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On the possibility of metabolic control of replicon "misfiring": Relationship to emergence of malignant phenotypes in mammalian cell lineages

(extrachromosomal nuclear DNA/double minute chromosomes/firones/tumor promoters)

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ABSTRACT **Constraints of a multireplicon chromosomal organization and of the necessity to maintain constant gene dosages demand that each origin of replication in a eukaryotic cell "fire" (initiate replication) only once per cell cycle. The central idea of this work is that a low probability of an extra ("illegitimate") round of DNA replication (called below "replicon misfiring") within any given chromosomal domain could be increased by certain substances of either intra- or extracellular origin. The term "firone" is proposed for such a substance. It is shown that existence of firones could greatly speed up evolution of cellular systems under selection pressure, a developing tumor being one example of such a system. Experimentally testable predictions of the firone hypothesis are discussed.**

One feature of eukaryotic DNA replication is tight control of the number of initiation events per origin of replication. Apart from a few special cases (2–5), each origin of replication in a eukaryotic cell fires only once per cell cycle; repetitive firings at the same origin are prevented, apparently by a special mechanism (6–8). Reasons for the tight control of the frequency of replication initiation include constraints imposed by a multireplicon chromosomal organization, by complex mitotic mechanisms, and by the necessity to maintain constant gene dosages for at least some of the genes. Molecular mechanisms for suppression of extra rounds of DNA replication are unknown and may include specific chromatin structures at the replication origins or termini and possibly replication-dependent chemical modifications of DNA segments involved in replication initiation.

The important point, however, is that the probability of an extra ("illegitimate") round of DNA replication (called below "replicon *misfiring*") within any given chromosomal domain, although generally very low, is not zero, as indicated by the following lines of evidence.

Multiple rounds of DNA replication at certain chromosomal loci in the polytene chromosomes of an insect *Rynchosciara* result in so-called DNA puffs (4). This is an example of a developmentally controlled repeated firing of a specific replicon. Other examples are the phenomenon of ribosomal DNA amplification in many different species (3) and the recently discovered amplification of genes for chorion proteins during oogenesis in *Drosophila* (5). Recent work by Botchan *et al.* (2) indicates that locus-specific replicon misfiring is responsible also for production of free circular simian virus 40 (SV40) DNA molecules after fusion of permissive green monkey cells with nonpermissive SV40-transformed mouse cells. The replication origin of integrated SV40 DNA does not function more than once per cell cycle (2, 9). However, upon fusion of a nonpermissive SV40-transformed mouse cell with a permissive monkey cell, a burst of SV40 replicon misfiring produces extrachromosomal copies of SV40 DNA (2).

That the phenomenon of replicon misfiring is not limited to locus-specific events and highly special cases is indicated by the following lines of evidence.

Cultured mammalian (10) and insect (*Drosophila*) cells (11) contain small but detectable amounts of their nuclear DNA sequences in the form of circular DNA molecules ranging in size from less than one to several kilobase pairs (kbp) and enriched in dispersed, middle repetitive DNA sequences (11). Although the available evidence (10, 11) is compatible with several different interpretations, one explanation is that small circular DNAs may be the result of infrequent events of replicon misfiring occurring preferentially within chromosomal regions containing stretches of dispersed repetitive DNA. In some cases the latter have been shown to behave as transposable elements (12).

In several cases of drug resistance, the cellular phenotype is known to be due to an overproduction of an enzyme that is the specific target of the drug in question (1, 13–21). For example, an overproduction of dihydrofolate reductase (DHFR; EC 1.5.1.3) in mouse cells resistant to the specific DHFR inhibitor methotrexate (MTX) was shown to be due to an increase in the *number* of DHFR genes per cell (13, 22). The extra DHFR genes are found either in small acentric chromosomes [double minute chromosomes (DMs) (23)] or integrated within specific regions of certain mouse chromosomes (24, 25). The extra copies of the DHFR gene accumulating in the course of selection for resistance to increasing concentrations of MTX are most likely the result of infrequent misfirings of the DHFR replicon, each misfiring producing an extra copy of the functional DHFR gene and its chromosomal domain. The increase in the DHFR gene dosage leads to an increase in the amount of DHFR and thus improves the cell's chances for survival in the presence of an otherwise lethal concentration of MTX (13). Incidence of mouse cells resistant to a low MTX concentration ($\approx$100 nM) is of the order of 10^{-6} (13), suggesting that the probability of the DHFR replicon misfiring within a single cell cycle is at least 10^{-6}.

Another example of a similar kind is the amplification of the CAD [CAD is the multifunctional protein containing the enzymatic activities carbamoyl-phosphate synthetase (EC 6.3.5.5), aspartate carbamoyltransferase (EC 2.1.3.2) (aspartate trans-

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Abbreviations: DHFR, dihydrofolate reductase; CAD, the multifunctional protein containing the enzymatic activities carbamoyl-phosphate synthetase, aspartate carbamoyltransferase (aspartate transcarbamoylase), and dihydro-orotase (1); DMs, double minute chromosomes (small acentric chromosomes); MTX, methotrexate; kbp, kilobase pair(s); TPA, 12-*O*-tetradecanoylphorbol 13-acetate; SV40, simian virus 40.

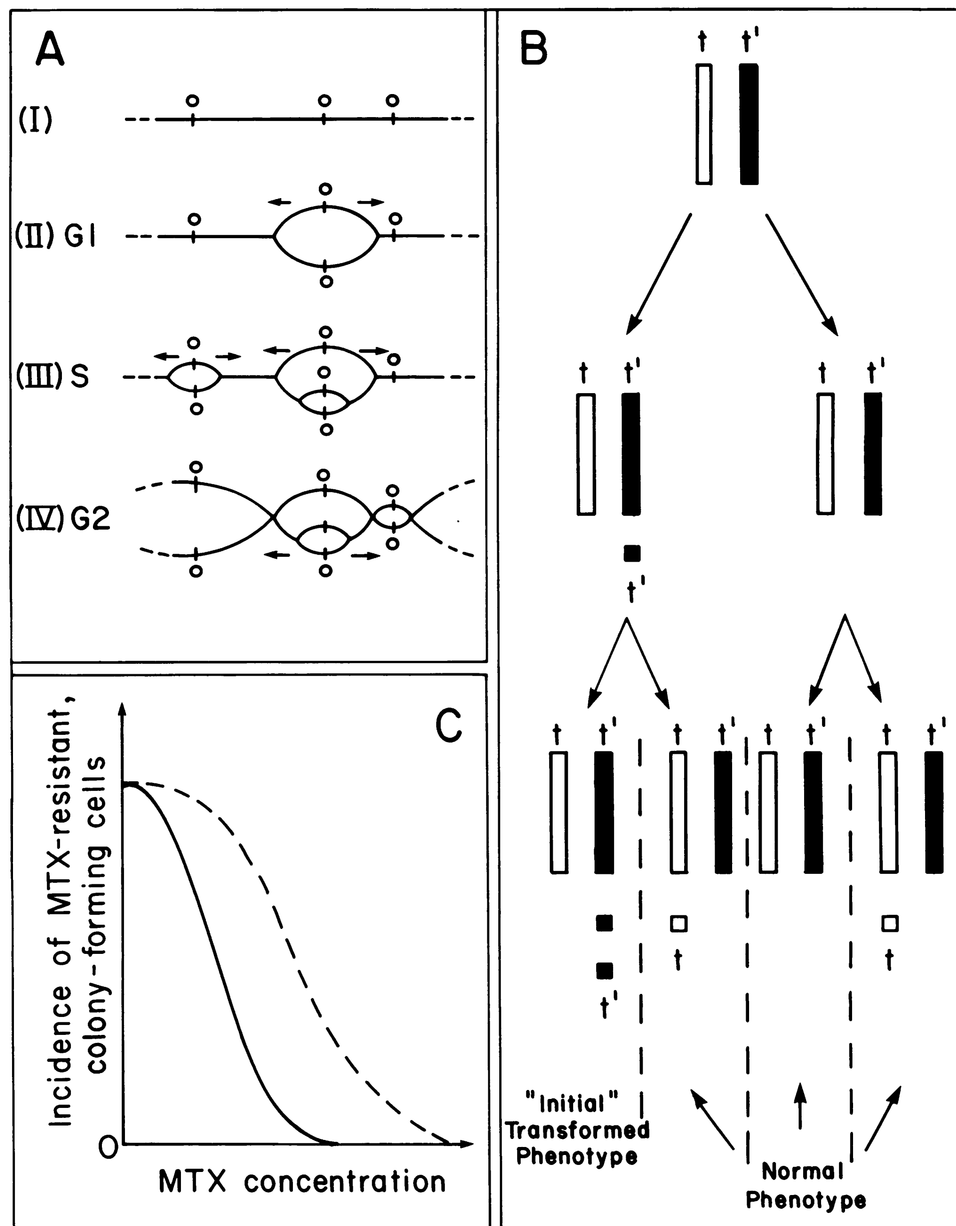

FIG. 1. Firone hypothesis. (*A*) Definition of replicon misfiring. I. A chromosomal region containing three origins of replication (○) is shown. II. Misfiring event in G_1 phase of the cell cycle is depicted as a single replication bubble growing from the middle origin. This event is classified as a misfiring because, by definition, no chromosomal origin of replication should be active in G_1 phase. Arrows denote directions of fork movement. III. Misfiring event in the S phase is depicted as an extra replication bubble growing within one of the two branches of an active middle replicon. Subsequent excision of an extra copy of a chromosomal domain produced may be carried out either by a general recombinational enzymatic system or by a special repair-type mechanism (1). IV. Misfiring event in the G_2 phase is analogous to that in the S phase except that a "legitimate" DNA replication is depicted finished, with replication forks meeting each other at points of subsequent DNA segregation. One additional assumption used to produce IV is that DNA segregation is a G_2-specific event; that is, no DNA segregation takes place in S phase, at least in a chromosomal region shown. It is not known whether the probability of a misfiring for any replicon differs strongly among G_1, G_2, and S periods of the cell cycle. Firones are postulated to increase the frequency of replicon misfiring either at specific chromosomal locations (locus-specific firones) or all over the genome (locus-unspecific firones).

(*B*) Possible role of replicon misfiring in facilitating an increase in dosage of a "transforming" gene. This diagram shows a pair of homologous chromosomes carrying a dominant normal allele (t) and a mutated recessive allele (t′) which is transforming. Before the first cell division, random replicon misfiring leads to a formation of an extra copy of t′, the copy being released as an acentric DM or as a smaller chromatin fragment (black square). At this stage, the greater-than-normal ratio of t′ to t (2:1) is presumed to be insufficiently high to overcome a dominant effect of the normal allele t. After the second cell division, however, one of the two daughter cells randomly acquires both extra copies of the transforming t′ allele and the increased ratio of t′ to t (3:1) is assumed to be sufficiently high to cause phenotypic alteration, denoted as initial transformed phenotype. Note that, unlike the first extrachromosomal copy of t′, the second extra t′ copy was formed not by a replicon misfiring but by a conventional, cell cycle-dependent replication of the first extra t′ copy (black squares). Other cells of the same lineage are shown to acquire extrachromosomal copies of the normal allele t (white squares) randomly, with no alteration in phenotype.

(*C*) Cellular gene dosage assay for firone detection. Incidence of MTX-resistant, colony-forming cells is determined for a range of MTX concentrations by seeding known amounts of cultured mammalian cells in the presence of different concentrations of MTX and determining the numbers of resistant cells. This can be done by scoring the colonies that MTX-resistant cells give rise to. The resulting dose–response curve (solid line) is then compared with an experimental curve (dashed line) derived from cells grown for several generations in the presence of a putative firone before

carbamoylase), and dihydro-orotase (EC 3.5.2.3)] genes which occurs in hamster cells resistant to *N*-(phosphonacetyl)-L-aspartate, a transition state inhibitor of aspartate transcarbamoylase (1). CAD, a multifunctional enzyme, catalyzes the first three reactions of *de novo* UMP biosynthesis. Relative incidence of resistant cells and other features of the phenomenon (1) are strikingly similar to those of the DHFR gene amplification (13, 22).

One can conclude that misfirings of eukaryotic replicons occur with detectable frequencies and, furthermore, that the sizes of duplicated elements can vary from those smaller than SV40 DNA (5.2 kb) (2, 10–12) to structures containing several chromosomal domains (100–1000 kb) (23, 27). At least some of the extra copies of chromosomal domains produced by infrequent acts of replicon misfiring (Fig. 1*A*) are converted into extrachromosomal chromatin fragments, either by a general recombinational enzymatic system or by a special repair-type mechanism. The existence of such an excision mechanism is strongly suggested by recent data on the formation of circular SV40 DNA molecules as a result of replication of SV40 DNA integrated into the mouse genome in SV40-transformed mouse cells (2) (see above).

Firone hypothesis

The central idea of the present work is that a low probability of a replicon misfiring within any given chromosomal domain (Fig. 1*A*) could be increased by certain substances of either intra- or extracellular origin. The term "firone" is proposed for such a substance. Firones, as shown below, are expected to influence strongly the *rate* of evolution of cellular systems under a selection pressure, a developing tumor being one example of such a system. The firone hypothesis leads to several testable predictions and to a new type of gene dosage assay.

Firones as tumor promoters

Tumor promoters are substances that increase the probability of a malignant transformation "initiated" by a previous exposure of cells to a carcinogen; by definition, tumor promoters have either very low or no carcinogenic activity when tested in the absence of an initiating carcinogen (28–41). They comprise a large variety of substances of which some are weak carcinogens by themselves and the others display neither carcinogenic nor detectable mutagenic activity when tested alone in bacterial or mammalian mutation assays (42–44). Some of the phorbol esters—in particular, 12-*O*-tetradecanoylphorbol 13-acetate (TPA)—are extremely potent tumor promoters in the mouse skin model (28, 29). TPA was also shown recently to increase the efficiency of carcinogen-, virus-, or radiation-induced transformation of cultured mouse cells (45–47).

In spite of the large amount of experimental work carried out, the primary mechanism of tumor promotion is not understood (28–55). TPA and related compounds have been found to produce a multitude of cellular effects that include stimulation of macromolecular metabolism and cell growth (28) and of various plasma membrane functions such as its phospholipid metabolism (28) and sugar transport (51), induction of enzymes such as plasminogen activator (33) and ornithine decarboxylase (52), either suppression or enhancement of terminal differentiation (41), induction of viral antigens (53), and alteration of cellular structure or shape (51). None of these effects immediately suggests molecular mechanisms for a heritable phenotypic change. Although epigenetic explanations of the tumor promotion effect (i.e., explanations that do not depend on changes of either DNA sequences or their relative abundances) are not precluded by the current data, difficulties with these explanations (56) compel one to look for a genetic mechanism of tumor promotion. One suggestion was that the promoter facilitates segregation of a recessive chromosomal lesion (transforming gene) through mitotic recombination (54). However, recent carefully executed attempts failed to confirm an effect of TPA on mitotic recombination (44, 55).

On the other hand, as shown below, one does not need to invoke mitotic recombination to achieve at least a partial segregation of a recessive transforming gene. Induction of replicon misfiring by a firone would lead to more frequent formation of extra copies of both a transforming gene and its dominant normal allele (Fig. 1*B*). These extra copies, in the form of small acentric chromosomes (DMs) or smaller chromatin fragments, would be distributed at random at each mitosis. As a result, some cells would acquire higher-than-average doses of the transforming gene. This would create conditions for expression of an initial transformed phenotype, assuming that the normal allele of a transforming gene is not of an overdominant type. Growth advantage of cells with increased doses of a transforming gene would lead to preferential retention of these cells in a population and to further evolution in the direction of a transformed phenotype. Reintegration of acentric chromosomal fragments carrying a transforming gene into one of the chromosomes would lead to formation of a stable lineage of transformed cells.

In summary, I suggest that locus-unspecific firones able to increase incidence of otherwise rare events of replicon misfiring should act as tumor promoters by facilitating segregation of a recessive transforming gene. The reverse suggestion, that all tumor promoters are firones, does not have to be true because there may be more than one primary mechanism of tumor promotion.

Firone hypothesis and emergence of malignant phenotypes in mammalian cell lineages

Evolution of tumor cell populations *in vivo* toward more malignant phenotypes is a well-known phenomenon (47, 56–60). In a developing tumor, those cells that have acquired a direct and heritable growth advantage—e.g., a shorter generation time—or a loss of dependence on a particular growth factor in short supply or an ability to secrete a factor promoting vascularization (61) will eventually outgrow their neighbors and give rise to a more actively expanding tumor.

A conspicuous feature of tumor cells is their frequent association with small DMs (62–65). DMs are rarely present in normal mammalian cells but are found frequently and in large numbers in naturally occurring malignant tumors (62–65). DMs are also found in certain types of drug-resistant cells created either in the laboratory (23) (see above) or in the course of cancer chemotherapy (16). DMs gradually disappear from tumor cells under nonselective conditions of *in vitro* growth but reappear upon reimplantation of the cells in a susceptible animal (62–64). This suggests that the presence of DMs, and possibly also of smaller chromatin fragments undetectable by conventional cytological methods, is of benefit to cells under conditions of

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exposing them to MTX. If the number of resistant survivors is significantly higher than the controls, the number of the DHFR genes per MTX-resistant cell is measured (13, 26), in order to verify that MTX resistance is due to an amplification of the DHFR gene rather than, for instance, to a mutation rendering DHFR less sensitive to inhibition by MTX. To minimize the possibility that firone activity of a tested substance is due to a specific disturbance of cellular metabolism caused by MTX, other single-step gene dosage assays of the same type but with different drugs—e.g., *N*-(phosphonacetyl)-L-aspartate (1)—can be used. Additional controls, in particular those measuring plating efficiencies in the presence and absence of a firone, would be also required for a gene dosage assay.

growth *in vivo* but not under conditions of growth in a cell culture. One explanation is that the presence of DMs disturbs a normal genotypic balance for the corresponding genes, thereby creating possibilities for phenotypic changes favorable for tumor cell multiplication and selection.

I suggest that firones of either extracellular or intracellular origin should influence the rates of tumor cell evolution by increasing the rate of formation of DMs through replicon misfiring.

The idea that changes in relative dosages of specific genes may lead to phenotypic changes is by no means new (reviewed in ref. 56). It is also known that a transcriptional activation of a transforming gene resulting in overproduction of certain proteins is one of the mechanisms of cellular transformation by RNA tumor viruses (reviewed in ref. 66). The idea of the present work is that the *rate* of gene amplification through replicon misfiring is specifically controlled by firones and that this mechanism could underlie the observed rapid cellular evolution in developing tumors and the action of at least some tumor promoters.

In considering the action of firones on cells it is important to distinguish between firones of extracellular origin and firones produced within a target cell. In the latter case the rate of cellular evolution becomes no longer dependent on a continuous supply of an extracellular firone and, in addition, a new evolutionary avenue is created, favoring overproduction of a firone by the cell. Thus, a chance emergence of a producer of an intracellular firone could set in progress an "autocatalytic" process in which an increase of firone activity facilitates evolution toward an even more efficient firone producer.

It should be noted that the postulated misfiring produced by firones would put an extra metabolic burden on both replication and repair systems and therefore would not be advantageous for a cell in the absence of a specific selection pressure.

Finally, because evolution of cellular phenotypes through replicon misfiring is certainly not the only existing evolutionary avenue, by selectively increasing the rate of misfiring-dependent evolution, firones would change the relative contributions of different evolutionary mechanisms to an overall process of heritable phenotypic change in a cellular population.

One of the testable predictions of the firone hypothesis as applied to evolution of malignant phenotypes is that tumor cells containing large amounts of DMs or smaller chromatin fragments should contain larger than normal amounts of an intracellular firone. Furthermore, if such a firone should be secreted from the cell, and be able to act on normal cells, it might be detected by exposing normal cells to a medium conditioned by the tumor cells and assaying the normal cells for replicon misfiring (see below).

Definition and classification of firones

Although identities of postulated locus-unspecific firones are unknown, substances that function as a locus-specific firone certainly exist. For example, an unidentified factor responsible for a formation of DNA puffs in *Rynchosciara* (4) clearly satisfies the definition of a locus-specific firone. Another example of a locus-specific firone is a combination of a SV40 large tumor antigen and a hitherto unidentified permissive factor for SV40 DNA replication. Both substances are apparently required for the burst of SV40 replicon misfiring that follows fusion of SV40-transformed nonpermissive mouse cell with a permissive green monkey cell (2).

Locus-specific firones are likely to be proteins that recognize and bind to specific DNA sequences. One possible origin of a partially or completely locus-unspecific firone would be through a mutation that diminishes the nucleotide sequence specificity of a locus-specific firone without destroying its firone activity. More generally, any substance that increases probability of replicon misfiring either directly (for example, by binding to a chromatin structure involved in suppression of misfiring) or indirectly (for example, by specifically disturbing metabolism of low molecular weight compounds important for a proper functioning of the replication machinery) would be classified as a firone.

Experimental approaches to firone detection

The most straightforward approach to firone detection would be a biochemical one. That is, one should be able to measure directly the frequency of replicon misfiring in several randomly chosen regions of the genome in the presence and absence of a putative firone. Such an assay appears feasible but would require a technique for detecting and fractionating DNA fragments intermediate in length between chromosome-sized DNAs ($>10^4$ kbp) and the largest DNAs resolvable in conventional agarose gels ($\approx 10^2$ kbp).

Another way to detect firones would be to use a new type of gene dosage assay described in the legend to Fig. 1*C*. The assay is based on the fact that most cases of resistance of mammalian cells to MTX are due to the acquisition of extra copies of DHFR genes (13). The incidence of cells resistant to given MTX concentrations can be measured in a single-step colony formation assay. In this assay, control cell populations are not treated with a putative firone. Experimental cells are allowed to grow for a few generations in the presence of the firone and are subjected then to single-step selections for resistance to a range of MTX concentrations. If the frequency of resistant cells is found to be increased, a set of additional control experiments is carried out to verify that the resistant phenotype is due to DHFR gene amplification.

The major point of this work is that the existence of partially or completely locus-unspecific firones would have important biological implications.

Note Added in Proof. Recent experiments of the type proposed in Fig. 1*C* have led to discovery of a dramatic effect of TPA on the incidence of mouse cells heritably resistant to MTX. These resistant cells have increased dosages of the DHFR gene (67).

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