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Adaptive Amplification

P. J. Hastings
Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas, USA

ABSTRACT Modern techniques are revealing that repetition of segments of the genome, called amplification or gene amplification, is very common. Amplification is found in all domains of life, and occurs under conditions where enhanced expression of the amplified genes is advantageous. Amplification extends the range of gene expression beyond that which is achieved by control systems. It also is reversible because it is unstable, breaking down by homologous recombination. Amplification is believed to be the driving force in the clustering of related functions, in that it allows them to be amplified together. Amplification provides the extra copies of genes that allow evolution of functions to occur while retaining the original function. Amplification can be induced in response to cellular stressors. In many cases, it has been shown that the genomic regions that are amplified include those genes that are appropriate to upregulate for a specific stressor. There is some evidence that amplification occurs as part of a broad, general stress response, suggesting that organisms have the capacity to induce structural changes in the genome. This then allows adaptation to the stressful conditions. The mechanisms by which amplification arises are now being studied at the molecular level, but much is still unknown about the mechanisms in all organisms. Recent advances in our understanding of amplification in bacteria suggests new interpretations of events leading to human copy number variation, as well as evolution in general.

KEYWORDS gene amplification, adaptation, stress response, duplication

INTRODUCTION

Amplification, the reiteration of a segment of a genome, is widespread in nature and can be induced in the laboratory. Adaptive genetic change is change that bestows an advantage to an organism in its present environment. The pattern of occurrence of amplification, as an apparent response to selection, leaves little doubt that it is an adaptive phenomenon. Examples of gene amplification are too numerous for a comprehensive report, so only selected references and reviews are cited. Despite the abundance of reports of amplification, studies on the mechanisms and adaptive nature of amplification are relatively few. This review concentrates on amplification as a stress response, and particularly on what we are learning from a model system in *Escherichia coli*. Note that multicopy amplification is distinct in several important respects (discussed below) from duplication of large segments of the bacterial chromosome, which is not reviewed extensively here. Readers are referred to Roth *et al.* (1996) for more comprehensive review of that topic.

Address correspondence to P. J. Hastings, Department of Molecular and Human Genetics, Baylor College of Medicine, 1 Baylor Plaza, Houston, Texas, 77030, USA.
E-mail: hastings@bcm.edu

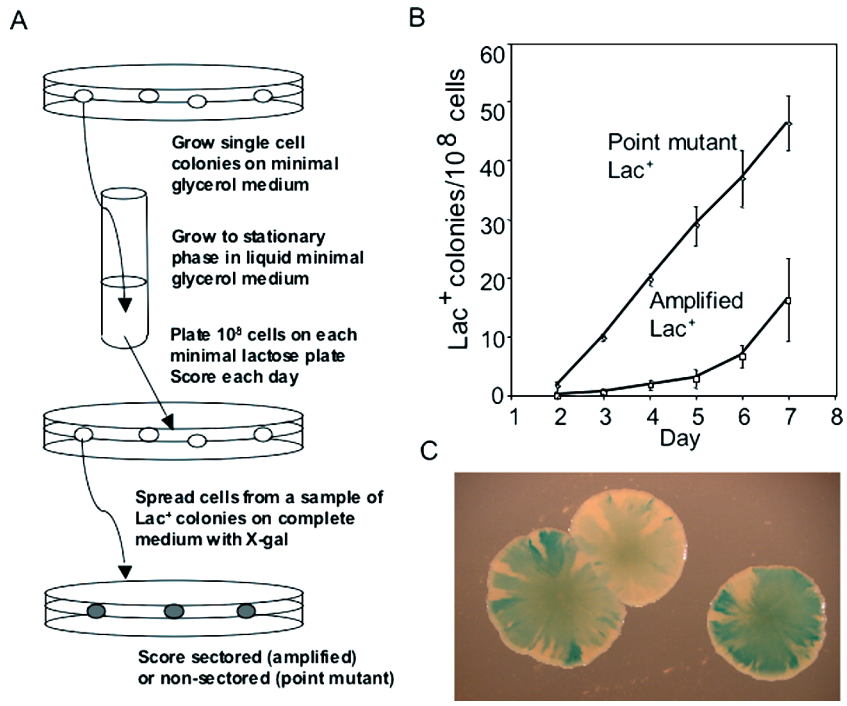

Figure 1 ***A*. Procedure for measuring adaptive amplification in the Lac-system. Lac$^-$ cells are plated as single cells on minimal glycerol medium. Small colonies are transferred to minimal glycerol liquid medium and grown to stationary phase. Approximately 10^8 cells per plate are plated on lactose minimal medium, and colonies arising are marked each day. Cells from a sample of colonies from each day are then plated on a rich medium with X-gal to score sectoring, and hence determine the frequency of *lac*-amplified colonies among total Lac$^+$ colonies. This frequency is used as a correction factor to determine the rate of amplification at *lac* per day. *B*. An example of the arising of *lac*-amplified and point mutant Lac$^+$ colonies during prolonged starvation on lactose minimal medium, illustrating the later occurrence of *lac*-amplified colonies. *C*. Colonies of cells of *Escherichia coli* amplified at *lac* grown on nonselective medium containing X-gal. The sectored colony morphology results from breakdown of the amplified array by homologous recombination. Figure 1C taken from Hastings *et al*. (2004).**

Amplification has traditionally been identified by Southern hybridization, measuring the copy number of the amplified sequences relative to unamplified regions of the genome. A second means of identification of amplification that can be used in some systems is the presence of an unstable phenotype, breaking down to the parental type in the absence of selection for multiple copies of a gene (Tlsty *et al.*, 1984; Whoriskey *et al.*, 1987). This instability is illustrated in Figure 1C. Presumably this loss of amplification also occurs in the presence of continued selection, but the cells that lose amplification then cease to grow, and do not contribute to the bulk of the colony. This likely explains the slow growth phenotype of colonies of amplified cells under selection (Hastings *et al*., 2000). Much more productive techniques are now available, including the use of microarrays for comparative genomic hybridization (CGH), quantitative polymerase chain reaction (qPCR), and high-resolution fiber fluorescent *in situ* hybridization (FISH) (reviewed by (Feuk *et al.*, 2006). These techniques allow all variation in copy number throughout a large part of large genomes to be detected (*e.g.*, Sharp *et al*., 2005). CGH is revealing more widespread amplification than previously expected, underscoring the importance of this phenomenon.

EVOLUTIONARY IMPLICATIONS OF ADAPTIVE AMPLIFICATION

A major role of amplification is that it extends gene control beyond the natural range of the systems that control gene expression. A cell adjusts to changed conditions by upregulating gene expression of the needed genes, but this occurs only within a limited range. Many organisms experience extreme variation in conditions, often in an episodic fashion. For example, enteric bacteria must be able to live both within the intestine and at ambient conditions outside the body when they are between hosts. Amplification is seen as a device to extend the range of gene expression to handle the extreme conditions (Anderson and Roth, 1977; Roth *et al.*, 1996; Romero and Palacios, 1997). It is expected that, if the extreme conditions occur repeatedly, eventually the organism would acquire selected

mutations that make the required amplification a more frequent event by, for example, acquiring sufficient homology spanning the segment that is to be amplified for unequal homologous recombination to be able to form the initial duplication (Mekalanos, 1983) or by evolving a site-specific recombination mechanism (Reams and Neidle, 2004b). Alternatively, the organism could develop an ability to exercise gene control over a broader range by point mutation. As a strategy to increase gene expression, amplification holds an advantage over point mutation or a rearrangement that changes the promoter region, because amplification is reversible, readily breaking down by unequal homologous recombination to a single copy when selection is relaxed (Roth *et al.*, 1996).

When there is selection for multiple copies of genes, there might be selection for retaining a high copy number. This requires a reduction in the recombination that continually changes the number. The copies might diverge from each other by mutation, or they might become separated by intervening sequences, yielding a cluster of like genes rather than a tandem array (Graham, 1995). The divergence will reduce unequal homologous recombination, which changes the copy number, and gene conversion, which tends to keep the repeated sequences alike. Eventually, the clustered genes might become unlinked, which could reduce gene conversion further and allows evolution of new functions (Graham, 1995).

Ohno (1970) suggested that repetition of genes would play a significant role in evolution. One copy of a gene retains the original function, while the other is free to change without selection until it encodes a new or modified function. This process would be aided if the evolving protein had a broad specificity range to begin (Aharoni *et al.*, 2005; Francino, 2005). As the repeated genes evolve, their domains can be reassorted by gene conversion, and eventually the most fit variant would replace the others by loss of copy number or by inactivation of the less fit copies, generating pseudogenes. These processes might be facilitated by the increase in mutation rate per cell that is expected to accompany the multiple copies, on the assumption that each copy has the same mutation rate as a single copy (Roth *et al.*, 1996).

Another aspect of the evolutionary impact of amplification is that it might be a major driving force in evolution of genome structure, leading to the clustering of related functions. Commonly, in adapting to a new environment, it would not be sufficient to increase production of only a single protein in a multicomponent pathway, because related functions would also need to be upregulated for the pathway to function efficiently. When related functions are clustered, they can be co-amplified so that all are upregulated and downregulated together (Reams and Neidle, 2004b). An example of this has been observed in *Acinetobacter*, where utilization of benzoate as a carbon source required amplification of two closely linked *cat* gene operons (Reams and Neidle, 2004a).

OCCURRENCE OF AMPLIFICATION

Amplification has been seen in archaea (Zusman *et al.*, 1989), as well as in many bacteria (reviewed by (Romero and Palacios, 1997) including bacteriophage (Wu and Black, 1987), yeast (Fogel and Welch, 1982; Huang and Campbell, 1995; Dunham *et al.*, 2002), insects (Raymond *et al.*, 1998), fish (Carginale *et al.*, 2004), plants (Gijzen *et al.*, 2006), plant cell cultures, *e.g.* (Widholm *et al.*, 2001), and mammals, in which it occurs widely in cancers (reviewed by (Lengauer *et al.*, 1998; Albertson, 2006).

Many of these examples occur in conjunction with a specific stress, and the amplification that results concerns specific parts of the genome that include genes encoding functions that are relevant to tolerance of the specific stress. For example, amplification has been associated with heat stress tolerance in *E. coli* (Riehle *et al.*, 2001), in which amplification of the genes for the stress-response transcriptional activator RpoS and a protein-repair protein Pcm occurred. Resistance to insecticides is bestowed on insecticide-resistant mosquitoes by amplification of the gene encoding an esterase that inactivates organochloride pesticides (Raymond *et al.*, 1998). Resistance to antibiotics can be achieved by amplification of the gene for the target protein; *e.g.*, amplification of *dhfr,* the gene for the target enzyme dihydrofolate reductase, bestows resistance to methotrexate in yeast (Huang and Campbell 1995) and in the archaean *Halobacterium volcanii* (Zusman *et al.*, 1989). Resistance to the herbicide glyphosate can be selected in plant cell cultures, and many of the resistant strains obtained show amplification of the gene encoding the target enzyme (Widholm *et al.*, 2001). Also, resistance to high concentrations of chloramphenicol arose on a plasmid in *Proteus mirabilis* by amplification of the *cat* gene, encoding a protein that inactivates

chloramphenicol (Perlman and Stickgold, 1977). Resistance to toxic metals can arise by amplification of the gene encoding metallothionein, which sequesters the metal (Palmiter, 2004). Limiting the supply of glucose during many generations of *Saccharomyces cerevisaea* growing in chemostats led, among other changes, to amplification of a hexose transporter gene (Dunham *et al.*, 2002). In many of these cases, the phenotype resulting from amplification is also produced in other individuals by point mutation (defined here as small changes to DNA including base-pair [bp] substitutions, and addition or deletion of one or a few bp). Amplification also occurs in association with pathogenicity (Mekalanos, 1983) and symbiosis (Moran and Baumann, 2000), and as a programmed developmental change in *Drosophila* (Spradling and Mahowald, 1980; Orr-Weaver, 1991; Calvi and Spradling, 1999).

The study of complete genomes is revealing extensive copy-number variation, including duplications and amplification, and much of the variation between individuals is being attributed to these copy-number polymorphisms both in humans (Sharp *et al.*, 2005; Feuk *et al.*, 2006) and in mice (Graubert *et al.*, 2007). In some cases, the genes involved seem to be adaptive, *e.g.*, concerned with resistance to infection (Hollox *et al.*, 2003). These findings suggest that amplification and is much more common than has been supposed.

AMPLIFICATION AS A STRESS RESPONSE

Many examples of amplification in response to stress appear to involve selection for a rare pre-existing variant. For example, amplification of esterase genes in *Culex pipiens* has occurred only once or a few times, and its present widespread occurrence was apparently caused by migration (Raymond *et al.*, 2001). Thus it is possible that a rare pre-existing spontaneous variant spread because it had a selective advantage. The alternative hypothesis is that the variant arose as part of a stress response, the stress occurring because the organism was maladapted, and therefore under the stress of the selective pressure.

It is of great interest to know whether such chromosomal structural changes happen routinely as part of the stress response: an increase in genomic instability in response to stress that accelerates the arising of amplification (Tlsty *et al.*, 1984; Hastings *et al.*, 2000). If this were the case, the stress might be inducing a condition of general genomic instability similar to that thought to occur in some cancers (Komarova *et al.*, 2002; Rajagopalan *et al.*, 2003; Michor, 2005). This question was first tackled by Tlsty *et al.* (1989), who studied the occurrence of PALA resistance in a mammalian cell line in response to challenge by PALA. In this case, it was concluded that all amplification was pre-existing; *i.e.*, amplification arose spontaneously in the cultures before selection began. The observation of only pre-existing amplification in this assay was not unexpected, however, because the selection used was lethal, so that cells had no opportunity to adapt to the presence of the drug after selection began. In a study of the evolution of R-plasmids to resistance to high levels of chloramphenicol, it was found by physical labeling techniques that amplification of the r-regions of the plasmids arose on plasmids that did not carry amplification at the beginning of the experiment. Nonamplified plasmids became amplified while the amplification was being selected (Perlman and Stickgold, 1977). Further evidence that amplification can be induced as part of a stress-response comes from the Lac assay system in *E. coli* of Cairns and Foster, (1991), and is discussed below.

THE LAC ASSAY SYSTEM IN *ESCHERICHIA COLI*

A well characterized Lac assay system in *E. coli* (Cairns and Foster, 1991), which has been used extensively to study stress-induced point mutation, also provides a model system for the study of adaptive amplification (Foster, 1994; Hastings *et al.*, 2000) (Figure 1A). The assay system involves a +1 frameshift mutation in a *lacIΩlacZ* fusion gene on an F' plasmid. Lac^+ mutants accumulate during prolonged starvation on lactose minimal medium in the absence of net growth of the cell population (Figure 1). After 5 days, they have a mutant frequency (10^{-6}), which is >100 times higher than that seen in growing cultures. The Lac^+ cells can either carry frameshift point mutations that restore the reading frame, or they can carry amplification of the *lac* region. *lac*-amplified cells are able to grow because the *lac* allele used in these studies is leaky, such that 20 or more copies of the lac^- gene provide sufficient β-galactosidase for growth on lactose medium. *lac*-amplified colonies are rare for the first few days, but rise to 40% of Lac^+ colonies appearing

on day 8 (Figure 1B) (Hastings *et al.*, 2000; Powell and Wartell, 2001). In this system, amplification is distinguished from point mutation by the instability of the Lac^+ phenotype seen as blue and white colony sectoring when colonies are grown on nonselective medium containing X-gal (Figure 1C).

Stress-inducibility

There are three points of evidence that amplification in the Lac system is stress induced: First, the events occur after the stress has begun. Reconstruction experiments were used to show that the event scored as amplification occurred after selection began (Hastings *et al.*, 2000). When cells from amplified Lac^+ isolates were replated under the conditions of the adaptive mutation experiment from which they were isolated, almost all of them formed visible colonies in less time than it took the Lac^+ colony to form in the first place. For example, cells from a colony appearing on day 9 of an experiment might reform visible colonies in 3 days, implying that the amplification event occurred on the sixth day of starvation (Hastings *et al.*, 2000). The same test was used to establish that Lac^+ point mutants in this system formed after plating on lactose (McKenzie *et al.*, 1998).

Second, no evidence of a clonal distribution, as would have been expected from pre-existing amplification, has been seen in this system (Hastings *et al.*, 2000).

The reconstruction experiment establishes that the amplification events occur after selection begins, but does not of itself suggest that the amplification was induced by starvation. The third point of evidence does that. Amplification in the *lac* system, like point mutation (Layton and Foster, 2003; Lombardo *et al.*, 2004), requires the stationary phase and general stress response sigma factor RpoS (σ^s) (Lombardo *et al.*, 2004); *i.e.*, mutation and amplification are not induced at a high rate by starvation if RpoS is unavailable to induce the RpoS regulon. RpoS is a subunit of RNA polymerase that activates transcription of specific promoters (reviewed by Hengge-Aronis, 2000, 2002). RpoS has been shown by transcriptome analysis to control about 350 genes that are induced in response to various different stresses in overlapping sets (Lacour and Landini, 2004; Weber *et al.*, 2005). It would be very instructive to know what RpoS-controlled function(s) are required for adaptive genetic change in this system. In the meantime, regardless of which RpoS-controlled functions are needed at induced levels for amplification to occur, it has now become difficult to argue that point mutation and amplification are not stress responses. Whatever RpoS-controlled function is required, it is only available in sufficient quantity for efficient point mutation and amplification when this stress-response regulator is induced.

Beyond the issue of whether genomic change is a stress response is the question of whether accelerated genetic change in response to stress is a selected trait, or whether genomic damage is an incidental byproduct of some other part of stress responses. We have no evidence on this point at present. A second, related, outstanding issue is whether, like point mutation (Torkelson *et al.*, 1997; Rosche and Foster, 1999; Godoy *et al.*, 2000), amplification stems from a cell-wide genome destabilization, or whether it is targeted to the region in which amplification would be beneficial. Although such targeting seems at first sight to be implausible, it is not impossible that long-term high rates of transcription, such as would occur when a gene product is needed in high quantity, might induce genomic change in the region of high transcription. At present, no evidence of unselected secondary genomic changes that would support models that specify genome-wide destabilization has been reported in conjunction with amplification.

MECHANISMS OF AMPLIFICATION

Study of the Mechanisms

Mechanisms of amplification have been studied in four distinct ways. First, description of the DNA sequence changes associated with amplification gives information about how amplification was formed. This is discussed below. Second, artificial constructs can lead to a high frequency of amplification, thus showing what situations promote amplification. For example, Petit *et al*. (1992) used this method to induce amplification by rolling-circle replication (discussed below) in *Bacillus subtilis*. Also, insertion of a restriction site has been used to show that double-strand breaks can lead to amplification in *E. coli* (Ponder *et al.*, 2005) and in mammalian cells (Pipiras *et al.*, 1998). The use of palindromic sequences and double-strand breaks to induce amplification was studied in yeast (Butler *et al.*, 1996; Narayanan *et al.*, 2006). Third, the genetic requirements of amplification reveal what proteins

need to be present for amplification to occur, and much about the mechanism can be inferred from the known enzymatic activities of these proteins. Use of this approach is described below. The fourth approach, one example of which was cited above (Perlman and Stickgold, 1977), is the physical analysis of DNA as the events occur.

A Variety of Mechanisms

Amplification arises by several different mechanisms in different systems, but descriptions of the events at a molecular level are usually not available. The simplest model, described as the canonical model (Romero and Palacios, 1997), is that a duplication is formed and subsequently expanded into an amplified array by multiple events of unequal crossing over among the repeated sequences (Figure 2A). Amplification in *E. coli* has been achieved by this mechanism using artificially constructed duplications (Hendrickson *et al.*, 2002; Slack *et al.*, 2006); however, we do not know that this is the usual mechanism of adaptive amplification in this system. In addition, the canonical model does not address the issue of the origin of the duplications.

An alternative mechanism of expansion is rolling circle replication (Young and Cullum, 1987; Petit *et al.*, 1992). In this model, a large amplified array could be formed in a single event (Figure 2B). In rolling-circle amplification, a circle is formed by annealing from a broken double-strand end (DSE) to a position behind the end. When the circle is closed by ligation, a replication fork can go round it repeatedly, making numerous tandem copies of the sequence that was circularized. Breakage of the circle restores the DSE, which will be reintegrated nonhomologously (Figure 2B). However, if a sister molecule is present or if the circle was formed in a pre-existing duplication, reintegration can be by homologous recombination.

Many mammalian amplification events arise by excision of circles, which can multiply by over-replication, and later reintegrate at their native site or ectopically (Cowell, 1982). This mechanism of generation of multiple copies is presumably unavailable to prokaryotes because almost all circles would lack origins of replication. What is known of amplification mechanisms in yeast and mammals was reviewed recently by Haber and Debatisse (Haber and Debatisse, 2006) and will not be covered in detail here. In yeast and mammals, a major mechanism is believed to be the breakage/fusion/bridge cycle (McClintock, 1942), generating arrays of direct and inverted repeats. The structure of amplified arrays in *E. coli* is simpler (described below), and the same mechanism does not seem to apply.

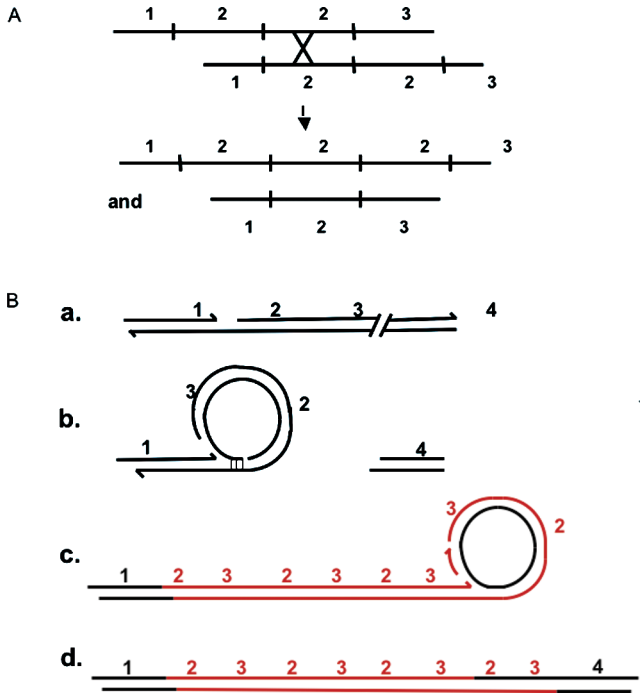

Figure 2 ***A.*** **Expansion to form an amplified array by unequal crossing-over between repeats. A crossover occurs by nonallelic homologous crossing-over between repeated segments in a duplication resulting in three copies of the segment on one molecule, and one on the other. Each line represents a duplex DNA molecule. Numbers 1 to 3 indicate specific DNA sequences.** ***B.*** **Expansion by rolling-circle replication.** ***a.*** **A DNA molecule showing both nucleotide chains (strands). The 3′ ends are marked by a half arrow. A double strand break occurred at the position marked by bars, and there is a single-strand gap on the same molecule.** ***b.*** **The 3′ end of one side of the broken molecule anneals in a gap on the same nucleotide chain behind the position of the break. The circle formed by this annealing is closed by ligation.** ***c.*** **A replication fork is established at the junction. Replication around this circle gives numerous tandem repeats of the sequence in the circle.** ***d.*** **Eventually the circle breaks and becomes rejoined at the original break. This rejoining would be a second non-homologous event unless their were already a duplication in this region, or unless it recombined with an intact sister chromatid, in which case double-strand end-repair could occur by homologous recombination. The numbers 1 to 4 indicate specific DNA sequences.**

Sequences of Novel Junctions Between Amplicons

Using the Lac assay system of Cairns and Foster (1991), we identified and sequenced the novel junctions

Figure 3 An example of a novel junction (*middle line*) formed between the *mhpT* locus (*upper line, red italic*) and the *prpR* locus (*lower line, blue roman*), spanning *lac* at a distance of 20.13 kb. Homologous base pairs are shown in upper case letters. The microhomology junction is marked by bold upper case letters.

of 31 different amplified isolates that formed under starvation (Slack *et al.*, 2006). Three of the sequences occurred twice. Two of these repeats came from different cultures, so that the repeat unit (amplicon) sequences do not provide evidence of a clonal distribution resulting from an event that occurred during growth before selection began. The sequences of the junctions revealed a 5 to 15 bp length of perfect homology, and amplicons identified are between seven and 32 kilobase-pairs (kb) in length. The mean length of amplicons in colonies formed from days 3 to 9 of an adaptive mutation experiment has been estimated to be 16 kb, with a modal value of 20 kb (Slack *et al.*, 2006). An example of a microhomology junction is shown in Figure 3. Some, but not all, of the junctions had a further short homologous region separated from the junction microhomology by one or more nonidentical bases. With one exception, there was only one novel junction present in each isolate, and all amplified arrays consist of direct tandem repeats. One isolate has more than one novel junction (discussed below). A further 48 junction sequences have been described recently (Kugelberg *et al.*, 2006). These authors used the same F′-borne Lac system inserted into *Salmonella enterica.* The amplification junction sequences of Kugelberg et al. (2006) are very similar to those described above in all respects except that they identified amplification involving amplicons of up to 90 kb. in length. The only unifying characteristic in the many junction sequences observed is that the junctions are guanine-rich.

Occurrence of Duplication and Amplification without Selection

In *E. coli* and *Salmonella*, large duplications are very common (Lehner and Hill, 1980; Lin *et al.*, 1984; Mahan and Roth, 1989; Sonti and Roth, 1989; Shyamala *et al.*, 1990; Segall and Roth, 1994; Galitski and Roth, 1997). Their occurrence is generally RecA dependent, suggesting that they form by homologous recombination, and repeated homologous regions (*e.g.*, ribosomal RNA genes (Lehner and Hill, 1980), IS or REP sequences (Shyamala *et al.*, 1990), or repeated genes (Lin *et al.*, 1984)) are found at the junctions (reviewed by (Roth *et al.*, 1996)). IS and REP are families of widespread repeated sequences in the *E. coli* genome. REP-mediated duplication can also occur independently of RecA (Shyamala *et al.*, 1990). This might be because heterogeneity between REP sequences of different families causes some REP-REP recombination to involve microhomology (15 bp. or less) (Slack *et al.*, 2006) or because the approximately 38 bp. length of REP sequences is marginal for RecA-mediated homologous recombination. Roth *et al.* (1996) estimate an average frequency of 10^{-3} for any given duplication and that, in an unselected population, 10% of cells carry some duplication. Smaller duplications that lack extensive homology at the junctions occur much less frequently (Roth *et al.*, 1996). A recent analysis of duplications at *lac* on an F′ plasmid in *Salmonella* (Kugelberg *et al.*, 2006) reported that duplications that included *lac* were present in 0.2% of unselected cells. However, the duplications analyzed were mostly (20/24) IS-mediated, with over 1 kb. homology, while the remainder were REP mediated, providing 20 to 36 bp. of homology. Microhomology-mediated duplications were not found in that sample. As reported above, only 7 of 86 amplicons recovered selectively that included the *lac* locus might have been pre-existing or formed very early under starvation, as determined by the day on which the colonies appeared compared with the speed of colony formation in reconstruction experiments (Hastings *et al.*, 2000). Thus there is little evidence at present to suggest that microhomology-mediated duplications commonly pre-exist in unselected cultures. There has been no report of large homology-mediated amplicons in amplified arrays, so it is likely that these cannot be expanded to sufficient number to give a Lac^+ phenotype. Another possible role for large duplications is discussed below.

Significance of Microhomology Junctions

Microhomology junctions in amplified arrays in *E. coli* have now been reported by several laboratories (Edlund and Normark, 1981; Whoriskey *et al.*, 1987;

Kugelberg *et al.*, 2006; Slack *et al.*, 2006). Microhomology junctions like these have also been seen in other kinds of nonhomologous recombination events, such as deletion formation (reviewed by Bzymek and Lovett, 2001). Microhomology-mediated non-homologous recombination has been attributed to a mechanism of template switching within replication forks (Bzymek and Lovett, 2001). The points of evidence taken to imply a possible template-switch mechanism are (i) Conditional mutations affecting DNA replication affect the frequency of nonhomologous recombination events, suggesting involvement of replication forks. (ii) It is difficult to find mutations affecting amplification induced by transposon insertion mutagenesis, implying that amplification is controlled by essential functions such as DNA replication proteins. (iii) Amplification responds to change in 3′-single-strand exonucleases, suggesting that the 3′ single-stranded ends might act as primers for DNA synthesis. (iv) Mutation of methyl-directed mismatch repair genes, which remove mismatches on nascent strands near to replication forks, affects the level of non-homologous recombination, again suggesting the involvement of replication. (v) There is a very strong distance dependence for recombination between small repeats, perhaps related to the dimensions of a replication fork. The aspect of this description that does not fit with the data for adaptive amplification is that our amplicons are much larger than is expected for the template-switch mechanism, indicating nonhomologous recombination at much greater distances. This point is discussed further below.

Structure of Amplicons

Most of the amplicons that have been reported in *E. coli* are simple, direct repeats. Slack *et al*. (2006) reported one complex junction out of 31 *lac*-amplified isolates analyzed. This isolate has at least two novel junctions. Each amplicon has a direct repeat of 16 kb that includes the *lac* region, and an inverted repeat of about 8 kb that overlaps the 16 kb region for most of its length. Kugelberg *et al.* (2006) reported five amplicons with complex junctions out of 48 analyzed. The precise structure of these complex amplicons is not known. One possible interpretation is that these complex amplicons have been formed by an inverted template switch followed by a second inverted template switch that rectified the direction. It is expected that a single inverted template switch in the Lac system would not lead to formation of an amplified Lac^+ colony because a large part of the F′ would have been deleted. Two of the junctions reported by Slack *et al.* (2006) had point mutations adjacent to the microhomology. The significance of this is not yet known.

All amplicon junctions described in the *lac*-system have been found by outward-facing PCR. The more complex junctions would be more difficult to locate by this technique (Kugelberg *et al.*, 2006), which is designed to identify direct repeats. Thus, the junctions described are not a truly random sample. Kugelberg *et al*. (2006) report that 8 of 56 amplified isolates were not characterized. If we assume, as they suggest, that these were complex junctions with inverted segments, we see that about 25% of all amplicons have two or more junctions. This suggests, first that direct and inverted duplication formation is more-or-less equally likely, and second, that duplication is not a rare event, at least in that subpopulation of cells that becomes amplified; it can happen twice in the same genomic region of the same cell at a frequency comparable to that with which it happens once.

Genetic Requirements of Amplification

In *E. coli*, the functions of many enzymes that act on DNA are known. We may, therefore, deduce aspects of the mechanism of any given process by finding which genes are needed. The mechanism of adaptive amplification in the Lac system in *E. coli* has been studied in this way (Slack *et al.* 2006). It was found that, as with stress-induced point mutation (Harris *et al.*, 1994; Foster *et al.*, 1996; Harris *et al.*, 1996), the proteins of double-strand break repair, RecA, RecBC, and RuvABC are all required. Because RecBC works exclusively at double-strand ends, the finding of a RecBC requirement implicates double-strand ends as an intermediate in the amplification process. Further evidence that double-strand ends promote the initiation of amplification comes from the finding that double-strand cutting near *lac* with the endonuclease I-*SceI* leads to a dramatic increase in amplification (Ponder *et al.*, 2005). Deletion of *xonA*, encoding Exo1, a 3′-specific single-strand nuclease, resulted in

an increase in amplification, while over-expression of *xonA* on a plasmid caused a decrease (Slack *et al.*, 2006). This suggests that 3′-ending single strands of DNA are involved, as reviewed above for microhomology-mediated deletions (Bzymek and Lovett, 2001). A further genetic requirement discovered is for *polA* (Hastings *et al.*, 2004b; Slack *et al.*, 2006). *polA* encodes DNA polymerase I (Pol I), which is involved in processing Okazaki fragments in the lagging-strand during replication, and in base-excision repair (BER) and nucleotide-excision repair (NER). It also encodes a 5′ single-strand endonuclease homologous to FEN1, the human flap endonuclease, and it is this domain that is required for amplification (Slack *et al.*, 2006). This, too, has a role in Okazaki fragment processing. Because other genes involved in BER and NER are not required for amplification, the need for the flap endonuclease implicates the lagging strand in amplification (Slack *et al.*, 2006).

Much recombination with short homology (less than 50 bp.) has been shown to be RecA independent (Matfield *et al.*, 1985; Shyamala *et al.*, 1990; Mazin *et al.*, 1991; Lovett *et al.*, 1993; Lovett *et al.*, 1994). Finding a requirement for RecA in short homology-mediated amplification is not in conflict with this, because amplification is a more complicated process, involving both duplication and expansion into many copies. RecA is required for the breakdown of amplified arrays (Tlsty *et al.*, 1984; Slack *et al.*, 2006). On the assumption that increase and decrease in copy number occur by the same mechanism, unequal crossing-over (Tlsty *et al.*, 1984), we can postulate that RecA is needed for the expansion phase, and we cannot determine whether it is needed for duplication formation. The same consideration applies to the requirement for RecBC and for RuvC. These have also been shown to be required for loss of amplification (Slack *et al.* 2006), and so might or might not be involved in duplication formation. Neither absence of Pol I nor over-expression of ExoI reduces the instability of amplification, so the effects of these factors can be attributed to the duplication phase of amplification.

In summary, we can deduce from the genetic requirements for adaptive amplification in the Lac system that it involves double-strand ends (requirement for RecBC and Ponder *et al.* (2005), 3′ single-strand tails (increase in amplification when *xonA* is deleted), replication forks and lagging strands (requirement for the flap endonuclease of Pol I).

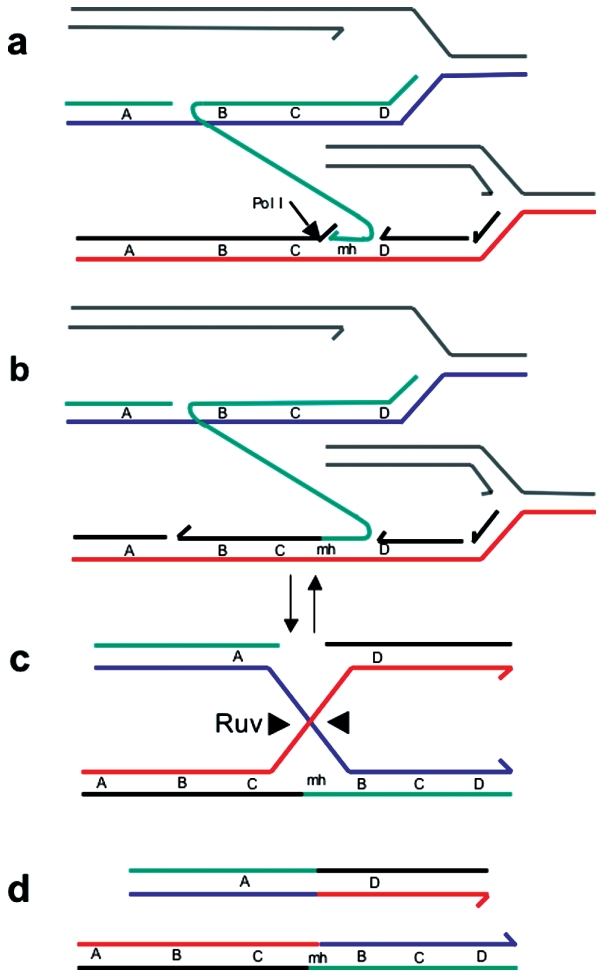

Figure 4 **The long-distance template-switch model of Slack *et al*. (2006). An example of how a duplication might be formed by template switching between replication forks. In *a*. a 3' end of a nascent lagging strand has switched templates to a region of microhomology (*mh*) on the lagging-strand template of a different replication fork running ahead of the first fork. *b*. The junction is stabilized by removal of the 5′ end of the recipient lagging strand's nascent DNA, using the flap endonuclease activity of DNA polymerase. (Pol I). The junction is further stabilized by extension by a DNA polymerase *c*. The structure in (*b*) after isomerization, reveals that it is a Holliday junction. Resolution by the resolvase RuvABC can lead to duplication of sequence BC, and a reciprocal deletion (*d*). Each line represents a single DNA nucleotide chain. 3′ ends are marked by half arrows. Strands are identified by various colors to show their origin, green and blue from one DNA molecule and black and red from another. Gray lines indicate those strands that were not involved in the event.**

MODELS FOR THE MECHANISM OF ADAPTIVE AMPLIFICATION

The Long-Distance Template-Switch Model

Slack *et al*. (2006) offered a model for the initial process leading to adaptive amplification: duplication formation. In this model (Figure 4), called the

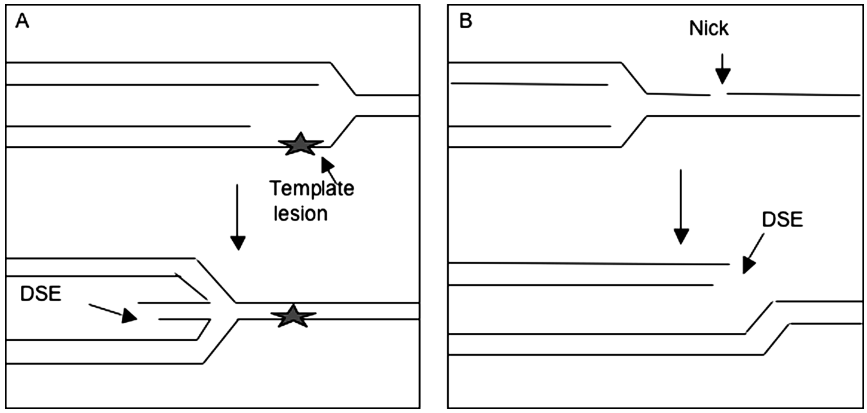

Figure 5 **Generation of double-strand ends (DSE) at stalled replication forks. *A*. Fork regression generates a DSE in response to a replication-blocking lesion on a template strand. *B*. When a replication fork encounters a nick in a template, fork collapse can ensue, leaving one DSE. Each line represents a DNA strand or nucleotide chain.**

long-distance template switch model, a nascent 3′ single-strand DNA end from the lagging strand of a stalled replication fork switches template to a non-homologous position on the lagging strand template of another stalled replication fork on a sister molecule (Figure 4a). This switch can lead to duplication either by re-replication of a length of sequence or, as shown in Figure 4, by capture of a length from the sister. The suggestion that the switch occurs between two forks is offered as an explanation for the relative distance-independence of the amplification event as compared with deletion events. The latter might show a strong distance dependence because of the need to have both a 3′ end and an exposed template to which it can bind, and these are most readily found together within a single replication fork (Bzymek and Lovett, 2001).

The most obvious alternative model to explain microhomology-mediated recombination is nonhomologous end joining (NHEJ) (Burma *et al.*, 2006). This mechanism has been cited routinely to explain mammalian rearrangement events that show microhomology at the junction point, (*e.g.*, Inoue *et al.*, 2002). NHEJ is a less likely event in *E. coli* because *E. coli* lacks the Ku proteins that mediate NHEJ in other organisms (Bowater and Doherty, 2006). It is still possible, however, that *E. coli* has NHEJ (Meddows *et al.*, 2004). NHEJ requires two double-strand ends. These are readily formed together by the action of agents that damage DNA to form a double strand break, *e.g.*, ionizing radiation. To form a duplication, however, the double strand ends would have to be in different positions. Thus, two breaks would be needed. Replication problems lead to double strand ends because of fork collapse or fork regression (see Figure 5), and this had been thought to be a frequent event: as often as once per replication in *E. coli* (Cox *et al.*, 2000), although this figure may be revised downward (Pennington and Rosenberg, 2007). Thus the presence of a single end, as used in the long-distance template-switch model, is expected to be much more common than the occurrence of two ends required for NHEJ.

Human Copy-Number Variations

In human, nonrecurrent duplications associated with genomic diseases also have dimensions that are much larger than a single replication fork, and these too show microhomology at the junctions (Lupski and Stankiewicz, 2005). In one case, these have been explained by the long-distance template-switch model (Lee, 2006).

Alternative Models

A variation on this model comes from the observation that in *S. enterica* the frequency of Lac^+ colonies is reduced if insertion sequences spanning the *lac* locus are deleted (Kugelberg *et al.*, 2006). These authors interpret this result to mean that the initial event in amplification is a pre-existing duplication formed by homologous recombination between insertion sequences. These duplications are too large (134 kb.) to form amplified arrays containing enough copies of *lac* to bestow a Lac^+ phenotype on the usual timescale of an adaptive mutation experiment (Hastings *et al.*, 2000). Kukelberg *et al.* (2006) suggest that a process of deletion occurs

to produce the short amplicons that we see from the large homology-mediated duplications. If this model is correct, it might be that the long-distance template switching described above is occurring not between sister molecules but within a duplicated segment on the same molecule.

Roth and colleagues (2006) have suggested that amplification is the precursor to adaptive point mutation, the multiple copies of the region increasing the chances of a beneficial mutation. This mechanism does not seem to apply to most point mutation in the Lac system (Hastings *et al.*, 2000, 2004b; Ponder *et al.*, 2005; Slack *et al.*, 2006; Stumpf *et al.*, 2007) (reviewed elsewhere in this volume by Galhardo *et al.*, 2007). However, there might be circumstances in which the mechanism does apply. Amplification might then lead to targeted point mutation as follows: The idea was mooted above that chronic intense transcription might serve to target amplification to regions of the genome where it would be adaptive. This might occur, for example by R-loop formation (Masai and Arai, 1996; Kogoma, 1997). If this were to occur, and were followed by amplification-mediated point mutation, then the point mutation might also appear to be targeted to sites where change would be beneficial.

CONCLUSION

Adaptive amplification is important in nature and in medicine as a mechanism of rapid reversible response that gives a cell a growth advantage under adverse conditions. It is also viewed as a mechanism that provides the feedstock for longer-term evolutionary processes. There are some clues as to the basic biological processes underlying the formation of adaptive amplification, but much is not yet understood. A major unanswered question is whether or not the alterations leading to amplification are part of a cell-wide destabilization of the structure of the genome. Given the widespread occurrence and the biological and medical significance of the phenomenon, it seems important to improve our understanding of it.

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