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Overexpression of an mRNA Dependent on Rare Codons Inhibits Protein Synthesis and Cell Growth

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λ's *int* gene contains an unusually high frequency of the rare arginine codons AGA and AGG, as well as dual rare Arg codons at three positions. Related work has demonstrated that Int protein expression depends on the rare AGA tRNA. Strong transcription of the *int* mRNA with a highly efficient ribosome-binding site leads to inhibition of Int protein synthesis, alteration of the overall pattern of cellular protein synthesis, and cell death. Synthesis or stability of *int* and ampicillin resistance mRNAs is not affected, although a portion of the untranslated *int* mRNA appears to be modified in a site-specific fashion. These phenotypes are not due to a toxic effect of the *int* gene product and can be largely reversed by supplementation of the AGA tRNA in cells which bear plasmids expressing the T4 AGA tRNA gene. This indicates that depletion of the rare Arg tRNA due to ribosome stalling at multiple AGA and AGG codons on the overexpressed *int* mRNA underlies all of these phenomena. It is hypothesized that *int* mRNA's effects on protein synthesis and cell viability relate to phenomena involved in lambda phage induction and excision.

The arginine codons AGA and AGG are the rarest codons in genes of *Escherichia coli* (1), and limitation of the respective tRNAs has been proposed to be a regulatory mechanism in genes enriched in these codons (6). Direct measurements of the level of the Arg-4 or *argU* (AGA) and Arg-5 (AGG) tRNAs have shown that they are present in extremely low concentrations during all phases of cell growth (11, 17, 32). Indirect experiments suggest that rare Arg tRNA may become limiting in mid-log phase (5, 6). Mutant forms of the Arg-4 tRNA gene, known as *argU* (or *dnaY*) (12, 16), strongly affect replication and cell growth.

The lambda integrase (*int*) mRNA is highly enriched in the rare arginine codons AGA and AGG, which represent 59% (20 of 34) of the total Arg codons and occur three times in tandem (9, 38). Translation of these codons has been demonstrated to be slower than that of the major arginine codons (2, 35). Previous studies have shown that efficient translation of *int* mRNA depends on the level of rare Arg tRNA (38).

The Int protein plays a critical catalytic role in integration of lambda phage DNA during lysogenization and also during the excision process accompanying phage induction (37). Transcriptional and posttranscriptional regulation of the bacteriophage lambda *int* gene is complex and has been the subject of intensive study (23). *int* is expressed from the N-antiterminated p_L transcript during excision and from the cII-activated p_I promoter during integration (27, 34). The two *int* transcripts show different patterns of termination and mRNA processing (23).

The major new result presented here is that high-level expression of *int* mRNA made from a plasmid-borne *tac* promoter (p_{tac}) and containing the T7 gene *10* translational enhancer (ε) and ribosome-binding site (RBS) produces inhibitory phenotypes which depend on the supply of rare Arg tRNA. In view of the other established inhibitory functions of lambda's *int* region, such an effect could function as an additional postexcision control in the lambda life cycle.

MATERIALS AND METHODS

Bacterial strains and plasmids. All experiments were performed with strain NM522 (F′ *lacI*^{q}) (13). A detailed description of the derivation of *int* expression plasmid pKZ280-6 is presented later in the text and in Fig. 1. From here on, this plasmid will be referred to as pInt. Plasmids p4, pAGA8, pAGA9, and pCL280 will be described elsewhere by Zahn and Landy (38). pGM4113, containing a fragment of T4 DNA coding for the tRNA cluster, was obtained from W. McClain, University of Wisconsin (21). Plasmid pKZ330 was constructed by cloning a 683-bp *Eco*RI fragment derived from pGM4113 into *Eco*RI-cleaved pACYC184 (4). Closely related plasmid pBS12 was derived from pKZ330 by deleting an internal 28-bp *Sau*3A fragment containing the anticodon loop from the cloned T4 *Eco*RI fragment (36a). pIntBamΔ was created by cutting pInt at the unique *Bam*HI site at λ position 27972 (9), filling in with deoxynucleoside triphosphates and Klenow fragment, and ligating with T4 DNA ligase. pIntStyIΔ was made by cutting pInt with *Sty*I, which cuts twice within the *int* sequence, at λ positions 27868 and 28793, isolating the larger resulting fragment, filling in the *Sty*I ends with Klenow DNA polymerase, and religating with T4 DNA ligase. This removed the major portion (825 bp) of the *int* coding region but left the amino-terminal rare Arg codons intact. All constructions were verified by restriction mapping and sequencing when necessary.

Media, cell growth, induction, and labeling. Competent NM522 cells (or NM522 cells containing pAGA8 or pAGA9) were electroporated with pInt by using a Bio-Rad Gene Pulser. Cells were plated on Luria broth (LB) plates plus ampicillin at 100 μg/ml; cells bearing pAGA8 or pAGA9 were plated on LB plates plus ampicillin and kanamycin (20 μg/ml). All cell growth was carried out at 37°C, and liquid cultures were incubated on a Cell-Gro Rotator. Overnight growth was in LB supplemented with ampicillin (100 μg/ml) or ampicillin plus kanamycin (20 μg/ml). Cells for induction and labelling experiments were subcultured by 50-fold dilution from LB into M9 medium with 0.25% Casamino Acids and 0.4% glucose. Cells were grown to an A_{600} of approximately 0.200, and 35 μM isopropyl-β-D-thiogalactopyranoside (IPTG) was added. Induction was for 1 h. If cells were to be labelled, prior to addition of L-[^{35}S]methionine, they were pelleted by brief centrifugation and resuspended in M9 medium plus glucose and a 0.1% 19-amino-acid mixture minus methionine. Labelling was carried out with 10 μCi of L-[^{35}S]methionine per ml for 5 min and terminated by chilling on ice, centrifugation, and rapid freezing on dry ice. For trichloroacetic acid-precipitable counts, the same cells were pelleted, frozen, resuspended in 50 μl of 0.1% sodium dodecyl sulfate (SDS), and boiled. A 10-μl volume of this lysate was precipitated with 10% trichloroacetic acid and collected on glass fiber filters which were washed, dried, and counted in a Packard Tri-Carb scintillation counter. For SDS-polyacrylamide gel electrophoresis (PAGE), cell pellets were resuspended in 50 μl of SDS sample buffer and lysed by boiling and 5 μl of the extract was separated on discontinuous SDS–12.5% polyacrylamide gels. Gels were fixed and stained by the silver staining method (25). Labelled gels were dried and exposed to Fuji X-ray film at room temperature. For transformation assay, after electroporation, cells were plated at appropriate dilutions on LB

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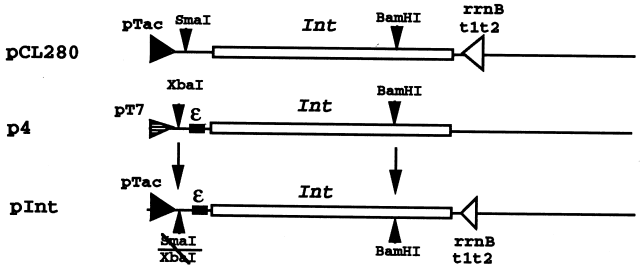

FIG. 1. Map and schematic representation (not drawn to scale) of the construction of *int* expression plasmid pInt. Plasmids pCL280 and p4 will be described elsewhere (38). A segment of plasmid pCL280 between the *Sma*I and *Bam*HI sites containing the translational regulatory sequences and the amino-terminal three-fourths of the *int* gene was replaced with the corresponding segment from p4. This was derived by sequential *Xba*I cleavage, limited S1 nuclease treatment and *Bam*HI treatment of p4, purification of the fragment, and ligation into the purified pCL280 *Sma*I-*Bam*HI backbone. Fusion at the *Sma*I-*Xba*I junction removed both sites. T7 and *tac* promoters and *rrnB* terminators are indicated by left- and rightward-pointing arrowheads, respectively. The *int* gene is shown as an open rectangle. Restriction sites are indicated by up- or downward-pointing arrows and marked accordingly. ε is the T7 gene *10* translational enhancer and RBS.

plates with ampicillin or with ampicillin plus kanamycin with or without 35 μM IPTG. Colony counts were made after 16 to 18 h at 37°C.

RNA isolation and analysis. After 1 h of growth as described above in the presence or absence of IPTG, a 50-ml volume of cells was pelleted and then frozen on dry ice. Cells were resuspended in lysis buffer containing 0.1% SDS and 0.1 M sodium acetate at 65°C as described by Plunkett and Echols (30) but with the following modifications. Extraction was done twice with hot phenol, once with chloroform-isoamyl alcohol, once again with hot phenol, and finally with chloroform. Nucleic acids were precipitated with ethanol, washed with 70% ethanol, and resuspended in 300 μl of distilled diethylpyrocarbonate-treated H_2O. A 30-μl volume of this mixture was removed, made to 40 mM Tris (pH 7.5)–6 mM $MgCl_2$, and treated with 20 μg of RNase-free DNase I (Worthington) per ml for 1 h at 37°C. The reaction was extracted once with phenol and then with chloroform, ethanol precipitated, and dried. The pellet was resuspended in 30 μl of H_2O, and portions were used for primer extension analysis and S1 mapping.

The S1 mapping techniques used were described by Plunkett and Echols (30). A 5′ ^{32}P-labelled restriction fragment was prepared by polynucleotide kinase labelling of pInt DNA cleaved with *Ava*II at a position about 90 nucleotides within the *int* gene (λ position 28798) (9). This fragment was subsequently recut with *Hin*cII at a position within the −35 region of the *tac* promoter to give a 195-bp probe which was isolated by electroelution after electrophoresis on a 6.25% acrylamide gel. Probe and RNA were mixed and then precipitated with ethanol. A 30-μl volume of formamide containing hybridization buffer was added, and the mixture was denatured at 85°C for 10 min and then incubated for 12 to 16 h at 60°C. This mixture was diluted 10-fold to S1 reaction conditions and treated with 30 U of S1 nuclease (BRL-Gibco) for 7.5 min at 30°C. The reaction was stopped by phenol-chloroform extraction and ethanol precipitation. S1 cleavage products were analyzed on 6% sequencing gels and aligned with Maxam and Gilbert (20) sequencing standards of the same restriction fragment.

For primer extension of *int* mRNA, the primer 5′CTCTTTACCCGTCCTT GGG was used, where the 5′ end corresponds to position 28781 in the lambda sequence (9), close to the *Ava*II site used for end labelling in the S1 experiments. For primer extension controls using the plasmid-encoded ampicillin resistance gene mRNA, the primer 5′GGAAATGTTGAATACTC was used, whose 5′ end corresponds to position 4137 in the pBR322 sequence. Reactions were assembled by mixing 10 pmol of the primer with various amounts of RNA. These mixtures were heated to 70°C for 10 min and moved to a 37°C water bath for 10 min, after which 5× reverse transcription buffer, deoxynucleoside triphosphates, dithiothreitol, and 200 U of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL Superscript) were added. Primer extension was carried out at 37°C for 30 min and stopped by ethanol precipitation, and the products were dissolved in formamide sequencing dyes. Extension products were analyzed on 6% sequencing gels, and alignment was obtained versus a dideoxy-sequencing ladder (U.S. Biochemicals Sequenase Kit) by using the same primers and a pInt DNA template. Quantitation of DNA bands was performed with a Molecular Dynamics PhosphorImager.

RESULTS

Construction of a high-level expresser of λ Int protein. To obtain high-level expression of the lambda *int* gene, plasmid pInt was constructed, in which the translational regulatory signals and the major amino-terminal portion of the *int* gene are contained on an *Xba*I-*Bam*HI fragment from plasmid p4 (38) and the plasmid backbone and carboxy-terminal portion of *int* are derived from an *Sma*I-*Bam*HI fragment from pCL280 (38). A simplified map of this construction is shown in Fig. 1. *int* mRNA synthesis is placed under *tac* promoter control, and the epsilon sequence and RBS from T7 gene *10*, a translational enhancer, are upstream of *int*. The ε-RBS segment of this RNA is known to enhance translation of unrelated mRNAs by up to 100-fold and is thought to function by providing an additional mRNA sequence homology with 16S rRNA (26). Sequences downstream of the *int* structural gene including *attP* and the translational and transcriptional regulatory regions *bar* and *sib* are replaced by the *rrnB* t_1t_2 terminators.

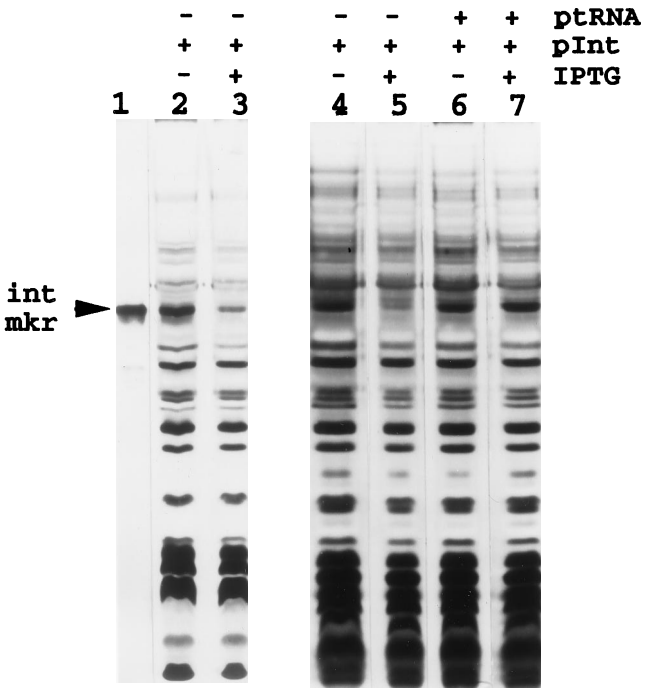

FIG. 2. Inhibition of Int protein synthesis by IPTG. Silver staining analysis of crude extracts separated on SDS–12.5% polyacrylamide gels is shown. NM522 cells containing only an *int* plasmid (pInt) (lanes 2 and 3) or this *int* plasmid and a second, compatible plasmid containing (pKZ330, lanes 6 and 7) or lacking (pBS12, lanes 4 and 5) an AGA tRNA gene insert were grown to an A_{600} of 0.20 and then incubated for 1 h with (lanes 3, 5, and 7) or without (lanes 2, 4, and 6) IPTG. Cells were harvested and lysed, and extracts were analyzed as described in Materials and Methods. int mkr, purified lambda Int protein.

Inhibition of Int synthesis from pInt upon IPTG induction. SDS-PAGE profiles of uninduced extracts from *lacI*^{q} strain NM522 containing pInt showed high levels of expression of Int protein (Fig. 2, lane 2), which do not occur in strain pCL280 (38), which lacks the 39-bp segment containing the T7 gene *10* translational control signals. Most remarkably, induction of the same cells with IPTG (Fig. 2, lane 3) resulted in strong inhibition of Int protein synthesis, again unlike the normal induction seen with pCL280.

To test whether the observed inhibitory effect of IPTG was due to depletion of the rare Arg tRNA, induction in the presence of a compatible plasmid which provides T4 AGA tRNA was tested. Figure 2, lanes 4 and 5, shows crude extracts, minus and plus the inducer, in which the second, tetracycline resistance-encoding plasmid carries the T4 AGA tRNA gene with

an inactivating 28-bp deletion in the anticodon loop (pBS12). Again, leaky Int synthesis was observed in the absence of IPTG and Int synthesis was inhibited in IPTG-treated cells. However, when the second plasmid contained the intact T4 AGA tRNA gene and the inducer was added (Fig. 2, lane 7), the inhibitory effect of IPTG was no longer seen. Identical results were obtained (data not shown) when the control and AGA tRNA plasmids used contained a different, kanamycin resistance-encoding plasmid backbone (pAGA8 and pAGA9) (38).

Since the sole difference between pCL280 and pInt is replacement of the lambda translational control region with that of T7, the apparent difference in inducibility and repression could relate to more efficient ribosome binding to the *int* mRNA or to fortuitous juxtaposition of new transcriptional regulatory sequence elements adjacent to p_{tac}.

Analysis of mRNA start point, level, and fine structure. Explanations based on novel effects at the transcriptional level were ruled out by mapping of the transcript start point and quantity in induced and uninduced cells by S1 nuclease and primer extension techniques.

For S1 mapping, a 5′ singly ^{32}P-labelled probe was prepared which covered the first 30 amino acids of *int* through the region upstream of the *tac* promoter elements. RNAs from uninduced and IPTG-induced cells were extracted by using the hot-phenol method, annealed with constant amounts of a radiolabelled probe, and then treated with S1 nuclease. A sequencing gel analysis of the S1 digestion products is shown in Fig. 3a.

Low levels of *int* mRNA were detected in the absence of an inducer, and the start points are consistent with low-level leakage from the *tac* promoter. Addition of the inducer resulted in strong mRNA synthesis from the same start point. Quantitation of the primer extension products by PhosphorImager analysis indicated greater-than-20-fold induction in the presence of IPTG. Repression and induction therefore appear normal in cells bearing pInt.

In experiments in which the same mRNA preparation was studied by both the S1 and primer extension methods, significant fine structure was seen in the primer elongation products which was not detected with the S1 mapping method (Fig. 3b). Especially striking was a strong (about 15% of the *int* mRNA) extension stop seen in the mRNA from IPTG-treated cells at codon 6 which did not occur in the mRNA from uninduced cells. This site is immediately downstream of the first dual rare Arg site and is predicted to be a site of severe ribosomal stalling (38). The differences in primer extension may reflect transcript modification such as methylation, covalent protein attachment, or deglycosylation (19) induced in response to stalled translation. The results of the S1 analysis indicate that the bulk of incompletely translated or untranslated *int* mRNA was stable. The in vitro translatability of this mRNA was not tested.

Controls of primer extension on the 5′ end of the ampicillin resistance mRNA from the same cells (Fig. 3c) revealed a small amount of this material that initiated from the predicted mRNA start point but no difference in its recovery from induced or uninduced cells. Within the limited resolution of this experiment, no heterogeneity in primer extension was seen with this mRNA from induced or uninduced cells as observed with *int* mRNA. Differences of mRNA recovery between induced and uninduced samples therefore cannot explain the differences in *int* mRNA levels. Thus, no parallel inhibition of RNA synthesis accompanied the effects on protein synthesis described above.

Generalized inhibition of protein synthesis during induction of pInt. To test whether the inhibition of synthesis of Int protein by IPTG as observed by silver staining extends to other proteins in the cell or to protein synthesis in general, total *E. coli* proteins were pulse-labelled under induction conditions for 5 min with L-[^{35}S]methionine. Trichloroacetic acid precipitation of labelled proteins and scintillation counting were used to quantitate relative incorporation under induced or uninduced conditions. The results are shown in Fig. 4A. IPTG treatment resulted in partial inhibition of protein synthesis such that incorporation did not increase during the course of the incubation. Depression of protein synthesis was also seen in the IPTG-treated pBR322 control. However, unlike that in the induced pInt cells, this effect was transient and synthesis recovered completely by 60 min. This inhibition was also not reflected by changes in the pattern of protein synthesis, again unlike pInt-bearing cells.

Analysis of ^{35}S-labelled *E. coli* lysates by SDS-PAGE confirmed the inhibition of Int synthesis observed by silver staining. In addition, general protein synthesis was reduced and the pattern of synthesis was perturbed by IPTG induction. The

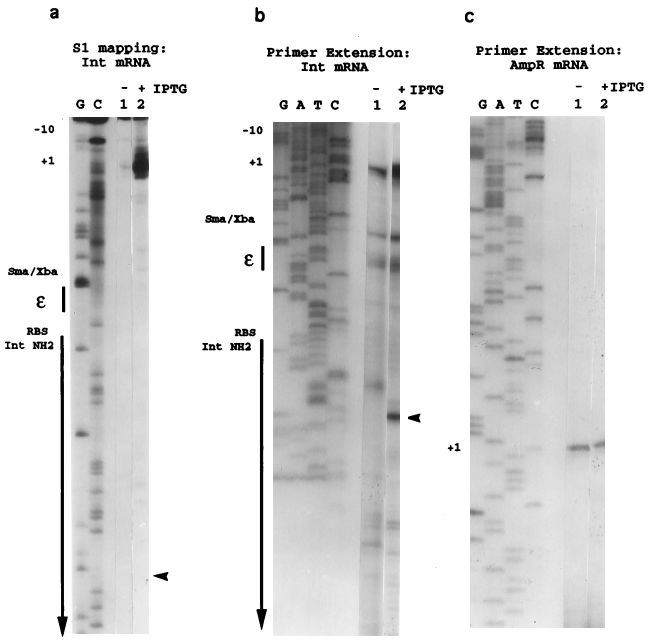

FIG. 3. S1 nuclease and primer extension analysis of *int* and ampicillin resistance mRNAs from induced and uninduced cells. Autoradiographs of 6% sequencing gels are shown. (a) Equivalent A_{260} amounts of total RNAs from uninduced (lane 1) or induced (lane 2) cells were annealed with a constant amount of a singly 5′ end ^{32}P-labelled *Ava*II-*Hin*cII restriction fragment. DNA-RNA hybrids were S1 treated, and the products were electrophoresed alongside Maxam and Gilbert (20) G and C sequencing products (lanes G and C) of the same end-labelled fragment. The positions of various features of the DNA sequence are indicated on the left. −10, −10 bases from the start point of *tac* promoter transcription; +1, start point of transcription; ε, T7 gene *10* translational enhancer; downward-pointing arrow, coding portion of the *int* gene; arrowhead, equivalent sequence position of modification shown in panel b. (b) Results of primer extension with the same RNA preparations as used in panel a. Approximately 3.5 times the A_{260} amount of RNA was used for the reaction mixture loaded in lane 1 (−IPTG) versus lane 2 (+IPTG). The primer was complementary to an amino-terminal portion of the *int* mRNA. All lane designations and sequence features are as shown in panel a, except that a full set of dideoxy sequencing standards (lanes G, A, T, and C) was used. In addition, the sequencing gel was run for a shorter time than for panel a. The arrowhead indicates the position of a major sequence modification occurring immediately downstream of *int* mRNA sequence AGA AGG CGA UC (within *int* codon 6) in IPTG-induced cells. (c) Results of primer extensions with the same RNA preparations used in a and b. In this case, extension was performed with a primer complementary to the amino terminus of the plasmid-encoded ampicillin resistance mRNA and the same A_{260} amounts of RNA were used for primer extension in lanes 1 and 2 as were used in panel b, lane 1.

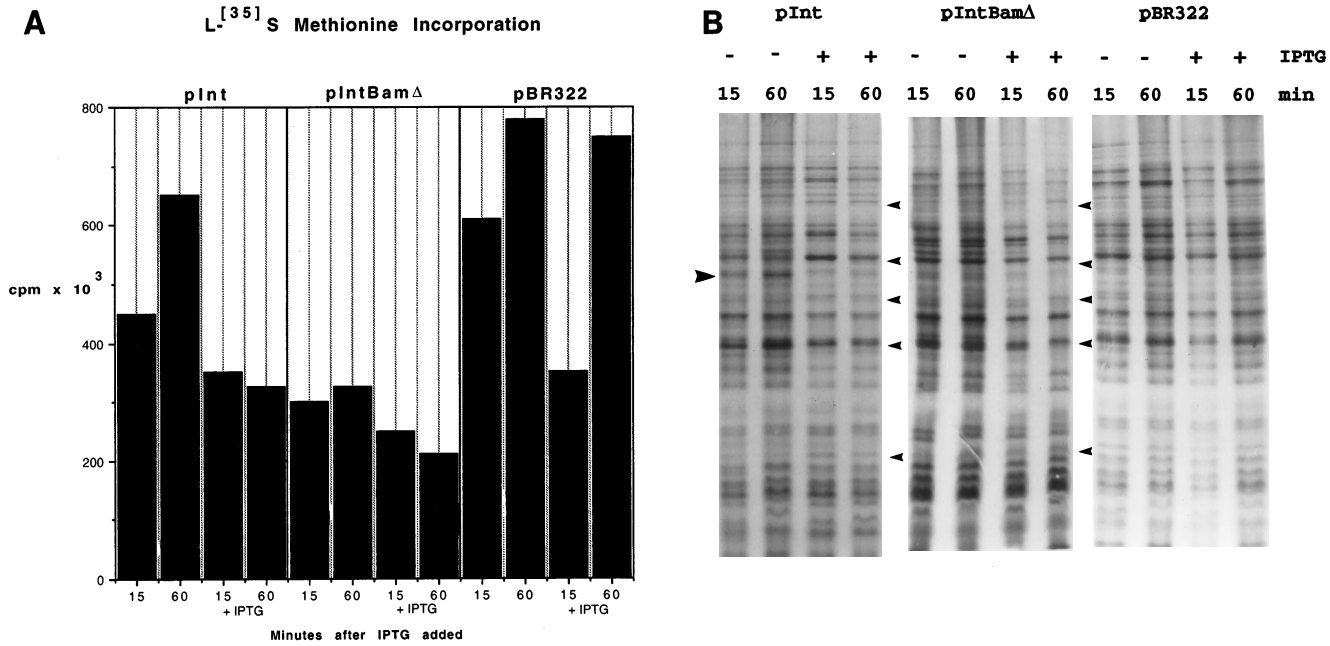

FIG. 4. Analysis of L-[^{35}S]methionine incorporation in induced versus uninduced cells. (A) Graphic representation of trichloroacetic acid-precipitable counts sampled at 15 and 60 min into the induction after 5 min of labelling with 10 μCi of L-[^{35}S]methionine per ml of cell culture. The induction protocol was as described in the legend to Fig. 2; see Materials and Methods and panel B (below) for precise details. (B) Autoradiographs of SDS–12.5% PAGE analysis of L-[^{35}S]methionine-labelled proteins synthesized in the presence (+) or absence (−) of the inducer IPTG. Cell cultures were sampled 15 and 60 min into the induction protocol after being labelled for 5 min with 10 μCi of L-[^{35}S]methionine per ml. From left to right, each group of four lanes was derived from pInt (*int* plasmid), pIntBamΔ (carboxy terminally deleted *int* gene plasmid), or pBR322 (vector alone). The large, rightward-pointing arrowhead indicates the position of the Int protein; the small, leftward-pointing arrowheads indicate the positions of other proteins induced by IPTG treatment.

observed perturbation could be interpreted as the shutdown of several proteins and the induction of others. No evidence of biosynthetic intermediates of the Int protein (due to stalled synthesis at rare Arg sites) was detected. This new pattern of protein synthesis does not reflect any general toxic effect of IPTG, since despite the transient drop in ^{35}S incorporation, pBR322-containing cells showed no similar qualitative alteration in the pattern of synthesis.

When analogous ^{35}S labelling experiments were carried out with a tRNA-supplementing plasmid (data not shown), the patterns of uninduced and induced protein synthesis were indistinguishable. This indicates that supplemental rare Arg tRNA can completely suppress the new pattern of protein synthesis induced by *int* mRNA. This is in agreement with the effects of supplemental tRNA on Int protein synthesis demonstrated with silver-stained gels (see above).

A plasmid derivative coding for a COOH-terminal deletion, pIntBamΔ, was tested for an IPTG-inhibitory effect. This plasmid, obtained by cleaving and filling in the *Bam*HI site within *int*, contains a 4-bp insertion and lacks the last 51 amino acids of the Int protein, including the active-site tyrosine at position 342 (28). This mutation results in translation termination in the +1 frame, 16 codons downstream of the insertion site. Under the same conditions of growth and IPTG induction, extremely similar patterns of protein synthesis (and IPTG-dependent inhibition) were seen (with the exception of Int). Interestingly, although the overall inhibition of protein synthesis was quantitatively similar, the kinetics of appearance and disappearance of particular proteins was slower. This may reflect a weaker inducing signal from this mRNA (i.e., due to lower stability) or some additional effect of the Int protein. The four-nucleotide insertion would be predicted to destabilize the base of a stable mRNA secondary structure which is proposed to protect against 3′-end degradation of the *int* mRNA (30). No truncated COOH-terminal deletion Int polypeptide was detected by staining or ^{35}S labelling. This may be due to destabilization of the frame-shifted protein, lability of the mutant mRNA, or both.

The inhibitory effect of IPTG therefore relates to expression of the *int* mRNA but not the Int protein. It appears that the cell can tolerate relatively high levels of the Int protein but that synthesis of high levels of *int* mRNA results in a pleiotropic effect on protein synthesis.

Effects of IPTG induction of *int* mRNA on cell growth. The effects of IPTG induction on growth of pInt-bearing cells in the presence or absence of a supplementing AGA tRNA plasmid are shown in Fig. 5. Cells were grown to an optical density of about 0.20 and then treated with 35 μM IPTG. Addition of the inducer had strikingly different effects, depending on the presence of the AGA tRNA plasmid.

In the absence of the inducer, pInt-bearing cells carrying a kanamycin resistance-encoding plasmid without a tRNA insert showed a faster growth rate than those with one. However, once IPTG was added the two populations of cells show a reversal in the pattern of growth. Cells with an AGA tRNA plasmid showed faster growth, whereas those without the tRNA plasmid slowed down. Cells bearing only an AGA tRNA plasmid or the control plasmid alone showed no differences in growth rate with or without the inducer (data not shown).

The IPTG-induced slow growth of pInt-bearing cells without a tRNA-supplementing plasmid can be understood in the context of the already described inhibitory effects of *int* mRNA on protein synthesis. It may be speculated that in the presence of the inducer, these cells enter the stationary phase sooner because of depletion of their rare Arg tRNA. The presence of a tRNA plasmid led to efficient Int protein synthesis and seemed

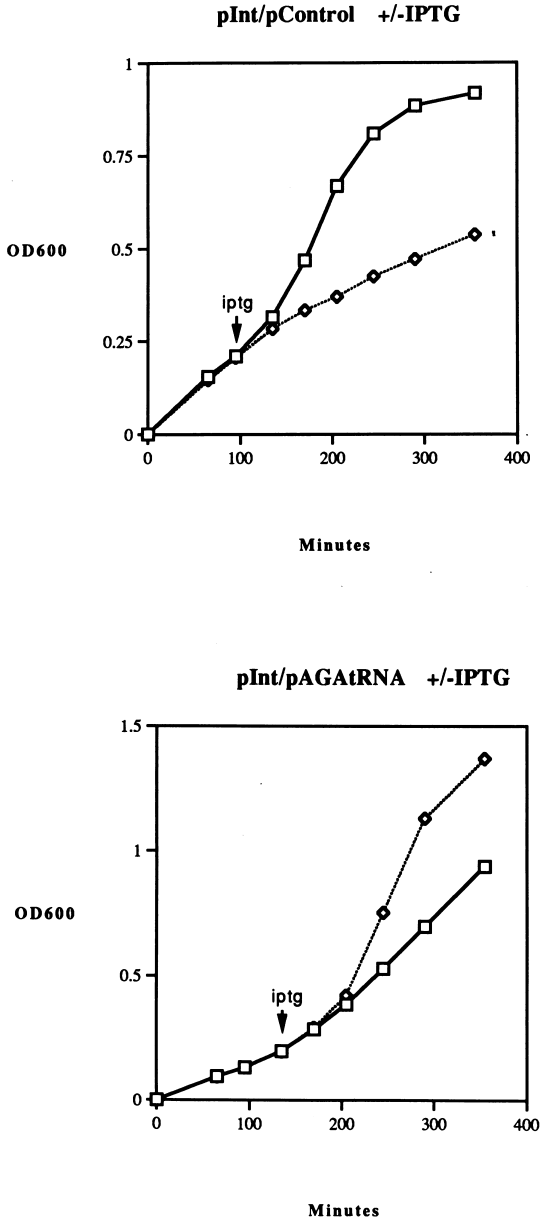

FIG. 5. Growth curves of cells grown in the presence or absence of an inducer and an AGA tRNA plasmid. The upper graph describes cells grown in the presence of *int* plasmid pInt and kanamycin resistance-encoding plasmid pControl (pAGA8), which does not have an AGA tRNA insert. The lower graph describes cells grown in the presence of *int* plasmid pInt and plasmid pAGAtRNA (pAGA9), which contains an AGA tRNA insert. Parallel cell cultures were grown to an A_{600} of 0.200, and then 35 μM IPTG was added to one culture. Growth was monitored by sampling the cultures and measuring A_{600} at the indicated time points. Symbols: □, plasmids without IPTG; ◇, plasmids with IPTG. OD600, optical density at 600 nm.

to slow growth initially but also protected against IPTG-induced entry into the stationary phase, allowing the cells to grow to a higher density.

These observations may relate to those of Chen and Inouye (5, 6) of growth phase-dependent expression of plasmid-borne β-galactosidase fusion proteins carrying blocks of AGG codons. In their experiments, limitation of expression of the AGG-containing tester protein was more extreme later in the growth cycle; however, no effect of any of the plasmid constructs on the growth rate of host cells was observed.

Growth of the induced pInt-bearing cells in the presence of the AGA tRNA plasmid is paradoxical. This strain grew more slowly early on, before the inducer was added, and faster after the inducer was added. An explanation for this phenomenon could be that expression of Int protein has a selective toxicity for the cell (i.e., because of DNA binding or topogenic effects) when produced early, at a certain low level, and this results in slowed growth. However, once enough protein is produced, inactive aggregates form which are no longer inhibitory. Since the Int protein is known to aggregate strongly (18a), this might be expected to occur later in the growth cycle, once sufficient protein has been synthesized. Uninduced cells lacking the AGA tRNA plasmid may produce this inhibitory level of Int protein later in the growth cycle, and combined with tRNA depletion, this may limit the ultimate cell density.

Cell killing upon transformation. To determine if the inducer had inhibitory effects on the transformability of this strain, electroporation into NM522 was carried out and cells were plated directly onto medium containing or lacking inducing levels of IPTG. Results of this experiment are shown in Fig. 6.

Plasmids with a wild-type or largely intact *int* gene coding sequence (pInt and pIntBamΔ) produced efficient cell killing in the presence of IPTG. This effect was completely reversed by transforming the same strain having a resident AGA tRNA plasmid. In addition, removal of the bulk of the *int* coding region by internal deletion with the enzyme *Sty*I (pIntStyIΔ) reversed the killing effect, although these colonies still grew slowly.

These results suggest a quantitative effect of the extent of the *int* mRNA sequence on cell viability. This effect may be due to a superabundance of rare Arg codons in the *int* mRNA or to the presence of another mRNA element exposed by stalled translation. Reducing the size of the *int* mRNA reduced AGA tRNA depletion and consequently the cell-killing effect.

The killing effect of *int* mRNA appears to occur at the level of plasmid establishment. IPTG-dependent toxicity was much weaker when exponentially growing cells containing an established *int* plasmid were plated on IPTG-containing plates (approximately 80% killing), and this killing was not suppressed by the presence of a second AGA tRNA plasmid (data not shown). Electrocompetent cells may be exquisitely sensitive to *int* mRNA toxicity, perhaps because of leakage or uncharging of rare *arg* tRNA.

DISCUSSION

As shown by SDS-PAGE and silver staining of uninduced extracts of cells containing plasmid pInt, *E. coli* can accumulate significant amounts of Int protein because of leaky transcription from p_{tac} and highly efficient translation of the resulting *int* mRNA, dependent on the T7 gene *10* ε sequence and RBS. In this situation, efficient translation of the *int* mRNA does not greatly hinder growth via the topogenic effects of the integrase protein (18) or formation of covalent DNA-Int protein complexes (24). Thus, the observed inhibitory phenomena do not correlate with Int protein expression or resemble those caused by gratuitous overproduction of certain proteins in *E. coli* (10).

int translation and transcription from the pInt plasmid appear to be uncoupled by IPTG induction of *int* mRNA, an effect which is proposed to be caused by dilution of a limited amount of rare AGA arginine tRNA on a large number of new ternary complexes. This is likely to be the result of efficient translation initiation conferred by the T7 ε sequence and RBS

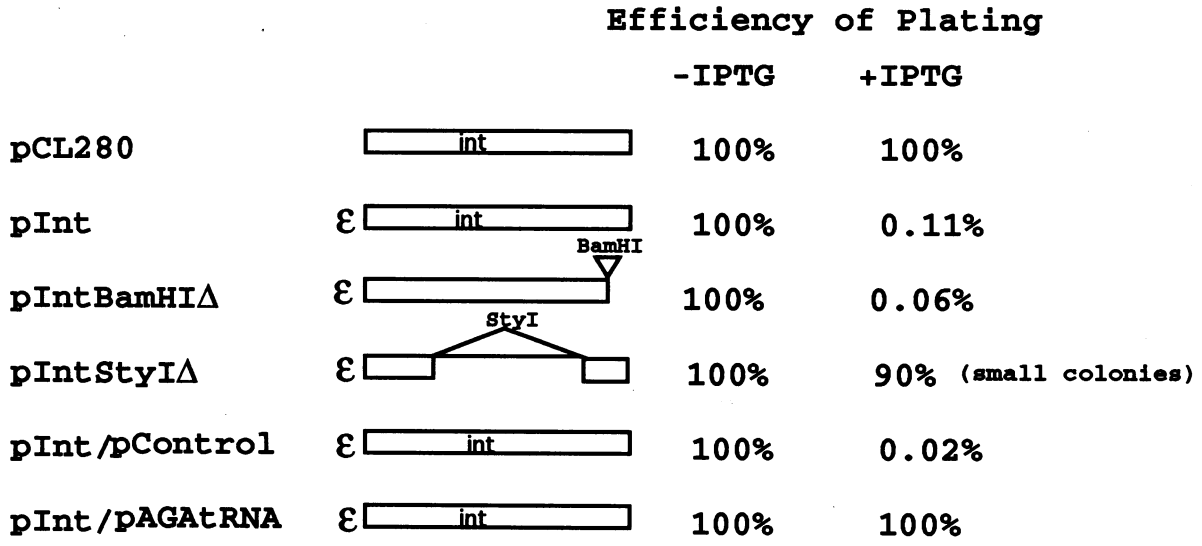

FIG. 6. Cell killing upon transformation with plasmids containing complete *int* genes or *int* gene fragments. All *int* gene plasmids were derived from pCL280 (see Fig. 1) and thus consist of a transcription unit bounded upstream by the *tac* promoter and downstream by *rrnB* terminators. The *int* gene is represented schematically by a rectangle, and variants are represented by truncated (pIntBamΔ) or interrupted (pIntStyIΔ) rectangles. Plasmid constructions carrying the T7 translational enhancer and RBS have ε preceding the *int* gene. Transformation into cells containing a second kanamycin resistance-encoding plasmid is indicated as either pControl (pAGA8) or pAGAtRNA (pAGA9). Results are presented as percentages of surviving colonies on IPTG-containing plates versus efficiency of plating without the inducer.

combined with *int*'s highly unfavorable rare Arg codon usage, resulting in inefficient elongation. Translational stalling at rare Arg codons ensues, and a variety of inhibitory effects are observed. Cell growth slows or ceases, protein synthesis is quantitatively decreased and qualitatively altered, and competent cells die upon transformation.

One proposed explanation of the effects on growth and transformation observed here is that another cellular mRNA(s), perhaps coding for a replication protein(s), competes for the same tRNA substrate and that the synthesis of this protein(s) (and consequently replication) is blocked under these conditions. This may be analogous to the results of Walker et al. (12, 16), who identified the Arg-4 tRNA gene as a *dna* gene (*dnaY*). In these experiments, the replication gene(s) controlled by the rare Arg tRNA was not identified. *int* mRNA overexpression may create a phenocopy of a *dnaY* mutant. Although slowed growth was observed under conditions inducing *int* mRNA, DNA synthesis was not examined.

In support of this idea, the novel pattern of protein synthesis seen in the presence of the inducer could be interpreted as an effect of tRNA depletion which causes several proteins which depend on the AGA (or AGG) cognate tRNA to migrate at new positions because of premature termination (22) or frameshifting (36). Such effects have been previously described as a result of overexpression of genes (22) or heterologous fusion proteins (36) in *E. coli*. However, analogous Int protein termination or frameshifting products corresponding to tRNA depletion effects on *int* mRNA translation have not been seen (37a; this report).

Brinkmann et al. (3) have shown that expression of certain eukaryotic genes rich in AGA and AGG codons in *E. coli* can result in growth inhibition and synthesis of ppGpp. Furthermore, the growth defect and ppGpp synthesis can be suppressed by supplementing AGA tRNA from a plasmid carrying the *E. coli* Arg-4 tRNA gene (*argU*). They therefore argue that rare Arg tRNA depletion due to heterologous gene expression leads to a stringent response. However, direct studies on depletion of the Arg-4 tRNA in temperature-sensitive *argU* mutant strains (7) do not show strong general inhibition of protein synthesis suggestive of a stringent response.

The apparent result of synthesis of a new set of proteins and suppression of synthesis of other proteins observed under conditions inducing *int* mRNA could indicate the induction of new mRNAs in addition to *int* mRNA and possibly a shutdown of others. One interpretation of such an effect is that it constitutes a novel general effect of stress on protein synthesis which depends on ribosome stalling on the *int* mRNA. Labelling studies analogous to those described here but employing heat shock protocols or amino acid starvation indicate that the precise nature of this alteration in protein synthesis does not resemble a stringent or heat shock response in the same cells (37a).

Cells experiencing transient amino acid starvation resulting in a stringent response can rapidly adapt their biosynthetic capacity and recover. Cells overproducing the *int* mRNA do not rapidly recover from this stress. Perhaps the quantity and slow decay of the abundant *int* mRNA prolong these effects. Synthesis of ppGpp or other effectors under conditions inducing *int* mRNA has not been examined.

While it is clear that sequences specific to the *int* mRNA can produce these effects, whether others rich in rare Arg (or other) codons suffice is unknown. Segments of the *int* mRNA contain the sequences AGAAGG, AGGAGA, and AGGAGG at codons 3 and 4, 108 and 109, and 176 and 177, respectively, which are perfect or nearly perfect RBSs but lack initiation codons. This provides the potential for internal binding of 30S ribosomal subunits. The consequences of 30S ribosome binding at these sites (if any) and their contribution to the inhibitory phenomena are unknown.

The possibility that covalent modification of the untranslated *int* mRNA occurs, dependent on stalled translation, is intriguing. The occurrence of this effect at a position predicted to be a translational stall (38) makes this phenomenon more remarkable. Does translational stalling induce a modification by the ribosome or some auxiliary factor, and could this specifically inactivate *int* mRNA? Are other cellular mRNAs or

other portions of the *int* mRNA modified under the same conditions? These are subjects for future experiments.

Are there naturally occurring conditions in which phage lambda uses the signal generated by stalled translation of *int* mRNA to its own advantage? The most likely case would be during excision, in which *int* mRNA translation is naturally uncoupled because of antitermination of the p_L transcript by the λ N protein, which causes RNA polymerase to process rapidly ahead of the first ribosome (31). Under these conditions, abundant synthesis of *int* mRNA from the p_L promoter could mimic the inhibitory effect of IPTG-induced *int* gene transcription described here, leading to partial inhibition of protein synthesis. Highly processive transcription by the N-modified RNA polymerase could contribute further to these effects by exposing large segments of untranslated *int* mRNA ahead of the first stalled ribosome.

The DNA rearrangement accompanying excision would place the additional λ inhibitory elements *sib* and *bar* immediately downstream of the *int* coding region. *sib* inhibition of Int protein synthesis (14, 33) and *bar* effects on host growth and protein synthesis (15, 29) depend on expression of segments of mRNA from the p_L operon downstream of *att*. These elements could function in concert with the *int* mRNA effects described here in a system of postexcision inhibition which prepares the cell for lysis, aids in the production of phage particles, and also prevents reinsertion of lambda DNA into *attB*. Since complete inhibition of cellular protein synthesis would be counterproductive to the phage burst, other cellular protein synthesis unrelated to phage particle production might be shut down by the *int* mRNA and *bar* effects.

Cohen and Chang (8) found a strong negative effect of a region which includes the amino terminus of *int* on expression of host β-galactosidase synthesis by using a set of prophage deletions. The effect described here may be related to their observations.

These results indicate that caution should be taken in attempts to clone and express heterologous (or bacteriophage) genes with codon biases which diverge greatly from those of *E. coli*. Cell killing and other inhibitory phenotypes may limit the ability to obtain the necessary clones or obtain high-level expression of certain genes. In this regard, codon-optimized derivatives of the lambda *int* gene like those to be described elsewhere by Zahn and Landy (38) (i.e., pLV and p5) cannot be constructed in the same plasmid background as pInt (37a).

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