Multiple Translational Products from a *Mycoplasma hyorhinis* Gene Expressed in *Escherichia coli*

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We analyzed protein expression from a cloned *Mycoplasma hyorhinis* genomic fragment that produces in *Escherichia coli* a set of related polypeptides of 110, 100, 65, and 55 kilodaltons from a coding region of just over 3.0 kilobases. Expression of these multiple products resulted from a mechanism operating at the translational level but not from truncation at UGA termination codons, which are known to encode tryptophan in several mycoplasma species. The structural relatedness of the proteins was demonstrated by two-dimensional tryptic peptic mapping, but their generation by posttranslational processing was ruled out by pulse-chase labeling analysis. Examination of proteins expressed from plasmid constructs and tryptic peptide analysis of these polypeptides and the original set of proteins revealed that they share carboxy-terminal regions, an observation inconsistent with truncation at UGA codons. Expression of proteins from this cloned fragment was not dependent on vector sequences and was observed when the coding region was placed under control of a T7 promoter, suggesting that all products were translated from a single message. Expression of related products in mycoplasmas was examined by immunoblot analysis of *M. hyorhinis* proteins with antiserum against overexpressed recombinant proteins. A single 115-kilodalton mycoplasma protein was detected, which is larger than any of the related proteins expressed in *E. coli*. Our analysis indicated that translation initiation sites are used in *E. coli* that are not active in mycoplasmas, thereby defining differences between the translational regulatory signals of mycoplasmas and eubacteria.

The study of gene structure and function in mycoplasmas and other organisms in the class *Mollicutes* has been inhibited by the lack of a classic genetics systems and by current limitations in expression of cloned genes in a native mycoplasma host (16). Therefore, cloning mycoplasma genes in eubacterial hosts and vectors is an essential tool for analyzing their organization and expression (1, 20, 25, 28-30). *Escherichia coli* and its genetic vectors have proved useful for the cloning, sequencing, and structural comparison of untranslated mycoplasma rRNA and tRNA genes (12, 22, 23, 27, 32, 33). However, expression of cloned mycoplasma genes for studying specific translated products and their biological functions has been less successful, due in part to fundamental genetic differences between mycoplasmas and eubacteria that preclude efficient expression of mycoplasma genes in otherwise versatile hosts such as *E. coli*. One such characteristic now shown to be common among several mycoplasma species is the use of the UGA codon to encode tryptophan rather than translation termination (9, 14a, 21, 30, 34). In eubacterial hosts, this would lead to the truncation of mycoplasma gene products at the first tryptophan encoded by UGA.

Results of some studies suggest indirectly that additional factors affect expression of mycoplasma gene products in eubacteria. For example, while the mycoplasma gene encoding spiralin contains no UGA codons (C. Saillard, C. Chevalier, and J. M. Bové, Abstr. 7th Congr. Int. Org. Mycoplasma., 1988, P166), multiple proteins are expressed in *E. coli* from this coding sequence (20) by mechanisms not yet defined (3). Expression of multiple proteins exceeding the coding capacity of a cloned genomic fragment from another mycoplasma species has also been described (1). An understanding of the factors involved in the expression of myco-

plasma proteins in eubacteria is essential for the study of mycoplasma protein structure and function and for interpreting features of mycoplasma gene expression in these hosts.

This report analyzes protein expression in *E. coli* from a 12-kilobase (kb) *Mycoplasma hyorhinis* genomic insert contained in a recombinant bacteriophage, MhrG27. This phage expresses high-molecular-mass protein products in *E. coli* totaling 430 kilodaltons (kDa), the approximate coding capacity of this chromosomal region. A subset of these proteins was identified with a total size far exceeding the coding capacity of the 4-kb subcloned DNA fragment which directed their synthesis in *E. coli*. Since these proteins were shown to be structurally related, studies were pursued to determine the mechanism underlying the expression of these multiple products. Results reported here indicate that in addition to differences in UGA codon usage, *E. coli* and *M. hyorhinis* use different initiation sites for translation of a mycoplasma structural gene. Analysis of this cloned region allowed us to identify novel translational effects on the expression of mycoplasma genes in an eubacterial host and will be valuable in further comparing translational processes in mycoplasmas with those of gram-negative or gram-positive eubacteria (13, 14).

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophage. *E. coli* strains used in this study were χ 984 (10), HB101 (5), and TB1 (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). *M. hyorhinis* GDL-1 has been described in detail elsewhere (6). Recombinant plasmids were constructed in the vectors pUC18, pUC19 (35), pGEM3Z (Promega Biotec, Madison, Wis.), and pT7-7 (provided by S. Tabor, Harvard Medical School, Boston, Mass.), and the resulting constructs were designated pUG, pUGS, pZG, and pTG, respectively. Plasmid pGP1-2 encodes T7 RNA polymerase and confers kana-

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mycin resistance (26). Recombinant bacteriophage were isolated from an *M. hyorhinis* genomic library constructed in the vector λ Charon 4A as described previously (28).

Media, chemicals, and enzymes. *E. coli* cultures for bacteriophage, plasmid, and minicell propagation were grown with shaking at 37°C in Luria-Bertani broth (19). Bacteria used for protein expression under T7 promoter control were grown with shaking in T broth (1.2% tryptone [Difco Laboratories, Detroit, Mich.], 2.4% yeast extract [Difco], 0.4% glycerol, 0.017 M KH_2PO_4 , 0.072 M K_2HPO_4) (Bethesda Research Laboratories, Focus 9:2, 1987). *M. hyorhinis* GDL-1 was cultured in modified Hayflick broth as previously described (31). Restriction enzymes were purchased from Promega Biotec and Bethesda Research Laboratories. DNA polymerase I large (Klenow) fragment, T4 DNA ligase, and 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal) were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). DNA restriction and modification enzymes were used according to the recommendations of the manufacturers. [^{35}S]methionine (specific activity, 1,200 Ci/mmol) was purchased from Dupont, NEN Research Products (Boston, Mass.). Trypsin, ampicillin, kanamycin, and rifampin were purchased from Sigma Chemical Co. (St. Louis, Mo.). Universal translation terminator (UTT), a 16-base-pair palindromic oligomer with the protein synthesis termination codon TAA in all reading frames, was purchased from Pharmacia, Inc. (Piscataway, N.J.).

Molecular genetics techniques. The isolation of bacteriophage DNA has been previously described (17). Plasmid DNA was isolated by the procedure of Birnboim and Doly (2). *E. coli* was transformed by the CaCl_2 method as described by Cohen et al. (8). Procedures for cloning and analysis of plasmid and bacteriophage DNA were performed as described by Maniatis et al. (17). Plasmids containing mycoplasma DNA inserts under the control of the T7 promoter were constructed in the vectors pGEM3Z and pT7-7. In-frame fusions in pT7-7 constructs were created by fill-in end repair with deoxynucleoside triphosphates and DNA polymerase (Klenow enzyme) of endonuclease-cleaved gel-purified fragments and religation.

Analysis of protein expression in *E. coli* minicells. Minicells were isolated by sucrose gradient centrifugation from stationary-phase cultures of *E. coli* χ 984 as described previously (10). To identify proteins expressed from recombinant bacteriophage, minicells were infected at a multiplicity of infection of approximately 1 PFU per cell and incubated at 37°C in 100 μl of labeling medium (24) for 15 min to allow adsorption of the phage to the minicells. [^{35}S]methionine was then added to a specific activity of 1.0 mCi/ml, and incubation continued for 1 h. Plasmid-containing minicells were isolated from cultures grown under selective conditions and were labeled with [^{35}S]methionine as described above for bacteriophage expression. Labeled minicells were rinsed and collected by centrifugation (12,000 $\times g$, 2 min). The pellet was dissolved and heated under reducing conditions in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and electrophoresed in a 9 to 14% linear gradient polyacrylamide gel containing 3% urea as described previously (15, 31). Direct autoradiography was performed as described elsewhere (7).

Tryptic peptide mapping. Whole lanes from 9 to 14% linear gradient gels containing separated radiolabeled minicell proteins or individual protein bands were excised and placed in digestion buffer (0.1% SDS, 0.16 M Tris, pH 6.8) with or without trypsin (1 mg/ml) and incubated at 37°C with gentle

shaking for 30 min. Strips (or individual bands) were then rinsed once with digestion buffer, heated at 100°C for 5 min in SDS-PAGE sample buffer, and placed horizontally (or in individual wells) on the stacking portion of a second 9 to 14% gel for second-dimension separation by SDS-PAGE. The gel was fluorographed or autoradiographed as described previously (4, 7). To compare proteins from two different minicell preparations, the samples were combined and electrophoresed in a single lane in the first dimension.

Pulse-chase labeling. Minicells containing recombinant plasmids were incubated for 2 min at 37°C in labeling medium containing 5 mCi of [^{35}S]methionine per ml. The minicells were washed by centrifugation at 4°C and immediately suspended in prewarmed (37°C) medium containing 0.05 M unlabeled methionine. Samples taken at 0, 2, 5, 10, and 20 min were immediately heated at 100°C for 5 min in sample buffer and analyzed by SDS-PAGE and autoradiography.

Protein expression under T7 promoter control and purification of recombinant protein. *E. coli* HB101(pGP1-2) carrying the temperature-controlled T7 RNA polymerase gene was transformed with pZG27.31, pTG-92, or pTG-44. The resulting transformants were grown in selective medium containing 100 μg of ampicillin and 50 μg of kanamycin per ml at 30°C to $A_{590} = 0.5$. For selective induction of T7 expression, cells from 200 μl of culture were washed in 0.05 M NaCl, suspended in 250 μl of M9 medium (17), and incubated for 1 h at 30°C. The temperature was then shifted to 42°C for 15 min, rifampin was added (final concentration, 200 $\mu\text{g}/\text{ml}$), and incubation continued for 10 min. The temperature was then shifted to 30°C for 20 min, [^{35}S]methionine was added to a final concentration of 25 $\mu\text{Ci}/\text{ml}$, and incubation continued for 5 min. Samples were then prepared for SDS-PAGE analysis as described above. For overexpression and subsequent purification of recombinant protein, cultures were incubated at 30°C to an $A_{590} = 1.5$ and then at 42°C for 25 min, rifampin was then added to a final concentration of 100 $\mu\text{g}/\text{ml}$, and the cultures were incubated at 37°C for 2 h. Cells were then harvested and subjected to SDS-PAGE as described above. The gel band containing the recombinant protein was excised, and the protein was electroeluted in buffer containing 0.015 M ammonium bicarbonate and 0.1% SDS in an Elutrap apparatus (Schleicher & Schuell Inc., Keene, N.H.) in a field of 5.5 V/cm for 24 h. Eluted protein was collected and analyzed by SDS-PAGE.

Antibody production and immunoblotting. BALB/c mice were immunized at weekly intervals by three intraperitoneal injections, each containing approximately 150 μg of gel-purified recombinant protein in incomplete Freund adjuvant. Serum was obtained by tail bleeding before each injection and at weekly intervals thereafter. To identify antigenic proteins, we subjected mycoplasmas or *E. coli* to SDS-PAGE on a 9% gel, and separated components were electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell) and immunostained as described previously (6) with mouse antiserum at a dilution of 1:400. Relative molecular weights were determined with prestained protein standards (Diversified Biotech, Newton Centre, Mass.) transferred directly onto membranes.

RESULTS

Expression of proteins in *E. coli* minicells from recombinant mycoplasma DNA. Six recombinant λ Charon 4A bacteriophage, selected from a previously described *M. hyorhinis* GDL-1 genomic library by immunoscreening (28), were

FIG. 1. Restriction analysis and protein expression from recombinant bacteriophage containing *M. hyorhinis* genomic DNA. (A) *Eco*RI restriction enzyme digests of recombinant λ Charon 4A phage DNA analyzed by agarose gel electrophoresis and ethidium bromide staining. Numbered lanes designate recombinant phage (MhrG) isolates containing *Eco*RI restriction fragments of *M. hyorhinis* origin; the vector λ Charon 4A (lane 4A) is shown for comparison; *Hind*III digested wild-type λ DNA was used as size markers (indicated at right in kilobases). Arrowheads indicate the positions of the vector arms common to all clones. (B) Autoradiograph of [35 S]methionine-labeled proteins synthesized in phage-infected *E. coli* minicells and subjected to SDS-PAGE as described in Materials and Methods. Lanes correspond to phage described in panel A. Positions of molecular size markers are indicated in kilodaltons (kd) at the right of the autoradiograph. The arrows in lane 27 indicate the high-molecular-mass mycoplasma DNA-specific proteins of 110, 100, 65, and 55 kDa synthesized from recombinant phage MhrG27.

shown by restriction enzyme analysis to contain distinct mycoplasma DNA inserts (Fig. 1A). [35 S]methionine-labeled proteins synthesized in *E. coli* minicells from these phage were examined by SDS-PAGE; each clone produced mycoplasma insert-specific proteins (Fig. 1B). Two clones, MhrG22 and MhrG27, expressed high-molecular-weight proteins. Phage MhrG27, which contained a 12-kb mycoplasma DNA insert, produced four prominent and discrete proteins of 110, 100, 65, and 55 kDa as well as additional insert-specific proteins. Together these products totaled about 430 kDa, the approximate coding capacity of this cloned chromosomal region. Expression of an entire mycoplasma DNA region of this size and synthesis of high-molecular-weight proteins were surprising in light of UGA codon usage differences between mycoplasmas and *E. coli*. Therefore, protein expression directed by this insert DNA was analyzed in detail to determine the basis for expression of these proteins in *E. coli*.

Gene localization by subcloning mycoplasma DNA fragments and analysis of plasmid-directed protein synthesis in minicells. To localize the DNA sequences encoding the high-molecular-weight proteins expressed by MhrG27, the 5- and 7-kb *Eco*RI fragments (Fig. 1A, lane 27) were separately subcloned into the plasmid vector pUC18 to generate plasmids pUG27.1 and pUG27.2 (which contained the 5-kb fragment in opposite orientations) and pUG27.3 (Fig. 2). Protein synthesis directed by each recombinant plasmid was examined in *E. coli* minicells (Fig. 3). The four high-molecular-weight proteins (110, 100, 65, and 55 kDa) expressed in phage MhrG27 were shown to be encoded entirely on the 7-kb *Eco*RI fragment (Fig. 3, lane 4), whereas none of these proteins was expressed from the 5-kb fragment (Fig. 3, lanes 2 and 3). The DNA region expressing these four proteins in

minicells (Fig. 3, lane 5) was further localized to a 4.0-kb fragment by deleting the 3.0-kb *Bgl*II-*Eco*RI fragment from pUG27.3 to form plasmid pUG27.31 (Fig. 2).

Since the DNA coding capacity needed to express four unique gene products of this size from distinct DNA coding regions is about 8.8 kb, and because the 4.0-kb *Eco*RI fragment only slightly exceeds the 3.0 kb required to encode the largest 110-kDa protein, at least some of the smaller (100-, 65-, and 55-kDa) proteins must originate from common regions of this mycoplasma DNA fragment. They could represent distinct polypeptides expressed from alternate reading frames or from opposite DNA strands, or they could arise by differential processing mechanisms operating in *E. coli*, including (i) partial suppression of UGA codons; (ii) generation of multiple transcripts from different regions of the DNA insert acting as promoters; (iii) precise posttranslational processing of a single, large mycoplasma polypeptide; or (iv) utilization of multiple ribosome-binding sites to initiate translation from multiple positions on a single transcript. These possibilities were investigated to determine the basis for the expression in *E. coli* of multiple gene products from this mycoplasma chromosomal region.

To deduce the direction of transcription and to further identify DNA regions corresponding to specific translational products in this set of proteins, we constructed several plasmids and examined their protein products in minicells. pUG27.31-1, containing the 2.2-kb *Eco*RV-*Bgl*II fragment (Fig. 2), produced no mycoplasma proteins in minicells (Fig. 3, lane 7). The 1.8-kb *Eco*RI-*Eco*RV fragment (Fig. 2) contained in pUG27.31-2 produced a 68-kDa protein (Fig. 3, lane 8) which was capable of alpha complementation of the *lacZ* lesion in the *E. coli* host TB1 (18). This implied that the 1.8-kb mycoplasma DNA fragment produced a polypeptide

FIG. 2. Restriction maps of *M. hyorhinis* DNA inserts in phage MhrG27 and pUC plasmid subclones. MhrG27 is a λ Charon 4A recombinant phage containing a 12-kb mycoplasma DNA insert; the positions of *EcoRI* cloning sites and vector arms (open rectangles) are indicated. The *EcoRI* fragments from MhrG27 were subcloned into the *EcoRI* site of pUC18. Plasmids pUG27.1 and pUG27.2 contain the 5.0-kb *EcoRI* fragment in opposite orientations, and plasmid pUG27.3 contains the 7.0-kb *EcoRI* fragment. Plasmid pUG27.31 contains the 4.0-kb *EcoRI*-*Bgl*II fragment from pUG27.3 cloned into the *EcoRI* and *Bam*HI sites of pUC18. Plasmid pUG27.31-1 contains the 2.2-kb *Bgl*II-*EcoRV* fragment of pUG27.31 directionally cloned into the *Bam*HI and blunt-ended (by end repair with Klenow fragment and deoxynucleoside triphosphates) *EcoRI* sites of pUC18. Plasmid pUG27.31-2 contains the 1.8-kb *EcoRI*-*EcoRV* fragment of pUG27.31 directionally cloned into the *EcoRI* and *Sma*I sites of pUC18. Abbreviations: E, *EcoRI*; B, *Bgl*II; R, *EcoRV*; C, *Clal*; S, *Sma*I; H, *Hpa*I. Plasmids not shown: pUGS27.31, which contains the same insert as pUG27.31 but in the vector pUC19; pUG27.3UTT, which has a translation termination oligonucleotide cloned into the *EcoRV* site of pUG27.3.

that was translated toward the *EcoRV* site and fused in phase at that point to the *lacZ* alpha peptide of the vector (pUC18). Plasmid pUG27.3UTT was constructed by the insertion of a universal translation terminator (UTT) oligonucleotide into the *EcoRV* site of pUG27.3 (refer to Fig. 2). This plasmid produced a single insert-specific protein of 60 kDa (Fig. 3, lane 9). Expression of the related proteins larger than 60 kDa (110, 100, and 65 kDa) was abrogated, and the 55-kDa protein was not detected. This result is not compatible with a mechanism involving partial suppression of UGA codons, which would have left the 55-kDa protein intact in both pUG27.31-2 and pUG27.3UTT.

Analysis of the relationship among the set of proteins by peptide mapping and pulse-labeling experiments. The possibility that the original set of 110-, 100-, 65-, and 55-kDa proteins are structurally related was assessed by two-dimensional SDS-PAGE tryptic peptide mapping of radiolabeled products expressed in *E. coli* minicells by pUG27.3. After separation of labeled products in one dimension of SDS-PAGE, in situ treatment of excised gel strips with trypsin was used to generate partial tryptic peptides that could be identified by SDS-PAGE in a second dimension. Peptides generated from each labeled protein migrated below the diagonal and could be compared directly by fluorography (Fig. 4A). A control omitting enzymatic treatment between electrophoresis steps formed the predicted diagonal pattern in the second-dimension gel (Fig. 4A, inset). In trypsin-treated samples, however, the high-molecular-weight mycoplasma proteins expressed from this clone were shown to share a distinctive set of common labeled peptides ranging from approximately 25 to 30 kDa (indicated by brackets in Fig. 4A). The existence of a shared set of peptides indicated structural similarity among these proteins and argued strongly for the expression of these products from common coding regions rather than from alternate reading frames or opposite DNA strands.

Multiple related proteins might arise by posttranslational processing such as degradation in *E. coli*, converting a single large mycoplasma product into smaller, discrete, stable

polypeptides. To examine temporal processing of these protein products, we performed pulse-chase analysis in *E. coli* minicells containing recombinant plasmid pUG27.3 as described in Materials and Methods. The relative labeling intensities of the [35 S]methionine-labeled mycoplasma proteins remained constant at each time point, with each product accumulating at the same rate (Fig. 5). In contrast, the β -lactamase gene products expressed from the same plasmid served as an internal control and readily demonstrated conversion of the precursor to its product form. Therefore, within the resolution of this experiment, no posttranslational conversion of proteins expressed from the mycoplasma DNA insert could be demonstrated.

Peptide mapping of proteins produced from insertion and deletion plasmid constructs. To determine which portions of the larger 110- and 100-kDa proteins were represented in the 65- and 55-kDa products, we assessed the structural relationships among this set of proteins and the truncated peptides produced by pUG27.3UTT (60-kDa peptide, Fig. 3, lane 9) and pUG27.31-2 (68-kDa fusion protein, Fig. 3, lane 8). Two-dimensional SDS-PAGE analysis was used as before, except that the [35 S]methionine-labeled minicell products to be compared were mixed and loaded in a single channel for separation in the first dimension. Comparison of the 60-kDa protein produced by pUG27.3UTT with the complete set of proteins expressed from pUG27.3 (Fig. 4B) indicated that trypsin treatment of the 60-kDa protein (Fig. 4B, dashed arrow) did not produce the characteristic group of 25- to 30-kDa peptides common to the original set of related proteins. The 60-kDa protein did, however, generate a cluster of small (8- to 10-kDa) peptides which were also produced from the 110- and 100-kDa proteins but not from the 65- and 55-kDa proteins (Fig. 4B, lower bracket). The DNA region encoding this set of small tryptic peptides was confirmed by comparative peptide mapping of the 60-kDa pUG27.3UTT product and the 68-kDa fusion protein produced by pUG27.31-2. These two proteins showed very similar tryptic peptide patterns, including the set of small peptides specifically present in the 110- and 100-kDa proteins

FIG. 3. Expression of mycoplasma-encoded proteins in *E. coli* minicells containing recombinant plasmids. Minicells purified from transformed parental χ 984 *E. coli* cultures were labeled with [35 S]methionine and analyzed by SDS-PAGE and autoradiography. Autoradiographs represent proteins encoded by plasmids described in Fig. 2: pUC18 (lane 1), pUG27.1 (lane 2), pUG27.2 (lane 3), pUG27.3 (lane 4), pUG27.31 (lane 5), pUGS27.31 (lane 6), pUG27.31-1 (lane 7), pUG27.31-2 (lane 8), and pUG27.3UTT (lane 9). Small arrowheads indicate the complete set of mycoplasma proteins, 110 and 100, 65, and 55 kDa, in lanes 4, 5, and 6. The open arrow (lane 8) indicates the 68-kDa *lacZ* fusion product. The solid arrow (lane 9) indicates the 60-kDa protein created by insertion of the universal translation terminator. Molecular mass markers (in kilodaltons) shown for the first panel correspond to lanes 1 to 4; markers were present in the separate gels represented in the other panels of this composite figure.

(data not shown). Because both the 60- and 68-kDa fusion products are primarily encoded within the 1.8-kb *EcoRI-EcoRV* region of the insert and transcription and translation proceed left to right (refer to Fig. 2), this region must encode the amino-terminal portion of the largest proteins. Moreover, because these truncated polypeptides shared tryptic peptide fragments with the 110- and 100-kDa proteins but no significant relatedness could be demonstrated to either the 65- or 55-kDa protein, it could be concluded that the smaller (65- and 55-kDa) proteins share sequences contained within the carboxy-terminal portion of the larger (110- and 100-kDa) proteins of the original set.

Promoter placement experiments. The above results indicated that the production of this set of related proteins is controlled at the transcriptional or translational level. To determine whether transcription was under the control of the *lacZ* promoter of the vector or was directed by mycoplasma sequences within the insert located close to the left *EcoRI* site (refer to Fig. 2, pUG27.31), the 4-kb *EcoRI-BglII* fragment was subcloned into the vector pUC19 cut with *EcoRI* and *BamHI*, thereby reversing its orientation with respect to the *lacZ* promoter. When protein production from this plasmid, pUGS27.31, was examined in minicells, the same set of high-molecular-weight proteins was observed (Fig. 3, lane 6), suggesting that the *lacZ* promoter is not required for their expression.

Multiple transcription initiation events occurring at dif-

ferent sites within a single large open reading frame of the mycoplasma insert could lead to the production of smaller related proteins. Accordingly, transcription initiation was evaluated in a strictly controlled system. The 4-kb *EcoRI-BglII* fragment from pUG27.3 (Fig. 2) was cloned into the vector pGEM3Z cleaved with *EcoRI* and *BamHI*, placing the coding region for the related proteins in the proper orientation behind the T7 promoter of the vector. The resulting plasmid, pZG27.31 (Fig. 6A), when transcribed exclusively from the T7 promoter, synthesized the complete set of high-molecular-weight proteins (Fig. 6B, lane 1). This implies that a single transcription initiation event is sufficient to generate the complete set of mycoplasma proteins in *E. coli*.

To investigate the alternative possibility that initiation of translation from multiple sites could generate the proteins synthesized from this mycoplasma gene, we constructed a series of expression plasmids in the vector pT7-7. This plasmid contains a T7 promoter sequence, a consensus *E. coli* ribosome-binding site, and an ATG translational start codon next to a multiple cloning site for creating in-frame fusions (S. Tabor, unpublished data). To construct pTG-92 (Fig. 6A), we subcloned the 6.3-kb *EcoRI-ClaI* fragment of pUG27.3 (refer to Fig. 2) into pT7-7 cut with *EcoRI* and *ClaI* to produce pTG27.3 (not shown), and the 0.5-kb *EcoRI-StyI* fragment was then deleted. This placed the T7 promoter and translational control site of the vector into a region of the

FIG. 4. Peptide mapping of mycoplasma-encoded proteins by two-dimensional SDS-PAGE. Proteins expressed and labeled with [35 S]methionine in *E. coli* minicells were applied to SDS-PAGE for separation in the first dimension (1stD). Autoradiographs of duplicate first-dimension gel lanes are shown at the top of each panel. The second-dimension SDS-PAGE, performed after trypsin treatment of the excised gel strip, is indicated (2ndD). The positions of the set of mycoplasma-encoded proteins (110 and 100, 65, and 55 kDa) are indicated above the first-dimension gels and along the diagonals of the second-dimension gels. (A) Peptide map of original set of mycoplasma proteins expressed from pUG27.3. The fluorograph of the second-dimension gel shows partial digestion products generated by trypsin treatment of the mycoplasma proteins after their separation in the first dimension. Brackets indicate a set of common tryptic peptides produced from each of the mycoplasma proteins. The inset shows a fluorograph of a control gel without trypsin treatment between electrophoretic separations. (B) Comparison of the insertionally terminated pUG27.3UTT protein product with the family of mycoplasma proteins expressed in *E. coli* by plasmid pUG27.31. This gel was more heavily loaded than that in panel A and was electrophoresed for a shorter time. The 60-kDa product of pUG27.3UTT and its tryptic peptides are indicated by dashed arrows. The upper bracket indicates the set of peptides common to all members of the family (as in panel A). The lower bracket indicates a second set of peptides, common to the 60 kDa (insertionally terminated)- and 110- and 100-kDa proteins.

insert downstream from the start of this gene but upstream from the region where translation of the 65- and 55-kDa members of the set was expected to begin. This placement was based on the sizes of the expressed proteins, the coding capacity of the insert, and the demonstrated relatedness of carboxy-terminal regions of the 110-, 100-, 65-, and 55-kDa proteins. Under conditions for exclusive T7 promoter expression, this plasmid produced a 92-kDa protein but also produced 65- and 55-kDa proteins indistinguishable from those of the original set (Fig. 6B, lane 2). This indicated that even under the exclusive control of a single strong promoter with a highly specific RNA polymerase and a consensus *E. coli* ribosome-binding site, translation in *E. coli* of this mycoplasma-derived message generates multiple distinct proteins. In addition, this result confirmed that the proteins of this set are not related at their amino termini, since the 65- and 55-kDa proteins would otherwise have been truncated in the same manner as the 110- and 100-kDa proteins.

An additional expression plasmid, pTG-44 (Fig. 6A), was constructed by deleting the 1.8-kb *EcoRI-EcoRV* fragment from pTG27.3, thus replacing any mycoplasma transcriptional control sequences as well as the region where translation of the 65- and 55-kDa proteins would be expected to initiate with the T7 promoter and *E. coli* ribosome-binding

site. Under T7 expression conditions, this construct did not produce any of the proteins of the original set but did produce a 44-kDa protein (Fig. 6B, lane 3). Tryptic peptide mapping of this protein revealed the characteristic cluster of 25- to 30-kDa peptides common to the original set of four proteins (Fig. 6C). This confirmed that the related regions shared by this set of proteins were in their carboxy-terminal portions.

The pTG-44, pTG-92, and pZG27.31 constructs also produced a 29-kDa insert-specific protein (Fig. 6B, lanes 1, 2, and 3). This product was probably related to the original set of proteins but was obscured in autoradiographs by comigrating vector-encoded proteins expressed in minicells (refer to Figs. 1B, 3, and 4A).

Immunological detection of native mycoplasma protein. Having determined that multiple translation initiation sites within the cloned mycoplasma protein-coding region are used in *E. coli* to produce a structurally related set of proteins, we then determined whether analogous proteins were expressed in mycoplasmas. The ability to generate, in vivo, large quantities of transcript under T7 promoter control has allowed overexpression of proteins encoded by these transcripts (26). The 44-kDa protein from plasmid pTG-44 (corresponding to the carboxy-terminal region of the

FIG. 5. Pulse-chase [35 S]-methionine labeling of proteins produced in *E. coli* minicells containing pUG27.3. Plasmid-containing minicells were pulse-labeled for 2 min and placed in unlabeled medium as described in Materials and Methods. Postchase samples were taken at 0 min (lane 1), 2 min (lane 2), 5 min (lane 3), 10 min (lane 4), and 20 min (lane 5), and the proteins were analyzed by SDS-PAGE and autoradiography. The positions of the mycoplasma proteins (in kilodaltons) are indicated to the right of the autoradiograph. The β -lactamase precursor is indicated by the bold arrow to the left of the autoradiograph, and the product is indicated by the open arrow to the right. A shorter autoradiographic exposure of the same gel is shown below to better delineate the processing of β -lactamase synthesized from this plasmid.

original four proteins) was therefore overexpressed in *E. coli*, purified, and used to generate antibodies recognizing this protein and others having common epitopes. Immunological staining of *M. hyorhinis* proteins by Western blot (immunoblot) analysis revealed a single protein of about 115 kDa (Fig. 7A, lane 1) recognized by the antibodies to this 44-kDa recombinant protein. Only this mycoplasma component and the recombinant 44-kDa mycoplasma protein expressed in *E. coli* (Fig. 7A, lane 2) reacted with the antiserum; no other *E. coli* or *M. hyorhinis* proteins were recognized by this immune serum (Fig. 7A) or by preimmune serum (Fig. 7B), even when up to 80 μ g of *M. hyorhinis* protein was used in a single lane of the Western blot analysis. This indicated that a single protein is produced in *M. hyorhinis* from this genomic region but also suggested that even the largest (110-kDa) form of the protein produced in *E. coli* from this same fragment does not represent the entire mycoplasma product. This was confirmed by examining SDS-PAGE separation of radiolabeled proteins expressed from pUG27.31 in *E. coli* minicells in the same channel with unlabeled *M. hyorhinis* proteins, followed by immunoblotting with the antiserum to the 44-kDa protein and autoradiography (data not shown). The 110-kDa protein

expressed in *E. coli* migrated slightly but distinctly faster than its mycoplasma counterpart.

DISCUSSION

The use of gram-negative hosts is currently an important tool in the study of mycoplasma molecular genetics. Until more versatile systems are available for studying mycoplasma protein expression and gene structure in a more natural environment, an understanding of the mechanisms operating in this heterologous gene expression system is essential.

The results of this study indicated that the use of multiple translation initiation sites can be an important factor in the expression of proteins from mycoplasma genes cloned in *E. coli*. This conclusion is based on analysis of protein expression from a cloned mycoplasma genomic fragment. A minimal model that describes a mechanism by which four major proteins are expressed in *E. coli* from a cloned chromosomal fragment of *M. hyorhinis* is shown in Fig. 8. This model includes the following salient features. (i) All four high-molecular-mass proteins (110, 100, 65, and 55 kDa) are encoded on a 4-kb *EcoRI*-*Bgl*II fragment; (ii) the coding region for each protein spans the *EcoRV* site; (iii) a single transcript originates from a sequence within the 4-kb mycoplasma insert in the proximity of the *EcoRI* site and transcription proceeds toward the *Bgl*II site; (iv) multiple translational start sites on the mycoplasma-encoded transcript are recognized and utilized in *E. coli*, resulting in the production of a set of proteins with common carboxy-terminal regions. The latter feature requires that nucleotide sequences resembling *E. coli* ribosome-binding sites and corresponding translation initiation codons are present in the appropriate regions of the mycoplasma gene sequence and are effectively utilized by the *E. coli* translation apparatus. Nucleotide sequence analysis of the entire structural gene has revealed such potential signals in the predicted locations throughout the gene (unpublished data) and therefore supports this important feature of the overall model.

It is particularly noteworthy that the generation of a set of related proteins with discrete and markedly different sizes occurs by this model from differences in the sites of translation initiation in *E. coli*, rather than by termination at specific UGA codons distributed in the mycoplasma protein-coding region. This latter phenomenon is well documented in mycoplasmas (9, 14a, 21, 30, 34), but our results specifically rule out translational termination as a mechanism generating the multiple proteins synthesized from the cloned region studied. It should also be emphasized that expression of cloned mycoplasma genes in *E. coli* may involve both promiscuous translation initiation and premature termination at UGA codons. This has the important implication that expression of protein products from cloned mycoplasma genes may well be observed even when UGA codons are present, owing to downstream initiation of new translational products. This has a practical importance for expression of segments of mycoplasma gene products in eubacteria, which should not be ruled out, as recently suggested (14a), based only on the presence of in-frame UGA codons derived from DNA sequence analysis. In this regard, it is notable that even the largest product of this cloned mycoplasma gene expressed in *E. coli* (110 kDa) is smaller than the native mycoplasma protein (115 kDa), as judged by relative migration in SDS-PAGE. While this difference may be due to a posttranslational modification in *M. hyorhinis* which results in an increase in apparent molecular weight, an alternative

FIG. 6. Expression and tryptic peptide mapping of mycoplasma-encoded proteins under T7 promoter control. (A) Plasmid maps showing the placement of the T7 promoter (T7) in pZG27.31 and the T7 promoter and consensus *E. coli* ribosome-binding site (RBS) in plasmids pTG-92 and pTG-44. Open regions represent mycoplasma DNA; hatched regions in pTG-92 and pTG-44 represent pT7-7 vector sequences including the ribosome-binding site; restriction sites are as indicated in Fig. 2. (B) Autoradiograph of SDS-PAGE analysis of plasmid-encoded [35 S]methionine-labeled proteins expressed in *E. coli* HB101(pGP1-2) containing plasmid pZG27.31 (lane 1), pTG-92 (lane 2), or pTG-44 (lane 3). Arrowheads in lane 1 indicate the set of related proteins, 110 and 100, 65, and 55 kDa. In lane 2, the open arrow indicates the 92-kDa protein and the small arrowheads indicate the 65- and 55-kDa proteins. The open arrow in lane 3 indicates the 44-kDa product of pTG-44. Positions of the molecular size standards are indicated (in kilodaltons). (C) Autoradiograph of partial tryptic digests of the gel-excised 44-kDa protein produced by pTG-44 (lane 1) and gel-excised p110 produced by pZG27.31 (lane 2). Bracket indicates set of common peptides, as shown in Fig. 4A.

explanation could be the presence of a UGA codon located close to either end of the transcript that would prevent production of the complete protein in *E. coli*. In fact, the nucleotide sequence suggests that a UGA tryptophan codon is present at residue 47 in the amino terminus of this coding region and is followed by translational signals that could give rise to the 110-kDa protein in *E. coli* (S. M. Notarnicola, M. A. McIntosh, and K. S. Wise, Abstr. Annu. Meet. Am. Soc. Microbiol., 1990, G-21, p. 139).

An aspect of our model which remains unresolved is the failure to detect the predicted truncated versions of the 100-, 65-, and 55-kDa proteins in the analysis of protein expression from pUG27.31-2 and pUG27.3UTT (Fig. 3, lanes 8 and 9). A 50-kDa version of the 100-kDa protein should be synthesized; however, since the 100-kDa protein is expressed at the lowest level of any of the members of the set, a truncated version may not be produced in detectable quantities. Because these experiments rely on the incorporation of labeled methionine for detection, the distribution of this amino acid in this coding region may further impair detection of these truncated products. The predicted truncation products of the 65- and 55-kDa proteins, approximately 15 and 5 kDa, respectively, would not have been easily resolved in SDS-

polyacrylamide gels. In addition, these small peptides may be unstable and rapidly degraded in *E. coli*.

Differential translation initiation may be a general phenomenon for mycoplasma genes cloned into eubacterial vectors. Other cloned DNA fragments from *M. hyorhinis* and *M. hyopneumoniae* have been observed to produce numerous mycoplasma-specific proteins in *E. coli* which exceed the coding capacity of the insert; some of these have also been shown to be structurally related by two-dimensional peptide mapping and may well arise from mechanisms similar to those summarized here (unpublished observations). Results from other reports also suggest that translation initiation is a major factor in expression of mycoplasma genes in *E. coli*. A cloned *Mycoplasma* strain PG50 genomic fragment has been reported to produce proteins in excess of its coding capacity in *E. coli* minicells (1). The cloned gene for spiralin, a major membrane protein of *Spiroplasma citri*, now known not to contain in-frame UGA codons (Saillard et al., Abstr. 7th Congr. Int. Org. Mycoplasma., 1988), was reported to produce three forms of the protein in *E. coli* which differed slightly in molecular weight (3, 20). While it has been proposed that this was a membrane translocation event in *E. coli* mirroring its processing in *Spiroplasma* (20),

FIG. 7. Immunological identification of native mycoplasma protein. Immunoblots represent *M. hyorhina* GDL-1 (lane 1), *E. coli* HB101(pGP1-2) containing induced T7 expression plasmid pTG-44 (lane 2), and *E. coli* HB101(pGP1-2) (lane 3). Protein samples were separated by SDS-PAGE and transferred to nitrocellulose. Panel A was immunostained with immune serum against the recombinant 44-kDa mycoplasma protein from pTG-44; panel B was immunostained with preimmune serum. Molecular size standards are indicated at the left in kilodaltons.

examination of the fates of these proteins in *E. coli* has not supported this possibility (3). Although the exact nature of the mechanisms operating in each of these examples has not been reported, it is quite possible that they represent further occurrences of differential translation initiation, as described in this report.

E. coli has been utilized extensively for molecular cloning and gene expression studies of gram-positive bacilli, and it has been demonstrated that the promoters from gram-positive organisms function effectively in gram-negative hosts but that the opposite configuration is inefficient (11). Because of strong evidence that mycoplasmas are evolutionarily related to gram-positive eubacteria (22, 32), it is perhaps not surprising that transcriptional expression of this set of mycoplasma proteins in *E. coli* occurs independently of known vector promoters. However, significant differences also exist in the abilities of gram-positive and gram-negative organisms to translate mRNA derived from one another (13). This phenomenon may be partially explained by the reported differences in binding stringency between their respective ribosomes and mRNA (14). In *E. coli*, ribosome binding to

mRNA requires fewer base pairings between the Shine-Dalgarno sequence and the 3' end of 16S rRNA, and has a lower average free energy of binding than that seen between ribosomes and mRNAs of gram-positive bacteria, such as *Bacillus subtilis* (13, 14). Translational controls in mycoplasmas might be expected to differ from those of *E. coli* at least as much as those of other gram-positive bacteria. In this context, the synthesis in mycoplasma of a single protein related to the set expressed in *E. coli* may reflect the ability of *E. coli* ribosomes to initiate translation on mycoplasma-derived mRNA at sites that do not serve a similar function in *M. hyorhina*.

One aspect of our results on mycoplasma protein expression in *E. coli* suggests an apparent inefficiency of translation, which may be analogous to results from in vitro protein translation studies showing that gram-positive *B. subtilis* bacteriophage mRNA was poorly translated by *E. coli* ribosomes (13). While we were able to detect mycoplasma protein expression in *E. coli* by radiolabeling, we were unable to detect overexpressed mycoplasma proteins by gel-staining methods, even from pZG27.31, which utilized the strong T7 promoter-polymerase system. In contrast, the pT7-7 expression constructs, with a consensus *E. coli* ribosome-binding site, were able to overexpress regions of this mycoplasma gene. This may indicate that in the former constructs, the ribosome-binding sites on the mycoplasma transcript that were recognized by *E. coli* ribosomes were not used optimally.

While the function of the *M. hyorhina* p115 protein encoded by this DNA fragment is not presently known, data base searches for regions of amino acid similarity have revealed very interesting relationships with nucleoside triphosphate-binding domains of proteins that are involved in recombination repair functions (Notarnicola et al., Abstr. Annu. Meet. Am. Soc. Microbiol., 1990, G-21, p. 139). Current efforts are directed toward examining the function of the p115 protein.

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FIG. 8. Proposed model for production of the related mycoplasma-encoded proteins in *E. coli*. The mycoplasma DNA insert of plasmid pUG27.31 (4.0 kb) is shown at the top of the figure. Arrows indicate the direction of transcription of the mycoplasma DNA and represent the deduced locations of the 110-, 100-, 65-, and 55-kDa translational products relative to their respective coding regions in the DNA. Restriction sites are abbreviated as in Fig. 2.

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