Insertions of Transposon Tn5 into Ribosomal Protein RNA Polymerase Operons

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The genetic organization and interrelationships between the two ribosomal protein transcription units (the L11 and L10 operons) from near 89 min on the *Escherichia coli* chromosome were studied by using insertional mutations generated by the kanamycin-resistant transposable element Tn5. The polar effects of Tn5 insertions on the expression of the L11, L1, L10, and L12 ribosomal protein genes and the β RNA polymerase subunit gene were examined (i) by the level of β-galactosidase activity generated from L10-*lacZ* and β-*lacZ* gene fusions, (ii) by direct sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the proteins specified by plasmid ribosomal protein genes in UV-irradiated maxicells, and (iii) by urea-polyacrylamide gel electrophoresis of plasmid- and chromosome-specified L12 protein. The results confirmed the organization of these genes into two transcription units as follows: P_{L11}, *rplK* (L11), *rplA* (L1), P_{L10}, *rplJ* (L10), *rplL* (L12), *rpoB* (β). . .; they also localized the position of the P_{L10} promoter within an 80-nucleotide region near the end of the L1 gene. The results also support the idea that the translational regulatory proteins for the L11 and L10 operons are L1 and L10, respectively, and that the expression of the L12 gene is closely linked to L10 gene expression.

The region near 89 min on the *Escherichia coli* chromosome contains a gene cluster which specifies four 50S ribosomal proteins and the two large subunits of RNA polymerase (for a review, see reference 15). Early experiments utilizing recombinant λ phage or plasmids 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: promoter (P_{L11}), *rplK* (L11), *rplA* (L1), promoter (P_{L10}), *rplJ* (L10), *rplL* (L12), *rpoB* (β), *rpoC* (β′) (14, 16, 17, 22, 30). The two promoters and the mRNA start sites were established from DNA sequence analysis, in vitro transcription mapping, and RNA polymerase binding assays, using short restriction enzyme fragments from this region (24, 28). 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 regions between L10 and L12 and L12 and β, which could play an important role in gene regulation (1, 2, 18).

More recently, S1 nuclease mapping experiments have suggested that some of the L11 operon transcripts may read into the L10 operon to produce an RNA specifying the synthesis of all four ribosomal proteins (7). However, inter-pretation of these experiments may be complex, particularly if the distal end of the L11 operon transcript were in overlap with the proximal end of the L10 operon transcript. From the DNA sequence there is no apparent transcription termination site in the region between the L1 and L10 genes. Our own S1 mapping experiments indicated that the L10 promoter is indeed functional in vivo, with transcription starts in the vicinity of nucleotides 1,347 and 1,366 (S. Hastrup, thesis, University of Copenhagen, Copenhagen, Denmark, 1982); there are good −10 and −35 regions associated with each of these start sites.

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 (27). 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 an RNase III processing site located between the L12 and the β genes (2, 3, 9). In exponentially growing cells, approximately 80% of the mRNA transcripts are terminated at the attenuator and the unattenuated 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 ColE1 plasmids to amplify the copy number of the L10 transcription unit suggested that the synthesis of L10, L12, β, and β' proteins is subjected to post-transcriptional regulation (10). Amplification of the L10 transcription unit resulted in a sixfold elevation in L10 operon transcription, but only a 30% increase in L12 synthesis rate and a twofold increase in the β and β' synthesis rates. The noncoordinate effects on L12, compared with β and β', suggest that the ribosomal proteins and the RNA polymerase subunit proteins are regulated by separate post-transcriptional control mechanisms.

More recent experiments have indicated that the L11 and L10 operons are subject to autogenous regulation at the level of mRNA translation (11, 31, 32; M. Johnsen, T. Christensen, N. P. Fiil, and P. P. Dennis, EMBO J., in press). The L1 protein regulates translation of the bicistronic L11 operon mRNA; the target site for L1 translational repression was inferred to be within the proximal 160 nucleotides of the mRNA. The L10 protein (or the L10-L12 complex) regulates translation of the proximal ribosomal protein sequences of the L10 mRNA. An association between the regulatory protein and a sequence within the 370-nucleotide leader region of L10 operon mRNA has been demonstrated (Johnsen et al., in press). The translational inhibition by both L1 and L10 (i.e., the binding to their respective mRNAs) is reversed by the presence of rRNA sequences.

In this study the organization and interrelationships between the ribosomal protein and RNA polymerase genes in the *rif* cluster were examined in vivo by isolating and characterizing Tn5 insertions into plasmids carrying these ribosomal protein operons (4). The polar effects of Tn5 insertions were used to confirm the general organization of the genes into two operons, and the in vivo site of the L10 operon promoter was localized. The polar effects and physiological changes caused by gene inactivation after insertion were studied by examining: (i) β-galactosidase activity in fusions of the *lacZ* gene to the L10 ribosomal protein and β RNA polymerase genes; (ii) synthesis of plasmid-specified proteins in UV-irradiated maxicells; and (iii) synthesis of plasmid-specified L12 proteins in a mutant strain producing an electrophoretically altered L12 protein from the chromosomal gene.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used are listed in Table 1. Bacteria were grown in NY liquid or solid medium containing, when appropriate, tetracycline (20 μg/ml) and kanamycin (20 μg/ml). Medium NY contains 10 g of N-Z-amine (Humko Sheffield Co.), 5 g of yeast extract (Difco Laboratories), and 5 g of NaCl per liter. For protein labeling, strains were grown in M9 minimal medium supplemented with glucose (2 mg/ml), thiamine (0.5 μg/ml), and required amino acids (50 μg/ml). For β-galactosidase measurements, strains were grown in M9 medium supplemented with glucose, Casamino Acids (8 mg/ml), and thiamine.

Isolation and characterization of Tn5 transpositions. Insertions of Tn5 in the Tet^r plasmids pNF1344, 1571, and 1962 (Fig. 1) were isolated from plasmid-containing strains of MC1000 after infection with λ *c*I857 *rex*::Tn5 O29(am) P80(am) *b*221. Plasmids containing a Tn5 transposition were identified by their ability to confer both kanamycin and tetracycline resistance after retransformation into MC1000. The approximate positions of Tn5 insertions were localized by restriction enzyme mapping with EcoRI, HindIII, and BglII (6). The precise sites of many of the insertions were determined by Maxam and Gilbert DNA sequence analysis (19).

Plasmid-phage recombination. Cultures of bacterial strains carrying Kan^r Tet^r plasmids were infected with λ1910 or λ1962 at 37°C. After 3 h the cells were concentrated by centrifugation and lysed with CHCl$_3$. Progeny were used to transduce strain MC4100, and kanamycin-resistant lysogens were selected at 30°C. Phage DNA was prepared from single lysogens by phenol-CHCl$_3$ extraction and ethanol precipitation and analyzed by digestion with EcoRI, HindIII, and BglII to localize the precise positon of the Tn5 insertion on the λ DNA.

The constructions and structures of λ1910 and λ1962 are described and illustrated in Fig. 1. These phage contain the distal portion of the *lacZ* gene fused in phase to the proximal portion of the L10 ribosomal protein gene or the β RNA polymerase gene, respectively. Lysogens of both fusions give essentially the same level of β-galactosidase activity per unit of bacterial mass in spite of the fact that the L10 fusion occurs in front and the β fusion occurs behind the attenuator located in the L12-β intergenic space. The explanation for this observation is not completely understood. It may be related to the observation that post-transcriptional control on ribosomal protein mRNA sequences is stronger than control on RNA polymerase mRNA sequences (10).

β-Galactosidase measurements. Strains of MC4100 lysogenic for the various λ1910- and λ1962-containing Tn5 insertions were grown exponentially in defined medium. At mid-log phase (optical density at 460 nm =

TABLE 1. Genotypes of bacterial strains

Strain	Genotype
MC1000	*araD139 Δ(araBCOIC-leu)7697 Δ(lacIOPZY)X74 galU galK rpsL thi*
MC4100	*araD139 ΔlacU169 rpsL thi mot recA*
JF1039	*ilvD argH rplL*
CSR603	*thr leu thi pro arg his*

0.3 to 0.4), triplicate samples (0.1 ml) were removed from the cultures and assayed for β-galactosidase activity in a final reaction volume of 2.8 ml (20). Units of enzyme activity are as follows: (absorbance at 420 nm $\times$ 10^2)/(t $\times$ optical density at 460 nm), where t is the incubation time of the assay in minutes and optical density at 460 nm is a measure of the mass of the bacterial culture at the time of sampling.

Synthesis of plasmid-specific proteins in UV-inactivated cells. Plasmids, with and without Tn5 insertions, were transformed into strain CSR603. After UV irradiation and overnight incubation, cultures were labeled in minimal medium with [^{35}S]methionine (5 μCi/ml; specific activity, ~1,500 Ci/mmol) for 1 h, and radioactive proteins were fractionated by electropho-

resis on 12.5% sodium dodecyl sulfate-polyacrylamide gels (25). The gels were dried and subjected to autoradiography.

Synthesis of L7/L12 protein specified by plasmid and chromosomal rpIL genes. The plasmids with Tn5 insertions were transformed into strain JF1039. This strain carries a mutation in the chromosomal rpIL (L12) gene which results in production of a fully functional but electrophoretically altered protein. In vivo the L12 protein is partially acetylated at the N-terminal position before assembly into ribosomes (29); in JF1039 about 80% of the L12 product is acetylated to the form designated L7. Strains were grown exponentially in minimal medium and labeled with [^{35}S]methionine (10 μCi/ml) for 30 min. Labeled L7 and L12 proteins
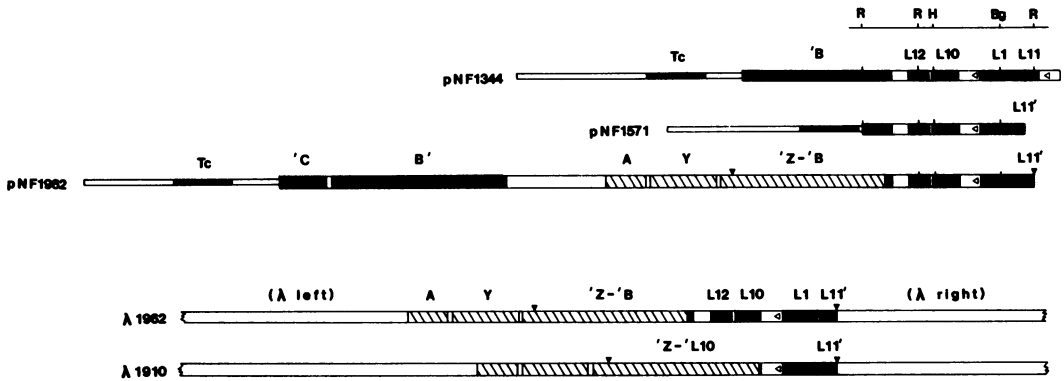

FIG. 1. Structures of recombinant plasmids and phage. The composite plasmids used were the pBR322 derivatives pNF1344 and pNF1571 and the pACYC184 derivative pNF1962. Plasmid pNF1344 contains a 6.0-kb fragment from λrifD18 inserted into the PstI site of the vector, whereas pNF1571 contains a 3,037-base bair PstI (nucleotide 487)-EcoRI (nucleotide 3,524) fragment inserted between the PstI and EcoRI sites of the vector DNA. Plasmid pNF1962 contains a 14.2-kb insert 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), B (rpoB), C (rpoC), and Tc (tetracycline resistance). The genes illustrated as hatched areas are Z (lacZ), Y (lacY), and A (lacA). All of these genes are expressed in a right-to-left direction and, with the exception of the plasmid tetracycline gene, are believed to be expressed utilizing one of the two promoters P_{L11} and P_{L10} (14); the probable mRNA start sites for these promoters are nucleotides 79 and 1,348, respectively (22). Plasmids pNF1571 and pNF1962 commence, respectively, at nucleotides 487 (PstI site) and 280 (EcoRI site); consequently, they do not contain the P_{L11} promoter and therefore do not express the fragment of the L11 or the intact L1 genes. The presence of (') to the right or left of a gene designates, respectively, the absence of the N-terminal or C-terminal coding portion of the gene. The presence of (-) between two genes represents a gene fusion and results in production of a hybrid polypeptide. The nucleotide boundaries for designated genes are: L11 (172 to 597); L1 (604 to 1,305); L10 (1,721 to 2,215); L12 (2,285 to 2,647); B (2,969 to ~7,500). In the RNA polymerase β-lacZ fusion the BamHI fragment from pMC901 was inserted into the Sau3A site of β at nucleotide 3,092. This fusion is in frame and produces a hybrid protein containing the N-terminal 42 amino acids of β fused to amino acid 8 of β-galactosidase. The restriction sites illustrated above plasmid pNF1344 are: R, EcoRI (280, 2,444); Bg, BglII (897); H, HindIII (2,154). An additional EcoRI site occurring late in the lacZ gene on plasmid pNF1962 is also illustrated. These sites were utilized for mapping the Tn5 insertion into the plasmid and phage DNAs. The two hybrid λ phages λ1910 and λ1962 are illustrated in the lower portion of the figure. The right junction of the bacterial DNA with λ DNA occurs at the EcoRI site in L11 (nucleotide 280). The RNA polymerase β-lacZ fusion on λ1962 is identical to that on plasmid pNF1962. The L10-lacZ fusion in λ1910 fuses the BamHI site in lacZ (preceding codon 8) to a PstI site (nucleotide 1,791) at codon 25 in the L10 gene. The fusion was achieved and the proper reading frame was established by using a 28-nucleotide-long PstI-BglII linker fragment derived from the L1 gene (nucleotides 869 to 897). Bacterial strains lysogenic for these phages exhibit β-galactosidase activity. The λ (right arm) is derived from a derivative of λgt[C] where the EcoRI restriction sites in the right arm have been removed by mutation. The λ (left arm) is derived from λMC209. This arm contains at the right end lac'A lacY lacZ'; the end of the fragment is the EcoRI site late in the lacZ gene. The intermediate phage in this construction was λNF1955, which contains λ left arm from λgt[C] fused to λ (right arm) from λMC209 through the single EcoRI site. The recombinant phage λ1910 and λ1962 were constructed by insertion of appropriate EcoRI fragments containing the indicated L10-Z or B-Z fusions into the single EcoRI site of λNF1955 and selecting for lysogens with β-galactosidase activity.

specified by the mutant chromosomal and the wild-type plasmid genes were extracted from lysates and fractionated by electrophoresis on urea-polyacrylamide gels (13). The gels were dried and subjected to autoradiography.

RESULTS

Isolation of Tn5 insertions. The Tn5 transpositions into tetracycline-resistant plasmids pNF1344, pNF1962, and pNF1571 were isolated by infecting plasmid-containing cells with λ::Tn5 and subsequently screening for plasmids which conferred resistance to both tetracycline and kanamycin. The sites of the Tn5 insertions were located by restriction enzyme analysis of plasmid DNA with EcoRI, HindIII, and BglII. Many of the insertions within or near the ribosomal protein gene cluster were positioned precisely by DNA sequence analysis (Fig. 2 and 3).

The insertion of Tn5 results in duplication of the 9-base-pair target DNA sequence at the ends of the insertion element (21). Table 2 presents these target sites for insertions which have been located by DNA sequence analysis. These sequences within or near the ribosomal protein gene cluster appear to be unrelated, suggesting that the Tn5 insertion event occurs at random positions.

Polarity of Tn5 insertions 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 (Fig. 1). The Tn5 insertions on plasmids pNF1344, 1571, and 1962 were moved to homologous positions on λ1910 and λ1962 by in vivo recombination during lytic phage infection. The resulting λ1910::Tn5 and λ1962::Tn5 recombinant and transposition phages were identified as Kan^r transductants in MC4100.

Bacteriophages carrying Tn5 insertions generated after legitimate recombination events with Tn5-containing plasmids were distinguished from those generated after Tn5 transpositional events by parallel restriction enzyme analysis of plasmid and bacteriophage DNA. The analysis was carried out with EcoRI, HindIII, and BglII and allowed unambiguous location of the position of Tn5 sequences on the λ DNA. Figure 4 illustrates the BglII digestion pattern for Tn5 insertions at three different positions within the 2.2-kilobase (kb) EcoRI fragment which spans from nucleotide 280 to 2,444 in the ribosomal protein gene cluster (see Fig. 1). Digestion of plasmid and phage DNA with BglII generates a common 2.7-kb fragment from the central portion of the Tn5 insertion sequence. In addition,
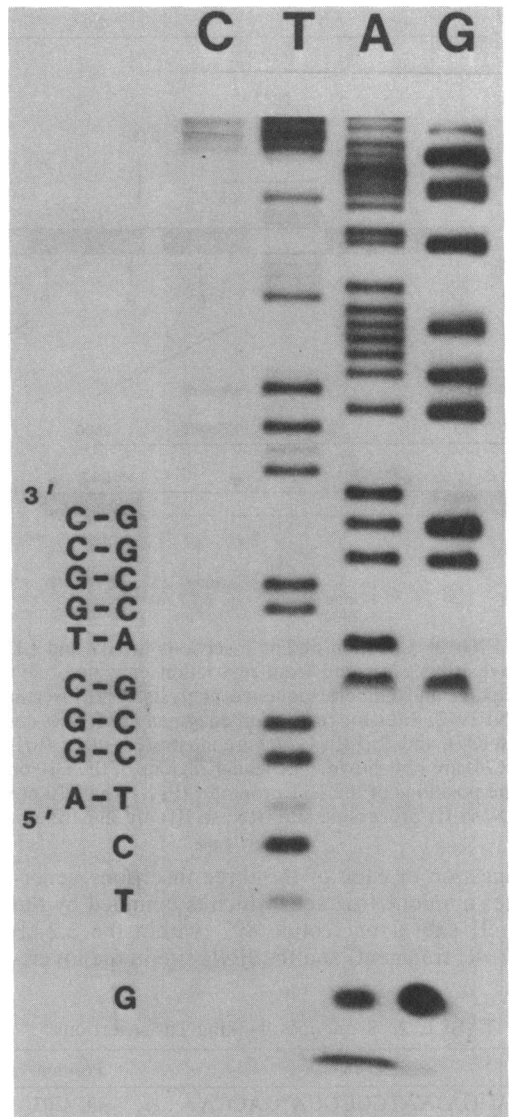

FIG. 2. Autoradiography of a 24% DNA sequencing gel to localize the precise site of Tn5 insertion on plasmid pNF1571 for strain PD342. The DNA sequence analysis was carried out with the Maxam and Gilbert method on a HinfI-HinfI fragment. One of the HinfI sites was cut from the end of the inverted repeats of Tn5 (G|ACTC), and the other is from nucleotide 1,416 in the leader region of the L10 operon (G|AGTC). This fragment was preferentially labeled at the 3' end of the HinfI site, which is four nucleotides from the Tn5 end, with [α-^32P]dCTP in the presence of cold dATP. The position of insertion was found to be at nucleotide 1,280 for strain pD342. The G residue at the bottom of the gel corresponds to the G at the 5' position in the HinfI site. The 9-base-pair direct repeat at the insertion site is indicated together with its complementary sequences. Similar procedures were used to sequence other Tn5 insertions.
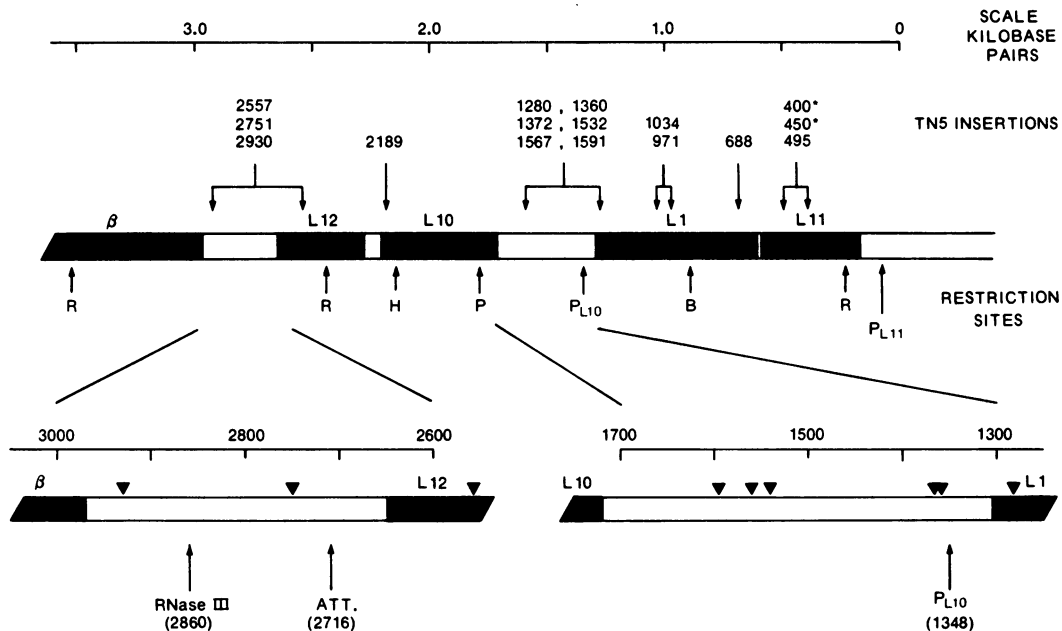
FIG. 3. Location of Tn5 insertions in L11 and L10 transcription units. Insertion positions marked with (*) have been estimated from restriction enzyme analysis only; other insertion positions have been located by Maxam and Gilbert sequence analysis. The insertions were isolated on plasmids pNF1344, pNF1571, and pNF1962. Sites for restriction enzymes EcoRI (R; nucleotides 280, 2,444, and 3,524), HindIII (H; 2,154), BglII (B; 897), and PstI (P; 1,791) are illustrated; other PstI sites in the region (nucleotide positions 202, 487, 869, and 3,674) are not shown. The leader regions of the L10 operon and the L12-β intergenic space are expanded below. The positions of the L11 promoter (P_{L11}), the L10 promoter (P_{L10}), the transcription attenuator (ATT), and the RNase III processing site (RNase III) are illustrated.

digestion of each of the three insertions generates a unique fragment which is bounded by the BglII site at nucleotide 897 (within the 2.2-kb EcoRI fragment) and the BglII site in the invert-

TABLE 2. Sequences flanking Tn5 insertions[a]

Direct repeat	Position
CAGGA*AATCGCGCA*GACCA......	495–503
TCGCA*CTGCTGAAA*GAGCT	688–696
GCGCG*TTGTTGGCC*AGCTG.......	971–979
GGGTA*CTGTAACAC*CGAAC	1,034–1,042
ACCAG*GCTGGCCTG*AGCGC	1,282–1,290
ATAAT*GCTTAATGC*AGACG	1,360–1,368
GCAGA*CTGATATCC*GAGAT	1,372–1,380
ATCCA*GGCCTCCGT*GCAAG	1,532–1,540
AAGAA*ACTTAATCC*CCTGC......	1,567–1,575
CGGTG*ACAGAACGC*TAAGA......	1,591–1,599
TGGCT*GCTGTACGC*GATGC.......	2,189–2,197
TCTGC*ACCGGCTGC*TCTGA......	2,557–2,565
ACACT*GTTTGACTA*CTGCT	2,751–2,759
TCAAT*GGACAGATG*GGTCG	2,930–2,938

[a] 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 (19), show very little homology. This suggests that the transpositional events of Tn5 are relatively random.

ed repeat of Tn5 (located 1,515 nucleotides from the end of the transposon sequence). The sizes of the unique fragments in the three respective insertions were estimated to be 1.65, 1.95, and 2.15 kb. These estimates were consistent with insertion positions at nucleotides 1,034, 1,360, and 1,567 as determined by DNA sequence analysis on the plasmid DNAs. All other λ1910::Tn5 and λ1962::Tn5 phage were analyzed in this manner, using both BglII and HindIII.

The effects of Tn5 insertion upon the expression of β-galactosidase activity in the L10-lacZ and β-lacZ fusions were measured in strain MC4100 lysogenic for λ1910::Tn5 or λ1962::Tn5. Table 3 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 before nucleotide 1,300 are not polar and exhibit normal expression of β-galactosidase activity in the L10-lacZ and β-lacZ fusion lysogens. Included in this class is an insertion at nucleotide 1,280, which is located 25 nucleotides from the C-terminal end of the L1 gene.

Insertions of Tn5 located beyond nucleotide 1,300, including the insertion at 1,360, exhibit a
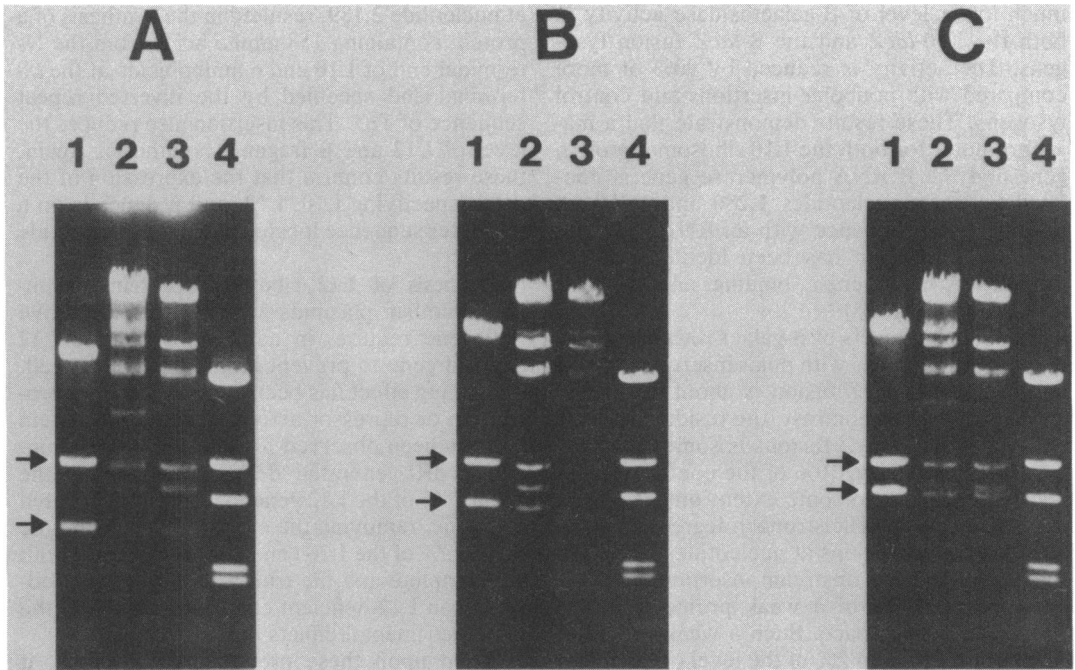
FIG. 4. Restriction enzyme mapping of Tn5 insertions into homologous position on plasmids and transducing phages. Plasmid and phage DNAs were digested with BglII, and fragments were separated by electrophoresis on 0.7% agarose gels. The three panels represent the digests of DNA from three separate insertions into the ribosomal protein region. The Tn5 transposon is about 5.7 kb in length and contains two BglII sites symmetrically located 1,515 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 the L1 gene (nucleotide 897) is evident 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 1,034; 2, λ1910::Tn5 at nucleotide 1,034; 3, λ1962::Tn5 at nucleotide 1,034; 4, size marker; (B) 1, pNF1344::Tn5 at nucleotide 1,360; 2, λ1910::Tn5 at nucleotide 1,360; 3, λ1962::Tn5 at nucleotide 1,360; 4, size marker; (C) 1, pNF1344::Tn5 at nucleotide 1,576; 2, λ1910::Tn5 at nucleotide 1,576; 3, λ1962::Tn5 at nucleotide 1,576; 4, size marker.

TABLE 3. Expression of L10-lacZ and β-lacZ fusion proteins with Tn5 inserted at various positions in the L11 and L10 transcription units[a]

Strain	Tn5 insertion (nucleotide)	Plasmid origin	λ1910::Tn5		λ1962::Tn5	
			Strain	β-Gal	Strain	β-Gal
PD179	~450	p1344	PD376	46.3	PD318	54.5
PD287	495	p1344	PD377	47.3	PD363	49.4
PD290	688	p1344	PD364	47.2	PD378	68.0
PD343	971	p1571	PD357	50.8	PD358	48.7
PD225	1,034	p1571	PD379	54.1	PD197	49.0
PD342	1,282	p1571	PD360	46.8	PD359	42.2
PD118	1,360	p1344	PD310	2.0	PD311	5.3
PD116	1,372	p1344	PD309	2.4	PD203	5.0
PD119	1,567	p1344	PD312	1.5	PD204	4.5
PD120	2,189	p1344			PD365	5.6
PD207	2,557	p1962			PD323	4.6
PD211	2,751	p1962			PD193	3.8
PD216	2,930	p1962			PD196	3.1
			MC4100(λ1910)	44.2	MC4100(λ1962)	56.9
			MC4100	0.8	MC4100	0.8

[a] The effect of Tn5 insertions on the expression of L10 and β fusion proteins was examined in strain MC4100. Strains were grown exponentially in M9-glucose-Casamino Acids medium at 30°C, and β-galactosidase (β-Gal) activity per unit of bacterial mass was determined during mid-log phase as described in the text.

much lower level of β-galactosidase activity in both the L10-*lacZ* and the β-*lacZ* fusion lysogens. The activity is reduced by 90% or more compared with nonpolar insertions and control lysogens. These results demonstrate that a major promoter for both the L10 ribosomal protein gene and the β RNA polymerase gene is confined between nucleotides 1,280 and 1,360. A promoter-like sequence with an RNA start site at nucleotide 1,348 has been identified by in vitro RNA polymerase binding and in vitro transcription (24, 28).

The residual levels of β-galactosidase activity from λ1910 lysogens with polar insertions of Tn*5* in front of L10-*lacZ* fusion is about 5% of the control values. In contrast, the residual level of activity of the β-*lacZ* fusions is somewhat larger, representing 5 to 10% of the control values, and is dependent to some extent on the site of the Tn*5* insertion. The stronger degree of polarity of the two insertions at nucleotides 2,751 and 2,931 compared to upstream insertions may reflect the presence of a weak promoter in the L12-β intergenic space. Such a weak promoter functioning at about 2% of the level of the major β promoter has recently been identified and characterized (18). The physiological significance of this low-level promoter is not known.

Polarity of Tn*5* insertions on ribosomal protein gene expression. The pNF1344 plasmids containing Tn*5* insertions were introduced into strain CSR603 to directly examine the effects of insertion upon the expression of ribosomal protein genes. Strain CSR603 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 plasmid DNA (25).

Plasmid pNF1344 contains both the P_{L11} and the P_{L10} promoters and in the absence of any Tn*5* insertion specifies the synthesis of ribosomal proteins L11, L1, L10, and L12 and RNA polymerase β fragment protein (Fig. 5). 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 pNF1344 and in the Tn*5* insert at 688). This result corroborates previous observations that the L11 and L1 genes form a common transcription unit and that L1 regulates L11 synthesis (32).

Insertion of Tn*5* into the leader region of the L10 operon, at nucleotide 1,360 or 1,567, reduced the synthesis of L10, L12, and β fragment but had no effect on L11 and L1 synthesis. An insertion into the distal portion of the L10 gene,

at nucleotide 2,189, resulted in the synthesis of a protein containing 158 amino acids from the N-terminal end of L10 and 6 amino acids at the C-terminal end specified by the inverted repeat sequence of Tn*5*. This insertion also reduces the level of L12 and β fragment synthesis. Again, these results confirm that the expression of the genes specifying L10, L12, and β depends on a promoter sequence located in front of nucleotide 1,360.

Synthesis of L12 ribosomal proteins. High-copy-number plasmids which carry an active L10 gene require, in addition, an active L12 plasmid gene to prevent killing of the host cell. The killing effect has been attributed to overproduction or repressor activity of the L10 protein and has been observed for plasmids which contain *Eco*RI-generated deletions, removing the distal 50% of the L12 gene, or *Hind*III-generated deletions, removing the entire L12 gene and the distal 10% of the L10 gene (15). Apparently both the complete and the truncated L10 gene products 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 Tn*5* 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 2,557, have been obtained on the lower-copy-number plasmid pNF1962. In contrast, a relatively high number of Tn*5* insertions into the distal region of the L10 gene were unexpectedly obtained. 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.

In *E. coli* the L12 protein is partially acetylated at the N-terminal position before assembly into ribosomes (29). 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::Tn*5* or pNF1962::Tn*5* plasmids were introduced into strain JF1039. Transformants carry both a mutant chromosomal L12 gene and a wild-type plasmid L12 gene and thus have the potential to produce both mutant and wild-type L7 and L12 proteins. The effect of Tn*5* insertions on the synthesis of wild-type L7 and L12 was examined by labeling exponential-phase cells with [^{35}S]methionine (Fig. 6). The
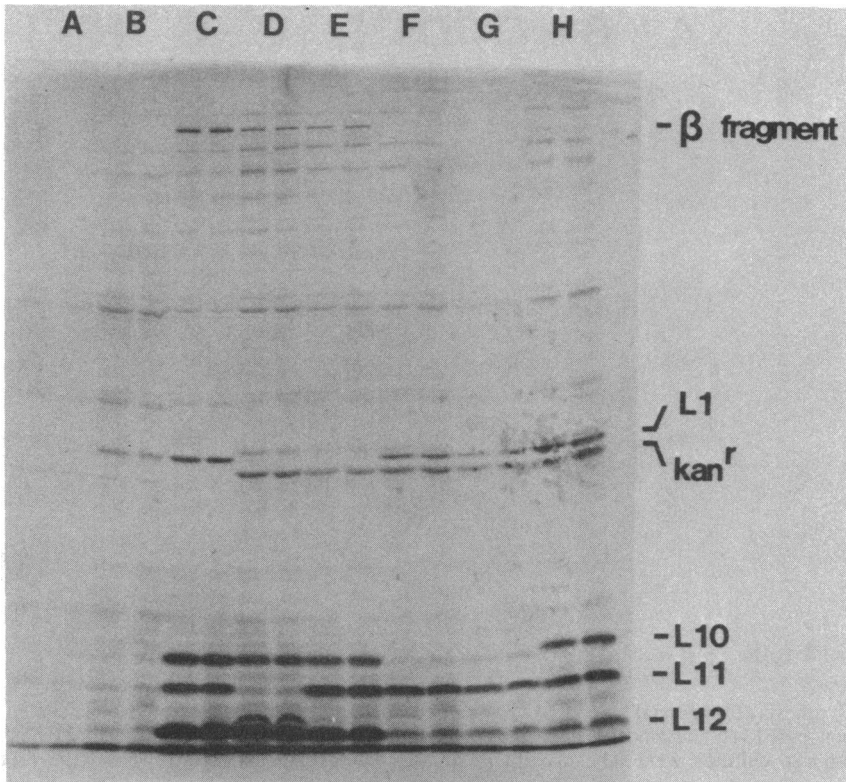
FIG. 5. Autoradiography of proteins labeled after UV inactivation of strain CSR603. Plasmid pNF1344 derivatives containing Tn5 insertion at various positions were introduced by transformation into strain CSR603. After inactivation of chromosomal genes by UV irradiation, proteins specified by plasmid genes were labeled with [^{35}S]methionine, fractionated by electrophoresis on 12.5% sodium dodecyl sulfate-polyacrylamide gels, and subjected to autoradiography. The ribosomal proteins L1, L10, L11, and L12 and the RNA polymerase β fragment are illustrated. Duplicate samples are as follows: A, CSR603; B, CSR603/pBR322; C, CSR603/pNF1344; D, CSR603/pNF1344::Tn5 (L11; nucleotide 495); E, CSR603/pNF1344::Tn5 (L1; nucleotide 688); F, CSR603/pNF1344::Tn5 (leader; nucleotide 1,360); G, CSR603/pNF1344::Tn5 (leader; nucleotide 1,567); H, CSR603/pNF1344::Tn5 (L10; nucleotide 2,189).

ribosomal proteins, including L7 and L12, were extracted with glacial acetic acid and separated on urea-polyacrylamide gels (13). In extracts from the parental JF1039 strain, only the mutant forms of L7 and L12 were apparent, whereas in strains containing 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 2,189 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. Plasmid pNF1962 lacks the L11 promoter. The Tn5 insertion in the intergenic region between L12 and

β (at nucleotide 2,751) on plasmid pNF1962 had no effect on the synthesis of the plasmid L7 protein. An insertion in the plasmid rplL gene (at nucleotide 2,557 on pNF1962) gave rise to a truncated protein with an altered mobility.

DISCUSSION

In an analysis of a large number of different pNF1344 Tet^r Kan^r plasmids generated from a single experiment, approximately 30% of the insertions were found to be into the 2.2-kb EcoRI fragment which contains the ribosomal protein genes. The DNA sequence analysis of the target sites indicated that insertions were not sequence specific (Table 2). However, the distribution of the insertions along the 2.2-kb fragment was clearly not random; no insertions were recovered in the intervals between nucleotides 700 and 1,350 or between 2,200 and 2,444 (Fig. 7). In total, we have examined more than 60
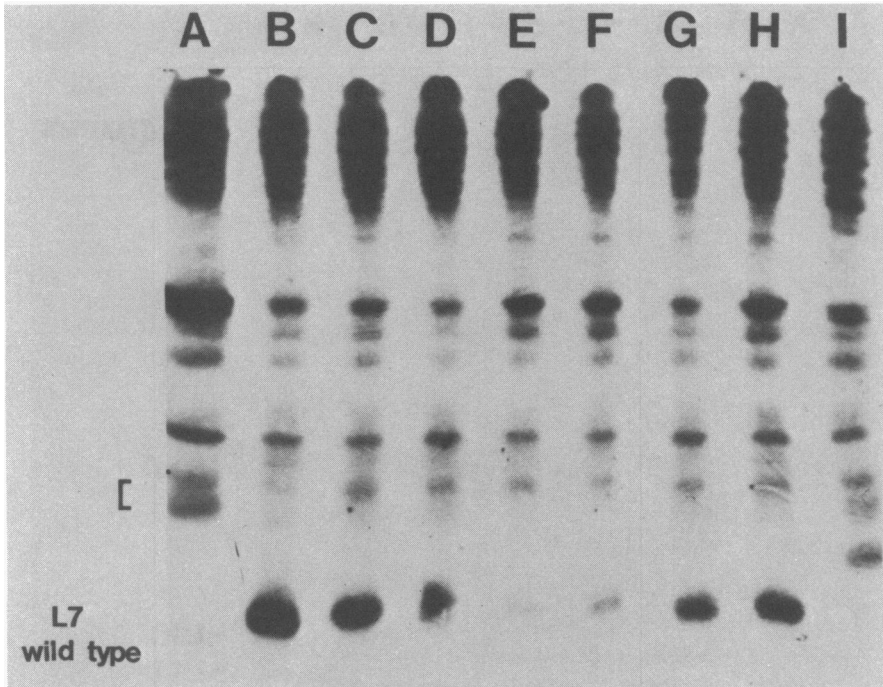
FIG. 6. Synthesis of ribosomal L7 and L12 proteins specified by plasmid and chromosomal copies of the *rplL* gene. Various pNF1344 and pNF1962 plasmids containing Tn*5* insertions were transformed into JF1039. Exponential-phase cultures were labeled with [³⁵S]methionine (10 μCi/ml) for 1 h, and the proteins were extracted and separated by electrophoresis on urea-polyacrylamide gels. The position of the plasmid-specified 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. These bands are not clearly resolved. Samples are as follows: A, JF1039; B, JF1039/pNF1344; C, JF1039/pNF1344::Tn*5* (L11; nucleotide 495); D, JF1039/pNF1344::Tn*5* (L1; nucleotide 688); E, JF1039/pNF1344::Tn*5* (leader; nucleotide 1,360); F, JF1039/pNF1344::Tn*5* (leader; nucleotide 1,567); G, JF1039/pNF1344::Tn*5* (L10; nucleotide 2,189); H, JF1039/pNF1962::Tn*5* (between L12 and β; nucleotide 2,751); I, JF1039/pNF1962::Tn*5* (L12; nucleotide 2,557).

independent insertions into the 2.2-kb fragment of pNF1344 generated in four separate experiments and have failed to identify insertions into these two intervals. This suggests that insertions into these regions may create an imbalance or disruption in the normal regulation and expression of the ribosomal protein genes and result in selection against such plasmid insertions.

Ribosomal protein L1 has been identified as the regulatory protein for the L11-L1 transcriptional unit (8, 32). Consistent with this is the observation that the Tn*5* insertional inactivation of the L1 gene on plasmid pNF1344 results in about a twofold elevation in the synthesis of L11 protein relative to that of the pNF1344 control (Fig. 5). The position of this Tn*5* 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 6 amino acids at the C-terminal end specified by the inverted repeat sequence of Tn*5*. That this plasmid is apparently not detrimental to the host cell implies that

expression of an 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 twofold (12, 26).

Over 60 independent Tn*5* insertions into the 2.2-kb *Eco*RI fragment on pNF1344 have been screened for insertions within the 702-nucleotide-long L1 gene; only one insertion at nucleotide 688 has been identified. The additional Tn*5* insertions which occur further downstream in the L1 gene at nucleotides 971, 1,034, and 1,282 were isolated on plasmid pNF1571; this plasmid lacks the promoter for the L11 operon and a portion of the L11 gene (Fig. 1). This failure to isolate more inserts of Tn*5* 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 syn-

thesis of L11 but not be 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. Such insertions would result in a peptide 102 amino acids in length, the proximal 98 amino acids being identical to the L1-proximal sequence.

Regulation of the L10 transcription unit. The expression of an L10 gene in the absence of an 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 HindIII-generated deletions, which remove the entire L12 gene and the C-terminal 19 codons of the L10 gene. These observations suggest that the L10 plasmid-specified 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 plasmids. However, 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 plasmid L12 expression in such instances? A previous study has identified a weak promoter located within 186 nucleotides of the ends of the inverted repeats of Tn5 (5). This promoter presumably accounts for the residual 5 to 10% level of β-galactosidase activity in the L10-lacZ and β-lacZ fusion lysogens carrying polar Tn5 insertions (Table 3). Yates and co-workers (31) have suggested that the fourfold-elevated level of translation of the 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 the L12 mRNA sequence. The insertion of Tn5 into the distal region of the L10 gene 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 low-level promoter within the Tn5 element would be very efficiently translated. Therefore, the continued expression of the L12 protein would be a consequence of (i) the low level of transcription from the Tn5 promoter and (ii) the efficient translation of the L12 mRNA sequence. This would result in sufficient synthesis of L12 to compensate for L10 fragment synthesis.

It seems unlikely that the elevated levels of L12 expression observed in these inserts into the distal end of the L10 gene are the result of increased transcription of the L12 gene because all inserts into the L10 operon, irrespective of position, give essentially the same low level of β-galactosidase activity in the λ1962 β-lacZ fusion lysogens. Examination of the nucleotide sequence of the L10 operon has failed to reveal
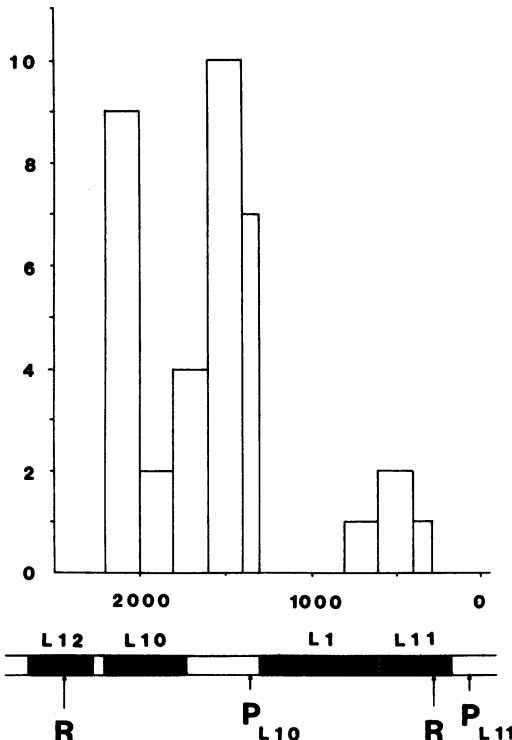

FIG. 7. Distribution of Tn5 insertions in the 2.2-kb EcoRI fragment of plasmid pNF1344. About 30% of the Tn5 insertions into plasmid pNF1344 in a single analysis were found on the 2.2-kb EcoRI fragment. The transpositional events do not appear to be random; in fact, there were three clusters of Tn5 insertions around nucleotide positions 280 to 600, 1,300 to 1,500, and 2,000 to 2,200. No Tn5 insertions were found in the middle and the distal portion of the L1 gene, from nucleotides 700 to 1,300, or in the L12 gene. The horizontal axis of the graph is the nucleotide position within the 2.2-kb EcoRI fragment as indicated.

any obvious region which could interact with and sequester the L12 initiation region.

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