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Synthesis and Overproduction of the 5A Protein of Insertion Sequence IS5

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We have demonstrated both the synthesis and overproduction of the 5A protein encoded by the longest open reading frame of the bacterial insertion sequence IS5. Expression was obtained in vitro and in *Escherichia coli* maxicells from plasmids containing IS5 in either orientation, as well as in vitro from a restriction fragment containing exclusively IS5 DNA. When IS5 was cloned in the appropriate orientation downstream of a strong *tac* promoter, production of the 5A protein was increased to 10 to 20% of the total protein synthesized in vitro.

IS5 is a 1,195-base-pair (bp) insertion sequence which has 16-bp terminal inverted repeats and creates a 4-bp direct repeat upon its insertion into the target site DNA (Fig. 1). The sequence of IS5 (8, 9, 18) contains three open reading frames (ORFs), which have been associated with functional promoter and terminator signals (9, 13, 14). The 5A ORF located on one DNA strand is capable of encoding a protein with a molecular size of 37.8 kilodaltons (kDa), while the 5B and 5C ORFs, located on the opposite strand, are capable of encoding proteins with molecular sizes of 11.1 and 9.4 kDa, respectively. Production of small amounts of proteins from each of these ORFs has been detected in vitro or in *Escherichia coli* minicells (13, 14). In this report, we confirm synthesis of the 37-kDa 5A protein both in vitro and in *E. coli* maxicells, and we demonstrate overproduction of the 5A protein by use of a strong *tac* promoter.

Synthesis of the 5A protein in vitro and in maxicells. Plasmids pDI15 and pDI40 (18), which contain IS5 at the same site but in opposite orientations within the cloned *Hha*II restriction and modification genes, were used in a cell-free bacterial transcription-translation system (6, 7) to direct in vitro synthesis of plasmid-encoded proteins. A protein with an apparent molecular size of 37 kDa on denaturing sodium dodecyl sulfate-polyacrylamide gels was synthesized by pDI40 but not by the control plasmids pBR322 (3) and pDI14 (18), which do not contain IS5 (Fig. 2). A protein migrating at the same position was produced by pDI15 (data not shown). Since its production was specific for IS5 DNA and independent of the orientation of the element, the gene for this protein must be wholly contained within IS5. Of the three ORFs within this element, only the 5A ORF was capable of specifying a protein of the observed size (Fig. 1). When pDI40 was linearized by cleavage at a *Bst*EII restriction site within the 5A ORF, the 37-kDa product was no longer made (data not shown). Thus, the 5A ORF was expressed in vitro as an approximately 37-kDa protein which we will refer to hereafter as the 5A protein. This protein amounted to <5% of the total protein synthesized in vitro.

Use of the maxicell technique (17) confirmed the in vivo synthesis of the 5A protein from *E. coli* cells containing pDI15 and pDI40 but not from cells containing the control plasmid pDI14 (Fig. 3A). This protein amounted to 5 to 10% of the total protein accumulated. Other workers have detected the synthesis of very low levels of a 37-kDa 5A protein in vitro and in minicells by using an independent copy of IS5 which had inserted into the λ *c*I gene in a single orientation (13). These observations are consistent with the low level of production seen for other insertion sequence-encoded proteins (13–16, 19).

Overproduction of the 5A protein in vitro. To increase production of the 5A protein, IS5 was cloned downstream of both the *tet* promoter and the *tac* promoter. The IS5-containing 1,554-bp *Bam*HI-*Aha*III fragment of pDI40 was gel isolated (12) and ligated (12) to the 3,766-bp pBR322 *Bam*HI-*Nru*I fragment carrying the ampicillin resistance gene to yield pJC100, which contains the 5A ORF downstream of the pBR322 *tet* promoter. The IS5-containing 1,199-bp *Mae*I fragment of pJC100 was gel isolated, blunt ended by a fill-in reaction with the Klenow fragment of *E. coli* DNA polymerase I (12), and inserted into the filled-in *Eco*RI site of the *tac* promoter expression vector ptac11 (1). This resulted in the plasmids pJE160, in which the 5A ORF is downstream of the *tac* promoter, and pJE161, in which the 5C and 5B ORFs are downstream of the *tac* promoter.

pJC100 and pJE161 directed the synthesis of low levels of 5A protein in the in vitro transcription-translation system (Fig. 2). This was the expected result, since the 5A gene can be transcribed only from its own promoter in pJE161 and because the additional upstream promoter in pJC100 is the relatively weak *tet* promoter of pBR322. Plasmid pJE160, however, produced approximately 100-fold more 5A protein than did pJE161 or pJC100 and approximately 10-fold more protein than did pDI40, which contains the relatively strong pBR322 anti-*tet* and β-lactamase promoters upstream of the 5A gene. This amounted to 10 to 20% of the total synthesized proteins and was probably due to increased transcription from the strong *tac* promoter. It should be noted that all of the plasmids containing the 5A ORF produced 5A protein, whereas those lacking this ORF did not. This was true regardless of the orientation of the IS5 element or the DNA sequences which flanked it. The *Mae*I fragment, which contained exclusively IS5 DNA (Fig. 1), produced large amounts of the 5A protein. This is conclusive evidence that this protein can be expressed from IS5 in the absence of external promoters.

We have noticed the occurrence of a minor polypeptide species which migrates just below the 37-kDa protein and

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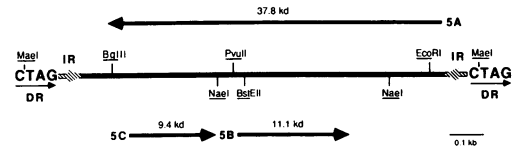

FIG. 1. Structure of the 1,195-bp bacterial transposable element IS5. The 4-bp 5′ CTAG direct repeats (DR) created upon insertion of the element into the host genome are cleavage sites for the *Mae*I restriction enzyme. The terminal inverted repeats (IR) are designated by the hatched arrows. The location and orientation of the three ORFs of IS5 are indicated by solid arrows above and below the element, with the predicted sizes of the corresponding gene products given in kilodaltons. The approximate scale of the diagram is indicated by the bar at the lower right. kb, Kilobases.

appears to be correlated with its presence in vitro. Although this product cannot be readily explained by translation initiation at a downstream ATG within the gene, it could be due to proteolytic degradation of the 5A protein. Close examination of the data indicates that several polypeptides with molecular sizes of 17 to 21 kDa were also consistently produced from plasmids or restriction fragments containing

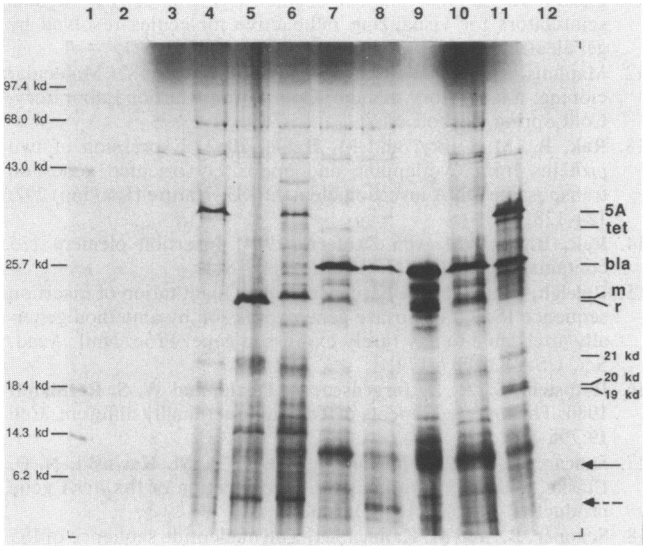

FIG. 2. Synthesis and overproduction of the 5A protein in vitro. Proteins labeled with [^{35}S]methionine were synthesized from substrate DNAs (0.5 to 1.5 μg) by using an in vitro transcription-translation system from Codon Co. (Mundelein, Ill.). The products were analyzed on a 15% sodium dodecyl sulfate-polyacrylamide gel (10), and the gel was treated with En^{3}Hance (Dupont, NEN Research Products, Boston, Mass.), dried, and autoradiographed (4, 11). Lanes: 1, high-molecular-weight protein markers; 2, no DNA and no S30 extract; 3, no DNA; 4, *Mae*I fragment; 5, pDI14; 6, pDI40; 7, pBR322; 8, pJC100; 9, ptac11; 10, pJE161; 11, pJE160; 12, low-molecular-weight protein markers. Positions of bands corresponding to the 5A protein (5A), the tetracycline resistance gene product (tet), β-lactamase (bla), the *Hha*II methylase (m), and the *Hha*II restriction enzyme (r) are indicated on the right, along with those of unidentified polypeptides with approximate molecular sizes of 21, 20, and 19 kDa, 12 kDa (⟵), and 5.5 kDa (⟵ - -). The molecular sizes of the following radiolabeled protein markers (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) are given in kilodaltons on the left: phosphorylase *b* (97.4), bovine serum albumin (68.0), ovalbumin (43.0), α-chymotrypsinogen (25.7), β-lactoglobulin (18.4), lysozyme (14.3), bovine trypsin inhibitor (6.2), and insulin (3.0).

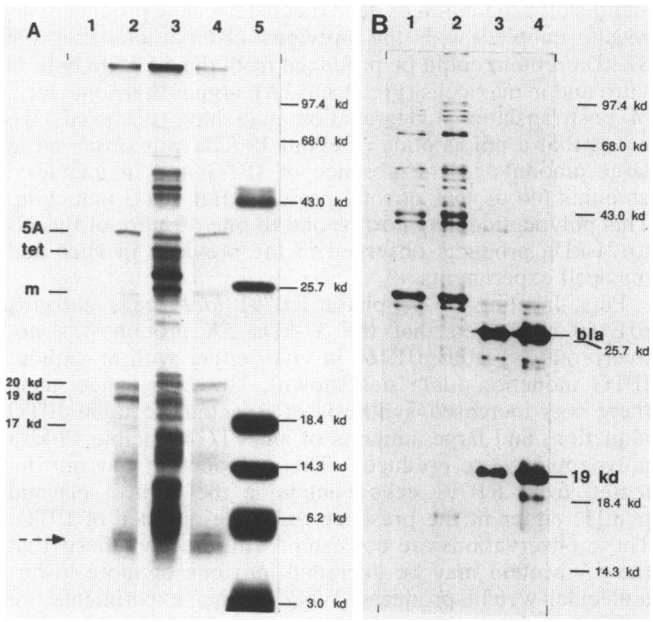

FIG. 3. (A) Synthesis of the 5A protein in maxicells. HB101 *recA13* (5) or DR1984 *recA1 uvrC34* (17) cells with or without plasmids were used for the synthesis of [^{35}S]methionine-labeled proteins by the maxicell technique. Products were analyzed as for Fig. 2. Lanes: 1, DR1984 (no plasmid); 2, DR1984(pDI40); 3, DR1984(pDI14); 4, HB101(pDI15); 5, low-molecular-weight markers. HB101 cells without a plasmid gave results similar to those seen in lane 1. Lane 3, which was taken from elsewhere on the same gel, has been placed adjacent to lanes 2 and 4 for better comparison. Positions of several proteins identified in Fig. 2, as well as that of an unidentified 17-kDa polypeptide (17 kd), are indicated. (B) Evidence that the 5A protein is not overproduced from pJE160 in maxicells. RB791 *lacI*^{q} cells (1) with or without pJE160 were used for the synthesis of [^{35}S]methionine-labeled proteins by the maxicell technique. Prior to labeling, each culture was divided in half, and one half was induced for 30 min with 1 mM IPTG. After being labeled, all samples were supplemented with a 3,000-fold excess of unlabeled methionine and placed on ice for 5 to 10 min. Labeled protein products were analyzed as for Fig. 2. Lanes: 1, RB791 (no plasmid); 2, RB791 plus IPTG; 3, RB791(pJE160); 4, RB791(pJE160) plus IPTG. The positions of β-lactamase (bla), a 19-kDa polypeptide (19 kd), and molecular weight markers (not shown) are indicated.

the 5A gene. For example, the *Mae*I fragment, pDI40, and pJE160 all produced polypeptides with approximate molecular sizes of 21, 20, and 19 kDa, which were not produced by the parental plasmids pDI14, pBR322, and ptac11. These proteins were too large to originate from the 5B or 5C ORFs, and their levels appeared to be proportional to those of the 5A protein. Several species with molecular sizes of 17 to 21 kDa were also produced in maxicells containing pDI40 and pDI15 (Fig. 3A). These observations suggest that the 5A protein may be degraded into one or more products of lower molecular size.

The 37-kDa 5A protein is not overproduced in vivo. When the maxicell technique was used to examine in vivo protein synthesis, *E. coli* RB791 *lacI*^{q} cells (1) containing pJE160 produced little, if any, 37-kDa 5A protein, even after addition of isopropyl-β-D-thiogalactoside (IPTG) to induce the *tac* promoter (Fig. 3B). Synthesis of β-lactamase, however, increased significantly upon induction. Since the 5A gene is located between the *tac* promoter and the ampicillin resistance gene, transcription initiated at the *tac* promoter must proceed through the 5A sequences without resulting in

significant production of the expected 5A gene product. This result, coupled with the previous observations that the 37-kDa protein could be produced from the 5A gene both in vitro and in maxicells (Fig. 2 and 3A), argues that some form of posttranslational degradation may have occurred. We noted that a polypeptide of about 19 kDa was observed in large amounts in the absence of IPTG and in increased amounts (40 to 45% of total protein) after IPTG induction. This polypeptide may correspond to one or more of the 17- to 21-kDa products observed in the previous in vitro and maxicell experiments.

Pulse-labeling of log-phase RB791 *lacI*^{q} cells carrying pJE160 confirmed that the 37-kDa 5A protein was not overproduced from pJE160 in vivo, either with or without IPTG induction (data not shown). However, once again there was increased synthesis of β-lactamase upon IPTG induction, and large amounts of an IPTG-inducible 19-kDa polypeptide were produced. This polypeptide was not detected from RB791 cells containing the control plasmid ptac11, either in the presence or in the absence of IPTG. These observations are consistent with the hypothesis that the 5A protein may be degraded into one or more lower-molecular-weight products. While further experiments are needed to clarify the nature and origin of these species, it is interesting to note that both the Mu transposase and the putative IS*4* transposase may be processed to lower-molecular-weight forms (2, 19).

In contrast to our results with the 5A gene, we have been unable to obtain clear evidence for the in vitro or maxicell synthesis of specific products from the 5B or 5C ORFs, either by using their native promoters alone (pDI15, pDI40, and pJC100) or in concert with an upstream *tac* promoter (pJE161). However, we have recently succeeded in overproducing a protein from the 5B ORF by using a specially designed synthetic ribosome-binding site in conjunction with a *tac* promoter (J. M. Chernak, Ph.D. thesis, The Johns Hopkins University, Baltimore, Md., 1987).

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