EXPRESSION OF THE HUMAN db1-ONCOGENE AND PROTO-ONCOGENE PRODUCTS IN INSECT CELLS USING A BACULOVIRUS VECTOR

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Summary. - Among the expression vectors, the baculovirus system has been successfully developed and it has been shown to be suitable as a helper-independent viral expression vector for high level of production of recombinant proteins in cultured insect cells. The high efficiency of this system derives from the viral strong promoter of the polyhedrin gene. Recombinant viruses containing foreign DNA inserted into the polyhedrin gene, will no longer produce polyhedrin and will form plaques morphologically different from the plaques produced by the wild type virus. We have expressed, at high level, the db1 and proto-db1 proteins using the baculovirus system. db1 and proto-db1 gene products produced in insect cells resulted to have the same post-translational modifications and subcellular localization observed in db1 and proto-db1 transfectedants.

KEY WORDS: baculovirus vector, insect cells, oncogenes.

Riassunto (Espressione dei prodotti del proto-oncogene e dell’oncogene db1 umani in cellule di insetto per mezzo di un baculovirus come vettore). - Tra i vettori di espressione, il sistema del baculovirus è stato di recente messo a punto e si è dimostrato estremamente utile come vettore di espressione di tipo virale, non dipendente da "helper" virus, per la produzione di proteine ricombinanti in cellule di insetto. Il notevole grado di efficienza di questo sistema deriva dalla presenza di un potente promotore virale, quello del gene della polyedrina. Virus ricombinanti contenenti frammenti di DNA esogeni, inseriti nel gene della polyedrina, non sono più in grado di produrre questa proteina e danno luogo alla formazione di placche di morfologia diversa da quelle formate dal virus "wild type". Mediante il vettore di espressione del baculovirus abbiamo espressi in quantità notevole il prodotto dell’oncogene db1 (p66) e del corrispondente proto-oncogene (p115). p66 e p115 prodotti nelle cellule di insetto infette dai rispettivi virus ricombinanti, presentano le stesse modificazioni post-translazionali e la stessa localizzazione subcellulare osservate nei transfectanti di NIH/3T3.

PAROLE CHIAVE: vettore di espressione del baculovirus, cellule di insetto, oncogeni.

General properties of the baculovirus vector

The Baculovirus Autographa California Nuclear Polyhedrosis Virus (AcNPV) has been shown to be suitable as a helper independent viral expression vector for functional mammalian proteins [1-9]. Prokaryotic cells can be successfully used only when the gene product has a simple structure and when post-translational modifications such as accurate signal peptide cleavage, glycosilation or phosphorylation are not required for its biological activity. One of the advantages of this expression system compared to prokaryotic expression systems is that heterologous proteins produced in insect cells generally retain their original physical and biological properties, since insect cells possess the appropriate machinery needed for proper post translational modification of the eukaryotic proteins.

Moreover it has been shown that baculovirus infected insect cells are able to perform the removal of intron by proper splicing [10]. This system utilizes a cell line established from the Fall Army worm Spodoptera Frugiperda (SP9) or larvae of the silkworm, which can be infected by the naturally occurring baculovirus AcNPV [7]. AcNPV is a non-defective, cytopathic, DNA virus exclusively pathogenic for insect cells. The virus can incapacitate the viral genome with large pieces of additional foreign DNA. During the AcNPV infection two forms of viral progeny are produced: extracellular virus particles and occluded viral particles, which are embedded in proteinaceous nuclear occlusions called polyhedra. AcNPV genome includes a gene (polyhedrin) which is not essential for the production of infectious extracellular viral particles in cultured cells and encodes a protein which accounts for the nuclear occlusions present in the infected cells, in which the viral particles are embedded. These nuclear occlusions are important for the lateral transmission of the virus since they serve to protect the virus from inactivation by environmental factors [9]. Polyhedrin gene transcription is driven by a strong promoter: late in lytic infection, polyhedrin represents 60% of the total cellular proteins. Since this gene is not essential for the production of the extracellular
forms of the virus, it can be easily replaced by another gene, that, being under the transcriptional control of the polyhedrin promoter, will be expressed at very high level. Moreover the recombinant virus will result to be an occlusion negative virus, which forms plaques morphologically different from those of the wild type occlusion positive virus, providing an easy way to screen for the recombinant virus.

In summary the properties that make the baculovirus suitable as an expression vector for the large scale production of biologically active proteins are: a) the virus is not pathogenic to vertebrate; b) the virus can inactivate together with the viral genome foreign genes; c) the virus genome contains non essential regions in which to insert foreign DNA's; d) the virus genome contains a very strong promoter which directs transcription late in infection when most of the host and viral genes are run over; e) the virus contains a genetic marker to select for recombinant virus.

Construction of \texttt{dbi} and \texttt{proto-dbi} recombinant viruses

Many eukaryotic proteins have been produced using baculovirus vectors. These proteins include human interferon \texttt{I} [1], human interferon \texttt{II} [2], human interleukin 2 [3], human interleukin 5 [11], human interleukin 6 [4], human erythropoietin [12], oncogene products [5, 13, 14], growth factor receptors [6, 15-19] and virus antigens [20-26]. All these proteins produced in insect cells retain their original structural and functional characteristics. We have studied the expression of the human \texttt{dbi} oncogene and proto-oncogene using the baculovirus expression system. For this purpose we have introduced the cDNA fragments encoding the \texttt{dbi} oncogene and proto-oncogene proteins into the baculovirus genome adjacent to the polyhedrin strong promoter. \texttt{dbi}-oncogene was initially isolated by transfection of NIH/3T3 cells with a DNA derived from a human diffuse B cell lymphoma [27]. Subsequently the \texttt{dbi} oncogene [28] and proto-oncogene (\texttt{proto-dbi}) [29] cDNAs have been isolated and their nucleotide and aminoacidic sequences determined. \texttt{dbi} and \texttt{proto-dbi} proteins have been previously characterized in \texttt{dbi} [30] and \texttt{proto-dbi} [31] transfecants of NIH/3T3. The \texttt{dbi} oncogene encodes a 66 kDa (p66) whose first 50 amino acids, derived from a different human locus, are fused to 428 amino acids encoded by the 3' end of the \texttt{dbi} proto-oncogene [28, 29]. p66 is a cytoplasmic phosphoprotein which is localized in both the membrane and soluble fractions of the cells [30]. On the other hand the \texttt{proto-dbi} product is a 115 kDa (p115) protein, which shares with p66 the same subcellular distribution and post-translational modification. However p115 is phosphorylated to a lesser extent than p66 and it has an extremely short half life, while p66 is a very stable protein.

Although both \texttt{dbi} and \texttt{proto-dbi} oncogenes have transforming potential, the transforming activity of the \texttt{dbi} oncogene is much higher than that of its normal counterpart. Therefore, while overexpression is sufficient to activate \texttt{proto-dbi} as transforming gene, gross structural alterations such as those occurring in the \texttt{dbi} oncogene enhance its transforming activity. The level of expression of p66 and p115 in NIH/3T3 transfecants is low, therefore in order to better understand the biochemical and biophysical properties of these proteins, we have expressed them at high level in the baculovirus system.

Recombinant baculoviruses were obtained by cloning the \texttt{dbi} oncogene and proto-oncogene cDNAs into a plasmid transfer vector (PAC373), which contains the baculovirus polyhedrin promoter and a unique BamHI site at -8bp from the initiation codon of the polyhedrin gene and 50 base downstream of the transcription start site, leaving the polyhedrin promoter and polyadenylation sequences intact [3]. PAC373 contains also an appropriate length of each 5' and 3' flanking sequences of the polyhedrin gene in the wild type virus which facilitates the homologous recombination when PAC373 is cotransfected with wild type viral DNA. Moreover ampicillin resistance gene and a replication origin from a bacterial plasmid are also present. The resulting constructs PAC373-dbi and PAC373 proto-dbi contain all but eight nucleotides of the polyhedrin 5' untranslated leader sequence fused to the complete coding sequences of the \texttt{dbi} genes including the first ATG codon (Fig. 1). Since the presence of the non-coding flanking regions of the foreign genes seem to reduce the expression of the genes inserted in the baculovirus vectors [7], all but few nucleotides from the 5' and 3' non coding sequences of the proto-dbi and \texttt{dbi} were removed.

Transfection and detection of the recombinant viruses

After determining the right orientation of the insert by standard restriction endonuclease mapping, the baculovirus transfer vectors containing the \texttt{dbi} or proto-\texttt{dbi} genes were amplified and purified using CsCl-ethidium bromide gradient centrifugation [32].

PAC373 \texttt{dbi} and PAC373 proto-\texttt{dbi} were cotransfected with wild type AcNPV total viral DNA into SF9 cells by the calcium phosphate coprecipitation method [7].

In vivo recombinational events usually lead to the generation of a recombinant baculovirus with the polyhedrin gene replaced by foreign DNA sequences. Thus, in order to identify whether a baculovirus population containing the \texttt{dbi} sequences was present, virus particles released in the supernatant fluids were harvested 4 days after transfection, lysed and viral DNA was analyzed by dot blot hybridization to a \(^{32}\)P-labeled \texttt{dbi}-specific DNA probe. By the fourth day post-transfection many of the cells should have viral occlusion in the nuclei; in fact the recombinant virus obtainable after transfection usually represents only a small percentage (0.1%-5%) of the viral population, mainly constituted by wild type virus. In order to purify the \texttt{dbi} and proto-\texttt{dbi} recombinant virus, the culture superna-
Fig. 1. **Construction of the baculovirus expression vectors.** The \( dbl \) (---) and proto-\( dbl \) (--:: complete cDNAs were inserted into the BamHI site of PAC373 downstream from the polyhedrin (-----) promoter in a 5' to 3' orientation; the left and right arms (-----) of this vector are homologous to the flanking sequences of the polyhedrin gene in the wild type virus.

S revertants, positive for the presence of the recombinant virus, were collected 4 days after transfection and used at several dilutions to inoculate monolayers of SF9 cells. After subsequent overlaying with 1.5% low melting agarose, recombinant baculoviruses were visually selected by identifying occlusion negative plaques with an inverted phase microscope [33]. The occlusion negative phenotype (Fig. 2A), resulting from the replacement of polyhedrin with the \( dbl \) gene, makes it possible to distinguish between the recombinant plaques and the wild type plaques, which, on the other hand are characterized by the nuclear occlusions (Fig. 2B). Recombinant plaques were picked and terminally diluted in 96-well insect cell monolayers. 3 days later monolayers were examined for the absence of polyhedra, media was removed and transferred to a duplicate 96 well plate and stored at 4°C. Cells were lysed and screened by dot blot hybridization to nick translated \( dbl \) DNA (Fig. 3). Among the recombinant virus-positive preparations, the one showing the strongest hybridization signal with the lowest contamination by wild type virus was chosen for further purification (Fig. 3, C-4 and F-14). Three rounds of plaque purification were thus performed in order to obtain a high titered wild type free recombinant virus population.

An alternative way for identifying recombinant virus is by plaque hybridization [7]. The agarose overlay is removed and stored at 4°C degree, and the cell monolayer is blotted onto a nitrocellulose disk, which is then hybridized to the \( 32P \) labeled specific probe. Areas on the agarose overlay corresponding to the part of the nitrocellulose disk showing hybridization signal is used for infection of insect cells monolayers cultures until no wild type virus contamination is detected (Fig. 4).

Purified recombinant viruses were then propagated using a single plaque to infect a monolayer culture in a Petri dish. After 4 days of infection the supernatant is harvested and used to infect a large number of cells in order to obtain a high-titered stock virus.

**Expression of the \( dbl \) and proto-\( dbl \) genes**

To determine whether cells infected by each of the recombinant viruses synthesized the appropriate \( dbl \) and proto-\( dbl \) proteins respectively purified \( dbl \) recombinant baculoviruses were used to infect monolayers of insect cells. Cells were harvested after 1, 2 and 3 days after infection and cell lysates were examined for expression of the \( dbl \) products by western blot analysis using polyclonal antibodies raised in rabbits against \( dbl \) synthetic peptides [27]. Alternatively cells were labeled with \( 35S \)-cysteine before immunoprecipitation with specific antiserum, followed by gel electrophoresis and autoradiography. \( dbl \) and proto-\( dbl \) NIH/3T3 transfectedants were used as controls. p66 and p115 levels of expression were much higher in insect cells than in \( dbl \) and proto-\( dbl \) NIH/3T3 transfectedants (Fig. 5). \( dbl \) products could be detected as early as 24 h after infection; moreover, there was a continuous increase in the level of p66 up to 72 h. On the other hand, the synthesis of the proto-\( dbl \) product started at 24 h after infection, reached the peak at 48 h and showed a marked reduction between 60 and 70 h (data not shown). \( dbl \) oncogene product p66 was expressed at much higher level than p115 and unlike p115, a significant amount of p66 was detected even after 72 h, when cell lysis was evident.
Fig. 2. - Comparison of wild type polyhedrin positive and dbl polyhedrin negative plaques. Monolayer cultures of SF9 cells were infected with dbl recombinant virus (A) and wild type virus (B), overlayed with agar and incubated for 4 days at 28 °C. Polyhedrin negative (A) and polyhedrin positive (B) plaques are shown x 200.

Fig. 3. - Identification of recombinant viruses by plaque hybridization. After performing a plaque assay (see text for details), agarose was lifted from the plate and stored at 4 °C. Nitrocellulose filter was placed on top of cells remaining in the plate and treated with a 0.5 NaOH, 1.5 NaCl solution. After neutralizing, filter was baked and hybridized with dbl specific 32P-labeled probe.
Fig. 4. - Dot blot analysis to identify the recombinant virus. Insect cells were infected using a 96 well plate. After 3 days, media was removed and stored at 4°C. Cells were lysed in the wells by adding 0.5N NaOH. After neutralizing the solution by adding 1M NH₄ acetate, lysates were spotted onto nitrocellulose filters and hybridized with dbi specific ³²P-labeled probe.

A second band was present in the proto-dbi recombinant virus infected cells (Fig. 5) that may represent either a degradation product of proto-dbi or an abnormally processed form of proto-dbi. None of these proteins were present in wild type infected cells. Although the level of expression of the eukaryotic proteins expressed so far by the baculovirus system is high, it never reaches that of the polyhedrin protein expressed by the wild type in virus-infected insect cells. Similar results have been obtained with the dbi and proto-dbi gene products. In fact the quantities of p66 and p115 present in insect cells infected by the corresponding recombinant virus resulted to be less than a few percent of the polyhedrin. Factors affecting the efficiency of the expression of foreign gene products are the sequences between the promoter and the translation initiation signal [34], as well as the characteristics of the foreign protein, being the nuclear and non structural proteins expressed at higher levels than the secreted or enveloped proteins [8].

We have previously established in NIH/3T3 transfectants that p66 and p115 are phosphorylated in serine residues, while we did not detect other post translational modifications such as glycosylation or fatty acid acylation for either of the dbi proteins. Therefore, in order to determine whether p66 and p115 were post translationally modified and thus phosphorylated when expressed in insect cells, 48 h after infection cells were labeled for 3 h with ³²P-orthophosphate and cells were analyzed for the presence of phosphorylated p66 and p115. The results of these experiments indicated that the dbi proteins produced in insect cells are phosphorylated (data not shown).

Subcellular localization studies in NIH/3T3 transfectants showed that p66 and p115 are cytoplasmic proteins, present in both the soluble and membrane fractions of the cells. Therefore experiments were performed in order to determine whether these proteins, when expressed in insect cells, showed the same subcellular distribution detec-
ted in NIH/3T3 transfectants after hypotonic shock of 35S labeled infected insect cells, nuclei were removed by low speed centrifugation and cytosolic and membrane fraction were prepared by ultracentrifugation of the low-speed supernatants. Although db1 and proto-dbl proteins were detected in both cytosolic and membrane fractions as it occurs in NIH/3T3 transfectants, in insect cells these proteins were mostly on the membranes (data not shown). A small amount of p66 and p115 was also detected in the supernatants collected after the labeling time: this observation doesn't indicate a real secretion of the protein in the media, but most likely the release of the proteins in the media due to the initial lysis of the cells (data not shown). In fact western blot analysis of conditioned media didn't show any significant amount of db1 or proto-dbl proteins in the media (data not shown).

In conclusion, we were able to express in insect cells the db1 and proto-dbl proteins at high levels and with the same post translational and subcellular localization characteristics as the db1 gene products expressed in mammalian cells.

We are now in the process of analyzing whether the db1 proteins expressed in insect cells are biologically active. Furthermore expression of the db1 proteins in the insect cells at high level will allow the purification of these proteins for analysis of possible catalytic functions or other biochemical properties.

REFERENCE


