Amplification–mutagenesis: Evidence that ''directed'' adaptive mutation and general hypermutability result from growth with a selected gene amplification

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When a particular *lac* mutant of *Escherichia coli* starves in the presence of lactose, nongrowing cells appear to direct mutations preferentially to sites that allow growth (adaptive mutation). This observation suggested that growth limitation stimulates mutability. Evidence is provided here that this behavior is actually caused by a standard Darwinian process in which natural selection acts in three sequential steps. First, growth limitation favors growth of a subpopulation with an amplification of the mutant *lac* gene; next, it favors cells with a lac^+ revertant allele within the amplified array. Finally, it favors loss of mutant copies until a stable haploid lac^+ revertant arises and overgrows the colony. By increasing the *lac* copy number, selection enhances the likelihood of reversion within each developing clone. This sequence of events appears to direct mutations to useful sites. General mutagenesis is a side-effect of growth with an amplification (SOS induction). The F′ plasmid, which carries *lac*, contributes by stimulating gene duplication and amplification. Selective stress has no direct effect on mutation rate or target specificity, but acts to favor a succession of cell types with progressively improved growth on lactose. The sequence of events—amplification, mutation, segregation—may help to explain both the origins of some cancers and the evolution of new genes under selection.

A selection system developed by John Cairns (1, 2) provided evidence that bacteria might sense growth-limiting stress and direct mutations to sites that enhance growth (adaptive mutation). The behavior of Cairns' system does not contradict classical demonstrations that some mutations arise independent of selection (3, 4). However, the behavior raises the possibility that another fraction of mutations might be induced or even directed by growth limitation. Stress-induced mutations (general or directed) would contradict the neo-Darwinian view that agents of selection play no role in causing mutations, but affect only the relative reproductive success of organisms with different genotypes.

Experiments supporting directed mutation used an *Escherichia coli* strain with a *lac* deletion on the chromosome and a revertible *lacZ* (+1) frameshift mutation on an F'_{128} plasmid (2). During growth, the *lac* point mutation reverts at a rate of 10^{-8} per cell per division. When 10^8 of these Lac^- cells are starved in the presence of lactose, about 100 revertant colonies accumulate over a period of 6 days, during which the plated population does not grow and does not accumulate unselected mutations (5). This behavior suggested that selection directs mutations to growth-promoting sites (2, 6).

Subsequent studies showed that lac^+ revertants have a 10- to 100-fold higher probability of carrying an unselected mutation than do unselected cells or starved nonrevertant cells (7). Thus, the revertants (but not the starved population as a whole) were generally mutagenized in the process of reversion. Once isolated, the revertants show a normal mutation rate. This finding suggested that stress induces a temporary general hypermutable state. This general mutagenesis requires induction of the SOS system of DNA repair with its error-prone polymerase DinB (8). Induction of SOS mutagenesis was attributed to a beneficial regulatory mechanism that evolved under selection because it facilitates genetic adaptation.

We have suggested (9) that apparently directed mutation could be explained if the leaky *lac* allele used in this experiment were amplified so as to allow slow growth of a clone on lactose. Reversion would then occur when the growing clone has accumulated enough mutant *lac* copies. What appears to be a directed single step mutation in a nongrowing cell can result from selection acting on growing cells within a colony. The required growth will not be evident in the lawn between colonies, but can be revealed by appropriate analysis of developing colonies (9).

Evidence is presented that amplification is essential for Lac^+ reversion in the Cairns system and that associated general mutability is an unavoidable side effect of growth with a selected gene amplification. These results suggest that several previously proposed attributes of the Cairns system are incorrect; specifically, (*i*) stress does not direct mutation to valuable sites (1, 6), (*ii*) stress has no direct effect on the general mutation rate (7), (*iii*) reversion does not occur in stationary-phase cells (2, 5, 10), (*iv*) there is no reason to suppose that mutation reflects error-prone recombination (5, 11), and (*v*) reversion is actually initiated before selection by formation of duplication-bearing cells during pregrowth; these cells, when placed on selection medium, grow and evolve by a conventional Darwinian process.

The amplification–mutagenesis model suggests a general mechanism by which selection may enhance the rate of genetic change during growth under strong selection without altering the rate or specificity of mutation. The model may be applicable to origin of some cancers and to the evolution of new genes.

Materials and Methods

Strains. All strains are derived from *E. coli* K-12; the tester strain FC40 (=TR7178) and scavenger FC29 (=TR7177) were provided by Patricia Foster (2). A Tn*10* insertion near *his* was obtained from Sidney Kushner (SK2651) and transduced into strain FC40 (producing TT23242). Insertion *nadB210*::Tn*10* was introduced into a *lac* deletion mutant to make the tetracycline-resistant (Tc^R) scavenger TT23241. When this scavenger was used, nicotinic acid was added to the selection plate. By P22-mediated crosses in *Salmonella enterica*, Tn*10* insertions were added to plasmid F'_{128} (from strain FC40) and their positions were mapped; plasmids were returned to a plasmid-free *E. coli* strain isogenic with FC40 (TT23250).

Reversion Experiments. Testers (FC40) and scavenger cells (FC29 or TT23241) were pregrown in NCE medium (34) containing glycerol (0.2%), thiamin (50 μM), and (for TT23241) nicotinic

Abbreviations: Tc, tetracycline; CTc, chlortetracycline; TetA, tetracycline-inducible exporter.

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acid (100 μM); cells were pelleted and resuspended in sterile saline. Each reversion plate [NCE, 0.2% lactose, 25 mg 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal)] was seeded with about 2×10^9 scavenger cells and incubated for 24 h to remove contaminating carbon sources before 10^8 tester cells were plated. For Fig. 4*A*, cells were pregrown with tetracycline (Tc; 10 μg/ml) to induce the *tetA* (resistance) gene before plating on minimal lactose medium containing Tc. This preinduction proved unnecessary and was not included in the experiment in Fig. 4*B*.

Testing Stability of Lac$^+$ Phenotypes. Revertants in Fig. 2 were identified (some with a dissecting microscope) as new colonies not present on the previous day. Each colony was removed from the selection plate with a plug of agar. Cells were suspended and stored at 4°C. Samples were diluted and plated on nutrient broth (Difco) plates containing rifampicin (50 mg/liter) to eliminate scavengers and X-Gal (40 mg/liter) to visualize *lac* phenotypes; this medium and X-Gal concentration facilitate visualization of the sectored colonies that indicate an unstable Lac$^+$ phenotype.

Testing the Clonal Relatedness of Stable and Unstable Lac$^+$ Cells. The two testers FC40 (TR7178) (Tc^S) and TT23242 (Tc^R) were plated at a 30:1 ratio (total 10^8) on plates with the scavenger strain TT23241 (Tc^R) as described above. Of 500 Day 6 revertants, 7 contained a substantial number of Tc^R, Lac$^+$ cells. These 7 suspensions were diluted and plated to determine the Lac$^+$ stability and genetic background of individual cells.

Testing Amplifications by Pulsed-Field Gel Electrophoresis. Unstable Lac$^+$ cells were mated with a *recA* recipient (TT23249) and Pro$^+$ exconjugants were selected. DNA was prepared (12) and cut with either *Bln*I or *Sfi*I before pulse-field gel electrophoresis. The F′ plasmid has one *Bln*I site 50 kb from *lac* and thus not within the amplified *lac* region. The size increase in the single plasmid band gave the total material added by the amplification (copy number × copy size). The F′ plasmid has one *Sfi*I site near *lac* (and within the amplified region) and others more than 50 kb away (outside the amplified region). Each tandem repeat is cut once by *Sfi*I, generating a heavy band whose size is the length of the amplified segment (copy size). The *lac* copy number per plasmid is obtained by dividing the increase in plasmid size by the size of the repeated unit.

Results and Discussion

The Amplification–Mutagenesis Model. This model (Fig. 1) proposes that growth of cells carrying an amplification explains all of the properties of the Cairns selection system, including both directed and general mutagenesis. Specific features are listed below (Fig. 1 *Left*).

Part I: Adaptive Mutation. During nonselective growth before plating, a few cells acquire a simple duplication of the leaky mutant *lac* allele. After plating, such cells initiate slowly growing clones. Most plated cells lack this duplication and cannot grow.

Within each successful clone, cell(s) arise that have further amplified the mutant *lac* allele. These cells grow faster and dominate the colony.

The growth rate attainable by *lac* amplification alone is limited by frequent loss of *lac* copies caused by recombination between repeats (segregation).

Eventually, a cell in the clone experiences a −1 frameshift mutation that creates a single *lac*$^+$ allele in a tandem array of mutant alleles.

Selection then favors descendents of this cell that maintain the *lac*$^+$ allele and lose the mutant copies, which now impede growth and destabilize the revertant allele.

Stable *lac*$^+$ segregants carrying only the revertant allele arise,

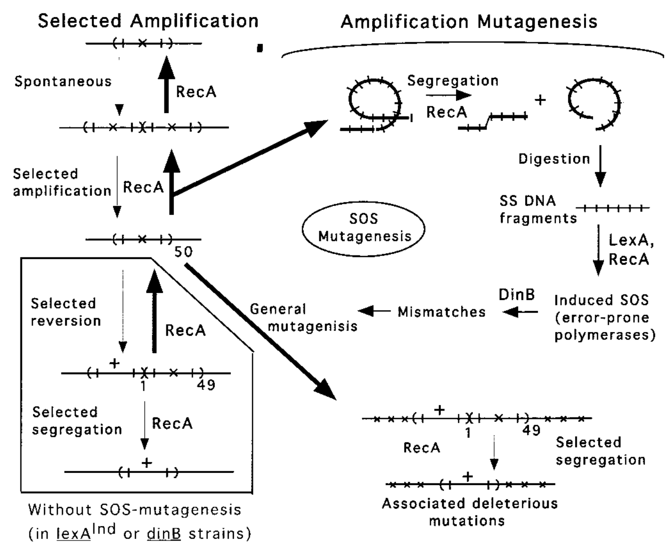

Fig. 1. Amplification–mutagenesis model for reversion during growth under selection and associated hypermutability. (*Left*) The basic amplification model without general mutagenesis. (*Right*) Induction of SOS mutagenesis is a side effect of growth with an unstable amplification.

overgrow the clone, and thus ultimately predominate in the mature revertant colony.

In this process, the probability of a reversion event increases with the number of replicating *lac* targets added to the clone.

The process is accelerated because the *lac* gene is on an F′ plasmid, which stimulates gene duplication and amplification.

During this process the lawn as a whole may not grow, but each visible colony (10^6 cells) results from one cell going through 20 generations under selection. If one is not aware of the process above—growth, amplification, reversion and segregation—then selective stress appears to specifically convert a nongrowing mutant cell into an adaptive revertant. The above process can enhance reversion without any change in rate or target specificity of mutation. However, a small temporary increase in general mutation rate does occur (refs. 7 and 13, and E.S.S., unpublished data). This is explained below (Fig. 1 *Right*).

Part II: General Mutagenesis. Recombination between repeated sequences releases DNA fragments from the array (segregation). These fragments are degraded to single strands that induce the SOS system. Therefore, growth under selection for a large amplification causes prolonged or repeated SOS induction.

The SOS response induces an error-prone DNA polymerase, whose normal purpose is to bypass damaged bases. This polymerase (DinB) leaves mismatches when it copies normal DNA (14, 15). To the extent that these extra mismatches exceed the capacity of the mismatch repair system (MMR), a genome-wide general mutagenesis ensues.

Growth with mutagenesis continues until a *lac*$^+$ reversion occurs and selection favors cells that have lost the mutant *lac* copies, and therefore the source of SOS induction. Once SOS (and DinB) is repressed, mutagenesis stops. Thus, although mutagenesis is temporary, it may continue for multiple generations before reversion.

Evidence for Amplification in Every Revertant Clone. In the model, reversion under selection occurs in microclones growing with a selected *lac* amplification. This parental microclone should remain buried within every revertant colony after *lac*$^+$ haploids have overgrown. The frequency of cells with an amplification is expected to be highest in tiny colonies and to be lower in large

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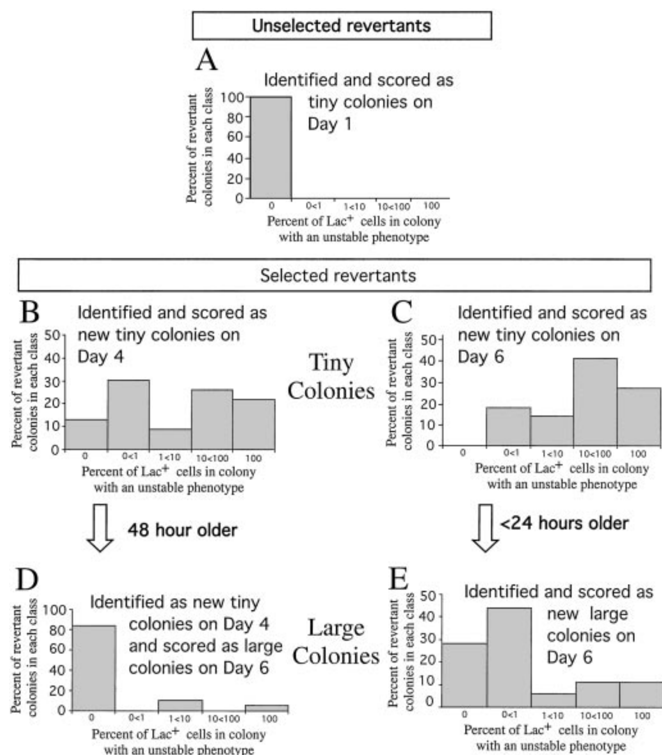

Fig. 2. Frequency of unstable Lac$^+$ cells in revertant colonies. For each histogram, about 20 revertant colonies were classified based on the percentage of Lac$^+$ cells (3,000 from each colony) with an unstable phenotype. (*A*) Early colonies formed by full revertants that arose before selection. (*B* and *D*) A set of microscopic new colonies were identified on day 4; half were picked and scored immediately (*B*) and the other half remained under selection for 2 more days before scoring (*D*). (*C* and *E*) A set of new colonies identified on day 6 (not visible on day 5). The colonies in *C* were tiny at the moment of identification and scoring; those in *E* were large. The tester strain used was FC40 = TR7178 [*ara thiA* Rif^R Del13(*lac proB*)/F′$_{128}$ *lacI33*(fs) *lacIZ*fusion(Ω) *pro*$^+$] and the scavenger strain was FC29 = TR7177 [*ara thiA* Rif^S Del13(*lac proB*)/F′$_{128}$ Del(*lacI*, *lacZ*) *pro*$^+$].

colonies whose size has increased by growth of stable *lac*$^+$ cells. In contrast, reversion events that occur under nonselective conditions (i.e., before plating) generate a *lac*$^+$ allele in cells that have not been under selection to amplify. These revertant cells, when placed on selective medium, should form colonies that appear early and include only stable haploid Lac$^+$ cells.

These predictions were verified by using the *E. coli* strains of Cairns and Foster (2). At appropriate times after plating (Fig. 2), revertant colonies were tested for content of unstable *lac*$^+$ cells. Each colony was suspended, diluted, and plated on rich medium containing X-Gal (a chromogenic β-galactosidase substrate). On this medium, Lac$^+$ cells form blue colonies and Lac$^-$ cells form white colonies. Cells carrying an amplification (with or without a revertant *lac* allele) have an unstable *lac*$^+$ phenotype and form blue colonies with multiple white sectors.

Revertant cells that arose during nonselective pregrowth formed colonies on selective medium that were tiny on day 1 and large by day 2. These colonies included no unstable Lac$^+$ cells (<1/3,000; Fig. 2*A*). In contrast, almost every colony that arose at later times, reflecting reversion events that occurred under selection, contained some unstable Lac$^+$ cells. As predicted, microscopic colonies showed the highest frequency of amplification-bearing cells (Fig. 2 *B* and *C*), whereas larger colonies exhibited a lower frequency (Fig. 2 *D* and *E*).

We previously observed amplification-containing cells within every Lac$^+$ revertant colony formed in a *Salmonella*-based analogue of the Cairns system (9). Attempts of others to repeat this in *E. coli* led to the conclusion that very few revertant colonies included cells with an amplification (16). In these experiments, only the most amplification-rich clones could be detected because too few cells were examined from each colony (50 rather than 3,000). Macroscopic colonies were tested and very few such cells are amplification-rich (see Fig. 2 *D* and *E*). Given the methods used, the reported results are fully consistent with the amplification model.

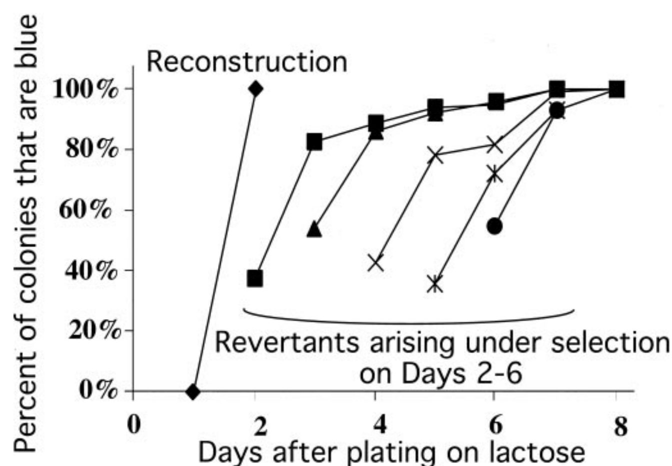

Fig. 3. Variable maturation of microclones during reversion under nonlethal selection. For the reconstruction, 400 Lac$^+$ revertants of FC40 were plated with scavengers on selective medium. Each of the other lines describes new revertant colonies (>100) that arose on 20 plates on each of a series of days (absent the previous day); each set was scored for color over several days.

Microcolonies Mature with Variable Delays. Amplification–mutagenesis proposes that reversion under selection depends on a series of sequential stochastic events. This predicts a highly variable maturation time. Such variability is apparent in Fig. 2.

Variability is also seen in the time needed for a visible microclone to develop its Lac$^+$ phenotype (Fig. 3). A 20-plate reversion experiment was performed on medium that included X-Gal. On each day after plating, all new microclones (those absent on the previous day) were identified by microscope and then followed over the ensuing several days by scoring increase in size and development of blue color (by hydrolysis of X-Gal). Colonies that were white when first identified subsequently attained blue color (and large size) after a variable delay—some did this within the next 24 h, whereas others required as many as 5 additional days. Colonies that were already blue when first identified as microclones had attained color while becoming large enough to see in the microscope.

A reconstruction experiment showed that heterogeneous maturation times are seen only for colonies initiated under selection. When 400 stable Lac$^+$ revertant cells (derived from FC40) were plated under standard reversion conditions with scavenger cells and X-Gal, they gave rise to white microcolonies on Day 1, all of which became blue within 18 h of their first appearance.

Unstable Lac$^+$ Cells Carry a Large Amplification. Leaky *lac* mutations on an F′ *lac* plasmid are known to revert by amplification. Such revertants carry 10 to 100 copies of a 10- to 40-kb sequence including the *lac* operon (9, 16, 17). Such amplifications were also found in the unstable Lac$^+$ cells taken from the tiny colonies described in *Materials and Methods*. Each amplification included 10 or more tandem repeats of a 10- to 40-kb sequence (data not shown). To estimate the total *lac* copy number within a cell, this *lac* amplification must be multiplied by F′ copy number, which is normally two to three, but increases in starved cells (18).

Stable and Unstable Lac+ Cells Within a Single Colony Are Clonally Related. According to the amplification model (Fig. 1), unstable and stable Lac+ cells (with and without an amplification) in a single colony arise sequentially within a clone initiated by a single duplication-bearing cell, and in this sense should be clonally related. To test this prediction, a 30:1 mixture of two distinguishable tester strains was plated on selection medium with scavengers. Reversion to *lac*+ can occur in either of the two testers or in a scavenger cell that received a mutant F′*lac* plasmid by conjugation after plating. If stable and unstable *lac*+ revertants are clonally related, a colony initiated by a cell with a rare genetic background (the rare tester or the scavenger) should contain stable and unstable *lac*+ revertant cells in that same rare background. Alternatively, if stable and unstable revertants arise independently, a single colony should rarely include both stable and unstable revertants of the same rare genetic background.

Of 500 new Lac+ revertant colonies appearing on day 5, seven occurred in one of the rare genetic backgrounds; these revertants showed the Tc^R marker that was carried by these parental strains but not by the majority tester. For six of these colonies, both the stable and unstable Lac+ cells appeared in the same rare genetic background (three in the minority tester cells, three in F′-containing scavenger cells). This clonality is inconsistent with the hypermutable state model, by which reversion and amplification are claimed to be independent phenomena (16). A clonal relationship between stable and unstable Lac+ cells was previously demonstrated in *S. enterica* (9).

Amplification of *lac* Copy Number Is Essential for Reversion Under Selection. The amplification–mutagenesis model predicts that growth with a *lac* amplification is a necessary precursor to reversion under selection. This prediction was tested by assessing reversion under conditions that specifically inhibit growth of cells with a *lac* amplification (Fig. 4).

Transposon Tn*10* encodes a Tc-inducible exporter (TetA), which normally confers Tc^R. However, when Tn*10* is present at high copy number, cells again become sensitive to Tc. This change occurs because an overabundance of TetA transporter compromises proton-motive force and prevents growth (19). Cells with an amplification that includes Tn*10* are specifically inhibited by Tc (20); the same effect can be seen with the analogue chlortetracycline (CTc), which is not toxic but can induce expression of the *tetA* gene (21).

These unusual phenotypes allow one to inhibit growth of cells with an amplification that includes Tn*10*. A strain carrying *Tn10* near *lac* should amplify the Tn*10* with the *lac* operon. If *lac* amplification is essential for reversion, addition of Tc or CTc to the selection plate should prevent growth of these cells and therefore severely reduce the number of Lac+ revertants. In contrast, these same agents should have no effect on reversion in strains carrying Tn*10* at sites (far from *lac*) that do not amplify with *lac* during selection.

A control strain with Tn*10* inserted at a chromosomal site (not amplified) allowed normal reversion of the *lac* mutation (on the F′ plasmid) with or without Tc (Fig. 4*A*). Thus, the presence of a Tn*10* element and exposure to Tc do not in themselves alter reversion behavior. In contrast, Tc strongly reduces the yield of revertants in a strain with Tn*10* near *lac*, as expected if reversion requires amplification. As predicted, this strain reverts normally in the absence of Tc. These effects were also seen with CTc, the nontoxic inducer of the *tetA* gene (Fig. 4*B*).

The nontoxic analogue CTc was used to examine the effect of distance between *lac* and Tn*10* on drug inhibition of reversion (Fig. 4*B*). When Tn*10* is placed on the F′ plasmid at increasing distances from *lac*, the probability decreases that an amplification will span both *lac* and Tn*10*. Correspondingly the fraction of amplifications that are inhibited by CTc should also decrease. Reversion was strongly inhibited by CTc for the strain whose

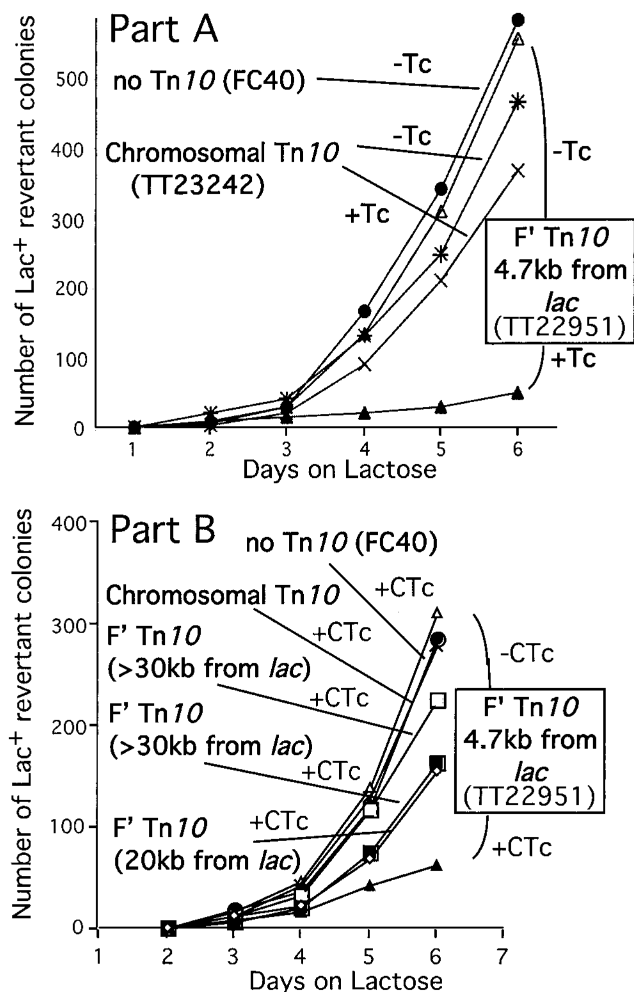

Fig. 4. Reversion is reduced by inhibiting growth of cells with a *lac* amplification. (*A*) Tester strains were FC40 (no Tn*10*), TT23242 (Tn*10* in the chromosome), TT22951 (Tn*10* in the plasmid *mhpC* gene 4.6 kb from *lac*). Strain TT23241 served as scavenger. "+Tc" and "−Tc" indicate presence or absence of Tc (10 μg/ml) in selection plates. (*B*) Tester strains were FC40 (no Tn*10*), TT23242 (Tn*10* in chromosome), TT22951 (Tn*10* 4.6 kb from *lac*). Strains TT23243, TT23244, and TT23245 carried Tn*10* >30 kb, >30 kb, and 20 kb from *lac*, respectively. Without chlortetracycline (CTc), all strains showed reversion like FC40.

Tn*10* was close to *lac*. Reversion was inhibited less when Tn*10* was farther from the *lac* gene. The slight reduction seen in the latter strains reflects the smaller fraction of *lac* amplifications that include these more distant Tn*10* insertions. As predicted by the model, the frequency of day 2 revertants was unaffected by either Tc or CTc, even in strains having Tn*10* near *lac* (Fig. 4 *A* and *B*, respectively). These revertants arise before selection and require no *lac* amplification for their formation.

Temporary Hypermutability Is a Side Effect of *lac* Amplification. In the amplification–mutagenesis model, amplification allows growth and therefore DNA replication. Beyond this, it enhances reversion in two ways. First, it increases the *lac* copy number (more mutation targets). Second, it releases DNA fragments that induce SOS and increase the frameshift mutation rate (more mutations per target).

These two separate contributions can be evaluated under particular conditions. First, a *recA* mutation should block both amplification (by removing recombination) and SOS induction (by eliminating the coprotease of *lexA* repressor) and should therefore eliminate both sources of selection-induced mutations.

Second, preventing growth of cells carrying an amplification (by Tc or CTc as above) should also eliminate both effects and thus should confer a reversion defect comparable to that caused by a *recA* mutation. A contrasting third prediction is that elimination of the DinB function or SOS induction (by a *dinB* or a $lexA^{Ind}$ mutation) should block only the contribution of general mutagenesis while allowing selection to continue enhancing reversion by the increase in *lac* copy number. Thus, a *dinB* or a $lexA^{Ind}$ mutation should have a smaller effect on lac^{+} reversion under selection than either of the first two conditions.

All three predictions are confirmed. A *recA* mutation causes a 10- to 15-fold reduction in revertant number (2, 11). Inhibiting the growth of amplification-containing cells with Tc or CTc also causes at least a 10-fold reduction in reversion (Fig. 4). In contrast, $lexA^{Ind}$ and *dinB* mutations, which strongly reduce general mutagenesis (30- to 50-fold), reduce Lac^{+} reversion only 2- to 4-fold (8). This finding implies that general mutagenesis is a minor factor in *lac* reversion.

Further evidence for the dispensability of general mutagenesis is provided by the observation that a *S. enterica* $lexA^{Ind}$ mutant, which cannot induce SOS mutagenesis, still shows a 25-fold increase in Lac^{+} colonies over a 6-day reversion experiment. Moreover, this residual effect of selection is RecA-dependent and associated with an increased number of cells with a *lac* amplification in each colony (E.S.S., unpublished data). These results imply once again that selection can enhance *lac* reversion purely by allowing growth with more *lac* targets.

How Does Amplification Confer a Transient Hypermutable State? The transient nature of SOS-dependent general mutability suggests that the SOS system is induced during stress and repressed after reversion occurs (7). A great deal is known about control of the SOS regulon (22). Single DNA strands (a signal of damage) interact with RecA protein, which stimulates autocleavage of the LexA repressor and thereby derepresses the SOS genes.

Fig. 1 (*Right*) suggests how SOS, and thus temporary hypermutability, might be induced in cells with an amplification. In brief, recombination between repeat units (in the same chromosome or in different sister chromosomes) releases double-strand fragments or end(s) that are then degraded to reveal the single strands that induce SOS.

This explanation assumes that recombination events between repeats do not always rejoin all DNA ends to produce covalently closed product molecules. The available evidence supports this view. Evidence has been reported that duplication segregation is only "half-reciprocal," i.e., it can either break the chromosome and release a circle or maintain chromosome integrity and release a linear fragment (23); both possibilities produce DNA ends that can be resected to yield single strands. Similarly, unequal sister-strand exchanges between separated direct repeats are seldom reciprocal (M. Carter and J.R.R., unpublished) and thus are likely to produce DNA ends. Finally, considering the magnitude of the unstable amplified material (0.2–2 megabase pairs), even occasional release of ends is likely to be sufficient for SOS induction.

Numerical Considerations Favor the Amplification–Mutagenesis Model. The amplification–mutagenesis model suggests that 100 preexisting duplication cells placed on a selection plate each initiate a colony that grows (about 20 generations) under selection into a microclone containing 10^5 amplification-bearing cells (10^7 cells per plate). Reversion events (which normally occur at 10^{-8} per cell per division) can be accounted for by the following estimates. Each of about 10^5 cells in microclone has $\approx$30 *lac* copies and grows with a mutation rate elevated about $\approx$35-fold by SOS induction. Each such microclone produces a lac^{+} revertant cell and develops into full-sized revertant colony. That is, 10^5 cells per microclone $\times$ 30 *lac* copies per cell $\times$ 35 $\times$ 10^{-8} revertants per *lac* copy = 1 revertant per microcolony.

The proposed value of each parameter is supported by direct measurements. About 10^5 unstable lac^{+} cells were seen in typical microclones (Fig. 2). Amplification of 30-fold is within the observed range (9, 16, 17). The 35-fold increase in mutation rate during SOS induction is the average of two experimental measurements—a 20-fold increase seen in *E. coli* (13) and a 50-fold increase in *Salmonella* (E.S.S., unpublished).

In contrast, the hypermutable state model cannot explain the behavior of the Cairns system. We described that model mathematically and tested it independently in a Monte Carlo simulation. Both approaches included shut-off of mutagenesis on reversion to lac^{+} (J.R.R., E. Kofoid, F. Roth, O. Berg, and D.I.A., unpublished data). By both tests, the model could not explain even one *lac* revertant if one assumed the measured increase in mutation rate (35$\times$) and a hypermutable population of 10^5 cells, as suggested by others (7, 13). The model predicts a maximum of 20 revertants, when mutagenesis of the same population is increased ($10^5\times$) to a level at which lethal mutations kill all cells except *lac* revertants (as is suggested by the model). When larger populations are mutagenized just sufficiently to give 100 revertants, nonrevertant cells are not all killed and the model predicts that the nonrevertant lawn will show more than a 100-fold increase in unselected mutations. This conflicts with experimental observations of little or no mutagenesis of nonrevertant cells (1, 6, 7, 24).

The Role of the F′ Plasmid in Amplification–Mutagenesis. For optimal reversion under selection, the Cairns system requires that the *lac* mutation be located on the F'_{128} plasmid, rather than in the chromosome (ref. 25 and E.S.S., unpublished data). The stimulation provided by the F′ plasmid requires expression of the plasmid *tra* operon, which encodes conjugative (transfer) functions (26, 27). It is not clear whether actual transfer of DNA between cells is needed (20, 27–29).

We tested the reversion behavior of the same mutant *lac* operon placed at each of several hundred positions in the genome of *S. enterica* (E.S.S., unpublished data). These results suggested that both duplication and high amplification (and therefore also reversion) are more difficult to achieve in the chromosome than on a conjugative plasmid. Previous work of others has demonstrated frequent amplification of genes on conjugative plasmids (17, 30, 31) and has shown the difficulty of amplifying chromosomal genes (32).

We suggest that the F′ plasmid contributes to reversion under selection because it frequently creates DNA ends when its transfer origin fires (whether or not conjugation occurs). These ends stimulate both legitimate and illegitimate recombination on the plasmid, thereby increasing the frequency of gene duplication, amplification, and segregation. Thus the F′ helps generate the copy-number variants that are the targets of selection in the amplification–mutagenesis model.

Revertants Arise During Growth and Not in Stationary Phase. Reversion in the Cairns system has been called "stationary-phase mutagenesis" (5, 7, 10, 24), because the parent lawn between revertant colonies grows very little. Growth is needed in the amplification model, but occurs within individual clones as they develop into full-sized revertant colonies. Evidence for this growth is the observation of many ($\approx 10^5$) amplification-bearing cells within virtually every revertant colony. Beyond the few duplication-carrying cells that initiate growing clones, the plated population does not need to contribute to the revertant number and is not required to grow. If growth of the general population is sufficiently restricted, the lawn between colonies would show growth only in the separated microclones that have not yet become visible. The model predicts that the observed 100 revertants can be explained if growth (mostly within colonies) adds as few as 10^7 cells to the plate (a 10% increase over the plated population). Thus, the observed absence of growth between revertant colonies is consistent with the amplification

model and does not constitute evidence for "stationary-phase mutagenesis."

The amplification model does not require selection to block all growth. It requires only that growth be sufficiently limited that natural selection can detect the progressive improvement provided by duplication, amplification, reversion and segregation of the lac^{+} allele. Thus, slow growth of the parent strain should not preclude adaptive mutation or general hypermutability. This prediction is fulfilled.

Reversion conditions that support no background growth of the *E. coli* tester strain allow cells of an analogous *S. enterica* strain carrying the same F′*lac* plasmid to divide about once per day, a rate that is easily detectable but insufficient (in itself) to explain reversion. Despite its slow background growth, *S. enterica* behaves like *E. coli*. In both organisms, reversion requires recombination, SOS induction, and the F′ plasmid; in both, reversion is associated with *lac* amplification and general mutagenesis (refs. 9 and 26, and E.S.S., unpublished data).

Unique Features of the Cairns System. The Cairns system demonstrates, within the period of a single week, an evolutionary succession of stochastically determined events that provide progressive growth improvement. This growth is possible because the *lac* allele used has just enough residual function to support growth when duplicated. Placement of the *lac* gene on an F′ plasmid increases the frequency of duplication, amplification, reversion, and segregation events and thereby accelerates a normally slow evolutionary process.

Implications of the Amplification–Mutagenesis Model for Cancer. Cancer requires that a single cell accumulate multiple particular mutations that release it from growth control. The conjunction of these mutations occurs at a frequency vastly exceeding the product of the their independent probabilities (33). This finding has been explained by a combination of factors, including successive clonal expansions and mutators. Thus in cancer, as in *lac* reversion, mutations arise in cells that are under strong selection to grow and those mutations appear to be directed preferentially to sites that improve growth. The amplification–mutagenesis model may help explain this shared behavior.

For example, a gene for a positive growth regulator (a protooncogene) could be progressively amplified under selection, thereby stimulating progressively faster cell growth. The added copies of the gene increase the likelihood of a mutational alteration that allows the factor to work more efficiently. Mutations that destabilize the genome may promote amplification, as does the F′ in the Cairns system. Amplification could elevate mutation rates either when released fragments induce error-prone polymerases, as in the Cairns system, or by increasing the dosage of genes for such polymerases. Thus the amplification–mutagenesis model suggests several ways in which slow growth under selection might accelerate the accumulation of the genetic changes that lead to cancer and do so with very little apparent growth.

Amplification–Mutagenesis May Solve an Evolutionary Puzzle. New genes clearly arise by duplication and divergence of preexisting genes. A difficulty in imagining this process is that initially the duplicate gene is not under selection, yet it must rise to high frequency in the population and be held there long enough to acquire the rare mutations that create a new selectable function. During this period, the duplicate must avoid common loss-of-function mutations.

These problems would be solved if each step in the process could be accomplished under positive selection. This selection would occur if the original gene (before duplication) has a very minor secondary function, which, at some point, becomes selectively valuable. (Abundant evidence for secondary gene functions is provided by experimentally induced high copy number suppression.) Natural selection then favors individuals in which the level of the original minor activity has been increased by duplication or amplification. Selection for the new function brings the extra copies to a high frequency in the population and holds them there through the subsequent slow process of mutational improvement. The process is analogous to *lac* reversion by amplification–mutagenesis in that both rely only on growth under continuous selection.

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