Amplified Synthesis and Stability of Tn5 Polypeptides in *Escherichia coli*

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대장균에서의 Tn5 단백질 증폭생합성 및 안정성

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ABSTRACT: Plasmid DNA molecules containing strong promoter upstream from IS50L or IS50R, the two insertion sequences that flank Tn5, were constructed to amplify the synthesis of Tn5-encoded polypeptides. When proteins made by cells that contain these plasmids were analyzed on polyacrylamide gels, enhanced synthesis of IS50R polypeptides could be detected. Synthesis of this polypeptide apparently is initiated within the large open reading frame of this element. In addition, the stability of IS50L-and IS50R-encoded polypeptides was analyzed. It was found that IS50L polypeptides are relatively unstable *in vivo*. This instability could account for the observed inability of this element to promote transposition.

KEY WORDS ⊐ Tn5 polypeptides; IS50; transposase; protein stability; tryptic peptides.

Transposition is a homology-independent recombinational process by which a discrete segment of DNA moves from a site to various sites. It does not require involvement of *rec*A protein in *Escherichia coli*. Instead, transposition process is generally dependent on a transposase that is usually encoded by the transposable element itself.

Tn5 is a prototype bacterial transposon that carries a kanamycin resistant gene. It contains 5,820 nucleotides that include two inverted repeat IS50 sequences at its terminal ends (IS50L and IS50R in Fig. 1).

IS50R encodes functions absolutely required for Tn5 transposition while IS50L is non-functional with regard to transposition (Rothstein *et al.*, 1980; Berg *et al.*, 1980). Proteins encoded by Tn5 have subsequently been identified by experiments using minicells containing various parts of the Tn5 DNA sequence (Rothstein *et al.*, 1980; Rothstein and

Reznikoff, 1981). Five polypeptides by Tn5 have been visualized; two from IS50R, two from IS50L, and one from the flanking region. Tryptic digest patterns of IS50 polypeptides (this work) as well as DNA sequence data (Auersward *et al.*, 1980), have confirmed that IS50 polypeptides are translated in the same reading frame. P3 and P4 are truncated versions of P1 and P2 respectively, resulting from an ochre mutation in IS50L at residue 1443. This mutation simultaneously creates a good promoter sequence for the NPTII transcript. Thus, the functional difference between IS50L and IS50R results from the single base pair nonhomology between these elements (Rothstein and Reznikoff, 1981).

RNA polymerase binding experiments with restriction fragments of Tn5 DNA have identified three sites of DNA-RNA polymerase complex formation (Rothstein *et al.*, 1980). These sites are
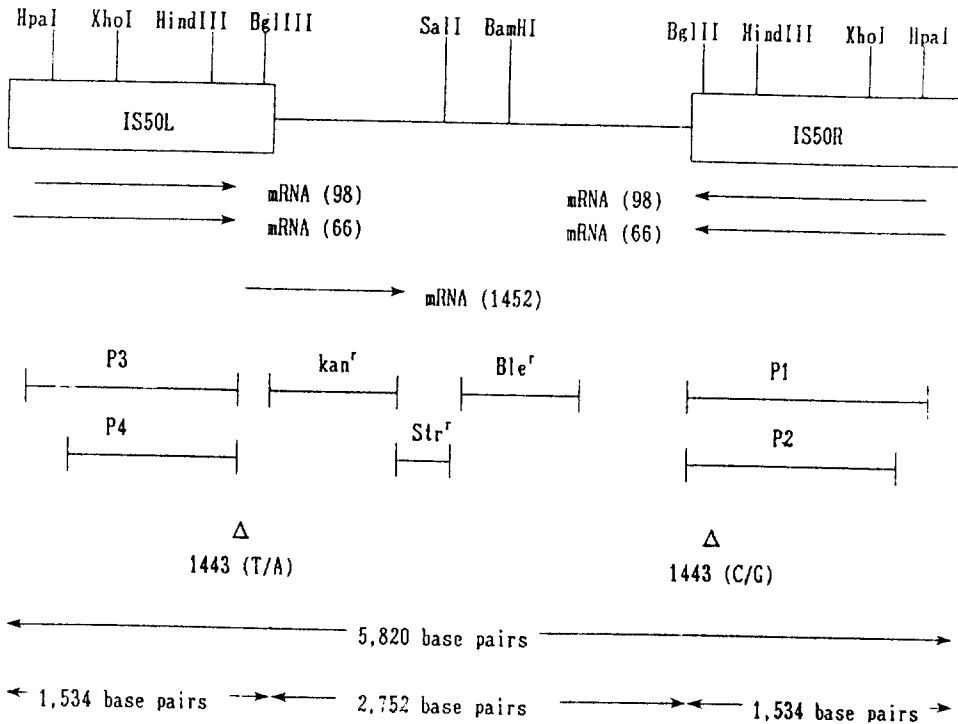
Fig. 1. *Physical and genetic structure of Tn5.*

Tn5 contains two inverted repeats of IS50 elements at its ends. These IS50's flank a sequence that encodes resistance to kanamycin, streptomycin, and bleomycin. However, only kanamycin resistance is expressed in *Escherichia coli*. Arrows indicate the transcripts synthesized from Tn5. Numbers in parentheses indicate the starting position of mRNA synthesis. Also indicated by bars are open reading frames deduced from the DNA sequence as well as tryptic peptide mapping. Triangles are the position of single base difference between IS50L and IS50R.

located near the two ends of Tn5 and at the NPTII promoter. Transcriptional initiation sites of three major messages were determined at the nucleotide sequence level by *in vitro* transcription experiments using Tn5 DNA as a template (Johnson and Reznikoff, 1981). There are two mRNA's starting from 98 position of both IS50's and NPTII mRNA starting from 1,452 position of IS50L. It was later found that two additional transcripts starting from position 66's of IS50L and IS50R are also synthesized at a reduced level (Krebs and Reznikoff, 1986). It is now believed that P1 and P3 are synthesized from mRNA whose 5' end is at position 66 of IS50R and IS50L, respectively, using AUG's at 93-95 as initiation codons while P2 and P4 are synthesized from mRNA whose 5' end is at position 98 of IS50R and IS50L, respectively, using AUG's at 258-260 as initiation codons.

Tn5 transposition is regulated by a diffusible negative regulator. The frequency of Tn5 transposition from newly introduced phage DNA onto the *Salmonella typhimurium* chromosome is dramatically reduced if the cell already contains Tn5 (Biek and Roth, 1980). Subsequently, it has been shown that the transposition frequency of Tn5 DNA newly introduced into cells already containing P2 is severely reduced. It has thus been concluded that P2 is a *trans*-acting inhibitor of Tn5 transposition. The inhibition is Tn5-specific and is exerted at neither the transcriptional nor translational level. Rather, P2 inhibits transposition by inhibiting the activity of the transposase enzyme (Johnson *et al.*, 1982; Isberg *et al.*, 1982).

The current understanding on the mechanism of Tn5 transposition is not as clear as that of Tn3 family or bacteriophage Mu in which *in vitro* systems for assaying the activities of the purified

transposases have been developed. Thus, in this study, plasmids have been constructed that synthesize large amounts of Tn5 polypeptides upon induction of a strong promoter, P_L, on the plasmids. These plasmids could be used for the purification of the Tn5 transposase.

It has been shown that these plasmids synthesize more P2 than P1 in both whole cells and minicells. It has been suggested that this differential synthesis of P1 and P2 is due to differential transcriptional and translational efficiencies. In addition, a pulse and chase experiment has been performed to determine the stability of IS50 polypeptides. It has been shown that IS50L polypeptides are not as stable as IS50R polypeptides. It has thus been proposed that the inability of IS50L to catalyze transposition is due to the stability of IS50 polypeptides.

MATERIALS AND METHODS

Bacterial strains

Escherichia coli K12 derivatives were used throughout the experiments. MO (F-, *rps* L, *thi*) is isogenic with Hfr Hays (Maquat and Reznikoff, 1978). Mo (λ+) is a wild type λ PAPA lysogen of MO. ΔH1 (M72 F*lac rps*L, cI857 *N7N53* ΔHI*bio*; Bernard *et al.*, 1979)was used to control the expression of genes in P_L-containing plasmids. A *mal+* derivative of the minicell strain 984 (F-, *thi, ara, leu, lac, gal, mal, min*A, *min*B, *rps*L; Frasier and Curtiss III, 1975) was constructed by P1 transduction. When P_L-containing plasmids were maintained in the minicell strain, the λ *cI857* Sam7 lysogen of the *mal+* minicell strain was used as a host.

Media and drugs

Bacterial strains were routinely grown in LB media (Miller, 1972). When needed, AB supplemented with glucose (0.48% w/v) and vitamin B1 (0.5 ug/ml) were used (Miller, 1972). Solid media were prepared with 1.5% agar (Difco). Concentrations of antibiotics in the media were (per ml); kanamycin 50 ug, tetracycline 10 ug, and streptomycin 100 ug.

Enzymes and buffers

Restriction enzymes used throughout these experiments were purchased from Bethesda Research Laboratories and New Elgland Biolabs.

Buffers for the enzymes used in this study were as described by Maniatis *et al.* (1982) or technical data sheets from the enzyme manufacturers.

TE buffer; 10 mM Tris-HCl, pH 7.8 and 1 mM EDTA. 1X DNA gel buffer for agarose gel electrophoresis; 20 mM Tris-acetate, pH 8.0, 7.3 mM NaCl, 10 mM Na $(OAc)_2$ 2 mM Na_2EDTA. 1X DNA gel buffer for acrylamide gel electrophoresis; 80 mM Tris-borate, pH 8.0, 2 mM Na_2EDTA, pH 8.0.

Plasmid purification

The alkaline denaturation method (Birnboim and Doly, 1979) or the boiling method (Holmes and Quigley, 1981) was used for small scale plasmid DNA purification. In some cases, Sephadex G-50 (Maniatis *et al.*, 1982) or Elutip-d columns (Schleicher and Schuell) were used for further purification. A method by Humphreys *et al.* (1975) was used for large scale plasmid DNA purification.

Analysis of polypeptides by labelling with 35 [S]-methionine

E. coli strain ΔH1 (Castellazzi *et al.*, 1972) containing plasmid DNA was grown overnight to saturation in AB glucose medium supplemented with vitamin B1, biotin, and amino acids (0.5 mM each), except methionine, diluted 50 fold in the same medium, and shaken at 30° for 1 hr. A one ml aliquot of the culture was transferred to a flask in a 42° shaker, and a thermal induction was performed for 10 min. The induced sample was then labelled with 20 uCi of 35[S] methionine for 2 min and an excess of nonradioactive methionine was added to stop labelling. The incorporation of radioactive methionine was measured using a scintillation counter. The cells were washed with 0.05 M Tris-HCl, pH 8.0, and the pellet was resuspended in 1X sample buffer (10% gylcerol, 0.7 M 2-mercaptoethanol, 3% SDS and 8 mM Tris HCl, pH 6.8). The sample was placed in boiling water for 2 min and an appropriate amount of radioactivity of the sample was loaded SDS polyacrylamide gel. Nonradioactive samples were prepared by the procedure of Lindahl *et al.* (1977). Electrophoresis on a $300 \times 140 \times 1.5$ mm polyacrylamide slab gel in the presence of SDS was performed as described by Laemmli (1970). Fifteen, 10 and 8.75% acrylamide gels were used. The gel was dried on a slab gel dryer and autoradiographed on Kodak X-ray film SB-5.

Purification and radiolabelling of minicell strain.

Purification: The *mal+* minicell strain with or without λ *cI*[857] *Sam7* was transformed with various plasmids. A single colony from a selection plate was used to purify minicell buds. The previously described procedures for minicell purification (Roozen *et al.,* 1971) was modified as follows. Two hundred fifty m*l* of LB in a 2 liter flask was inoculated with 5 m*l* of overnight culture and shaken for 7-9 hrs at 30° or at 37°. Succeeding procedures were done at 4° unless otherwise mentioned. The culture was centrifuged briefly (3,000 rpm, 5 min in GSA) and the pellet was discarded, in order to enrich for minicell buds. The minicell buds were pelleted in a GSA rotor (8,500 rpm, 15 min), resuspended in 3 m*l* TSG (20 mM Tris-HCl, pH 7.4; 0.85% NaCl and 0.01% gelatin), and layered on a sucrose step gradient (5, 12 and 20% in sucrose gradient buffer; 0.05 M Tris, 0.01% SDS). After centrifugation in the Beckman SW27 rotor at 5,000 rpm for 15 min, a minicell band from the 5%/12% interface was collected, diluted two fold with TSG and centrifuged at 11,000 rpm for 15 min in a Sorvall SS34 rotor. The pellet was resuspended into 4 m*l* of TSG and layered again on a sucrose step gradient. After another centrifugation and wash, the concentration of minicell buds was adjusted to O.D.$_{620}$ = 0.2 with AB glucose supplemented with vitamin B1 and amino acids (0.1 mM each) except methionine. A 4 m*l* aliquot of the purified minicell preparation was transferred to 25 m*l* flask and shaken at 37° for 1 hr in the presence of 100 ug/m*l* ampicillin, which selectively kills any remaining contaminating cells, while sparing the non-dividing minicell buds. One m*l* of the pre-incubated minicell culture was labelled with 25-50 uCi of [35][S] methionine for 2 min and labelling was stopped by adding an excess of nonradioactive methionine. When P$_L$-containing plasmids were tested, pre-incubation was performed at 30° for 1 hr and the sample was split into an appropriate number of flasks for the labelling of the polypeptides synthesized by the thermal induction of P$_L$. For the preparation of the induced samples, minicells were shaken at 42° for 5 min to equilibrate the temperature of the flask before labelling. The labelled cells were transferred to a 3 m*l* pyrex tube

on ice, and centrifuged in an SS34 rotor at 11,000 rpm for 10 min. The pellet was washed once with 0.05 M Tris-HCl, pH 8.0, and resuspended in 100 u*l* of 1X SDS sample buffer. In some cases, however, samples were prepared by DNase and RNase treatment as described above. Samples were applied on an SDS polyacrylamide gel after 2 min in boiling water.

**Tryptic digest of radioactive polypeptides**

The band on a dried polyacrylamide gel was cut out using autoradiogram as a guide. The tryptic peptides of the band were prepared as described by Lindahl *et al.* (1977) and separated by paper ionophoresis on 57 × 46 cm Whatmen 3 MM paper in 5% acetic acid, 0.05% pyridine (pH 3.5). The paper was electrophoresed for 4,600 volt hr, cut into 3 × 1 cm strips and the radioactivity was determined in a scintillation counter.

RESULTS

Construction of plasmids that overproduce Tn5 polypeptides

To construct plasmids that contain a strong promoter upstream from IS50 sequences, a restriction fragment of λ kanl DNA which contains Tn5 downstream from P$_L$ was cloned into pBR322, and restriction fragments of the cloned plasmid were rearranged in appropriate orientations for the stimulated synthesis of IS50R or IS50L polypeptides.

The plasmid, pKPG12, was constructed by cloning the *Eco*RI fragment of λ kanl (Berg *et al.,* 1975) that contains the kanamycin resistance gene into the *Eco*RI site of pBR322 (Fig. 2). Kanamycin resistance conferred by this plasmid is temperature sensitive in cells containing a temperature sensitive repressor, presumably because amplified transcription from P$_L$ interferes with replication of the plasmid at 42° (Bernard *et al.,* 1979). Restriction analysis proved that the orientation of Tn5 in pKPG12 is IS50R-IS50L-P$_L$ (data not shown). Thus, Tn5 was oriented on λ kanl and pKPG12 such that the known genes of IS50L encoding P3 and P4 could be transcribed from P$_L$. Because there is only a single base pair difference between the IS elements of Tn5 at base pair 1443 (Auersward *et al.,* 1980), inverting the Tn5 *Hind*III fragment would functionally change the orientation of the
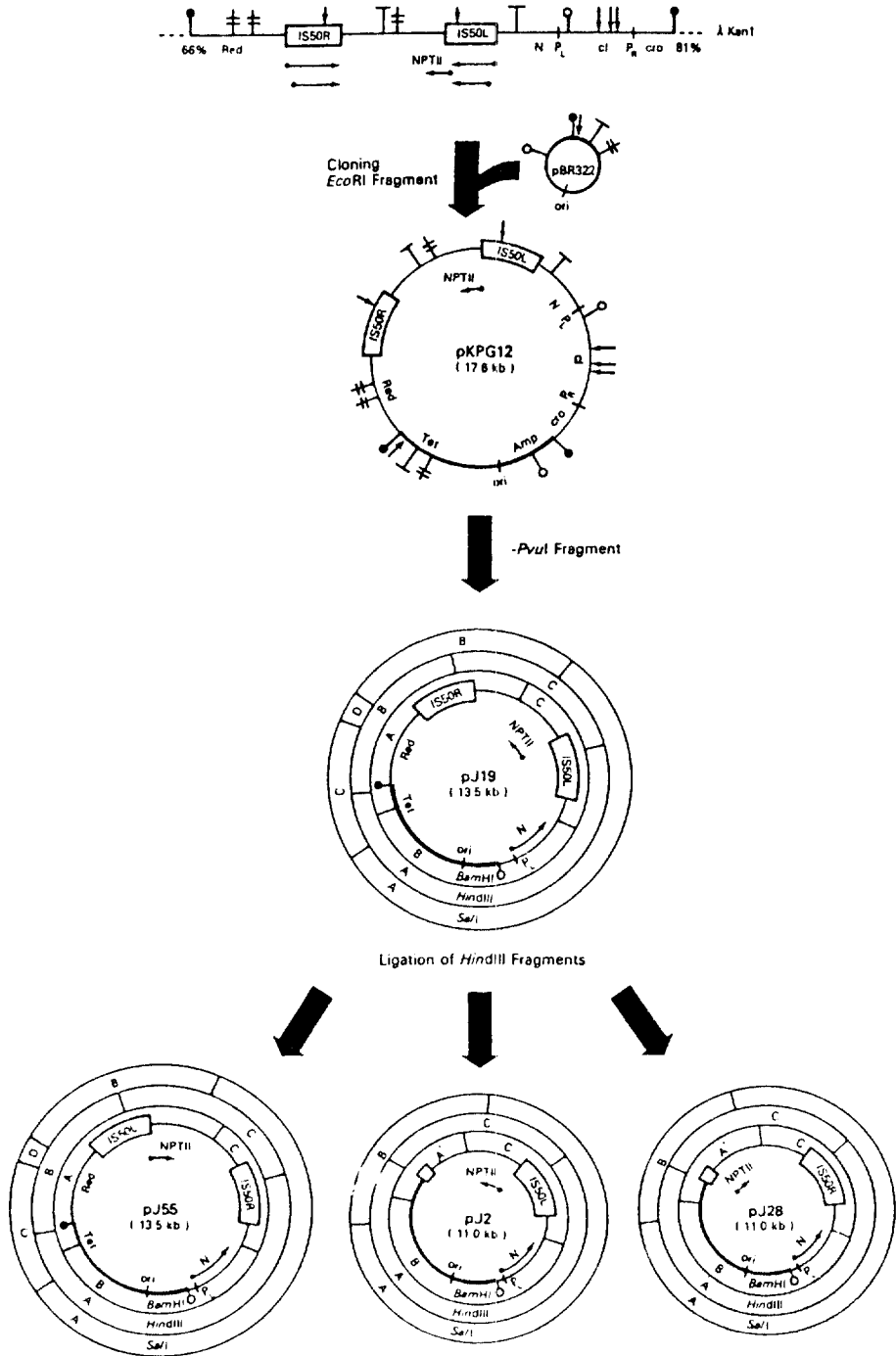
Fig. 2. *Construction of pKPG12 and restriction map of resulting plasmids.*

The kanamycin resistance gene in the 7.5kb EcoRI fragment of λ kanl DNA was cloned into the EcoRI site of pBR322. The plasmid pKPG12 was then digested with PvuI and ligated to eliminate the P_R containing fragment. The resulting plasmid, pJ19, was digested with HindIII and ligated to obtain different combinations of HindIII fragments. pJ2 and pJ19 have IS50L downstream from P_L, while pJ28 and pJ55 have IS50R downstream from P_L (T, BamH; ‡, SalI, ↓, HindIII; ●, EcoRI; ○, PvuI).

entire Tn5 in pKPG12. The 4.3 kb *Pvu*I fragment of pKPG12 was eliminated to facilitate the rearrangement of the *Hind*III fragment, since this 4.3 kb piece has three *Hind*III sites. Additionally, this elimination removed the *cl* and *cro* genes. The resulting plasmid has been designated pJ19 (Fig. 2).

The deletion palsmid pJ19 contains three *Hind*III fragments, each of which contains a different selectable marker. These are a 6.8 kb fragment containing the origin of pBR322 replication (fragment A), a 3.3 kb fragment containing kanamycin resistance (fragment C), and a 3.2 kb fragment containing the promoter for the *tet* protein (fragment B). A *Hind*III digest of pJ19 was religated, and kanamycin resistant transformants were isolated. A plasmid designated pJ55 was obtained that is identical to pJ19 except that the *Hind*III C fragment has been inverted so that P_L is now able to direct trascription into IS50R. At the same time, two other plasmids were also obtained that contain the pJ19 *Hind*III A and *Hind*III C fragments but lack *Hind*III B. These plasmids are pJ2 and pJ28 in which P_L directs transcription into IS50L and IS50R, respectively.

Fig. 2 illustractes the genetic and restriction maps of these four plasmids. pJ2 and pJ28 are tetracycline sensitive because the promoter for the *tet* protein is missing in these plasmids. The orientation of each *Hind*III fragment in each plasmid was determined by restriction enzyme analysis (data not shown). pJ2 has the same orientations of the 3.3 kb *Hind*III C fragment as pJ19, while pJ28 and pJ55 have different orientations of the 3.3 kb *Hind*III C fragment. Two classes of plasmids, therefore, were obtained; one for the transcription of IS50L from P_L (pJ2 and pJ19) and the other for the transcription of IS50R from P_L (pJ28 and pJ55).

Stimulated synthesis of polypeptides P1 and P2 upon thermal induction of P_L

Since these plasmids contain a strong promoter, P_L, upstream from IS50 sequences, cells containing these plasmids would be expected to synthesize IS50 polypeptides upon derepression of P_L. The stimulated synthesis of IS50 polypeptides has been demonstrated on a polyacrylamide gel in this experiments.

The *E. coli* K12 strain ΔHI (Bernard *et al.*, 1979) contains the thermolabile *cl*857 allele on a cryptic prophage in the chromosome. This strain was used to control the P_L promoter on the plasmids, pJ2, pJ19, pJ29, and pJ55. The synthesis of P2 was only detected in the ΔHI (pJ28) sample that was induced when whole cells were labelled with 35[S] methionine. However, no distinct P2 bands appeared in the ΔHI (pJ55) induced sample, although pJ55 has the same structure as pJ28 with respect to the P1 and P2 reading frames (data not shown). One possible explanation for this result is that the putative secondary structure of the pJ55 message interferes with its translation. To be specific, the pJ55 transcript initiated at P_L contains the IS50 sequences complementary to the IS50R message. The message would be expected to extend into the *tet* gene region since the plasmids contain the antiterminator N protein. The formation of a secondary structure by pairing of the complementary IS50 region in the transcript might inhibit translation of the IS50R message. This explanation is compatible with the appearance of P1 and P2 bands in the induced ΔHI (pJ28) sample, because no secondary structure could inhibit the translational initiation of the P1 and P2 message in the pJ28 transcript from P_L. A similar result was obtained when unlabelled proteins synthesized following the induction of P_L were electrophoresed and stained with Coomassie blue (Fig. 3). This experiment was intended to show the accumulation of IS50R polypeptides as well as the stability of these polypeptides.

Part of the inverted IS50L is still present on pJ28 (Fig. 2). In order to test whether this remaining complementary region also decreases the synthesis of P1 and P2, the *Sal*I fragment containing IS50L sequences in pJ28 was inverted, generating pJ28ISal. It was found that the amounts of P1 and P2 in the induced ΔHI (pJ28 ISal) sample did not exceed those in the induced ΔHI (pJ28) sample (Fig. 3, lane 13 and 10, respectively). Thus, the remaining inverted IS50L sequences in pJ28 did not appear to significantly decrease the synthesis of P1 and P2.

Neither the ΔHI (pJ2) nor the ΔHI (pJ19) induced samples exhibited an accumulation in the synthesis of P3 or P4. This result is probably due to the instability of these proteins which will be ex-

1   2 3 4 5 6 7 8 9 10 11 12 13   14

P1
P2

P3
P4
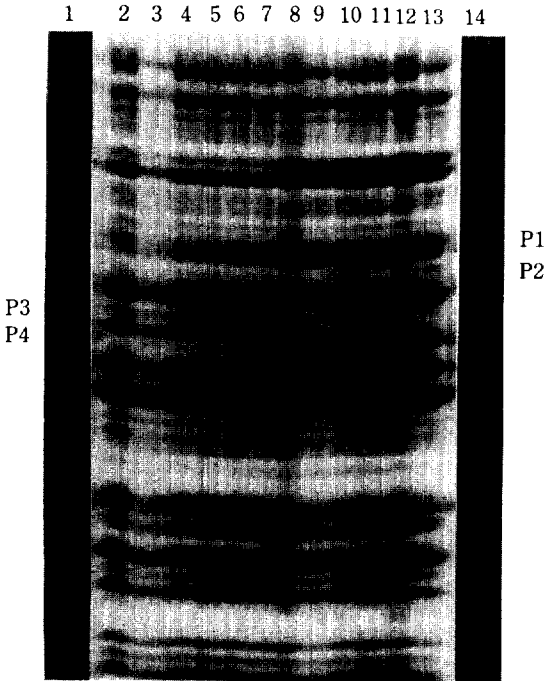
Fig. 3. *Accumulation of IS50R polypeptides synthesiz-ed in vivo.*

E. coli ΔH1 cells with and without plasmids were induced at 42° for 20 min, and grown at 37° for 2 hrs. Cell extracts were electrophoresed in 8.75% SDS polyacrylamide gel. Lane 1 and 14 indicate positions of IS50 polypeptides as re-ferenced from ^{35}S-labelled pJ2 and pJ28 poly-peptides, respectively, for size markers. Lanes 2 through 7 are uninduced samples from 2)ΔH1, 3) ΔH1 (pJ2), 4)ΔH1 (pJ28), 5)ΔH1 (pJ19), 6)ΔH1 (pJ55), 7)ΔH1 (pJ28ISal). Lane 8 through 13 are induced samples from 8)ΔH1, 9)ΔH1 (pJ2), 10) ΔH1 (pJ28), 11)ΔH1 (pJ19), 12)ΔH1 (pJ55), 13) ΔH1 (pJ28ISal).

plained in the next section.

Synthesis and stability of Tn5 polypeptides in minicells

Because the polypeptides P3 and P4 are ochre derivatives of P1 and P2, respectively, P3 and P4 might be expected to be unstable. This instability of P3 and P4 could readily explain the failure to detect accumulated bands of IS50L polypeptides. Therefore, in this experiment, the relative amounts of IS50 polypeptides synthesized in minicells con-taining pJ2, pJ28, pJ19, pJ55 and pJ55red were visualized and their stability was examined by a pulse and chase experiment.

Minicells containing various plasmids were purified as described in Materials and Methods, pregrown at 30° for an hr and labelled with [35][S] methionine. For pulse labelling, samples were shaken at 42° for 5 min before adding [35][S] methionine. Chases were done at 42° for 30, 50, 100, or 200 min after the addition of an excess of nonradioactive methionine.

An additional plasmid, pJ55red, was con-structed and the plasmid was used for identifying proteins since it is missing two adjacent SalI fragments of pJ55 which extent from the *red* gene of λ to the middle of the *tet* gene of pBR322.

Fig. 4a is an autoradiogram of 8.75% SDS polacrylamide gels. Each three lane set contains uninduced, 5 min pulse, and 5 min pulse plus 30 min chase samples. Fig. 4b is an autoradiogram of a longer pulse and chase experiment with minicells containing pJ2 and pJ28, showing the stability of each polypeptide encoded by the plasmids. Chases up to 200 min were performed to determine the stability of IS50 polypeptides. The synthesis and stabilities of proteins appeared as follows.

1) IS50 polypetides

Two polypetides encoded by each IS50 have been identified in minicell experiments (Rothstein *et al.*, 1980), P1 and P2 from IS50R, P3 and P4 from IS50L. The translational reading frames of these IS50 polypeptides have been deduced from the sequences of the IS50 elements (Auerswald *et al.*, 1981).

Fig. 4a shows that all 4 polypeptides encoded by the IS50 elements on pJ2, pJ28, pJ19, pJ55, and pJ55red appeared even in uninduced samples as well as in induced samples. The appearence in unin-duced samples is presumably due to IS50's own constitutive promoter. There was an appreciable increase in the synthesis of these polypepdies upon induction. As was expected, the polypeptides P3 and P4 are very unstable, while P1 and P2 are not (compare lanes 2 and 7 with lanes 5 and 10, respec-tively, of Fig. 4b). In general, the smaller the sizes of the plasmids, the more intense the protein bands. For example, the relative sizes of the plasmids are pJ19/pJ55 > pJ55red > pJ2/pJ28 and the relative intensities of polypeptide bands was pJ19/pJ55 < pJ55red < pJ2/pJ28. A possible inter-pretation of this observation is that the genes of
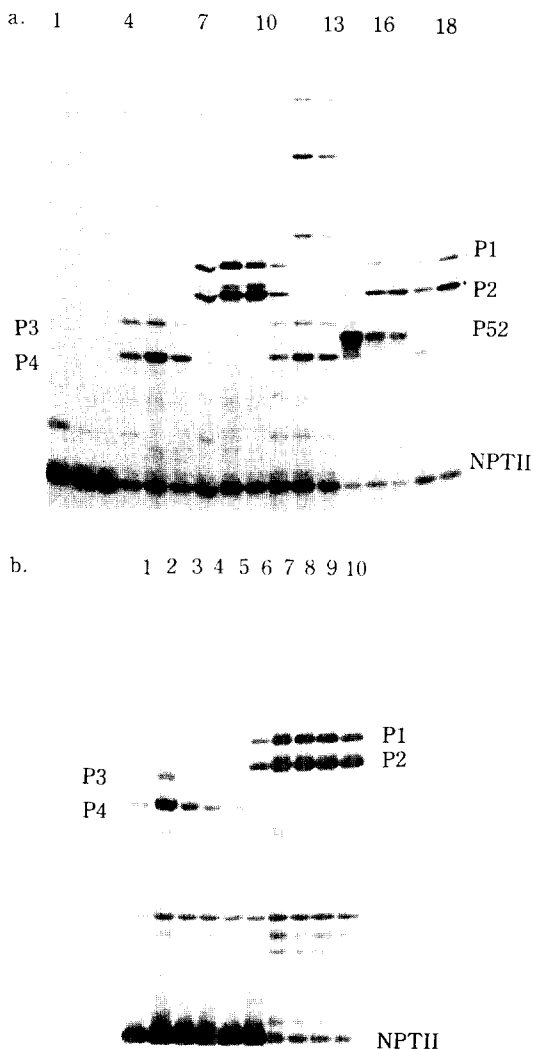
a.

b.

Fig. 4. Autoradiogram of 8.75% and 10% polyacryl-
amide gels of minicell samples.
a. Purified minicells containing plasmids were
labelled after 1 hr incubation at 30 °C. Pulse
labelling was performed 5 min after incubation
at 42 °C. Chase samples were perpared by in-
cubation at 42 ° for 30 min after stop-labelling.
Each three lane set contains minus-induction,
pulse, and chase samples. 1)-3), -plasmid; 4)-6),
pJ2; 7)-9), pJ28; 10)-12), pJ19; 13)-15), pJ55;
16)-18), pJ55red. (pJ55 red is a deletion deriva-
tive of pJ55 that has lost SalI C and D
fragments.). b. Minicells containing pJ2 and
pJ28 were pulse labelled and chased for 50, 100,
and 200 min. Lanes 1)-5); pJ2 samples 1)- induc-
tion, 2) pulse label, 3) 50 min chase, 4) 100 min
chase, 5) 200 min chase. Lanes 6)-10); pJ28
samples 6)-induction, 7) pulse label, 8) 50 min
chase, 9) 100 min chase, 10) 200 min chase.

large plasmids are not as effectively expressed as
small plasmids. Another observation is that the
derepression of P_L greatly reduced the synthesis
of polypeptides whose transcriptional orientations
are opposed to P_L. For example, the P1 and P2
bands of pJ19 almost disappeared upon derepres-
sion of P_L. It should pointed out that transcription
initiated from P_L overcomes the strong transcrip-
tional termination sites within Tn5 with the aid of
N gene product. This is based on observations that
downstream genes beyond Tn5 was not express-
ed when N gene was truncated (data not shown).

2) NPTII

Neomycin phosphotransferase II consists of 264
amino acids (Beck et al., 1982) and its estimated
size by mobillity on an SDS polyacrylamide gel is
26,000 daltons (Rothstein et al., 1980). The pro-
moter for NPTII transcription is between the Hin-
dIII and BglII sites of IS50L (Fig. 2). Therefore,
it was expected that NPTII synthesis would be
stimulated by induction of P_L in pJ19 and pJ2.

This polypeptide was easily identified in
polyacrylamide gels (Fig. 4a and 4b). As was true
of P1 and P2 in pJ19, its synthesis was decreased
by the induction of P_L in pJ28 or pJ55. On the
other hand, there was an increase in NPTII syn-
thesis upon induction of pJ2 (compare lane 1 and
2, Fig. 4b). Also the polypeptide was shown to be
quite stable upon 200 min chase.

3) P52

Another polypeptide of unknown origin was
found whose estimated molecular weight on a
polyacrylamide gel is 52,000 dalton (P52 in pJ55
samples of lanes 13, 14 and 15 in Fig. 4a). Because
there was no possible translational reading frame
to accomodate this large polypeptide in pJ55, an
explanation could be that P52 might be a degrada-
tion product of P1 or P2.

To test possibility, P52 bands along with P1,
P2 and P4 bands were eluted from the gel and in-
cubated with trypsin. Ionophoresis of tryptic
digests of these polypeptides on 3 MM Whatman
Paper was cut into strips at 1 cm intervals and the
pattern of P52's tryptic peptides was shown to be
different from that of other protein's tryptic pep-
tides, while P1, P2 and P4 share a similar pattern
(Fig. 5). This figure not only demonstrates that P52
is not related IS50's polypeptides but also direct-
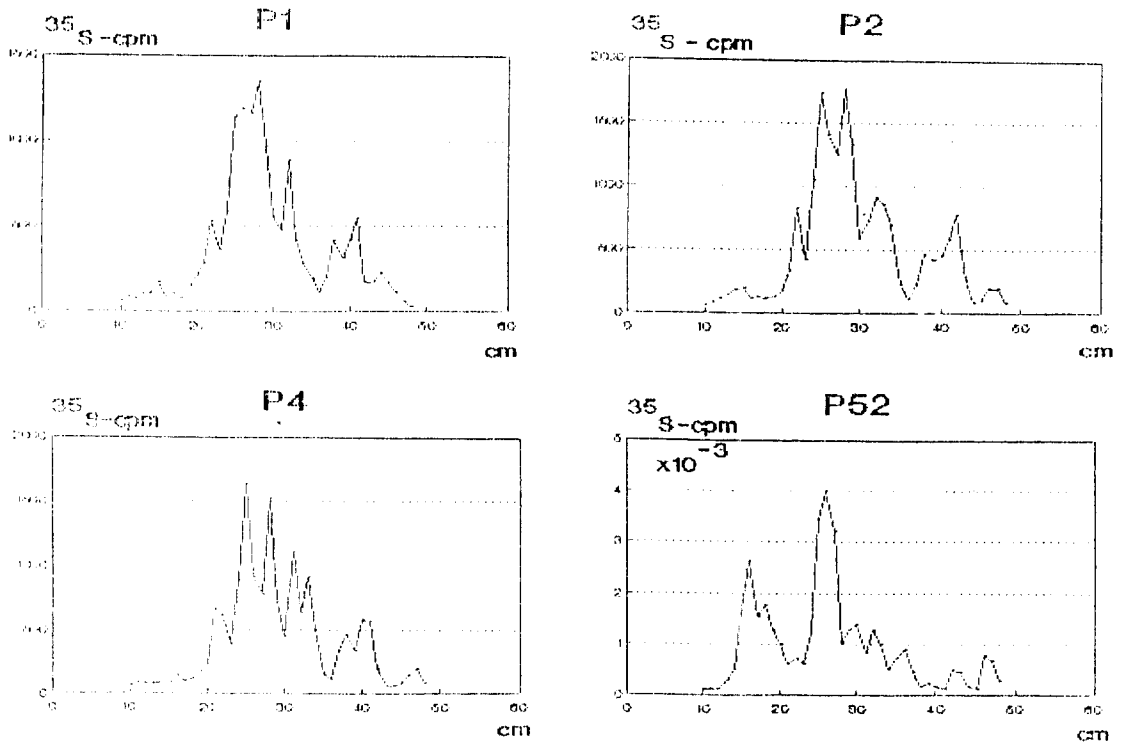ly confirms that the IS50 polypeptides are

Fig. 5. *Tryptic digests of P52 and IS50 polypeptides.*
The ^{35}S-methionine labelled polypeptides were eluted from dried gels and digested with trypsin. The digested peptides were separated by paper ionophoresis, cut into strips and radioactivities were determined. P52 is distinct from P1, P2, and P3, suggesting that P52 is not related to IS50 polypeptides.

translated from the same reading frame. However, the origin of P52 was not studied further.

DISCUSSION

Plasmids have been constructed that direct the synthesis of appreciable amounts of IS50R polypeptides *in vivo*. The ΔH1 strain containing pJ28 or pJ28ISal exhibits accumulated synthesis of IS50R polypeptides, when a strong promoter, P_L, on the plasmid is derepressed by temperature shift (Fig. 4a). This regulatable synthesis was further confirmed by minicell experiments (Fig. 4b). Thus, these plasmids could be valuable tools for purifying Tn5 transposase, P1, and its inhibitor, P2, for *in vitro* experiments investigating Tn5 transposition and its regulation. In addition, several properties of IS50 polypeptides were studied which relate to Tn5 transposition.

Inspection of the radiolabelled protein bands in

Fig. 4a and 4b indicates that different amounts of IS50R-encoded polypeptides P1 and P2 are synthesized in minicells. Approximately four times as much P2 as P1 is made. The ratio of P1 and P2 synthesis in minicells reflects *in vivo* ratio of P1 and P2 synthesis in a whole cell (P1:P2 = 1:4; Johnson and Reznikoff, 1984). Although Reznikoff and his coworkers have reported the contrasting result that less P2 than P1 is synthesized in minicells (Johnson *et al.*, 1982), their previous results (Rothstein *et al.*, 1980) as well as other result obtained from maxicell experiments (Isberg *et al.*, 1982) are consistent with the results obtain in this study. A possible explanation for the discrepancy between these two contrasting results could be that in the case where a larger amount of P1 appeared to be made the sample preparation contained a ColE1 protein which comigrates with P1 on a polyacrylamide gel. Thus the intensity of the 58,000 dalton band was misleading.

Examination of the DNA sequences proceeding the translational start signal for the P1 and P2 reading frames suggests that the P2 reading frame has a better 16S rRNA binding sequence than the P1 reading frame. Also, RNA polymerase binding and *in vitro* transcription experiments indicate that the P2 message (98 start mRNA; Fig. 1) is, in fact, transcribed at a greater frequency thar the P1 message (66 start mRNA, Krebs and Reznikoff, 1986).

Thus it appears that two distinct features of the IS50R open reading frame contribute to the level of expression of P1 and P2. These are, the rate at which transcription initiates at the promoter for each protein and the relative efficiency of translation of each mRNA molecule. In the expriments reported here, the unbalanced synthesis of these proteins appears to be primarily due to the efficiency of their translation, not to the differential synthesis of P1 message (66 start mRNA) and P2 message (98 start mRNA), since a single transcript synthesized from P_L is expected to constitute the majority of mRNA for the synthesis of IS50R polypeptides when P_L is derepressed.

A pulse and chase experiment performed to determine the stability of IS50 polypeptides demonstrated that IS50L polypeptides are relatively unstable while IS50R polypeptides are quite stable upon 200 min chase. The stability of IS50R polypeptides was also confirmed by showing that these proteins are accumulated upon 2 hr induction. Thus, it is not likely that *cis*-preferential action of Tn5 transposase is due to the instability of the enzyme. Since P3 retains the unique coding sequence of P1's amino-terminus region, it was suggested that the inability of IS50L to promote transposition is due to the observed instability of P3, rather than truncation at the carboxy-terminus. An observation consistent with the hypothesis that the amino-terminus of P1 is the catalytically active region is that several mutation, including missense, nonsense, frameshift, and deletion mutations, within the amino-terminus region of P1 in IS50R all abolished the ability of the element to catalyze transposition (Johnson and Reznikoff, 1984). Also Meyer *et al.* (1979) have shown that a Tn5 derivative in which the *trp* operon replaces the 3,500 base pair *Hind*III fragment of Tn5 can mediate the cointegration process but is defective in cointegrate resolution. Therefore, the amino-terminus of P1 and/or P3 is likely to be responsible for the recognition and/or breakage of Tn5-specific sequences.

적   요

Transposon Tn5의 단백질 합성을 *E. coli* 내에서 증폭 합성시키기 위하여 Bacteriophage의 P_L 촉진유전자가 Tn5의 두 개 module인 IS50L과 IS50R을 전사시킬 수 있도록 plasmid를 재구성 하였다. P_L 촉진유전자로부터의 전사를 탈억제 시켰을 경우, IS50R 으로부터 합성되는 두 개의 단백질은 모두 그 세포내 축적량이 SDS-polyacrylamid gel에서 확인될 정도로 증폭합성되었으나 IS50L 의 두 개 단백질은 동일 gel 상에서 확인되지 않았다. Minicell system 에서 합성양상과 각 단백질들의 안정성을 조사한 결과 IS50R 단백질은 모두 안정하게 유지되었으나 IS50L 단백질은 모두 불안정하여 생분해 되어 진다는 사실을 밝혔다. 이러한 IS50L 단백질의 불안정성은 IS50L 이 transposition에 있어서 불활성을 나타내는 원인이라 추정된다. 또한 IS50L 과 IS50R 의 단백질은 모두 동일한 open reading frame에 의하여 합성되어짐을 tryptic peptide 양상을 통하여 알 수 있었다.

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