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Use of juvenile hormone esterase as a novel reporter enzyme in the baculovirus expression system

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Abstract

Juvenile hormone esterase (JHE) has a number of characteristics favorable for use as a reporter enzyme. It is exiremely stable under a variety of adverse conditions including in organic solvent. JHE is easily detected by a rapid and sensitive colorimetric assay, and detection is facilitated by export of the enzyme from the cell. Its use is illustrated in the baculovirus expression system by promoter studies and evaluation of culture conditions necessary for optimal production of recombinant proteins. From this, it was found that protein yields were greater for *Autographa californica* nuclear polyhedrosis virus (AcNPV) expression vectors using the basic protein promoter to drive production of JHE than for the p10 protein or polyhedrin promoters. This has significant implications for current baculovirus expression methodologies. The effect of multiplicity of infection on protein yield was found to be insignificant between 0.1 and 10 for expression under the basic protein promoter. Yields of JHE were about 40% higher from the cell line Tn5B1–4 ('High Five') relative to the Sf21 cell line under optimized conditions for each cell line, with maximum yields obtained at 2–3 days, and 3–5 days post-infection for the two cell lines respectively when cultured in ExCell 401 medium. The presence of fetal calf serum in the cell culture medium enhanced protein yields from both cell lines. These studies demonstrate the use of JHE as a reporter enzyme for optimizing high yields of protein from the baculovirus expression system. JHE also has a potential application as a reporter enzyme in other eukaryotic systems. The advantages and use of JHE over other reporter enzymes are discussed.

Keywords: Juvenile hormone esterase; Reporter enzyme; Baculovirus expression

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1. Introduction

Juvenile hormone esterase (JHE) is involved in regulating the titer of juvenile hormone (JH) in Lepidoptera, which is critical for the successful progression of larval development (Hammock, 1985). JHE is a soluble 66 kDa enzyme characterized by a high substrate affinity and moderate k_{cat} (Wing et al., 1984), and has the unique feature of being active in some organic solvents (Croston et al., 1987). The cDNA for JHE derived from the moth *Heliothis virescens* was isolated (Hanzlik et al., 1989) and subsequently inserted into various baculovirus constructs for improving the insecticidal efficacy of the virus (Bonning and Hammock, 1994). In addition to application as insecticidal agents, baculoviruses have been used widely for high yield production of both prokaryotic and eukaryotic proteins (O'Reilly et al., 1992). The baculovirus expression system has a number of advantages over other expression technologies, including correct post-translational processing of recombinant proteins. Numerous new baculovirus transfer vectors have recently been constructed facilitating baculovirus expression under control of different viral promoters, and for expression of multiple proteins by the same virus (O'Reilly et al., 1992; Bishop, 1992). Despite the increasing use of baculoviruses for high level production of foreign proteins, few analyses have been carried out using a quantifiable reporter enzyme to address the myriad of variables which influence protein yield. Evaluation of the effect of such variables as the promoter, cell line, culture conditions etc. on recombinant protein production is aided by the use of a reporter protein such as JHE which is stable at room temperature (Ward et al., 1992) and easily assayed. In addition to a commercially available, high specific activity radiolabeled substrate, JHE activity can now be assayed quickly, easily and precisely using a newly developed chromogenic substrate. Alternatively JHE can be assayed by a classical enzyme-linked immunosorbent assay (ELISA) or an affinity amplified ELISA (Székács et al., 1992). In this report we illustrate for the first time, the use of JHE in a colorimetric assay to evaluate the production of recombinant protein in a baculovirus expression system. We assess (a) the yields of enzyme produced from the basic, p10 and polyhedrin protein promoters of *Autographa californica* nuclear polyhedrosis virus (AcNPV), (b) the effect of multiplicity of infection (MOI) on protein yield and (c) the relative merits of the two cell lines IPBLSf-21AE (Sf21) (Vaughn et al., 1977) and 'High Five' BTI-Tn5B1–4 (Tn) in the presence and absence of fetal calf serum (FCS). These experiments were initiated to optimize production of a modified JHE with enhanced insecticidal activity (Bonning and Hammock, 1994).

2. Materials and methods

2.1. Construction and propagation of viruses

Cell culture techniques used were as described previously (King and Possee, 1992). The cDNA sequence of JHE derived from *H. virescens* (Hanzlik et al., 1989) was excised from 3hv16B (Hammock et al., 1990b) by restriction endonuclease digestion, and ligated into the *Bgl*II cloning site of the transfer vector pAcMP1 (Hill-Perkins and

Possee, 1990) downstream from the basic protein promoter. Correct insertion of the cDNA was ascertained by restriction endonuclease analysis. The recombinant virus AcMP1.JHE was made by cotransfection of Sf21 cells with the plasmid DNA (pAcMP1.JHE) and virus DNA, AcUW1-PH (Weyer et al., 1990), to produce a polyhedrin protein-positive, p10 protein-negative virus, AcMP1.JHE. The *lacZ* gene in AcUW1-PH was replaced by the 1.73 kb insert encoding JHE by homologous recombination on cotransfection of the insect cells. Subsequently, AcMP1.JHE was purified by blue/white selection for recombinants lacking the *lacZ* gene using *o*-nitrophenyl β-D-galactopyranoside (ONPG) as substrate for β-galactosidase.

The 1.73 kb insert encoding JHE was also ligated into the plasmids pAcUW2(B) under control of the p10 protein promoter (Bonning et al., 1992) and pAcRP23 under control of the polyhedrin promoter (Matsuura et al., 1987; Possee and Howard, 1987), as described previously (Hammock et al., 1990a). The recombinant virus AcUW2(B).JHE was made by cotransfecting Sf21 cells with DNA of pAcUW2(B).JHE and DNA from the virus AcRP8 to produce a p10 protein-positive, polyhedrin protein-positive virus (Bonning and Hammock, 1994; Bonning et al., 1992). AcUW2(B).JHE and AcRP23.JHE were purified by selection of polyhedrin-positive or -negative virus respectively, and by monitoring JHE activity produced by recombinant virus in viral plaques (Bonning et al., 1992; Hammock et al., 1990a). All of the vectors in this study are commonly used, and have been optimized for maximum expression (Matsuura et al., 1987).

2.2. Analysis of expression of JHE in vitro

For comparison of expression from different viral promoters, cells of the cell line Sf21 (derived from *Spodoptera frugiperda*; Vaughn et al., 1977) were set up at 10^6 cells/35 mm Petri dish. After 2 h at 28°C, the cells were infected with 10 plaque forming units (pfu)/cell of AcMP1.JHE, AcUW2(B)JHE or AcRP23.JHE. After 1 h at room temperature, the inoculum was removed and 2 ml medium (ExCell 401 [JRH Biosciences, Woodland, CA] with 1% Penicillin Streptomycin (PS) and 3% FCS [Intergen]) was added. The dishes were maintained at 28°C for 4 days. A single aliquot was taken from each dish for enzyme analysis at various time points. Samples were stored at −20°C prior to assay of JHE activity.

To investigate the effect of MOI on protein yield, Sf21 cells were set up in 35 mm Petri dishes as described above and infected at 0.1, 1 or 10 pfu/cell with AcMP1.JHE or AcUW2(B).JHE. Aliquots were taken at various times post-infection over a period of 3 days, and stored at −20°C prior to assay of JHE activity.

For analysis of protein production in larger scale cultures, cells were maintained in ExCell 401 medium with 1% PS with or without 3% FCS. Cells were adapted to serum-free medium using standard procedures (O'Reilly et al., 1992). Sf21 cells were cultured in 50 ml spinner flasks stirred at 80 rpm, whilst Tn cells (BTI-Tn5B1–4, Invitrogen^R) were cultured in 100 ml volumes in 250 ml Erlenmeyer flasks shaken at 100 rpm. These conditions were found to be optimal for protein production in these two cell lines. Cells were infected with AcMP1.JHE at 5 pfu/cell, at 5×10^5 cells per ml for 1 h in a minimum amount of medium. Aliquots of medium were taken at various

times post-infection of the cells and stored at −20°C prior to analysis for expression of JHE. All time course experiments were repeated three times.

2.3. Assay of JHE activity

A colorimetric assay has recently been developed for rapid monitoring of JHE activity (McCutchen et al., 1993). This assay was modified from Ellman et al. (1961) such that 278 μl of 0.015% 5'5-dithiobis-2-nitrobenzoic acid (DTNB) in 0.05 M sodium phosphate buffer pH 7.4 with 10% sucrose and 20 μl enzyme (diluted appropriately) was added per microtiter plate well. Each sample was assayed in triplicate. Reactions were started by addition of 2 μl of the colorimetric substrate HEPTAT (methyl 1-heptylthioacetothioate $[CH_3(CH_2)_6SCH_2C(O)SCH_3]$ in ethanol) to give a final concentration of 0.2 mM. Esterase activity was measured continuously for 2 minutes by monitoring the increase in yellow coloration (5-thio-2 nitrobenzoic acid) at 405 nm and converted to mOD/min. Hydrolytic rates were linear over this time period at 23°C.

3. Results

3.1. Expression of JHE under control of different viral promoters

The cDNA sequence of JHE derived from *Heliothis virescens* was inserted into three AcNPV constructs under control of the p10, polyhedrin or basic protein promoters. JHE was employed as a reporter enzyme to test the expression characteristics of these promoters. During infection of Sf21 cells, JHE activity was detected 13 h post-infection (hpi) for AcMP1.JHE (basic protein promoter), 17 hpi for AcUW2(B).JHE (p10

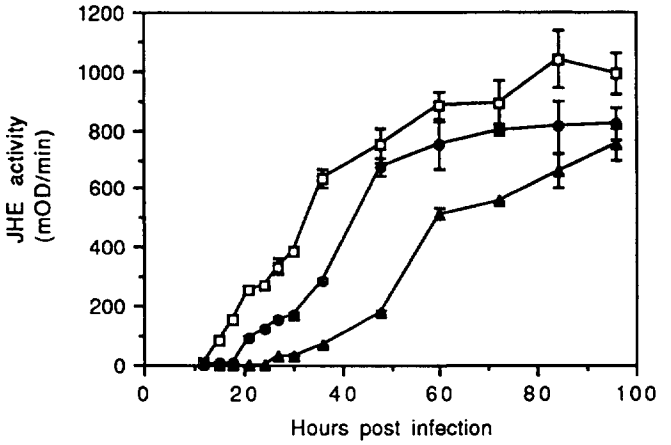

Fig. 1. Time course of JHE production: Expression of JHE in Sf21 cell culture under control of the basic, p10 or polyhedrin promoters by AcMP1.JHE, AcUW2(B).JHE and AcRP23.JHE respectively. 1000 mOD/min corresponds to a rate of 2.09 μmol substrate hydrolyzed/min/ml, or 16 mg catalytically active JHE/liter. Symbols: □, AcMP1.JHE; ●, AcUW2(B).JHE; ▲, AcRP23.JHE.

promoter) and 27 hpi for AcRP23.JHE (polyhedrin promoter; Fig. 1). Expression of JHE was optimal under control of the basic protein promoter (Fig. 1). There were substantial differences in protein yield from these viruses up to 48 hpi, and the trend of relative protein yield for the three viruses remained the same throughout infection. About 20% more protein was produced from the p10 promoter than from the polyhedrin promoter at 84 hpi. The maximum activity of 1000 mOD/min for expression under the basic protein promoter is equivalent to 16 mg JHE/liter. The polyhedrin promoter, which is commonly used for baculovirus expression, produced 30% less JHE than the basic protein promoter, and the least JHE of the 3 promoters tested.

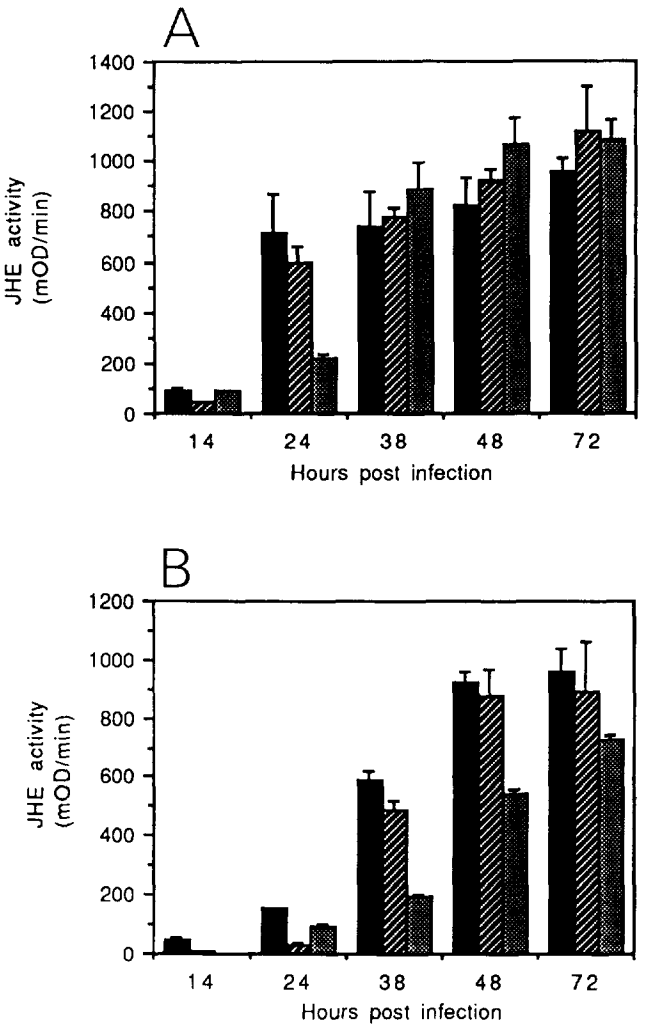

Fig. 2. Effect of different MOI on protein yield. Cells were infected at 10 (black bars), 1.0 (striated bars) or 0.1 (grey bars) pfu/cell with (A) AcMP1.JHE (basic protein promoter) or (B) AcUW2(B).JHE (p10 promoter). Standard deviation bars are shown.

3.2. Effect of multiplicity of infection on protein yield

To assess the effect of different MOI on protein yield, cells of Sf21 were infected at 10^6cells per 35 mm Petri dish with 0.1, 1.0 or 10 pfu/cell with AcMP1.JHE or AcUW2(B).JHE. Samples were assayed for JHE activity. The data show that there is no significant difference among these MOIs for AcMP1.JHE after 24 hpi (Fig. 2A). However, 20% to 50% less JHE is produced on infection with AcUW2(B).JHE at an MOI of 0.1 relative to infection at MOIs of 1 or 10 after 24 hpi (Fig. 2B).

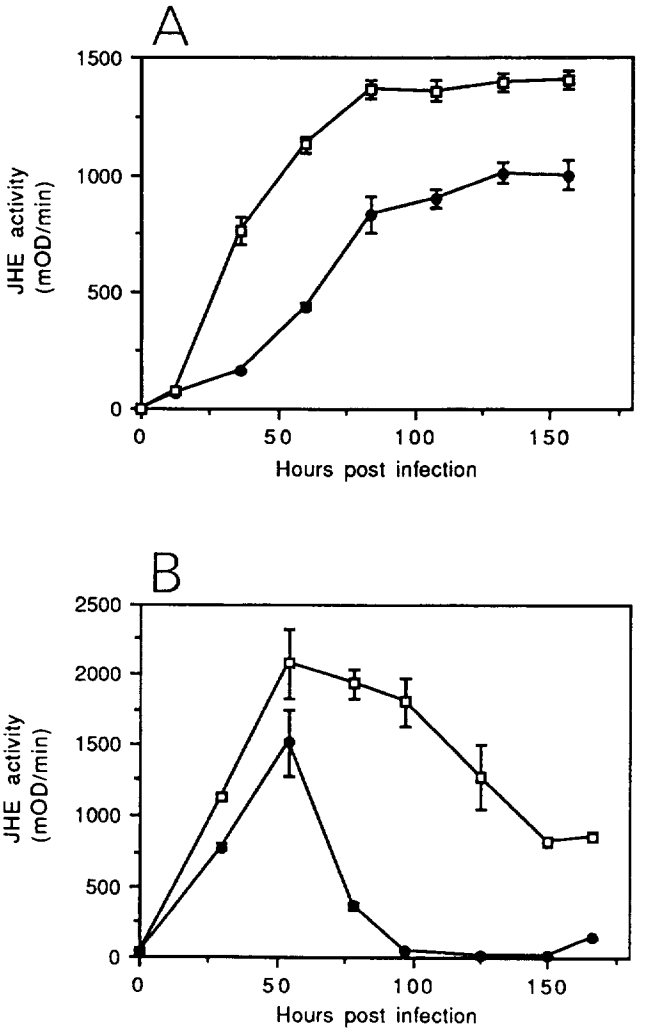

Fig. 3. Comparison of expression of JHE in Sf21 and Tn cells grown in media with (□) or without (●) 3% FCS. Cells were cultured in ExCell 401 medium, and infected at 5×10^5 cells per ml at an MOI of 5. (A) Expression of JHE by AcMP1.JHE infected Sf21 cells. (B) Expression of JHE by AcMP1.JHE infected Tn cells.

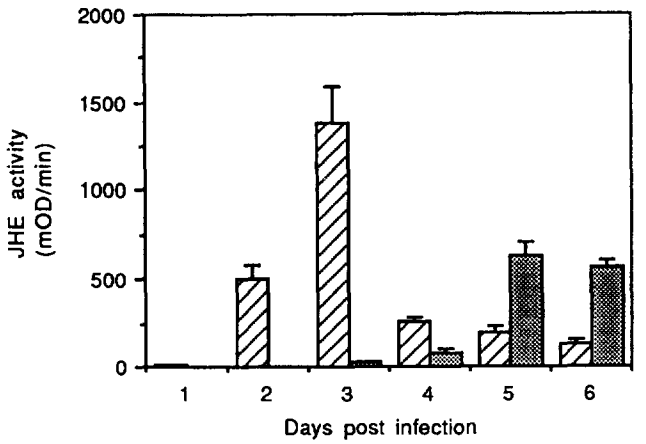

Fig. 4. Comparison of expression of JHE in Sf21 cells (grey bars) and Tn cells (striated bars) infected with AcMP1.JHE. Cells were maintained in ExCell 401 in the absence of FCS. Standard deviations are shown.

3.3. Production of JHE with and without FCS on infection of Sf21 and Tn5B1–4 cells

Production of JHE by AcMP1.JHE in Sf21 cells in 50 ml spinner cultures was compared using ExCell 401 medium in the presence and absence of 3% FCS. Higher yields of JHE were consistently detected from cells cultured in the presence of FCS (Fig. 3A). Maximum expression occurred after 3 days for Sf21 cells in the presence of FCS and after 5 days for Sf21 cells in the absence of FCS.

Comparison of yields of JHE in 100 ml shaker flasks of Tn cells infected with AcMP1.JHE showed that higher and more rapid expression of JHE was attained on infection of Tn cells in the presence of FCS than in the absence of FCS (Fig. 3B). Maximum expression by the recombinant baculovirus AcMP1.JHE was 32 mg/l at 52 hpi for Tn cells. However, active JHE was rapidly lost from Tn cell culture after day 3, particularly in the absence of FCS.

A direct comparison of expression of JHE by cells of Sf21 and Tn cells following infection with AcMP1.JHE is shown in Fig. 4. Cells were infected at a MOI of 5 and cultured in the absence of FCS. This figure illustrates the more rapid and higher expression yields from Tn cells relative to Sf21 cell culture.

4. Discussion

4.1. JHE as a reporter enzyme

Currently the most commonly used reporter enzymes in molecular biology are β-galactosidase (β-Gal) and chloramphenicol acetyl transferase (CAT). A number of novel reporter enzymes have also recently been brought to light, including a modified alkaline phosphatase (Davis et al., 1992) and luciferase (Karp et al., 1992). Important characteristics of a reporter enzyme are stability, assay sensitivity, speed, ease and cost

of assaying. A colorimetric assay for JHE activity has recently been devised (McCutchen et al., 1993). This is a continuous assay whereby samples in 96-well microtiter plates are monitored for increase in absorbance at 405 nm by a Softmax microtiter plate reader. This colorimetric assay for JHE is more sensitive than that for β-Gal because the JHE assay has a much higher signal to noise ratio. Use of JHE is also facilitated because this enzyme is exported from the cell. The cDNA of JHE incorporated into these viruses includes the natural JHE export leader sequence. Hence, lysis of cells for analysis of reporter enzyme activity is unnecessary.

Despite inferiority to CAT or luciferase in terms of the sensitivity of the colorimetric assay for JHE, the speed and ease of the assay which requires neither radioactivity nor specialized equipment makes JHE an attractive alternative. A second, more sensitive assay for JHE which employs a radiochemical substrate (Hammock and Roe, 1985) has been used commonly for titering levels of JHE in insect hemolymph and can be adapted to detect extremely small amounts (picograms) of JHE (Ward et al., 1992). Since JHE and the conjugated ester of JH are very stable under most incubation conditions, this assay can be modified to increase sensitivity $> 1000 \times$ over the standard method (Ward et al., 1992). Use of alkaline phosphatase as a reporter enzyme is often hampered by background problems associated with endogenous phosphatase activity. Since JH is an α/β conjugated ester it is over 100-times more resistant to the hydrolysis catalyzed by base or most esterases than nonconjugated substrates. Even the colorimetric substrate is relatively selective for JHE, and JHE activity is distinguishable from non-specific esterase activity because it is inhibited by 3-octylthio-1,1,1-trifluoropropan-2-one (OTFP) but not diisopropyl fluorophosphate (DFP) or methyl or ethyl carbamates such as carbaryl (Hammock et al., 1987). There may also be advantages associated with the fact that JHE was derived from a noctuid species closely related to *S. frugiperda* from which the Sf21 cell line was isolated; therefore JHE cDNA is likely to have the optimum codon usage for use in the baculovirus expression system.

In this study we have demonstrated the use of JHE assayed colorimetrically to characterize viral promoters, cell lines, and culture conditions relevant to the expression of recombinant proteins in the baculovirus expression system. With regard to promoters, the viral basic protein promoter produced more protein than either the p10 or polyhedrin promoters. This trend is also seen for intracellular enzymes such as β-galactosidase (Bonning et al., 1994). Reduced yields from the p10 promoter compared to the basic protein promoter may be related to competition within the cell for resources to produce the very late p10 and polyhedrin proteins produced by AcUW2(B).JHE, because AcMP1.JHE is a p10 negative virus with the basic protein promoter driving expression of the foreign gene (Bonning and Hammock, 1994). To date, the polyhedrin promoter is the most commonly used promoter for baculovirus expression. Our data suggest that use of the basic or p10 promoters may be better for maximum protein production. However, conclusions regarding promoter strength determined for one protein are not necessarily directly applicable to all other proteins. Baculovirus-infected cells are commonly harvested at 72 hpi for recovery of recombinant proteins. After 84 hpi, yields from the different viruses begin to converge (Fig. 1), suggesting that other factor/s, beside use of different promoters, may be a rate limiting step in the production of recombinant proteins from these cells.

The effect of the MOI on the yield of JHE in Sf21 cells was found to be insignificant for AcMP1.JHE (basic protein promoter) after 24 h. However, the JHE yield was substantially less (by 20% to 50%) at 0.1 for AcUW2(B).JHE (p10 promoter) relative to MOIs of 1 and 10. Previous studies showed no significant difference for infection between the MOIs of 0.1 and 1.0 for viruses using the polyhedrin promoter to drive expression (Shuler et al., 1990). This observation indicates that high yield protein production may be achieved by use of lower MOIs for the earlier viral promoters for baculovirus expression of foreign proteins.

Recently, a number of different serum-free media have been developed (Maiorella et al., 1988; Inlow et al., 1989) with the aims of reducing the cost of cell culture and facilitating purification of secreted recombinant proteins from a low protein background. Protein production by recombinant baculovirus-infected cells in serum-free medium has been compared to production in other media in the presence of serum (Wang et al., 1992). Here we compared expression of JHE in the serum-free medium ExCell 401, in the presence and absence of 3% FCS to assess the efficacy of this medium for high protein yields from the baculovirus expression system. Our data show that protein yields from cell cultures maintained in the serum-free medium ExCell 401, are 30% to 40% lower than yields from cells cultured in the presence of FCS. The maximum yield of JHE from Tn cells in shaker flasks was 32 mg/l. Similar results have been obtained on comparison of non-secreted β-galactosidase expression from Sf9, Sf21 and TN368 cell lines, in the presence and absence of FCS (Wang et al., 1992). In each case the cells were adapted to the media or culture comditions before monitoring yield.

A dramatic reduction in the yield of JHE from Tn cells was seen on day 3 post-infection. Rather than reaching a plateau as seen on infection of Sf cell culture (Bonning et al., 1992), the yield of JHE dropped completely in the absence of FCS, and by 50% in the presence of 3% FCS. This drop in recombinant protein yield may be due to the action of proteases in the medium resulting from lysis of Tn cells on day 3 and day 4. Tn cells maintained in ExCell 401 are routinely passaged every 3 to 4 days because cells begin to lyse after this time. This lysis may be caused by limitations of the medium ExCell 401, which was developed for the Sf9 rather than the Tn cell line. The loss of JHE activity is reduced in the presence of FCS (Fig. 3B). This observation may be due to the FCS (i) providing an alternative substrate for the proteolytic enzymes (Luckow and Summers, 1989) or (ii) inhibiting protease enzymes (King et al., 1991).

Comparison of data obtained for production of JHE on infection of Sf21 and Tn cell cultures indicates that the Tn cell line gives consistently higher protein yields than the more commonly used Sf cell line under the conditions described. Given that larvae of the cabbage looper *Trichoplusia ni* are significantly more susceptible to AcNPV infection than are larvae of *Spodoptera frugiperda* (Payne, 1986), it might be expected that Tn cells would support baculovirus infection more efficiently than Sf cells in vitro. However, the difference in susceptibility of these larvae to virus infection may also be affected by processes involved at the pre-infection stage. The expression of a large number of both intracellular and extracellular recombinant proteins has proved to be consistently and significantly higher on infection of Tn cells relative to Sf cells (data not shown).

In conclusion, JHE has a number of advantages over other reporter enzymes in terms

of stability and ease of assay, for use in baculovirus research in particular, and may also be applied to yeast, and other eukaryotic systems. JHE may also be used for expression and purification of fusion proteins, analogous to the glutathione-*S*-transferase fusion system (Davies et al., 1993). An affinity purification method for JHE has already been developed (Abdel-Aal and Hammock, 1986) and would facilitate such an approach.

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