Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 5427–5431, June 1992
Cell Biology

Fusions near telomeres occur very early in the amplification of *CAD* genes in Syrian hamster cells

[*N*-(phosphonoacetyl)-L-aspartate/dicentric chromosomes/bridge–breakage–fusion/recombination/unstable amplicons]

KATHLEEN A. SMITH, MARY B. STARK, PATRICIA A. GORMAN, AND GEORGE R. STARK

Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London, WC2A 3PX, United Kingdom

Contributed by George R. Stark, March 4, 1992

ABSTRACT **Previous analyses by fluorescence *in situ* hybridization of structures present 20–30 cell generations after the primary events of mammalian gene amplification have shown that tens of megabases of DNA separate each copy of the selected gene in chromosomal arrays that contain up to 15 copies. Since these structures are very unstable, it is necessary to study amplified DNA as soon as possible after it has been formed to relate the structures observed to the primary mechanisms that generated them. Previously, new amplifications of the *CAD* gene were analyzed in colonies of 10^5 *N*-(phosphonoacetyl)-L-aspartate-resistant Syrian hamster BHK cells. *CAD* is on the p arm of chromosome B9 and the amplified genes were usually found in large extensions of B9p, with one copy in its normal position. We now report that dividing drug-resistant cells have been physically separated from static drug-sensitive cells, to allow the amplified structures to be observed only a few cell generations after they have been formed. The most informative results are that about one-third of the newly formed chromosomes carrying amplified *CAD* genes are dicentric and that about half of these carry two B9q arms. These observations reveal that recombination between the p telomeric regions of two B9 sister chromatids is an important primary event of amplification in this system. The resulting dicentric chromosomes can then enter bridge–breakage–fusion cycles that provide the means to increase the number of *CAD* genes per cell in successive generations by an asymmetric distribution at each cell division.**

Several recent papers in which fluorescence *in situ* hybridization has been used to look at structures formed early in gene amplification have shed light on this process and have dramatically changed our ideas about the mechanisms involved. Trask and Hamlin (1) studied methotrexate-resistant populations of Chinese hamster cells derived from a single cell by serial selection with increasing concentrations of drug over a period of about 8 weeks. The amplified dihydrofolate reductase genes were chromosomal and spaced tens of megabases apart. They were located on extended structures derived from the same chromosome arm that carries the single gene in unselected cells. Trask and Hamlin (1) concluded that replicative mechanisms were unlikely to be responsible and favored recombinational mechanisms such as sister chromatid exchange.

We studied (2) amplifications of the carbamoyl-P synthetase, aspartate transcarbamylase, dihydroorotase (*CAD*) gene in Syrian hamster cells resistant to *N*-(phosphonoacetyl)-L-aspartate (PALA) at a somewhat earlier stage, when colonies had reached about 10^5 cells. We also saw (2) chromosomally amplified genes tens of megabases apart, usually on the same chromosome arm that carried the *CAD* gene in unselected cells. Furthermore, there were two strong indications that the structures formed in the initial event had changed considerably by the 10^5-cell stage: individual cells within a single clone had quite different numbers of *CAD* genes and some cells had up to 15 copies of *CAD*, many more than are needed to resist the low concentration of PALA employed (2). Furthermore, when resistant clones were maintained longer in the original concentration of PALA, the amplified genes were found in more condensed chromosomal structures. Like Trask and Hamlin (1), we also concluded that replication-based mechanisms were unlikely and favored a recombination-based mechanism such as sister chromatid exchange. We considered telomeric fusions to be an alternative possibility since ≈3% of the amplified *CAD* genes were found in dicentric chromosomes (2).

Windle *et al.* (3) studied amplification of dihydrofolate reductase genes in Chinese hamster cells but looked earlier in the amplification process than did Trask and Hamlin (1). Somewhat different results were obtained, with examples of both chromosomal and extrachromosomal amplification observed. The most likely mechanism was considered to be deletion of a chromosomal region to form extrachromosomal acentric elements, which then reintegrated. Consistent with this idea, the extrachromosomal copies were rarely observed in cells kept for a longer time in the same selective medium.

Tolédo *et al.* (4) studied amplifications of the AMP deaminase genes in Chinese hamster cells at the 10^5- to 10^6-cell stage. Tandem repeats in which the genes were separated by tens of megabases were found, originally on the chromosome arm carrying the normal copy of the gene. These structures were overtaken by more condensed ones when selection was prolonged. More recent work of this group, with two different probes, clearly reveals inverted repeat structures and suggests a mechanism that involves chromatid breakage, followed by fusion–bridge–breakage cycles (12).

In summary, the four studies available to date indicate that recombination (not replication) of very large chromosomal regions is an important early event in mammalian gene amplification. All the studies show that the amplified DNA was unstable at the time it was examined, clearly indicating the importance of examining the structures formed as soon after the primary event as possible. We have tried to do this in the current work by using a method that does not require waiting until colonies of drug-resistant cells are formed. The results show that a much higher proportion of the chromosomes carrying amplified *CAD* genes are dicentric than we observed (2) by studying colonies of 10^5 cells. We conclude that telomeric fusions are an important primary event of *CAD* gene amplification in Syrian hamster cells.

MATERIALS AND METHODS

Two similar protocols were used. First we exposed 5×10^5 cells to PALA in T flasks (Table 1, method F) but found that it was easier and better to do the experiment with smaller

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: PALA, *N*-(phosphonoacetyl)-L-aspartate.

Table 1. Dicentric and nondicentric marker chromosomes in BHK cells soon after the initial event of amplification

Days between amplification event and start of selection	Days in PALA selection	Chromosomes banded, no.		Chromosomes not banded, no.	
		Dicentric	Not dicentric	Dicentric	Not dicentric
4 (F)	3	1	0	1	2
5 (F), 2 (D)	4	3	5	2	5
5 (F)	5	1	2	0	3
5 (F), 3 (D)	6	1	9	1	10
3 (F), 2 (D)	7	0	0	1	1
3 (F)	8	1	3	1	2
3 (D)	10	2	0	2	2
3 (F)	11	3	2	0	5
4 (F)	12	1	1	1	9
4 (F)	13	0	0	0	6
Totals		14 (39)	22 (61)	9 (17)	45 (83)

A total of 137 marker chromosomes were observed. Ninety could be interpreted as dicentric or not, and 47 were not clear enough to be scored. Letters in parentheses refer to the method used as follows: F, flask method; D, dish method. Numbers in parentheses are percent of total.

populations of 10^5 cells in dishes (Table 1, method D). Since the results from the two methods were similar (Table 1), we give results only from method D. Individual populations of 10^3 Syrian hamster BHK cells were grown to $\approx 10^5$ cells in wells of a 24-well plate without selection. Each group of cells was then dispersed with trypsin and seeded independently into 10-cm dishes containing Dulbecco's modified Eagle's medium with 10% (vol/vol) dialyzed fetal calf serum and 20 μM PALA (2). After several days, Colcemid (150 ng/ml) was added to the medium for 1.75 hr at 37°C. Most of the medium was then carefully removed but retained. Cells arrested in metaphase were dislodged by shaking each dish and tapping it against the bench top. The medium was then returned to the dish; mitotic cells in medium were collected with a wide-bore pipette, placed in a conical tube, and centrifuged to pellet the cells. These cells were resuspended in 0.5 ml of the supernatant solution to which 4.5 ml of 75 mM KCl, prewarmed to 37°C, was added. Mitotic chromosome spreads were prepared as described (2). The cells remaining attached to the dish were washed twice with excess phosphate-buffered saline and placed in fresh medium containing 20 μM PALA. After selection as usual (2), the number of PALA-resistant colonies was scored and used to calculate when the initial amplification event had occurred.

RESULTS AND DISCUSSION

***In Situ* Analyses of Newly Amplified Structures.** In both the previous (2) and the present studies, to ensure that the amplified structures observed were new, we grew many independent populations of 10^3 Syrian hamster BHK cells to 10^5 (or 5×10^5) cells without selection and then dispersed the cells before exposing them to 20 μM PALA. If an initial population were to contain one cell with a preexisting amplification of *CAD* (unlikely, since the frequency is $\approx 10^{-5}$), one would expect to see $\approx$100 colonies after a 100-fold expansion to 10^5 cells. Alternatively, if an amplification were to occur in the last cell generation before selection, there should be only one PALA-resistant colony. Thus, the number of colonies in each population is a clock, revealing when the amplification arose during the period of unselected growth. At various times, beginning 3 days after selection with PALA was imposed, we isolated groups of metaphase cells from each dish by shaking them off the surface. Since dividing cells are attached much more loosely than the others, this procedure gives a few metaphase spreads highly enriched for resistant cells that continue to grow and divide in PALA. About 1 in 5–10 such spreads showed amplified *CAD* genes, an enrichment of $\approx$10,000-fold compared to the frequency of 10^{-5} in the initial population. The attached cells in each dish were returned to selective medium and colonies were scored after $\approx$2 weeks. Results from a set of experiments in which the exposure to PALA was prolonged for up to 13 days are summarized in Table 1 and examples are shown in Fig. 1. In the left columns of Fig. 1, each yellow spot of hybridization probably represents a single *CAD* gene (2). The most dramatic finding is that a large proportion of the chromosomes carrying amplified *CAD* genes were dicentric (Fig. 1). Many such marker chromosomes, stained with propidium iodide, showed constrictions at the two presumptive centromeres. When the chromosomes were banded with Wright's stain after *in situ* analysis, we could be more sure that two centromeres were indeed present because the two constrictions were more clear. Data from all the marker chromosomes that could be scored are summarized in Table 1. A large fraction (39%) of the banded chromosomes was dicentric. Furthermore, a significant fraction (17%) of the marker chromosomes from spreads that were not banded could also be scored as dicentric by noting the positions of the centromeric constrictions. In our previous work (2), in which PALA-resistant cells were taken from 10^5-cell colonies 22–24 days after the initial event, only 7 of 230 (3%) of the marker chromosomes were dicentric. One example from this earlier work (Fig. 1*B*) shows $\approx$10 copies of *CAD* in the region between the two centromeres, more copies than in any of the 137 marker chromosomes scored in the present study. The banding pattern of this very long marker shows a q arm of B9 at each end. In the present study, 6 of the 14 dicentric chromosomes identified by banding carried a B9q arm at both ends (Fig. 1*A*) and the remaining 8 had a B9q arm at one end only, with chromatin that could not be identified by banding at the other (Fig. 1*C*). To substantiate further that some of the marker chromosomes were dicentric, some spreads that had been hybridized with a CAD probe and then banded with Wright's stain were also analyzed by C-banding (5). Because this was the third procedure used, not all such spreads were informative. Two examples that could be interpreted are shown in Fig. 1 *A*, row 2, and *C*, row 4. Of the 22 banded marker chromosomes that were not dicentric (Fig. 1*D*), 11 had one B9q arm, a result similar to that obtained previously (2).

In the present work, the number of days between the initial event and analysis ranged from $\approx$7 (4 days before PALA and 3 days in PALA) to $\approx$17 (4 days before PALA and 13 days in PALA) (Table 1). The 137 marker sister chromatids scored had a total of 489 spots of hybridization (average, 3.6 spots

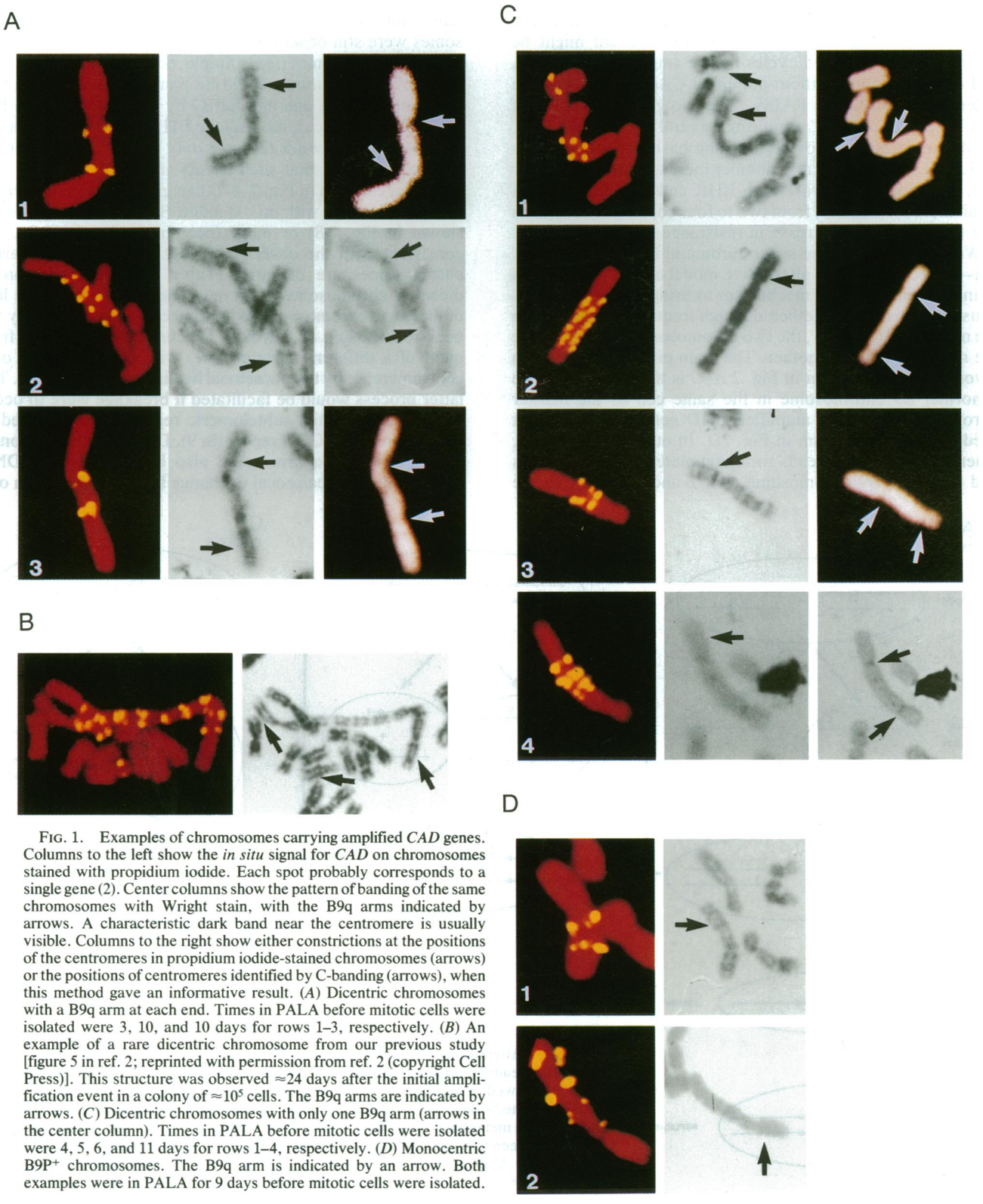

FIG. 1. Examples of chromosomes carrying amplified *CAD* genes. Columns to the left show the *in situ* signal for *CAD* on chromosomes stained with propidium iodide. Each spot probably corresponds to a single gene (2). Center columns show the pattern of banding of the same chromosomes with Wright stain, with the B9q arms indicated by arrows. A characteristic dark band near the centromere is usually visible. Columns to the right show either constrictions at the positions of the centromeres in propidium iodide-stained chromosomes (arrows) or the positions of centromeres identified by C-banding (arrows), when this method gave an informative result. (*A*) Dicentric chromosomes with a B9q arm at each end. Times in PALA before mitotic cells were isolated were 3, 10, and 10 days for rows 1–3, respectively. (*B*) An example of a rare dicentric chromosome from our previous study [figure 5 in ref. 2; reprinted with permission from ref. 2 (copyright Cell Press)]. This structure was observed ≈24 days after the initial amplification event in a colony of $\approx 10^5$ cells. The B9q arms are indicated by arrows. (*C*) Dicentric chromosomes with only one B9q arm (arrows in the center column). Times in PALA before mitotic cells were isolated were 4, 5, 6, and 11 days for rows 1–4, respectively. (*D*) Monocentric $B9P^+$ chromosomes. The B9q arm is indicated by an arrow. Both examples were in PALA for 9 days before mitotic cells were isolated.

per chromatid), 122 markers had 2–5 (89.1%), 15 had 6–9 (10.9%), and none had 10 or more spots. In our previous study (2), all but one of the analyses were done after 21 days in PALA (clone 50-2 was in PALA for 27 days). There is no dramatic difference in average copy number when the two studies are compared, except that cells with 10 or more copies of *CAD*, found in 6 of 230 (2.6%) of the marker chromosomes in the previous work, were not observed at all in the present study of earlier events.

Alternative Mechanisms of Telomeric Fusion. Fusion of two B9p telomeric regions might occur between sister chromatids (Fig. 2 *A–C*) or between homologous chromosomes (Fig. 2*D*). In either case, the dicentric chromosomes formed would be expected to enter bridge–breakage–fusion cycles (6). These cycles can lead to gene amplification when a dicentric chromosome breaks in such a way that both copies of *CAD* segregate into one of the daughter cells (Fig. 2 *B* and *D*) and not when breakage leads to equivalent transfer of *CAD* into

the two daughter cells (Fig. 2*A*). Amplification will also result if an unbroken dicentric chromosome segregates into one daughter cell (Fig. 2*C*). The initial fusion event might be followed by several cell cycles before amplification occurs, and of course cells without amplified *CAD* genes would be killed or arrested by PALA. The slow accumulation of amplified *CAD* genes, requiring several cell generations, would nevertheless provide a strong basis for selection by PALA as soon as the copy number increased above two per cell. In our previous work with BHK cells, we found that a small increase in CAD, from two to four copies per cell, gives rise to an ≈20-fold increase in resistance to PALA (7).

We strongly favor the sister chromatid fusion model (Fig. 2 *A–C*) because, in the alternative model of Fig. 2*D*, one has to invoke an unknown mechanism to bring the two homologous chromosomes together at a high frequency, whereas, in the model of Fig. 2 *A–C*, the two chromosomes that must fuse are normally bound together. The main piece of evidence in favor of the mechanism in Fig. 2 *A–C* is that we usually see a normal B9 chromosome in the same cell as the marker chromosome carrying amplified *CAD* genes, a finding not predicted by mechanism in Fig. 2*D*. In our previous study, where many of the spreads were complete, most of the cells had a normal B9 chromosome (ref. 2 and Table 1). In the present study, where many of the spreads were incomplete, due to differences in the methods used, normal B9 chromosomes were still observed, often in the same spreads as the marker chromosomes (for example, see Fig. 1*C1*). A cell with three B9 chromosomes could be imagined to give rise to a resistant cell with one normal B9 by the mechanism in Fig. 2*D*. However, since almost all the BHK cells observed in this and the previous work (2) have only two B9 chromosomes, it is very unlikely that such events are significant.

The mechanism in Fig. 2 *A–C* can give rise to monocentric sister chromatids with an extended B9p arm having two (as shown in Fig. 2*B*) or more (not shown) copies of *CAD*, consistent with the observation that not all of the marker chromosomes are dicentric (Table 1). The formation of dicentric chromosomes with only one B9q arm and the loss of dicentric chromosomes with time can be explained by the occasional fusion of a broken B9 chromosome with a fragment of a different chromosome, with or without its own centromere, or by the occasional healing of a broken end. The latter process would be facilitated if breakage were to occur within an interstitial subtelomeric region closely related to the terminal TTAGGG repeat (8, 9). Dicentric chromosomes with a single B9q arm could also be formed after DNA replication by a reciprocal exchange between the p arm of a

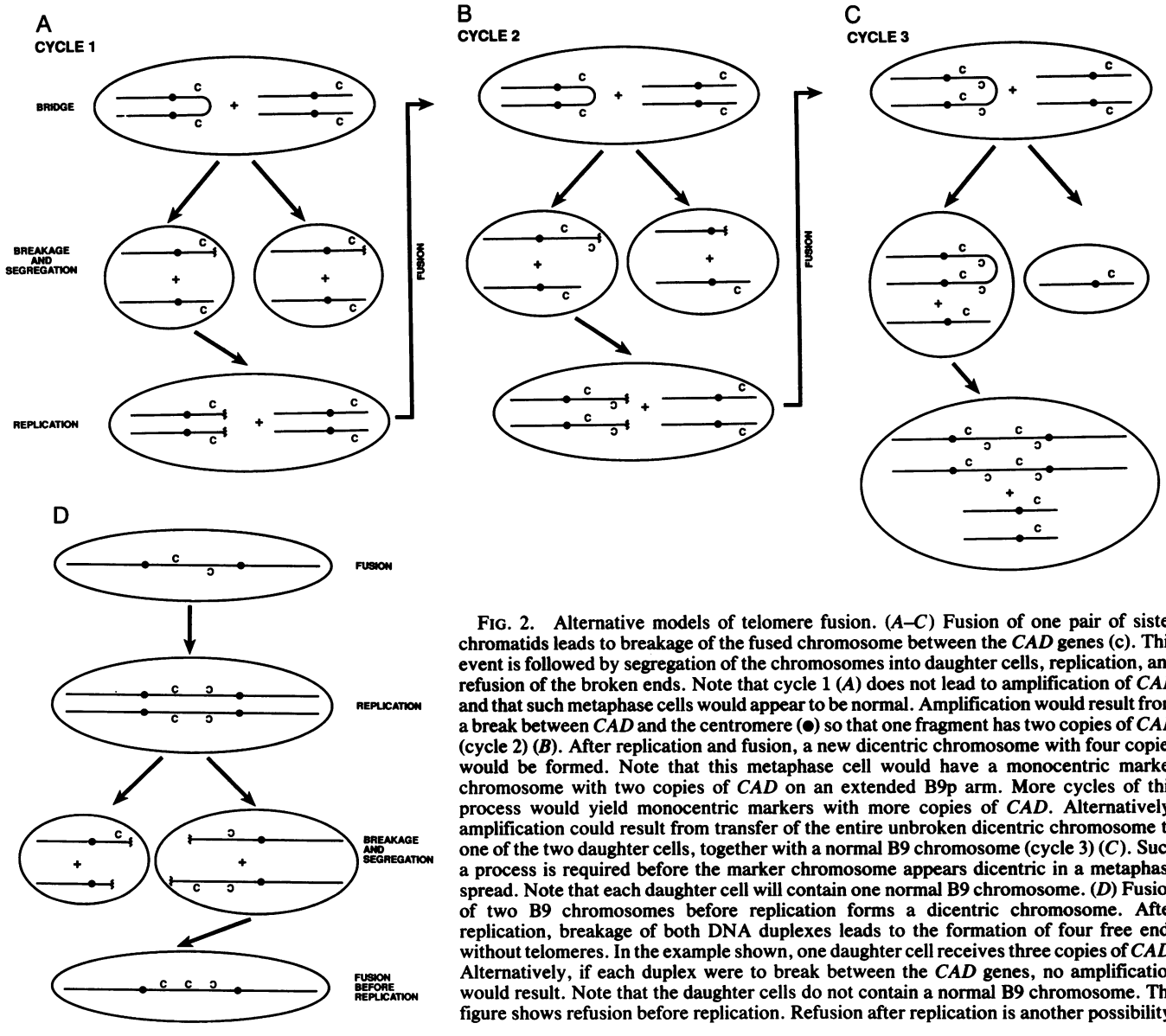

FIG. 2. Alternative models of telomere fusion. (*A–C*) Fusion of one pair of sister chromatids leads to breakage of the fused chromosome between the *CAD* genes (c). This event is followed by segregation of the chromosomes into daughter cells, replication, and refusion of the broken ends. Note that cycle 1 (*A*) does not lead to amplification of *CAD* and that such metaphase cells would appear to be normal. Amplification would result from a break between *CAD* and the centromere (●) so that one fragment has two copies of *CAD* (cycle 2) (*B*). After replication and fusion, a new dicentric chromosome with four copies would be formed. Note that this metaphase cell would have a monocentric marker chromosome with two copies of *CAD* on an extended B9p arm. More cycles of this process would yield monocentric markers with more copies of *CAD*. Alternatively, amplification could result from transfer of the entire unbroken dicentric chromosome to one of the two daughter cells, together with a normal B9 chromosome (cycle 3) (*C*). Such a process is required before the marker chromosome appears dicentric in a metaphase spread. Note that each daughter cell will contain one normal B9 chromosome. (*D*) Fusion of two B9 chromosomes before replication forms a dicentric chromosome. After replication, breakage of both DNA duplexes leads to the formation of four free ends without telomeres. In the example shown, one daughter cell receives three copies of *CAD*. Alternatively, if each duplex were to break between the *CAD* genes, no amplification would result. Note that the daughter cells do not contain a normal B9 chromosome. The figure shows refusion before replication. Refusion after replication is another possibility.

single chromatid of B9 and a chromatid from a different chromosome. Although such events have been observed in Chinese hamster cells treated with the topoisomerase II inhibitor VM-26 (10), they would probably be too infrequent in untreated BHK cells to account for our results.

Region-Specific Recombinations as Primary Events in Gene Amplification. For amplification of the *CAD* gene in BHK cells, the data strongly support the hypothesis that fusion of sister chromatids at or near telomeres (T–T events), to generate an inverted dicentric chromosome (Fig. 2 *A–C*), is an important initiating event. However, our data do not rule out the possibility that additional mechanism(s) contribute substantially. For example, we have considered (2) that unequal sister chromatid exchanges between pericentromeric and peritelomeric regions (C–T events) might initiate an amplification cascade by generating a direct repeat of an entire chromosome arm. To distinguish direct from inverted repeats, one could use two widely separated probes from the same chromosome arm labeled with different fluorochromes. This approach would be difficult in the Syrian hamster experimental system because the B9p arm is relatively short and especially because no probe other than the CAD probe is available for this arm. For these reasons, we have elected to do such analyses in other systems where appropriate probes are available. For example, an early event in *CAD* gene amplification in human cells, assayed using two probes well-spaced along the p arm of chromosome 2, has been observed to generate a new marker chromosome carrying two copies of all or most of the p arm, inverted about a single centromere (C–C event; K.A.S., M.B.S., G.R.S., and R. Groves, unpublished data).

In a study of the amplification of AMP deaminase genes in Chinese hamster cells (4, 12) use of two probes has provided strong support for a mechanism in which breakage of one sister chromatid is an important early event, generating large-scale inverted repeats. However, the symmetrical dicentric chromosomes expected for a T–T fusion event were not observed by Tolédo *et al.* (4) at the 10^5- to 10^6-cell stage, making it uncertain whether the breakage is caused by such an event or by another mechanism.

From the limited data now available, many of the initial events of amplification seem to involve nonrandom recombinations between the pericentromeric and peritelomeric regions of sister chromatids. There are three possible combinations (T–T, C–T, or C–C events). It is pertinent to note that long sequences closely related to the TTAGGG repeats of mammalian telomeres are found not only in the large subtelomeric regions of virtually all chromosomes but also near many centromeres (11). The T–T and C–T events generate unstable structures that would evolve rapidly (see Fig. 2 and ref. 2). It is also possible that intrachromatid recombination between telomeric and centromeric sequences could generate acentric or monocentric circles, perhaps related to the extrachromosomal amplified DNA observed in at least one system (3). Further work will be required to substantiate the validity and generality of the region-specific recombinations we now propose and to evaluate which mechanisms initiate the amplification of different genes in cells of different species.

We appreciate the help and advice of Charles O'Neill, Denise Sheer, and Tania Jones.

1. Trask, B. J. & Hamlin, J. L. (1989) *Genes Dev.* **3,** 1913–1925.
2. Smith, K. A., Gorman, P. A., Stark, M. B., Groves, R. P. & Stark, G. R. (1990) *Cell* **63,** 1219–1227.
3. Windle, B., Draper, B. W., Yin, Y., O'Gorman, S. & Wahl, G. M. (1991) *Genes Dev.* **5,** 160–174.
4. Tolédo, F., Smith, K. A., Buttin, G. & Debatisse, M. (1992) *Mutation Res.*, in press.
5. Salamanca, F. & Armendares, S. (1974) *Ann. Genet.* **17,** 135–136.
6. McClintock, B. (1951) *Cold Spring Harbor Symp. Quant. Biol.* **16,** 13–47.
7. Kempe, T. D., Swyryd, E. A., Bruist, M. & Stark, G. R. (1976) *Cell* **9,** 541–550.
8. Harrington, L. A. & Greider, C. W. (1991) *Nature (London)* **353,** 451–454.
9. Morris, G. B. (1991) *Nature (London)* **353,** 454–456.
10. Charron, M. & Hancock, R. (1991) *Chromosoma* **100,** 97–102.
11. Meyne, J., Baker, R. J., Hobart, H. H., Hsu, T. C., Ryder, O. A., Ward, O. G., Wiley, J. E., Wurster-Hill, D. H., Yates, T. L. & Moyzis, R. K. (1990) *Chromosoma* **99,** 3–10.
12. Tolédo, F., Le Roscouet, D., Buttin, G. & Debatisse, M. (1992) *EMBO J.*, in press.