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Genetics

Open reading frame expression vectors: A general method for antigen production in *Escherichia coli* using protein fusions to β-galactosidase

(gene cloning/genetic engineering/*lacZ*/*ompF*/gene fusion)

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ABSTRACT **We have developed an *Escherichia coli* plasmid vector for the identification and expression of foreign DNA segments that are open reading frames (ORFs). The 5′ end of *ompF*, an *E. coli* gene encoding an abundant outer membrane protein, is used to provide a strong, regulated promoter, translation initiation site, and signal sequence for export from the cytoplasm. This sequence is coupled to the *lacZ* gene of *E. coli* so that expression of β-galactosidase requires *ompF* transcription and translation signals. However, this hybrid gene is LacZ⁻ because *lacZ* is out of frame with respect to *ompF*. Restriction enzyme recognition sites are located between *ompF* and *lacZ* to allow convenient insertion of DNA fragments. If an insert is an ORF of the correct length, *ompF* and *lacZ* become realigned in frame, resulting in a LacZ⁺ gene that produces a tribrid protein with the translation product of the insert sandwiched between OmpF and β-galactosidase. The LacZ⁺ phenotype thus identifies clones containing an expressed ORF. To demonstrate the vector's utility we inserted a fragment from the herpes virus thymidine kinase gene and used the resulting tribrid protein to raise antibodies that precipitate thymidine kinase from herpes virus-infected cells. We also inserted a fragment from the *E. coli lexA* gene to produce a tribrid protein that is precipitated by antiserum raised with LexA protein. Thus, tribrid fusion proteins can be used to produce or detect antibodies and also to identify the product of a cloned gene.**

One of the goals of genetic engineering is the expression of all or part of a gene to produce an antigenic protein segment that can be used as a vaccine or to raise or detect antibodies for research and diagnosis. Expressing foreign DNA sequences in *Escherichia coli* requires properly attached bacterial signals for initiating transcription and translation and a method for detecting clones that make the desired product. Several powerful methods can accomplish this, using recombinant DNA techniques to join bacterial signals to foreign DNA and immunological methods to detect the desired clone. However, these techniques are limited because they require knowledge of the gene's DNA sequence or an immunoassay for the gene's product.

In this paper we describe a method for expressing foreign DNA in *E. coli* that does not have these limitations. The method is based on the fact that a DNA sequence encoding a polypeptide contains an open reading frame (ORF) that can be translated without encountering chain termination codons. We have constructed vectors that confer a readily identifiable phenotype, LacZ⁺, when an ORF is properly joined to bacterial signals and expressed. The ORF is expressed as part of a hybrid protein with sequences from the *E. coli* proteins, OmpF and β-galactosidase. Hybrid proteins containing β-galactosidase have previously been used as immunogens (1) and here we extend this technology and demonstrate that short sequences present between OmpF and β-galactosidase are also antigenic.

MATERIALS AND METHODS

Strains. *E. coli* strains are MH3000: *araD*139 Δ(*ara, leu*)7697 Δ(*lac*)X74 *galU galK rpsL* (str^R) *ompR*101 and TK1046: *araD*139 Δ(*argF-lac*)U169 *rpsL*150 (str^R) *relA*1 *flbB*5301 *deoC*1 *ptsF*25 *malPQ*::Tn5 *ompB*cs1. The *ompR*101 mutation (2) drastically decreases *ompF* expression, whereas the *ompB*cs1 mutation decreases *ompF* expression at temperatures near 30°C and shows increased *ompF* expression above 37°C (unpublished data). MH3000 was used to construct LacZ⁺ clones because it is highly transformable and prevents the overproduction lethality of *ompF–lacZ* fusions (see *Results and Discussion*). We also observed that ompR⁺ cells containing the pORF vectors formed light blue colonies on 5-bromo-4-chloro-3-indolyl-β-D-galactoside (XG) plates, even though *ompF* and *lacZ* were out of frame, whereas with MH3000 the colonies were white, allowing clones to be more clearly identified. TK1046 transformed poorly and was not suitable for the initial construction of clones; its use was therefore limited to testing for overproduction lethality and producing high levels of tribrid proteins. The cs mutation in TK1046 is either in *ompR* or *envZ* and allows more expression of *ompF* at 30°C than does *ompR*101. Vero cells grown in Eagle's minimal essential medium and 10% heat-inactivated calf serum were used to propagate herpes simplex virus type 1 (HSV-1). We used HSV-1 strains 14012 (wild type) and AV-5, which is a thymidine kinase (TK)-deficient virus obtained by BrdUrd mutagenesis of strain 14012.

DNA. The source of *lacZ* DNA was pMLB1034 (unpublished data), a derivative of pBR322 containing sequences from the ninth codon of *lacZ* to the *Ava* I site at nucleotide 208 of *lacY* (3). This plasmid was derived from pMC871 (4) and is similar to pMC1403 (4) except that it is deleted for part of *lacY* and additional downstream material. The source of *ompF* DNA was pMLB841, in which a 447-base pair (bp) *Taq* I–*Bgl* II fragment containing the 5′ end of the *ompF* gene was cloned between the *Cla* I and *Ava* I sites of pBR322 (unpublished data). To construct the pORF vectors, this *ompF* fragment was cloned upstream from *lacZ* and appropriate restriction enzyme recognition sites were introduced from pUC71K (a gift from J. Messing

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Abbreviations: ORF, open reading frame; HSV-1, herpes simplex virus type 1; bp, base pair; XG, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; TK, thymidine kinase.

and J. Vieira). The source of the TK gene (*tk*) was plasmid pX1 (5) and the *lexA* gene was derived from pLC44-14 (6). pGE43 is a $LacZ^+$ derivative of pORF2 in which *ompF* and *lacZ* are in frame; pGE109 and pGE126 are the $LacZ^+$ plasmids derived from pORF2 by insertion of the *lexA* and *tk* segments as described below.

Media and Reagents. L broth and M63 medium were as described (7). Ampicillin was present at 150 μg/ml in plates and liquid media. One-tenth milliliter of a solution of XG (in dimethylformamide) at 10 mg/ml was added to plates to detect $LacZ^+$ clones.

DNA Manipulations. T4 DNA ligase and restriction enzymes were used as specified by the supplier. DNA transformation of $CaCl_2$-treated cells (8) and DNA sequence analysis (9) were as described.

Use of Tribrid Protein for Immunization. A 25-ml culture of TK1046 harboring a $LacZ^+$ plasmid was grown at 25–28°C in L broth containing ampicillin until the A_{600} reached 0.2. The culture was then shifted to 37°C and incubated for 1–2 hr. The cells were centrifuged and resuspended in loading buffer (125 mM Tris·HCl, pH 6.8/2% $NaDodSO_4$/9% glycerol/0.7 M 2-mercaptoethanol/0.0025% bromophenol blue) at an A_{600} of 14. This concentrated suspension was incubated in boiling water for 5 min and centrifuged, and the supernatant was loaded on two 9% $NaDodSO_4$/polyacrylamide gels (10). Electrophoresis was performed at 35 mA until the dye reached the gel bottom. The tribrid protein band was visualized by staining and excised, and the protein (100–150 μg) was electroeluted into at least 2 ml of the $NaDodSO_4$ gel buffer. This solution was emulsified with an equal volume of Freund adjuvant and injected into a goat. Similar suspensions were used for two booster injections 2 wk apart.

Labeling of Cells Infected with HSV-1. Vero cells were infected with strain 14012 or AV-5 at a multiplicity of infection of 20. After virus adsorption for 1 hr the inocula were removed, and the cells were labeled for 18 hr with 100 μCi of [^{35}S]methionine (1,200 Ci/mmol; 1 Ci = 3.7×10^{10} Bq; Amersham) per ml in Eagle's minimal essential medium containing 5% dialyzed heat-inactivated fetal calf serum and 10% of the normal concentration of methionine. The cells were washed twice with ice-cold Tris-buffered saline (pH 7.4) and scraped into extraction buffer [0.1 M Tris·HCl, pH 8.0/10% (vol/vol) glycerol/0.5% Nonidet P-40/0.5% sodium deoxycholate/0.2 mM phenylmethylsulfonyl fluoride]. After incubation for 1 hr at 4°C, the extracts were clarified by centrifugation at 60,000 × *g* for 1 hr.

Antibodies and Immunoprecipitations. Rabbit antiserum to LexA protein was provided by John Little (University of Arizona). Rabbit antiserum to HSV-1 TK (11) was provided by Gertrude B. Elion. The broad-spectrum rabbit antibodies to HSV-1 were produced by DAKO Immunochemicals (Denmark) and obtained from Accurate Chemical and Scientific (Westburg, NY). [^{35}S]Methionine-labeled protein was precipitated from HSV-1-infected cell extracts and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis as described (12). To label the OmpF–LexA–β-galactosidase tribrid protein a culture of TK1046/pGE109 was induced in M63 glucose medium and 0.5 ml was labeled for 5 min with 100 μCi of [^{35}S]methionine per ml. The labeled cells were chilled and washed once with chilled 10 mM Tris·HCl (pH 8.0) and then resuspended in 25 μl of 1% $NaDodSO_4$/1 mM EDTA. The suspension was incubated in boiling water for 2 min, then 0.65 ml of TSET (50 mM Tris·HCl, pH 8.0/150 mM NaCl/0.1 mM EDTA/2% Triton X-100) was added, and the cell debris was removed by centrifugation. One-half milliliter of the supernatant was incubated with anti-LexA serum for 30 min at 0°C, then 10 μl of a 10% (wt/vol) *Staphylococcus aureus* protein A suspension in TSET was added. After incubation for 30 min at 0°C, the sample was washed twice with TSET and finally with 10 mM Tris·HCl (pH 8.0). The pellet was resuspended in 50 μl of loading buffer, incubated 5 min in boiling water, and centrifuged, and 35 μl of the supernatant was electrophoresed through a 9% $NaDodSO_4$/polyacrylamide gel. The gel was treated with sodium salicylate (13) and exposed to x-ray film.

RESULTS AND DISCUSSION

Fig. 1 illustrates the method for expressing ORFs in *E. coli*. The ORF expression vector contains the 5′ end of the *ompF* gene of *E. coli* to provide signals for initiating transcription and translation. The *ompF* gene produces a major outer membrane porin protein of *E. coli*, present at about 100,000 molecules per cell (14). The promoter is regulated by environmental factors and requires the *ompB* locus (consisting of *ompR* and *envZ*) as a positive regulator for expression (2, 15). This 5′ segment of *ompF* contains the promoter and regulatory region and encodes the 21-amino acid signal sequence and first 12 amino acids of the mature OmpF protein (unpublished data). Next to this is part of the *lacZ* gene, which encodes the enzymatically active carboxyl terminus of β-galactosidase, starting at amino acid 9 of the complete protein. β-Galactosidase retains activity with virtually any amino-terminal polypeptide fused to this fragment so that its activity can be used as an indicator of gene expression (4, 16). Because the *lacZ* segment lacks its normal 5′ sequences, its expression depends upon the signals provided by the upstream *ompF* fragment. However, in the vector, the *lacZ* sequence is out of frame with respect to *ompF* so that translation initiating in *ompF* does not produce β-galactosidase. As a result, the vector is $LacZ^-$.

Between these two regions are recognition sequences for re-

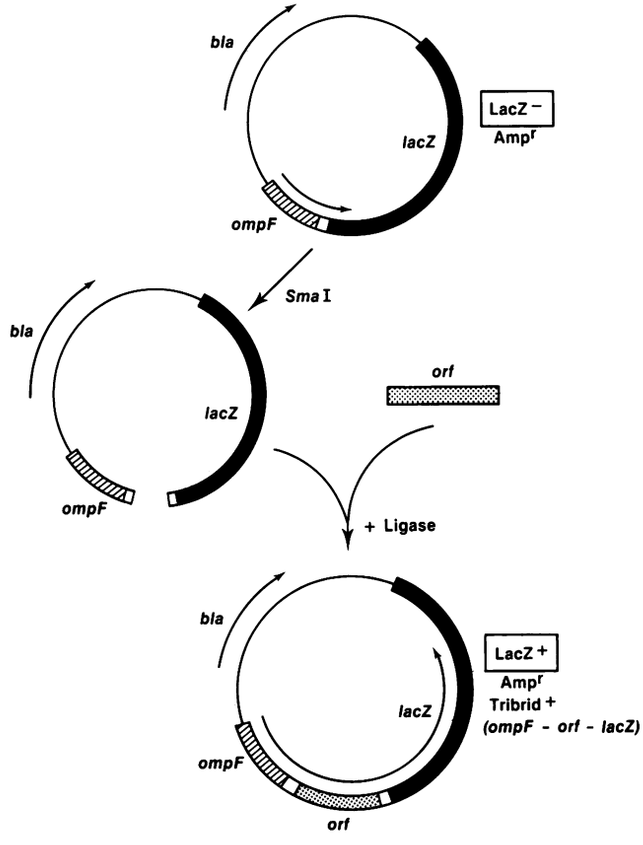


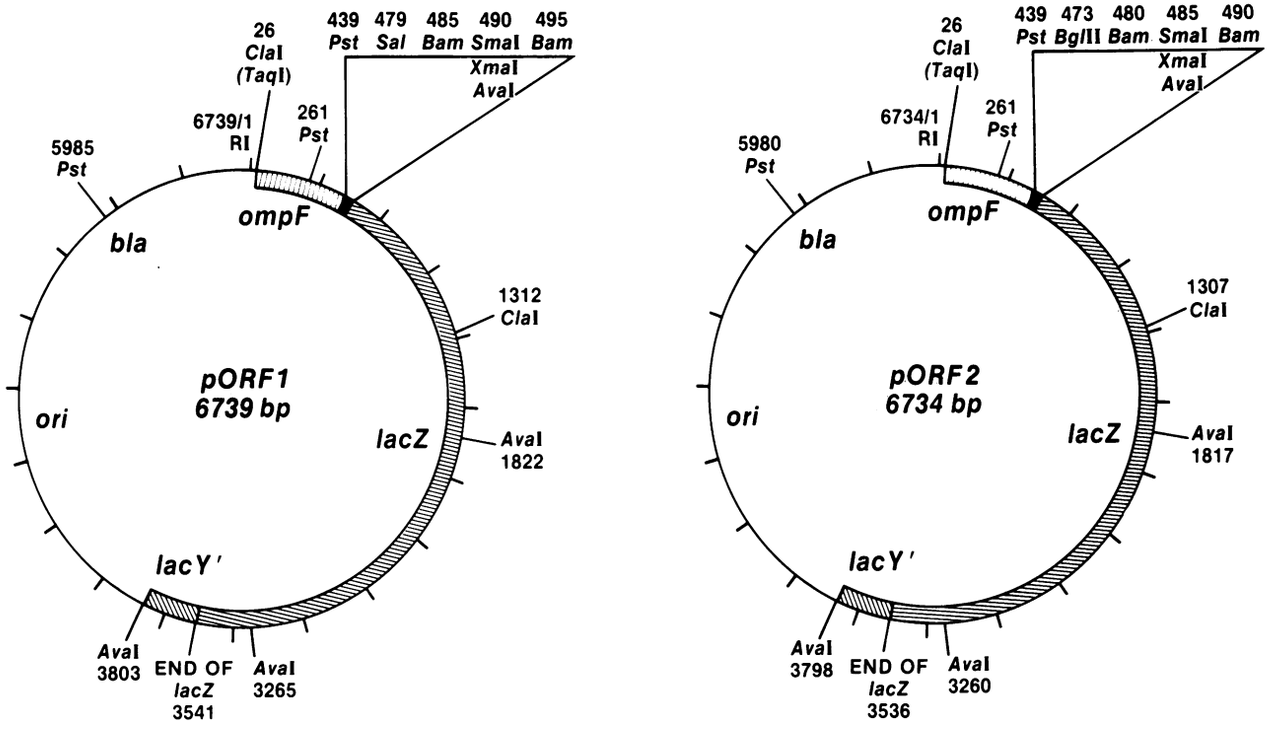
FIG. 1. Use of ORF expression vectors. See text for details.

striction enzymes, such as *Sma* I, to provide sites for inserting foreign DNA. Insertion of a DNA fragment can realign the *lacZ* reading frame with that of *ompF* if the length of the fragment is the proper compensating nonintegral number of codons. If the inserted DNA is also an ORF and is inserted so that the ORF is in frame with both the *ompF* and *lacZ* sequences, then the vector will produce a "tribrid" protein containing the polypeptide encoded by the ORF sandwiched between the OmpF and β-galactosidase sequences. This tribrid protein will have β-galactosidase activity and thus the vector will now confer a $LacZ^+$ phenotype. β-Galactosidase will not usually be produced if the ORF is translated in the wrong reading frame because chain-termination codons will be encountered. Thus, conversion of the vector from $LacZ^-$ to $LacZ^+$ identifies clones containing an expressed foreign coding sequence. Although most random fragments from an ORF sequence inserted in the vector will not produce a $LacZ^+$ phenotype (theoretically only 1 of 18 clones will be $LacZ^+$), those that do can be easily identified with the indicator XG. Furthermore, if the size of the fragments is long enough, the possibility that the ORF is a fortuitous sequence, rather than a natural coding sequence, will be small (of a random collection of 300-nucleotide-long fragments, <1% will be ORFs).

We constructed two ORF vectors utilizing the 5′ end of the *ompF* gene (Fig. 2). *lacZ* and *ompF* are out of frame in both vectors but the frameshift differs. The *Sma* I site allows insertion of blunt-ended fragments, which can then be excised from the neighboring *Bam*HI sites. The structure of the *ompF–lacZ* joint was verified by DNA sequence analysis. In addition, digestion of pORF1 with *Bam*HI or pORF2 with *Bam*HI and *Bgl* II, followed by ligation of the sticky ends, put *lacZ* in frame with *ompF* and resulted in $LacZ^+$ plasmids.

The *ompF* gene, because it encodes an exported protein, provides a signal sequence, and this has several important implications. The tribrid protein may be exported from the cytoplasm, which can increase the protein's stability (17) and possibly allow detection of antigens at the surface of intact cells. In addition, overproduction of OmpF–β-galactosidase fusion proteins is lethal to the cell (unpublished data), a phenomenon observed with fusions of β-galactosidase to other exported proteins (14). This lethality can be prevented by mutations in the positive regulator *ompR*, which decrease ompF expression enough to spare the cell while still allowing the $LacZ^+$ phenotype to be observed. The lethality of a fusion in $ompR^+$ cells provides a convenient test to demonstrate that translation starts within *ompF* and proceeds through the insert. Inserts that are not in frame with *ompF* can be $LacZ^+$ if translation starts at an ATG codon within the insert that is in frame with *lacZ*. However, the protein produced would not contain the *ompF* signal sequence and would not cause the lethal phenotype. Thus, the

a



b

FIG. 2. Structure of ORF expression vectors with the 5′ end of the *ompF* gene. (*a*) Structure of ORF vectors. The thin line represents material from pBR322 including the β-lactamase gene (*bla*) and origin of replication (*ori*). The *Taq* I site is not unique. (*b*) Sequences in the region of the *ompF–lacZ* fusion joint.

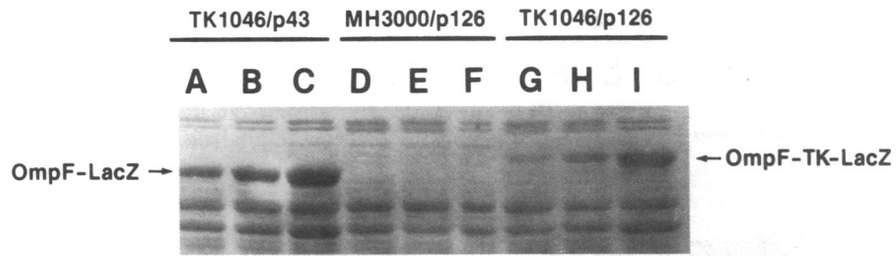


FIG. 3. Production of hybrid proteins by ORF expression vectors in TK1046 or MH3000. Cells were grown in L broth at 25°C to an A_{600} of 0.2 (lanes A, D, and G); then half of the culture was incubated at 42°C for 2 hr (lanes C, F, and I), whereas the other half was maintained at 25°C (lanes B, E, and H). Whole-cell extracts were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis. Only the high molecular weight region of the gel is shown.

observation of overproduction lethality indicates that the complete insert is being expressed as an ORF.

Insertion and Expression of Defined Fragments in pORF2. To test the efficacy of this system, we inserted segments of the HSV-1 *tk* and *E. coli lexA* genes into pORF2. A 301-bp fragment of the *tk* gene (18), from the *Bgl* II cleavage site 57 bp upstream from the start of the structural gene to the *Ava* I cleavage site 244 bp into the gene, was cloned between the *Bgl* II and *Xma* I cleavage sites of pORF2. Inspection of the *tk* DNA sequence predicted that this fragment was an ORF of an appropriate length to realign *ompF* and *lacZ* and to be read in the proper reading frame. Although the first 57 bp of this fragment precede the structural gene and are not normally translated, there are no chain-termination codons in the *tk* reading frame in this region. Translation of this *tk* segment should yield a polypeptide containing 100 amino acids, of which 82 correspond to those of the amino terminus of the TK protein. Insertion of this fragment into pORF2 resulted in a $LacZ^+$ plasmid (pGE126) whose structure was verified by DNA sequence analysis. Similarly, a 526-bp fragment from the *E. coli lexA* gene (19), between the *Hin*cII cleavage sites located 11 and 537 bp from the start of the structural gene, was inserted into the *Sma* I cleavage site of pORF2. This fragment contains an ORF that realigned *ompF* and *lacZ* to produce a $LacZ^+$ plasmid, pGE109. This *lexA* segment encodes a sequence of 175 amino acids internal to the LexA protein.

Both pGE126 and pGE109 were constructed in the *ompR*101 mutant MH3000 to prevent lethality. When transferred to the *ompB*cs mutant, TK1046, the cells were viable at 30°C but died at 42°C due to the lethality of high-level production of the fusion protein. Thus, both clones most likely initiate translation from the *ompF* gene, rather than from within the inserted sequence.

Using TK1046 we were able to produce significant amounts of the tribrid proteins. As shown in Fig. 3, TK1046 cells harboring pGE126 produced a high molecular weight polypeptide whose synthesis was enhanced at higher temperature. This protein was not produced from the plasmid in MH3000 at any temperature. The $LacZ^+$ derivative of pORF2, pGE43, generated by the *Bgl* II–*Bam*HI deletion described above, also showed temperature-dependent synthesis of a high molecular weight protein in TK1046 that was smaller than that produced from pGE126. By these criteria, the inducible polypeptide is the OmpF–TK–LacZ tribrid protein. Similar results were obtained with pGE109.

Use of the OmpF–TK–LacZ Protein to Raise Antibodies Against TK. To show that tribrid proteins can be used to raise antibodies against the inserted sequence, we used the tribrid protein produced from pGE126 to raise antibodies against TK. Because of the large size of β-galactosidase, tribrid proteins migrate in $NaDodSO_4$/polyacrylamide gels in a region where there are few other *E. coli* proteins (Fig. 3). We were thus able to purify the tribrid protein produced from pGE126 simply by excising the band from a gel and electroeluting the polypeptide. This preparation was used to immunize a goat, and the resulting serum was tested for antibodies specifically directed against TK. As shown in Fig. 4, the serum precipitated only the M_r 41,000 TK protein from cells infected with HSV-1. This protein was identified as TK because it was also precipitated by antibodies prepared against intact TK and a broad-spectrum antiserum against HSV-1 proteins, whereas none of these sera precipitated this protein from cells infected by the tk^- mutant AV-5. The titer of the anti-TK antibody in serum raised with the tribrid protein appeared to be lower than in the other sera. It is not known if this is a general property of sera raised with tribrid proteins. In any case, it is clear that the anti-tribrid serum effectively precipitated TK. Because a relatively short sequence from TK was immunogenic, it is apparent that large inserts are not necessary to raise antibodies with tribrid proteins.

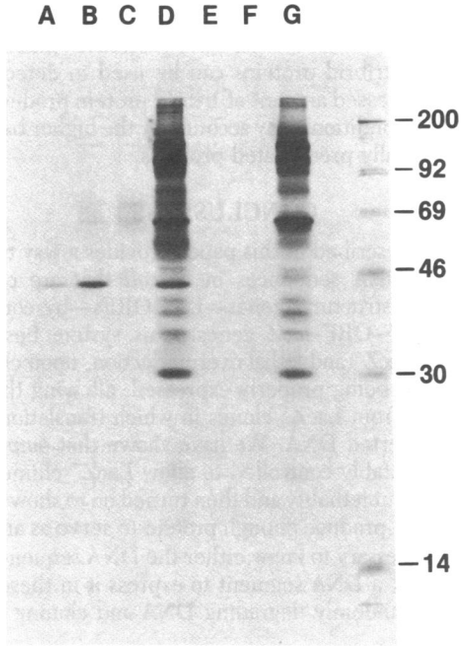


FIG. 4. Immunoprecipitation of TK from cells infected with HSV-1. The precipitated labeled proteins were separated on a $NaDodSO_4$/5–20% polyacrylamide gel gradient. Molecular weight markers are shown as $M_r \times 10^{-3}$. Extracts of cells infected with HSV-1 strains 14012 (lanes A–D) or AV-5 (lanes E–G) were reacted with goat antiserum against the OmpF–TK–LacZ protein (lanes A and E), rabbit antiserum against TK (lanes B and F), preimmune rabbit serum (lane C), and broad-spectrum antibody against HSV-1 proteins (lanes D and G).

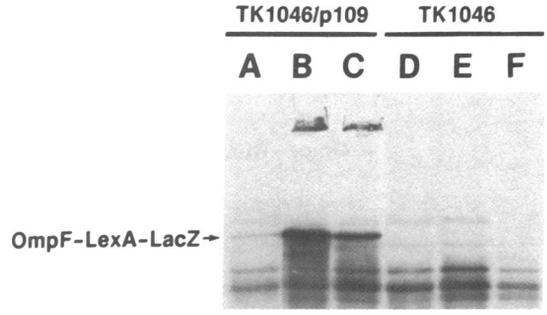


FIG. 5. Immunoprecipitation of OmpF–LexA–LacZ tribrid protein by antiserum against LexA. Cells were TK1046 with (lanes A–C) or without (lanes D–F) pGE109. Lanes A and D, whole-cell extracts not precipitated; lanes B and E, precipitation with anti-LexA serum; and lanes C and F, precipitation with anti-β-galactosidase serum. Only the high molecular weight region of the gel is shown.

Precipitation of the OmpF–LexA–LacZ Tribrid Protein by Antibodies Directed Against LexA. To show that antibodies raised against a complete protein can recognize the partial sequence in a tribrid protein, we used the tribrid protein produced from pGE109. To provide a source of antigen, this plasmid was introduced into TK1046 and the tribrid protein was induced and labeled in minimal glucose medium. Although TK1046/pGE109 produced a high level of tribrid protein in L broth, similar to that from pGE126 (data not shown), significantly less was made in minimal medium (Fig. 5, lane A). We believe that this results from media effects on *ompF* expression, rather than from the specific insert, because we have observed the same effect with all *ompF* fusions in TK1046. Despite this, the level of tribrid protein produced was sufficient to demonstrate its antigenicity. Antisera directed against either LexA or β-galactosidase precipitated the tribrid protein (Fig. 5), which was identified as the high molecular weight polypeptide that was absent in cells that did not carry pGE109. This result demonstrates that tribrid proteins can be used to detect an antibody. The decreased amount of tribrid protein produced under these growth conditions may account for the higher background of nonspecifically precipitated proteins.

CONCLUSIONS

The method described in this paper provides a way to identify and express DNA sequences in *E. coli* that are coding sequences from structural genes—i.e., ORFs—by constructing chimeric *ompF*–ORF–*lacZ* genes. This system bestows two phenotypes, $LacZ^+$ and lethal overproduction, upon clones with ORFs that are being properly expressed, allowing them to be distinguished from $LacZ^+$ clones in which translation initiates within the inserted DNA. We have shown that *ompF* expression can be suitably controlled to allow $LacZ^+$ chimeras to be detected without lethality and then turned on to show the lethal phenotype and produce enough protein to serve as an antigen.

It is not necessary to know either the DNA sequence or protein product of a DNA segment to express it in these vectors. Methods for randomly degrading DNA and cloning the small fragments have been described and should make the system generally applicable to either genomic DNA or cDNA (20). Because the relatively short sequences in the tribrid proteins produced by this system are immunogenic, these proteins can serve as a reagent to produce antibodies that can be used to identify the product of an intact gene, as demonstrated here with TK. This sytem thus provides a method to identify both a gene and its product without requiring prior knowledge of either. In addition, because the tribrid proteins react with antibodies raised with a complete protein, as shown with LexA, it should also be possible to use this system to identify the gene coding for a known protein. Existing antibodies against the protein can be used to identify clones expressing its coding segments and those clones can serve as probes to identify the gene.

Since this work was completed several papers have described additional vectors for identifying ORFs in *E. coli* (21–23). These reports further demonstrate and extend the validity and applications of this approach for antigen production.

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