Identification of the site of translational frameshifting required for production of the transposase encoded by insertion sequence IS 1

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Summary. Previous genetic analyses indicated that translational frameshifting in the -1 direction occurs within the run of six adenines in the sequence 5'-TTAAAAAACTC-3' at nucleotide positions 305-315 in IS 1, where the two out-of-phase reading frames insA and B'-insB overlap, to produce transposase with a polypeptide segment Leu-Lys-Lys-Leu at residues 84-87. IS/ mutants with a 1 bp insertion, which encode mutant transposases with an amino acid substitution within the polypeptide segment at residues 84-87, did not efficiently mediate cointegration, except for an IS/ mutant which encodes a mutant transposase with a Leu-Arg-Lys-Leu segment instead of Leu-Lys-Lys-Leu. An IS 1 mutant with the DNA segment 5'-CTTAAAAAACTC-3' at positions 305-315 carrying the termination codon TAA in the B'-insB reading frame could still mediate cointegration, indicating that codon AAA for Lys corresponding to second, third and fourth positions in the run of adenines is the site of frameshifting. The β-galactosidase activity specified by several IS 1-lacZ fusion plasmids, in which B'-insB is in-frame with lacZ, showed that the region 292-377 is sufficient for frameshifting. The protein produced by frameshifting from the IS 1-lacZ plasmid in fact contained the polypeptide segment 5'-TTAAAAAAACTC-3', indicating that -1 frameshifting does occur within the run of adenines.

Key words: Adenine run – Amino acid sequencing – Cointegration – LacZ fusion protein

Introduction

Insertion sequence IS 1 is the smallest active IS element in bacteria (Ohtsubo and Ohtsubo 1978; Johnsrud 1979) and is involved in various kinds of genomic rearrangements, including the cointegration event between two replicons (Iida and Arber 1980; Ohtsubo et al. 1980, 1981). IS 1 encodes two out-of-phase reading frames, insA and B'-insB, where B' is an open reading frame extending in-frame from the initiation codon ATG of the insB frame for 126 bp. The B' reading frame, which overlaps the 3' end of the insA frame, is in the -1 frame with respect to insA. Previous genetic analyses indicated that a frameshifting event occurs in the -1 direction within a run of six adenines, which lies in the overlap region between insA and B', to fuse insA and B'-insB by translation, producing the InsA-B'-InsB fusion protein that has IS 1 transposase activity (Sekine and Ohtsubo 1989). The InsA protein, which is produced unless the frameshifting event occurs, may play a role as a negative regulator of transposition (Machida and Machida 1989; Sekine and Ohtsubo 1989; Zerbib et al. 1990). Since the efficiency of frameshifting determines the ratio between the amount of InsA and that of transposase, frameshifting is thought to be a mechanism which controls transposition of IS 1 (Sekine and Ohtsubo 1989; Escoubas et al. 1991). The production of transposase encoded by other IS elements, such as IS3 and perhaps those related to IS 3, has been suggested to depend on -1 frameshifting within a run of adenines in these elements (Sekine and Ohtsubo 1991). Recently, frameshifting in IS 150 which is related to IS 3, has been demonstrated (Vögele et al. 1991).

We present here further genetic analyses which support the concept of frameshifting in IS 1 and show that the precise site of frameshifting is codon AAA for Lys in the run of adenines in insA. We also present here the result of amino acid sequencing analysis showing that -1 frameshifting does occur in the run of adenines to produce transposase with the polypeptide segment Leu-Lys-Lys-Leu.

Materials and methods

Bacterial strains and plasmids. Bacterial strains used were Escherichia coli K12 derivatives, MV1184 (A/lac–
Plasmid construction. Plasmid pSEK17 has only one cleavage site for PstI in the insA coding region in IS1, and was constructed by self-ligation of plasmid pSEK15 after digestion with Spel and SalI and treatment with DNA polymerase I (Klenow) to remove the PstI site flanked by the Spel and SalI sites present in the pUC18 sequence. Each pSEK17 derivative carrying an IS1 mutant with a substitution(s) or a 1 bp insertion was then constructed as follows. The KpnI—HindIII fragment in the cloning site segment in vector plasmid pUC19 was replaced with the KpnI—HindIII fragment of pSEK17 containing the entire IS1 sequence, yielding pSEK117. Using pSEK117 as template and oligodeoxyribonucleotides synthesized using a DNA synthesizer 380B (Applied Biosystems) as primers, the IS1 sequence in pSEK17 was mutagenized by site-directed mutagenesis according to Kunkel et al. (1987). The sequences mutated were confirmed by DNA sequencing. Then the PstI—BstEII fragment of IS1 in pSEK17 was replaced with the PstI—BstEII fragment of each of the resulting pSEK117 derivatives.

Plasmids pSEK2055, pSEK207, pSEK9000 and pSEK6000 are IS1—lacZ fusion plasmids having a DNA fragment of wild-type IS1. These were constructed as follows. Using pSEK117 as template, two BglII recognition sites were introduced into appropriate positions (see Fig. 3) flanking the run of adenines within the IS1 sequence, by site-directed mutagenesis as described above. The sequences mutated were confirmed by DNA sequencing. Each of the BglII fragments was then inserted into the BamHI site of vector plasmid pR-pMLB. Plasmids pSEK2055-I, pSEK207-I, pSEK9000-I and pSEK6000-I are IS1—lacZ fusion plasmids having a DNA fragment of IS1 with a single adenine insertion in the run of adenines. These were constructed in the same way as the IS1—lacZ fusion plasmids, such as pSEK2055 etc. using, however, plasmid pSEK131, which carries IS1-31 with a single adenine insertion in the run of adenines as template; pSEK131 itself was obtained by replacing the KpnI—HindIII fragment in the cloning site segment of vector plasmid pUC119 with the KpnI—HindIII fragment of pSEK31 which includes IS1-31 (Sekine and Ohtsubo 1989).

Purification of β-galactosidase (LacZ) fusion proteins and amino acid sequencing. Strain YS202, harboring the IS1—lacZ fusion plasmid pSEK9000 or pSEK9000-I, was grown in L-rich broth (30°C for pSEK9000 and 250 ml for pSEK9000-I), containing 0.2% (w/v) glucose at 30°C until the OD600 reached 0.5–0.6, and then the culture was incubated with aeration at 40°C for 60 min to induce the LacZ fusion protein. The protein was purified from these cells according to the procedure of In-
amoto and Ohtsabo (1990), except that a French press was used to disrupt the cells instead of sonication. After lyophilization, the protein was solubilized in water, subjected to SDS-polyacrylamide gel electrophoresis, and subsequently electroblotted onto PVDF-type membrane (ProBlott, Applied Biosystems), according to the method of Aebersold et al. (1986). The protein band of interest was excised and subjected to amino acid sequencing analysis using an Applied Biosystems model 470A sequencer or model 477A sequencer fitted with an on-line Applied Biosystems 120A high performance liquid chromatography analyzer. Here, to avoid possible degradation of the protein we used strain YS202. This strain carries a mutation in the lon gene, which encodes a protease involved in the degradation of some unstable proteins (for a review, see Gottesman 1989), and a second mutation in the ompT gene, which encodes an outer membrane-associated protease responsible for in vitro cleavage of several proteins, including the SecY protein (Akiyama and Ito 1990), during their purification. Indeed when an ompT" strain was used as a host, cleavage of the protein between two consecutive basic amino acids was observed.

Cointegration assay. Each of the ampicillin-resistance pSEK plasmids carrying wild-type IS1 or mutant IS1 was introduced by transformation into the E. coli K12 strain JE5519, which already harbored the tetracycline-resistance plasmid pHS1. Cointegration between a pSEK plasmid and pHS1 was assayed according to the method described in Sekine and Ohtsubo (1989).

LacZ assay. Each of the ampicillin resistance plasmids carrying the IS1-lacZ fusion gene was introduced by transformation into MC1000. Liquid cultures of MC1000 harboring a plasmid were incubated overnight at 30°C in L-rich broth containing 100 μg ampicillin/ml, and diluted 1/100 into L-rich broth. After shaking at 30°C until turbidity at 600 nm had reached 0.15-0.2, the temperature was shifted to 40°C. When the turbidity of the culture was 0.8-0.9, LacZ activity was measured by the method described by Miller (1972).

Results

Determination of the site of frameshifting of IS1 by genetic analyses

Our previous analyses of the cointegration ability of IS1 mutants carrying a nonsense mutation in the insA or B'-'insB reading frame have suggested that the run of six adenines at nucleotide positions 307-312, where the two frames overlap (see Fig. 3), is likely to contain the possible frameshift site (Sekine and Ohtsubo 1989). Also, our analyses of several IS1 mutants with single bp insertions within or close to the run of adenines which results in the placement of insA and B'-'insB in the same reading frame, have shown that one IS1 mutant with a single adenine insertion in the run of adenines (see mutant IS1-31 in Fig. 1) can mediate cointegration at a much higher frequency than does either wild-type IS1 or any of the other mutants with a 1 bp insertion neighboring the run of adenines (see IS1-32 and IS1-33 in Fig. 1) (Sekine and Ohtsubo 1989). The mutant IS1-31 is considered to produce, without frameshifting, active transposase in which the amino acid sequence at residues 84-87 is Leu-Lys-Lys-Leu (LKKL in Fig. 1), but the other mutants produce inactive transposase with FKKL or LKNL (see Fig. 1). These results have suggested that wild-type IS1 produces transposase having the polypeptide segment LKKL without frameshifting. In the other IS1 mutants, which produce mutant transposases, only the codons altered and the amino acids substituted are indicated. Boldface letters indicate the mutated nucleotides. The frequency of cointegration (per division cycle) mediated by wild-type IS1 or each IS1 mutant is shown with its relative value in parenthesis by taking the frequency for IS1-31 as 100. Cointegration frequencies mediated by IS1-31, IS1-32 or IS1-33 were taken from Sekine and Ohtsubo (1989).

![Fig. 1. Frequency of cointegration mediated by wild-type IS1 or each IS1 mutant with a 1 bp insertion. The nucleotide sequence of a critical region of IS1 (305-315) of IS1 and of each IS1 mutant is shown together with putative amino acids encoded by the two reading frames, insA and B'-'insB. The leucine residue (L) on the left of the amino acid sequence is actually amino acid residue 84 of the InsA protein, where the first methionine residue is defined as residue 1. The 85th lysine residue (85K) is actually encoded by codon 308AAA in insA as described below (see Fig. 2). IS1-31 with a single adenine insertion is supposed to produce wild-type IS1 transposase with a polypeptide segment LKKL without frameshifting. In the other IS1 mutants, which produce inactive transposases, only the codons altered and the amino acids substituted are indicated. Boldface letters indicate the mutated nucleotides. The frequency of cointegration (per division cycle) mediated by wild-type IS1 or each IS1 mutant is shown with its relative value in parenthesis by taking the frequency for IS1-31 as 100. Cointegration frequencies mediated by IS1-31, IS1-32 or IS1-33 were taken from Sekine and Ohtsubo (1989).](image-url)
Analysis of frameshifting using IS1 - lacZ fusion plasmids

To determine the nucleotide sequence required for and the efficiency of frameshifting in IS1, we constructed several IS1 - lacZ fusion plasmids having a DNA fragment of wild-type IS1 containing the run of adenines. The fragment is flanked by the ATG of cro of bacteriophage λ and the lacZ gene, such that insA is fused with the initiation codon ATGcro in-frame and B' - insB is fused with lacZ in-frame (Fig. 3). The transcription of the fusion gene from the λ pr promoter for cro is under the control of a thermosensitive repressor, the product of e857 which is also carried by the fusion plasmid. The occurrence of -1 frameshifting during translation of insA would therefore result in the synthesis of the InsA - B' - InsB - LacZ fusion protein with β-galactosidase (LacZ) activity. We also constructed IS1 - lacZ fusion plasmids having a DNA fragment of IS1 with a single adenine insertion in the run of adenines, so that insA is fused with B' - insB - lacZ in-frame. The ratio of LacZ activity specified by the out-of-frame plasmid to that specified by the corresponding in-frame plasmid is considered to reflect the efficiency of frameshifting in the out-of-frame plasmid.

The LacZ activity measured after heat induction in lysates of cells harboring pSEK2055, having a DNA insert corresponding to IS1 coordinates 63 - 377 and containing the entire insA and B' coding frames, was 1.73 units (Fig. 3). On the other hand, LacZ activity specified by pSEK2055-I, a derivative of pSEK2055 with a single adenine insertion in the run of adenines, was 811 units (Fig. 3). Thus, the efficiency of frameshifting in pSEK2055 was estimated to be 0.21% (Fig. 3). LacZ activity specified by plasmid pSEK9000 having the region 292 - 377 was 17.9 units, while the LacZ activity specified by the corresponding in-frame plasmid pSEK9000-I was 5220 units (Fig. 3). The efficiency of frameshifting in pSEK9000 was thus estimated to be 0.34% (Fig. 3), which was almost the same level as that in pSEK2055. This suggests that the region 63 - 291, which is upstream of the run of adenines, is not required for frameshifting. The increase of LacZ activity specified by pSEK9000 (and pSEK9000-I), when compared with that specified by pSEK2055 (and pSEK2055-I) (see Fig. 3), may reflect instability of a portion of the InsA
Fig. 3. Structures of IS1-lacZ fusion plasmids and the activity of LacZ produced in cells harboring each of the plasmids. IS1 (with coordinates 1–768; Ohtsubo and Ohtsubo 1978) has two overlapping open reading frames, insA and B'-insB, shown by two arrows within IS1 at the top. Each plasmid carries a DNA fragment of IS1 containing a part of the insA and B'-insB frames, each of which is in-frame with ATGcro and lacZ, respectively. pSEK2055-I, etc. are the plasmids carrying a DNA fragment with a single adenine insertion in the run of adenines which results in fusion between the two frames to give in-frame expression of lacZ. Nucleotide positions of the ends of each IS1 fragment are indicated. Filled portions indicate a frame(s) required to give the InsA—B'—InsB—LacZ fusion protein. Production of the fusion protein is controlled at promoter PR by a thermosensitive repressor, the product of ci857. Broken lines show regions of IS1 deleted in each plasmid. The LacZ activity in the lysate of the cells harboring each plasmid was assayed at least three times; standard errors were less than 15%. Efficiency of frameshifting (%) was obtained by calculating the ratio of LacZ activity specified by the out-of-frame plasmid to that specified by the corresponding in-frame plasmid.
Fig. 4. A Nucleotide sequences of a critical region in the IS \( I \) – \( lacZ \) fusion plasmids and amino acid sequences of the proteins encoded by the region. Plasmid pSEK9000 has an IS \( I \) fragment (coordinates 292–377) containing a part of \( \text{insA} \) and of \( B' \)-\( \text{insB} \) fused with \( \text{ATG}_o \) and \( \text{lacZ} \), respectively. Plasmid pSEK9000-I is a derivative of pSEK9000 with a single adenine insertion in the run of adenines (shown in \textit{boldface}) which results in in-frame fusion between \( \text{insA} \) and \( B' \)-\( \text{insB-lacZ} \). Numbers above the nucleotide sequence represent IS \( I \) coordinates. Recognition sites of restriction endonucleases used to construct the plasmids are shown. The amino acids encoded by the two reading frames are shown. The amino acids indicated in \textit{boldface} are those determined by sequencing the purified LacZ fusion protein produced; numbers above or below the amino acids indicate cycle numbers of Edman degradation of the LacZ fusion protein. The 9th lysine residue (K) of the protein specified by pSEK9000 is encoded by codon 3°AAA in \( \text{insA} \) as described above (see Fig. 2). B Critical phenylthiohydantoin (PTH)-amino acids (in pmol) detected during each sequencing cycle of Edman degradation of the purified LacZ fusion protein produced from cells harboring pSEK9000 (a) or pSEK9000-I (b). The data are not corrected for injection, base line, and tailover. A number over a vertical line represents the major PTH-amino acid recovered from that cycle protein or a portion of the transcript encoded by pSEK2055 (and pSEK2055-I).

The efficiency of frameshifting in pSEK6000 carrying the region 292–353 was 0.25% (Fig. 3) which was reduced relative to that in pSEK9000; a similar reduction has been reported by Escoubas et al. (1991). The degree of the reduction, however, seemed not to be so great, and the efficiency in pSEK6000 is the same as that in pSEK2055. Moreover, the efficiency in pSEK6100 having the region 292–332 was the same as that in pSEK2055 and pSEK6000 (data not shown). In the two plasmids, pSEK6000 and pSEK6100, several possible secondary structures seen in the region downstream of the run of adenines (Sekine and Ohtsubo 1992) are deleted. These show that the region 333–377 is not essential for frameshifting, suggesting that the contribution of the secondary structures downstream of the run of adenines to the efficiency of frameshifting is small (Sekine and
Ohtsubo 1992). Note here that the efficiency of frameshifting in pSEK207 having the region 63–314 was, however, 0.047% (Fig. 3), a 4.5-fold decrease compared with that in pSEK2055. This suggests that the region 315–377, which is located downstream of the run of adenines, is required to stimulate frameshifting. The reduction of efficiency of frameshifting seen in pSEK207 was considered to be caused mainly by the elimination of the termination codon of insA, but not by the elimination of secondary structures, as described above and by Sekine and Ohtsubo (1992).

Analysis of the amino acid sequence encoded by an IS1 segment containing the run of adenines

To obtain direct evidence for frameshifting in IS1, we determined the amino acid sequence of the protein produced in the form of the InsA–B′–InsB–LacZ fusion protein from plasmid pSEK9000, which contained a region of IS1 sufficient for efficient frameshifting, as described above. In this plasmid, the codon for Leu at the 84th residue in IS1 transposase becomes the 8th codon in the coding region for the LacZ fusion protein, where the ATG of cro is the first codon (see Fig. 4A). The LacZ fusion protein, which was overproduced by heat induction in cells harboring pSEK9000, was readily purified using a LacZ-specific affinity column (see Materials and methods). The purified protein was subjected to 21 cycles of Edman degradation (Fig. 4A), and the relevant phenylthiohydantoin (PTH)-amino acids detected in the first 13 cycles are shown in Fig. 4B (a). This shows that translation of the LacZ fusion protein was initiated at the ATGcro and continued in-frame along insA, but shifted into B′–insB–LacZ. The amino acid sequence detected at cycles 8–11, which was produced from the sequence 5'-TTAAAAAAGCT-3', was LKKE (Tsuchihashi and Kornberg 1990), indicating that frameshifting occurs at one of the consecutive codons, AAA and AAG, for lysine (K) in the 0-frame. In IS150, the amino acid sequence of the protein produced by frameshifting from the DNA segment 5'-CUAAAAAGCU-3' was LKKA (Vögele et al. 1991), indicating that frameshifting occurs at either codon CUA for leucine (L) or one of the consecutive codons, AAA and AAG, for lysine (K) in the 0-frame. Since the exact site for frameshifting is not clear in dnaX or IS150, it is unknown at present whether the site of frameshifting in these genetic systems is the same as that in IS1 or not.

Secondary structures downstream of the frameshift site have been demonstrated to stimulate frameshifting in many genetic systems (Jacks et al. 1987, 1988; Brierley et al. 1989; Flower and McHenry 1990; Tsuchihashi and Kornberg 1990; Dinman et al. 1991; Vögele et al. 1991), but such structures seem not to be responsible for efficient frameshifting in IS1 (for further experiments on, and discussion of the role of the secondary structures in IS1, see Sekine and Ohtsubo 1992). We have shown here that the efficiency of frameshifting in IS1 is 0.2–0.3%. This is very low when compared with other cases, for example, 5–25% in retroviruses (for a review, see Varmus and Brown 1989), 40–50% in dnaX (Flower and McHenry 1990; Tsuchihashi and Kornberg 1990), and 30% in IS150 (Vögele et al. 1991). The lack of the secondary structures which stimulate frameshifting might result in such a low efficiency of frameshifting in IS1. It is reasonable to assume that IS1 adopts a low level of frameshifting, which results in a low level production of transposase, to avoid deleterious rearrangement of the host chromosome containing IS1.

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