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Production and simple purification of a protein encoded by part of the *gag* gene of HIV-1 in the *Escherichia coli* HB101F$^+$ expression system inducible by lactose and isopropyl-β-D-thiogalactopyranoside

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Abstract

The development of the *Escherichia coli* expression system, which was prepared by transferring the F′ episome from strain 71/18 to a highly transformable F$^-$ strain HB101, is described. These new HB101 (F$^+$) cells, which produced high levels of *lac* repressor, were capable of taking up lactose and grew under strict selection conditions. A relatively simple two-step purification of part of a protein (M_r 27 000) encoded by the *gag* gene of HIV-1 in this expression system is described. The supernatant prepared by removal of cell debris was precipitated by 30% saturation of ammonium sulphate. The protein spectrum was characterized by gel electrophoresis, immunoblotting and ion-exchange titration curves. Optimum separation was achieved using a strong anion exchanger (Mono Q) at pH 8.0. The purified protein did not cross-react with antibodies to *E. coli*.

1. Introduction

The development of methods for the introduction of recombinant DNA into different prokaryotic and eukaryotic cells has created novel possibilities for expressing heterologous genes with the assistance of several cell types and in a broad range of unconventional expression systems. Techniques for expressing cloned genes introduced into *Escherichia coli* are probably the most popular and they have been routinely used for the preparation and functional analysis of many proteins of scientific interest [1–14].

Large amounts of recombinant protein can be produced in *E. coli* by placing a cloned gene in an expression vector under the control of a strong, regulated promoter. The genetically manipulated operator–repressor system of the *lac* operon can efficiently regulate the transcription of heterologous genes which are placed in appropriate expression plasmid under the control of the *lac* operator. The interaction *lac* operator–repressor has been thoroughly characterized [15] and has been successfully used to control the expression of genes cloned in both prokaryotic [1,5–8,11,12,16] and eukaryotic cells [17].

In this paper, we report the production and simple purification of protein encoded by part of the *gag* gene of human immunodeficiency virus

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type 1 (HIV-1) in *E. coli*. We describe the preparation of the *E. coli* expression system, which was developed by transferring the F′ episome from strain 71/18 to a highly transformable F^- strain HB101. These new HB101 (F^+) cells, which produced high levels of *lac* repressor, were capable of taking up lactose and grew under strict selection conditions. After transformation by the plasmid pUC18 with the part of the *gag* gene and induction by lactose, the expression of viral proteins was demonstrated by enzyme-linked immunosorbent assay (ELISA) and Western blotting. The kinetics and optimum conditions of the expression were determined. In addition, we used a simple method for the elimination of antibodies to *E. coli*, commonly present in human and animal sera. We also established a simple technique for final purification of soluble recombinant proteins.

2. Experimental

2.1. Media, antibiotics and reagents

Luria–Bertani (LB) medium [18], A Minimal Medium (supplemented with thiamine and L-leucine) [18] and A Minimal Medium plates (15 g of agar per 1000 ml of A Minimal Medium) [18] were used. Streptomycin (sodium salt) (40 μg/ml), ampicillin (sodium salt) (20 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (20 g/ml) and isopropyl-β-D-thiogalactopyranoside (IPTG) (1 m*M*) were obtained from Sigma.

2.2. Bacterial cells and plasmid

E. coli strains 71/18, genotype *supE thi* (*lac-proAB*) F′ [*proAB*$^+$ *lacI*^{q} *lacZ M15*) [19], a donor of F′ episome and HB101, genotype F^- *supE44 hsdS20* ($r_B^- m_B^-$) *recA56 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1* [20], a highly transformable acceptor of F′, were used. The expression vector was a gift from Dr. M.I. Bukrinsky (D.I. Ivanovski Institute of Virology, Moscow, Russian Federation). The viral insert, placed in the plasmid type pUC18 under the control of the *lac* promotor, had been described as containing a segment of *gag* gene encoding p24 (core) protein of human HIV-1 of the BH10 strain [21].

2.3. Bacterial conjugation of HB101 and 71/18 strains of E. coli

Overnight cultures of the donor (71/18) and acceptor (HB101) cells were inoculated (1%) in LB medium and grew (37°C, intensive aeration) until the middle of the exponential growth phase ($A_{550} = 0.4$). Then both cultures were mixed (1:1) and cultivated for 60 min under the same conditions to allow for the conjugation process. After conjugation, the bacterial cells were selectively plated (A Minimal Medium plates supplemented with L-leucine and streptomycin) and colonies of HB101F$^+$ were isolated after 48 h of cultivation at 37°C.

Preparation of fresh competent cells HB101F$^+$ using $CaCl_2$ and transformation by expression plasmid were performed by modification of procedure of Cohen *et al.* [22] and as also described by Maniatis *et al.* [14].

2.4. Induction of expression

A culture of *E. coli* strain HB101F$^+$ transformed by the expression vector pUC18 containing part of *gag* gene of HIV-1 was grown in LB medium (supplemented with streptomycin and ampicillin) in a shaking incubator at 37°C. Aqueous lactose solution (stock solution, 200 mg/ml) was added up to a final concentration of 1 mg/ml at the beginning of the exponential phase of growth ($A_{550} = 0.2$). Cultivation was continued under the same conditions for the desired period of time. A pilot study of the kinetics of the recombinant protein expression revealed a maximum concentration of p24*gag* protein in bacterial cells 7–8 h after induction by lactose and then the content of this protein slowly decreased (ELISA). Similar results were obtained after induction by IPTG using a final concentration of 1 m*M* (data not shown). Expression of the recombinant protein and cell growth were always stopped 7.5 h following

induction by transferring the culture to 4°C, and the cells were harvested by centrifugation, washed with cold phosphate-buffered saline (PBS) and stored at −20°C.

2.5. Partial purification of the recombinant viral proteins

Induced cells were pelleted from medium by centrifugation at 8000 *g* for 15 min and washed twice with cold PBS. The bacterial pellet from 500 ml of medium was resuspended in 50 ml of PBS and frozen and defrozen slowly three times (maximum temperature 4°C). The partially disrupted bacterial suspension was sonicated repeatedly for 10 × 2 min (Soniprep 150, amplitude 14–18 μm) on ice. The lysate was clarified by centrifugation at 100 000 *g* for 30 min at 4°C. The resulting supernatant was very slowly mixed with a saturated solution of ammonium sulphate to reach a final concentration of 30% and chilled overnight under intensive mixing at 4°C. The precipitate was centrifuged at 100 000 *g* for 15 min at 4°C, carefully drained, redissolved in 5 ml of cold 0.1 *M* ammonium carbonate (pH 7.5) and dialysed against an abundance of the same solution (supplemented with 15 m*M* sodium azide) at 4°C. After dialysis, the protein content was measured by the method of Lowry *et al.* [23] and the protein cocktail was adjusted with 0.1 *M* ammonium carbonate (pH 7.5) up to 10 mg/ml, lyophilized and stored under nitrogen at 4°C.

2.6. Preparation of the rabbit anti-E. coli HB101F$^+$ polyclonal sera

To generate a specific post-immune rabbit serum with a high level of antibodies against *E. coli*, a dose of 10^9 HB101 (F^+) cells, washed twice in PBS, sonicated (Soniprep 150, 14–18 μm, 4°C) and suspended in complete Freund's adjuvants, was used for primary immunization. Animals were boosted on days 21 and 42 with the same portion (without adjuvants) and serum was collected 52 days after the first immunization. High levels of specific antibodies were confirmed by an indirect ELISA test (immobilized antigen–lysate of *E. coli* HB101F$^+$).

2.7. Removal of anti-E. coli antibodies present in human sera

The human HIV-1 positive sera used for detection of the recombinant viral p24*gag* protein during the purification procedure exhibited different levels of anti-*E. coli* antibodies which resulted in false positivity or a strong background in several immunochemical assays. These undesirable antibodies were successfully eliminated by saturation of sera with lysate of *E. coli* cells (HB101F$^+$). In brief, a twice-washed (PBS) pellet of HB101F$^+$ was resuspended and sonicated in 1 m*M* ammonium carbonate (pH 7.5) (Soniprep 150, amplitude 14–18 μm) at 4°C and clarified by centrifugation at 100 000 *g* for 15 min at 4°C. The protein content was adjusted with 1 m*M* ammonium carbonate solution to 10 mg/ml and lyophilized. The freeze-dried proteins were solubilized (5–8 mg/ml) immediately before use in sample diluent with human or animal serum (diluted 20–100-fold) and all detectable anti-*E. coli* HB101F$^+$ antibodies were blocked within 30 min of incubation at 37°C. This simple procedure completely eliminated the activity of all detectable anti-*E. coli* HB101F$^+$ antibodies presented in a panel of human HIV-1 positive sera in ELISA and Western blot assays.

2.8. Enzyme-linked immunosorbent assay

The examination for the presence of the recombinant viral proteins during the purification procedure consisted of indirect ELISA. The recombinant viral *gag* proteins were detected with the assistance of human HIV-1 positive serum (characterized by Western blotting, Biotech DuPont) and the presence of residual *E. coli* proteins was monitored with rabbit hyperimmune anti-*E. coli* HB101F$^+$ serum. Usually 1 μg of purified or 10 μg of semipurified recombinant proteins were diluted in 100 μl of carbonate buffer (pH 9.6) (15 m*M* Na_2CO_3–35 m*M* $NaHCO_3$–3 m*M* NaN_3) and coated on one well of a microtitre plate (Immunolon 2; Dynatech) overnight at 4°C. Residual binding capacity of the well was blocked with 200 μl of blocking buffer (6% non-fat milk–0.05% Tween 20 in

binding buffer) within 120 min at 37°C. The well was then washed twice with washing buffer (6% non-fat milk–0.05% Tween 20 in PBS and human or rabbit serum was added after dilution (20–100-fold) in washing buffer (60 min, 37°C). The human serum for detection of the viral proteins was saturated with *E. coli* lysate as described previously. The well was rinsed three times as before and conjugate (peroxidase-labelled anti-human or anti-rabbit IgG antibodies) in washing buffer (1000×diluted) was added (60 min, 37°C). The final rinsing was performed three times with washing buffer, twice with PBS and once with substrate buffer (pH 5.0) (40 m*M* citric acid–70 m*M* Na_2HPO_4). A 100-μl volume of development solution (0.04% *o*-phenylenediamine and 0.02% H_2O_2 in substrate buffer) was added and the reaction stopped with 50 μl of 2 *M* sulphuric acid. The absorbance was measured at 492 nm using a microplate reader.

2.9. Protein analyses

Recombinant proteins were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [24,25], immunoblot analysis [26] and 2D PAGE [27]. Polyacrylamide gels (exponential gradient, 5–15% of acrylamide) were prepared and the gels were stained with Coomassie Brilliant Blue or with ammoniacal silver according to Sammson *et al.* [28]. Standard proteins transferred to nitrocellulose sheets were stained with colloidal silver [29].

2.10. Chromatographic purification of viral recombinant proteins

Partially purified recombinant viral proteins (after ammonium sulphate precipitation) were dialysed against 20 m*M* Tris–HCl buffer (pH 8.0) and loaded up to 20 mg in 1.0 ml on a HR5/5 Mono Q column (Pharmacia) pre-equilibrated with the same buffer. The fractions were eluted at a flow-rate of 1.0 ml/min with a linear gradient from 0 to 350 m*M* NaCl in 25 min in the same buffer. Chromatographic fractions of 1.0 ml were collected and tested by SDS-PAGE, by immunoblotting and by ELISA for the presence of both viral recombinant *gag* proteins and *E. coli* proteins.

In the preparative mode, Q Sepharose Fast Flow (11 × 2.4 cm I.D.) (Pharmacia) was used, up to 500 mg of partially purified recombinant viral proteins were loaded and elution was performed with a linear gradient from 0 to 350 m*M* NaCl in 40 min at a flow-rate of 5.0 ml/min.

3. Results

The *lac* repressor gene is transcribed at an extremely low rate and it synthesizes only about 5–10 molecules each generation [30]. In order to obtain a bacterium that produced high amounts of the *lac* repressor, we transferred F′ episome from *E. coli* 78/18 (donor) to HB101 (acceptor) by bacterial conjugation. In strain 78/18 the *lac* repressor is overproduced by a factor of *ca.* 10 owing to the I^q promoter mutation on the transmissable F′ episome [19].

The resulting *E. coli* cells, HB101F$^+$, exhibited a unique phenotype in contrast to the parental strains 78/18 and HB101. They grew on an A Minimal Medium plate supplemented by L-leucine, thiamine and streptomycin. Overproduced *lac* repressor could be regulated by IPTG and more importantly by inexpensive lactose (Table 1). After transformation of the competent *E. coli* cells HB101F$^+$ by expression vector pUC18 with part of *gag* gene of HIV-1, these cells displayed resistance to ampicillin.

Growth and induction of larger amounts of cells were performed in a fermenter (volume 7.5 l) under the same cultivation and induction conditions as on a laboratory scale. The typical harvest was about 0.5 g of dry bacterial paste from 1000 ml of medium, which represented *ca.* 100 mg of semipurified recombinant protein fraction and *ca.* 500 μg of HPLC purified viral proteins (0.5% of semipurified protein fraction).

The protein fraction from induced cells obtained after precipitation by ammonium sulphate and containing recombinant viral antigens was analysed in SDS–polyacrylamide gels and the proteins were revealed by staining with Coomassie Brilliant Blue as shown in Fig. 1. The

Table 1
Bacterial growth and induction of *lac* operator–repressor system by IPTG or lactose of *E. coli* strains 71/18, HB101 and HB101F$^+$ on a selective A Minimal Medium plate supplemented by L-leucine and thiamine

A Minimal Medium plates	Donor F$^+$ 71/18	Acceptor F$^-$ HB101	New cell (F$^+$) HB101F'
X-gal	+	–	+
X-gal, IPTG	+	–	**
X-gal, IPTG, streptomycin	–	–	**
X-gal, lactose	+	–	**
X-gal, lactose, streptomycin	–	–	**

X-gal was used as a chromogenic substrate for β-galactosidase (blue colonies): – = no growth of the bacterium; + = growth of the bacterium, white colonies; ** = growth of the bacterium, blue colonies (binding of the *lac* repressor by IPTG or lactose and production of β-galactosidase).

position of recombinant viral proteins within the gel was located after comparison with Western blots, which were prepared from resolved protein aliquots.

Two-dimensional PAGE of crude recombinant proteins prepared from the supernatant of bacterial cell lysate by precipitation with ammonium sulphate revealed a complex mixture of proteins (data not shown).

An efficient one-step purification method based on ion chromatography on a Mono Q column was developed to achieve a high degree of immunochemical purity of recombinant proteins (Fig. 2). The recombinant viral proteins identified by the ELISA detection method were resolved from residual *E. coli* proteins as is shown in the representative chromatographic profile. Microelectrophoresis on polyacrylamine gels and Western blotting revealed three protein bands with relative molecular masses of 27 000, 28 000 and 29 000, binding human HIV-1 positive serum (Figs. 1 and 3). An attempt to elute recombinant proteins in a void volume by a more acidic or a more concentrated starting buffer failed as it caused co-elution of the recombinant proteins with some amount of *E. coli* proteins.

The amount of HPLC-purified viral recombinant proteins usually represented 0.5–1.2% of

Fig. 1. SDS-PAGE or recombinant protein. Exponential gradient, 5–15%. Lanes: 1, 2 and 6 = crude recombinant proteins; 4 = purified recombinant proteins; 3 and 5 = low-molecular-mass standard (LMMS) (Pharmacia) (M_r 94 000, 67 000, 43 000, 30 000, 20 100 and 14 400). Lanes 1–3 stained with Coomassie Brilliant Blue; lane 4, Western blot stained with human HIV positive serum; lanes 5 and 6, stained with colloidal silver; lane 5, LMMS; lane 6, crude recombinant proteins.

the protein obtained after precipitation with ammonium sulphate.

The human and animal sera used for detection of recombinant proteins during the purification procedure exhibited different levels of anti-*E. coli* antibodies. This resulted in false positivity or a strong background in several immunochemical assays (ELISA, Western blotting). False reactivity was successfully eliminated by saturation of sera with lysate of *E. coli* cells (HB101F$^+$) (data not shown).

4. Discussion

The disadvantage of prokaryotically expressed proteins employed in an ELISA test is the

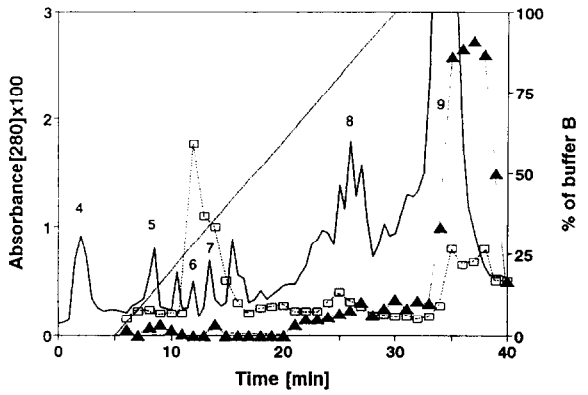

Fig. 2. Chromatography of semipurified recombinant proteins. Column, Mono Q HR 5/5; buffer A, 20 m*M* Tris–HCl (pH 8); buffer B, 0.35 *M* NaCl in buffer A; gradient from 100% buffer A to 100% buffer B in 25 min (dotted line); flow-rate, 1.0 ml/min; detection, absorbance at 280 nm (solid line). □ = ELISA for the presence of viral recombinant *gag* proteins; ▲ = ELISA for the presence of residual *E. coli* proteins.

potential for false-positive results due to the presence of antibodies to *E. coli* proteins. We overcame this problem in the preparation of HIV-1 *gag* fusion proteins by thorough purification of the recombinant product and/or by saturation of sera by crude bacterial lysates.

A simple and obvious solution to the problem

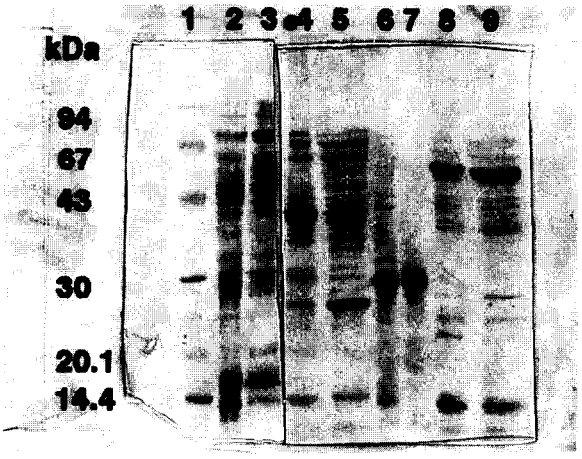

Fig. 3. SDS-PAGE of chromatographic peaks. Concentrated fractions from five consecutive separations. Electrophoretic conditions as in Fig. 1, staining with Coomassie Brilliant Blue. Lanes: 1 = low-molecular-mass standard; 2 = crude recombinant proteins; lane 3 = semipurified protein fraction; 4–9 = peaks indicated in Fig. 2.

of removing *E. coli* proteins by affinity chromatography on rabbit anti-*E. coli* HB101F$^+$ polyclonal sera immobilized on BrCN-activated Sepharose 4B was not successful, however, in our system (data not shown). The reason for this is not clear; it might be due to altered binding properties of our immunoglobulins after immobilization and/or to the fact that the polyclonal antibodies may not contain the appropriate amounts and titres of immunoglobulins specific for individual *E. coli* proteins.

We found that our recombinant proteins (their heterogeneity was probably the result of proteolytic degradation) were located at neutral pH, whereas the most abundant *E. coli* proteins in a crude recombinant preparation were slightly acidic (Fig. 2). The purification procedure based on this analysis using optimized chromatography on an anion exchanger (Mono Q) separated semipurified proteins efficiently in one step (Fig. 3).

A starting buffer with a low salt concentration and a slightly basic pH performed very well and recombinant proteins were well resolved from *E. coli* proteins. The chromatographic conditions enabled us to use relatively large volumes of samples without significant broadening of eluted fractions.

In conclusion, the inexpensive induction of recombinant product HIV-1 *gag* with lactose, a cheap medium, and a simple purification make the described system attractive for the production of other recombinant proteins on either a laboratory or an industrial scale.

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6. References

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