Belg. J. Zool. - Volume 127 (1997) - issue 1 - pages 35-45 - Brussels 1997

RECOMBINANT PROTEIN EXPRESSION IN INSECT CELL SYSTEMS

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Abstract. Since the introduction of baculovirus expression vectors, the suitability of insect cells for the expression of functionally active genes of eukaryotic origin has been exhaustively documented. Originally realization of a functional viral expression vector was laborious and depended on double homologous *in vivo* recombination success as well as successful purification of recombinant baculovirus by plaque assay. Using the commercial Bac to Bac™ expression system we confirm that transposon-assisted recombination and cloning of recombinant transfectable bacmid DNA in bacteria now allow fast productive expression of a gene of interest.

As an alternative to the expression by infected cells which sometimes suffers from the effects induced by cell lysis, we developed a stable *Drosophila* S-2 cell transformation protocol using the constitutive promoter of the immediate early gene of the silkworm baculovirus (BmNPV). Although baculovirus immediate early gene promoters are reported to be rather weak promoters, we routinely obtain expression levels up to the same range as obtained with the baculovirus system.

Keywords: transient expression, *baculovirus*, stable transformation.

INTRODUCTION

Less than two decades ago knowledge of protein diversity, functionality and structural organization mainly depended on, and was limited by, developments in protein identification, separation and purification technology. Hitherto, proteins of low abundance were only purified and structurally characterized when financial revenues could be *a priori* assured due to specific therapeutic (*e.g.* insulin), enzymatic (*e.g.* hirudin) or antigenetic (*e.g.* viral coat proteins for vaccine production) properties of the protein. Since the introduction of recombinant DNA and bacterial transformation technology it became clear that original limitations such as the need for an abundant natural source of the protein to be purified, were no longer valid. Due to the lack of posttranslational protein processing in bacterial hosts, restriction of expression to a prokaryotic environment in many cases results in the production of functionally inactive and/or structurally changed proteins.

Contribution to the 3th Benelux Congress of Zoology, Namur, November 1996.

Whereas bacterial hosts remain irreplacable for rapid gene cloning, amplification and manipulation, new alternatives for gene expression and mass production of particular recombinant proteins needed to be explored. Among the resulting eukaryotic expression systems those based on yeast cells (*Saccharomyces cerivisiae*, *Pichia pastoris*) are considered to be most efficient and cost effective but they still have peculiar processing properties which make them unsuitable for the expression of particular proteins (HERSCOVICS & ORLEAN, 1993; KALSNER *et al.*,1992). In those situations, the choice of a suitable expression system is mainly limited to either expression in insect (LUCKOW, 1991) or mammalian (JENKINS & CURLING, 1994) cells and, in specific situations, to production in transgenic animals (DAMAK *et al.*, 1996). One major drawback of the mammalian *in vitro* expression systems (*CHO* cells, hybridoma cell lines) is their limited productivity due to the lack of strong promoters. This limitation probably explains the wide range of recombinant proteins of mammalian origin that became preferentially produced in the heterologous insect cell based expression system. The availability of strong promoters for expression in insect cells and the resulting higher yields, together with the functionally active proteins obtained (JARVIS & SUMMERS, 1992) added to the world-wide use of this system which we will further elaborate in this paper.

TRANSIENT EXPRESSION OF RECOMBINANT PROTEINS IN INSECT CELLS USING BACULOVIRUS EXPRESSION VECTORS

Baculoviruses

The term baculovirus designates a particular group of viruses that only replicates in invertebrates and therefore can be considered safe for humans and vertebrates in general. In the context of *baculovirus* expression vectors, only a particular subgroup of so-called nuclear polyhedrosis viruses (NPV's) is considered. These NPV's are characterized by their double life cycle. Early in the infection cycle assembled virions are released by budding from the host cell membrane. Later in the infection cycle virions become enveloped inside the cell nucleus and end up embedded inside a crystalline matrix of viral encoded polyhedrin protein. This embedding results in microscopically visible polyhedrons or occlusion bodies inside the nucleus and an easily recognized cytopathogenic effect which is typical for all nuclear polyhedrosis viruses.

Rapid accumulation of polyhedrin protein late in the infection cycle points towards the presence of a strong promoter controlling this gene. Furthermore, in an *in vitro* situation, only extracellular viruses are needed for re-infection of new cells. These observations suggested the use of the polyhedrin gene as a favoured site for foreign gene insertion. Placing a foreign gene downstream of the strong polyhedrin gene promoter indeed resulted in a recombinant viral expression vector which, once used for insect cell infection, in most cases allowed the production of large amounts (up to µg/ml) of the recombinant protein.

Baculovirus expression in practice

Baculoviruses, having a double stranded circular DNA genome of +/-120 kb, are not suitable *per se* for direct *in situ* cloning of a foreign gene. As explained in Fig. 1 production of a recombinant baculovirus expression vector involves cloning the gene downstream of the polyhedrin promoter present in a so-called transfer plasmid. In this transfer plasmid the polyhedrin expression cassette is flanked at both sides by viral sequences which also flank the polyhedrin gene in the intact wildtype virus. By cotransfecting DNA of the recombinant transfer plasmid together with DNA of wildtype virus into insect host cells (*e.g.* Sf-9 cells) homologous recombination between corresponding flanking sequences present in both DNA's takes place and results in an exchange of the polyhedrin gene of the wildtype virus for the polyhedrin expression cassette. As a result of this cotransfection, within a few days both recombinant virions as well as wildtype virions will start to accumulate in the culture medium of such transfected cells. In a second important step the recombinant viruses need to be cloned from the mainly wildtype virus-contaminated pool by subsequent rounds of so-called plaque purification. Once an inoculum of pure recombinant virus is obtained and following a gradual scaling up of viral progeny by reinfection cycles of increasing cell numbers, the productive infection of insect cells can be started. Since expression of the gene of interest depends upon viral infection, the recombinant protein production though high in relative yield is short in duration due to lysis and death of the host cells. Reinfection of new host cells is the only means of obtaining larger amounts of the desired recombinant protein.

In terms of production it should be mentioned that the lepidopteran host cells can be cultured either in an adhering monolayer, which is most appropriate during transfection and plaques purification cycles, or in suspension cultures allowing higher cell densities. The use of suspension cultures is most suitable for this productive expression.

Pitfalls and improvements in the production of recombinant baculovirus expression vectors

Successful production of a recombinant baculovirus expression vector assumes both experience in insect cell culture technology and in basic viral handling practice. The former should not be a real drawback since culturing insect cells is not as demanding as culturing mammalian cells. Equipment needed is absolutely minimal, except for the summer period when a refrigerated incubator may become necessary since the lepidopteran cells have optimum growth characteristics below 28°C. On the other hand no atmospheric regulation is necessary and insect cells can be cultured in closed culture flasks. Several culture media are commercially available and usually need to be supplemented with 10% of insect cell qualified, heat inactivated, fetal calf serum for optimal growth performance. During productive expression the fetal calf serum supplemented medium can be replaced by serum free media which is also commercially available. Concerning viral handling, at least during the starting period, some practical assistance might be most welcome but it can be learned by trial and error as well. In terms of safety considerations, as mentioned earlier, baculoviruses *per se* are harmless for the experimenter. It should be mentioned however that although the viruses do not replicate in human cells, internalisation by non- permis-

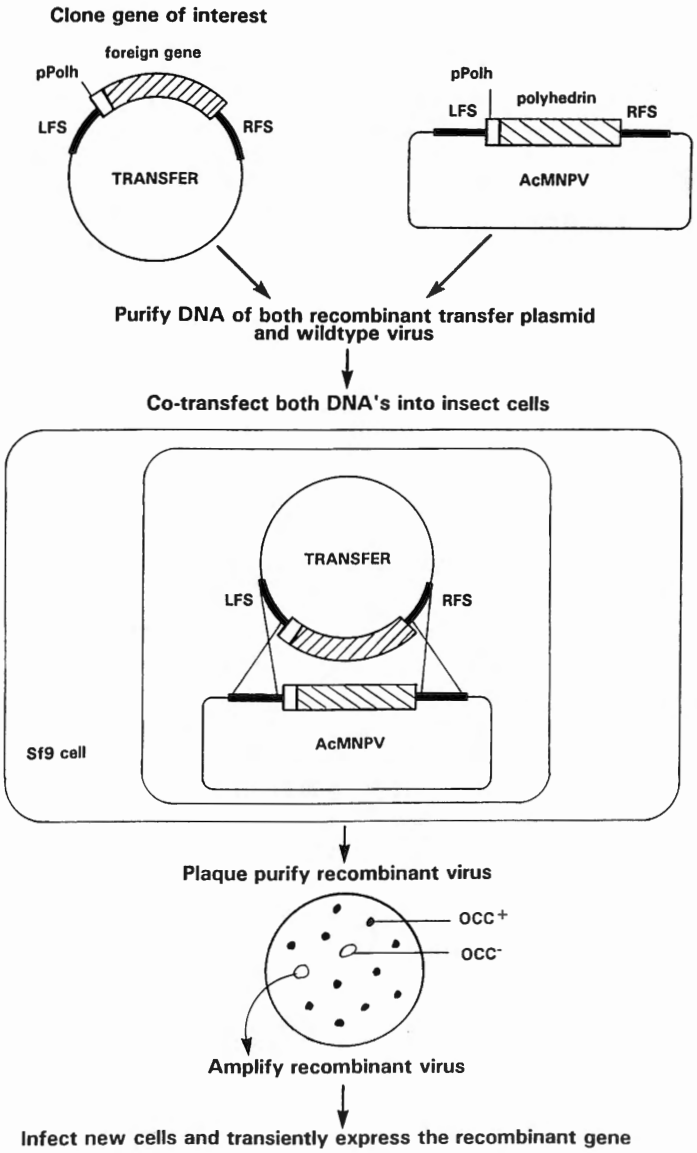

Fig. 1. – Schematic representation of the classical baculovirus expression system: The gene of interest (either gDNA or cDNA) is cloned in a polycloning site downstream of the strong polyhedrin promoter. Upon cotransfection of host cells with both the recombinant transfer plasmid and purified viral DNA, recombinant baculovirus generation is based upon a double homologous recombination event between polyhedrin flanking sequences that are present in both DNA's. In the absence of any recombination event wildtype virus is assembled and released as well. Several rounds of plaque purification are needed to obtain pure recombinant baculovirus suitable for productive expression during subsequent infection rounds.

sive cells is well known and transactivating effects by viral early genes can never be excluded. Especially in those situations in which baculoviruses containing human genes or genes of human pathogens are constructed those viruses should be handled with appropriate care and always in conformance with local biosafety regulations.

One major technical limitation of the classical production of recombinant baculovirus is the dependence upon the success of the double homologous recombination which needs to take place following co-transfection of both viral and recombinant transfer vector DNA. Depending on the DNA quality, the outcome of homologous recombination can result in about 3% of the viruses produced being good recombinants. However in most cases less than 1% of recombinants can be observed during subsequent plaques purification. This cloning of recombinant viruses is most tricky. In the classic situation the difference between wildtype or occlusion body positive plaques and recombinant or occlusion body negative plaques has to be established with the microscope. With a trained eye this is not really difficult. Newcomers are advised to pick-up as many plaque-resembling spots as possible. Following infection of cells in a multiwell plate with viruses present in those plaques, the identity of the plaques can then be simply determined by PCR using a combination of either universal polyhedrin based primers or more specifically using a combination of one gene specific primer with either one of both universal primers.

Suppliers of commercial kits (In Vitrogen, Pharmingen, GibcoBRL-Life Technologies,...) are aware of the inexperience of most researchers trying baculovirus expression. To overcome these difficulties several approaches have been introduced (DAVIES, 1994). Generally the wildtype virus DNA delivered with those kits is linearized by a single digestion in the polyhedrin region. In theory linear viral DNA should not replicate and in theory only recircularisation following recombination can result in virus production. In practice a high number of wildtype plaques is observed due to the need for double homologous recombination, usually preceded by single recombination. Alternatively a marker gene is integrated in either the viral DNA used for cotransfection or, preferably in the transfer vector used for making the desired recombinant construct. In both cases this marker gene helps with the recognition and identification of recombinant plaques. A better alternative is the use of an artificial deletion mutant of wildtype viral DNA for co-transfection. In such a mutant an essential gene is deleted and functionally repaired only in recombinants. As a result only recombinant viral progeny is produced and plaque purification can be omitted.

More recently LUCKOW and co-workers (1993) developed a baculovirus expression system in which most steps up to the production of transfectable recombinant viral DNA are performed in bacteria (see Fig. 2). By making use of the Tn7 bacterial transposase this system became independent of random recombination events. Extension of this system with a traditional blue/white selection of true recombinants makes the conventional cloning of the gene of interest in the donor plasmid the most difficult part of the entire procedure. Most importantly, with this bac to bac™ system a significant time saving is achieved making it the best choice in those situations in which high numbers of recombinant viruses need to be produced within a limited time period. Following some preliminary trials we are now using this bac to bac protocol with good results on a routine basis (POELS, 1996; HUYBRECHTS, unpublished).

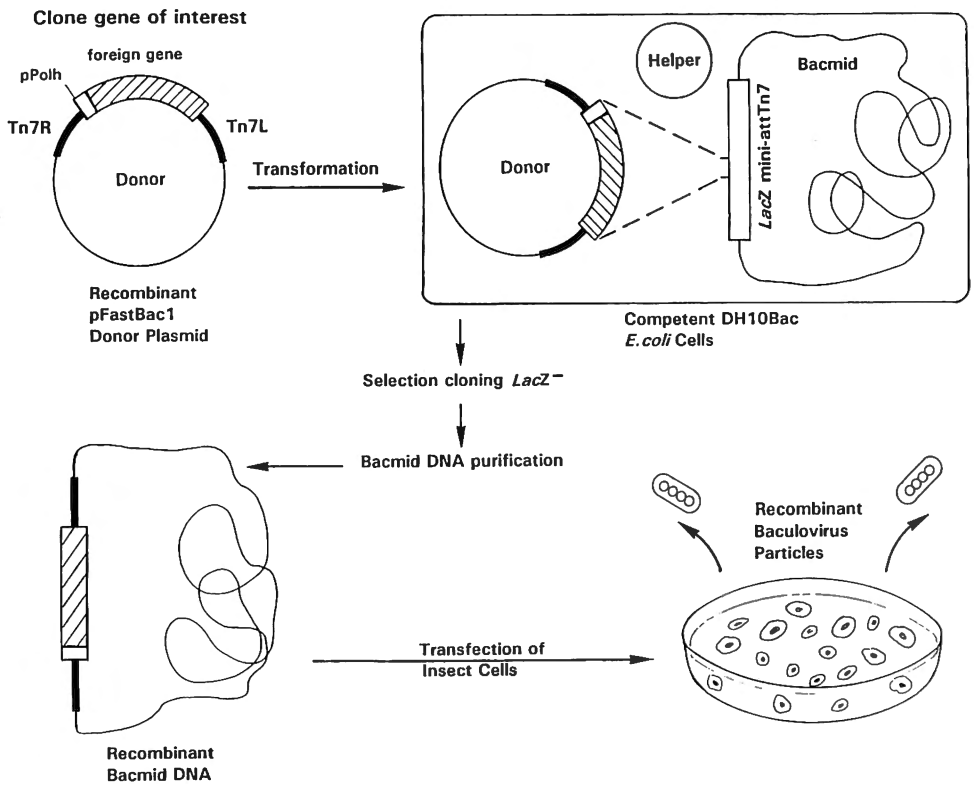

Fig. 2. – Schematic explanation of the Bac to Bac™ protocol for rapid production of recombinant baculovirus: Following cloning of the gene downstream of the polyhedrin promoter present in a donor plasmid, the purified recombinant donor plasmid is used for transformation of DH10 Bac cells. These host cells contain a replicating baculovirus genome having the polyhedrin open reading frame replaced by LacZ and being flanked with Tn7 attachment sites. A helper plasmid delivering the Tn7 transposase in trans is also present in these DH10Bac cells and will transpose the polyhedrin transcription unit of the donor plasmid (*cfr.* presence of flanking Tn7 donor sites) into the attachment site as present in the bacmid. After plating and growing of the transformed bacteria, a white colony is selected and bacmid DNA purified. This recombinant Bacmid DNA once transfected into insect host cells will generate recombinant baculovirus only. Since no contaminating wildtype baculovirus is released no plaque purification is needed and productive infection of host cells can be started immediately. (Bac to Bac™ is distributed by GibcoBRL-Life Technologies).

CONTINUOUS EXPRESSION IN INSECT CELLS FOLLOWING PRODUCTION OF STABLE TRANSFORMED CELL LINES

Although the baculovirus expression system became one of the most widely used methods for the quantitative production of a variety of recombinant proteins, it should be noted that protein expression is maximal in cells near death due to viral infection.

Obviously in many cases this is suboptimal for correct overall processing of the protein of interest and, more importantly may result in high levels of protein degradation due to the release of proteolytic enzymes during lysis of infected cells. On the other hand the advantage of high expression levels due to the functioning of strong extra late viral promoters (cfr. supra polyhedrin promoter but equally the well known P10 promoter) can still not be realized outside an intact viral and *in se* lytic environment.

In practice, good alternative expression using the advantages of an insect cell based expression environment can be realized making use of early baculovirus promoters for driving the expression of the gene of interest in a constitutive and therefore continuous way. In contrast to the extra late viral gene promoters which need a cascade of transactivating

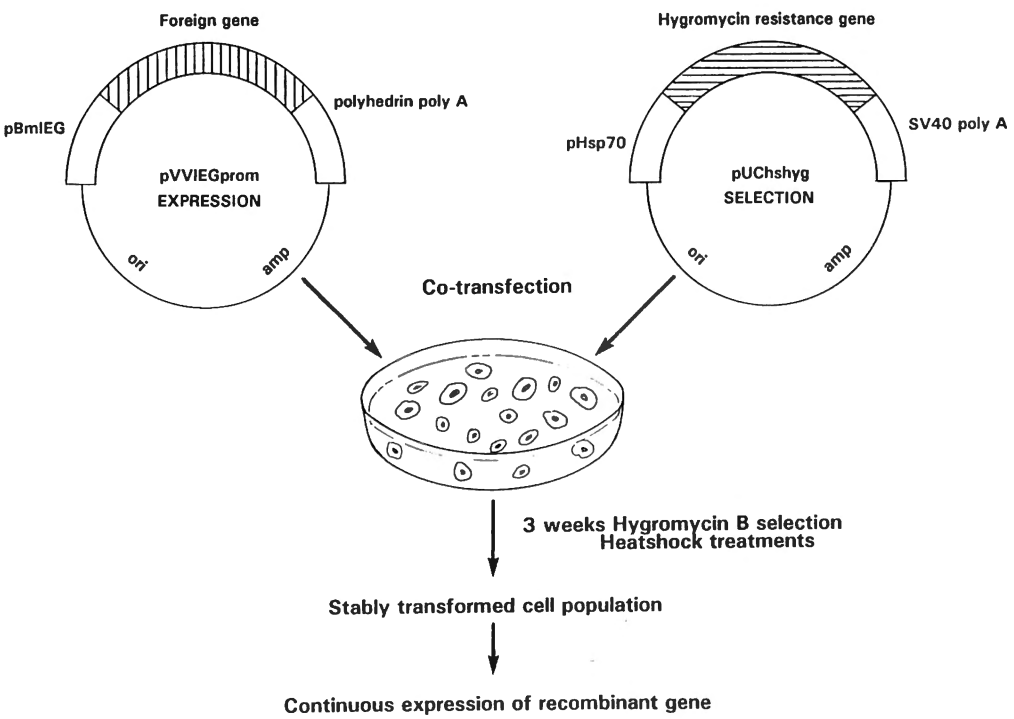

Fig. 3. – Overview of the protocol for the production of stable transformed insect cell lines for constitutive expression of a gene of interest. The gene to be expressed is inserted into a polycloning site downstream of the pBMIEG promoter present in the expression plasmid which contains appropriate transcription termination and poly-adenylation signals originating from the polyhedrin gene. When *Drosophila* S-2 cells are used as expression environment, selection for true chromosomal integration is best accomplished by cotransfection of the expression plasmid and a Hygromycin-B resistance gene containing plasmid (*e.g.* pUChshyg). Since here the resistance gene is situated downstream of the inducible *Drosophila* heatshock hsp70 promoter, repeated heatshock treatments are necessary as long as cells are grown in Hygromycin-B (240 µg/ml) containing medium. Usually a three week selection period is sufficient for obtaining stable transformed cells that contain both the selection and expression plasmid.

events by a still obscure array of viral encoded proteins in order to become functional, these early baculoviral genes use the host cells own polymerase II transcription activation machinery. As a consequence, simple plasmid constructs containing the gene of interest cloned downstream of an immediate early baculovirus gene promoter will, upon simple transfection into insect cells, even those originating from insects of non-lepidoperan orders, result in transient production of the corresponding recombinant protein (HUYBRECHTS *et al.*, 1992). Continuous expression can be achieved when transfected cells can be selected for genomic integration of the recombinant expression cassette. As illustrated in Fig. 3 this is realized in practice by cotransfecting a recombinant expression plasmid together with a second selection plasmid carrying an antibiotic resistance gene preferentially placed under control of an inducible promoter. It became empirically evident that selection for integration of the selection plasmid results in a resistant cell population carrying the expression plasmid in their genome as well (see Fig. 4 as well as VULSTEKE *et al.*,1993; VANDEN BROECK *et al.*, 1995; LAUWERS, 1996). It was further evidenced that such transformed cells remained stable even without continued selection. In Fig. 5 it is well documented that in contrast to earlier predictions concerning the rather low strength of such early baculovirus promoters this continuous expression system can become at least as productive as the classic polyhedrin promoter based expression system. In part this is explained by the possibility of obtaining extremely high cell densities with the Schneider S-2 cells compared to the Lepidoptera Sf9 cell line we use in the respective context. Additionally it can be concluded that in the continuous expression system, accumulation

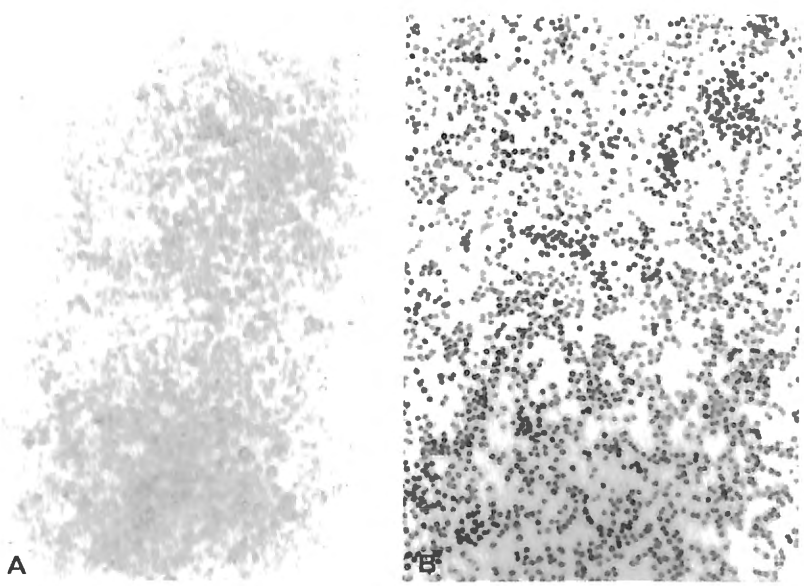

Fig. 4. – Illustration of a typical cotransfection and selection result during production of stable transformed S-2 cells. A lacZ coding sequence was used for constructing the recombinant expression plasmid (*cfr.*VULSTEKE *et al.* 1993 for specific details). Panel B clearly illustrates that all cells of the Hygromycin-B resistant population stain X-gal positive as well. Control cells (panel A) do not show any endogenous galactosidase activity. Differences in staining intensities between individual cells are explained by differences in integrated copy numbers of the expression plasmid.

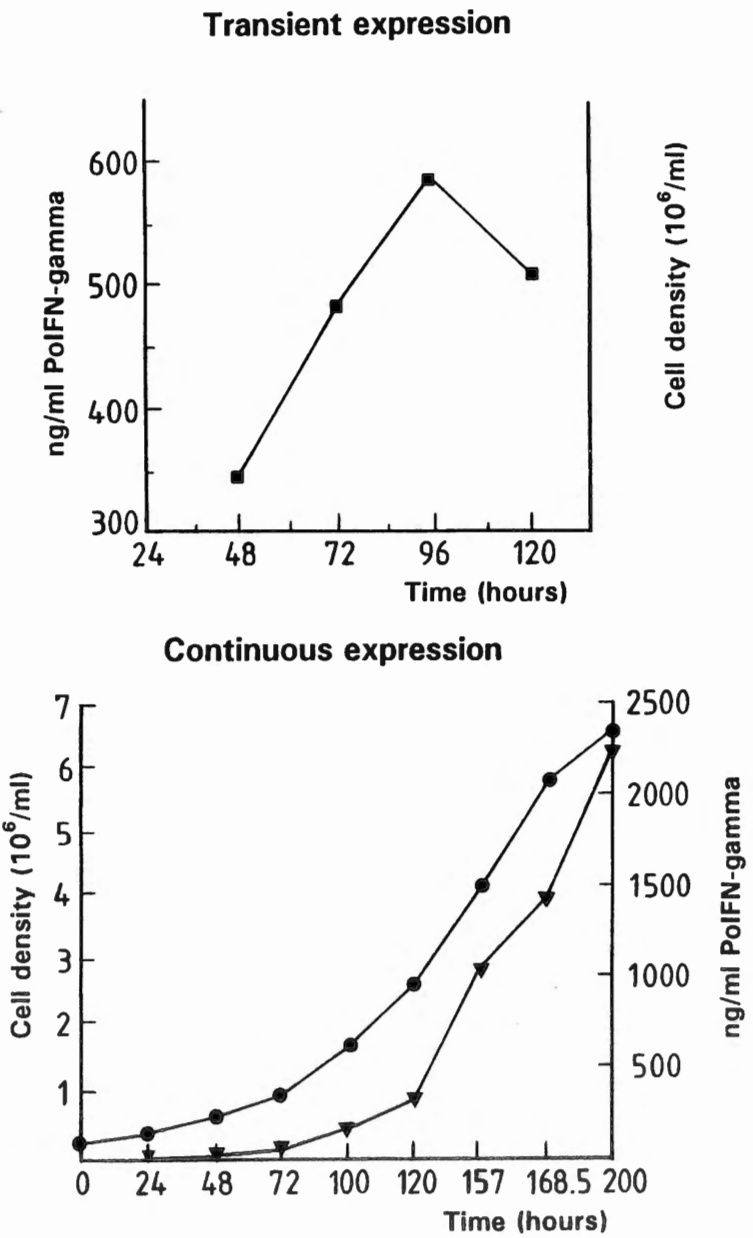

Fig. 5. – Comparison of porcine interferon-gamma production, as determined by a sandwich ELISA (*cfr.* VANDEN BROECK *et al.*, 1994) in a transient recombinant baculovirus based Sf-9 cell system (left panel) and in a continuously expressing, stable transformed S-2 cell system (right panel). In both situations the experiment started from a cell density of $2.5.10^5$ cells /ml. In contrast to the infected cells in which cell division is arrested, the continuously expressing cells (filled dots) continue dividing up to densities of almost 6.10^6 cells/ml without any apparent deleterious effect.

of recombinant protein over a far more extended period is possible without a decline, due to proteolytic damage, in immunologically recognized recombinant protein (LAUWERS, 1996). Comparing promoter activities at transcription level further demonstrated that polyhedrin as well as the early gene promoter results in comparable amounts of transcripts (VANDEN BROECK *et al.*, 1995). This last observation is most probably explained by the extremely high copy numbers of integrated recombinant genes that are usually observed following stable transformation (VULSTEKE, 1995).

CONCLUSIONS

Thanks to the success of baculovirus expression systems, insect cells are recognized as an acceptable environment for the recombinant expression of eukaryotic cDNA and of intron containing genes (IATROU *et al.*, 1988; GOPINATHAN personal communication). Functionality of the expressed proteins is guaranteed and major posttranslational processing events will occur. The original limitation of insect cells having only mannose-type glycosylation properties is now almost circumvented by both selection of cell lines with improved processing characteristics (OGONAH, 1996) as well as by co-expression of genes coding for the necessary enzymes (JARVIS & FINN, 1996). High yields of recombinant protein production and expression in an insect cell environment, both considered as major advantages of the baculovirus expression system, can now be achieved using the continuous S-2 cell and BmNPVIEG promoter based expression system (this paper).

ACKNOWLEDGEMENTS

We would like to thank the team of Prof. A. Billiau (Rega Instituut, K.U.Leuven) for providing the porcine interferon-gamma cDNA as well as the anti-interferon-gamma antibodies.

The «Onderzoeksraad K.U.Leuven (0T90.015), The EC (ERB-C11*-CT94-0092) and the (N)FWO (G.0260.96, G.2169.94) are acknowledged for financially contributing to the realization of this research.

The assistance of Ms. J. Puttemans in the realization of the illustrations is appreciated.

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