CONDITIONS RESULTING IN FORMATION OF PROPERLY ASSEMBLED RETROVIRAL CAPSIDS WITHIN INCLUSION BODIES OF *Escherichia coli*

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It has been generally accepted that inclusion bodies (IBs) formed in *Escherichia coli* consist of non-biologically active aggregated proteins, which are stabilized by non-productive interactions. We show here that bacterial expression of a retroviral capsid polyprotein results in formation of insoluble IBs that contain fully assembled viral particles connected with amorphous material. The efficiency of IBs formation and capsid assembly was not significantly affected by changes in induction temperature, pH of cultivation medium or the level of expression.

Key words: Native proteins; Bacterial expression; Inclusion body; Retroviral capsid assembly; Retroviruses.

Overproduction of proteins cloned downstream from a strong promoter leads to high levels of synthesis in *E. coli*. Yields of heterologous protein ranging from 20 to 50% of total cell protein have been reported[1,2]. Many recombinant proteins if expressed in such high concentrations do not fold properly and form large insoluble aggregates called inclusion bodies (IBs) (for reviews see refs[1,3–6]). Transmission electron microscopy has shown that IBs are amorphous aggregates that are not surrounded by membrane. Their size and density are distinguishable from the other structures within the cytoplasm. The insolubility of IBs is advantageous since it facilitates efficient purification of recombinant proteins. Most of the cellular proteins in a lysate can be eliminated by washing of IBs several times with diluted detergents such as Triton X-100 or deoxycholate[6]. Another advantage of proteins

accumulated in the IBs is the fact that they are protected from the action of bacterial proteinases. Finally, accumulation in inclusion bodies can often overcome the problem of the toxicity of proteins expressed in such a high amount[5].

Although IBs formation has been extensively studied, the mechanism of their formation has not been satisfactorily elucidated. Several factors influence formation of IBs. The effects of these factors often vary with different proteins. It has been shown that the reduced growth rate decreased accumulation of protein in the IBs (ref.[7]). Increased temperature resulted in stimulation of IBs formation and some proteins have been found preferentially in the soluble fraction when expressed under lower temperatures[8–10]. Changes in the pH of cultivation media were also reported to affect inclusion body formation[11]. Distribution between soluble and insoluble fraction is often dependent on a particular protein sequence. Mutations in p22 tailspike protein affect the protein aggregation and abolish its deposition into IBs (refs[12,13]). Point mutations in CheB and CheY signal transduction proteins can change the distribution of product into soluble and aggregated fractions[14]. Cloning of a gene of interest in frame with bacterial coding sequence and its expression as a fusion protein may be the tool to overcome inclusion body formation[15]. Proteins found in IBs have been characterized as inactive and misfolded[1,5,6,16–19]. Attempts to over-express retroviral proteins resulted either in formation of properly folded soluble proteins[19,20] or inactive protein aggregates within IBs (ref.[21]). No organized structures have been observed within IBs, with the exception of particles formed by the Gag polyprotein precursor of Mason–Pfizer monkey virus that we described previously[22]. In these studies we used an efficient T7-based expression system for the production of retroviral structural proteins in *E. coli*. The unique morphogenetic pathway of this virus, a D-type retrovirus, which assembles immature capsids within the cytoplasm of an infected cell provides an opportunity to assemble particles in the microbial cells[22].

EXPERIMENTAL

Expression vector. An *Nde*I restriction site in the 3′ terminus of M-PMV *gag* gene was created. A 0.7 kilobase-pair *Nde*I-*Sac*I fragment was cloned together with a *Sac*I-*Xho*I fragment containing the remainder of the M-PMV sequence into an *Nde*I-*Xho*I digest of pGEMEX-2 bacterial expression vector (Promega) to yield plasmid pG10MNX (8.4 kbp). pG10MNX was used to create plasmid pG10GAG (6 kbp) in which downstream sequence was deleted yielding M-PMV *gag* under the transcriptional control of the T7 RNA polymerase promoter[22].

Expression and purification of the M-PMV Gag *precursor*. The colonies of *E. coli* BL21 (DE3) transformed with pG10GAG were resuspended in LB (Luria–Bertani) medium in the presence

of 100 μg/ml ampicilin at 37 °C and to $O.D._{590} \approx 0.1$[+]. The cells were induced with 0.4 mM IPTG at an $O.D._{590}$ corresponding to 0.6–0.8. The cells were harvested 4–6 h post induction, lysed in lysis buffer (50 mM Tris-HCl pH 8.0; 1 mM EDTA; 150 mM NaCl; lysozyme 1 mg/ml) for 30 min at room temperature. The cells were disrupted with a French press. The lysate was then incubated for 30 min at 4 °C in the presence of sodium deoxycholate (0.1%), then for another 30 min at 37 °C with DNase I (1 mg/ml) and RNase A (10 μg/ml). The mixture was centrifuged at 12 000 *g* for 15 min. The pellet was washed three times with 0.5% Triton X-100 and 10 mM EDTA.

Electron microscopy (according to Compans *et al.*[23]). Cells were fixed for 1 h at room temperature with 1% glutaraldehyde and then washed in phosphate-buffered saline. After postfixation with 1% osmium tetroxide, cells were embedded in an epoxy-resin mixture, sectioned and stained with uranyl acetate and lead nitrate. Philips 301 electron microscope was used.

RESULTS

The rate of expression of Gag *polyprotein*. The time required for maximal expression of Gag was estimated over a period of 24 h (Fig. 1). A very limited amount of Gag protein was detected even in the uninduced cells. The level of Gag increased within the first hours of expression and it did not significantly change after 2 h following induction with IPTG (see Fig. 1, lane 5).

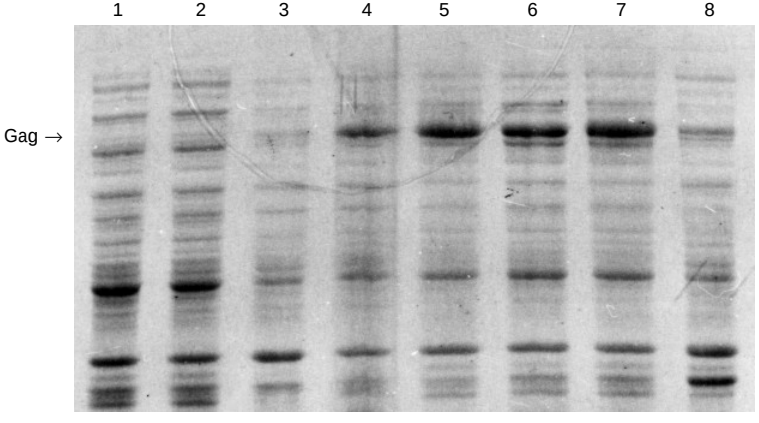

FIG. 1
SDS-PAGE (Coomassie Blue stained 12% gel) analysis of the time dependence of Gag formation in *E. coli* at 37 °C. Lanes represent whole cell lysates. Lane 1, uninduced cells; lanes 2–8, cells following induction (with 0.4 mM IPTG at $O.D._{590} \approx 0.6$) for 10 min, 30 min, 1 h, 2 h, 4 h, 6 h and 24 h, respectively

+ O.D. Optical density (absorbance).

Inclusion bodies were detected in the cells 30 min post-induction using a light microscope. All of the Gag protein could be pelleted as an insoluble fraction after mechanical disruption of cell lysate, indicating that the product aggregates immediately following expression. The same result was observed when minimal medium (containing only essential nutrients) was used instead of LB. The appearance of IBs was observed using the electron microscope. Inclusions were not detectable at 10 min post-induction but visible particles started to form after 30 min (Fig. 2C). The size of IBs increased during the 4–6 h period of incubation at which time they reached their maximal size. The inclusion bodies appeared as distinct areas of the bacterial cytoplasm without ribosomes (Fig. 2). However, the inclusions were not homogeneous as has been described previously in the literature and they were observed to contain partially or fully assembled capsids surrounded by amorphous material. These structures were indistinguishable from capsids assembled from the same retroviral polyprotein in mammalian cells[22].

The effect of temperature on production. The level of production of Gag was evaluated at four different temperatures, 22, 30, 37 and 42 °C. The rate of expression increased with the incubation temperature (Fig. 3, lanes 1 and 2), however, the total amount of Gag synthesized did not vary significantly after reaching a maximum at 4 h post-induction (Fig. 3). No Gag was detected in the soluble fraction of the cell lysate, consistent with its localiza-

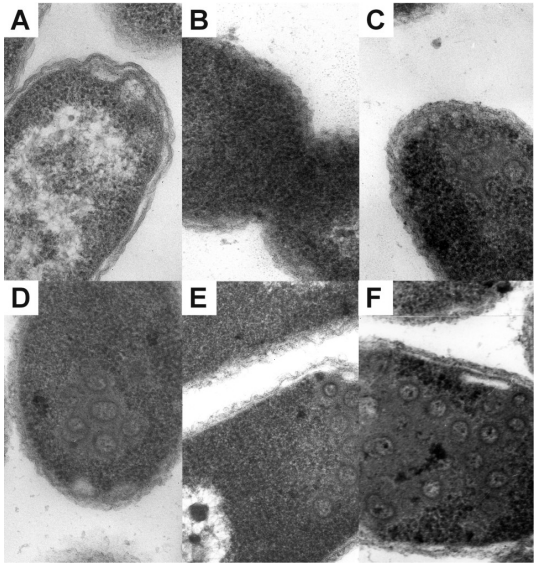

FIG. 2
Electron micrographs of *E. coli* expressing Gag at the same conditions as in Fig. 1. Thin sections of uninduced cells, panel A; cells following induction for 10 min, 30 min, 2 h, 4 h and 6 h, panels B–F, respectively

tion within the inclusion bodies (data not shown). Electron microscopy of thin sections of cells expressing Gag revealed the presence of capsid-like structures inside inclusion bodies at all temperatures tested. The majority of them had the same morphology and size as immature capsids assembled in virus-infected cells; however, a few of them were of irregular shape and variable size and appeared to have a spiral morphology (Fig. 6). No significant difference in capsid yield or structure was observed in bacteria incubated at temperatures in the range from 22 to 42 °C (Fig. 4, left and right panel, respectively).

The effect of pH on production. The pH of cultivation media in the range from 6 to 8 has no significant effect on the production and localization of Gag polyprotein in the inclusions (data not shown).

The composition of inclusion bodies. The inclusions released from the cells by mechanical disruption after lysozyme treatment were purified from contaminating proteins and other cellular material by multiple washings in detergent containing buffer. The majority of impurities were removed in the first step by washing in deoxycholate-containing buffer after the DNase and

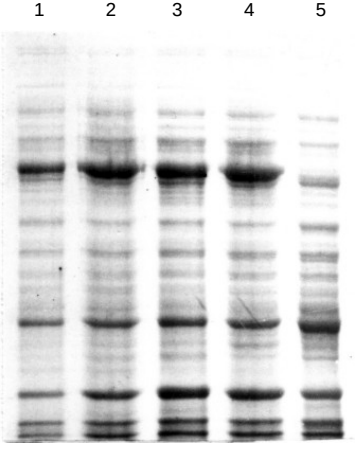

FIG. 3
SDS-PAGE (Coomassie Blue stained 12% gel) analysis of the temperature dependence of Gag formation in *E. coli*. The cells were induced with 0.4 mM IPTG at $O.D._{590} \approx 0.6$. Lanes represent whole cell lysates; 4 h post-induction. Lanes 1–4, total cellular proteins in *E. coli* cells incubated at 22, 30, 37 and 42 °C, respectively. Lane 5, uninduced cells

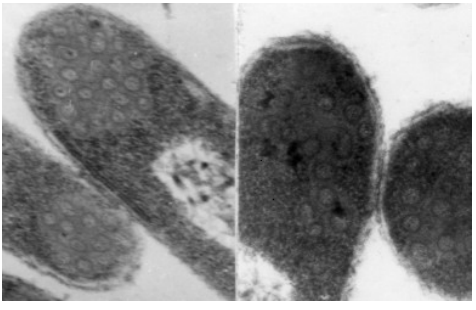

FIG. 4
Electron micrographs of *E. coli* expressing Gag at different temperatures. Left and right panels represent thin sections of the cells following induction for 4 h at 22 and 42 °C, respectively

RNase treatment (Fig. 5, lane 3). Determination of proteins has shown that approximately 40% of proteins of total lysate were removed in this step. Next three washing steps in Triton X-100 containing buffer resulted in elimination of approximately 10% of total contaminating proteins. According to Coomassie blue stained gels no Gag was eluted during this procedure and resulting material contained essentially pure Gag protein (see Fig. 5).

DISCUSSION

A unique feature of M-PMV as a D-type retrovirus is the formation of an immature capsid from polyprotein precursors within the cytoplasm. In contrast to other retroviruses there is no requirement for the N-terminal myristylated domain of the Gag polyprotein of D-type retroviruses to interact with the plasma membrane for capsid assembly. This together with the fact that N-terminal myristylation is not necessary for capsid formation[24] provides the opportunity for capsids to assemble in *E. coli* cells that are surrounded by a rigid cell wall. The fact that the capsids are assembled within inclusion bodies was rather unexpected because the insoluble inclusions have been generally described as insoluble aggregates folding intermediates that are not able to achieve their native conformation. The core-like particles of duck hepatitis B virus and hepatitis B virus were localized also at the

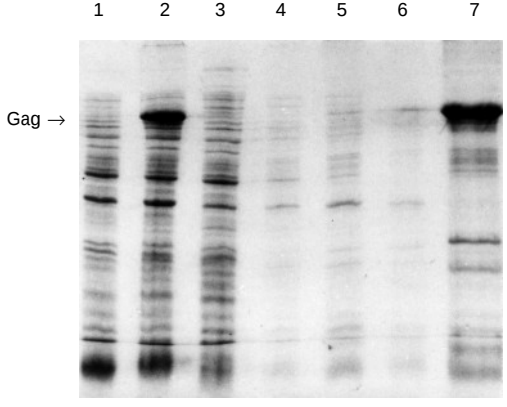

FIG. 5
SDS-PAGE (Coomassie Blue stained 12% gel) analysis of total proteins in *E. coli* expressing Gag 4 h post-induction with 0.4 mM IPTG and inclusions in individual purification steps. Lanes represent whole cell lysates. Lane 1, uninduced cells; lane 2, induced cells 4 h after induction; lane 3, supernatant after washing IBs with deoxycholate containing buffer; lanes 4–6, low-speed supernatants after subsequent washings in Triton X-100 containing buffer; lane 7, low-speed pellet after detergent washing

poles of *E. coli* but not as insoluble inclusions[25,26]. These particles were released from the cells into the soluble fraction by lysozyme-detergent treatment followed by digestion with RNase and DNase.

We addressed the question: are capsids assembled within the cytoplasm and subsequently targeted into the inclusions? This seems to be the more likely pathway than capsid assembly from protein aggregates within the inclusions. We focused on a search of conditions that could prevent targeting of the capsids into the inclusions. Higher pH values have been described to limit formation of IBs. However, changes of pH of cultivation media in the range from 6 to 8 did not result in formation of soluble Gag product or abolishing of capsid formation. The effect of temperature is known to prevent the IB formation and to facilitate formation of native, biologically active proteins. However, we have found that the expression temperatures within the range from 22 to 42 °C affected only the synthesis rate but not the targeting of the expressed protein into the IBs. Also the efficiency of capsid formation was not changed by a temperature shift as was documented by electron microscopy (Fig. 4). However, no isolated capsids outside of the IBs were observed at any temperature. The only exception, when capsids were not surrounded or connected with an apparent compact material without ribosomes, was during the early phase of expression. As was documented by the electron microscopy, some of the capsids were assembled as early as 30 min post-induction (Fig. 2). Most of them developed only as small fragments of capsids located at the cellular poles. These "early" capsids pelleted as insoluble inclusions during the low-speed centrifugation of disrupted cells treated with DNase and RNase; no isolated capsids that might be expected to remain in the supernatant if these condi-

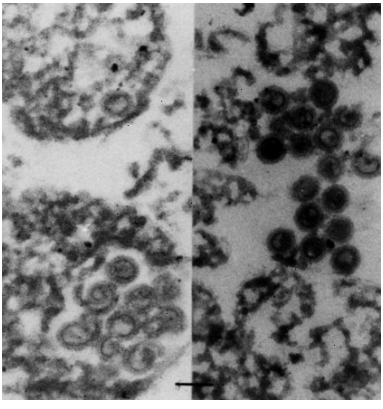

FIG. 6
Electron micrographs of inclusion bodies. Thin sections of washed inclusions. Bar represents 100 nm

tions yielded free, assembled structures, were obtained. This indicates that Gag assembles immediately after reaching the necessary concentration. It can also be hypothesized that the amorphous material connecting the capsids starts to form when the rate of protein synthesis reaches a critical value.

Electron microscopy of the sections of released and washed inclusion bodies showed localization of capsids within the aggregated material (Fig. 6, left panel). Capsids separated from most of the aggregated material were also observed (Fig. 6, right panel). The results from the SDS-PAGE suggested that both the assembled and aggregated materials consisted of Gag as no significant amount of another protein was detected within the inclusions (Fig. 5, lane 7). However, the purification procedure did not release capsids from their clusters that were pelleted by the low-speed centrifugation (Fig. 5, lanes 3–6).

Thus we conclude that in certain instances, inclusion bodies can contain a significant fraction of proteins that retain their native structure and are capable of assembling into high-order multimeric structures.

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