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Enhanced Heterologous Gene Expression in Novel *rpoH* Mutants of *Escherichia coli*

MARK G. OBUKOWICZ,* NICHOLAS R. STATEN, AND GWEN G. KRIVI

Monsanto Corporate Research, Monsanto Company,
700 Chesterfield Village Parkway,
St. Louis, Missouri 63198

Received 28 October 1991/Accepted 25 February 1992

Extragenic temperature-resistant suppressor mutants of an *rpoD800* derivative of *Escherichia coli* W3110 were selected at 43.5°C. Two of the mutants were shown to have a phenotype of enhanced accumulation of heterologous proteins. Genetic mapping of the two mutants showed that the mutation conferring temperature resistance resided in the *rpoH* gene. P1-mediated transduction of the *rpoD*$^+$ gene into both of the *rpoD800 rpoH* double mutants resulted in viable *rpoH* mutants, MON102 and MON105, that retained temperature resistance at 46°C, the maximum growth temperature of W3110. The complete *rpoH* gene, including the regulatory region, from MON102, MON105, and the parental W3110 was cloned and sequenced. Sequencing results showed that a single C→T transition at nucleotide 802 was present in both MON102 and MON105, resulting in an Arg(CGC)→Cys(TGC) substitution at amino acid residue 268 (R-268-C; this gene was designated *rpoH358*). Heterologous protein accumulation levels in both MON102 and MON105, as well as in *rpoH358* mutants constructed in previously unmanipulated W3110 and JM101, were assessed and compared with parental W3110 and JM101 levels. Expression studies utilizing the *recA* or *araBAD* promoter and the phage T7 gene 10L ribosome-binding site (g10L) showed that increased accumulation levels of a number of representative heterologous proteins (i.e., human or bovine insulin-like growth factor-1, bovine insulin-like growth factor-2, prohormone of human atrial natriuretic factor, bovine placental lactogen, and/or bovine prolactin) were obtained in the *rpoH358* mutants compared with the levels in the parental W3110 and JM101. The mechanism of enhanced heterologous protein accumulation in MON102 and MON105 was unique compared with those of previously described *rpoH* mutants. Pulse-chase and Northern (RNA) blot analyses showed that the enhanced accumulation of heterologous proteins was not due to decreased proteolysis but was instead due to increased levels of the respective heterologous mRNAs accompanied by increased synthesis of the respective heterologous proteins. The plasmid copy number remained unaltered.

The heat shock response occurs in *Escherichia coli* when cells are shifted abruptly from a low (30°C) to a high (42°C) temperature (20, 36, 38). Transcription of all of the heat shock genes characterized to date is controlled by an alternate RNA polymerase sigma factor, σ^{32}, the product of the *rpoH* (*htpR*) gene (20, 21, 22, 25, 47). Mutations in the *rpoH* gene which curtail heat shock gene expression (6, 49, 54, 55) have been characterized. Such mutants might have practical value for the synthesis of heterologous proteins because inhibition of the heat shock response causes an overall decrease in proteolysis mediated directly or indirectly by the heat shock proteins La, ClpP, DnaK, DnaJ, GrpE, and GroEL (16, 17, 28, 29, 36, 48). The most commonly used *rpoH* mutant allele, *rpoH165*, is an amber mutation that renders the cell temperature sensitive for growth (8). Except for *rpoH111*, all of the other characterized *rpoH* mutations are temperature sensitive in an *rpoD*$^+$ strain background (6, 23, 49, 54). Thus, one drawback of using *rpoH* mutant strains is that the choice of mutant alleles is limited and a temperature shift to the nonpermissive temperature of 42°C must be performed to maximize heterologous protein accumulation. Nonetheless, the use of *rpoH* mutant strains, particularly *rpoH lon* double mutants, has been reported to increase heterologous protein accumulation levels (16, 28).

The *rpoD800* (*rpoD285*) mutant allele has an in-frame internal deletion of 42 bp (27) and gives a temperature-sensitive phenotype because the mutant σ^{70} subunit is rapidly degraded at temperatures of >40°C (19). Previous experiments showed that extragenic, temperature-resistant suppressor mutants of strains containing *rpoD800* that impaired proteolytic degradation of the mutant σ^{70} subunit could be obtained. Selection at 41.5°C yielded predominantly *lon* mutants (19), whereas selection at 43.5°C yielded predominantly *rpoH* mutants (23).

E. coli W3110 is a strain typically used for high-level expression of heterologous genes. The objective of this study was to isolate and characterize novel *rpoH* mutants of W3110 that accumulated higher levels of heterologous proteins. This report describes the isolation and characterization of a novel *rpoH* mutant allele, *rpoH358*, obtained by selecting for temperature-resistant suppressor mutants of an *rpoD800* derivative of W3110 at 43.5°C. The *rpoH358* mutant accumulated higher levels of a number of heterologous proteins by a mechanism that did not involve impaired proteolysis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and antibiotics. The bacterial strains and plasmids used in this study are listed in Table 1.

All of the plasmid expression vectors were derived from pBR327 and encode resistance to either ampicillin or spectinomycin. Expression of the prohormone of human atrial natriuretic factor (pro-ANF), human or bovine insulin-like growth factor-1 (IGF-1), bovine insulin-like growth factor-2

* Corresponding author.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype and/or relevant features[a]	Source or reference
Strains		
CAG440	*lac*(Am) *trp*(Am) *pho*(Am) *supC*(Ts) *rpsL mal*(Am) *lon*$^+$ *rpoH*$^+$	C. Gross, University of Wisconsin—Madison
CAG630	*lac*(Am) *trp*(Am) *pho*(Am) *supC*(Ts) *rpsL mal*(Am) *lon*$^+$ *rpoH165*(Am)-Tn*10*	C. Gross, University of Wisconsin—Madison; *zhg*::Tn*10* approximately 85% linked to *rpoH165*(Am)
CAG1130	F$^-$ *thi-1 thr-1 leu-6 lacY1 tonA21 supE44 galK rpoD800*-Tn*10*	C. Gross, University of Wisconsin—Madison; *rpoD800*-Tn*10* derivative of C600; Tn*10* approximately 90% linked to *rpoD800*
JM101	Δ(*pro-lac*) *supE thiA*/F′ *traD36 proAB lacI*^{q} *lacZ*ΔM15	32
MON100	*rpoD800*-Tn*10*	P1(CAG1130) × W3110
MON101	*rpoD800*-Tn*10 rpoH358*	Temperature-resistant suppressor mutant no. 1 of MON100, selected at 43.5°C
MON102	*rpoD*$^+$ *rpoH358*	P1(W3110) × MON101
MON103	*rpoD800* Δ*tet rpoH358*	Fusaric acid-resistant, tetracycline-sensitive derivative of MON101
MON104	*rpoD800*-Tn*10 rpoH358*	Temperature-resistant suppressor mutant no. 2 of MON100, selected at 43.5°C
MON105	*rpoD*$^+$ *rpoH358*	P1(W3110) × MON104
MON106	*rpoD800* Δ*tet rpoH358*	Fusaric acid-resistant, tetracycline-sensitive derivative of MON104
MON107	*rpoH*$^+$-Tn*10*	P1(CAG630) × CAG440; tetracycline-resistant, temperature-resistant transductant at 43.5°C
MON108	*rpoD800* Δ*tet rpoH358*-Tn*10*	P1(MON107) × MON103; tetracycline-resistant, temperature-resistant transductant at 43.5°C
MON109	*rpoD800* Δ*tet rpoH*$^+$-Tn*10*	P1(MON107) × MON103; tetracycline-resistant, temperature-sensitive transductant at 43.5°C
MON110	*rpoD800* Δ*tet rpoH358*-Tn*10*	P1(MON107) × MON106; tetracycline-resistant, temperature-resistant transductant at 43.5°C
MON111	*rpoD800* Δ*tet rpoH*$^+$-Tn*10*	P1(MON107) × MON106; tetracycline-resistant, temperature-sensitive transductant at 43.5°C
MON114	*rpoH358*-Tn*10*	P1(MON110) × JM101
MON115	*rpoH*$^+$-Tn*10*	P1(MON111) × JM101
MON116	*rpoH358*-Tn*10*	P1(MON110) × W3110
MON117	*rpoH*$^+$-Tn*10*	P1(MON111) × W3110
TST3	F$^-$ *araD139* Δ(*argF-lac*)*205 flbB5301 ptsF25 relA1 spoT1 rpsL150 malTS4*::Tn*10 deoC1* λ$^-$	G. Bogosian, Monsanto Co.
W3110	F$^-$ λ$^-$ IN(*rrnD-rrnE*)*1*	1
Plasmids		
pXT4	Ap^r; pBR322 derivative that has deletion in *tet* promoter	G. Bogosian, Monsanto Co.
pMON6717	Ap^r; *rpoH* gene from MON102 cloned into pUC118	This study
pMON6718	Ap^r; *rpoH* gene from MON105 cloned into pUC118	This study
pMON6719	Ap^r; *rpoH* gene from W3110 cloned into pUC118	This study
pMON5589	Ap^r; P*recA*-g10L–pro-ANF–T7$_{ter}$	13
pMON2320	Ap^r; P*recA*-g10L–IGF-1–T7$_{ter}$; synthetic IGF-1 gene with *E. coli*-preferred codons	52
pMON2368	Ap^r; P*recA*-g10L–IGF-1–f1*ori*$_{ter}$; synthetic IGF-1 gene from pMON2320 that is AT rich in first 14 codons to *Pst*I site	This study
pMON2360	Ap^r; P*recA*-g10L–bIGF-2–f1*ori*$_{ter}$; synthetic bIGF-2 gene that is AT rich in first 16 codons to *Pst*I site	9
pMON3401	Sp^r; P*recA*-g10L–bPL–T7$_{ter}$	This study
pMON3859	Ap^r; P*recA*-g10L: 40–bPRL:4–f1*ori*$_{ter}$; g10L:40 has AT-rich spacer region between the SD sequence and AUG initiator codon and a C nucleotide at position −5 (see ref. 40); bPRL:4 is the bPRL cDNA clone with the first 10 codons made A-T rich	This study
pMON3843	Ap^r; P*araBAD*-g10L:40–bPRL:4–f1*ori*$_{ter}$; P*araBAD* derivative of pMON3859	This study
pFN97	Ap^r; wild-type *rpoH* gene (deleted of P1 promoter) cloned into pBR322	37

[a] Abbreviations: f1*ori*$_{ter}$, phage f1 *ori* transcription terminator sequence; g10L, phage T7 gene 10 leader sequence; P*araBAD*, *araBAD* promoter; P*recA*, *recA* promoter; T7$_{ter}$, phage T7 gene 10 transcription terminator sequence.

(bIGF-2), and bovine placental lactogen (bPL) was under control of the *recA* promoter. Expression of bovine prolactin (bPRL) was under control of the *recA* or *araBAD* promoter. The bacteriophage T7 gene 10 leader sequence (g10L) (40) or a g10L variant sequence was positioned 5′ to the coding sequence for efficient translation initiation. The bacteriophage T7 gene 10 terminator or the bacteriophage f1 *ori* region was positioned downstream of the heterologous gene for transcription termination. Genes optimized for *E. coli* codon usage (18) were previously synthesized for IGF-1 (52) and bIGF-2 (9) expression. For expression of pro-ANF (13), bPL (44), and bPRL (35), cDNA clones or cDNA clones slightly modified at the 5′ end were used.

The antibiotics used and their respective concentrations (micrograms per milliliter) were as follows: ampicillin, 200 (agar plates) or 100 (liquid); tetracycline, 10; and spectinomycin, 50.

Shake flask expression analysis. Cells were grown as described previously for shake flask analysis of heterologous gene expression (39). M9 induction medium was used for *recA* promoter induction, whereas a modified M9 induction medium containing 0.05 instead of 0.2% glucose was used for *araBAD* promoter induction. Expression from the *recA* promoter was induced by adding nalidixic acid to a final concentration of 50 μg/ml at approximately 150 Klett units. Expression from the *araBAD* promoter was induced by adding arabinose to a final concentration of 0.2% at approximately 150 Klett units, a point at which glucose had been depleted from the medium. One-milliliter samples were taken for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis just prior to the addition of inducer (preinduction) and at various time points after induction (postinduction).

Relative plasmid copy number estimation. Just prior to induction and at 2 and 4 h after induction, pMON2368 (IGF-1 expression) or pMON3401 (bPL expression) DNA was isolated according to the alkaline lysis method of Sambrook et al. (42) and the concentration was normalized to cell density. The relative plasmid copy number was determined by densitometric scanning of agarose gels containing plasmid DNA samples stained with ethidium bromide. Each sample contained approximately 50 ng of plasmid DNA, an amount within the range (10 to 200 ng) in which mass is proportional to the fluorescence of ethidium bromide (7).

SDS-PAGE and Western blot analysis. SDS-PAGE and detection of IGF-1 by Western blot (immunoblot) analysis were performed as described previously (39). Quantitative estimations of the relative accumulation levels of the heterologous proteins were determined by densitometric scanning of Coomassie blue-stained protein gels or Western blot autoradiograms.

P1*vir* transduction. Standard P1*vir*-mediated transductions were performed by the method of Silhavy et al. (46). All transductants were purified by isolation as single colonies on the selective medium before testing them for their growth phenotype.

Selection for loss of Tn*10*-encoded tetracycline resistance in W3110 derivatives. Selection for loss of Tn*10*-encoded tetracycline resistance was performed by plating MON101 and MON104 on a fusaric acid-containing medium (31). Representative tetracycline-sensitive, temperature-resistant derivatives of MON101 and MON104, designated MON103 and MON106, respectively, were picked and used for Hfr and P1 mapping of the mutation responsible for the temperature-resistant phenotype and for later strain construction.

Hfr mapping. Standard Hfr matings were performed (33) by using a collection of 16 Hfr donor strains obtained from B. Bachmann, *E. coli* Genetic Stock Center, via G. Bogosian, Monsanto Company. The Hfr donors have a Tn*10* element downstream of the origin of transfer. The recipient strain, MON106, was transformed with plasmid pXT4 encoding ampicillin resistance in order to provide an ampicillin-resistant phenotype to counterselect against the ampicillin-sensitive Hfr donor strains. After mating, the cells were spread on Luria-Bertani–tetracycline–ampicillin (LB-tet-amp) agar plates and incubated at 30°C to select for tetracycline-resistant, ampicillin-resistant recombinants. The frequency of spontaneous tetracycline-resistant, ampicillin-resistant mutants was undetectable ($<10^{-7}$). All recombinants were purified by isolation as single colonies on the selective medium at 30°C and then tested for growth (i.e., formation of single colonies) on LB agar at 43.5°C.

P1*vir* cotransductional mapping. P1*vir* lysates were made from the following two donors having a Tn*10* element linked to *rpoH*$^+$: (i) TST3 (*zhf721*::Tn*10*) and (ii) MON107 (*zhg*::Tn*10*). The Tn*10* element in TST3 and MON107 is located at kb 75 and 76, respectively (23). MON103 and MON106 served as recipients. Tetracycline-resistant transductants were selected at 30°C on LB-tet agar and then scored for growth (i.e., colony-forming ability) on LB agar at 43.5°C.

Complementation mapping. Plasmid pFN97 (37) is a pBR322 derivative containing the wild-type *rpoH* gene fragment that has been used previously for complementation studies (11). MON101 and MON104 were transformed with pFN97 or pBR322 (negative control). Transformants were selected at 30°C on LB-amp agar plates. Two transformants each were purified by isolation as single colonies on LB-amp agar plates at 30°C. Single colonies were then restreaked on both LB and LB-amp agar plates and tested for growth (i.e., colony-forming ability) at 43.5°C. Growth was tested on both LB and LB-amp agar plates in order to eliminate any negative effects ampicillin may have on growth at 43.5°C. Complementation was scored as positive only when both transformants were temperature sensitive.

Cloning and sequencing of the wild-type and mutant *rpoH* genes. The entire *rpoH* gene (6, 14), including the regulatory region (approximately 0.2 kb), was isolated by the polymerase chain reaction (PCR) from genomic DNAs of W3110, MON101, MON102, MON104, and MON105 as a 1.2-kb *Bam*HI fragment (Fig. 1). The synthetic primer used to initiate forward strand synthesis of the *rpoH* gene contained a *Bam*HI site 5 nucleotides from the 5′ end and then 20 complementary nucleotides that hybridized to the *rpoH* gene at a position 50 bases upstream of the −35 region of the P1 promoter (11, 14). The synthetic primer used to initiate reverse strand synthesis of the *rpoH* gene also contained a *Bam*HI site 5 nucleotides from the 5′ end and then 20 complementary nucleotides that hybridized at the very end of the *rpoH* gene sequence reported by Calendar et al. (6), being 29 bases downstream of the UAA termination codon.

Plasmid DNA was prepared from CsCl-ethidium bromide gradients (42) or from Qiagen columns (Qiagen, Inc.) for double-stranded plasmid DNA sequencing. A total of 10 synthetic primers were used for sequencing both strands of the *rpoH* gene (Fig. 1). The universal forward and reverse primers for pUC vectors were also utilized for DNA sequencing of the 5′ and 3′ ends of the *rpoH* gene. Dideoxy sequencing of both strands of the entire *rpoH* gene was performed by using the Sequenase 2.0 kit (United States Biochemical Corp.). DNA sequence data were generated by using either conventional autoradiography or the Genesis 2000 automated DNA sequencer (DuPont). The presence of

a given mutation was confirmed by sequencing the immediate area containing the mutation of a second *rpoH* clone, whereby the *rpoH* gene fragment was obtained from an independent PCR.

^{35}S labeling of proteins. (i) Pulse-chase analysis. Cells were grown in M9 induction medium, and heterologous gene expression was induced by the addition of nalidixic acid. Five milliliters of culture was removed 30 min postinduction and pulse-labeled with 100 μCi of [^{35}S]cysteine (10 mCi/ml; Amersham) for 5 min. After labeling, cold cysteine (5 mg/ml, final concentration) was added as a chase. Samples (0.5 ml) were removed immediately after addition of the chase (0 min) and at various time points afterwards. Proteins were precipitated with trichloroacetic acid (1/10 volume of a 50% solution containing 2 mg of sodium deoxycholate per ml). After centrifugation, the protein pellets were resuspended in 1× sample buffer and normalized to cell density by adding one μl of sample buffer per Klett unit equivalent. Ten Klett unit equivalents (10 μl) was loaded per lane for SDS-PAGE analysis. The gels were stained with Coomassie blue in order to assess equal lane loadings, after which they were dried and subjected to autoradiography with Kodak XAR-5 film. Relative protein levels were quantified by densitometric scanning of the autoradiograms.

(ii) ^{35}S labeling of proteins after induction of the heat shock response. Cells were grown to log phase in [^{35}S]methionine labeling medium at 30°C. Two-milliliter portions were transferred to each of three 50-ml flasks, which had been prewarmed at 30, 42, or 50°C. After 10 min, the cells were pulse-labeled with 20 μCi of [^{35}S]methionine (10 mCi/ml; Amersham) for 1 min and then chased with cold methionine (500 μg/ml, final concentration) for an additional minute. Samples (0.5 ml) were removed immediately and treated as described above.

Puromycyl fragment degradation. An analysis of puromycyl fragment degradation was performed as described by Straus et al. (48). Three independent replicates were performed, and average values (percent protein degraded) at each time point were calculated.

Northern blot analysis. RNA extraction and Northern (RNA) blot analyses were performed as described by Miller and Wu (34). IGF-1 mRNA, bPL mRNA, and 16S rRNA (internal standard) were detected with synthetic oligodeoxyribonucleotide probes. The probes were derived from the anti-sense strand of the coding region of each respective gene as follows: (i) IGF-1 mRNA probe, nucleotides 4 to 43; (ii) bPL mRNA probe, nucleotides 333 to 349; and (iii) 16S rRNA probe, nucleotides 134 to 158. The probes were 5′ end labeled with ^{32}P by using [γ-^{32}P]ATP (>5,000 Ci/mmol; Amersham) and polynucleotide kinase (New England Biolabs) by the method of Sambrook et al. (42). The relative level of heterologous mRNA was quantified by densitometric scanning of the autoradiograms and was normalized to the corresponding level of 16S rRNA.

Construction of *rpoH358* derivatives of previously unmanipulated W3110 and JM101. By using *zhg*::Tn*10* that is approximately 85% linked to the *rpoH* gene (23), the *rpoH358* allele was cotransduced from MON110 into W3110 and JM101 by selecting for tetracycline-resistant transductants. The resulting *rpoH358*-Tn*10* transductants of W3110 and JM101 are referred to as MON116 and MON114, respectively. The *zhg*::Tn*10* element was also transduced from MON111 into the respective *rpoH*$^+$ parental strain to eliminate the possibility that the Tn*10* element affected the synthesis and accumulation of heterologous proteins. The resulting *rpoH*$^+$-Tn*10* transductants of W3110 and JM101 are referred to as MON117 and MON115, respectively. Since *rpoH358* provides no phenotype in an *rpoD*$^+$ strain background, the presence of *rpoH358* in tetracycline-resistant transductants of W3110 and JM101 was confirmed by sequencing a portion of the 3′ end of the *rpoH* gene which contained the mutation. Single-stranded template DNA for sequencing was amplified directly from the chromosome of tetracycline-resistant transductants by an asymmetric PCR protocol (J. Wild, University of Wisconsin—Madison). Approximately 25 nucleotides flanking either side of nucleotide 802, the site of the C→T transition in *rpoH358*, were sequenced to confirm the presence of *rpoH358* or *rpoH*$^+$ in the respective tetracycline-resistant transductants.

Construction of *rpoH*$^+$ derivatives of MON103 and MON106. Since the *rpoH358* allele causes a temperature-resistant phenotype in an *rpoD800* mutant, it was straightforward to construct *rpoD800 rpoH*$^+$ and *rpoD800 rpoH358* isogenic pairs. The *zhg*::Tn*10* element (23) was used to cotransduce *rpoH*$^+$ into the recipient strains. By scoring tetracycline-resistant transductants for temperature sensitivity at 43.5°C, *rpoH*$^+$ was cotransduced from MON107 into MON103 and MON106 (*rpoD800 rpoH358* derivatives of W3110), resulting in MON109 and MON111 (*rpoD800 rpoH*$^+$-Tn*10* derivatives of W3110), respectively. For controls, the *zhg*::Tn*10* element was transduced from MON107 into MON103 and MON106 (*rpoD800 rpoH358* derivatives of W3110) by scoring for tetracycline-resistant, temperature-resistant transductants, resulting in MON108 and MON110 (*rpoD800 rpoH358*-Tn*10* derivatives of W3110), respectively. Thus, all of the *rpoH358* and *rpoH*$^+$ transductants contained *zhg*::Tn*10* in order to maintain isogenic strains for comparing heterologous gene expression levels.

RESULTS

Selection of extragenic temperature-resistant mutants of MON100, an *rpoD800* derivative of W3110. The *rpoD800* mutation was transduced into W3110 by utilizing *zhg*::Tn*10* (23), which is approximately 90% linked to *rpoD800*. Transductants were selected for tetracycline resistance and then scored for temperature sensitivity. One tetracycline-resistant, temperature-sensitive transductant (referred to as MON100) was chosen for subsequent studies.

The selection for temperature-resistant suppressor mutants was carried out at 43.5°C because *rpoH* mutants were expected to be predominant (23). Temperature-resistant suppressor mutants from two different cultures of MON100 appeared at a frequency of approximately 10^{-7}. Twenty-one nonmucoid colonies (5 from culture no. 1 and 16 from culture no. 2) were picked at random and purified at 43.5°C two additional times. Mucoid colonies were not chosen because of the likelihood that they were *lon* mutants (19). All 5 mutants analyzed from culture no. 1 and 14 of 16 mutants analyzed from culture no. 2 retained temperature resistance. Five mutants from each culture were then assessed for heterologous protein accumulation levels (see below).

Previous results have shown that the accumulation of heterologous proteins is sometimes increased in *rpoH* mutants (3, 16, 28). Accumulation levels of two relatively labile heterologous proteins, IGF-1 and pro-ANF, were therefore examined in the 10 putative *rpoH* mutants of MON100. Results showed that >twofold higher levels of IGF-1 (encoded by pMON2320) and pro-ANF (encoded by pMON5589) accumulated in only 2 of the 10 putative *rpoH* mutants of MON100 (designated MON101 and MON104) compared with the levels in MON100 (data not shown).

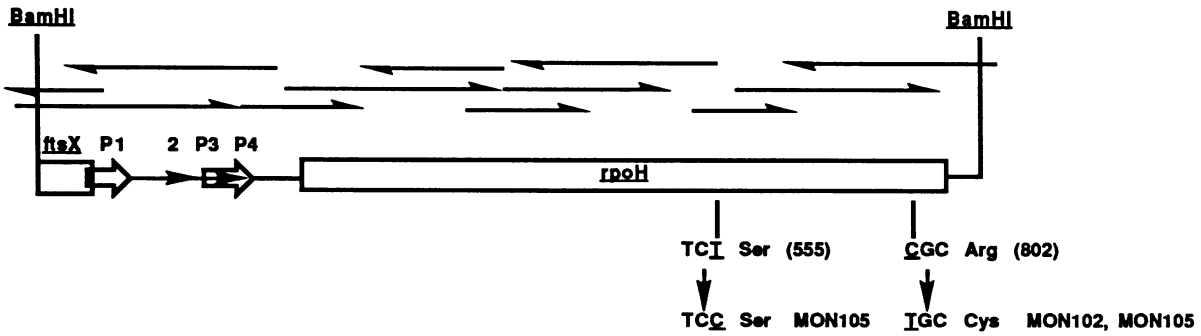

FIG. 1. Schematic representation of the cloned *rpoH* gene. The upstream regulatory region is composed of three tandem promoters (11): P1 (wide, open arrow), P3 (closed arrowhead), and P4 (narrow, open arrow). The promoter for transcript 2 (small, dark arrow) has not been identified by consensus sequence comparisons (11). In the *rpoH358* mutants, MON102 and MON105, a C→T transition was present at nucleotide 802 (amino acid 268), causing an Arg(CGC)→Cys(TGC) nonhomologous amino acid substitution (R-268-C) near the C terminus of σ^{32}. A silent mutation was also present in the *rpoH* gene of MON105. A T→C transition at nucleotide 555 was present, changing the TCT serine codon at amino acid 185 to a TCC serine codon. Horizontal arrows above indicate the DNA sequencing strategy using a series of synthetic DNA primers. The 3′ end of the upstream cell division gene, *ftsX* (14), was included because it overlaps with the P1 promoter. The entire *rpoH* gene from all three strains was sequenced on both strands, except for a short stretch to the right of the mutation at nucleotide 555.

MON101 and MON104 were derived from the same MON100 culture, making it possible that they were siblings. Even so, both mutants were characterized further by genetic mapping.

Mapping of the temperature-resistant suppressor mutations to the *rpoH* gene. Genetic mapping data (Hfr, P1, and complementation) showed that the mutation responsible for the temperature-resistant phenotype in MON101 and MON104 resided in the *rpoH* gene.

Hfr mapping. On the basis of a previous report (23), it was suspected that the temperature-resistant suppressor mutation was in the *rpoH* gene, which maps at kb 76 on the *E. coli* chromosome. Interpretation of the mating results was somewhat complicated because strain W3110 has a chromosomal inversion [IN(*rrnD-rrnE*)*1*] between kb 72 and 90 (1), encompassing the *rpoH* gene at kb 76. However, results showed conclusively that the mutation conferring the temperature-resistant phenotype in MON106 mapped between kb 75 and 85 (data not shown).

P1*vir* cotransductional mapping. Results with both recipients, MON103 and MON106, showed that the mutation responsible for the temperature-resistant phenotype was approximately 75% linked with *zhg*::Tn*10* (15 of 20 tetracycline-resistant transductants were temperature sensitive), indicating that it probably was present in the *rpoH* gene. No linkage of the temperature-resistant suppressor mutation with *malTS4*::Tn*10* was detected with either recipient (0 of 20 tetracycline-resistant transductants were temperature sensitive). Presumably, not enough transductants were scored since the *malTS4*::Tn*10* element is <8% linked with the *rpoH* gene (23).

Complementation mapping. Results showed that each of the two transformants of MON101(pFN97) and MON104 (pFN97) was temperature sensitive on both LB agar and LB-amp agar plates at 43.5°C. All of the transformants containing pBR322 retained temperature resistance at 43.5°C. These results demonstrated conclusively that a recessive *rpoH* mutant allele was responsible for the temperature-resistant phenotype in both MON101 and MON104.

Construction of MON102 and MON105, *rpoD*$^+$ *rpoH* derivatives of W3110. *rpoD*$^+$ derivatives of MON101 and MON104 were constructed for two reasons: (i) to assess whether higher accumulation levels of heterologous proteins could be obtained in an *rpoD*$^+$ strain background containing the *rpoH* mutation and (ii) to determine whether the *rpoH* mutation would be compatible (i.e., viable) with the *rpoD*$^+$ gene and, if so, whether it would still provide a temperature-resistant phenotype. The maximum growth temperature at which W3110 formed colonies on LB agar was 46°C, whereas MON101 and MON104 failed to form colonies at temperatures of >43.5°C.

A P1*vir* lysate of W3110 was used to transduce *rpoD*$^+$ into MON101 and MON104. The *rpoD*$^+$ *rpoH* transductants, MON102 and MON105, respectively, were selected for their ability to grow and form colonies at 46°C on LB agar. Since the *rpoD800* mutant allele is approximately 90% linked to a Tn*10* element encoding tetracycline resistance, loss of the tetracycline-resistant phenotype in the *rpoD*$^+$ *rpoH* transductants, MON102 and MON105, served to verify the presence of the *rpoD*$^+$ gene. Temperature-resistant, tetracycline-sensitive transductants were obtained with both recipients, indicating that the *rpoH* mutant allele was compatible with the *rpoD*$^+$ gene. None of the four transductants tested retained tetracycline resistance.

Cloning and sequencing of the *rpoH* gene from MON102, MON105, and W3110. In order to locate the mutation in the *rpoH* gene, the entire *rpoH* gene, including the complex regulatory region, was isolated from the genome of the *rpoD*$^+$ *rpoH* mutants, MON102 and MON105, and from parental W3110 (*rpoH*$^+$), and the DNA sequence was determined (Fig. 1). The sequence of the regulatory and structural portions of the *rpoH* gene from W3110 was identical to the published sequence of the *rpoH*$^+$ gene (6). In contrast, the *rpoH* gene from both mutants, MON102 and MON105, had the identical structural gene mutation (Fig. 1), hereinafter referred to as *rpoH358* (B. Bachmann). A C→T transition was present at nucleotide 802, causing an Arg(CGC)→Cys(TGC) nonhomologous amino acid substitution at amino acid 268 (R-268-C) near the C terminus of σ^{32}. This is a unique mutation not previously described (5, 6, 49, 54). In addition, the *rpoH* structural gene mutation in MON102 and MON105 was confirmed to be present in the original temperature-resistant suppressor mutants, MON101 and MON104, by DNA sequence analysis of the analogous

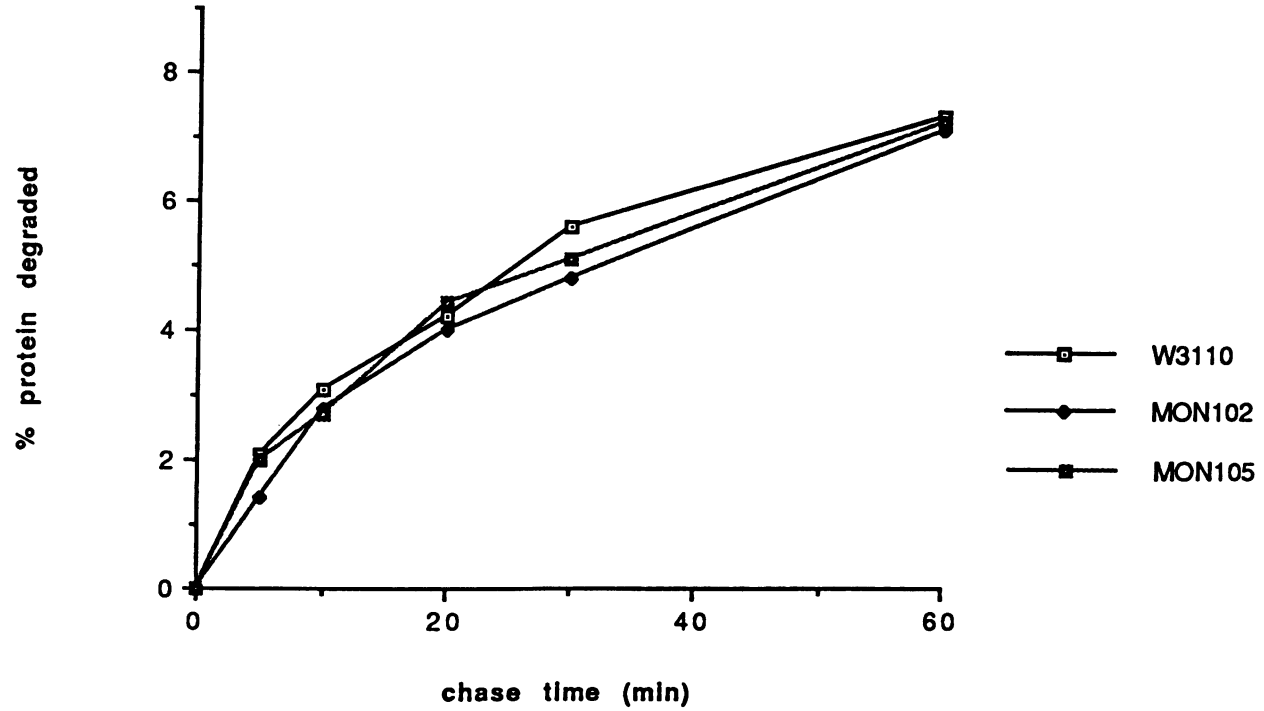

FIG. 2. Rate of puromycyl fragment degradation in the *rpoH358* mutants, MON102 and MON105, compared with that in parental W3110 at 37°C. Cells were grown to mid-log phase in ^{35}S-labeling medium, after which puromycin (Boehringer-Mannheim) was added (100 μg/ml) and the cultures were incubated for 10 min. Thirty microcuries of [^{35}S]methionine (10 mCi/ml; Amersham) was added, and the cultures were incubated for an additional 3 min. The cells were harvested by centrifugation, washed twice in saline, and resuspended in fresh ^{35}S-labeling medium containing cold methionine (400 μg/ml). Samples (0.5 ml) were removed at 0, 5, 10, 20, 30, and 60 min and added to an Eppendorf tube containing 55 μl of 50% trichloroacetic acid (containing 2 mg of sodium deoxycholate per ml) and 200 μg of bovine serum albumin as a carrier. Trichloroacetic acid-soluble ^{35}S counts were measured in a liquid scintillation counter. The rate of proteolysis was calculated by subtracting the background of trichloroacetic acid-soluble counts present in the 0-min chase sample from the trichloroacetic acid-soluble counts present in the respective chase samples and dividing by the total number of counts present in the 0-min sample. The rate of puromycyl fragment degradation is expressed as the percent protein degraded per minute after addition of the cold methionine chase.

1.2-kb *Bam*HI fragment. Also, PCR cloning and sequencing were repeated for the two *rpoH358* mutants, MON102 and MON105, and yielded identical results.

An additional silent mutation was also present in the *rpoH* gene of strain MON105. A T→C transition at nucleotide 555 was present, changing the TCT serine codon at amino acid 185 to a TCC serine codon. Both TCT and TCC are highly preferred *E. coli* serine codons (18). PCR cloning and sequencing were repeated for this mutant and yielded the identical result. However, DNA sequencing of the *rpoH* gene present in the original temperature-resistant suppressor mutant, MON104, showed that the wild-type sequence (TCT serine codon) was present. It remains unclear why a silent mutation occurred during the construction of MON105 from MON104. The relevance of this silent change is unknown, although previous reports in the literature showed there to be disagreement regarding the amino acid present at this position because of an altered nucleotide in the codon (6).

Since both MON101 and MON104 were initially selected as temperature-resistant suppressor mutants from the same culture of MON100 and both contained the *rpoH358* mutant allele, it was very likely that they were siblings. Nonetheless, characterization of the mutant phenotype of both MON102 and MON105 was performed, especially with regard to the effects of an altered σ^{32} subunit on the enhanced accumulation of heterologous proteins. Ensuing results showed that, as expected, MON102 and MON105 behaved identically.

Puromycyl fragment degradation. Puromycin added to growing bacterial cultures causes premature translation termination, generating unstable puromycyl polypeptides. The rate of degradation of these polypeptides into trichloroacetic acid-soluble products has been used to measure the ability of strains to carry out the degradation of abnormal proteins (15).

Experiments examining the rate of puromycyl fragment decay were performed with the *rpoH358* mutants, MON102 and MON105, and parental W3110 in order to determine whether the *rpoH358* mutation caused a decreased rate of proteolysis. There were no differences in the rates of proteolysis among parental W3110, MON102, and MON105 at 37°C, the growth temperature routinely used for heterologous gene expression (Fig. 2).

Heat shock response in MON102, MON105, and W3110. All previously characterized *rpoH* mutants are defective in the heat shock response (5, 6, 49, 54, 55). ^{35}S-labeling experiments showed that the *rpoH358* mutants, MON102 and MON105, were not defective in the heat shock response (Fig. 3). The profiles of newly synthesized heat shock proteins were nearly identical in parental W3110, MON102, and MON105, regardless of whether the cultures were maintained at 30°C or temperature shifted from 30 to 42°C or from 30 to 50°C (Fig. 3). At 50°C, heat shock proteins are synthesized exclusively (10). Judging by the intensity of the newly synthesized protein bands at 42 and 50°C, higher levels of most of the predominant heat shock proteins were

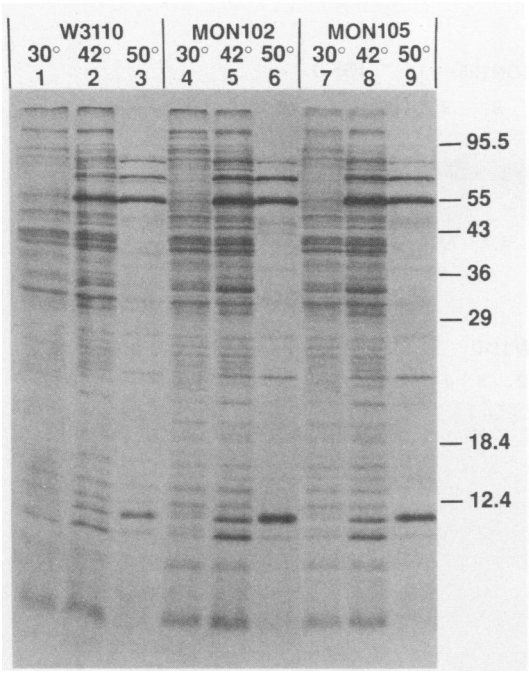

FIG. 3. Heat shock protein synthesis in *rpoH358* mutants, MON102 and MON105, compared with that in parental W3110. In vivo protein synthesis in MON102, MON105, and W3110 was compared at 30°C and after induction of the heat shock response at 42 or 50°C. At each temperature, cells were pulse-labeled with [^{35}S]methionine (see Materials and Methods). The autoradiogram was obtained after exposing the dried gel to Kodak XAR-5 film for 5 days at −70°C with an intensifying screen. Coomassie blue staining of the protein gel prior to autoradiography showed that all of the lanes had approximately equal loadings of cell protein (data not shown). Molecular weight markers (10^3) are indicated.

synthesized in MON102 and MON105 compared with parental W3110 (Fig. 3). Also, parental W3110 did not synthesize any observable heat shock protein at a higher level than MON102 or MON105 (Fig. 3).

Enhanced heterologous protein accumulation in the *rpoH358* mutants, MON102 and MON105. Several heterologous proteins (i.e., IGF-1, bIGF-2, bPRL, and pro-ANF) were used in combination with two unrelated promoters, *recA* and *araBAD*, in order to ascertain whether enhanced protein accumulation in the *rpoH358* mutants, MON102 and MON105, was heterologous protein independent and promoter independent. Not all of the heterologous proteins tested were totally unrelated. IGF-1 and bIGF-2 have approximately 60% homology (26), whereas bPL and bPRL have approximately 50% homology (35, 44). However, there is no similarity among pro-ANF, IGF-1–bIGF-2, and bPL–bPRL. The *recA* promoter is a relatively strong σ^{70}-dependent promoter that has strong consensus in the −35 and −10 regions with other σ^{70}-dependent promoters (24). In contrast, the *araBAD* promoter is unrelated to other σ^{70}-dependent promoters in that it lacks any recognizable consensus sequence at both the −35 and −10 regions, and, instead, transcription is regulated positively and negatively by the AraC protein (24, 43).

Enhanced accumulation of all the heterologous proteins was reproducibly obtained in the same relative amounts in the *rpoH358* mutants, MON102 and MON105, compared with parental W3110 (Fig. 4). The differences in accumulation levels depended on the heterologous protein in particular and the sampling time. IGF-1 encoded by pMON2368 accumulated to levels approximately eightfold higher at 2 h after induction (Fig. 4A). Approximately 1.5-fold more bIGF-2 encoded by pMON2360 accumulated at 3 h after induction (Fig. 4B) (9). bPL encoded by pMON3401 accumulated to levels approximately twofold higher at 4 h after induction (Fig. 4C). The *araBAD* promoter, as well as the *recA* promoter, was used to control the expression of bPRL. Under *araBAD* promoter control, bPRL encoded by pMON3843 accumulated to levels approximately twofold higher at 2 h postinduction (Fig. 4D), showing that enhanced heterologous protein accumulation was not specific for *recA* promoter-controlled expression. In contrast, bPRL encoded by pMON3859 (*recA* promoter control) accumulated to similar levels in the mutants and parent, presumably because high-level accumulation (approximately 30% of total cell protein) was readily obtained in parental W3110 (data not shown). Lastly, pro-ANF encoded by pMON5589 accumulated to levels approximately twofold higher at 2 h postinduction (Fig. 4E).

Mechanism of enhanced heterologous protein accumulation in the *rpoH358* mutants, MON102 and MON105. Pulse-chase, Northern blot, and plasmid copy number analyses were performed to assess whether enhanced heterologous protein accumulation in the *rpoH358* mutants, MON102 and MON105, occurred by decreased proteolysis or by increased synthesis and whether the gene dosage (i.e., plasmid copy number) was affected. On the basis of previous reports (2, 16, 17, 23, 28), it was presumed that increased heterologous protein accumulation levels in the *rpoH358* mutants, MON102 and MON105, would be due to an increased protein half-life. This explanation, however, was not supported by the observation that the rate of puromycyl fragment degradation was the same in MON102, MON105, and parental W3110 at 37°C (Fig. 2), the temperature normally used for the induction of heterologous protein synthesis.

Pulse-chase experiments were performed to compare the relative synthesis levels and half-lives of IGF-1, bPL, and pro-ANF in the *rpoH358* mutants, MON102 and MON105, and parental W3110. Densitometric quantification of the autoradiograms showed that the relative synthesis levels of all three proteins were 1.3- to 1.7-fold higher in the mutants than in parental W3110 (Fig. 5). Densitometric quantification of the chase sample lanes in the autoradiograms also showed no significant differences in the half-lives of all three proteins in the three strains (Fig. 5). In contrast to the relative lability of IGF-1 and pro-ANF (Fig. 5A and C, respectively), bPL was very stable in all three strains, showing very little degradation after a 32-min chase (Fig. 5B).

IGF-1 and bPL mRNA levels were higher in the *rpoH358* mutants, MON102 and MON105, than in parental W3110 (Fig. 6). IGF-1 mRNA levels were approximately fourfold higher at 30 min after induction and approximately two- to threefold higher at 2 h after induction (Fig. 6A). bPL mRNA levels were similar at 30 min after induction but were nearly threefold higher at 2 h after induction (Fig. 6B).

Increased plasmid copy number did not appear to be responsible for the increased IGF-1 and bPL mRNA levels. The plasmid copy number of pMON2368 (IGF-1 expression) and pMON3401 (bPL expression) remained unaltered in MON102, MON105, and parental W3110 prior to induction and at 30 min, 2 h, and 4 h after induction. At every sampling point with all three strains, the difference in ethidium bromide fluorescence of the pMON2368 or pMON3401 samples was <10% (data not shown).

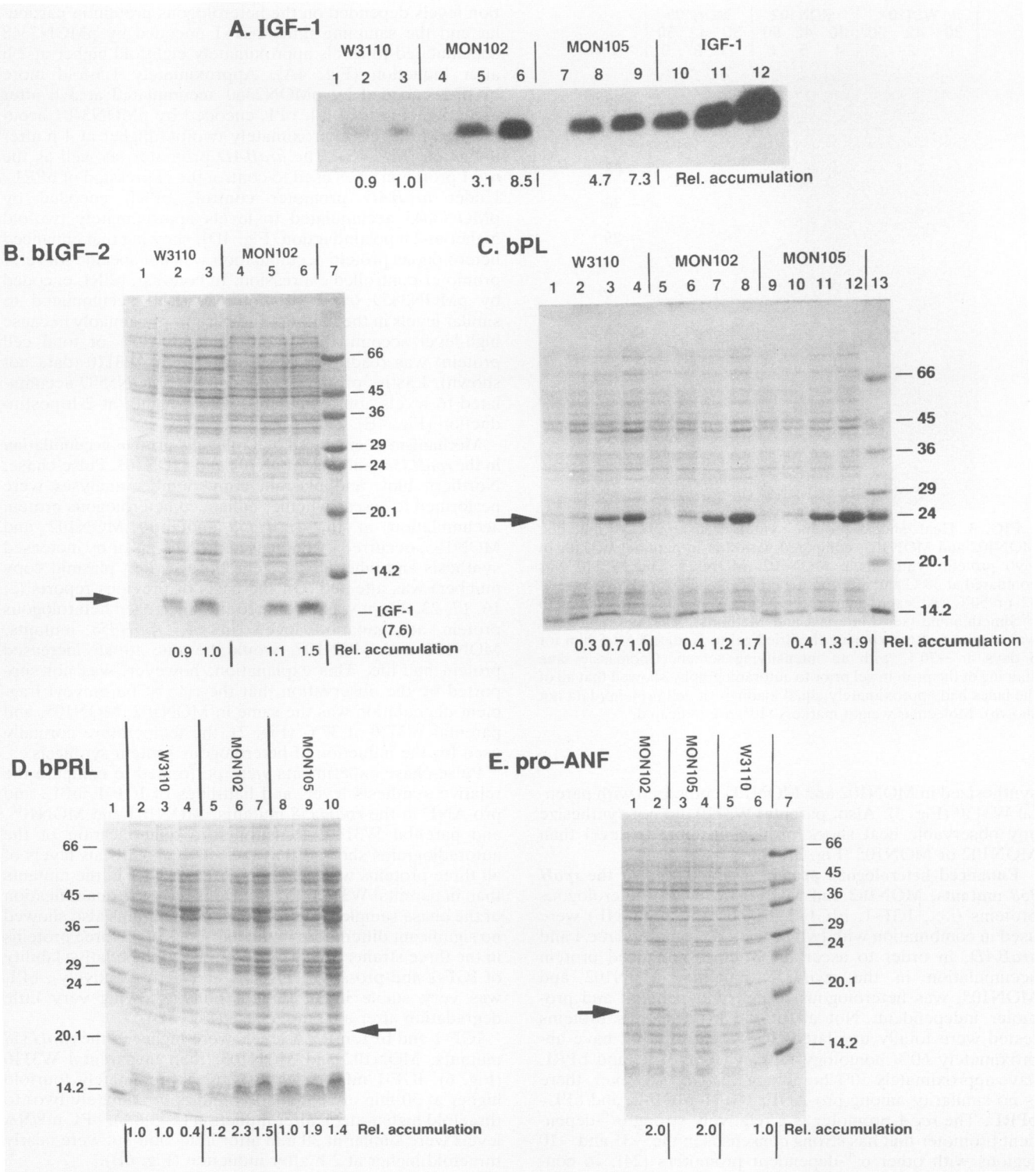

FIG. 4. Enhanced accumulation levels of heterologous proteins in *rpoH358* mutants, MON102 and MON105, compared with parental W3110. Except for IGF-1 (panel A), all of the heterologous proteins were detected by Coomassie blue staining of protein gels as distinct, inducible bands. IGF-1 accumulation levels were estimated by Western blot analysis. Expression of IGF-1, bIGF-2, bPL, and pro-ANF was under *recA* promoter control, while expression of bPRL was under *araBAD* promoter control. The cells were grown and induced in M9 induction medium (see Materials and Methods). Samples were taken preinduction and at various time points postinduction. Densitometric scanning of the Western blot autoradiogram (A) or the Coomassie blue-stained protein gels (B to E) was used to quantify the relative amounts of the heterologous proteins which accumulated postinduction (numbers below lanes). In each case, the amount of heterologous protein which accumulated to the highest level in parental W3110 was arbitrarily set at 1.0. Molecular weight markers (10^3) are indicated. (A) Lanes 1 to 3, W3110(pMON2368) preinduction (lane 1), 30 min postinduction (lane 2), and 2 h postinduction (lane 3); lanes 4 to 6, MON102(pMON2368)

***rpoH358* is responsible for enhanced heterologous protein accumulation.** Genetic mapping results clearly showed that *rpoH358* is a recessive mutation responsible for the temperature-resistant phenotype of MON101 and MON104. Since extragenic temperature-resistant suppressor mutations have been isolated in temperature-sensitive *rpoH* mutants (30, 50, 53), it was possible that a second mutation, acting separately or in concert with *rpoH358*, was responsible for enhanced heterologous protein accumulation in the *rpoH358* mutants, MON102 and MON105. The possibility also existed that the altered σ^{32} factor encoded by *rpoH358* would not function to enhance heterologous protein accumulation in a strain other than W3110. To show that *rpoH358* was necessary for enhanced accumulation of heterologous proteins, two sets of *rpoH358* and *rpoH*$^+$ isogenic strains were constructed (see Materials and Methods for details of strain construction) and accumulation levels of two relatively labile heterologous proteins, IGF-1 and pro-ANF, were compared. With all of the strain constructions, *zhg*::Tn*10* was used to cotransduce *rpoH358* or *rpoH*$^+$ into recipient strains because it is approximately 85% linked to the *rpoH* gene (23). In the first set of strains constructed, *rpoH358* was transduced into previously unmanipulated W3110 as well as into JM101, resulting in strains MON116 and MON114, respectively. MON114, an *rpoH358* derivative of JM101, was constructed to assess whether enhanced accumulation of heterologous proteins occurred in an *rpoH358* mutant strain background besides that of W3110. For controls, *zhg*::Tn*10*-containing *rpoH*$^+$ derivatives of W3110 and JM101, MON117 and MON115, respectively, were constructed. In the second set of strains constructed, *rpoH*$^+$ was transduced back into the existing *rpoD800 rpoH358* derivatives of W3110, MON103 and MON106, recreating the parental *rpoD800 rpoH*$^+$ strains, MON109 and MON111, respectively. For controls, *zhg*::Tn*10* was transduced into MON103 and MON106, resulting in MON108 and MON110, respectively.

If *rpoH358* were necessary for the enhanced accumulation of heterologous proteins, then all of the strains containing *rpoH358* should accumulate more pro-ANF and IGF-1 compared with the corresponding *rpoH*$^+$ parental strains. In the first set of expression experiments, both pro-ANF and IGF-1 accumulated to higher levels in the *rpoH358* derivative of W3110 (MON116) or JM101 (MON114) compared with the corresponding *rpoH*$^+$ derivative of W3110 (MON117) or JM101 (MON115). In MON116 and MON114, pro-ANF accumulated to similar levels, being approximately two- to threefold higher than in MON117 and MON115, respectively (data not shown). IGF-1 accumulated to levels approximately fivefold higher in MON116 than in MON117 (data not shown). The degree of enhanced accumulation of pro-ANF and IGF-1 in MON116 compared with MON117 paralleled very closely that obtained in the original *rpoH358* mutants, MON102 and MON105, compared with that obtained in parental strain W3110 (see Fig. 4A and E). For unknown reasons, IGF-1 accumulated to levels approximately 10-fold lower in MON114 compared with MON116. However, IGF-1 accumulation was undetectable in MON115. These results demonstrated that the *rpoH358* allele was responsible for enhancing the relative accumulation levels of heterologous proteins in two different strain backgrounds. In the second set of expression experiments, pro-ANF accumulation levels were compared in the *rpoD800 rpoH358* derivatives of W3110, MON108 and MON110, and the corresponding *rpoD800 rpoH*$^+$ derivatives, MON109 and MON111. Pro-ANF accumulated to levels of approximately 3% of total cell protein in both MON108 and MON110, whereas it was virtually undetectable (<1% of total cell protein) in MON109 and MON111 (data not shown). These results also demonstrated that the *rpoH358* allele was responsible for the enhanced accumulation of heterologous proteins.

DISCUSSION

The major objective of this study was to isolate novel *rpoH* mutants of W3110 that accumulated higher levels of a broad range of heterologous proteins, while retaining desirable fermentation properties. The isolation of the novel *rpoH* allele, *rpoH358*, and construction of MON102 and MON105, both *rpoH358* derivatives of W3110, met the objective. The enhanced accumulation of several heterologous proteins, including pro-ANF, IGF-1, bIGF-2, bPL, and bPRL, was obtained. In addition, MON102 and MON105 still retained desirable fermentation properties for optimal productivity of heterologous proteins, including (i) cell viability coupled with a relatively fast growth rate within a range of temperatures (25 to 42°C), (ii) lack of a mucoid phenotype, and (iii) enhanced accumulation of heterologous proteins without a temperature shift. Also, the enhanced accumulation of heterologous proteins caused by the presence of *rpoH358* was both strain and promoter independent. The enhanced accumulation of IGF-1 and pro-ANF was obtained in *rpoH358* derivatives of both W3110 and JM101 by using *recA* promoter-controlled expression plasmids, while the enhanced accumulation of bPRL was obtained by using an *araBAD* promoter-controlled expression plasmid. Other rather unique properties conferred by the *rpoH358* mutation include (i) a mechanism of enhanced heterologous protein accumulation that did not involve impaired proteolysis, (ii) an apparently fully functional heat shock response, (iii) a temperature-resistant phenotype, and (iv) compatibility with the *rpoD*$^+$ gene. In most of the previously characterized *rpoD800* (*rpoD285*) *rpoH* double mutants constructed in an identical

preinduction (lane 4), 30 min postinduction (lane 5), and 2 h postinduction (lane 6); lanes 7 to 9, MON105(pMON2368) preinduction (lane 7), 30 min postinduction (lane 8), and 2 h postinduction (lane 9); lanes 10 to 12, IGF-1 standard: 25 ng (lane 10), 50 ng (lane 11), and 100 ng (lane 12). (B) Lanes 1 to 3, W3110(pMON2360) preinduction (lane 1), 2 h postinduction (lane 2), and 3 h postinduction (lane 3); lanes 4 to 6, MON102(pMON2360) preinduction (lane 4), 2 h postinduction (lane 5), and 3 h postinduction (lane 6); lane 7, molecular weight markers. (C) Lanes 1 to 4, W3110(pMON3401) preinduction (lane 1), 30 min postinduction (lane 2), 2 h postinduction (lane 3), and 4 h postinduction (lane 4); lanes 5 to 8, MON102(pMON3401) preinduction (lane 5), 30 min postinduction (lane 6), 2 h postinduction (lane 7), and 4 h postinduction (lane 8); lanes 9 to 12, MON105(pMON3401) preinduction (lane 9), 30 min postinduction (lane 10), 2 h postinduction (lane 11), and 4 h postinduction (lane 12); lane 13, molecular weight markers. (D) Lane 1, molecular weight markers; lanes 2 to 4, W3110(pMON3843) 30 min postinduction (lane 2), 2 h postinduction (lane 3), and 4 h postinduction (lane 4); lanes 5 to 7, MON102(pMON3843) 30 min postinduction (lane 5), 2 h postinduction (lane 6), and 4 h postinduction (lane 7); lanes 8 to 10, MON105(pMON3843) 30 min postinduction (lane 8), 2 h postinduction (lane 9), and 4 h postinduction (lane 10). (E) Lanes 1 and 2, MON102(pMON5589) preinduction (lane 1) and 2 h postinduction (lane 2); lanes 3 and 4, MON105(pMON5589) preinduction (lane 3) and 2 h postinduction (lane 4); lanes 5 and 6, W3110(pMON5589) preinduction (lane 5) and 2 h postinduction (lane 6); lane 7, molecular weight markers.

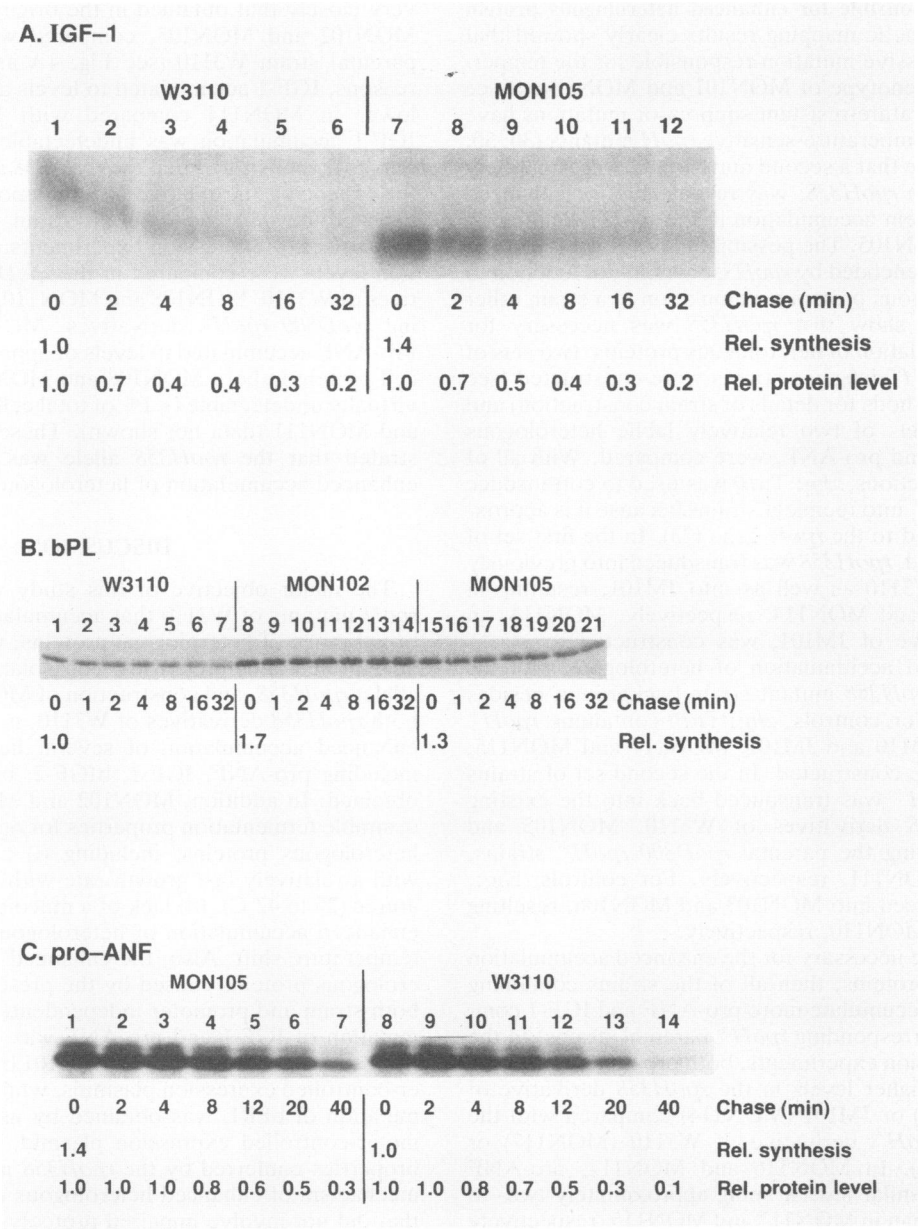

FIG. 5. Comparison of relative synthesis levels and half-lives of IGF-1 (A), bPL (B), and pro-ANF (C) in *rpoH358* mutants, MON102 and MON105, and parental W3110. Results for these heterologous proteins were determined by pulse-chase analysis with [^{35}S]cysteine (see Materials and Methods). Protein levels at the zero time point (initial synthesis) and at various time points during the chase were quantified by densitometric scanning of the autoradiograms and are indicated beneath lanes. Since bPL was very stable after a 32-min chase, only the relative synthesis level was quantified by densitometric scanning. For the given heterologous protein, the initial protein level at the zero time point in the W3110 parental strain was arbitrarily assigned a value of 1.0. Coomassie blue staining of the protein gel prior to autoradiography showed that all of the lanes had approximately equal loadings of cell protein (data not shown). (A) Lanes 1 to 6, W3110(pMON2368); lanes 7 to 12, MON105(pMON2368). (B) Lanes 1 to 7, W3110(pMON3401); lanes 8 to 14, MON102(pMON3401); lanes 15 to 21, MON105 (pMON3401). (C) Lanes 1 to 7, MON105(pMON5589); lanes 8 to 14, W3110(pMON5589).

manner (6, 23), the *rpoH* mutant alleles were not compatible with the *rpoD*$^+$ gene. In examples in which the *rpoH* mutant alleles were compatible with the *rpoD*$^+$ gene, a temperature-sensitive phenotype resulted in nearly every case. Previously characterized *rpoH* mutations similar to *rpoH358* thus appear to be relatively rare (23, 49, 54). In this sense, *rpoH358* is similar to the previously characterized *rpoH111* mutant allele (6, 23) in that it does not have a conditional phenotype in an *rpoD*$^+$ strain background at temperatures of >25°C. Compatibility of the *rpoD*$^+$ gene with a mutant *rpoH* gene is thus allele specific (23).

DNA sequence analysis of the *rpoH358* gene showed that a novel C→T transition at nucleotide 802 was present, causing a single amino acid substitution, Arg→Cys, at amino acid position 268 (R-268-C). Since both of the original *rpoH* mutants, MON101 and MON104, were isolated from the

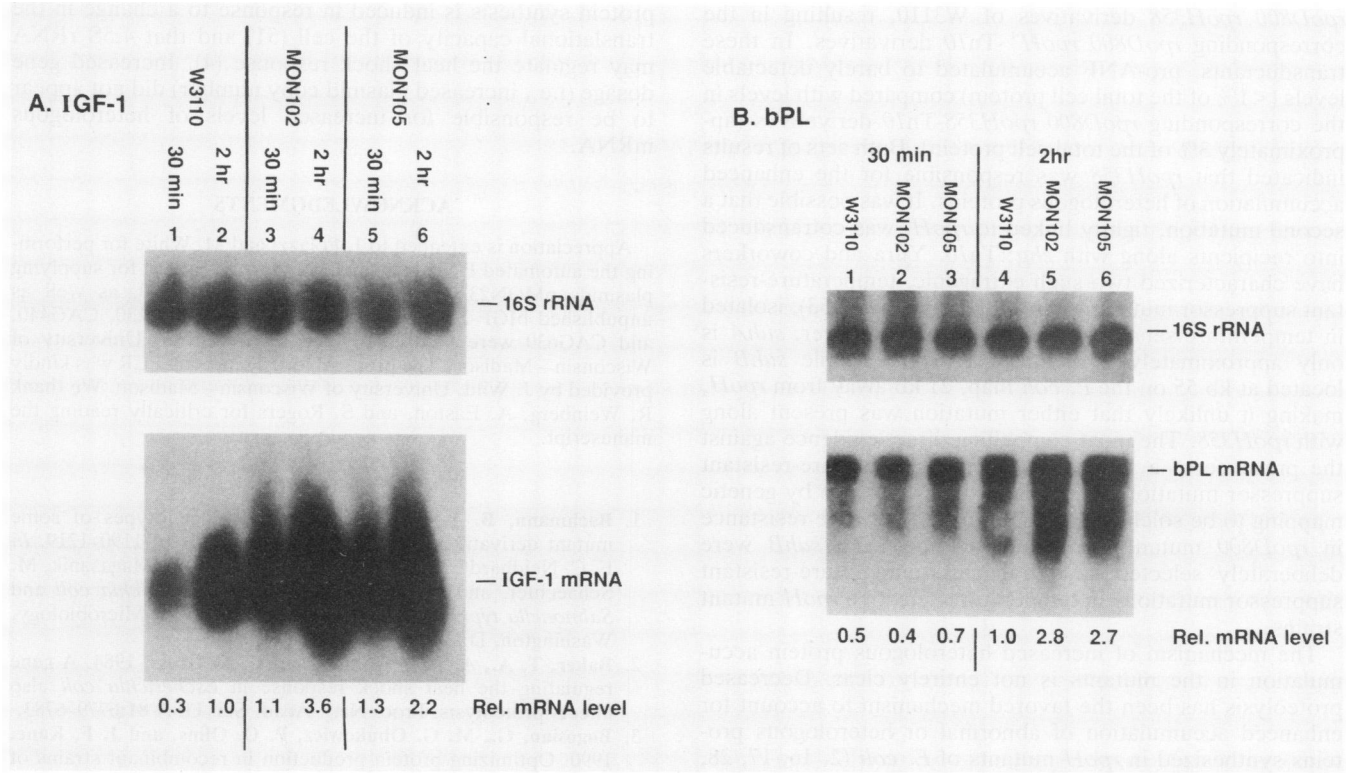

FIG. 6. Comparison of the relative mRNA levels of IGF-1 (A) and bPL (B) in *rpoH358* mutants, MON102 and MON105, and parental W3110. Cells were grown in M9 induction medium, and heterologous gene expression was induced by the addition of nalidixic acid. Relative mRNA levels were determined at 30 min and at 2 h postinduction. The relative mRNA level from each sample (number below each lane) was normalized to the corresponding level of 16S rRNA. The relative amount of mRNA which was present at the highest steady-state level in parental W3110 was arbitrarily set at 1.0. (A) Lanes: 1 and 2, W3110(pMON2368); 3 and 4, MON102(pMON2368); 5 and 6, MON105 (pMON2368). (B) Lanes: 1, W3110(pMON3401); 2, MON102(pMON3401); 3, MON105(pMON3401); 4, W3110(pMON3401); 5, MON102 (pMON3401); 6, MON105(pMON3401).

same culture and possessed the identical *rpoH* mutation, it is likely that they were siblings. The Arg→Cys substitution at amino position 268 is present in region 4.2 (25) of σ^{32}. Region 4.2 contains a helix-turn-helix motif, which presumably interacts directly with the −35 promoter region (25). The most compelling evidence for the interaction of region 4.2 with the −35 promoter region comes from a mutational analysis of σ^{70} of *E. coli*, in which the Arg residues at positions 584 and 588 were implicated in making contacts with specific bases in the −35 promoter region (12, 45). Interestingly, the *rpoH358* mutant allele (R-268-C) is analogous to the *rpoD*-RH588 mutant allele (R-588-H) described by Gardella et al. (12). In both cases, an amino acid substitution occurred at the conserved Arg residue present in the second helix of the helix-turn-helix motif of region 4.2. With both σ^{70} and σ^{32}, this specific Arg residue has been implicated in making contact with the third position (G) of the −35 consensus sequence, TTGA, which is common to both σ^{70} and σ^{32} promoters (12, 45). For comparison, only two other *rpoH* mutant alleles, *rpoH112* (L-278-F) (6) and *rpoH15* (E-265-K) (54), have mutations in region 4.2, neither of which are implicated in contacting bases within the −35 region of heat shock promoters. The R-268-C mutation in *rpoH358* might therefore be expected to affect heat shock gene expression. Previously described *rpoH* mutants do not induce the heat shock response efficiently (5, 49, 54). However, no dramatic effects on heat shock protein synthesis were observed between parental W3110 and the *rpoH358* mutants, MON102 and MON105 (Fig. 3), suggesting that the R-268-C substitution did not markedly affect the interaction between the mutant σ^{32} and the −35 heat shock gene promoter region. It is possible that subtle and therefore undetectable differences in heat shock protein synthesis exist because a one- rather than two-dimensional SDS-PAGE analysis was performed. In any case, an unaltered heat shock response should perhaps not be surprising because the temperature-resistant suppressor mutants, MON101 and MON104, were selected at 43.5°C, a temperature requiring a relatively high level of heat shock protein synthesis for cell viability (49, 54, 55).

Genetic mapping results showed that *rpoH358* was responsible for temperature resistance in MON103 and MON106. In order to show that *rpoH358* was also responsible for the enhanced accumulation of heterologous proteins, both forward and reverse genetic experiments were performed. In the forward genetic experiments, *rpoH358* was cotransduced with a tightly linked Tn*10* element, *zhg*::Tn*10* (approximately 85% linkage), into previously unmanipulated strains of W3110 and JM101. Both IGF-1 and pro-ANF accumulated to levels severalfold higher in the *rpoH358*-Tn*10* transductants from either strain compared with the levels in corresponding *rpoH*$^+$-Tn*10* control transductants. These results also demonstrated that *rpoH358* functioned in enhancing heterologous protein accumulation in a strain other than W3110. In the reverse genetic experiments, *rpoH*$^+$ was cotransduced with *zhg*::Tn*10* into two

rpoD800 rpoH358 derivatives of W3110, resulting in the corresponding *rpoD800 rpoH*$^+$-Tn*10* derivatives. In these transductants, pro-ANF accumulated to barely detectable levels (<1% of the total cell protein) compared with levels in the corresponding *rpoD800 rpoH358*-Tn*10* derivatives (approximately 3% of the total cell protein). Both sets of results indicated that *rpoH358* was responsible for the enhanced accumulation of heterologous proteins. It was possible that a second mutation, tightly linked to *rpoH*, was cotransduced into recipients along with *zhg*::Tn*10*. Yura and coworkers have characterized two such extragenic, temperature-resistant suppressor mutations, *suhA* (50) and *suhB* (53), isolated in temperature-sensitive *rpoH* mutants. However, *suhA* is only approximately 10% linked to *rpoH*, while *suhB* is located at kb 55 on the *E. coli* map, 21 kb away from *rpoH*, making it unlikely that either mutation was present along with *rpoH358*. The most compelling direct evidence against the presence of a second extragenic temperature-resistant suppressor mutation is that *rpoH358* was shown by genetic mapping to be solely responsible for temperature resistance in *rpoD800* mutants. In contrast, *suhA* and *suhB* were deliberately selected as extragenic, temperature-resistant suppressor mutations in temperature-sensitive *rpoH* mutant strains.

The mechanism of increased heterologous protein accumulation in the mutants is not entirely clear. Decreased proteolysis has been the favored mechanism to account for enhanced accumulation of abnormal or heterologous proteins synthesized in *rpoH* mutants of *E. coli* (2, 16, 17, 28, 41). The *rpoH358* mutants used as host strains for heterologous gene expression provided unanticipated results showing that a different mechanism was responsible for the enhanced accumulation of the heterologous proteins. That is, enhanced accumulation of heterologous proteins did not result from a decreased rate of proteolysis. Rather, the enhanced accumulation of heterologous proteins was correlated with enhanced levels of heterologous mRNA and enhanced levels of heterologous protein synthesis. Enhanced heterologous protein synthesis could conceivably be due to increased transcription initiation from the σ^{70} promoters, *recA* and *araBAD*, resulting in higher mRNA levels. Since both σ^{70} and σ^{32} compete for RNA polymerase core enzyme (36), an altered σ^{32} subunit might compete less effectively with σ^{70}, allowing more σ^{70} to bind to RNA polymerase core enzyme. Increased transcription initiation from the σ^{70}-specific promoters, *recA* and *araBAD*, in the *rpoH358* mutants could result in higher levels of heterologous mRNA and, in turn, higher levels of heterologous protein synthesis and ensuing accumulation. This interpretation is tenuous. The *rpoH358* mutants possess a functional heat shock response, eliminating any correlation between an increase in heterologous protein accumulation and a defective heat shock response. Western blot results showed that the respective steady-state levels of σ^{32} and σ^{70} were similar in parental W3110 and the *rpoH358* mutants at various time points during growth at 30 or 37°C or after a temperature shift from 30 to 42°C (unpublished data). It is possible, though, that subtle differences in transcription initiation were present that were manifested by σ^{70} promoters present on high-copy-number expression plasmids. Alternatively, the presence of higher levels of heterologous mRNA may be only coincidental. The mRNA might be protected from degradation by an increased number of associated ribosomes because of higher levels of translation initiation in the *rpoH358* mutants. Such a translational mechanism is also possible because of recent reports suggesting that heat shock protein synthesis is induced in response to a change in the translational capacity of the cell (51) and that 4.5S rRNA may regulate the heat shock response (4). Increased gene dosage (i.e., increased plasmid copy number) did not appear to be responsible for increased levels of heterologous mRNA.

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