TN5 INSERTIONS IN RIBOSOMAL PROTEIN GENES IN

ESCHERICHIA COLI

by

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THE UNIVERSITY OF BRITISH COLUMBIA

April, 1982

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ABSTRACT

The genetic organization and interrelationship between the two ribosomal protein transcription units (the L11 and the L10 operons) from near 89 min on the *Escherichia coli* chromosome were studied using insertional mutations generated by the kanamycin resistant transposable element Tn5.

The polar effects of Tn5 insertion on the expression of these ribosomal genes were examined by:

(1) The level of β-galactosidase generated from L10-*lacZ* and β-*lacZ* fusion proteins;

(2) Direct SDS-polyacrylamide gel electrophoresis of the proteins specified by the plasmid ribosomal genes in UV-inactivated maxicells;

(3) Urea-polyacrylamide gel electrophoresis of plasmid and chromosome specified L12 proteins in an L12 electrophoretic mutant (Strain JF1039).

The above experiments localized the major promoter for the L10 operon between nucleotide 1280 and 1360. These investigations also confirmed that the translational regulatory proteins for the L11 and L10 operons are L1 and L10 respectively.
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I also wish to thank Dr. K. L. Maltman for his encouragement and invaluable discussions, and Miss E. Dunstan-Adams for her technical assistance.
The *Escherichia coli* ribosome has a highly specialized and complex structure; it consists of three species of RNAs (16S, 23S, and 5S) and over fifty distinct species of proteins. Each ribosomal component is present in one copy per ribosome with the exception of ribosomal protein L7/L12 (hereafter referred to as L12), which is present in four copies per ribosome (38). Early studies indicated that in exponentially growing cells, the synthesis of ribosomal components is balanced and that the pools of free ribosomal RNA and free ribosomal proteins are negligibly small (9, 12). Changes in nutritional conditions including shift ups, shift downs, and amino acid insufficiencies, have no effect on the coordinate synthesis of ribosomal components (28, 29). Enriched nutrient conditions result in enhanced synthesis whereas deficient nutrient conditions result in reduced synthesis of ribosomal components.

To understand the molecular mechanism of control and regulation of ribosome biosynthesis, a detailed knowledge of the genetic organization of the ribosomal genes is required. Genetic mapping using λ-transducing phages and antibiotic resistance mutants have localized a large number of ribosomal genes on the *E. coli* chromosome (Fig. 1; Ref. 13, 14, 20).
Figure 1: Genetic Map of *E. coli* K12 Chromosome Showing Locations of Genes for Ribosomal RNAs, Ribosomal Proteins, RNA polymerase Subunits, and Peptide Elongation Factors (21).

Where it is known, the direction of transcription is indicated by arrows. Ribosomal RNA operons are indicated by rrn"X"; the gene structure of each of the transcription units is 16S gene, spacer transfer RNA, 23S gene, and 5S gene. Genes coding for protein are indicated by the names of the products. Transcription units in the two major ribosomal gene clusters, str and rif, are identified by the operon "names".
I. Genetic Organization and Regulation of the L11 and L10 Transcription Units:

A. Organization of the Ribosomal Genes:

The region near 89 min on the *E. coli* chromosome contains a gene cluster which specifies four 50S ribosomal proteins and the two large subunits of RNA polymerase (Fig. 2). Early experiments utilizing recombinant λ phage or plasmid for infection of UV-irradiated cells and *in vitro* DNA dependent protein synthesis indicated that these six genes were arranged into two transcription units with the following organization: P_rplK (L11), rplA (L1); P_rplJ (L10), rplL (L12), rpoB (β), rpoC (β') (20, 22, 23, 27). The two promoters and the mRNA start sites were established (Fig. 2) from DNA sequence analysis, *in vitro* transcription mapping, and RNA polymerase binding assays using short restriction enzyme fragments from this region (30, 39). In addition to the two major promoters, several genetic experiments have suggested that this region may contain at least two additional minor promoters, in the intergenic region between L10-L12 and L12-β, which could play an important role in gene regulation (2, 11, 15).
Figure 2: Organization of Ribosomal Genes in the rif Region
Near 89 min of the E. coli Chromosome (21).

The genes are identified by the names of their products and are indicated by boxes whose lengths are approximately proportional to the expected length of each gene based on molecular weights of the corresponding protein products. The two transcription units in this region are indicated in the lower part of the figure by horizontal arrows. Their promoters are designated by "P".
In vitro transcription experiments have identified a transcription start site for the major L10 promoter about 45 nucleotides beyond the end of the L1 gene (30). The presence of this promoter was demonstrated only under conditions in which the L11 promoter was not functioning. Examination of the nucleotide sequence at the end of the L1 gene fails to reveal any terminator-like sequence for the L11-L1 mRNA transcript (15). Recently, it has been suggested that expression of the entire gene cluster may under certain conditions take place utilizing the promoter for the L11 operon with no termination or reinitiation in the region between L1 and L10 genes (30).
B. Transcriptional and Post-Transcriptional Controls:

During balanced growth, the stoichiometry of the proteins produced from the L11 and L10 operons is 1:1:1:4:0.2:0.2, for L11, L1, L10, L12, β and β' respectively. The mechanism responsible for the elevated production of L12 is unknown whereas the reduced synthesis of the β and β' RNA polymerase subunits has been attributed to the presence of a transcription attenuation site and a RNase III processing site located between the L12 and the β gene (Fig. 11, Ref. 2, 3). In exponentially growing cells, approximately 80% of the mRNA transcripts are terminated at the attenuator and the un-attenuated full length transcripts are cleaved by RNase III. This implies that the translation of RNA polymerase mRNA is separated from the translation of ribosomal protein mRNA.

Early experiments utilizing composite ColEI plasmids to amplify the copy number of the L10 transcription unit (10) suggested that the synthesis of L10, L12, β and β' proteins is subjected to post-transcriptional regulation. Amplification of the L10 transcription unit resulted in six fold elevation in L10 operon transcription but only a 30% increase in L12 synthesis rate and a two-fold increase in the β and β' synthesis rate. The non-coordinate effects on L12, compared to β and β' suggest that the ribosomal proteins and the RNA polymerase subunit proteins are regulated by separate post-
transcriptional mechanisms.

Experiments with other ribosomal protein operons have confirmed this original observation and lead to the general hypothesis that certain key ribosomal proteins regulate translation of their own operon mRNAs (41). It has been suggested that these regulatory ribosomal proteins can bind to homologous sequences on ribosomal RNA and their own mRNA. When the protein is present in excess over ribosomal RNA, it binds to the homologous sequences on its mRNA and prevents further translation. Inhibition is relieved when ribosomal RNA again becomes available.

Using both in vitro and in vivo experiments, Nomura et al. (8, 41) have demonstrated that L1 is the regulatory inhibitor of the L11 operon mRNA. The target site for the L1 translational repressor action was inferred to be within the first 160 bases of the bicistronic mRNA (42), and the presence of 23S ribosomal RNA, but not 16S ribosomal RNA, relieved translational inhibition by L11. It seems likely that L1 recognizes structural features on its mRNA target that are homologous to the L1-binding site on the 23S ribosomal RNA. The translation of the mRNA is thus coupled to the availability of 23S ribosomal RNA which acts as a continuous drain on the accumulating L1 regulatory protein. A "sequential translation" mechanism, in which the synthesis of L1 requires translation of the preceding
L11 cistron, has been suggested as a means to guarantee coordinate regulation of both proteins from a single mRNA target site.

Similarly, several independent studies (17, 43) have demonstrated that the ribosomal protein L10 (or the L10-L12 complex) regulates the L10 operon. It acts by binding to the unique region in the noncoding leader sequences of the mRNA, and thereby inhibits translation. This is the only ribosomal protein operon where it has so far been possible to demonstrate, by nitrocellulose binding assays, the physical association between the regulatory protein and its own mRNA. This interaction is reduced or abolished by deletions which remove all or part of the leader sequence of the mRNA or by the presence of 23S ribosomal RNAs. The binding site on the mRNA shows extended sequence homology to the 23S ribosomal RNA.

Mutations which reduce the translational efficiency of the L10 operon mRNA in vivo have also been isolated (11). These mutations apparently alter the secondary structure of the leader sequence of the mRNA and are located near or within the binding sequences for the L10 regulatory protein. Again it appears likely that there is translational coupling between the L10 and L12 cistrons but there is no apparent feature to explain how the extra translations of the L12 sequence occurs (32).
II. The Transposable Element Tn5:

A. Structure and Genetic Organization of Tn5:

Transposon Tn5 is a discrete movable DNA element approximately 5700 bp in length, and contains a gene encoding resistance to kanamycin (1, 34). It is a natural component on an R-factor plasmid isolated originally from Klebsiella and has no homology with sequences normally present in the chromosome of E. coli K12 (4, 5). The structure and genetic organization of Tn5 is shown in Figure 3 and 4 respectively. It has two 1.5 kb terminal inverted repeat sequences that flank a central unique region of about 2.3 kb. Restriction endonuclease and DNA sequence analyses have shown that the left and right inverted repeats of Tn5 are identical except for a single-base-pair difference. The left inverted repeat contains a "stop" codon (TAA), whereas the right contains a "glutamine" codon (GAA). Analysis of insertion and substitution derivatives of Tn5 have shown that Tn5 codes for the production of five different polypeptides. The inverted repeats of Tn5 are functionally different (33). The right inverted repeat codes for polypeptides which have trans-acting functions necessary for the transposition of the element to a new site (transposases); the left inverted repeat codes for two polypeptides of unknown functions. These polypeptides are similar to those from the right inverted repeat but
Figure 3: Restriction Map of Tn5 (34).

The inverted repeats which have identical restriction enzyme sites are indicated by thick solid lines. The boxed regions were DNA fragments that were found to form heparin-resistant complexes with RNA polymerase.
Figure 4: The Genetic Organization of Tn5 (34).

The locations of the inverted repeats and the promoter for the kanamycin resistant gene are shown. There are five proteins encoded by Tn5. Polypeptide 1 and 2 are different from that of 3 and 4 due to the single-base-pair nonhomology. One or both of the proteins encoded by the right inverted repeat (1 and 2) are known to be required for the transposition process. Polypeptide 5 is coded for by the Neomycin gene (NPTII) which confers kanamycin resistance.
are smaller in sizes due to the presence of the stop codon generated from the single-base-pair difference. The fifth polypeptide, responsible for the expression of kanamycin resistance, is coded for by the central unique region of Tn5. Its promoter is generated by the GAA to TAA mutation in the left inverted repeat (see above) which generates the Pribnow box sequence TAAGGTT (31) for transcription of the kanamycin resistance gene.

B. Properties of Tn5 Insertions:

Transposon Tn5 can insert into many sites on a replicon. Transposition is promoted by transposon encoded polypeptides and is entirely recA independent. The transposition appears to proceed by insertion of the Tn5 sequence into a staggered cleavage of a 9 bp target sequence. The final result of the transposition is a direct duplication of the 9 base pair target sequence at the ends of the inserted Tn5 sequence. The cointegrate model for transposition proposed by Shapiro is shown in Figure 5 (36). Insertion of Tn5 in either of the two possible orientation is functionally equivalent.
Figure 5: Cointegrate Model for Transposition (36).

I. Various DNA regions may be brought into close physical proximity for the subsequent cleavage and ligation events. (Solid lines indicate donor DNA; dashed lines indicate target DNA; the small boxes indicated the oligonucleotide target sequence. The letters a, b, c, and d in the duplex arms flanking the transposable elements and target oligonucleotide serve to indicate the genetic structure of the various duplex products.)

II. Four single-stranded cleavages. The donor molecule is cleaved on either strand at the extremity of the transposable element. The target molecule is cleaved at sites chosen with greater or lesser specificity (depending on the element) to yield 5- or 9-bp cohesive ends. Following cleavages, the donor and target strands are ligated to generate a x-shaped structure held together by the transposable element. (The arrowheads indicate 3'-hydroxyl ends of DNA chains and the dots indicate 5'-phosphate ends.)

III. Filling in of the unpaired oligonucleotide sequences at the ends and semiconservative replication of the transposable element. The result of this replication will be two recombinant duplexes each containing a semiconserved transposable element adjacent to the target 5- or 9-bp sequence. If the donor and target molecules are both intact replicons, a fused replicon is formed. (The heavy bars are parental DNA of the transposable element, and open bars are newly synthesized DNA.)

IV. Site-specific reciprocal recombination takes place between the two transposable elements to generate both the original donor molecule and the target molecule containing an inserted element flanked by a 5- or 9-bp repeat.
Transposons such as Tn5 are very useful tools for genetic engineering in vivo because of the following properties (18, 26):

1. The drug-resistance gene permits a positive selectable marker for inheritance of the insertion and any mutation caused by it.

2. The integration into a gene of an operon, not only abolishes the function of the gene, but also leads to a reduction in the activities of all other genes downstream in the direction of transcription (polarity effects).

3. Transposons can generate deletions near the insertion site.

4. Insertion mutations can be obtained at high frequency after a low level of "mutagenesis" by exposure to a translocatable element.
III. The Present Investigation:

In this study the organization and interrelationships between the ribosomal protein and RNA polymerase genes in the \textit{rif} cluster were examined \textit{in vivo} by isolating and characterizing Tn5 insertions into plasmids carrying these ribosomal protein operons. The polar effects of Tn5 insertions were used to confirm the general organization of the genes into two operons and the site of the L10 operon promoter was localized. The polar effects and physiological changes caused by gene inactivation following insertion were studied by examining:

(1) \textit{\beta}-galactosidase activity in fusions of the \textit{lacZ} gene to the L10 ribosomal protein and \textit{\beta} RNA polymerase genes;

(2) The synthesis of plasmid specified proteins in UV-irradiated maxicells (35);

(3) The synthesis of plasmid specified L12 proteins in a mutant strain producing an electrophoretically altered L12 protein from the chromosomal gene.
MATERIALS AND METHODS

I. Abbreviations and Chemicals:

A. Abbreviations:

- Bis-acrylamide: N,N'-methylene-bisacrylamide
- Bis-tris: \([\text{bis}(2\text{-hydroxyethyl})\text{imino-tris} (\text{hydroxymethyl}) \text{methane}]\)
- DNA: deoxyribonucleic acid
- EDTA: disodium ethylene diamine tetraacetate
- MES: \([2(\text{N-morpholine})\text{ethanesulfonic acid}]\)
- mRNA: messenger ribonucleic acid
- ONPG: o-nitrophenyl-\(\beta\)-D-galactoside
- SDS: sodium dodecyl sulfate
- TEMED: N,N,N',N'-tetramethylethylenediamine
- TRIS: tris(hydroxymethyl)aminomethane
- XGAL: 5-bromo-4-chloro-3-indoly1-\(\beta\)-D-galactoside
B. **Chemicals and Radioisotopes:**

Acrylamide, Bis-acrylamide, TEMED were obtained from Eastman Corp.

Agarose, Bis-tris, (NH₄)₂SO₄, MES, ONPG, kanamycin, tetracycline were obtained from Sigma.

XGAL was obtained from Bachem.

Radioactive isotopes: L-\(^{35}\)S-methionine, α-\(^{32}\)P-dCTP were obtained from New England Nuclear.
II. Media and Solutions:

A. Media:

**M9 minimal medium:** per liter

- NH₄Cl 1.0 g
- Na₂HPO₄ 7.0 g
- KH₂PO₄ 3.0 g
- NaCl 0.5 g

After autoclaving, add 0.1 ml and 0.01 ml of 1M MgSO₄ and 1M CaCl₂ respectively.

**NY broth/agar:** per liter

- N-Z-amine 10 g
- Yeast extract 5 g
- NaCl 5 g
- 1M NaOH 2 ml

NY agar: 15 g agar per liter of NY.

B. Buffers and Solutions:

1. Plasmid DNA preparations:

**Solution I:** per 100 ml final conc.

- EDTA 0.37 g 10 mM
- Glucose 0.9 g 50 mM
- Tris 0.3 g 25 mM

Prepare fresh with 2 mg/ml lysozyme and store at 0°C.
**Solution II:** per 100 ml final conc.

- NaOH: 0.8 g 0.2 M
- SDS: 1.0 g 1 %

**Solution III:** per 100 ml final conc.

- NaOAc: 40.8 g 3 M
Dissolve NaOAc in minimal volume of water and titrate to pH 4.8 with glacial acetic acid. Then bring up to 100 ml with water.

**Tris-EDTA buffer:** per 100 ml final conc.

- 1M Tris pH 7.5: 1 ml 10 mM
- 0.5M EDTA pH 8.0: 0.2 ml 1 mM

2. Competent cell preparations:

**Tris-Mg buffer:** per 100 ml final conc.

- 1M Tris pH 7.5: 0.5 ml 5 mM
- 1M MgCl₂: 0.5 ml 5 mM

**Tris-Mg-Ca buffer:** per 100 ml final conc.

- 1M Tris pH 7.5: 0.5 ml 5 mM
- 1M MgCl₂: 0.5 ml 5 mM
- 1M CaCl₂: 10 ml 100 mM
3. λDNA preparations:

<table>
<thead>
<tr>
<th>B buffer</th>
<th>per 100 ml</th>
<th>final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M Tris pH 7.5</td>
<td>1.0 ml</td>
<td>10 mM</td>
</tr>
<tr>
<td>1M MgSO₄</td>
<td>0.5 ml</td>
<td>5 mM</td>
</tr>
<tr>
<td>5M NaCl</td>
<td>1.0 ml</td>
<td>50 mM</td>
</tr>
</tbody>
</table>

4. β-galactosidase assays:

<table>
<thead>
<tr>
<th>Z buffer</th>
<th>per 100 ml</th>
<th>final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂HPO₄</td>
<td>0.85 g</td>
<td></td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>0.55 g</td>
<td>1 M</td>
</tr>
<tr>
<td>KCl</td>
<td>0.075 g</td>
<td>10 mM</td>
</tr>
<tr>
<td>After autoclaving, add:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1M MgCl₂</td>
<td>0.1 ml</td>
<td>1 mM</td>
</tr>
<tr>
<td>100 mg/ml CM</td>
<td>0.2 ml</td>
<td>200 µg/ml</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>0.27 ml</td>
<td>38 mM</td>
</tr>
</tbody>
</table>

5. Agarose gel electrophoresis:

<table>
<thead>
<tr>
<th>10X AGB buffer</th>
<th>per liter</th>
<th>final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOAc</td>
<td>27.2 g</td>
<td>0.2 M</td>
</tr>
<tr>
<td>Tris</td>
<td>48.4 g</td>
<td>0.4 M</td>
</tr>
<tr>
<td>EDTA</td>
<td>3.7 g</td>
<td>10 mM</td>
</tr>
</tbody>
</table>

Adjust pH to 8.0 with about 12 ml of glacial acetic acid.
### 0.7% agarose gel:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
<td>1.05 g</td>
<td>0.7 %</td>
</tr>
<tr>
<td>10X AGB</td>
<td>15 ml</td>
<td>1 X</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>135 ml</td>
<td>-</td>
</tr>
</tbody>
</table>

Heat to dissolve agarose. Cool to about 60°C and add 4 μl of 10 mg/ml ethidium bromide.

### 6. SDS-polyacrylamide gel electrophoresis:

#### Cracking buffer:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25M Tris pH 6.8</td>
<td>5 ml</td>
<td>0.0625 M</td>
</tr>
<tr>
<td>10% SDS</td>
<td>20 ml</td>
<td>2 %</td>
</tr>
<tr>
<td>Glycerol</td>
<td>10 ml</td>
<td>10 %</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>5 ml</td>
<td>5 %</td>
</tr>
<tr>
<td>0.1% bromophenol blue</td>
<td>1 ml</td>
<td>0.001 %</td>
</tr>
</tbody>
</table>

### 12.5% SDS-polyacrylamide slab gels:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5M Tris pH 8.8</td>
<td>10 ml</td>
<td>0.375 M</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.4 ml</td>
<td>0.1 %</td>
</tr>
<tr>
<td>30% acrylamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8% bis-acrylamide</td>
<td>16.6 ml</td>
<td>12.5 %</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>6.4 ml</td>
<td>-</td>
</tr>
<tr>
<td>-- degas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1% TEMED</td>
<td>1.0 ml</td>
<td>0.1 %</td>
</tr>
<tr>
<td>1% (NH$_4$)$_2$S$_2$O$_8$</td>
<td>2.8 ml</td>
<td>0.07 %</td>
</tr>
</tbody>
</table>
3% SDS-polyacrylamide stacking gels:

<table>
<thead>
<tr>
<th></th>
<th>per 10 ml</th>
<th>final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25M Tris pH 6.8</td>
<td>1.0 ml</td>
<td>0.125 M</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.1 ml</td>
<td>0.1 %</td>
</tr>
<tr>
<td>30% acrylamide :</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8% bis-acrylamide</td>
<td>1.0 ml</td>
<td>3.0 %</td>
</tr>
<tr>
<td>H₂O</td>
<td>6.2 ml</td>
<td></td>
</tr>
<tr>
<td>-- degas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1% TEMED</td>
<td>1.0 ml</td>
<td>0.1 %</td>
</tr>
<tr>
<td>1% (NH₄)₂SO₄</td>
<td>0.7 ml</td>
<td>0.07 %</td>
</tr>
</tbody>
</table>

10X running buffer:

<table>
<thead>
<tr>
<th></th>
<th>per liter</th>
<th>final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>30.9 g</td>
<td>0.25 M</td>
</tr>
<tr>
<td>Glycine</td>
<td>144 g</td>
<td>1.92 M</td>
</tr>
<tr>
<td>SDS</td>
<td>10 g</td>
<td>1 %</td>
</tr>
</tbody>
</table>

The pH should be at 8.3 without adjusting.

7. Urea-polyacrylamide gel electrophoresis:

10X upper buffer:

<table>
<thead>
<tr>
<th></th>
<th>per 500 ml</th>
<th>final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bis-tris</td>
<td>20.92 g</td>
<td>20 mM</td>
</tr>
</tbody>
</table>

Adjust pH to 7.0 with about 44 ml of 8 g of MES in 50 ml of water.
### 10X lower buffer:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bis-tris</td>
<td>30.34 g</td>
<td>30 mM</td>
</tr>
</tbody>
</table>

Adjust pH to 6.0 with about 7 ml of glacial acetic acid.

### 2X gel buffer:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>9M urea</td>
<td>50 ml</td>
<td>9 M</td>
</tr>
<tr>
<td>Bis-tris</td>
<td>1.19 g</td>
<td>50 mM</td>
</tr>
</tbody>
</table>

Adjust pH to 5.0 with about 0.95 ml of glacial acetic acid.

### 4% urea-polyacrylamide gels:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>9M urea</td>
<td>18.25 ml</td>
<td>3.3 %</td>
</tr>
<tr>
<td>30% acrylamide :</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8% bis-acrylamide</td>
<td>6.67 ml</td>
<td>4.0 %</td>
</tr>
<tr>
<td>2X gel buffer</td>
<td>25 ml</td>
<td>1 X</td>
</tr>
<tr>
<td>TEMED</td>
<td>50 μl</td>
<td>0.01 %</td>
</tr>
<tr>
<td>10% (NH₄)₂S₄O₆</td>
<td>250 μl</td>
<td>0.05 %</td>
</tr>
</tbody>
</table>
III. Methods:

A. Bacterial Strains and Growth Conditions:

The characteristics of the bacterial strains used are listed in Table I.

The bacterial strain MC1000 was used as a general recipient for plasmids in the course of the investigation. Bacteria were grown in NY broth or agar containing, when appropriate, tetracycline (20 μg/ml) and kanamycin (20 μg/ml). Lysogens of recombinant phage were grown in M9 minimal medium supplemented with 0.2% glucose, 0.8% casamino acids, thiamine (0.5 μg/ml), and the required antibiotics.

The synthesis of plasmid specified proteins was investigated in E. coli CSR603 (maxicells), carrying recA and uvrA mutations. These cells are defective in photoreactivation and hence unable to repair UV-induced damaged DNA (35). Bacterial cells were grown in M9 minimal medium supplied with amino acids threonine, leucine, proline, histidine and arginine (50 μg/ml).

The synthesis of plasmid specified L12 protein was examined using an L12 electrophoretic mutant, strain JF1039, in M9 minimal medium containing the required amino acids valine, isoleucine and arginine. This strain produces functionally active L12 proteins which have different electrophoretic properties from the wild type L12 protein on urea polyacrylamide gels.
Table I: Genotypes of Bacterial Strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genetic Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC1000</td>
<td>araD139, (araBCOIC leu)7697, (lacIOPZY)X74, galU, galK, strA, thi</td>
</tr>
<tr>
<td>MC4100</td>
<td>araD139, lacU169, strA, thi, mot, recA</td>
</tr>
<tr>
<td>JF1039</td>
<td>ilvD, argH, rplL</td>
</tr>
<tr>
<td>CSR603</td>
<td>thr, leu, thi, pro, arg, phr-1, recA1</td>
</tr>
<tr>
<td>(\lambda ex::Tn5)</td>
<td>(\lambda CI857 rex::Tn5), O29(am), P80(am), b221</td>
</tr>
</tbody>
</table>
B. Isolation and Characterization of Tn5 Transpositions:

1. Transposition of Tn5 from λCI857 rex::Tn5 onto Plasmids pNF1344, pNF1571 and pNF1962:

Recipient MC1000 carrying plasmids of interest were inoculated into 2 ml of NY broth in the presence of 0.01M MgCl₂ and tetracycline at 37°C. After 16 hours of growth, 0.5 ml of λrex::Tn5 phage stock was added and infection was allowed to proceed at 20°C for 30 min. The λrex::Tn5 phage is a cryptic bacteriophage which is unable to replicate or lysogenize the host bacterium. The cells were harvested, resuspended in 20 ml of the above fresh medium without MgCl₂ and Tn5 transposition events were permitted by growing the infected cells for 3 hours at 37°C. Cells were concentrated by centrifugation, plated onto selective NY plates containing kanamycin and tetracycline, and incubated at 37°C, to obtain kanamycin and tetracycline resistant bacteria.
2. Isolation of Plasmids Containing Tn5 Insertions:

The Tn5 infected, kanamycin and tetracycline resistant bacteria obtained have Tn5 elements transposed or hopped from the DNA to either the plasmid DNA or the chromosomal DNA. Plasmids which confer both kanamycin and tetracycline resistance were isolated and used to transform a kanamycin and tetracycline sensitive bacterium to obtain kanamycin and tetracycline resistant clones.

a. Small scale plasmid DNA preparations:

Plasmid screening and small scale plasmid preparations were carried out following the Birnboim rapid procedure (7). Cultures were inoculated into 2 ml of NY broth with 0.2% glucose and the appropriate antibiotics, and allowed to grow overnight at 37°C. Cells were harvested and resuspended into 100 μl of lysozyme solution (Solution I) for 30 min. at 0°C. The cells were lysed and the DNA was denatured by the addition of 200 μl of NaOH-SDS, Solution II, (5 min., 0°C). Chromosomal DNA was precipitated with 150 μl of acetic acid, Solution III (60 min., 0°C). The total renatured plasmid DNA was separated from the bulk of cellular debris and chromosomal DNA by centrifugation and was precipitated by two volumes of ethanol (30 min., -80°C). The DNA pellet was
resuspended into 400 μl of 0.1M NaOAc, 0.05M Tris pH 8.0 and precipitated again with ethanol (10 min., -20°C). The final purified DNA pellet was resuspended into 40 μl of Tris-EDTA buffer.

b. Preparation of competent cells and transformation of competent cells with plasmid DNA:

Recipient cells were inoculated into 50 ml of NY glucose broth and allowed to grow to a density of 0.5-0.6 A_{600}. The cells were harvested by centrifugation at 10K for 3 min. (4°C). The pelleted cells were first treated with 30 ml of Tris-Mg buffer (15 min., 0°C), then harvested and treated with 30 ml of Tris-Mg-Ca buffer (20 min., 0°C). The final competent cells were harvested and resuspended in 1 ml of Tris-Mg-Ca buffer and kept on ice.

Transformation was allowed by mixing 100 μl of competent cells to 1 μg (10 μl) of plasmid DNA from Birnboim's method. The cells were kept on ice for 1 hour and then spreaded onto nitrocellulose filter on non-selective NY plates at 37°C. After 2 hours of incubation, the nitrocellulose filter was transferred onto selective NY plates with the appropriate antibiotics.
c. Characterization of Tn5 Transpositions:

Purified plasmids from individual kanamycin and tetracycline resistant clones were examined for the position of Tn5 insertion by restriction enzyme mapping. The restriction maps and the construction of the parental plasmids being studied are shown in Figure 6. The approximate positions of insertion were initially localized with restriction enzyme EcoRI, and further with BglII and HindIII. The precise sites of some of the insertions were determined by DNA sequence analysis, using the Maxam and Gilbert procedure (24).
Figure 6: Construction of Plasmids pNF1344, pNF1571, and pNF1962.

The composite plasmids employed were pBR322 derivatives pNF1344, and pNF1571, and the pACYC184 derivative pNF1962. Plasmid pNF1344 contains a 6.0 kb fragment from \( \lambda \) rifd18 inserted into the PstI site of the vector whereas pNF1571 contains a 3037 bp long fragment, PstI (nucleotide 487)-EcoRI (nucleotide 3524), inserted between the PstI and EcoRI sites of the vector DNA. Plasmid pNF1962 contains a 14.2 kb fragment inserted into the EcoRI site of the vector plasmid. The genes illustrated as solid areas on the respective plasmids are L11 (\( rplK \)), L1 (\( rplA \)), L10 (\( rplJ \)), L12 (\( rplL \)), \( \beta \) (\( rpoB \)), \( \beta' \) (\( rpoC \)), and Tc (tetracycline resistance). The genes illustrated as hatched areas are Z (\( lacZ \)), Y (\( lacY \)) and A (\( lacA \)). All these genes are expressed in a right to left direction, with the exception of the plasmid tetracycline gene, and are believed to be expressed utilizing the two promoters \( P(L11) \) or \( P(L10) \) (20). The mRNA start sites for these promoters are nucleotides 79 and 1348 respectively. Plasmids pNF1571 and pNF1962 commence respectively at nucleotides 487 (PstI site) and 280 (EcoRI site) and consequently do not contain the \( L11 \) promoter and therefore do not express the fragment of the \( L11 \) or the intact \( L1 \) genes. The presence of a (') to the right or the left of a gene designates respectively the absence of the N-terminal or the C-terminal coding portion of the gene. The presence of a (-) between two genes represents a gene fusion and results in production of a hybrid polypeptide. The nucleotide boundaries for designated genes are: L11 (172-597); L1 (604-1305); L10 (1721-2215); L12 (2285-2647); \( \beta \) (2969-). In the RNA polymerase \( \beta-lacZ \) fusion the BamHI fragment from pMC901 was inserted into the Sau3A site of \( \beta \) at nucleotide 3092. This fusion is in frame and produces a hybrid protein containing the N-terminal 42 amino acids of \( \beta \) fused to the eighth amino acid of \( \beta \)-galactosidase. The restriction sites are illustrated.
C. Plasmid-Phage Recombination and the Expression of lacZ Fusion Products:

The effect of Tn5 insertion on the expression of ribosomal protein L10 and RNA polymerase β subunit was examined utilizing λ1910 and λ1962 respectively. The construction of these two transducing phages is described in Figure 7. The Tn5 insertions on plasmids were moved to homologous position on the bacteriophages by in vivo recombinations (Fig. 8) and the expression of L10-lacZ and β-lacZ fusion proteins were determined by measuring the level of β-galactosidase activity.

1. Plasmid-phage recombination and transduction:

Strains carrying kanamycin and tetracycline resistant plasmids were inoculated into 10 ml of M9 minimal medium supplemented with 0.2% maltose, 0.8% casamino acids, thiamine (0.5 μg/ml) and antibiotics. When cell density was about 1.0 A₆₀₀, the cells were harvested, washed with 10 ml of M9 minimal medium in presence of 0.01M MgCl₂. The final cell pellet was resuspended into 1 ml of fresh M9-Mg minimal medium and UV-irradiated in the dark for 30 seconds at a distance of 40 cm. The UV-irradiated cells (0.5 ml) were infected in the dark with 5 μl of λ1910 or λ1962 in 4.5 ml NY glucose broth with 0.01M MgCl₂, tetracycline and kanamycin at 37°C. After three hours of infection, the cells were concentrated by
centrifugation and resuspended into 0.5 ml of B buffer. Cells were lysed with 25 μl of CHCl₃ and 1 μl of DNase at 37°C for 15 min. Cell debris were removed by centrifugation and the supernatant which contains the phage particles was stored over CHCl₃ at 4°C.

The phage isolated were used to transduce strain MC4100, which were grown to early saturation in M9 minimal medium supplemented with maltose, casamino acids and thiamine. To 100 μl of the phage solution, 150 μl of the recipient cells was added and infection was allowed to proceed at 20°C for 30 min. The cells were grown in 0.5 ml of NY glucose broth for 3 hours at 30°C, and then plated onto selective NY plates containing XGAL and kanamycin at 30°C.
The right junction of the bacterial DNA with $\lambda$ DNA occurs at the EcoRI site in L11 (nucleotide 280). The RNA polymerase $\beta$-lacZ fusion on $\lambda$1962 is identical with that on plasmid pNF1962 (see Fig. 6). The L10-lacZ fusion on $\lambda$1910 fuses the BamHI site in lacZ (preceding codon 8) to a PstI site (nucleotide 1791) at codon 25 in the L10 gene. The fusion was achieved and the proper reading frame established using a 28 nucleotide long PstI-BglII linker fragment derived from the L1 gene (nucleotide 869-897). Bacterial strains lysogenic for these phages exhibit $\beta$-galactosidase activity. The $\lambda$ (right arm) is derived from a derivative of $\lambda$gt(C) where the EcoRI restriction sites in the right arm have been removed by mutation. The $\lambda$ (left arm) is derived from $\lambda$MC209. This arm contains 'lacAlacYlacZ' and the right end of this fragment is the EcoRI site located late in the lacZ gene. The intermediate phage in these construction was $\lambda$NF1955 which contains $\lambda$ (left arm) from $\lambda$gt(C) fused to $\lambda$ (right arm) from $\lambda$MC209 through the single EcoRI site. The recombinant phage $\lambda$1910 and $\lambda$1962 were constructed by insertion of EcoRI fragments containing the indicated L10-lacZ or $\beta$-lacZ fusions into the single EcoRI site of NF1955 and selecting for lysogens with $\beta$-galactosidase activity.
Figure 8: Recombinational Transfer of Tn5 from Plasmids to Homologous Positions in the Transducing Phage.

The Tn5 insertions into the ribosomal protein region on plasmids pNF1344, pNF1571, and pNF1962 were transferred to homologous positions on λ1910 and λ1962 during lytic phage infection. Selection was made for progeny phage which transduced kanamycin resistance, and the position of the Tn5 transferred was confirmed by restriction enzyme mapping. An example of recombinational transfer of Tn5 (indicated by open triangle) from the leader region of the L10 gene on plasmid pNF1344 to λ1962 is illustrated.
BY BACTERIAL RECOMBINATION
2. Preparation and characterization of λDNA:

Lysogens were grown in 50 ml of NY glucose broth with 0.01M MgCl\textsubscript{2} and kanamycin at 30°C. When cell density was about 1.0 A\textsubscript{600}, the temperature was shifted up to 42°C for 15 min., and then to 37°C for 2 hours. Cells were harvested, resuspended in 0.5 ml B buffer, and lysed with 50 μl CHCl\textsubscript{3}, and 1 μl DNase (15 min., 37°C). Phage particles were separated from cell debris by centrifugation and stored over CHCl\textsubscript{3} at 4°C.

To 0.5 ml of the phage solution, 200 μl of Tris-EDTA saturated phenol and 50 μl of 0.5M EDTA were added to disrupt the phage particles (10 min., 20°C). The aqueous layer containing phage DNA was extracted once with phenol:CHCl\textsubscript{3} (100 μl:100 μl), and twice with 200 μl CHCl\textsubscript{3}. The phage DNA was precipitated with two volumes of ethanol in the presence of 0.3M NaCl and resuspended into 50 μl Tris-EDTA buffer.

The phage DNA prepared from single lysogens was analyzed by restriction enzyme digestions, using EcoRI, BglII and HindIII. The position of Tn5 insertion on the λDNA was compared to that of the respective parental plasmids.
3. \(\beta\)-galactosidase measurements:

Strains of MC4100 lysogenic for various \(\lambda 1910\) and \(\lambda 1962\) containing Tn5 insertions were grown exponentially in the defined minimal media. At cell density of 0.3-0.4 \(A_{600}\), triplicate samples of 0.1 ml were removed from the cultures and assayed for \(\beta\)-galactosidase activity. The assay was done in 1.4 ml of Z buffer, 50 \(\mu\)l of 0.1% SDS and 100 \(\mu\)l of CHCl\(_3\) at 28\(^\circ\)C. The incubation time was started by the addition of 0.3 ml of 4 mg/ml ONPG and stopped by 1 ml of 1M Na\(_2\)CO\(_3\). The final reaction volume was 2.8 ml. Absorbance at 420 nm was taken to measure the concentration of the ONP product. Units of enzyme activity are \((A_{200}\times 100)/(t\times A_{600})\), where \(t\) is the incubation time of the assay in minute and \(A_{600}\) is a measure of the mass of the bacteria culture at the time of sampling.
D. Synthesis of Plasmid Specific Proteins in UV-inactivated Cells:

Plasmids, with and without Tn5 insertions, were transformed into strain CSR603. The transformed CSR603 strains were grown in defined minimal medium at 37°C. At cell density of 0.35-0.45 A₆₀₀, 10 ml of cells were taken for UV-irradiation for 15 seconds at a distance of 56 cm. The irradiated cells were incubated for 16 hours at 37°C, centrifuged and resuspended into 5 ml of fresh medium. Protein labelling was done in 2.5 ml of cells with (³⁵S)-methionine (5 µCi/ml; spec. act. ~1500 Ci/mmol) for 1 hour. Cells were harvested and lysed by boiling in 0.25 ml cracking buffer for 3 min. Radioactive proteins were fractionated by electrophoresis on 12.5% SDS-polyacrylamide gels at 40 mA for 7-8 hours. The gels were stained for 15 min. with 0.1% Coomassie blue in 50% TCA and then destained in 7% acetic acid and 5% methanol. The gels were dried and autoradiograms were obtained using Kodak XRP-1 X-ray film.
E. Synthesis of L12 Protein Specified by Plasmid and Chromosomal rplL Genes:

Plasmids, with and without Tn5 insertions, were transformed into strain JF1039. The transformed strains were grown in 1.5 ml of the defined minimal media. When cell density is about 0.3 A_{460}, the cells were labelled with (\textsuperscript{35}S)-methionine (10 \mu Ci/ml; spec. act. \sim 1500 Ci/mmol) in presence of 0.1 \mu g/ml cold methionine for 1 hour. Cells were harvested, washed with 1 ml 50 mM Tris pH 8.0 and resuspended in 100 \mu l of 10mM EDTA, 50mM Tris pH 8.0. The cells were disrupted by 3 cycles of freezing and thawing in the presence of 100 \mu g/ml lysozyme. The magnesium concentration was adjusted to 0.2M by the addition of 20 \mu l of 1M MgCl\textsubscript{2} and 20 A_{260} units of carrier ribosomes were also added. The proteins were extracted with 2 volumes of glacial acetic acid at 0°C for 1 hour. Following centrifugation, the acid soluble fraction was dialyzed for 18 hours against 8M urea, 0.05M \textmu -mercaptoethanol. The volumes of the extract was measured, and 10X upper buffer and bromophenol blue were added to achieve a final concentration of 1X and 0.01% respectively.
The acid soluble proteins were separated on 4% polyacrylamide slab gels in the presence of 8M urea without cooling at 40 mA for 5-6 hours, with continuous circulation of the upper buffer with a 1 liter external reservoir. The gels were fixed with 50% methanol, 10% acetic acid for 30 min., and soaked in 1M sodium salicylic acid, pH 5.5, for 20 min. to enhance subsequent autoradiography. The gels were dried and subjected to autoradiography.
RESULTS

I. Isolation of Tn5 Insertions:

The Tn5 transpositions into tetracycline resistant plasmids pNF1344, pNF1571, and pNF1962 were isolated by infecting plasmid containing cells with λCI857 rex::Tn5 and subsequently screening for plasmids which conferred resistance to both tetracycline and kanamycin. The approximate sites of the Tn5 insertions were located by restriction enzyme analysis of plasmid DNA with EcoRI, BglII and HindIII. Many of the insertions were precisely mapped by DNA sequencing (Fig. 9). In one experiment where over 100 different pNF1344::Tn5 kanamycin and tetracycline resistant colonies were screened, approximately 30% of the insertions were into the 2.2 kb EcoRI fragment which contains the cloned ribosomal gene cluster (Fig. 10). The frequency of Tn5 insertions along the 2.2 kb EcoRI fragment was non-random; three regions favored for insertion between nucleotide positions 280-700, 1300-1500 and 2000-2200 were observed. In contrast, no insertions between nucleotide 700-1300 or 2200-2444 were recovered on pNF1344; this was probably the result of negative selection on insertion into regions of the L1 and L12 gene as described below. Plasmid pNF1571 which lack the promoter for the L11 operon was utilized to obtain Tn5 insertions within the L1 gene between nucleotides 700-1300.
Figure 9: Autoradiography of a 24% DNA Sequencing Gel to Localize the Precise Site of Tn5 Insertion for Strain PD342.

The DNA sequence analysis was carried out using the Maxam and Gilbert method (24) on a HinfI-HinfI fragment. One of the HinfI site was cut from the end of the inverted repeats of Tn5 (G|ACTC) and the other is from nucleotide 1416 in the leader region of the L10 operon (G|AGTC). This fragment was preferentially labelled at the 3' end of the HinfI site, which is four nucleotides from the Tn5 end, with γ-\((^{32}\text{P})\)-dCTP in the presence of cold dATP. The position of insertion was found to be at nucleotide 1280 for strain PD342. The 9 base pair repeat at the insertion site is indicated together with its complementary sequences.
Figure 10: Distribution of Tn5 Insertions in the 2.2 kb EcoRI Fragment of Plasmid pNF1344.

About 30% of the Tn5 insertions into plasmid pNF1344 in a single analysis were found on the 2.2 kb EcoRI fragment. The transpositional events do not appear to be random; in fact, there were three clusters of Tn5 insertions around nucleotide position 280-600, 1300-1500 and 2000-2200. No Tn5 insertions were found in the middle and the distal portion of the L1 gene, from nucleotide 700 to 1300 or in the L12 gene from nucleotide 2200 to 2444. (The horizontal axis of the graph is the frequency of Tn5 insertion; the vertical axis is the nucleotide position with respect to the 2.2 kb EcoRI fragment as indicated.)
The insertion of Tn5 results in duplication of the 9 base pair target DNA sequence at the ends of the insertion element. Table II presents these target sites for insertions which have been precisely located by DNA sequence analysis (Fig. 11). These sequences appear unrelated, suggesting that the transposition of Tn5 showed very little, if any, sequence specificity at the target site. Randomness in the target sequence has also been observed for insertions into the lacZ gene (25).
Table II: Sequences Flanking Tn5 Insertions.

Insertion of Tn5 results in a direct duplication of the 9 base pair target DNA at the ends of the Tn5 sequence (represented between two asterisks "*"). The nucleotides at the insertion site, sequenced by the Maxam and Gilbert method (24), show very little homology. This suggests the transpositional events of Tn5 is relatively random.
<table>
<thead>
<tr>
<th>Direct Repeat</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAGGA<em>AATCGCGCA</em>GACCA</td>
<td>495-503</td>
</tr>
<tr>
<td>TCGCA<em>CTGCTGAAA</em>GAGCT</td>
<td>688-696</td>
</tr>
<tr>
<td>GCGCG<em>TTGTTGGCC</em>AGCTG</td>
<td>971-979</td>
</tr>
<tr>
<td>GGGTA<em>CTGTAACAC</em>CGAAC</td>
<td>1034-1042</td>
</tr>
<tr>
<td>TGACC<em>AGGCTGGCC</em>TGAGC</td>
<td>1280-1288</td>
</tr>
<tr>
<td>ATAAT<em>GCTTAATGC</em>AGACG</td>
<td>1360-1368</td>
</tr>
<tr>
<td>GCAGC<em>CTGATATCC</em>GAGAT</td>
<td>1372-1380</td>
</tr>
<tr>
<td>ATCCA<em>GCCCTCCGT</em>CGAAG</td>
<td>1532-1540</td>
</tr>
<tr>
<td>AAGAA<em>ACTTAATCC</em>CCTGC</td>
<td>1567-1575</td>
</tr>
<tr>
<td>CGGTC<em>ACAGAACC</em>TAAGA</td>
<td>1591-1599</td>
</tr>
<tr>
<td>GTCGC<em>CTTTGAAGG</em>TGAGC</td>
<td>2056-2064</td>
</tr>
<tr>
<td>TGGCT<em>GCTGTACGC</em>GATGC</td>
<td>2189-2197</td>
</tr>
<tr>
<td>TCTGC<em>ACCGGCTGC</em>TCTGA</td>
<td>2557-2565</td>
</tr>
<tr>
<td>ACACT<em>GTGGACTA</em>CTGCT</td>
<td>2751-2759</td>
</tr>
<tr>
<td>TCAAT<em>GGACAGATG</em>GGTCG</td>
<td>2930-2938</td>
</tr>
</tbody>
</table>
Figure 11: Location of Tn5 Insertions in L11 and L10 Transcription Units.

Insertion position marked with (*) have been estimated from restriction enzyme analysis only; other insertion positions have been located by Maxam and Gilbert sequence analysis. The insertions were isolated on plasmids pNF1344, pNF1571, and pNF1962. Sites for restriction enzymes EcoRI (R, nucleotide 280, 2444, 3524), HindIII (H, 2154), BglII (B, 897), and PstI (P, 1791) are illustrated; other PstI sites in the region (nucleotide position 202, 487, 869 and 3674) are not shown.
II. Polarity of Tn5 Insertion on the Expression of lacZ Fusion Products:

The polar effects of the Tn5 insertions on the expression of the L10 ribosomal protein gene and the RNA polymerase β gene were examined utilizing fusions of the distal portion of the lacZ gene to the proximal portion of the L10 and β genes, respectively. The L10 fusion is carried on λ1910 and the β fusion is carried on λ1962 as indicated on Figure 7. The Tn5 insertions on plasmids pNF1344, pNF1571 and pNF1962 were transferred to the homologous positions in the transducing phages by in vivo recombination during lytic phage infection (Fig. 9). The resulting recombinant and transposition phages were identified as kanamycin resistant transductants in MC4100.

Bacteriophages carrying Tn5 insertions generated from legitimate recombinational events were distinguished from those generated from Tn5 transpositional events by parallel restriction enzyme analysis of plasmid and bacteriophage DNA. The analysis was carried out using EcoRI, HindIII and BglIII, and the DNA fragments were separated on 0.7% agarose gels, allowing unambiguous location of the position of Tn5 sequences on the λDNA. Figure 12 illustrates the BglIII digestion pattern for Tn5 insertions at three different positions within the 2.2 kb EcoRI fragment which spans from nucleotide 280 to 2444 in the ribosomal protein gene cluster.
Figure 12: Restriction Enzyme Mapping of Tn5 Insertion into Homologous Position on Plasmids and Transducing Phages.

Plasmid and phage DNAs were digested with BglII and fragments were separated by electrophoresis on 0.7% agarose gels. The three panels represent the digests of DNA from three separate insertions into the ribosomal protein region. The Tn5 transposon is about 5.7 kb in length and contains two BglII sites symmetrically located 1515 nucleotides from the ends of the inverted repeats. Digestion with BglII generates a 2.7 kb fragment from the internal position of Tn5. The upper arrows illustrate the position of this common Tn5 fragment. A unique junction fragment, illustrated by the lower arrows, representing 1.5 kb of Tn5 DNA fused to DNA terminating at the BglII site in L1 (nucleotide 897) is evidence for each of the three insertions. The size marker fragments in lane 4 of each panel are 1.1, 1.2, 2.2, 2.7, and 6.4 kb in size.

The lanes are as follows:
A. 1. pNF1571::Tn5 at nucleotide 1034;
   2. λ1910::Tn5 at nucleotide 1034;
   3. λ1962::Tn5 at nucleotide 1034;
   4. Size marker;
B. 1. pNF1344::Tn5 at nucleotide 1360;
   2. λ1910::Tn5 at nucleotide 1360;
   3. λ1962::Tn5 at nucleotide 1360;
   4. Size marker;
C. 1. pNF1344::Tn5 at nucleotide 1576;
   2. λ1910::Tn5 at nucleotide 1576;
   3. λ1962::Tn5 at nucleotide 1576;
   4. Size marker.
Digestion of plasmid and phage DNA with BgII generates a common 2.7 kb fragment from the central portion of the Tn5 insertion sequence; in addition, it generates a unique fragment which is bounded by the BgII site at nucleotide 897 (within the 2.2 kb EcoRI fragment) and the BgII site in the inverted repeat of Tn5 (located at 1515 nucleotide from the end of the transposon sequence). The sizes of the unique fragments in the three respective insertions were consistent with insertion positions at nucleotides 1034, 1360 and 1567 as determined by DNA sequence analysis on the plasmid DNAs. All other λ1910::Tn5 and λ1962::Tn5 phage were analyzed in this manner using BgII as well as HindIII and EcoRI.

The effects of Tn5 insertion upon the expression of β-galactosidase activity in the L10-lacZ and β-lacZ fusions were measured in MC4100 strains lysogenic for λ1910::Tn5 or λ1962::Tn5. Table III summarizes the positions of the respective Tn5 insertions, the plasmid on which the insertion was originally isolated and the β-galactosidase activity in lysogens carrying these insertions. Insertions at or before nucleotide 1280 were not polar and exhibited normal expression of β-galactosidase activity in the L10-lacZ and β-lacZ fusion lysogen. In contrast, those insertions at or after nucleotide 1360 exhibited a much lower level of β-galactosidase activity in both types of lysogens. The activity was reduced by 90% or more compared to non-polar insertions and control lysogens. These results demonstrated that a major promoter for both the L10
ribosomal protein gene and the RNA polymerase $\beta$ gene is confined between nucleotides 1280 and 1360. Previously a promoter-like sequence with an RNA start site at nucleotide 1348 was identified by in vitro RNA polymerase binding and in vitro transcription (23, 39).

The residual levels of $\beta$-galactosidase activity from $\lambda$1910 lysogens with polar insertions of Tn5 in front of L10-lacZ fusion was about 5% of the control values. In contrast, the residual level of activity of the $\beta$-lacZ fusions was somewhat larger, representing 5-10% of the control values and is dependent to some extent on the site of Tn5 insertion. The stronger degree of polarity of the two insertions at nucleotides 2751 and 2931 compared to upstream insertions may reflect the presence of a weak promoter in the L12-$\beta$ intergenic space. Such a weak promoter functioning at about 2% of the level of the major L10 promoter has recently been identified and characterized (15). However, the physiological significance of this low level promoter is not known.
Table III: The Expression of L10-lacZ and β-lacZ Fusion Proteins with Tn5 Inserted at Various Position in the L11 and L10 Transcription Units.

The effect of Tn5 insertions on the expression of L10 and β fusion proteins was examined in strain MC4100. Insertions at or before nucleotide 1280 have β-galactosidase activities comparable to that of the control (without Tn5 insertions), whereas insertions at or after nucleotide 1360 have strong polar effects (90-95%) on the expression of both L10 and β fusion proteins indicating the major promoter for the L10 operon is between nucleotides 1280 and 1360. A weak promoter functioning at less than 2% of the major promoter level may be present in between nucleotides 2557 and 2751 in the intergenic region of L12 and β genes. In contrast to expectation, the level of β-galactosidase in λ1962 was of the same order as that of the λ1910 in spite of the presence of an attenuator. The reason for this is still unknown.
<table>
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<th>Strain No.</th>
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<th>Plasmid Origin</th>
<th>λ1910::Tn5 Strain No.</th>
<th>p-gal.</th>
<th>λ1962::Tn5 Strain No.</th>
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MC4100 λ1910 44.2 MC4100 λ1962 56.9
MC4100 0.8 MC4100 0.8
III. Polarity of Tn5 Insertions on Ribosomal Protein Gene Expression:

The pNF1344 plasmids containing Tn5 insertions were introduced into strain CSR603 in order to directly examine the effects of insertion upon the expression of ribosomal protein genes. Strain CSR603 is a highly UV-sensitive strain and is unable to repair UV-induced DNA damage. Moderate levels of irradiation inactivate genes located on the bacterial chromosome. Genes located on plasmids escape inactivation because of the high copy number and the small target size of the plasmid DNA.

The plasmid pNF1344 contains both the L11 and L10 operon promoters; in the absence of any Tn5 insertions, it specifies the synthesis of ribosomal proteins L11, L1, L10, L12 and RNA polymerase β fragment protein (Fig. 13). An insert at nucleotide 495, within the L11 gene, abolished the synthesis of L11 and L1 but not the synthesis of L10, L12 and β fragment proteins. An insert at nucleotide 688, within the L1 gene, abolished synthesis of L1 and enhanced synthesis of L11 (compare the L11 and L10 band intensities in the pNF1344 control and in the Tn5 insert at 688). This result substantiates previous observations that the L11 and L1 genes form a common transcription unit and that L1 regulates the synthesis of L11 (42).
Figure 13: Autoradiography of Proteins Labelled Following UV-Inactivation of Stain CSR603.

Plasmid pNF1344 derivatives containing Tn5 insertion at various positions were introduced by transformation into strain CSR603. Following inactivation of chromosomal genes by UV-irradiation, proteins specified by plasmid genes were labelled with \(^{35}\)S-methionine, fractionated by electrophoresis on 12.5% SDS-polyacrylamide gels and subjected to autoradiography. The ribosomal proteins L1, L10, L11, and L12, and the RNA polymerase \(\beta\) fragment are illustrated.

Samples are loaded in duplicates as follows:
A. CSR603;
B. CSR603/pBR322;
C. CSR603/pNF134;
D. CSR603/pNF1344::Tn5 (L11, nucleotide 495);
E. CSR603/pNF1344::Tn5 (L1, nucleotide 688);
F. CSR603/pNF1344::Tn5 (leader, nucleotide 1360);
G. CSR603/pNF1344::Tn5 (leader, nucleotide 1567);
H. CSR603/pNF1344::Tn5 (L10, nucleotide 2189).
Insertion of Tn5 into the leader region of the L10 operon, at nucleotide 1360 or 1567, abolished or reduced the synthesis of L10, L12 and \( p \) fragment but had no effect on L11 and L1 synthesis. An insertion into the distal portion of the L10 gene, at nucleotide 2189, resulted in the synthesis of a protein containing 158 amino acids from the N-terminal end of L10 and six amino acids at the C-terminal end specified by the inverted repeat sequence of Tn5. This insertion also reduces the level of L12 and \( p \) fragment synthesis. Again, these results confirm that the expression of the genes specifying L10, L12 and \( p \) depends on a promoter sequence located in front of nucleotide 1360.
IV. Synthesis of L12 Ribosomal Proteins:

High copy number plasmids which carry an active L10 gene require, in addition, an active L12 plasmid gene in order to prevent killing of the host cell. The killing effect has been attributed to over production of the L10 protein, and has been observed for plasmids which contain EcoRI generated deletions, removing the distal 50% of the L12 gene, or HindIII generated deletions, removing the entire L12 gene and the distal 10% of the L10 gene (11). Apparently both the complete and the truncated L10 gene product in an L12 deficient environment exhibit the same detrimental effects on cell viability.

Based upon these previous observations it was not surprising that insertions of Tn5 into the L12 gene of the high copy number plasmids pNF1344 and pNF1571 were not observed. A few insertions into the L12 gene including the one at nucleotide 2557 have been obtained on the lower copy number plasmid pNF1962. In contrast, a relatively high number of Tn5 insertions into the distal region of the L10 gene were unexpectedly obtained (Fig. 10). The survival of cells carrying these plasmids suggests that the expression of the intact plasmid L12 gene continues in the presence of the insertion at a level sufficient to balance the production of the L10 protein fragment. 7

In E. coli the L12 protein is partially acetylated at the N-terminal position prior to assembly into ribosomes (40). The acetylated and unacetylated forms are designated
L7 and L12 respectively; acetylation is not essential or required for any known function of the protein. Strain JF1039 carries a mutation in the chromosomal rplL (L12) gene and produces a fully functional but electrophoretically altered protein of which about 80% is acetylated to the L7 form.

To test the hypothesis stated above relating to plasmid expression of the rplL (L12) gene, various pNF1344::Tn5 or pNF1962::Tn5 plasmids were introduced into strain JF1039. Transformants carry both a mutant chromosomal L12 gene and a wild type plasmid L12 gene have the potential to produce both mutant and wild type L7 and L12 proteins. The effects of Tn5 insertions on the synthesis of wild type L7 and L12 was examined by labelling exponential phase cells with \( ^{35}\text{S} \)-methionine. The ribosomal proteins, including L7 and L12, were extracted with glacial acetic acid, and separated on urea-polyacrylamide gels (Fig. 13, Ref. 19). In extracts from the parental JF1039 strain, only the mutant forms of L7 and L12 were apparent, whereas in those from the strains which contained the pNF1344 plasmid, the wild type L7 proteins predominated because of the amplified copy number of the plasmid gene. Insertion of Tn5 within the L11 or L1 gene failed to affect the plasmid specified synthesis of wild type L7 protein whereas insertion into the leader region in front of the L10 gene greatly diminished the synthesis of wild type L7. The most important observation however was that the insertion at nucleotide 2189 near the
distal end of the L10 gene failed to diminish the synthesis of wild type L12; this correlates with the unexpected viability of this plasmid although it is difficult to explain based on the generally accepted organization of these genes. Plasmid pNF1962 lacks the L11 promoter, and as expected, the Tn5 insertion in the intergenic region between L12 and p (at nucleotide 2751) on plasmid pNF1962 had no effect on the synthesis of the plasmid L7 protein. An insertion in the plasmid rplL gene (at nucleotide 2557 on pNF1962) gave rise to a truncated protein with an altered mobility.
Figure 14: Synthesis of Ribosomal L7 and L12 Proteins Specified by Plasmid and Chromosomal Copies of the rplL Gene.

Various pNF1344 and pNF1962 plasmids containing Tn5 insertions were transformed into JF1039. Exponential phase cultures were labelled with ($^{35}$S)-methionine (10 μCi/ml) for one hour and the proteins were extracted and separated by electrophoresis on urea-polyacrylamide gels. The positions of the plasmid specified wild type ribosomal L7 protein is indicated. The bracket ([]) indicates in descending order the positions of chromosome specified L12, plasmid specified L12 and chromosome specified L7 proteins. The bands shown here are not clearly resolved.

Samples are as follows:
A. JF1039;
B. JF1039/pNF1344;
C. JF1039/pNF1344::Tn5 (L11, nucleotide 495);
D. JF1039/pNF1344::Tn5 (L1, nucleotide 688);
E. JF1039/pNF1344::Tn5 (leader, nucleotide 1360);
F. JF1039/pNF1344::Tn5 (leader, nucleotide 1567);
G. JF1039/pNF1344::Tn5 (L10, nucleotide 2189);
H. JF1039/pNF1962::Tn5 (between L12 and p, 2751);
I. JF1039/pNF1962::Tn5 (L12, nucleotide 2557).
DISCUSSIONS

I. Regulation of the L11 Transcription Unit:

Ribosomal protein L1 has been identified as the regulatory protein for the L11-L1 transcriptional unit (8, 42). Consistent with this is the observation that the Tn5 insertional inactivation of the L1 gene on plasmid pNF1344 results in about a two-fold elevation in the synthesis of L11 protein relative to that of the pNF1344 control (Fig. 13). The position of this Tn5 insertion is very early in the L1 gene and presumably results in the synthesis of a peptide fragment consisting of 30 amino acids specified by the 5' terminal end of L1 gene and six amino acids at the C-terminal end specified by the inverted repeat sequence of Tn5. The fact that this plasmid is apparently not detrimental to the host cell implies that expression of the L11 plasmid gene in the absence of an intact L1 plasmid gene is inconsequential to the survival of the bacterial cell. Recently, several mutant strains of *E. coli* which completely lack L1 protein in their ribosomes have been isolated; these mutants appear to overproduce L11 by about two-fold (16, 37).

Over 100 independent Tn5 insertions into the 2.2 kb EcoRI fragment on pNF1344 have been screened for insertions within the 702 nucleotide long L1 gene; only one insertion at nucleotide 688 has been identified (Fig. 10, and Dennis and Fiil unpublished results). The additional Tn5 insertions
which occur further downstream in the L1 gene at nucleotide 971, 1034 and 1282 were isolated on plasmid pNF1571; this plasmid lacks the promoter for the L11 operon and a portion of the L11 gene (see Fig. 6). This failure to isolate more inserts of Tn5 into the L1 gene on pNF1344 could be a statistical phenomenon, or alternatively, an indication that large protein fragments of L1 might be detrimental and affect survival of the bacterial cell. For example, larger L1 fragments might be able to repress the synthesis of L11 but not effectively incorporated into ribosomes. Consistent with this second possibility is the observation that DNA polymerase I generated 4 base pair insertions into the middle of the L1 gene at the BglII site (nucleotide 897) cannot be recovered on plasmid pNF1344. In contrast, these BglII insertions have been isolated on pNF1571 which does not express the L1 gene (Dennis unpublished results).
II. Regulation of the L10 Transcription Unit:

The expression of a L10 gene in the absence of a L12 gene on a high copy number plasmid has been observed to be detrimental to the survival of the host cell (11). This effect was observed for both EcoRI generated plasmid deletions which remove the C-terminal half of the L12 gene and for HindIII generated deletions which remove the entire L12 gene and the C-terminal 19 codons of the L10 gene. These observations suggest that the plasmid specified L10 fragment as well as the complete protein, is detrimental to host cell survival in the absence of compensating plasmid L12 synthesis. From this it was reasonable to expect that insertion of Tn5 into the plasmid L12 gene or the distal region of the plasmid L10 gene should be detrimental on high copy number plasmid. However, several insertions into the distal region of the L10 gene have been recovered whereas no insertions of Tn5 into the L12 gene on pNF1344 have been identified.

How does a Tn5 insertion into the distal region of the L10 gene survive? Survival very clearly results from the fact that such insertions continue to express the L12 plasmid gene at a level sufficient to compensate for the expression of the truncated L10 plasmid gene. How does the cell maintain L12 expression? A previous study by Berg et al. (6) has identified a weak promoter located within 186 nucleotides of the ends of the inverted repeats of Tn5. This promoter presumably accounts for the residual 5-10% level of
\( \beta \)-galactosidase activity in the L10-lacZ and \( \beta \)-lacZ fusion lysogens carrying polar Tn5 insertions (Table III). Nomura and his coworkers (43) have suggested that the four fold elevated level of translation of L12 mRNA sequence on L10 operon mRNA requires concomitant translation of the proximal L10 mRNA sequence; presumably it is the ribosome movement through the L10 sequence that opens up the downstream initiation site and allows ribosomes to bind and begin translation of L12 mRNA sequence. The insertion of Tn5 into the distal region of the L10 genes might alter or physically remove the sequences which normally sequester the L12 ribosome binding site. In the absence of sequestering sequences, the L12 mRNA initiated from the promoter within the Tn5 element would be very efficiently translated. Therefore, the sufficient expression of L12 to regulate the synthesized L10 fragments would be a consequence of:

1. The high copy number of the plasmid;
2. The low level of transcription from the Tn5 promoter;
3. The efficient translation of the L12 sequence.

On the other hand, insertions of Tn5 into the leader region of the operon do not produce high levels of L12 and \( \beta \) proteins as indicated in Figure 13 because the Tn5 initiated transcripts presumably contain the sequestering sequences. Unfortunately examination of the nucleotide sequence has failed to reveal an obvious candidate for a sequestering sequence which would interact with the L12 initiation region.
In summary the experiments reported here have confirmed the organization of the four ribosomal protein and two RNA polymerase genes into two transcription units as illustrated in Figure 2. The sequence representing major promoter for the L10 operon was localized between nucleotide 1280 and 1360.

Evidence has been presented which supports the idea that L1 is the translational regulatory protein for the L11 operon and that larger fragments of the L1 protein produced by Tn5 insertion late in the gene can have detrimental effects on the growth of the bacterium. Analysis of insertions into the L10 operon have supported the idea that L12 plasmid gene expression is required to balance L10 plasmid gene expression and lead to the suggestion that a sequence within the L10 mRNA may interact with and sequester the ribosome binding site of the L12 mRNA.
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