BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **242,** 39–45 (1998)
ARTICLE NO. RC977910

Suppression of Cell Growth by Heavy Chain Ferritin

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Received November 4, 1997

While producing recombinant rat liver H and L chain ferritin homopolymers using the baculovirus expression system, we noticed that rat liver H chain ferritin, but not L chain ferritin, had a suppressive effect on the growth of *Spodoptera frugiperda* (Sf-21) cells. Suppression was observed immediately after infection with recombinant H chain ferritin baculovirus prepared from lysed infected cells. Immediate suppression was observed when purified with either recombinant H chain apoferritin or various holoferritins (loaded with 1,970 ± 50 or 2,520 ± 90 atoms of iron/ferritin) indicating that suppression was not due to sequestration of iron required for cell growth. Suppression by H chain ferritin was also observed upon attempting to express the protein in *Escherichia coli.* Strategies for expression of recombinant rat liver H and L ferritin homopolymers in both prokaryotic and eukaryotic expression systems were developed.

Key Words: **recombinant ferritin; iron; cell suppression.**

Ferritin is a ubiquitous protein composed of 24 subunits of H and L chains which is considered to be the intracellular storage protein for iron (1). The 24 subunits form a protein shell that sequesters and stores up to 2,500 atoms of iron per molecule of ferritin (2). We have cloned the genes of H and L chains of rat liver ferritin into expression vectors to investigate the functions of the two chains. In the process we noticed that H chain had a strong suppressive effect on *Escherichia coli* growth. The yield of recombinant H chain ferritin (rH-Ft) was poor due to the limitation of cell growth when rH-Ft was present. This was not observed when recombinant L chain ferritin (rL-Ft) was expressed.

Our first attempt involved expression of rH-Ft in *E. coli* using expression vector pDR540 (from Pharmacia) which contains a very strong fusion promoter, the *tac* promoter, for expression of inserted genes. The growth of *E. coli* stopped upon transformation with the recombinant pDR540 vector containing rat liver H chain ferritin gene. The *tac* promoter of pDR540 may be so strong such that some rH-Ft protein was expressed, even though the inducer, isopropyl-β-D-thiogalactoside (IPTG), had not been added. Under this situation, the inhibition of cell growth by the rH-Ft resulted in very low yield of protein. This prompted us to consider other expression systems which may effectively postpone the time of expression so that the host can grow before rH-Ft expression is initiated. The baculovirus expression system was also used to investigate the suppression of eukaryotic cell growth during expression of rH-Ft. The results provide important information regarding expression strategy for rH-Ft production in both prokaryotic and eukaryotic expression systems.

MATERIALS AND METHODS

Materials. The baculovirus expression system was purchased from PharMingen (San Diego, CA) and insect cell medium EX-CELL 401 was purchased from JRH Biochemicals (Lenexa, KS). The plasmid pBR322 was obtained from Pharmacia Biotech (Alameda, CA). The genes for T7 promoter and T7 terminator were obtained from plasmid pET-14b which was from Novagen, Inc. (Madison, WI). The *E. coli* hosts, JM109 and JM109(DE3), were purchased from Promega Corporation (Madison, WI). The expression vector pET-11a and the *E. coli* host B834(DE3)pLysS were purchased from Novagen, Inc. (Madison, WI). The *pfu* DNA polymerase and polymerase chain reaction (PCR) reagents were from Stratagene Cloning Systems (La Jolla, CA). Other enzymes used for DNA manipulations were purchased from Stratagene, Boehringer Mannheim Biochemicals (Indianapolis, IN) or United States Biochemicals (Cleveland, OH) and used according to manufacturers' instructions. Other chemicals were of reagent grade and obtained from Sigma (St. Louis, MO) or Aldrich (Milwaukee, WI).

Baculovirus expression system for rH-Ft and rL-Ft. Construction of recombinant baculovirus and expression of rH-Ft and rL-Ft homopolymers have been previously described (3). Briefly, rat liver ferritin H and L chain cDNA were inserted individually at *Bgl*II site on the pAcUW21, a baculovirus transfer vector containing a p10 promoter upstream of the cloning site. The inserts were sequenced by the dideoxy chain termination method using Sequenase (United States Biochemicals, Cleveland, OH). Sf-21 cells maintained in EX-CELL

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0006-291X/98 $25.00

401 medium were transfected with recombinant pAcUW21 containing rat liver H or L chain gene or wild type baculovirus using Lipofectin (Gibco BRL, Gaithersburg, MD). Recombinant virus (Occ^+) were purified by three rounds of plaque screening. Confirmation of rat liver ferritin H or L chain gene inserts was corroborated by PCR analysis. Cultured Sf-21 insect cells were then infected with isolated rH-Ft recombinant baculovirus (H-virus) or rL-Ft recombinant baculovirus (L-virus). Cell medium supernatants harvested 2 d post-infection (p.i.), at which time the recombinant ferritins had not been expressed (3), were utilized as the source of recombinant virus. Recombinant baculovirus stocks of H-virus and L-virus were also prepared 7 d p.i., at which time all the infected cells were lysed and the expressed recombinant proteins were released to the medium. The concentration of virus stocks from the non-lysed cells (2 d p.i.) or the lysed cells (7 d p.i.) were adjusted to 2.3×10^8 virus/ml by dilution with insect cell medium EX-CELL 401.

Suppression of insect Sf-21 cells during expression of ferritin. Insect Sf-21 cells, about $(1.1 \pm 0.2) \times 10^6$ cells, were cultured in 6 ml EX-CELL 401 media at 27°C. The high titer virus stocks (approximately 0.1 ml) prepared from the non-lysed 2 d p.i. infected cells or the lysed 7 d p.i. infected cells were added into cell cultures at a multiplicity of infection of 20 per cell. The cells were counted every 24 hours using a Hausser *Hy-Lite* hemocytometer (from Fisher Scientific, Pittsburgh, PA).

Effect of ferritin on the baculovirus expression system. The methods for purification of rH-Ft, rL-Ft, and ceruloplasmin were as described earlier (3). Neither rH-Ft or rL-Ft, examined by total iron assay (4), contained iron. Holoferritin was prepared by loading iron into apoferritin using ceruloplasmin (3). Some rH-Ft was loaded with a similar amount of iron as found in native rat liver ferritin and other was maximally loaded with iron. These two holoferritins con-

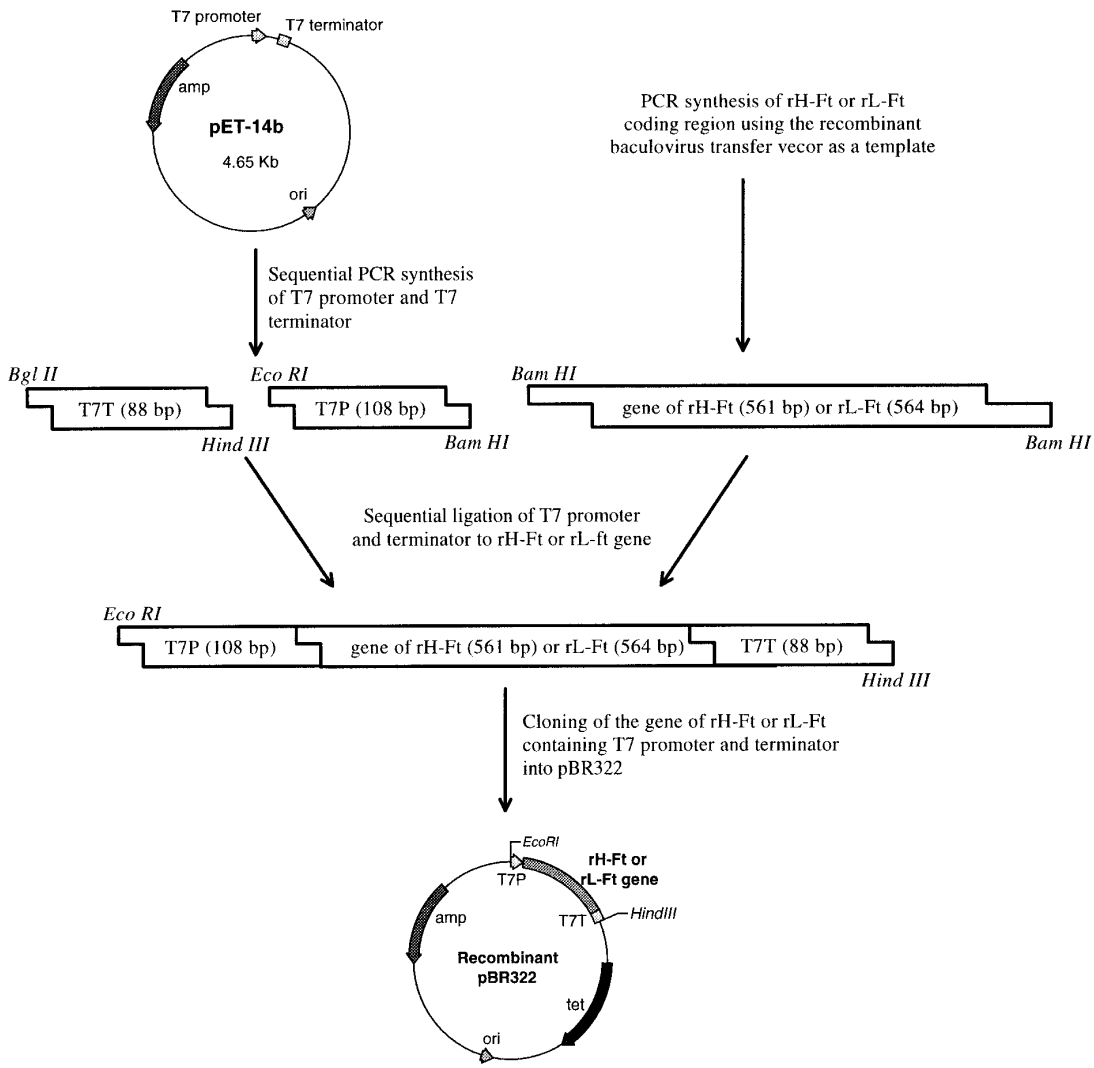

FIG. 1. Schematic illustration of the *E. coli* expression vectors constructed for rat liver rH-Ft and rL-Ft expression. The T7 promoter (T7P) and T7 terminator (T7T) were amplified by PCR, using pET-14b as a template, to generate a *BglII-HindIII* fragment and a *EcoRI-BamHI* fragment, respectively. Both rat liver H and L chain genes were also amplified by PCR, using the previous recombinant baculovirus transfer vectors (3), to generate a *BamHI-HindIII* fragment. The T7 promoter and the T7 terminator were sequentially ligated onto the 5′- and 3′-end, respectively, of both ferritin genes. The gene fragments of rat liver rH-Ft and rL-Ft containing T7 promoter and terminator were inserted into *EcoRI* + *HindIII* cut pBR322 to produce recombinant rH-Ft and rL-Ft plasmids, respectively.

tained 1970 ± 50 and 2520 ± 90 atoms of iron per molecule, respectively. To investigate the suppressive effect on the Sf-21 cell growth, 0.1 mg of rH-Ft, rL-Ft, or holoferritin of rH-Ft, which contained the different amounts of iron, were added individually into the 6-ml insect cell cultures. The number of cells were quantitated every 24 hours as described above.

Suppression of E. coli during expression of ferritin. The plasmids constructed for this study are diagrammed in Fig. 1. Rat liver H (561 bp) and L (564 bp) chain ferritin genes were amplified from the recombinant baculovirus transfer vectors, which were described earlier (3), by PCR using *pfu* DNA polymerase (5). The T7 promoter (108 bp) and T7 terminator (88 bp) genes were also amplified from plasmid pET-14b using the same PCR polymerase. The T7 promoter was ligated upstream and the T7 terminator downstream of ferritin H chain and L chain genes. The DNA products were then cloned into plasmid pBR322. The sequences of the cloned DNAs were confirmed by DNA restriction mapping and sequencing.

The expression of rat liver H or L chain ferritin in this system is driven by T7 RNA polymerase. The polymerase is highly selective for its own promoters, the gene for which does not exist naturally in *E. coli* (6) or in pBR322 (7). The host, *E. coli* strain JM109 (DE3), derived from JM109, contains a chromosomal copy of the gene for T7 RNA polymerase (6). Expression of T7 RNA polymerase by the host can therefore regulate expression of inserted ferritin genes on the derived pBR322. The gene for T7 RNA polymerase in the host chromosome has been placed under the control of *lac UV5* promoter which can be induced by IPTG. In the absence of IPTG, little or no T7 RNA polymerase should be produced by JM109 (DE3) therefore preventing expression of ferritin genes.

Seed cultures of JM109 or JM109(DE3), containing either H or L chain ferritin expression vector, were grown in 100 ml Luria-Bertani (LB) medium containing 100 μg/ml ampicillin overnight (16 h) at 37°C and 200 rpm in a orbital incubator shaker. The seed cultures were transferred to 100 ml fresh LB medium containing 100 μg/ml ampicillin to a final cell density of approximate 0.035 $OD_{600\,nm}$ and incubated at 37°C and 200 rpm in a orbital incubator shaker. Cell densities were quantitated spectrophotometrically at 600 nm every 30 min. IPTG was added to the cell cultures to a final concentration of 5 mM at an early stage (2 h after the seed culture transfer) or at a late stage (4 h after the seed culture transfer) of exponential growth.

Construction and expression of ferritin H chain homopolymer using E. coli B834(DE3)pLysS. The rat liver ferritin H chain gene described above was cloned into a pET-11a vector which contained a *Lac* operator between the T7 promoter and the cloned gene for additional regulation of gene expression. *E. coli* B834(DE3)pLysS, which contains the T7 RNA polymerase gene in its genome under the control of *lacUV*5 and a plasmid containing the gene for T7 lysozyme, was transformed with the recombinant pET-11a vector for expression of rat liver ferritin H chain homopolymer. The T7 lysozyme is a natural inhibitor of T7 RNA polymerase (8) which should suppress any basally expressed T7 RNA polymerase. Expression of ferritin H chain homopolymer using the *E. coli* B834(DE3)pLysS cells was conducted similarly as for *E. coli* JM109(DE3), as described above.

RESULTS

Suppression of insect Sf-21 cells during expression of ferritin. The average doubling time for the growth of Sf-21 cells was approximately 18 h (Fig. 2). Upon addition of medium from lysed H-virus infected cells (7 d p.i.), the growth of Sf-21 was suppressed from the first day of infection (Fig. 2). The suppression of cell growth was not observed with media from lysed cells (7 d.p.i.) infected with either wild-type baculovirus or L-virus (the doubling time of cell growth was about 19 ± 1 h). When media prepared from non-lysed cells (2 d.p.i.) infected with wild-type baculovirus, H-virus, or L-virus were used for infection, the growth of Sf-21 cells during the first two days post infection was similar to non-infected cells. The expression and accumulation of polyhedrin in the cell nuclei was observed on the third day after infection indicating expression of the inserted ferritin gene. At that time the growth of cells infected with H-virus ceased (Fig. 2). Cells infected with either wild-type baculovirus or L-virus prepared from the non-lysed infected cells (2 d p.i.) did not exhibit the suppressive effect (Fig. 2). All infected cells started to lyse on the fourth day following infection.

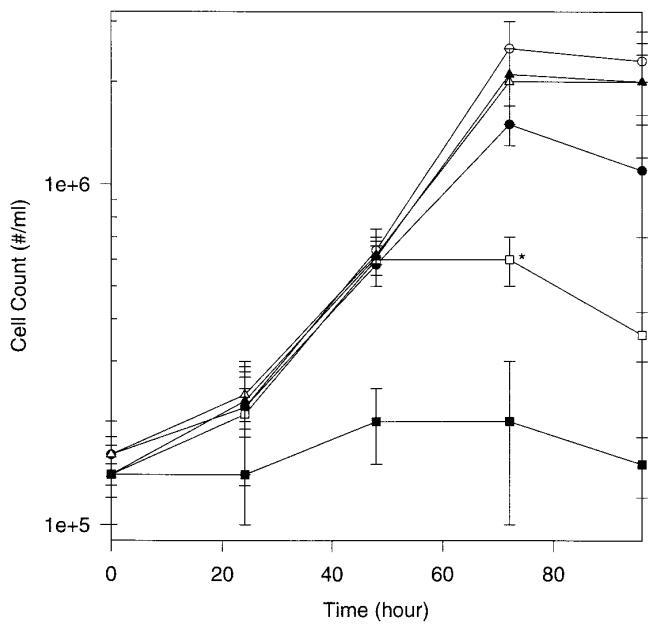

FIG. 2. Suppression of Sf-21 cell growth during the expression of rH-Ft. Sf-21 cells (1.1×10^6) were infected with various recombinant baculoviruses prepared from non-lysed infected cells (2 d p.i.) or lysed infected cells (7 d p.i.) and incubated at 27°C. The viable cells were counted using a hemocytometer and the trypan blue exclusion method. Each point and bar represents the mean and SD of three experiments done in triplicate. The growth curve of Sf-21 cells infected with 7 d p.i. H-virus (■) was significantly different from that of Sf-21 cells alone (○) ($p < 0.01$). No statistically significant difference ($p < 0.05$) was shown among the growth curves of Sf-21 cells alone (○), cells infected with wild-type baculovirus (●), and cells infected with 2 d p.i. L-virus (△) or 7 d p.i. L-virus (▲). The asterisk shows the time of which a statistically significant difference ($p < 0.01$) occurred for cells infected with 2 d p.i. H-virus (□). The data for 96-hour (4 d p.i.) were not included for statistical analyses because the cells were lysing at this time.

Direct addition of rL-Ft into the Sf-21 cultures caused no suppressive effect on the cell growth (the doubling time of cell growth was about 18 ± 1 h) (Fig. 3). However, the addition of rH-Ft showed strong sup-

pression of cell growth (the doubling time of cell growth was increased to 64 h) (Fig. 3). The suppressive effect on cell growth was also observed, although to a lesser extent, when holoferritins of rH-Ft containing 1970 atoms of iron or 2520 atoms of iron were added (the doubling times were 39 h and 38 h, respectively) (Fig. 3).

Construction of an E. coli expression system for expression of rH-Ft and rL-Ft. Construction of expression vectors containing the genes of rH-Ft and rL-Ft was accomplished as described under Materials and Methods. The cloning orientation and DNA sequences for both were correct (data not shown). Preliminary analyses confirmed that the recombinant ferritin homopolymers were only expressed by the host JM109 (DE3) which was induced with 5 mM IPTG. Expression of recombinant ferritin homopolymers was not observed if IPTG was not added. Additionally, no ferritin was expressed when the expression vector was carried by a host, such as JM109, lacking the T7 polymerase gene (data not shown).

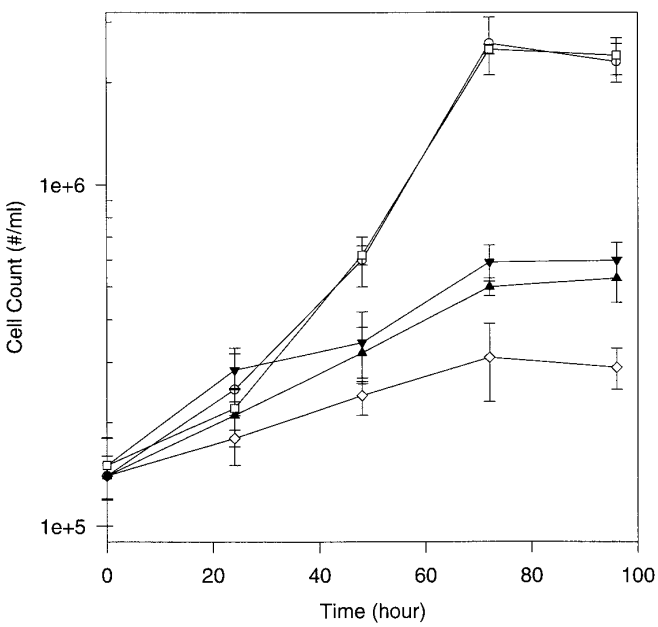

FIG. 3. The effect of various ferritins on Sf-21 cell growth. Sf-21 cells (1.1×10^6 in 6 ml) were incubated with 1 mg of various ferritins at 27°C and viable cells counted daily using a hemocytometer and the trypan blue exclusion method. Each point and bar represents the mean and SD of three experiments. The growth curves for Sf-21 cells grown with rH-Ft (♦), or with rH-Ft containing 1970 (▲) or 2520 (▼) atoms of iron were significantly different from that of Sf-21 cells alone (♦) ($p < 0.01$). The curves for cells grown with rH-Ft containing 1970 (△) or 2520 (▼) atoms of iron were also significantly different from that of rH-Ft (♦) ($p < 0.01$). No statistically significant difference was found between the growth curves of Sf-21 cells alone (○) and cells grown with rL-Ft (□) ($p < 0.01$) or between the growth curves of cells grown with rH-Ft containing 1970 (▲) and 2520 (▼) atoms of iron ($p < 0.05$).

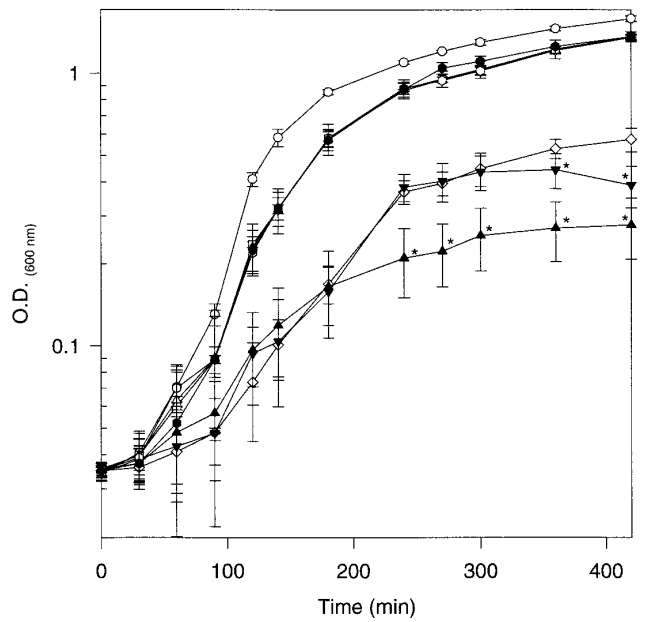

FIG. 4. Suppression of *E. coli* growth during the expression of rH-Ft. Recombinant *E. coli* JM109(DE3) (OD_{600} = 0.035) containing rH-Ft or rL-Ft plasmids were grown in 100 ml of LB medium shaken at 37°C. The inducer IPTG (5 mM) was added 2 h or 4 h after incubation. Cell density was monitored spectrophotometrically at 600 nm every 30 min. Each point and bar represents the mean and SD of three experiments done in triplicate. The growth curves for JM109, JM109(DE3), and JM109(DE3) with the rL-Ft plasmid were essentially identical. Only the curve for the latter (△) is shown. The growth curve for JM109(DE3) with the rH-Ft plasmid (♦) was statistically ($p < 0.01$) lower than the host without the plasmid. The asterisks show data points which were statistically significant lower ($p < 0.01$) when IPTG was added at 2 h (▲) or 4 h (▼).

Suppression of E. coli during expression of ferritin. The doubling time for JM109(DE3) was 35 min, only slightly longer than for JM109 (31 min). When JM109 (DE3) was transformed with the expression vector containing the rL-Ft gene and induced with IPTG, the growth rate remained the same as wild type JM109 (DE3). However, the doubling time of JM109(DE3) containing the rH-Ft expression vector approximately doubled (71 min) (Fig. 4). When 5 mM IPTG was added to this culture during the early stage of exponential growth (OD_{600} was 0.10 ± 0.04), expression of rH-Ft was observed within 30 min (data not shown) and cell growth was limited. When 5 mM IPTG was added during the late stage of exponential growth (OD_{600} was 0.40 ± 0.07), rH-Ft was expressed after 30 min (Fig. 5). Similar to when IPTG was added earlier, cell growth rate declined after one hour (Fig. 4). The suppressive effect on cell growth was not observed with the host containing the rL-Ft expression vector (Fig. 4). Cultures transformed with the expression vector containing the rH-Ft gene formed massive clumps of cells soon after the addition of IPTG. Therefore, the OD_{600}

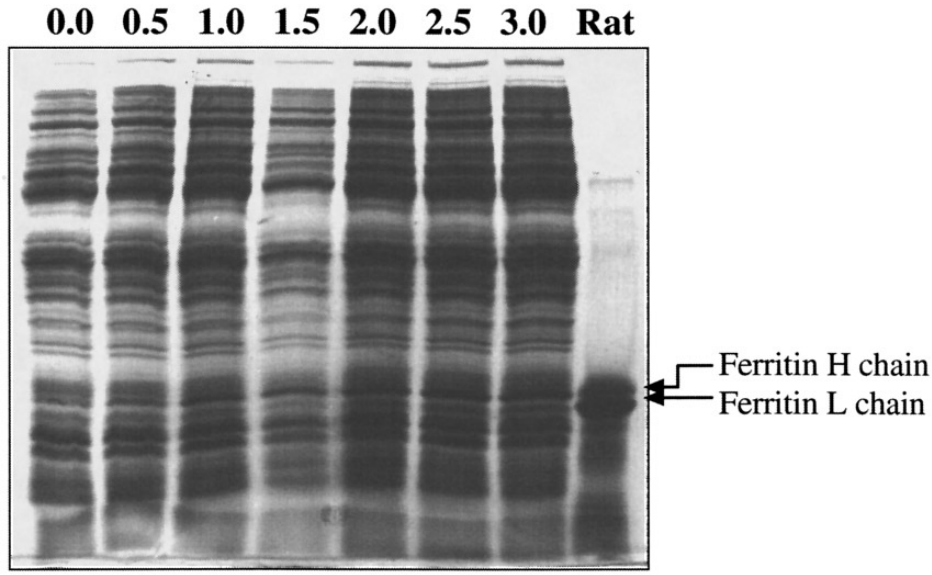

FIG. 5. Time course of expression of rat liver rH-Ft in recombinant *E. coli* JM109(DE3) upon induction with IPTG. IPTG was added 4 h after seed culture transfer. Samples (1 ml) were taken every 30 min after adding the IPTG and centrifuged at 3,000 *g* for 10 min. The resulting pellet was resuspended in 0.5 ml 50 mM Tris (pH 7.0) and 10 μl was subjected to denaturing polyacrylamide (12%) electrophoresis gel. After electrophoresis the gels were stained with Coomassie blue. Lanes 0, 0.5, 1, 1.5, 2, 2.5, and 3 represent the hours after adding the IPTG; lane Rat represents partially purified native rat liver ferritin.

readings after addition of IPTG was not a reliable determination of *E. coli* growth. The *E. coli* precipitated out of the medium completely if the culture were left unshaken for an hour.

Expression of ferritin H chain homopolymer using E. coli. *E. coli* B834(DE3)pLysS containing the LH-Ft expression vector was grown in LB medium and 5 mM IPTG was added after 4 h of incubation. No suppression of cell growth was observed before IPTG was added (data not shown). The induction of ferritin H chain expression was observed 30 min after IPTG induction and expression was greater (Fig. 6) than in *E. coli* JM109(DE3) using the recombinant M13 derived T7 promoter driven expression vector described above (Fig. 5).

DISCUSSION

The suppressive effect of ferritin H chain on the proliferation and functions of high iron-requiring cells, such as lymphocytes, granulocytes, and myeloid progenitor cells, has been observed previously (9–11). These effects were proposed to be associated with the sequestration of iron required by the cells (12). However, we found that rat liver rH-Ft with or without iron exhibited a suppressive effect on cell growth. This phenomenon indicated that the suppression could not be only by sequestration of iron required for growth. Furthermore, the rat liver rH-Ft expressed in the recombinant baculovirus system did not contain any detectable iron. Interestingly, we also found that the expression of human liver rH-Ft was less suppressive than the expression of rat liver rH-Ft in *E. coli* (unpublished data).

It has been reported that the ferritin H chain may associate with mRNA and inhibit *in vitro* translation of certain mRNA (13, 14). Mulvey *et al.* (15) demonstrated that Mengo virus infection induced apoferritin which effectively inhibited the translation of mRNA in reticulocyte lysates. They proposed that this inhibition may turn off the synthesis of certain proteins in the host, which might cause the suppression of cell growth, and liberate translation components for the synthesis of viral proteins. We also observed an inhibitory effect of rat liver rH-Ft on globin mRNA translation *in vitro* (unpublished data). These observations suggested that rH-Ft might serve other novel functions for the regulation of cell growth.

The baculovirus system expresses viral genes required for the initiation of viral DNA synthesis at an early stage, genes required for the assembly of virus particles at a late stage, and genes inserted in the recombinant viral genome at a very late stage of the viral cycle (16). This provided a strategy for production of rH-Ft, even though the protein was highly suppressive to cell growth. Baculovirus multiplies and is released as a budding virus into the medium in the first couple days post infection. Little or no rH-Ft was expressed during this time. Therefore, H-virus produced during the first 48 h of infection can be used for infection with

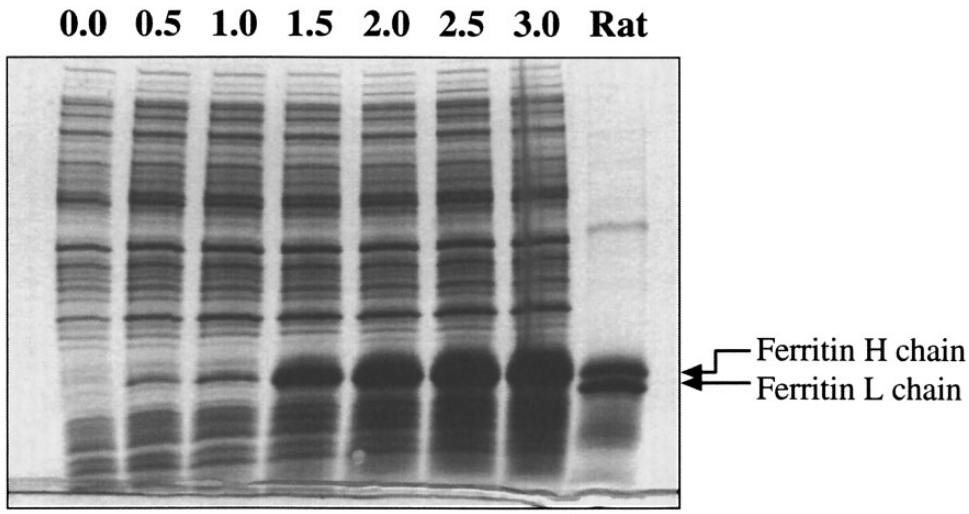

FIG. 6. Time course of expression of rat liver rH-Ft in recombinant *E. coli* B834(DE3)pLysS upon induction with IPTG. The expression induction and sample preparations were the same as Fig. 5. Ten microliters of the resuspended cells was subjected to denaturing polyacrylamide (12%) electrophoresis gel. After electrophoresis the gels were stained with Coomassie blue. Lanes 0, 0.5, 1, 1.5, 2, 2.5, and 3 represent the hours after adding the IPTG; lane Rat represents partially purified native rat liver ferritin.

little or no suppression of cell growth. The suppression of insect cell growth occurred approximately 48 h p.i., at which time the rat liver rH-Ft was expressed (3). The expressed rat liver rH-Ft was present in the 7 d p.i. media due to the lysis of cells. Therefore upon infection of Sf-21 cells with this H-virus media, the suppression of cell growth occurred immediately and the yield of rat liver rH-Ft was very low (less than 0.1 mg per 100 ml of cell culture). The initial cell density of Sf-21 for infection should be about 5×10^5 cells/ml. This condition allows the growth of insect cells to reach maximal density of 2×10^6 cells/ml before the rat liver rH-Ft is expressed. Therefore, the expression system may have enough cells for expression and achieve a higher yield of recombinant protein production.

The strategy for designing the *E. coli* expression system was to construct a vector that was under stringent control for expression of the inserted gene. Regulation of the expression of the RNA polymerase for the promoter of the inserted gene was considered better than to directly control the expression of the inserted gene because the former method could lengthen the processing time for expression of the inserted gene if regulation was not stringent. Prior to addition of IPTG, the growth of JM109(DE3) containing the expression vector of rH-Ft was relatively slow when compared to wild type JM109 (DE3) or JM109 (DE3) containing the expression vector of rL-Ft. This observation suggested that the regulation of T7 mRNA polymerase expression was leaking. However, the amount of rH-Ft expressed was not high enough to be observed upon SDS-PAGE, and was not enough to completely suppress growth of the host JM109(DE3). We were able to induce the expression of rH-Ft by adding the IPTG when the growth of the host JM109(DE3) reached a late stage of exponential growth. In order to obtain a higher yield of recombinant protein, we suggest that the inducer, IPTG, should be added when the OD_{600} of the cell culture reaches 0.4.

When *E. coli* B834(DE3)pLysS containing a recombinant pET-11a expression vector was used for expression of ferritin H chain homopolymer, no suppression was observed prior to the addition of IPTG and the system produced greater quantity of recombinant protein than the JM109(DE3) system. Since *E. coli* B834(DE3)pLysS included two additional constructs, a *Lac* operator downstream of the T7 promoter and the gene for T7 lysozyme, for stringent regulation of expression of the inserted gene, no suppression of cell growth was observed and the production of ferritin H chain occurred after addition of IPTG. These results demonstrate that suppression by the ferritin H chain may be avoided in a highly stringent expression system and provide for significant H chain ferritin expression in a such expression system.

ACKNOWLEDGMENTS

We thank Majid Abedi for construction of the T7 expression system using the M13 vector, Thomas B. Cardon and G. Ryan Berger for excellent technical assistance, and T. Maughan for secretarial assistance in preparation of the manuscript. This work was supported in part by NIH Grant ES05056.

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