Genetic rearrangements and gene amplification in *Escherichia coli*: DNA sequences at the junctures of amplified gene fusions

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We describe gene fusions that result from genetic duplications of 5–20 kb, which are amplified 50- to 100-fold. Because one end point of the fusion lies within the sequenced *lacI* gene, the new junctures created by the duplications are readily identified. Using a procedure for dideoxy sequencing of double-stranded DNA, we show that the duplications occur almost exclusively at short sequence repeats (less than 15 bp), sometimes involving broken homologies, in the 30 cases examined. Most of the duplications place the *lacI*-Z encoded hybrid repressor- β -galactosidase protein under the control of a downstream promoter, resulting in the production of a more complex hybrid protein with β -galactosidase activity. In some cases the fusion occurs with the *lacY* or the *lacA* gene, which suggests that silent promoters can be uncovered by gene fusion and subsequent amplification. In some ways this system represents a bacterial analog to chromosomal rearrangements of oncogenes in higher cells, since here the expression of a silent gene is the result of a genetic rearrangement that is followed by amplification during selected growth.

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Large genetic rearrangements have been studied for many years (Auerbach 1976), but relatively little is known about them at the DNA sequence level, and few of the enzymatic pathways involved have been elucidated. Although transposable genetic elements can mediate certain rearrangements (see Shapiro 1983), many rearrangements of the genome occur without the obvious participation of such elements. It is now recognized that rearrangements may play a central role in many biological processes, including development (Long and Dawid 1980; Spradling 1981; Bostock 1984; Haselkorn et al. 1986), control of gene expression (Varmus 1984; Murnane 1986), differentiation (Tonegawa 1983; Bostock 1984), antibody formation (Tonegawa 1983), antigenic variation (Van der Ploeg et al. 1984; Segal et al. 1985), and evolution (Riley and Anilionis 1978; Sager et al. 1985; Schimke et al. 1986). Recent work with oncogenes has strongly implicated genetic rearrangements in the origin of certain human cancers (Land et al. 1983; Leder et al. 1983; Marx 1984), an idea that had been postulated previously (see, for instance, Cairns 1981). With respect to specific types of rearrangements, gene amplification in mammalian cells is responsible for observed resistance to chemotherapeutic agents. For instance, methotrexate resistance results from amplification of the dihydrofolate reductase gene in cultured cells (Schimke 1984) and in human patients (Horns et al.

1984). Amplification has itself been implicated in the origin of cancer, as evidenced by correlation of the amplification of the *n-myc* oncogene with tumorigenesis and tumor progression for human neuroblastomas (Alitalo 1984; Schwab et al. 1984; Gilbert 1985). Clearly, it is important to understand the pathways that lead to different kinds of genetic rearrangements, and in particular those responsible for gene amplification.

The complexity of many eukaryotic systems has made it difficult to study certain mutational processes, and particularly genetic rearrangements, at the DNA sequence level. Bacterial systems offer the possibility of carrying out high-resolution genetic studies together with detailed DNA sequence analyses. Mutational pathways can be understood at the sequence level in bacteria and then be compared with their counterparts in eukaryotic systems. Also, bacterial genes can be introduced into mammalian cells via shuttle vectors and rearrangements can be analyzed subsequently in bacterial hosts (Calos et al. 1983; Miller et al. 1984).

We have characterized a series of high amplifications of 8- to 37-kb regions of the *Escherichia coli* chromosome surrounding the *lac* region on an F' plasmid (Tlsty et al. 1984). Restriction enzyme analysis demonstrates that the amplified units, which are repeated in tandem 40–200 times, can occur at many different points. This can be seen in Figure 1, which shows the extent of 50

amplified units that we mapped (S.K. Whoriskey and J.H. Miller, unpubl.), extending the data of Tlsty et al. (1984). Our goal is to determine the mechanism that causes these rearrangements. A necessary first step is to determine the sequences at the junctures of the amplified units. With an understanding of the DNA sequence requirements of gene amplifications we can then pursue the enzymatic pathway(s) involved in their formation. The task involved in determining juncture sequences is outlined in Figure 2, which depicts in more detail one of the amplification events portrayed in Figure 1. At the top of the figure the region to be duplicated (and subsequently amplified) is shown, together with surrounding sequences, indicated by the letters a, b, x, and y. In the wild-type, the sequence segment a borders segment b, and much further away, the segment labeled x borders segment y. In the middle of the figure the consequences of a duplication are shown. It can be seen that this rearrangement creates a unique "juncture" at which the sequence segment x now borders the sequence segment b. This x-b juncture is unique to every duplication event. Amplification of the duplication, represented in the bottom of Figure 1, results in the amplification of the x-b fused segment.

Our first aim is to elucidate the sequence at the junctures of numerous duplication-amplification events and to determine whether there are any distinguishing characteristics of these sequences. For instance, such re-

arrangements might occur at specific sequences, at sequences with secondary structure possibilities, or at short homologies such as seen in the case of deletions (see Albertini et al. 1982, and references therein). Alternatively, these events might be generated at sequences lacking any obvious characteristics. To determine the juncture sequences, however, we would have to detect and clone juncture fragments and compare them with the wild-type sequences on either side of the duplicated segment (see Fig. 2). Since the rearrangements detected so far are spread out over a 40-kb range (Fig. 1 and Tlsty et al. 1984), which has not been characterized at the DNA sequence level, elucidating the nature of the sequences at the junctures in 20–30 examples would be a formidable task. Therefore, we have sought a method that would generate duplication-amplifications with one end in a known DNA segment. We have devised a genetic selection that allows us to focus on such a subset of rearrangement events. Here we describe the sequence analysis of amplification events in which the junctures terminate in the *lacI* gene, which permits rapid determination of the sequence surrounding the juncture points.

Results

Selection for duplication-amplifications

Figure 3 diagrams the *lac* region carried on an F'*lacproB* episome in strain JM177, which also contains a chromo-

Figure 1. Extent of the amplified DNA in independently occurring mutants. DNA was isolated from several independent, unstable revertants of a *lacI*-Z₇ fusion strain carrying the amber mutation X13, and digested with *HincII*. The restriction pattern of the amplified DNA was compared with the *HincII* pattern of plasmid pGM11 to determine the amplified region. The top line represents the *HincII* pattern for pGM11. The lines below represent the amplified regions from several mutants, with the numbers in parentheses indicating the number of independent isolates with the same mapping pattern. Dotted lines indicate end points of the amplified DNA that fall outside of the mapped region of pGM11 (see also Tlsty et al. 1984).

Figure 2. Schematic representation of the formation and amplification of a novel juncture. A tandem duplication of DNA sequences, including the lactose operon, would result in the formation of a new juncture. Sequences b and x, widely separated in the wild-type chromosome, would become contiguous. A comparison of the DNA sequence at the x-b juncture with the wild-type x and b sequences would reveal the exact nucleotides at the point of rearrangement.

somal deletion for *lacproB*. Here, the *lacI* and *lacZ* genes are fused, as previously described (Müller-Hill and Kania 1974; Brake et al. 1978), resulting in the production of a hybrid repressor- β -galactosidase protein with full β -galactosidase activity, under the control of the *lacI^Q* promoter (Calos et al. 1978 and references therein). However, the promoter for *lacI* has been inactivated by a deletion of 4 bp (*UJ177*; Calos and Miller 1980). Therefore, transcription is not initiated at the *I* promoter, and the *lac* enzymes are not produced. Revertants to *Lac*⁺ must express both the hybrid *Z* gene and the *Y* gene, and thus must be under the control of a new promoter. When

Figure 3. The *lacI*-*Z* fusion carrying promoter mutation *UJ177*. A deletion described by Müller-Hill and Kania 1974 (see also Brake et al. 1978) results in an in-frame *I*-*Z* gene fusion, under the control of the *lacI^Q* promoter (see Calos 1978). The carboxy-terminal 4 amino acid residues of repressor and the amino-terminal 23 residues of β -galactosidase are absent from the resulting functional, hybrid protein. The *UJ177* mutation (Calos and Miller 1980) is a 4-bp deletion in the -10 region of the *lacI^Q* promoter. This mutation greatly reduces expression of the *lac* operon, resulting in a *Lac*⁻ phenotype when in combination with the *I*-*Z* fusion deletion.

strain JM177 is plated on lactose minimal medium, we detect *Lac*⁺ revertants at approximately 10^{-7} in the population. Some of the revertants are deletions, as shown by Southern analysis (data not shown), which probably fuse the *I* gene to an upstream operon. However, a major class of revertants appears somewhat unstable in the absence of the *Lac*⁺ selection, generating *Lac*⁻ colonies at a visible level when plated on indicator media. Further analysis, described below, demonstrates that most of the unstable revertants contain duplications of the type depicted in Figure 4.

It can be seen from Figure 4 that the duplications create a novel juncture that can result in fusion to a "downstream" operon oriented in the same direction. Fusions of this type have been previously reported by Anderson and Roth (1978a) in *Salmonella typhimurium*. If the expression of the new fused operon were strong enough, then a single copy of the fused operon would be sufficient to allow growth on lactose. However, in all of the cases we examined, the level of gene expression of a simple duplication is not sufficient, since examination of the DNA from the *Lac*⁺ unstable revertants demonstrates that the DNA is highly amplified.

Gene amplification

Figure 5 shows the *HincII* digestion pattern for some of the *Lac*⁺ revertants. The pattern of intense bands permits us to determine: (1) that the *lac* DNA sequences are amplified in tandem; (2) the size of the amplified re-

Figure 4. Schematic representation of a duplication that introduces a new promoter upstream of the *lac* operon. A tandem duplication of a DNA segment (the underlined portion), with one end point in the *lacI* gene and the other end point downstream in an unidentified operon *X* with promoter *P*, would result in the formation of a novel junction and an *X-I* gene fusion event. This genetic rearrangement would juxtapose a promoter upstream of the *lac* operon. Amplification of the duplicated segment could uncover low-level functional promoters.

Figure 5. Restriction enzyme digestion of the amplified DNA sequences. DNA from six *Lac*⁺ unstable revertants of JM177 was prepared, digested with restriction endonuclease *HincII*, separated by electrophoresis in a 1.4% agarose gel, and stained with ethidium bromide, as described in Materials and methods. Plasmid pGM11 is a pBR322 derivative containing the *lac* region and flanking sequences (see Galas et al. 1980). This plasmid does not contain the *lacI-Z* fusion deletion, but instead contains the *lac* promoter-operator region. Samples 42-5, 53-2, 44-2, 2-10, 3-1, and E9 are *Lac*⁺, unstable, amplification revertants of JM177. Sample UJ177 is the *Lac*⁻ starting strain.

gion; (3) the *HincII* end points of the amplified DNA; and (4) the size of the unique *HincII* junction fragment.

Figure 6 displays the end points of the duplication-amplifications found in JM177 *Lac*⁺ revertants. Most of the duplication-amplification junctions end within the *lacI* gene, as expected for operon fusions of the type shown in Figure 4.

Sequences at amplification junctions

We determined the DNA sequence surrounding each junction by the following strategy:

1. Partially purified *E. coli* DNA, which included both chromosomal and episomal DNA, was prepared from each revertant carrying an amplified *lac* region.
2. DNA preparations were used as a double-stranded template for dideoxy sequencing with a series of 5'-end-labeled synthetic oligonucleotide primers in *lacI*, using the enzyme AMV reverse transcriptase.
3. The resulting DNA sequences were compared with the known wild-type *lacI* (Farabaugh 1978), *lacY* (Büchel et al. 1980), and *lacA* (Hediger et al. 1985) sequences, or with sequences of the surrounding region obtained during this study.

We employed the following strategy. A series of synthetic primers 17–20 bp long were synthesized at approximately 150-bp intervals in the *I* gene, with the 5' to 3' direction pointing toward the fusion joint. Initial restriction mapping (see Materials and methods) predicted which 300- to 400-bp segment of the *I* gene contained the fusion point, allowing use of a limited set of primers for DNA sequence analysis. Our sequencing procedure for double-stranded DNA, described in detail in Materials and methods, is a modification of a procedure by Huibregtse and Engelke (1985). We use as template partially purified, digested, episomal DNA; no cloning into plasmid DNA is required. The DNA is denatured at 95°C and immediately transferred to 50°C. Standard dideoxy sequencing using 5'-end-labeled 17-mer oligonu-

Figure 6. Extent of amplified DNA sequences from several independently isolated mutants. The *HincII* restriction pattern was determined for 45 independent amplification revertants of JM177, by comparing the amplified bands present in a restriction enzyme digestion of the mutant to the pGM11 *HincII* map. The top line is the *HincII* restriction map of plasmid pGM11. In determining the extent of the amplified DNA in the mutants, the absence of the next distal fragment indicates that the end point of the amplified unit is within that fragment. The numbers in parentheses indicate the number of isolates with the same mapping pattern.

cleotides as primers is carried out at 50°C using AMV reverse transcriptase. The reaction is carried out in the absence of any carrier DNA. The resolution afforded by this modified procedure allows unambiguous sequence determination of double-stranded DNA. Figure 7 shows a sample sequencing gel from one of the fusion junctions. The DNA preparation was of total chromosomal and episomal DNA, digested with *HincII*, from a strain with highly amplified episomal DNA segments. DNA sequencing gels of similar resolution have been obtained with multicopy plasmid DNA and even with single-copy F' DNA (data not shown).

The size of the *HincII* restriction fragment containing the fusion junction allows us to predict the approximate end point of the duplication–amplification (Figs. 5 and 6). Using the known *lacI* sequence (Farabaugh 1978) we can determine at which nucleotide the sequence reflects a fusion of part of *lacI* to a new genetic region. We initially focused on those fragments that included the *lacY* and *lacA* regions, since a significant portion of the surrounding DNA has been sequenced (Büchel et al. 1980; Hediger et al. 1985). Subsequently, other regions were included in our analysis.

Twenty-eight of the 30 junctions that we analyzed were created at repeated sequences, as evidenced by the example shown in Figure 8. Here, a broken homology of 9 bp is found between a sequence in the *lacI* gene and a sequence downstream from *lac* within the 0.4 *HincII* fragment depicted in Figure 6. The duplication of a 9-kb segment occurred at this sequence, producing the arrangement shown in Figure 8. The duplicated segment is amplified 50–100 times in the Lac⁺ revertant. Figures 9

and 10 depict all of the sequences at the duplication–amplification junctions of 30 randomly sequenced independent isolates. In some cases more extended broken homologies can be defined. We have identified nine different junction sequences. There are repeat occurrences of rearrangements at four of the nine junctions.

Molecular consequences of gene fusions

The fusions created by the duplications studied here raise several interesting points concerning gene expression. For instance, where are the active promoters in the duplication fusions that end within *lacY* and *lacA*, and what is their function in the unamplified, wild-type chromosome? Mulligan and McClure (1986) have performed a computer analysis of the occurrence of consensus promoter sites in DNA, and have shown that two sites in particular in *lacY* have significantly high scores

Figure 7. DNA sequence of the junction of an amplification mutant obtained from double-stranded, total *E. coli* DNA. Total, *E. coli* DNA, digested with *HincII* restriction endonuclease, was heat-denatured and annealed to a 5'-end-labeled oligonucleotide complementary to nucleotides 363–347 of the *lacI* gene, as described in Materials and methods. The primer was extended using AMV reverse transcriptase in a chain-terminator reaction, across the fusion junction point (see Fig. 3). In lane 0, no dideoxynucleotide was added. The DNA sequence is wild-type *lacI* up to nucleotide 249 and then shifts to the wild-type sequence of the downstream *HincII* 0.4-kb fragment.

Figure 8. Sequences involved in the formation of a duplication. A duplication, shown in *A*, occurs between short, partial homologies. A match of 9 out of 10 bp is found both in the wild-type sequence of the *lacI* gene and downstream within a *HincII* 0.4-kb fragment, designated as part of operon *X* here. A 9-kb duplication between these homologies produces the rearrangement shown in *B*. The heavy black lines indicate the duplicated region and show that we cannot determine which one of the homologies at the end points of the duplication is the one present at the fusion junction. An *X-I* gene fusion is formed as a result of the duplication.

in their analysis. As can be seen from Figure 11, a promoter in *lacY* would result in two novel hybrid proteins (see legend to Fig. 11). Also, because there are no transcription termination signals within the fused segments, each transcript could proceed into the next segment, and potentially continue on into many following segments.

This would result in large transcripts, differing in size by one amplified segment. Translation of multiple transcripts would offer the possibility of a second level of amplification of gene expression. In the extreme case, the combination of amplified transcription and translation could lead to a several thousandfold magnification

Figure 9. Nucleotide sequence of the junctions in which both end points are within the *lac* operon. The wild-type nucleotide sequence of the leftward *lacI* end point and the rightward *lacY* or *lacA* end point from several different cases are shown, aligned before recombination (see Fig. 7). The regions of homologies between the end points are boxed; the arrows show the box in which recombination occurred in the amplification revertants. The number of independent isolates with the same juncture sequence is indicated in parentheses. The regions of homology between the end points are boxed. The arrows show the box in which an in-frame recombination event occurred in the amplification revertants.

Figure 10. Nucleotide sequences of the junctions with the right-hand end point within the *HincII* 0.4 fragment. The nucleotide sequence of the left-hand *lacI* end point and the right-hand end point in the downstream *HincII* 0.4-kb fragment are aligned exactly as shown in Fig. 9.

of gene expression. In this manner, low-level promoters and inefficient translation start sites could still be utilized to provide sufficient levels of the required gene products.

Discussion

Gene amplifications have been described in a number of bacterial systems (Anderson and Roth 1978b; Edlund et al. 1979; Mekalanos 1983; Tlsty et al. 1984; Albertini and Galizzi 1985; Deretic 1986), and it is hoped that analyzing these events at the sequence level will help to elucidate the mechanism of amplification. In higher cells, amplification has been postulated to occur via discoordinate replication (see below). However, in bacteria it is possible to envision amplification as occurring in at least two distinct steps. In this model, the initial event

generates a tandem duplication, and subsequent steps amplify the tandem copies by *recA*-mediated unequal recombination. Therefore, amplification cannot occur or is sharply reduced in *recA*⁻ strains. This idea is supported by the fact that the amplification events described here are not detected in *recA* strains (see also Tlsty et al. 1984). It has not been clear whether the initial duplication event utilizes a pathway involving *recA*. Although duplications involving very large homologies (on the order of 1000 bp) are targets for unequal recombination mediated by *recA* (Anderson and Roth 1981), the *recA* requirement for duplications that do not involve large homologies appears to differ in different systems and for different types of duplications. It is important, therefore, to define the sequences at the junctures of specific duplications, and to correlate the nature of these sequences with the dependency on different host pathways.

Figure 11. Molecular basis for the phenotypic consequences of gene amplification. Amplification of a low-level promoter (*P*) in *lacY* could result in multiple transcripts (mRNA), due to initiation at each promoter site. The lack of transcription termination signals in this type of rearrangement could result in large transcripts, differing in size by one amplified unit. Translation of these transcripts would result in the formation of two trihybrid proteins. One hybrid would contain a carboxy-terminal permease protein fragment and the repressor- β -galactosidase moieties. The other hybrid would contain the repressor- β -galactosidase chimera and almost all of the permease protein, except for the carboxyl-terminal few amino acids.

Normark and co-workers (Edlund and Normark 1981) have analyzed duplications and subsequent amplifications involving the *E. coli ampC* locus. The juncture point of one duplication event was shown to involve a 12-bp sequence that was repeated on each side of the *ampC* locus, 10 kb apart. We have employed *lacI*-Z fusion strains to detect amplifications of the *lac* region on an *F'lacproB* episome in *E. coli* (Tlsty et al. 1984). [Duplications that result in gene fusions have been described in *S. typhimurium* (Anderson and Roth 1978a)]. Here we describe amplification of gene duplications that fuse the *lacI* gene to a downstream sequence (see Fig. 4). Most of the duplication juncture points end within *lacI*, the sequence of which is known (Farabaugh 1978), thus facilitating the sequence analysis of the duplication events. By using double-stranded sequencing of amplified DNA with oligonucleotide primers corresponding to *lacI* sequences (Fig. 6), we can readily locate each duplication juncture. Comparison with the wild-type sequence for regions surrounding *lacI* allows determination of the sequence at which the duplications occurred. Sequencing directly from the chromosome eliminates anomalies that could be introduced by cloning out the juncture sequences.

We have sequenced junctures from 30 independently occurring duplication–amplifications and found that 28 of them involve short repeated sequences (less than 15 bp), sometimes involving broken homologies. It should be pointed out that although 2 of 30 junctures in the *lacI* fusions displayed no short homologies, the frequency of these events per available site is far less than this percentage indicates. As calculated in the Materials and methods sections, sites with homologies of 6 bp are approximately 10,000-fold more prone to duplication–amplifications of the type described in this work. The specific rearrangements detected are representative of the largest available homologies, as picked out by a computer analysis of the homologies between the beginning of the *lacI* gene and the end of *lacY* and all of *lacA* (data not shown). In summary, large duplications obey the same rules as deletions in *E. coli* (Farabaugh 1978; Albertini et al. 1982, and references therein), occurring at short sequence repeats, the extent of the repeat influencing the frequency of the event.

The plasticity of the genome displayed by bacterial cells is remarkable. In response to selective pressure, mutants with rearranged genomes emerge easily, fusing inactive genes to distant gene segments by duplication, and subsequently amplifying the number of duplicated copies to upwards of 100, in tandem array. What pathways allow such facile rearrangements? It is of great interest to study the cellular processes involved, since these types of rearrangements mimic those seen in higher eukaryotes in a number of ways. Gene amplification is involved in the expression of resistance to chemotherapeutic drugs in mammalian cells (Schimke 1984), and the same process is seen to occur during tumor progression in numerous types of cancer (Alitalo 1984; Schwab et al. 1984; Gilbert 1985). Also, oncogene rearrangements often result in a silent gene being placed

under the control of a more active promoter (Crew et al. 1982; Kohl et al. 1983; Leder et al. 1983), exactly as seen here in the duplications involving the *E. coli lacI*-Z fusion system.

The finding that most amplification events in bacteria occur at short repeated sequences provides strong evidence for a model in which the initial event is a duplication mediated by the sequence repeat. Studies in higher cells have led to conflicting models of gene amplification in eukaryotes. For instance, Mariani and Schimke (1984) have proposed that the initial event of amplification is the rereplication of DNA segments within a single cell cycle followed by recombination events. On the other hand, Hahn et al. (1986) suggest that selective treatment may induce aberrant segregation of chromosomal segments which are subsequently amplified, or may cause unequal sister chromatid exchange resulting in gene amplification. This latter model (see also Stark and Wahl 1984) is formally analogous to the sequence of events that we envision for bacterial gene amplification. At this point it is not known if there is a sequence specificity at the junctures of amplified DNA in mammalian cells.

The finding here that large duplications that are highly amplified occur preferentially at short sequence repeats, as do deletions in bacteria, stimulates the consideration of several possible models for the spontaneous generation of these rearrangements.

1. Errors of replication might result from slippage promoted by the short homologies (see Farabaugh et al. 1978; Albertini et al. 1982, and references therein).
2. The rearrangements might represent low-level recombination mediated by one of the known recombination pathways. Many of these pathways in *E. coli* share the requirement for *recA*. For instance, *recA*, *recB*, and *recC* are involved in general recombination, *recA*, *recF*, *recJ*, *ruv*, and *recN* in recombinational repair, and *recA* and *recE* in a supplementary recombination system (see Shen and Huang 1986, and references therein). The sharply reduced frequencies of deletions in *recA* strains has been reported (Albertini et al. 1982).
3. It is possible that a special enzyme system exists that catalyzes rearrangements at short repeated sequences. This system might also utilize the *recA* function, explaining the partial dependency of deletions and certain duplications on *recA*. One such system might involve *E. coli* DNA gyrase, which has been shown to mediate recombination and deletion formation in vitro (Ikeda et al. 1982; Jones et al. 1982; Michel and Erlich 1986). Marvo and co-workers have proposed that certain recombination events in vivo are catalyzed by DNA gyrase (Marvo et al. 1983). At this stage, attempts to correlate gyrase cutting sites with the junctures reported here have proven inconclusive.

Future experiments are aimed at distinguishing between the above possibilities.

Materials and methods

Isolation of *Lac*⁺ revertants of JM177

Overnight cultures of JM177 grown in LB medium (media as described in Miller 1972) were plated on lactose minimal plates. Colonies were picked after 48 hr and purified twice on lactose minimal medium. Single colonies were streaked on lactose–MacConkey plates to determine phenotypic stability. One unstable revertant per culture was analyzed further.

Rapid isolation of DNA for restriction enzyme digestion

DNA was prepared using a modification of a procedure described in Holmes and Quigley (1981). Bacteria were scraped off minimal lactose plates and thoroughly suspended in 0.35 μ l of a solution of 8% sucrose, 0.5% Triton X-100, 50 mM EDTA (pH 8.0) and 10 mM Tris-Cl (pH 8.0). After the addition of 25 μ l of freshly prepared lysozyme (10 mg/ml in 10 mM Tris-Cl, pH 8.0), the samples were boiled for 1 min and centrifuged at room temperature in an Eppendorf centrifuge for 15 min. The supernatant was transferred to a new tube and extracted twice with buffer-saturated phenol. The DNA was precipitated with ethanol on ice and resuspended in 20 mM Tris (pH 7.5), 1 mM EDTA. Care was taken throughout the procedure not to vortex at any step. The samples were digested with restriction endonucleases according to the conditions described by the supplier (Pharmacia).

Agarose and acrylamide gels

DNA restriction fragments were separated by electrophoresis on 1.4% agarose gels (agarose from Sigma) in Tris, borate, and EDTA buffer (Davis et al. 1980) at 100 volts for 5 hr. Acrylamide gels of 8% were made as described by Sanger et al. (1977).

Isolation of total *E. coli* chromosomal/episomal DNA for DNA sequence analysis

DNA was prepared according to slight modifications of a procedure described by Silhavy et al. (1984). The cells from a 100-ml lactose minimal culture were collected by centrifugation and resuspended in 5 ml of 50 mM Tris-Cl (pH 8.0), 50 mM EDTA and frozen at -20°C . A fresh lysozyme solution (10 mg/ml in 0.25 M Tris-Cl, pH 8.0) was prepared and 0.5 ml was added to the frozen cells. The cells were thawed at room temperature and then placed on ice for 45 min. To the chilled cells were added 1 ml of STEP solution (0.5% sodium dodecyl sulfate, 50 mM Tris-Cl, pH 7.5, 0.4 M EDTA, and 1 mg/ml of proteinase K). The mixture was heated at 55°C for 60–90 min with occasional gentle mixing. The mixture was extracted twice with Tris-buffered phenol and twice with chloroform/isoamyl alcohol (24:1). The DNA was precipitated on ice with 1/10 volume of 3 M sodium acetate and 2.5 volumes of cold ethanol. The precipitate was resuspended in 5 ml of 50 mM Tris-Cl (pH 7.5), 1 mM EDTA, and 200 μ g/ml RNase A and incubated in a shaker water bath at 37°C for 2 hr. The DNA was precipitated and resuspended in 1 ml of 10 mM Tris-Cl (pH 7.5), 1 mM EDTA. In a 200- μ l volume, 150 μ l of the DNA was digested overnight with *HincII* restriction endonuclease (Pharmacia). The digestion was phenol-extracted and precipitated with 1/2 volume of 7.5 M ammonium acetate and cold ethanol. The DNA was resuspended in 70 μ l 10 mM Tris-Cl (pH 7.5), 1 mM EDTA. Because this procedure yields partially purified DNA, an OD₂₆₀ reading is not accurate in quantitating DNA yields. Ethidium bromide staining of agarose gels of digested DNA was used to determine what fraction of the DNA prep was necessary for sequence anal-

ysis. (Cells grown in rich media result in much higher DNA yields.)

Oligonucleotides

Sequencing primers of 17 nucleotides were synthesized approximately 150 bp apart throughout the *lacI* gene and used to sequence towards the 5' end of the gene, across the fusion junction.

Radioactive kinasing of the DNA sequencing primers

The synthetic 17-mer oligonucleotides were kinased in a 10- μ l volume of: 2 μ l of 60 mM MgCl₂, 1 μ l of 50 mM DTT, 1 μ l of 0.5 M Tris (pH 7.6), 30 pmoles oligonucleotide, 5 units of T4 polynucleotide kinase (Pharmacia), and 2 μ l (300 μ Ci) of crude [γ -³²P]ATP (ICN Radiochemicals). The reaction was incubated for 1 hr at 37°C and transferred to 65°C for 10 min to heat-inactivate the enzyme. The kinased oligonucleotide was purified on a 100- μ l DE-52 column (Whatman) poured into a 1-cc syringe. The kinased oligonucleotide was loaded on the column in a 1-ml volume of 50 mM triethylamine bicarbonate (TEAB) (pH 8.0). The column was washed several times with 1 ml of 0.2 M TEAB (pH 8.0), until the eluate had low radioactivity. The collected fractions were centrifuged in a Speed Vac Concentrator until all the liquid was evaporated, and the labeled oligonucleotides were resuspended in total volume of 60 μ l of water.

Sequencing digested, double-stranded, *E. coli* DNA

The DNA sequencing reaction is a modification of a protocol by Huibregtse and Engelke (1986). In an Eppendorf tube, the following were combined per sequencing template: 16 μ l of *HincII*-digested total *E. coli* chromosomal/episomal DNA (isolated and digested as described above), 5×10^5 cpm ³²P-kinased oligo, and water to 65 μ l. The mixture was placed in a 95°C water bath for 5 min and immediately transferred to a 50°C water bath. After 30 min at 50°C , 10 μ l of $10\times$ AMV buffer (500 mM Tris, pH 8.0, 60 mM MgCl₂, 400 mM KCl) was added to the template mixture. The template mixture was equally aliquoted to five tubes labeled A, T, G, C, X. (The X tube served as a control for the sequence-specific pausing of the reverse transcriptase enzyme. This tube received all four deoxynucleotides and no dideoxynucleotides in the sequencing reaction.) A 2 : 1 mix of $10\times$ dATP with $10\times$ ddATP, $10\times$ dTTP with $10\times$ ddTTP, etc., was prepared from $10\times$ frozen stocks. ($10\times$ dNTPs: 6 mM nonlimiting dNTPs, 0.6 mM limiting dNTPs. $10\times$ ddNTPs: 0.2 mM ddNTP.) To the side of tube A was added 4 μ l of a 2 : 1 mix of $10\times$ dATP/ $10\times$ ddATP, and 0.5–1.0 unit of AMV reverse transcriptase (Pharmacia, IBI, or Seikagaku America, Inc.) diluted in water. Tube T received 4 μ l of a 2 : 1 mix of $10\times$ dTTP/ $10\times$ ddTTP and 0.5–1.0 units of enzyme; tube G received 4 μ l of a 2 : 1 mix of $10\times$ dCTP/ $10\times$ ddGTP and 0.5–1.0 unit of enzyme; and tube C received 4 μ l of a 2 : 1 mix of $10\times$ dCTP/ $10\times$ ddCTP and 0.5–1.0 unit of enzyme. The tubes were spun for 1 sec in an Eppendorf centrifuge to start the reaction and returned to the 50°C water bath. After 30 min, the reaction was stopped by the addition of 4 μ l of 0.4 M EDTA. Ethanol was added to the tubes and the samples were precipitated on ice for 5 min and spun in a centrifuge in the cold for 30 min. The pellets were resuspended in 4 μ l of loading dye (Sanger 1977), heated at 95°C for 2 min, and immediately placed on ice. The samples were separated by electrophoresis on an 8% acrylamide gel. The gel was transferred to Whatman 3MM paper, covered with plastic wrap, and exposed to Kodak XR5 film with an enhancer overnight at -70°C .

Cloning and sequencing the wild-type HincII 0.4-kb fragment

A *Bgl*III–*Pst*I fragment from plasmid pGM11 (Galas et al. 1980), which contains the *Hinc*II 0.4 fragment, was electroeluted from a 0.7% agarose gel (Sigma) and subcloned into the *Bam*HI–*Pst*I site in phage M13, mp18, and mp19 (New England Biolabs). Single-stranded DNA was sequenced as described by Sanger et al. (1977).

Computations

To determine the sequence homologies between *lacI* and the rest of the *lac* operon, the sequence was run through the Los Alamos SeqH computer program (Kanehisa 1982). We could discount approximately two-thirds of the homologies the computer identified, because recombinations at those homologies would alter the reading frame and therefore not show up in our genetic selection. We can consider potential homologies in several ways, and with several different criteria. As one example, let us consider the events occurring at homologies of 6 bp (in a row) or greater. In random sequences of DNA a 6-bp repeat will occur at $1/4^6$ the frequency of any sequence with less than a 6-bp repeat, or $1/4096$. If half of the duplications occurred at 6-bp repeats and half did not (a ratio of 1 : 1), then the average mutation frequency at a 6-bp repeat site would be 4096-fold higher than the average frequency at a site with less than a 6-bp homology. In reality, 23 of 30 of the duplications sequenced here occur at 6-bp repeats (a ratio of 3.29 : 1). This would give an estimate of a 13,458-fold higher rate per site (on the average), for sites with 6-bp homologies or greater. Of course, this number is even higher for the hotspots among the 6-bp repeat sites.

We can look at the sequences for *lacY* and *lacA* and compare them with *lacI* sequences for homologies in the parts of *I* and *Y* (and all of *A*) where we have been able to detect duplication–amplifications. By computer search we have identified 21 homologies of greater than 6 bp in which the resulting fusion would be in the correct translational reading frame. Using the number of bases in the first portion of *I* (258 bp) and the regions of *Y* and *A* (738 bp), we can show that the total number of possible duplication end points is $258 \times 738 \times 1/3 = 63,468$. If we divide the number of occurrences at each type of site by the number of available sites, we get, in the *Y* and *A* regions, 7/21 for 6-bp homologies, and 2/63,468 for nonhomologies, or 0.33 and 0.0000315, respectively. Dividing the first number by the second results in 10,476, which is the factor of increase of rate of duplications occurring at homologies of 6 bp or greater. This is in close agreement with the number calculated from random sequences.

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