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Perspectives in Cancer Research

The Importance of Circular DNA in Mammalian Gene Amplification[1]

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Despite a decade of intensive research, the molecular mechanisms of gene amplification have remained elusive. However, recent molecular analyses and cytogenetic investigations suggest that one frequently used mechanism for generating this important form of chromosome instability begins with the production of acentric, circular, extrachromosomal DNA molecules which replicate autonomously. Gene amplification then results from the unequal mitotic segregation and progressive accumulation of these elements under selective conditions. In many cases, the initial circles are too small to be visualized by light microscopy, but they enlarge over time to form heterogeneously sized double minute chromosomes. The molecular structures of the initial circular amplification intermediates, their production coincident with deletion of the corresponding chromosomal region in some cases, and the ability to increase the frequencies of both gene amplification and gene deletion with treatments which perturb DNA synthesis lead to the proposal that recombination within stalled replication intermediates may be an important mechanism for generating circular amplification precursors. Furthermore, recent molecular data demonstrate a molecular chronology for gene amplification in which the initial extrachromosomal elements integrate to generate intrachromosomal regions of gene amplification. A similar model was previously proposed to account for a wealth of cytogenetic observations (12–14). The substantial agreement between the molecular and cytogenetic data in human and rodent systems encompassing a variety of genes and selective conditions encourages the belief that the proposed model can account for many cases of gene amplification. In order to facilitate understanding of the interrelationships between the molecular and cytogenetic data presented later in this article, a model for the proposed chronology of gene amplification is presented in Fig. 1.

Gene Amplification as a Model System for Investigating Genetic Plasticity in Cancer Cell Chromosomes

Exposure to changing environments provides a constant challenge for the survival of cancer cells *in vivo*. The genetic instability which typifies cancer cells opens up vast possibilities for changing gene expression through alterations in gene structure or gene copy number and undoubtedly underlies the ability of such cells to rapidly acquire new phenotypes and to survive in suboptimal growth conditions. However, while abnormal karyotypes are a hallmark of neoplasia and specific chromosomal alterations provide molecular signatures for certain cancers, it is generally thought that such alterations occur at much lower frequencies, if at all, in normal somatic cells. Thus, understanding the molecular mechanisms by which chromosomal alterations occur in cancer cells, and whether they can be precipitated by specific environmental conditions, should provide insight into both the control mechanisms in normal cells which maintain genomic integrity and how these controls are abrogated in cancer cells.

What is the best approach for investigating genetic instability in cancer cells? Clearly, it is important to study an event which occurs frequently in cancer cells *in vivo* and which can also be analyzed *in vitro* under controlled laboratory conditions. There are four predominant manifestations of genetic instability which are candidates for such studies: (*a*) increases in gene copy number (gene amplification); (*b*) chromosomal translocation; (*c*) interstitial deletions; and (*d*) chromosomal aneuploidy. Among these, only gene amplification and gene deletions are amenable to genetic selections and biochemical, cytogenetic, and molecular analyses using mammalian cell lines. While there are now more than two dozen different genes which can be selected for amplification *in vitro* (see Ref. 10 for a review), only a few selective strategies have been used to isolate cells with gene deletion (15–18), and there are no reported examples in mammalian cells of selective strategies for the isolation of cells with specific chromosomal translocations. Thus, appropriate drug selections can be used to reveal the presence of local increases in copy number at many different chromosomal positions since the respective target genes are present at different genomic locations. The broad diversity of selectable target genes also enables analysis of the genomic consequences of selection under a variety of metabolic challenges. Furthermore, data to be summarized below indicate that deletion of chromosomal segments containing a replication origin and a selectable gene provides one means of producing circular intermediates of gene amplification. Thus, it is conceivable that recombination within a single type of chromosomal structure could generate an amplification precursor or a deletion, with the genetic outcome being determined by the nature of the selection imposed. Although it is not straightforward to analyze the mechanisms and conditions which lead to the generation of large deletions such as those associated with the elimination of "tumor suppressor" genes (*e.g.*, see Ref. 19 for a recent review), if gene amplification is frequently initiated by deletion of the corresponding sequences from the chromosome, then it could provide an experimental system for gaining insight into the process of deletion as well.

Extrachromosomal Elements, the Most Common Carriers of Amplified Genes in Human Tumor Cells

Two abnormal chromosomal structures, double minute chromosomes and expanded chromosomal regions, are the predom-

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[1] This article is not meant to be a comprehensive review of the amplification literature. Rather, it was solicited to provide a new perspective on the role of circular DNA in the amplification process. The brevity of discussion of other models was motivated purely on the basis of space constraints. I refer the reader to Refs. 1–11 for excellent reviews on gene amplification, which establish many of the basic principles in the field, and to Ref. 12 for an extensive literature citation list.

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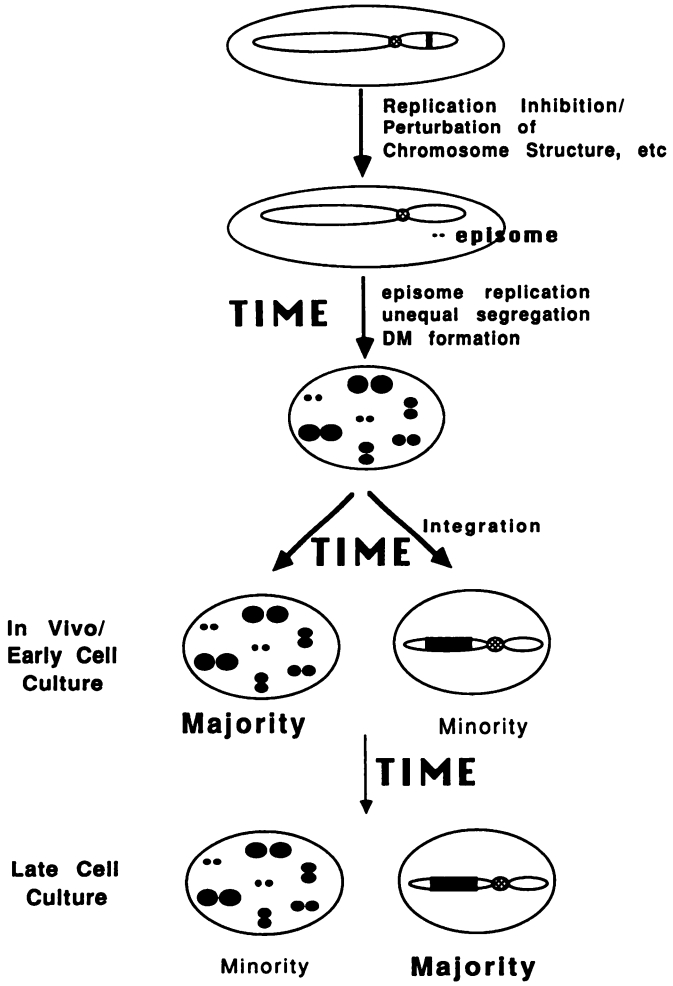

Fig. 1. Molecular chronology for gene amplification. The steps involved are: step 1, production of submicroscopic circular precursors of DMs called episomes; step 2, continued growth or selection produces cells with heterogeneously sized DMs and gene amplification; step 3, integration of DMs or episomes generating cells with expanded chromosomal regions in one or more chromosomes; step 4, showing that, *in vitro*, cells with expanded chromosomal regions will overtake the population under appropriate growth conditions. The formation of the episome in step 1 is shown to occur by deletion of the corresponding sequences from the chromosome, although retention of chromosomal sequences is also possible as described in the text. This chromosome is left out of the remaining cells in the figure for simplicity, not as an indication that it is lost as a consequence of episome formation. In some cases, DMs may form without an episomal precursor by "excision" of a chromosomal fragment exceeding $1-2 \times 10^6$ base pairs (the limit of resolution of the light microscope). In such cases, size heterogeneity of extrachromosomal elements may not develop as extensively as when episomes are involved.

inant sites of gene amplification in mammalian cells. DMs[3] are paired, acentric extrachromosomal chromatin bodies which replicate autonomously and once per cell cycle. DMs segregate randomly at each mitosis, and consequently are lost from the cell population over time in the absence of a selective pressure. By contrast, amplified sequences within expanded chromosomal regions (commonly referred to as homogeneously staining regions when they fail to exhibit trypsin-Giemsa bands[4]) segregate properly during mitosis because they are linked to a centromere and are generally not lost in the absence of selection (for a possible example of gradual loss of sequences from a HSR, see Ref. 12).

The existence of two such different cytogenetic structures which carry amplified sequences raises several important questions for understanding amplification mechanisms. For instance, is either the predominant carrier of amplified sequences *in vivo*? Are these structures produced by two mutually exclusive molecular pathways? Can one structure be the precursor of the other (*e.g.*, do DMs integrate to form HSRs, do HSRs break down to generate DMs, or is there an equilibrium between the two processes)? If there is a precursor product relationship, why are both structures not observed to coexist within a single cell? Relevant to the last question, does the commonly observed presence within a single cell of either DMs or HSRs, but never both, containing the same amplified sequences reflect selective overgrowth of cells containing only a single form of amplified region? If rapid shifts in the makeup of a population do occur, then the common practice of analyzing the cytogenetic consequences of gene amplification after multiple steps of selection or after extended passage in cell culture is likely to obscure the identity of the initial products.

We investigated whether extrachromosomal or intrachromosomal structures predominate in human tumors *in vivo* by reviewing published cytogenetic analyses of tumor samples which were made at the time of biopsy and had evidence of gene amplification.[5] We then assessed whether a shift in the cytogenetics of the population occurs after extended periods of growth *in vitro* by analyzing a large number of publications concerning tumor cells which had been passaged in culture.[5] The results were both dramatic and revealing. Analysis of 192 tumors (75 leukemias and myelodysplastic syndromes and 117 solid tumors derived from 21 different conditions) with cytogenetic and/or molecular evidence of gene amplification at biopsy revealed that 95% contained DMs and only 5% contained HSRs. A small percentage of samples consisted of mixed populations with cells containing either DMs or HSRs. By contrast, the proportion of tumors with DMs or HSRs shifted significantly when data from a broad diversity of tumor cell lines (109 reports) were analyzed; ~34% of the *in vitro* passaged samples contain HSRs, the percentage with DMs was approximately 66%, and 11% of all cell lines reported consisted of mixed populations containing cells with either DMs or HSRs. These data do not derive from the analysis of unrepresentative or restricted tumor types, and they cannot be explained by more facile detection of cells with DMs.[5] Interestingly, of 14 cases with HSRs in biopsy samples, 6 were from patients who had been treated previously with chemotherapy. These observations are consistent with HSR containing cells being clonal outgrowths from a population of cells in which the amplified sequences were initially localized to DMs. The data also strongly indicate that DMs are the predominant cytogenetic manifestation of gene amplification in human cancer *in vivo*.

Extrachromosomal Elements: Probable Initial Intermediates in Many Examples of Gene Amplification

The high incidence of DMs *in vivo* can be explained in three ways. First, DM formation could be more highly selected *in vivo* than is HSR formation. In this case, both DMs and HSRs could be present in cells within a population, but the cells containing DMs predominate due to a growth advantage *in*

[3] The abbreviations used are: DM(s), double minute chromosome(s); HSRs, homogeneously staining regions; MTX, methotrexate.

[4] Several different names have been used to describe expanded chromosomal regions containing amplified genes. For example, "ABR" (abnormally banding region) has been used to indicated a region which bands abnormally with trypsin-Giemsa, while "ECR" is a general acronym for "expanded chromosomal region." While I prefer the general abbreviation ECR because Giemsa banding is somewhat subjective, I will use the acronym " HSR" due to its general usage in the literature and familiarity to most readers.

[5] S. Benner, G. M. Wahl, and D. D. Von Hoff. Double minute chromosomes and homogeneously staining regions in human malignancy, submitted for publication.

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vivo. Second, the formation of DMs could represent an earlier event in gene amplification than HSR formation, and as previously suggested (12–14), HSRs could form subsequently by integration of DMs into chromosomes. A third possibility is that HSRs are an early step in gene amplification, but they continuously break down *in vivo* to form DMs (*e.g.*, see Refs. 1, 20).

Studies from rodent and human cell culture systems strongly indicate that DM formation is likely to be an earlier event in gene amplification than is HSR formation and that HSRs do not break down to form DMs at measurable rates, except in some special cases involving cell fusion (see Ref. 1 for further references). (*a*) Flow sorting analyses indicate that Chinese hamster ovary cells resistant to methotrexate are initially unstable and are only later replaced by cells with stably amplified sequences (21). (*b*) *In vitro* propagation of cell lines containing amplified genes initially in DMs resulted in eventual replacement of the DM population by cells containing one or more HSRs (13, 14). (*c*) *In situ* hybridization and Southern blotting have shown that a DM or its precursor (see below) could integrate into chromosomes (22, 23),[6] and no evidence was found to indicate that an HSR could disaggregate to form DM precursors *in vitro* (22). (*d*) Several studies (24, 25) show that a mouse ascites tumor cell line maintains DMs harboring c-*myc* genes when passaged *in vivo*, but a subpopulation of cells containing HSRs predominates after *in vitro* passage. Importantly, when a mixed culture which contained cells with either DMs or HSRs after *in vitro* growth was passaged *in vivo*, the DM containing cells dominated *in vivo*. However, *in vivo* passage of a clone containing HSRs failed to yield any DM containing cells (25). Taken together, the data indicate that a growth advantage probably does accrue to cells containing unstable extrachromosomal elements *in vivo*, but not *in vitro*, and they also indicate that HSRs do not break down to form DMs *in vivo* or *in vitro* at a measurable rate. The data also make it reasonable to conclude that when DMs are observed, they or the precursors described below represent the initial molecular products of the mechanisms which generate amplified sequences.

Formation of DMs from Submicroscopic Precursors

Since DMs are probably the most common early cytogenetic products of gene amplification in humans and possibly other species, it is clearly important to understand whether they are formed *de novo* or whether they are derived from smaller precursors. There are several reasons for entertaining the latter possibility: (*a*) DMs within a single cell often vary in size from particles which are barely visible under the light microscope (~1–2 × 10^6 base pairs) to structures the size of small chromosomes (*e.g.*, see Ref. 1); (*b*) analyses of the genetic complexity of DMs by in gel renaturation studies suggest that some DMs contain far less than the 10^6 base pairs minimally required for their detection by light microscopy (26, 27); (*c*) some reports describe examples of unstable amplification detectable by Southern blotting but not by light microscopy (*e.g.*, see Ref. 28).

If precursors of DMs exist, they should fulfill three criteria: (*a*) they should either be circular or be capable of forming circular structures since DMs have been shown to be circular by electron microscopic (7) and molecular analyses (29, 30)[7]; (*b*) they should replicate once per cell cycle, since DMs replicate in this fashion (31); (*c*) the precursors should enlarge over time to form structures which are microscopically indistinguishable from DMs. All three criteria have been fulfilled in one system (22, 28), and submicroscopic, autonomously replicating circular molecules have been reported in three other cases (see Table 1 for a summary and references). There are three additional systems in which submicroscopic extrachromosomal elements have been found, but their replication has not been documented (see Table 1). These studies encompass amplification of transfected genes, endogenous genes which engender resistance to several antineoplastic agents, and two examples involving the c-*myc* protooncogene. In one of the latter examples, the submicroscopic elements were found in early but not late passages of the human promyelocytic leukemia cell line HL60 (32). Importantly, since c-*myc* amplification and the presence of DMs in HL60 were reported prior to passaging the cells in culture (27), the submicroscopic elements containing amplified c-*myc* genes must have been formed *in vivo*. Thus, there is now ample evidence that molecules with the characteristics expected for DM precursors are formed in a diversity of systems involving gene amplification *in vivo* and *in vitro*. In several cases, the precursors have been shown to represent very early if not initial molecular products of the amplification process (22, 28, 32, 34).

I propose the term "episome" to describe submicroscopic DM precursors. The term is borrowed from the literature on bacterial extrachromosomal elements and is meant to signify that these elements originate from the chromosome and can integrate into chromosomes (see below and Ref. 22). Our finding of episomes in six of six cell lines which had heterogeneously sized DMs raises the question of whether DMs always originate from such precursors. The data at present indicate that episomes will almost certainly be found in cells containing heterogeneously sized DMs. It is unclear at present why episomes enlarge to form DMs, but it is reasonable to propose that the larger size confers a growth advantage accruing to a higher probability of transmission of the larger elements to daughter cells at mitosis (*e.g.*, by nonspecific attachment to metaphase chromosomes, by more efficient "trapping" within the reforming nuclear membrane subsequent to mitosis, etc.).

Molecular Mechanisms of Episome and DM Formation

The episomes analyzed thus far range in size from 120 to 750 kilobases, and all of those tested replicate semiconservatively and only once per cell cycle. These data indicate that episomes contain functional origins of DNA replication. Since the sizes of the episomes are within the sizes expected for the looped replication domains or "replicons" in mammalian cells (35), it is tempting to speculate that the mechanisms of episome formation are in some fashion linked to DNA replication and that they involve the higher order chromosome structure of replicons. Molecular analysis of the structures of mammalian episomes is beginning to provide suggestions about the molecular mechanisms involved in their formation. The preliminary molecular data indicate that episomes fall into at least two structurally distinguishable subtypes. Type I episomes are simple circles whose structure is the same as that of the corresponding chromosomal region. Type II episomes are formed by an inverted duplication of the corresponding chromosomal region since they contain one head-to-head and one tail-to-tail junction (see Fig. 2 for examples). Results similar to these have been derived for circular amplification products involved in MTX resistance in *Leishmania* (38, 39); "R region" episomes, which

[6] J. Ruiz and G. M. Wahl, manuscript in preparation.
[7] B. Windle and G. M. Wahl, unpublished observations.

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Table 1 *Episomes detected in mammalian cells*

Examples are listed in which submicroscopic elements harboring amplified genes have been detected thus far and the literature citations for published studies. There is only one case in which the shape of the element is in question (example 5). CHO is a Chinese hamster cell line, and examples 2–6 are from human tumor cells (Hep2 is a squamous carcinoma line).

Gene	Cell line	Selection	Shape	Size (kilobases)	Replication	Ref.
1. *CAD*	CHO	*In vitro*-PALA	O	~250	+	22, 28
2. c-*myc*	HL-60 (clones)	*In vivo*-?	O	~250	+	32
3. c-*myc*	COLO320DM (clones)	?-?	O	120, 160	ND[a]	32
4. *mdr1*	KB-V1	*In vitro*-VBL	O	600, 750	+	30
5. DHFR	HeLa	*In vitro*-MTX	?	600–700	ND	33
6. DHFR	Hep2	*In vitro*-MTX	O	200–300	+	[b]
7. *ADA*	Mouse	*In vitro*-AAU	O	500–1400	ND	Footnote 7

[a] ND, not determined; DHFR, dihydrofolate reductase; PALA, *N*-(phosphonacetyl)-L-asparatic acid; VBL, vinblastine.
[b] D. Van Devanter and D. Von Hoff, unpublished observations.

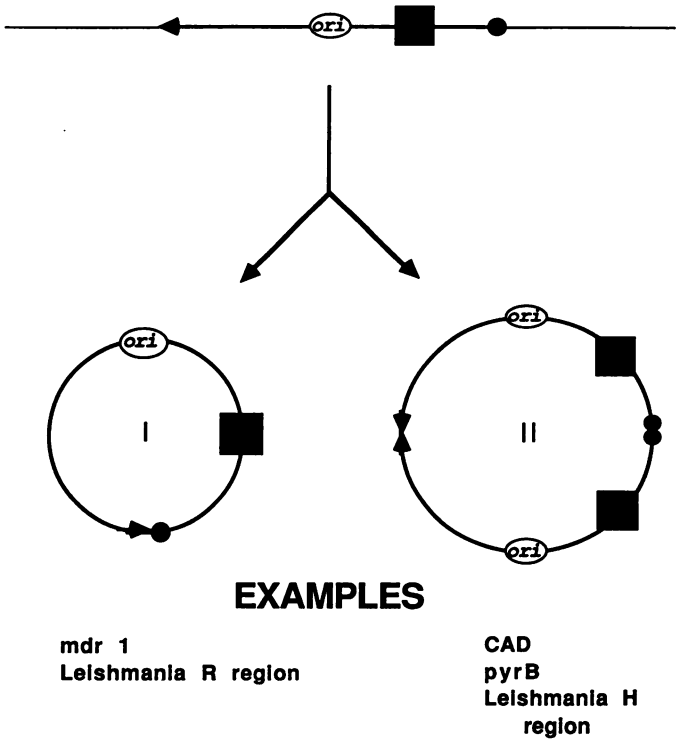

Fig. 2. Molecular structures of episomes. The structure at the *top* represents the chromosomal region corresponding to that contained in the various episomes. *Left*, a type I episome. These are circular molecules the structure of which is the same as that of the chromosomal region from which they were derived. *Right*, a type II episome. These episomes have a size which is twice their genetic complexity. They contain a duplication of cell sequences arranged as an inverted repeat with one head-to-head and one tail-to-tail junction. Asymmetrical recombination events produce type II episomes which do not contain perfect inverted repeats (*e.g.*, see Refs. 33, 36 and 37). Both structures are predicted by their replication properties to contain at least one replication origin. The *mdr1* episome in vinblastine resistant KB cells is inferred to be type I since its physical size determined by δ-irradiation is the same as that produced by cleavage with *Not1* or *Nru1* (33). Complete restriction maps confirm that the *Leishmania* R region episome is type I (38, 39), the *Leishmania* H region and *CAD* episomes are type II (38, 39),[8] and the pyrB episome is inferred to be type II because it has one head-to-head junction which is known to be part of a large inverted repeat (31).

contain the bifunctional thymidylate synthetase/dihydrofolate reductase target gene of MTX, are simple circles, while "H region" episomes contain one head-to-head and one tail-to-tail inverted repeat (38).

The two molecular structures presented above raise the question of whether both are created from a single molecular intermediate by alternative recombination events or whether two completely independent molecular mechanisms are involved. Several models can be envisioned to generate both episomal types from a common intermediate. For example, a single recombination event across a replication loop could generate a type I episome (Fig. 3) while a double recombination within the same structure would generate a type II episome (Fig. 3). Similar recombination events within a chromosomal region which has undergone re-replication (9) or "double rolling circle" replication (36, 40) could also generate either type of episome. Importantly, recombination within a standard replication loop predicts the generation of a chromosomal deletion coincident with episome formation, while the other two models are not expected to result in a loss of the native chromosomal locus. We have observed a deletion of chromosomal sequences corresponding to the episomal sequences in cells containing the type II *CAD* episome (22),[9] and in another case we observed that cells containing an extrachromosomally amplified structure with at least one inverted repeat also contained a deletion of the chromosomal locus (34).[6] Production of R region episomes in some MTX resistant lines of *Leishmania* has also been shown to be associated with deletion of the chromosomal R locus (39). Thus, in these examples, the data are consistent with a model involving recombination within a replicating structure. However, there are also examples of the production of R region circles with apparent retention of chromosomal sequences in different *Leishmania* cell lines (39). Such data are consistent with a conservative mechanism which preserves native chromosomal structure, although other alternatives have not been ruled out. Therefore, it is possible that multiple mechanisms may generate extrachromosomal, autonomously replicating circular DNA molecules.

The proposed relationship between episome formation and recombination of replication intermediates is intriguing in light of the substantial literature which indicates that a variety of treatments which interfere with DNA synthesis or damage DNA can increase the frequency of gene amplification (see Ref. 40 for an example and Ref. 11 for additional references) and can also increase the frequency of gene deletion (cited in Refs. 11 and 18). The increased frequency of gene amplification under such conditions was previously interpreted as evidence that these treatments induce reinitiation of DNA synthesis (11, 37). However, recent experiments argue strongly against such an interpretation (41). Another explanation is that these treatments result in the accumulation of replication intermediates which, because of gaps or nicks in incomplete nascent strands, are excellent substrates for recombination (42). As described above, one product of recombination within such structures could be a circle with one or more replication origins and the inverted repeats found in several episomes analyzed thus far. Yet another means by which such treatments could stimulate gene amplification is by the mobilization of transposable ele-

[8] B. Draper and G. M. Wahl, unpublished observations.

[9] B. Draper, B. Windle, and G. M. Wahl, unpublished observations.

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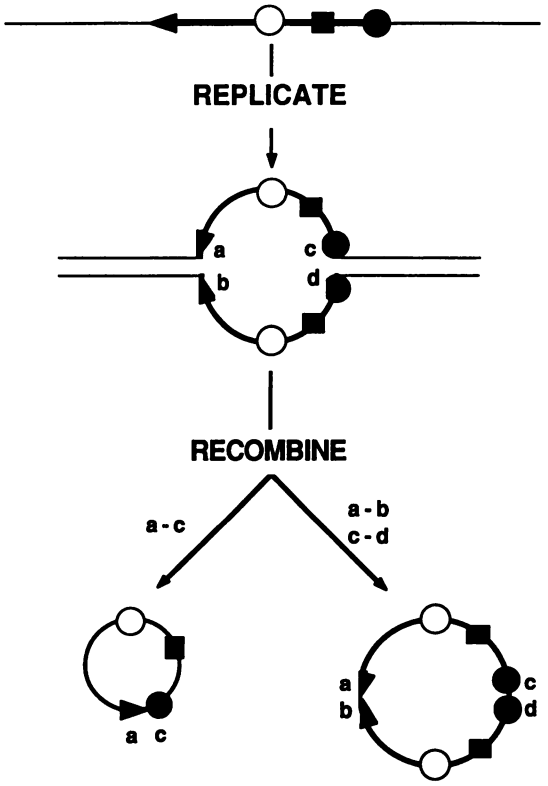

Fig. 3. Recombination within a replication loop can generate both type I and type II episomes. Under the appropriate conditions, progress of a replication fork may be impaired, leading to the accumulation of stalled replication intermediates. A crossover between *sites a* and *c* generates a type I episome, while recombination within the same structure between *points a* and *b* as well as *c* and *d* generates a type II episome. In the latter case, an acentric and a dicentric chromosome should also be generated.

ments which, after reintegration, destabilize the chromosome at the point of insertion. Experimental support for such a proposal comes from the observation that UV treatment can apparently "induce" the transposition of some repetitive sequences [*e.g.*, "Alu" (43)] and that DNA insertion in some genomic sites apparently precipitates the formation of extrachromosomal elements which lead to rapid amplification by unequal segregation (34).

There is a substantial literature on other mechanisms which could generate circular molecules, and these have been addressed in detail in previous reviews (*e.g.*, see Ref. 3) and other publications (36, 40). While such mechanisms can readily account for the production of amplification units less than 10^6 base pairs, molecular and cytogenetic analyses suggest that in some cases each "unit" of amplification may be far larger than 10^6 base pairs (*e.g.*, see Refs. 29, 44, and 45). If such expansive regions are the initial products of amplification in these cases, then it is unlikely that the simple mechanisms proposed above could account for their production. Other processes which may make chromosomes fragile and subject to random breakage may be involved. One possibility is suggested by the striking microscopic similarities between large DMs and the prematurely condensed chromatin which results from the fusion of S phase cells with metaphase cells (46).[10] It is conceivable that cells in metaphase fuse with interphase cells to produce hybrids with prematurely condensed chromatin which is mechanically fragile. The chromatin could break apart, liberating structures spanning several megabases or more and containing the selected gene and one or more replication origins.

[10] S. Sen, personal communication.

DM Integration and the Generation of Chromosomally Amplified Regions

Taken together, the experiments summarized above provide compelling support for the idea that episomes or DMs represent very early products of gene amplification, and they are likely to be precursors of HSRs in many cases. The inverted and direct repeats found in HSRs (47–49) could easily be explained by the integration of type I or type II episomes or of episome multimers generated by intra- or interepisome recombination events. Although the majority of examples indicate that chromosomally amplified genes are usually not at the site of the native gene and are often at multiple chromosomal sites in each cell, some reports have shown amplification near or at the site of the resident gene (see Ref. 3 for citations of original references). It is easy to envision random insertion of DMs generating multiple chromosomal sites of amplification, and recent data reveal that integration of extrachromosomal elements can generate multiple sites of chromosomal amplification within a single cell (23).[6] Is it reasonable that DMs also integrate by homologous recombination at a sufficiently high frequency to generate a HSR near or at the native locus? Recent data showing that the frequency of homologous insertion increases with the length of homology and is not influenced by repetitive sequences (50) make such a proposal feasible. Thus, integration of extrachromosomal elements could explain the observed chromosomal patterns of gene amplification in many of the reports presented thus far. However, I emphasize that observations on some examples of gene amplification suggest that HSRs may also form *de novo* by mechanisms which do not involve an extrachromosomal intermediate (see Refs. 36 and 44 for two potential examples and below for further discussion).

Speculation on Why DMs Predominate *in Vivo* and Disappear *in Vitro*

The above observations lead to the question of why cells with DMs should predominate *in vivo*, while they are apparently selected against *in vitro*. Clearly, cells with amplified sequences present in extrachromosomal elements must be continuously exposed to a selective pressure or else the amplified sequences would be lost over time. However, when a constant selective pressure is applied, a population consisting of cells with DMs should theoretically grow more slowly than a cell population containing the same sequences amplified in HSRs. The reason for this expectation is that unequal segregation of the acentric DMs at mitosis results in some daughter cells containing fewer copies of the amplified sequences than are required to sustain growth in the presence of selection. By contrast, amplification within HSRs guarantees that every daughter cell inherits the same number of amplified copies as the parent, and hence each cell should survive if the selection pressures remain constant. It is likely that a cell population with DMs is at an advantage *in vivo* because the selection pressures are inconstant. The high rate of copy number fluctuation in such cells ensures that some cells will contain an appropriate number of copies for optimal growth under the diverse conditions likely to prevail *in vivo*. The same reasoning can explain why the relatively constant environment imposed experimentally *in vitro* selects for cells containing HSRs; in this case, a faster population doubling time accrues to cells which maintain the same copy number at each cell division.

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Perspectives

Early studies of gene amplification produced the widely held view that large increases in the copy number of a selected gene could be achieved within a single cell cycle, as happens in infections by certain DNA viruses. This idea was bolstered by experiments which demonstrated the presence of substantial amounts of doubly replicated DNA within a single S phase under conditions which perturbed cell cycle progression (51). However, more recent experiments strongly indicate that if DNA rereplication occurs, it is at a level below the limits of current detection methods (41). The common observation that the most effective strategies for selecting cells with amplified sequences use stepwise selections with shallow increases in selective stringency at each step is also inconsistent with mechanisms which generate large increases in copy number rapidly. Rather, such experiments imply that amplified sequences build up gradually over multiple cell divisions. Our observations that extrachromosomal elements are the most common repositories of amplified sequences *in vivo*, and possibly also *in vitro*, and that they represent early if not initial steps in many examples of gene amplification provide a molecular explanation consistent with these latter findings.

At the present time, the molecular chronology proposed here for gene amplification is the only one for which direct molecular evidence has been obtained for each step. The high incidence of extrachromosomal elements in human biopsy specimens containing amplified sequences implies that the proposed model is likely to account for the majority of cases of gene amplification in human cancers. This raises the question of the number of other mechanisms utilized for gene amplification in mammalian cells and how frequently each is used. There is certainly good molecular evidence for unequal sister chromatid exchange being involved in gene duplications in the globin locus (52). Such events must have occurred in the germ line, but it is unclear how frequently they occur in mitotically dividing cancer cells. No direct molecular support has been obtained for such a mechanism participating in selected gene amplification in cancer cells. There is circumstantial evidence that *de novo* amplification occurs within chromosomes without an extrachromosomal intermediate in some cases (36, 44). It has further been proposed that in some cases the initial units of chromosomal amplification are unstable and very large (44, 45) and that the large units decrease in size and generate complex sequence rearrangements with continuing passage (44, 45). As emphasized above, there is no evidence that chromosomally amplified sequences can break down to liberate extrachromosomal elements; we have observed in many systems that small extrachromosomal elements increase in size over time, and we have never observed the reverse. It is conceivable that large, unstable chromosomal amplicons are used in the amplification of certain loci in rodents some percentage of the time, but this mechanism is not used very frequently in human gene amplification. Given the diversity of amplification mechanisms utilized in other organisms, it would be surprising if the model proposed in Fig. 1 constitutes the sole mechanism utilized in mammals; on the other hand, if extrachromosomal elements are as frequently involved in early stages of gene amplification in rodents as they appear to be in humans, then it may be the most commonly used strategy for achieving localized increases in copy number.

The molecular chronology of gene amplification proposed here raises several important questions and suggests exciting avenues for future basic and clinical research. First, it invites investigation of the mechanisms employed for producing the "first circle." The data suggest that recombination of replication intermediates may generate some episomes, but they also leave open the possibilities that mitotic recombination, or recombination of substrates generated by abnormal replication processes (*e.g.*, rereplication or double rolling circle), also contributes to circle formation at some frequency. If units exceeding several megabases are generated early during amplification, they may be formed by fundamentally different mechanisms. Analysis of the chromosomal region remaining after production of the initial amplification products should reveal the relative frequencies with which nonconservative (*e.g.*, deletion) and conservative (*e.g.*, rereplication) mechanisms are utilized for the production of circular amplification products. Moreover, the conclusion that extrachromosomal elements frequently mediate the early steps of gene amplification is based in part on analysis of human tumor biopsy samples which are difficult to define with regard to their proximity to the "first event." Therefore, it is important to design *in vitro* studies to determine the proportion of cases which initiate with the production of circles *versus* the *in situ* generation of chromosomally amplified regions. Due to the rapid rate at which population heterogeneity can develop under *in vitro* passage conditions and the tendency for some cell lines to reintegrate extrachromosomal elements (22, 53), it will be crucial to use sensitive techniques which enable analysis of gene amplification after a single step selection and after the minimum amount of time in culture. Such experiments must be designed to determine whether large apparently unstable units of chromosomal amplification are generated *de novo* or whether (submicroscopic) extrachromosomal intermediates are transiently involved. In addition, if extrachromosomal elements are the predominant early intermediates of gene amplification, it will be important to investigate whether the conditions which increase amplification frequency act by increasing the probability of episome production. Since episomes as large as $1–2 \times 10^6$ base pairs can be differentiated from chromosomal DNA and they can be detected rapidly and sensitively using molecular analyses, monitoring episome production provides an ideal molecular model for analyzing the impact of potentially genotoxic agents on chromosome stability. Finally, all of our data indicate that episomes replicate because they have one or more DNA replication origin—regions implicated in the control of initiation of DNA synthesis. Thus, episomes should provide an excellent and abundant source for the isolation of these previously elusive genomic regions and their molecular dissection should enable a better understanding of how mammalian cells control the initiation step of DNA synthesis.

It is appropriate to end this article with potential means of applying the observations reviewed here to the clinical arena. First, it is conceivable that the molecular chronology presented for gene amplification could be translated into a prognostic paradigm. The chronology implies that cancer cells with gene amplification mediated by episomes should be at an earlier stage of progression than those containing amplification of the same sequences in DMs or HSRs. Thus, in addition to using the existence of the amplification of a particular protooncogene as a prognostic indicator (*e.g.*, see Refs. 54 and 55), the existence of episomes containing that protooncogene may provide another level of precision for determining the stage of tumor progression. Second, consider that the products of the amplified genes must provide the cell with as yet undefined growth advantages, or else the amplified genes would not be retained. Thus, elimination of the amplified structures should theoretically reduce the growth advantages derived from their gene

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products and possibly reduce the tumor burden and enhance the efficacy of chemo- or immunotherapeutic intervention. It may be possible to develop strategies which preferentially eliminate episomes or DMs from the cell; since they are acentric their segregation may be compromised under conditions which do not affect that of centric chromosomes. The spectrum of cancers and number of cases for such treatment opportunities await the derivation of a larger data base to address how frequently gene amplification contributes to various types of cancer and how often episomes and DMs are involved in the amplification process. However, the existence of episomes in a variety of *in vitro* model systems involving protooncogenes and drug resistance genes makes such studies accessible to immediate investigation and encourages the testing of these ideas to determine whether they can provide a new and effective treatment strategy for the future.

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