplified. In 53 cases, exemplified in Figure 2C, several AMPD2 spots were flanked by two P3C4 spots. In two spreads this structure seemed to be duplicated (e.g. Figure 2D). Occasionally, the symmetry was imperfect: for example, Figure 2E shows a spread with an AMPD2 mixed ladder flanked by two P3C4 sequences, but a third P3C4 spot is visible near the telomere. Imperfect symmetries with AMPD2 genes at the telomere were also found (not shown). In two of the 62 spreads amplified for P3C4, a cluster of AMPD2 copies was flanked by two P3C4 sequences; however, most cells with clusters (and rare mixed ladders) were unamplified for P3C4. No duplications or large ladders were observed.

The study of pool B confirmed the co-amplification as well

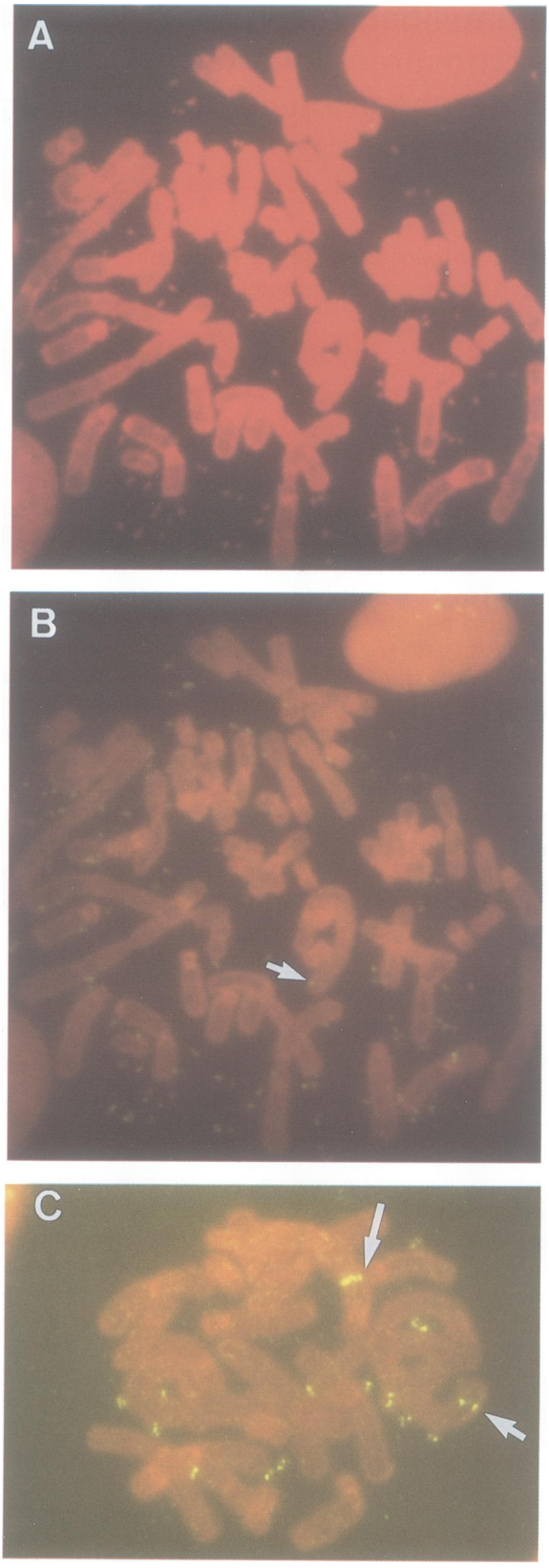

Fig. 1. Examples of extrachromosomal amplification. (**A**) Propidium iodide staining of a metaphase containing DMs (this cell is tetraploid). (**B**) AMPD2 hybridization of the same metaphase. Arrow indicates a chromosomal single copy. (**C**) Metaphase with simultaneous extra- and intrachromosomal AMPD2 amplification. Small arrows: chromosomal single copy. Large arrow: chromosomal multiple copies.

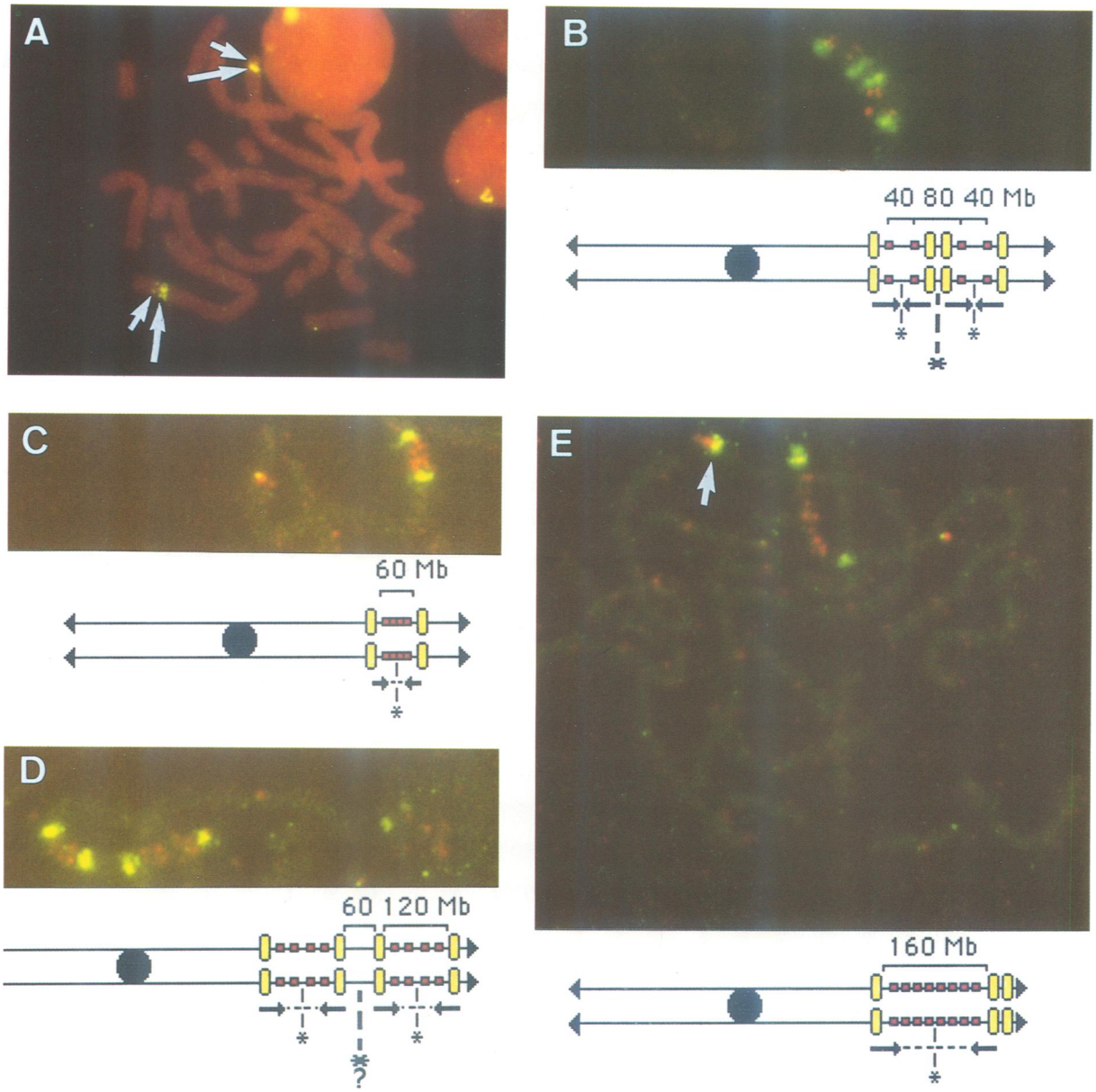

Fig. 2. Organization of P3C4 and AMPD2 sequences on metaphase chromosomes. (**A**) Single-colour FISH of an unamplified cell. Large arrows: P3C4; small arrows: AMPD2. As already reported (Toledo *et al.*, 1992), the chromosomes 1 of a wild-type GMA32 cell are not totally homologous. (**B–E**) Two-colour FISH of amplified cells or chromosomes displaying mixed ladders of AMPD2 genes. AMPD2 and P3C4 sequences are identified by rhodamine (red) and fluorescein (green–yellow) respectively. The arrow in panel E indicates the unamplified homologue. A diagram of the amplified chromosome is presented below each photograph with, when possible, approximate distances between hybridization spots. Red squares: AMPD2; yellow ovals: P3C4; circles: centromeres; arrowheads: telomeres; arrows: orientations of the amplified units; plain or bold asterisks indicate the symmetry planes inside or between domains.

as the striking organization of the markers in mixed ladders and the absence of P3C4 amplification in most clusters. Moreover, duplication or large ladder structures were observed in some cells of this pool. P3C4 was not amplified in these cells: one copy was present at its normal location on each chromosome 1. Moreover, in cells containing DMs, only the AMPD2 marker was present on the DMs (not shown).

In situ analyses of interphase nuclei amplified for P3C4 and AMPD2

The amplification of P3C4 and AMPD2 sequences is associated with distinctive alterations in nuclear shape. The nuclei of 290 cells of pool A, amplified for both markers, were analysed. Table I summarizes the frequencies of shape alterations of these nuclei compared with the nuclei of unamplified GMA32 cells. It is striking that only 65% of the nuclei in amplified mutants have an unaltered shape, compared with 95% in unamplified cells. We classified the alterations into two categories. Type 1, found in 13% of amplified nuclei and 4% of unamplified nuclei, consists of small bulges; these slight alterations, observed rather frequently even in normal cells, could result in part from spreading artefacts. As shown in Table I, large bulges, classified as type 2 alterations, are found almost exclusively in nuclei containing amplified sequences. Such nuclear

Table 1. Quantification of nuclear shape alterations in wild type cells and cells of pool A amplified for both markers

		Altered		
	Unaltered	Type 1	Type 2	Total
GMA32	422 (95.2%)	18 (4.1%)	3 (0.7%)	443
Pool A	187 (64.5%)	37 (12.7%)	66 (22.8%)	290

Guide to the shape of nuclei: unaltered,  ; altered (type 1),  ; altered (type 2),  ,  ,  .

anomalies are similar to those observed in several other systems (including established cell lines chromosomally amplified for DHFR or CAD genes and tumour cells) and have been called nuclear 'blebs' (Ruddle, 1962), 'projections' (Hsu *et al.*, 1974) or 'buds' (Miele *et al.*, 1989).

The topological distribution of amplified sequences in the same nuclei was studied with the two-colour FISH technique (Table II). Remarkably, in about half of unaltered nuclei the extra copies of each marker segregated in distinct domains (Figure 3A). In the other half the markers were, probably depending on the state of decondensation, either distributed more or less according to the organization found on

Table 2. Distribution of AMPD2 and P3C4 spots in nuclei amplified for both markers.

	Distribution of P3C4 and AMPD2 spots in unaltered nuclei		Labelling of blebs			
	Segregation	Others	P3C4	AMPD2	AMPD2 + P3C4	Unlabelled
Unaltered nuclei (187)	85 (45.5%)	102 (54.5%)	–	–	–	–
Type 1 alteration (37)	–	–	20 (54.1%)	3 (8.1%)	2 (5.4%)	12 (32.4%)
Type 2 alteration (66)	–	–	31 (47%)	5 (7.6%)	21 (31.8%)	9 (13.6%)

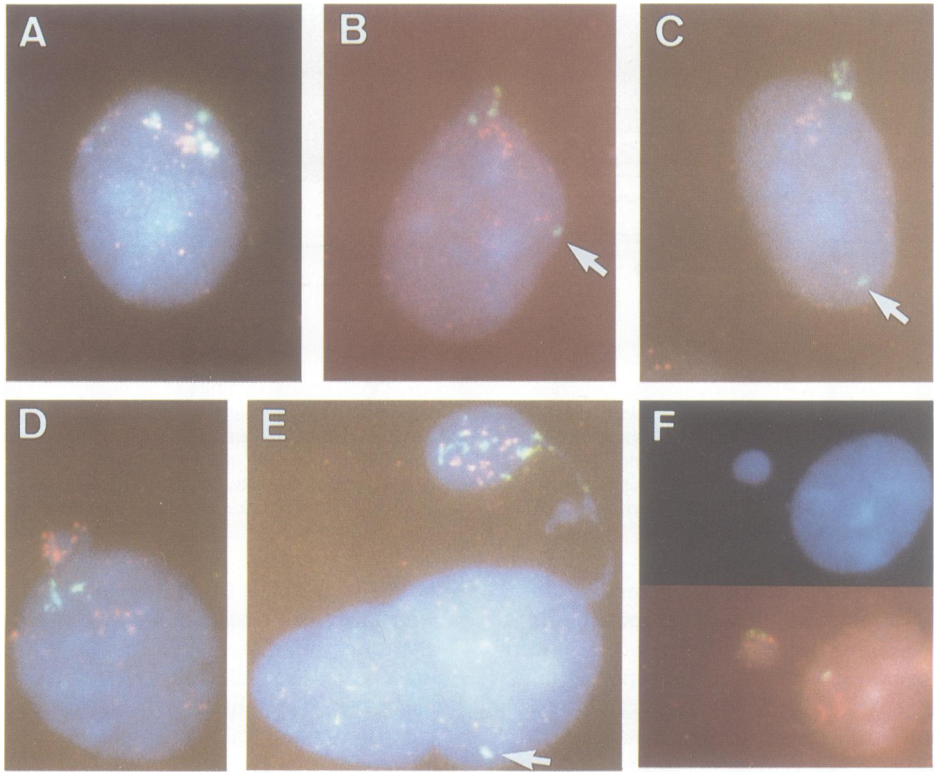

Fig. 3. Organization of P3C4 and AMPD2 sequences in interphase nuclei. (**A**) Unaltered nucleus with a segregation of copies of each marker. (**B–E**) Nuclei with type 2 blebs. Nuclei with blebs labelled with P3C4 only (B and C), AMPD2 only (D), or both markers (E) are presented. (**F**) micronucleus and a neighbouring nucleus. The micronucleus is labelled, the neighbouring nucleus displays an amplification of AMPD2 but only one P3C4 spot. Arrows indicate, when unequivocal (B, C and E), the labelling corresponding to the unamplified homologue. Although in the nuclei presented here the P3C4 and AMPD2 copy numbers are similar, nuclei with under-representation of the P3C4 marker display the same properties.

metaphase chromosomes or simply co-localized without any particular distribution (data not shown). The frequency of segregation is probably underestimated since we observed images resulting from the projection in two dimensions of three-dimensional structures.

In ~85% of the nuclei displaying shape alterations, the extra copies of P3C4 and/or AMPD2 are localized at or near the blebs (Table II). Since in such nuclei the two markers are also often segregated, blebs labelled with several copies of only one marker were observed frequently. Figure 3 panels B and C show examples of blebs labelled with P3C4 only, and Figure 3D shows a bleb labelled with AMPD2 only. In a few cases, the bleb is linked to the nucleus only by a thin chromatin fibre (Figure 3E). Miele *et al.* (1989) proposed that such a bleb may eventually separate from the nucleus and become a micronucleus. As illustrated in Figure 3F, labelled micronuclei were indeed observed in these cells. The same results were obtained when pool B was analysed (not shown).

Discussion

The chromatid type of breakage–fusion–bridge cycles explains the organization of amplified units in mixed ladder structures

Visualization of the arrangement of two co-amplified markers during the early stages of gene amplification has added a new level of resolution concerning this process. The striking images of head-to-head organizations, involving one or several orders of symmetry, are perfectly explained by the chromatid type of BFB cycles (McClintock, 1941a, 1951). According to this model, the sister chromatids produced after replication of a broken chromatid fuse at the location of the break, generating a dicentric chromatid. At anaphase the centromeres of the dicentric chromatid move to opposite poles of the mitotic spindle, creating a bridge which is later broken. Each daughter cell receives a broken chromatid which again replicates and forms another bridge, perpetuating the BFB cycles until the broken end is 'healed', probably by the addition of telomeric TTAGGG repeats by the telomerase (Blackburn, 1991). Recent studies have shown that telomerase is able to heal a chromosome only if particular sequences, partly homologous to the TTAGGG motif, are localized at or near the broken end (Harrington and Greider, 1991; Morin, 1991). Thus, once a chromatid is broken, it is expected to undergo several BFB cycles until such sequences are, by chance, localized near the broken end. The bridges formed by fusion of sister chromatids readily explain the perfect symmetries observed (Figure 4). Random breakage sites account for (i) the variable size of the amplified units, (ii) the creation of imperfect symmetries, (iii) the different distances observed from cell to cell between the most telomeric hybridization spot and the telomere and (iv) the transient heterogeneity of clonal populations at early stages of amplification, since each breakage site is unique in each cell and the number of cycles between the initial breakage and chromatid healing is variable. Thus, because the first break triggers rearrangement cascades, BFB cycles involving sister chromatids readily account for the rapid accumulation of AMPD2 genes in some cells. However, if all the amplified structures presented in Figure 2 can be explained by the chromatid type of BFB cycles (see Figure 4), the observation of differential amplification of the two markers in almost 90% of the mixed ladders is not predicted by this mechanism. We have to suppose either that anaphase breakages occur preferentially between the AMPD2 and P3C4 sequences, or that cells containing mixed ladders with a low P3C4 copy number rapidly overgrow the population because they have a strong selective advantage, or that an additional mechanism permits the differential amplification of the markers, or a combination of several of these factors. Some clarification of these issues will be presented in subsequent sections.

Though the chromatid type of BFB cycle has not been favoured as a model for explaining gene amplification in mammalian cells, several authors have proposed the intervention of BFB cycles in DNA amplification because dicentric or ring chromosomes have been observed frequently in metaphase cells (Cowell and Miller, 1983; Kaufman *et al.*, 1983; Miele *et al.*, 1989; Trask and Hamlin, 1989; Ruiz and Wahl, 1990; Smith *et al.*, 1990). Dicentric chromosomes were also observed in the AMPD2 system (Toledo *et al.*, 1992). However, dicentric chromosomes, which arise from the fusion of two chromosomes, are expected to undergo the chromosome type of BFB cycles (McClintock, 1941b, 1942). In the AMPD2 system, BFB cycles following the fusion of two chromosomes 1 are highly unlikely since we observed an intact unamplified chromosome 1 in every complete spread. BFB cycles of the chromosome type involving a chromosome 1 and a different chromosome would create direct repeats (McClintock, 1941b), which obviously does not fit our results. Thus the chromosome type of BFB cycle does not play a significant role in AMPD2 gene amplification at these early stages. Two other segregative mechanisms are also unlikely to create mixed ladders: unequal sister-chromatid exchanges (Trask and Hamlin, 1989; Smith *et al.*, 1990) would generate direct repeats, and targeted reintegration of DMs would not account for symmetrical distribution of the hybridization spots.

Organization of amplified sequences in interphase nuclei and shortening of amplified units

Since coamplification of P3C4 and AMPD2 was found mostly in mixed ladders, nuclei amplified for both markers give us insights into the interphase organization of this structure. The results show that copies of the two markers, which alternate along the amplified chromosomes, often cluster in distinct regions of the interphase nuclei. Each decondensed chromosome in an interphase nucleus has been shown to be attached to a network of non-histone proteins called the nuclear matrix, skeleton or scaffold. Moreover, interphase chromosomes are localized in discrete spatial regions of the nucleus and the homologous chromosomes are generally separated (Hubert and Bourgeois, 1986; Manuelidis, 1990); we observed such a separation for the amplified and unamplified chromosomes 1 (Figure 3B, C and E). Furthermore, the data reported here indicate that the amplified AMPD2 and P3C4 sequences are anchored to specific, distinct nuclear domains (Figure 3A–D). Figure 5A illustrates the suggested organization of an amplified chromatid during interphase.

Another distinctive feature of nuclei amplified for P3C4 and AMPD2 is the presence of nuclear blebs and

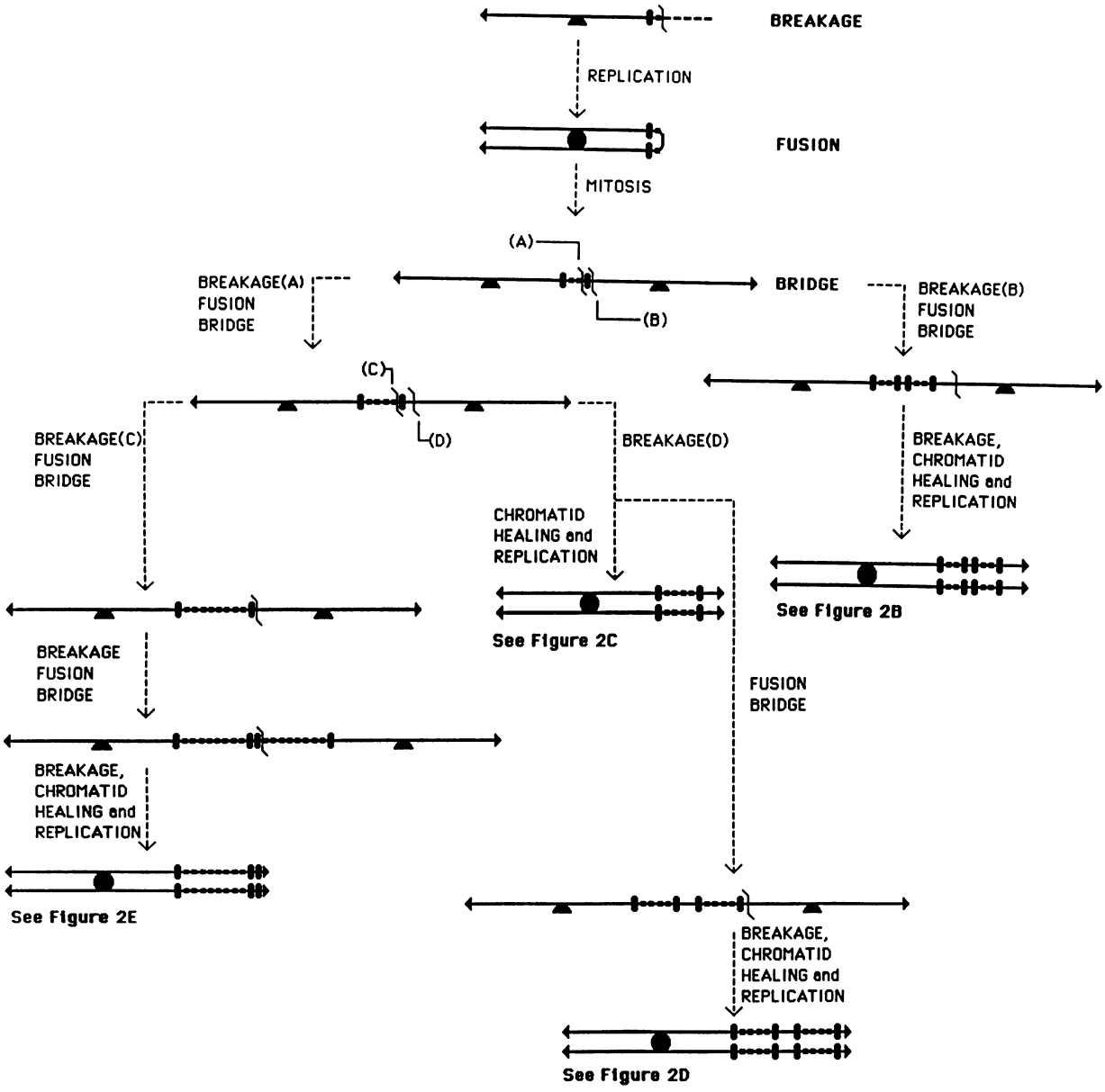

Fig. 4. Formation of symmetrical structures by the chromatid type of breakage–fusion–bridge cycles. Possible pathways leading to the structures shown in Figure 2B–E are presented. This is a diagrammatic representation: in fact, because the genesis of each structure imposes the destruction of the structure from which it derives, the pathways of all structures are shown in a single figure for convenience only. The first BFB cycle is shown in detail; for subsequent cycles, the fusion intermediates are not represented. The first bridge contains one domain with two AMPD2 copies flanked by two P3C4 sequences: a breakage outside this domain (breakage B) reinitiating a BFB cycle may lead to the duplication of this domain (observed in Figure 2B); a breakage inside it (breakage A) would lead to an increase in copy number for AMPD2 without reamplification of the P3C4 sequence. If a breakage were to occur inside one domain and were followed by chromatid healing, the perfect symmetry is lost (pathway leading to the structure of Figure 2E). ■: AMPD2; ▮ : P3C4; ⟩ : breakage site; ▶: telomere; ◓ , ●: centromere of one or two chromatids.

micronuclei. Blebs have been observed in several lines possessing an extra-long chromosome (Ruddle, 1962; Hsu *et al.*, 1974; Lo Curto and Fraccaro, 1974; Jackson and Clement, 1974; Miele *et al.*, 1989). In a cell line highly amplified for the DHFR gene after multiple selection steps, Miele *et al.* showed that 90% of the blebs contain DHFR genes, suggesting that bleb formation was specific to the amplified units. Our results agree with this observation: 85% of the type 2 blebs contain several copies of at least one of the two available markers (Table 2). However, <40% of type 2 blebs contain the selected AMPD2 gene; this is expected from the segregation of markers observed in interphase nuclei, provided that the blebs labelled only by the P3C4 probe contain the nuclear area in which amplified P3C4 sequences are localized during interphase but not the AMPD2 attachment domain (Figure 5B), and vice versa.

It is generally considered that micronuclei contain acentric chromosomal fragments and that their frequency reflects chromosomal damage (Fenech and Morley, 1985). Miele *et al.* (1989) proposed that a micronucleus would be formed during interphase after the separation of a nuclear bleb. In the AMPD2 system, the shape of some observed nuclei suggests indeed that blebs are precursors of micronuclei (Figure 3E). Moreover, most micronuclei, like blebs, are labelled by one or both amplified markers (Figure 3F). These results indicate that DNA breakage may occur during

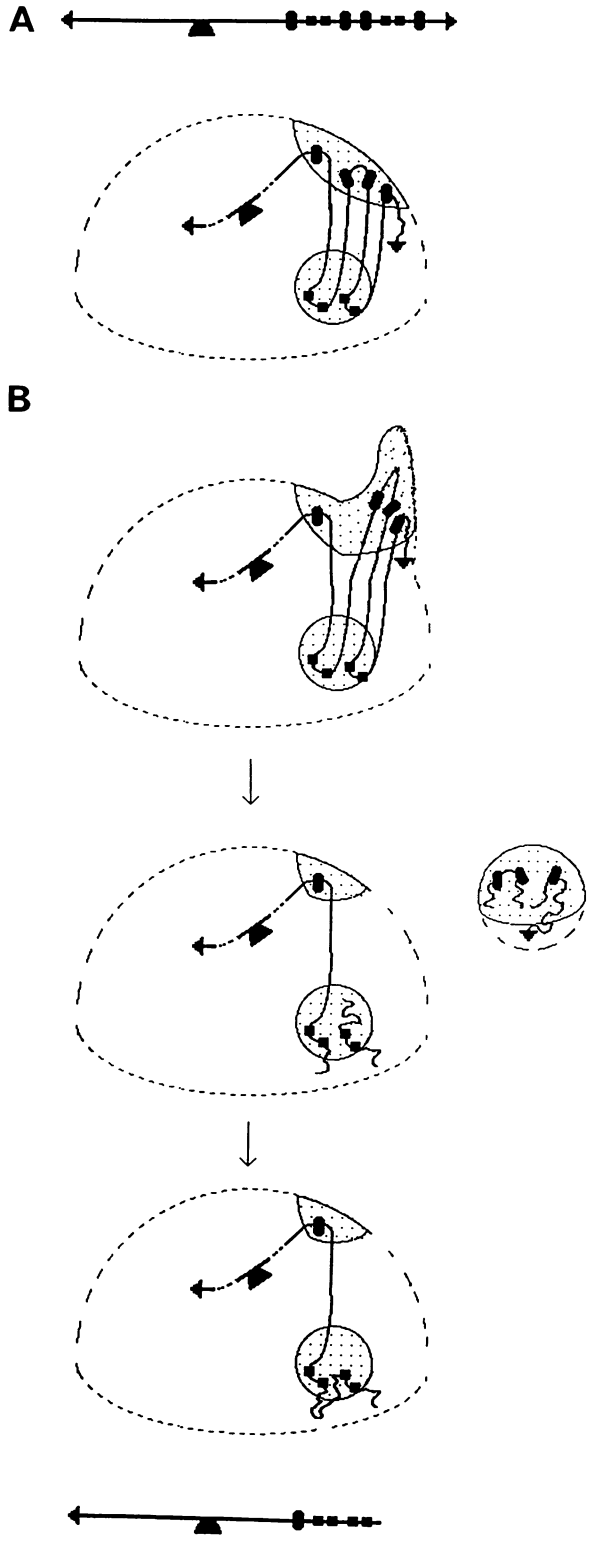

Fig. 5. Organization of interphase nuclei and the interphase breakage model. (**A**) Putative organization of an amplified chromatid during metaphase and interphase. The amplified chromatid is represented at mitosis (anaphase–telophase) and interphase (G_1 phase). (**B**) Interphase breakage of the amplified chromatid following the separation of a bleb containing only the extra copies of P3C4. These breakages may lead to shortening of chromosomal amplified units after intermolecular fusion of the broken molecules remaining in the nucleus, and may reinitiate BFB cycles of the chromatid type. Telomeres, centromeres, AMPD2 and P3C4 sequences represented as in Figure 5; ⊛ : nuclear domains of AMPD2 or P3C4 attachment.

interphase. If a bleb separates from the nucleus, the extra copies of the P3C4 sequence could be removed (Figure 5B) (the same process may indeed eliminate the AMPD2 genes but the affected cell would die in selective medium). In this example, the nucleus contains two broken molecules: one chromatid with one P3C4 and two AMPD2 sequences near its broken end and a DNA fragment with two AMPD2 copies. Broken ends clustered in the AMPD2 nuclear attachment domain are expected to fuse easily, creating an interphase chromatid with one P3C4, four AMPD2 sequences and a broken end. Thus DNA extrusion in micronuclei may correct for structural abnormalities of amplified chromosomes through the simultaneous shortening of most units, leading to differential deletion of the initially co-amplified markers while contributing to the progression of amplification through the generation of broken chromatids. All the properties of mixed ladders (their structure, rapid evolution, and the differential amplification of two markers in almost 90% of these structures) may be explained by the joint operation of two mechanisms: one amplification mechanism (the chromatid type of BFB cycle) and one deletion mechanism (interphase breakage). Some AMPD2 clusters were flanked by P3C4 sequences and are likely to be generated by the joint operation of BFB cycles and interphase breakages. Whether or not all clusters are formed by these mechanisms remains to be determined. Finally, micronucleus formation may be a key phenomenon in amplification processes even if it occurs infrequently, because cells containing small amplified units tend to overgrow the cell population (see Toledo *et al.*, 1992).

Formation of DMs

The DMs observed in this system are characterized by four major properties: (i) they are several Mb long; (ii) each contains one or few AMPD2 genes, but no P3C4 sequence; (iii) DMs of variable size are generally present within a single cell; and (iv) DMs may occasionally co-exist with extra copies of chromosomal AMPD2. In this system, DMs have been observed only when pools were studied. Since each pool may contain the progeny of several initial events, how DMs are related to the chromosomally amplified structures is not easily determined.

It has been proposed that megabase-long DMs may result from the fusion of several submicroscopic extrachromosomal molecules called episomes (Carroll *et al.*, 1988). This hypothesis postulates that the differences in the size of DMs within a single cell reflect the variation of episome copy number per DM. It does not account for the present results since the megabase-long DMs we observe contain one or a few AMPD2 genes. In this system, it seems more likely that the initial extrachromosomal molecule(s) is several megabases long. If the initial amplification event were to generate a large acentric extrachromosomal molecule, cells with many copies of this molecule would appear after several cycles of unequal segregation (Windle *et al.*, 1991) and the size heterogeneity of DMs could be generated by occasional fusion of such molecules. Alternatively, several large extrachromosomal molecules, heterogeneous in size, could have been created at the same time from a previously amplified chromosome. Since the size of the DMs compares well with the size of amplified units in ladders, DMs may derive from these structures through formation of micronuclei; if acentric molecules created after interphase

breakage circularize rather than fuse with the broken chromatid, DMs of various size could be created.

What is the initial event?

The present study shows that BFB cycles of the chromatid type are likely to be an essential part of the mechanism of progression of gene amplification as early as 20 cell divisions after this process has started. Such a mechanism may in fact operate from the very beginning: all chromosomal alterations in cells of pool A can be simply accounted for by this interpretation. In this hypothesis, the initial event of amplification itself could be a fusion of sister chromatids. The frequent occurrence of chromatid fusions at or near telomeres and their involvement in the earliest steps of amplification are strongly suggested by the properties of amplified dicentric chromosomes observed at a very high frequency a few cell generations after the initial event of CAD gene amplification in Syrian hamster cells (G.R.Stark, personal communication). On the other hand, the random breakage of a chromatid—or of a single chromosome— may occasionally serve as an initial event if healing is delayed.

An alternative hypothesis is that BFB cycles of the chromatid type propagate a different initial mechanism of amplification. We observed chromosomal repeats of two or more apparently identical large units (duplications and large ladders) not only in cells of most pools examined in this study but in clones observed at very early stages of AMPD2 gene amplification. Such unstable structures were first described in cells containing amplified CAD genes by Smith *et al.* (1990) who proposed, among other things, that they might correspond to direct repeats generated by unequal but homologous sister chromatid exchanges. The study of pool B by two-colour FISH revealed that the P3C4 marker is not coamplified in large ladders of AMPD2 genes. This observation is indeed consistent with the interpretation that the large ladders are direct tandems of identical units from which the P3C4 marker was excluded at the first unequal sister chromatid exchange. If so, these enlarged chromosomes are expected to be shortened by interphase breakages which would trigger BFB cycles. Further work will be needed to establish the contribution to the initiation of AMPD2 amplification of the hypothetical processes just considered; several may be operative and, as previously discussed, they may occasionally include the generation of extrachromosomal elements.

Conclusion

BFB cycles involving sister chromatids were first observed in maize (McClintock, 1939) and later in *Nicotiana* plants (Gerstel and Burns, 1966), and both genetic (McClintock, 1939) and cytological (McClintock, 1939; Gerstel and Burns, 1966) evidence of DNA amplification was obtained in these systems. Our two-marker study points to an important role of the chromatid type of BFB cycles in mammalian DNA amplification.

The organization of mixed ladders also shows that megabase-long inverted repeats are created very early in the AMPD2 amplification process. Inverted rearranged units of much smaller size are present in highly AMPD2 amplified stable cell lines derived from multiple selection steps (Hyrien *et al.*, 1988; Debatisse *et al.*, 1992) and small inverted repeats are a common feature in gene amplification (Ford *et al.*, 1985; Stark *et al.*, 1989; Fried *et al.*, 1991). Further analysis is required to know whether the inverted repeats found at late stages are derived directly from the large inverted repeats visualized here or whether they are generated by mechanisms that operate only later (Stark and Wahl, 1984; Schimke *et al.*, 1986; Passananti *et al.*, 1987; Hyrien *et al.*, 1988; Ma *et al.*, 1988; Wahl, 1989).

This study also reveals a more general importance of amplified systems as experimental models for the analysis of nuclear organization and genomic rearrangements. Formation of micronuclei may play a role in the evolution of the chromosomally amplified structures and possibly in extrachromosomal amplification. Remarkably, blebs have been observed in many tumours, particularly breast adenocarcinomas (Hsu *et al.*, 1974; Jackson and Clement, 1974; Lo Curto and Fraccaro, 1974), which are often characterized by amplification of the ERBB-2 (*neu*) oncogene (Bishop, 1991). The contribution of BFB cycles and micronucleus formation to the genomic instability of cancer cells deserves further investigation.

Materials and methods

Selection of AMPD2 amplified mutants

The wild-type GMA 32 cell line, as well as normal and selective culture media have been described elsewhere (Debatisse *et al.*, 1982, 1984). The method used to observe cells at early stages of AMPD2 amplification is derived from the one described for CAD amplification (Smith *et al.*, 1990). Seventeen subpopulations of 100 GMA32 cells, statistically devoid of pre-existing amplified cells [the frequency of amplified mutants is $\sim 10^{-4}$ with the conditions of selection used (Toledo *et al.*, 1992)] were expanded to 5×10^5 cells in normal medium and then independently plated in medium containing 0.5 μg/ml of coformycin. After 10 days, the surviving cells derived from each subpopulation were pooled, giving rise to independent cultures of resistant cells. Out of 17 subpopulations, three yielded no resistant colony. Since $P(0) = 3/17$, according to Poisson's distribution, the mean number of initial events in each cell population is 1.73; each pool is thus, on average, derived from two initial events. Cells of eight independent pools were treated with colcemid, then spread on slides as previously described (Toledo *et al.*, 1992) and used for FISH studies.

Single-colour in situ hybridization

Single-colour FISH was performed with biotinylated cosmids 61W14 and 56A1, spanning 60 kb of the AMPD2 locus (Debatisse *et al.*, 1988) and P3C4, isolated from a Chinese hamster chromosome 1-specific library (Debatisse,M., Labidi,B. and Metezeau,P. unpublished results). Cosmids were biotinylated by nick translation (Nick Translation System; BRL) with biotin-16-dUTP (Boehringer). The probes were recovered by filtration through Quick Spin Columns (Sephadex G-50; Boehringer). FISH was performed essentially as described by Pinkel *et al.* (1988) and Smith *et al.* (1990). Cells mounted on slides were treated with 100 μg/ml of RNase in 0.3 M sodium chloride, 30 mM sodium citrate (2×SSC), pH 7.0 at 37°C for 1 h, dehydrated in a 70%/85%/100% ethanol series, treated with proteinase K (Boehringer, 400 ng/ml in 20 mM Tris−HCl, 2 mM $CaCl_2$, pH 7.5 at 37°C for 7−8 min) and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), 50 mM $MgCl_2$, pH 7.0 for 10 min at room temperature. Chromosomes were denatured by immersion in 70% formamide, 2×SSC, pH 7.0 at 70°C for 2.5 min. The hybridization mixture (10 μl total volume per slide consisting of 50 ng of probe, 10 μg of sonicated GMA32 genomic DNA, 50% formamide, 1% Tween 20 (Pierce), 10% dextran sulfate (Pharmacia) in 2×SSC) was heated for 10 min at 70°C, placed at 37°C for 20 min, to allow pre-annealing of repetitive sequences, then applied to slides. Hybridization was at 37°C overnight. Slides were then washed at 42°C in three changes of 50% formamide, 2×SSC; then three of 2×SSC. Slides were treated with STM [4×SSC, 0.05% Tween-20, 5% non-fat dried milk (Carnation), pH 7.0] for 10 min at room temperature then with alternating layers of fluoresceinated avidin (Vector Labs) and biotin-conjugated goat anti-avidin (Vector Labs), both at 5 μg/ml in STM, for 20 min each at room temperature until two layers of avidin were applied. Each avidin or anti-avidin treatment was followed by three washes of 5 min each in ST (4×SSC, 0.05% Tween-20) at room temperature. Chromosomes were then stained with 0.1 μg/ml of propidium iodide (Sigma) in PBS and mounted in Citifluor, an antifading preparation (Citifluor, Ltd).

Two-colour in situ hybridization

Two-colour FISH was performed essentially as described (Tkachuk *et al.*, 1990). Cosmid P3C4 was biotinylated as before and cosmids 61W14 and 56A1 were labelled with digoxygenin using the DIG DNA labelling kit (Boehringer) according to the supplier's procedure. Slides were treated with RNase and proteinase K and then fixed as before. The probes were mixed, then denatured and preannealed as above. After incubation overnight at 37°C, slides were washed as before. Slides were then treated first with STM as above, then, for 30 min at 37°C each, successively with layers of: (i) fluoresceinated avidin (5 μg/ml) in STM, (ii) biotin-conjugated goat anti-avidin antibody (5 μg/ml) and mouse anti-digoxin antibody (Sigma, 1:500 dilution) in NTTbr (150 mM NaCl, 100 mM Tris–HCl, 0.05% Tween-20, 0.05% blocking reagent (Boehringer), pH 7.0), (iii) fluoresceinated avidin (5 μg/ml) and rhodamine-conjugated rabbit anti-mouse antibody (Sigma, 1:1000) in NTTbr and (iv) rhodamine-conjugated goat anti-rabbit antibody (Sigma, 1:1500) in NTTbr. After each layer, slides were washed (three times for 5 min each) with ST (for the first layer) or with 150 mM NaCl, 100 mM Tris, 0.05% Tween-20 (layers 2–4). Chromosomes were stained with 0.1 μg/ml diamidino-phenylindole (DAPI) in PBS and mounted as above.

Microscopy

In single-colour FISH experiments, slides were observed with a Photomicroscope III (Zeiss) equipped with the Zeiss filter combinations 487701, 487709 and 487715. For two-colour FISH experiments, slides were observed with an Axiophot (Zeiss) equipped with a precision pass-band filter, the Zeiss filter combinations 487709 and 487715 and a double band-pass filter (Omega opticals) or the Zeiss filter combinations 487701, 487709 and 487715. No image intensification or processing devices were used. Ektachrome ASA 400 colour films (Kodak) were used for all photographs.

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