A new method for the isolation of recombinant baculovirus

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ABSTRACT

An improved method for the isolation of baculovirus recombinants is described. The method involves the replication and maintenance of the baculovirus genome in the yeast Saccharomyces cerevisiae which was accomplished by the isolation of a baculovirus recombinant containing yeast ARS and CEN sequences ensuring stable replication in yeast and a URA3 selectable marker. The viral DNA maintained its ability to replicate in insect cells. An efficient and rapid selection system was set up, to isolate viral recombinants in yeast; DNA from selected yeast colonies was transfected into insect cells to obtain recombinant virus. We demonstrate the utility of this system by isolating recombinant viruses that express two different members of the CREB/ATF family of transcription factors.

INTRODUCTION

Baculoviruses have been widely used as eukaryotic vectors for the expression of a variety of foreign proteins (for review, see 1-3). In most cases such vectors have resulted in high levels of protein being produced which, with only a few exceptions, are correctly processed and biologically active. The expression system is based on replacing the highly expressed viral polyhedrin gene with a recombinant gene consisting of the polyhedrin promoter fused upstream to the desired foreign coding sequence. Since the polyhedrin gene is dispensable for viral replication, the ensuing recombinant virus is fully infectious and at late times of infection the activated polyhedrin promoter directs efficient transcription of the foreign gene.

Although the system has proved to be very efficient and useful, it remains time consuming and tedious to use because of the difficulties in identifying and isolating the recombinant virus. The most widely used baculovirus for expression of foreign genes is the Autographa californica nuclear polyhedrosis virus (AcNPV), whose genome consists of a circular dsDNA molecule of approximately 130 kb (4). Owing to this large genome size it is not possible to insert foreign DNA by conventional techniques such as the use of restriction endonuclease cleavage sites. The method used therefore, relies upon homologous recombination between viral DNA and an appropriate transfer vector when cotransfected into insect cells (5-9). The transfer vector consists of the recombinant gene inserted downstream of the polyhedrin promoter flanked by the same sequences that flank the polyhedrin gene in the intact virus. In vivo recombination between the homologous flanking sequences on the virus and transfer vector results in the replacement of the polyhedrin gene with the foreign gene. The difficulty arises from the low recombination frequencies generally obtained, which are typically between 0.1 and 1%. Therefore, the recombinant virus has to be identified over a very significant background of parental virus. Several methods have been used to identify the recombinant viruses including: (1) the identification by microscopy of viral plaques that consist of polyhedra-negative infected cells (10); (2) the identification of recombinant plaques by plaque hybridization to probes specific to the foreign DNA followed by visual screening (7,11); (3) antibody screening of plaques (12). In the case of all of these methods, multiple plaque assays are generally required before pure recombinant virus is obtained.

Recently, two modifications of the system have been described that facilitate identification of recombinant plaques. In one case, the transfer vector contains in addition to the polyhedrin promoter driven foreign gene, the bacterial lacZ gene driven either by a second viral promoter or a non-viral promoter (13). The recombinant virus arising through homologous recombination will also receive this lacZ gene and can therefore be identified by screening plaques for β-galactosidase production. In the second case, the parent virus contains a unique restriction endonuclease cleavage site allowing transfection of linear rather than circular viral DNA (14). As a result, the frequency of recombination is increased by approximately 10-fold.

We describe in this report an entirely novel method we have developed for generating recombinant viruses that is rapid and efficient, ensures that there is no background of parental virus and eliminates the need for time-consuming plaque assays. Additionally, multiple recombinants can be readily isolated simultaneously. The method involves the propagation of the viral genome in the yeast Saccharomyces cerevisiae where the appropriate recombinants can be more easily selected. Virus is then obtained by transfecting insect cells with DNA isolated from the selected yeast colonies. We show in this report how we have used this system to isolate recombinant viruses that express two members of the CREB/ATF family of transcription factors.

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MATERIALS AND METHODS

Cells

Maintenance of the insect cell line Sf9 (ATCC accession number CRL 1711) and the production of wild-type AcNPV (strain E2) has been described previously (10).

The *S.cerevisiae* strain used in this study was y657 (mat α his3-11,15 trpl-1 ade2-1 leu2-3,112 ura3-52 can1-100 his4-::HIS3) provided by Andy Newman (41). Growth of this strain or derivatives containing baculovirus/cen plasmids was in rich medium (yeast extract/petone/dextrose (YPD)) or synthetic minimum medium (SD) supplemented with histidine (20 μg/ml), tryptophan (20 μg/ml), adenine (20 μg/ml), leucine (60 μg/ml) and casamino acids (10 mg/ml) (42). Canavanine resistant transformants were selected on plates containing 2% agar, SD with appropriate amino-acid supplements and 60 μg/ml canavanine sulphate (Sigma). All yeast growth was at 30°C.

Construction of recombinant baculovirus, YCbv

A plasmid pBY3 was constructed by the insertion of TRP1/ARS1, CEN4 and URA3 sequences into bluescript (Stratagene) as follows: An 840 bp fragment containing the TRP1/ARS1 sequence was inserted between the EcoRI and HindIII sites of bluescript followed by the insertion of a 1.1 kb fragment containing the URA3 gene into the HindIII site and a 1 kb fragment containing CEN4 between the XhoI and KpnI sites. This yeast cassette was removed as a BamHI-KpnI fragment and inserted into the BamHI-KpnI sites of the transfer vector pAcC4 (9) to give pAcAuCh. This vector was co-transfected together with wild-type AcNPV DNA into Sf9 cells and recombinant baculovirus (YCbv) isolated as described previously (41). Viral DNA was prepared from extracellular virus and used to transform y657 yeast cells to give the yeast strain YGP1.

Construction of transfer vectors

The transfer vector pAcY1 was constructed as follows (Fig.2A): The 840 bp TRP1/ARS1 sequence was inserted into the EcoRI-HindIII sites of bluescript plasmid. This plasmid, pBY1, was then restricted with XbaI, filled-in with Klenow polymerase and further restricted with BamHI. pAcC4 DNA was restricted with BstEII, filled-in with Klenow polymerase, recut with BamHI and the 3 kb fragment containing the polyhedrin promoter and 5′ flanking sequences isolated. This was ligated to the cut pBY1 DNA described above and the DNA fragment contained between the NcoI and EcoRI sites replaced with a polylinker to give pACY1. The transfer vector pACY2 was constructed in the same way (Fig.2A) except that the BstEII-BamHI fragment was derived from the plasmid pAcYM1.

pACY2-S was constructed as follows: plasmid p464 was restricted with EcoRI to release a 300 bp fragment containing the ochre suppressor gene, SUP4-o. This fragment was inserted into the transfer vector pACY2 at the EcoRI site present just upstream of the yeast ARS1 sequence. When used for yeast transformation, pACY2-S was restricted at the BglII site contained within the ARS1 and at the Xhol site present in the baculovirus sequence of the transfer vector.

pACY2-CREBl was constructed by inserting a 1.6 kb EcoRI fragment containing the CREB1 sequence including 600 bp non-coding sequence at the ‘3′ end, at the EcoRI site of the transfer vector, pACY2.

To construct pACY2-CREB2, a HindII-ClaI, filled-in fragment containing the CREB2 coding region was inserted into the Smal site contained in the pACY2.

Yeast transformation and selection

Yeast spheroplasts were prepared and transformed by previously published methods (43). Briefly, a 50 μl culture of yeast cells was grown in appropriate medium to a density of OD600 of 0.5–1. Cells were pelleted at 2K for 5 min and washed once in distilled water and once in 1M sorbitol and resuspended in 20 μl of SCem at pH 5.8. 10 mM EDTA and 30 μM β-mercaptoethanol and 1000 units of lyticase (Sigma). The cell suspension was incubated at 30°C for 20–30 min until 90% of the cells had formed spheroplasts. The spheroplasts were gently pelleted at 1K for 6–7 min and washed with 20 μl of STC (1M sorbitol, 10 mM TRIS/HCl pH 7.5 and 10 mM CaCl2) and finally resuspended in 2 μl of STC. 5 μg of transfer vector containing the relevant foreign DNA was restricted within the polyhedrin sequences and either within ARS sequence or immediately downstream of ARS sequence. The restricted plasmid was ethanol precipitated and was used to transform 100 μl of the prepared spheroplasts. The yeast-plasmid DNA mixture was allowed to stand at room temperature for 10 min after which 1 ml of PEG solution (10 mM TRIS/HCl pH 7.5, 10 mM CaCl2 and 20% PEG 8000) was added and the incubation continued for a further 10 min. The spheroplasts were centrifuged at 1K for 6–7 min and the pellet resuspended in 150 μl of SDS buffer (1M sorbitol, 6.5 mM CaCl2, 0.25% yeast extract (Difco) and 0.5% bactopeptone (Difco)) and incubated at 30°C for 15–30 min. 8 ml of molten top agar containing relevant supplements was added to spheroplast suspension and immediately plated on top of an agar plate containing SD medium supplemented with histidine, leucine, adenine and tryptophan. For cotransformation, 0.5 μg of a TRP1 gene containing plasmid was added to the restricted transfer vector and the selection was in the absence of tryptophan in the medium.

YGP1 yeast cells were obtained by transforming y657 with purified YCbv DNA. Transformants were selected by growth in the absence of uracil. YGP2 cells were obtained by transforming YGP1 with the transfer vector pAcY2-S previously restricted with XhoI and BglII. Transformants were selected by growth in the absence of adenine and subsequently colony purified on SD-agar plates supplemented with histidine, tryptophan, adenine and leucine. Individual white colonies were tested for growth in the presence of canavanine and colonies that were sensitive were selected for further analysis. These colonies had the expected phenotype in the presence of the tRNA suppressor SUP4-o, namely adenine independence (due to suppression of the ochre ade2-1 mutation ade2-1) and canavanine sensitivity (due to suppression of the can1-100 mutation). The presence of an integrated SUP4-o gene was further verified by Southern hybridization analysis.

Replacement of the SUP4-o gene in YCbv::SUP4-o with CREB1 or CREB2 was as follows: The transfer vector pACY2-CREB1 was restricted with SalI and transformed into YGP2 spheroplasts together with a plasmid that contains the yeast TRP1 gene. After 2–3 days the faster growing TRP+ transformants were tested for growth in the presence of canavanine. Such resistant transformants would arise from homologous recombination between the resident YCbv::SUP4-o and pACY2-CREB1 DNA replacing SUP4-o with CREB1. A number of canavanine resistant colonies were verified for the presence of CREB1 sequences by Southern hybridization. A similar procedure was used to isolate CREB2 containing recombinants except that the pACY2-CREB2 vector was restricted with XhoI.
Preparation of yeast DNA

Total yeast DNA was prepared as described previously (44). Briefly, 250 mls of YGP3 or YGP4 yeast cells were grown in SD medium supplemented with histidine, leucine, adenine, tryptophan and casamino acids to an OD$_{600}$ of 1–2. Spheroplasts were prepared as described for the transformation procedure, and gently resuspended in 15 ml lysis buffer containing 4.5M GuHCl, 0.1M EDTA pH8, 0.15M NaCl and 0.05% Sarkosyl. After 10 min at 65°C, the suspension was cooled to room temperature and DNA precipitated with 15 mls of cold ethanol followed by centrifugation at 10,000 rpm for 10 mins in a Sorvall SS-34 rotor. The pellet was resuspended in 10 mls 0.1M Tris pH 7.5, 0.01M EDTA and 50 µl of 10 mg/ml RNase A. Incubation was for 60 mins at 37°C followed by the addition of 150 µl of 10 mg/ml proteinase K and further incubation at 65°C for 30 mins. The DNA was phenol extracted twice, ethanol precipitated and resuspended in TE (10mM Tris, 1mM EDTA).

For most of the experiments described, the total DNA was fractionated by sucrose gradient centrifugation. The DNA was layered on top of a 5%–20% sucrose gradient made up in 200 mM NaCl, 10 mM Tris pH 7.5 and 2 mM EDTA. The gradients were centrifuged for 3 hrs at 35K rpm in an SW40 rotor. Under these conditions, the bulk of baculovirus DNA migrates half-way down the gradient as assayed by polymerase chain reaction. The gradient was fractionated and 2 volumes of cold ethanol was added to the fractions containing the baculovirus DNA. The precipitated DNA was resuspended in TE (10 mM Tris, 1 mM EDTA) and used directly for insect cell transfections.

Transfection of insect cells

Sucrose gradient fractionated DNA was introduced into Sf9 cells using lipofectin (Gibco). Water was added to 5–10 µl of fractionated DNA to a total of 12 µl. An equal volume of lipofection buffer containing 8 µl lipofectin mixed with 4 µl water was added to the DNA. The DNA and buffer solutions were mixed gently and after 15 mins incubation at room temperature were added to a 35 mm tissue culture dish containing 1.5×10$^6$ Sf9 cells in 1 ml medium without serum. After 16 hrs at 28°C, the medium was removed and replaced with fresh medium containing 10% FCS. By day 3 or day 4 the cells were visibly infected at which time they were harvested for analysis and the supernatant saved for reinfection of fresh cells.

Electromobility Shift Assay

Infected or 'lipofected' Sf9 cells from a 35 mm dish were washed with PBS and the cells resuspended in 100 µl of extraction buffer containing 10 mM Hepes pH 7.9, 1.5 mM MgCl$_2$, 0.1 mM EGTA, 5% glycerol, 0.5 mM DTT and 0.5 mM PMSF. After 5 mins at 0°C, the lysate was centrifuged for 10 seconds and the supernatant transferred to a fresh tube. The pellet was resuspended in 50 µl extraction buffer containing 0.35M NaCl, incubated for 15 mins at 4°C and cell debris removed by centrifugation for 10 mins. 45 µl of the supernatant was mixed with the 100 µl of low salt lysate to give a whole cell extract with a final salt concentration of 0.1M. Electromobility shift assays using an oligonucleotide containing the fibronectin ATF/CREB binding site was as described previously (45). 1 µl of the cell extract was used in each assay. To test the effect of specific antibodies on the mobility of complexes, antibody was added 5 mins after initiation of the binding reaction. The antibodies used were as follows: The CREB1 antibody has been described previously (35) and was raised against the carboxy terminal 10 amino-acids of the human CREB1 protein. The N-terminal CREB2 antibody (NJ2) was raised against a peptide comprising amino acids 85–96 of human CREB2 protein and the C terminal antibody (NJ4) was raised against a peptide comprising of amino acids 490–505.

RESULTS

Strategy

The strategy we employed for the rapid and efficient isolation of recombinant baculoviruses is outlined in Figure 1. It is based on the ability to generate the recombinant viruses by homologous recombination in yeast. The main advantage afforded by using yeast as the host is that recombinants can be rapidly and easily selected. The success of the strategy depended upon the construction of a baculovirus derivative that could be stably maintained in yeast. This required the insertion into the viral genome of essential yeast DNA elements namely, a yeast ARS sequence necessary for autonomous replication of DNA in yeast (15,16), a CEN sequence that confers mitotic centromere function on DNA molecules and ensures a stable low copy number of plasmid DNA (17) and a selectable marker gene (URA3). We reasoned that if the viral DNA containing these elements could be stably maintained in yeast and moreover remained infectious for insect cells, then such hosts would provide an ideal environment for subsequent homologous recombination to generate appropriate recombinant DNAs. The insertion of a second selectable gene (SUP4-o) (18) which can be selected for or against in yeast would allow recombinants to be readily identified and isolated; if the desired recombination event replaced the SUP4-o gene with the polyhedrin promoter driven foreign gene, then the recombinants could be isolated by SUP4-o counterselection. DNA from the selected yeast colonies could then be isolated and used to transfect insect cells to allow the production of recombinant virus.

Construction of Baculovirus DNA that can Replicate in Yeast and Insect Cells

Homologous recombination in transfected Sf9 cells was used to isolate a recombinant baculovirus (YCbv) containing a cassette of three yeast DNA elements (Fig.1A). The yeast TRP1/ARS1 and CEN4 elements together with the URA3 gene were inserted into the previously described transfer vector pAcC4 (19). Following cotransfection into Sf9 cells together with wild-type baculovirus DNA, recombinants were visually identified and purified by repeated plaque assays. Recombinant viral DNA was prepared and used to transform a uracil dependent strain of *Saccharomyces cerevisiae* y657. A number of Ura$^+$ colonies were obtained, some of which were analyzed for the presence of baculovirus sequences by Southern blot hybridization using total yeast DNA as a probe. All the Ura$^+$ colonies tested contained the expected viral sequences (data not shown). Total DNA was isolated from one such transformant (YPG1), partially purified by sucrose gradient centrifugation and transfected into Sf9 cells. Three days later the culture supernatant was collected and used to infect fresh Sf9 cells. Microscopic examination of the cells indicated the presence of infectious virus which was subsequently confirmed by plaque assay. Viral DNA was prepared from the infected cells and shown to have maintained its ability to confer uracil independence when transformed into Ura$^-$ yeast. Clearly,
therefore, the recombinant baculovirus DNA, (YCbv) could be shuttled between yeast and insect cells with no apparent difficulty.

To facilitate the detection of YCbv derivatives that had undergone homologous recombination with the transfer vector containing the foreign gene to be expressed, a second selectable marker gene was inserted into YCbv DNA (Fig. 1A) replicating in YGP1. The gene chosen was the SUP4-o gene, an ochre-suppressing allele of a tRNA\textsubscript{Tyr} gene. In appropriate yeast strains (for example, y657; see Materials and Methods), SUP4-o can be placed under negative or positive selection. We used a strain containing ochre mutations in the ADE2 and CAN1 genes. ADE2 encodes an essential enzyme in the adenine biosynthetic pathway (20,21). Mutations in this gene not only render the host adenine dependent but also result in the accumulation of an intermediate product of the adenine pathway, (phosphoribosylaminimidazole, AIR), that gives rise to a pink colouration of cell colonies. The CAN1 gene encodes for the enzyme arginine permease and renders the cell sensitive to the toxic arginine analogue, canavanine (22). Can\textsuperscript{-} mutants are unable to synthesize functional arginine permease and are therefore canavanine resistant. A summary of the different phenotypes of y657 derivative cells that do or do not carry the SUP4-o gene is given in Fig.1B, the key features being that adenine independence provides a positive selection for the presence of SUP4-o whereas canavanine resistance provides a strong counter-selection.

To introduce the SUP4-o gene into YCbv DNA a transfer vector was constructed containing the SUP4-o gene flanked on one side by sequences that lie upstream of the polyhedrin translation initiation codon and downstream by the yeast TRP1/ARS1 sequences. Following transformation into YGP1 yeast, homologous recombination between YCbv and the vector

Figure 1A. A schematic outline of the strategy for the isolation of recombinant baculovirus using \textit{S.cerevisiae} as an intermediate host. See text for details. The ARS, URA and CEN yeast elements are designated as A, U and C respectively. 1B. The phenotype of y657 yeast derivatives that do or do not contain the SUP4-o gene. y657 is the starting yeast strain. YGP2 contains episomally maintained baculovirus DNA with the yeast SUP4-o, ARS, URA and CEN genetic elements inserted downstream of the polyhedrin promoter (figure 1A). YGP3 and YGP4 are derivatives of YGP2 where replicating genomes contain the CREB1 and CREB2 cDNA sequences have replaced the SUP4-o gene. The full genotype of the yeast strain is given in materials and methods.
would result in the insertion of SUP4-o into YCbv (Fig. 1A). Stable Ade\textsuperscript{+} and canavanine sensitive transformants were selected as described in materials and methods and correct integration of SUP4-o into YCbv confirmed by Southern hybridization. One such recombinant (YGP2) was chosen for subsequent experiments.

Construction of Transfer Vectors pAcY1 and pAcY2

The yeast strain YGP2 provides a convenient recipient for the insertion via homologous recombination of foreign sequences downstream of the polyhedrin promoter. To facilitate such transfer, two new vectors were constructed. pAcY1 is based on the previously described vector pAcC4 (19) (Fig. 2A). The essential features of this vector are that it contains the polyhedrin promoter together with 3 kb of 5' flanking baculovirus sequence, a polylinker containing a number of unique restriction sites immediately downstream of the promoter and polyhedrin 5' non-coding sequences followed by the yeast ARS sequences that were used for the construction of YCbv (Fig. 2B). The Ncol site of the polylinker incorporates the polyhedrin ATG translation initiation codon. The second transfer vector, derived from vector pAcYM1 (23), is very similar except for the polylinker used for the insertion of foreign sequences (Fig. 2A). The polyhedrin ATG codon is lost and thus an initiation codon has to be provided by the inserted foreign sequence (Fig. 2B). Therefore unlike previously described vectors where the insert is flanked on both sides by baculovirus DNA, these vectors result in the insert being flanked 5' with viral sequences and 3' by yeast ARS sequences.

**Figure 2A.** Construction of the transfer vectors pAcY1 and pAcY2. The solid, hatched and empty sections of each plasmid represent yeast ARS, baculovirus and plasmid backbone sequence respectively. **2B.** Restriction endonuclease map of the transfer vectors pAcY1 and pAcY2. The plasmids contain the baculovirus polyhedrin promoter and leader sequence preceded by flanking viral sequences and followed by the yeast TRP/ARS sequence. The sites downstream of the leader that are available for the insertion of foreign coding sequences are highlighted. In the case of pAcY1, the final version of this transfer vector contained an oligonucleotide of the indicated sequence inserted between the Ncol and EcoRI sites. **2C.** Restriction endonuclease map of pAcY1 derivatives containing inserts of the human CREB1 and CREB2 (CRE-BP1) cDNA coding sequences.
Overexpression of Human CREB Proteins

In order to test the efficacy of this novel system, we decided to isolate baculovirus recombinants containing two different members of the CREB/ATF family of transcription factors. Members of this family bind specifically to the DNA sequence 5′ TT/GACGTCG 3′, which can confer transcription activation via elevated levels of cAMP (24) and the adenovirus EIA protein (25). At least 10 different cDNAs have been described that encode members of this family (26–30); they can all bind to DNA as homodimers but many can also interact and bind as heterodimers. This provides very significant complexity, the unravelling of which may be facilitated by the availability of large amounts of individual member proteins. We decided to isolate recombinants containing CREB1 and CREB2 (CRE-BP1, ATF-2) cDNAs. They are of particular interest since CREB1 activity is modulated by cAMP or other conditions that elevate kinase A activity levels (31), and CREB2 activity is modulated by EIA protein (32–34). In both cases the cDNAs were inserted into the pAcY2 vector (Fig.2C) and subsequently introduced into YGP2 yeast cells (Fig.1A) along with a co-transforming plasmid containing the yeast TRP1 gene. To facilitate homologous recombination, CREB containing transfer vectors were cut with restriction endonucleases to release a linear fragment containing the CREB cDNA sequence and flanking sequences, in the case of pAcCREB1 with SalI and in the case of pAcCREB2 with Xhol. Although linearisation is not absolutely necessary it does increase the frequency of gene replacement. Transformants were selected by growth in the absence of tryptophan and CanR transformants were selected by testing individual colonies for growth on a canavanine containing plate. It is advisable not to directly select for CanR transformants since the level of revertants resistant to canavanine is high (approximately 10−6) and the majority of the yeast cells do not take up DNA during the transformation. Direct selection, therefore, would result in CanR colonies that arise both from reversion and gene replacement. This problem can be easily overcome by cotransforming with a circular plasmid that gives high frequency transformation (in our case a TRP1 gene containing plasmid) and screening the resulting transformants for canavanine resistance. Only those cells which have actually taken up DNA will therefore be screened and the vast majority of CanR colonies in this population will have arisen by gene replacement. We deliberately picked larger colonies since it is known that the presence of SUP4-o results in slower yeast growth. The majority of the larger colonies would therefore represent transformants lacking SUP4-o. Indeed we routinely found that about 80% of the picked colonies were CanR. In practise, therefore, it is only necessary to pick a few large colonies from the transformation plate and check their ability to grow on a plate containing canavanine. An alternative strategy is to pick colonies that become pink due to the accumulation of an intermediate product of the adenine pathway; this product does not accumulate in cells containing SUP4-o due to suppression of the ade2-1 ochre mutation and hence the pink colouration does not develop. Longer incubation of the transformation plates is often required in order for the pink colouration to be seen although this also depends upon the adenine concentration in the medium. 12 different resistant transformants were tested by Southern hybridisation for the replacement of the SUP4-o gene with CREB1 or CREB2 sequences. This was found to be the case in every single transformant tested demonstrating the efficacy of the selection system used. It should therefore prove to be unnecessary to routinely test resistant colonies by Southern hybridisation.

Total DNA prepared from YGP3 (containing YCbv::CREB1) and YGP4 (containing YCbv::CREB2) yeast cells was partially fractionated using sucrose gradient centrifugation. The location of the viral DNA was rapidly determined using PCR analysis. Although viral DNA could be found throughout the gradient, under the conditions we used and describe the majority of the

![Figure 3](https://example.com/figure3.png) **Figure 3.** Electromobility shift analysis of baculovirus synthesised CREB1 binding to an oligonucleotide containing the fibronectin CRE/ATF binding site. Sf9 cells were infected with a baculovirus-CREB1 recombinant and whole cell extracts prepared at 1, 1.5, 2 and 3 days post infection. Aliquots of each extract were incubated with [32P]-labelled CRE containing oligonucleotide in the absence or presence of CREB1 specific or non-specific antibody (A) or a 100-fold molar excess of fibronectin oligonucleotide or a non-specific oligonucleotide (B).

![Figure 4](https://example.com/figure4.png) **Figure 4.** Electromobility shift analysis of baculovirus synthesised CREB2 binding to a CRE/ATF containing oligonucleotide. Sf9 cells were infected with baculovirus-CREB2 recombinant and whole cell extract prepared 2 days later. The extract was analysed for binding to [32P]-labelled fibronectin oligonucleotide in the absence or presence of two different CREB2 specific peptide antibodies or a non-specific antibody. A similar analysis was performed on an extract prepared from uninfected cells.
DNA sedimented approximately in the centre of the gradient. This DNA was introduced into Sf9 cells by lipofection (Fig. 1A), and infected cells were readily apparent 3 days later. The supernatant was saved for subsequent infections and the cells tested for the presence of recombinant protein. Successful infection could also be obtained using the unfractionated, total DNA preparation. However, we found the success rate to be variable and a wide range of DNA concentrations needed to be tested in order to have some chance of success. We presume that the variability is due to inhibitory substances present in the yeast DNA preparation that interfered with the transfection process. In contrast, consistent results were obtained with the sucrose-gradient separated material and so although this step is not absolutely necessary it is recommended.

The expression of CREB1 and CREB2 protein in viral-infected Sf9 cells was analyzed by the electromobility shift assay. Total cell extracts were prepared and tested for specific DNA binding activity using a labelled oligonucleotide probe containing the fibronectin ATF/CRE binding site. As shown in Fig. 3A, even at 1 day post-infection CREB1 specific protein-DNA complex was seen; the mobility of the complex was retarded in the presence of an antibody raised against a C-terminal peptide of CREB1 (35) but was unaffected by a non-specific antibody. The level of binding activity significantly increased over the next two days. However, it was apparent that at times greater than 2 days post-infection, much of the CREB1 protein was degraded giving rise to a series of faster migrating complexes. On longer exposures, binding activity could be seen in uninfected extracts. However, this endogenous activity was not recognised by the CREB1 antibody. The specificity of the binding was confirmed by competition with an excess of unlabelled fibronectin oligonucleotide which competed well whereas a non-specific oligonucleotide had little affect (Fig. 3B). Analysis by western blotting confirmed the presence of CREB1 protein in infected extracts and its degradation at times later than three days post-infection (data not shown).

Extracts from cells infected with the CREB2 containing recombinant also contained highly elevated levels of binding activity which specifically interacted with the ATF oligonucleotide (Fig. 4). At 2 days post-infection a number of complexes were seen that were unique to the infected extract. The slowest migrating complex was almost certainly due to intact CREB2 protein since it was recognised and its mobility altered, by antibodies raised against peptides located close to the N-terminus and C-terminus of CREB2. Additionally, western blot analysis revealed the presence of protein migrating at a position expected of CREB2 (data not shown). The faster migrating complexes are due to proteolytic cleavage of the intact protein. They were not recognised by the N-terminal antibody but were recognised by the C-terminal antibody. As with CREB1, the smaller products were more apparent at later times post-infection (data not shown).

Although both CREB proteins were synthesised in recombinant virus infected cells, the accumulated levels of each was significantly different. After 2 days post infection CREB1 protein represented approximately 25% of the total protein whereas CREB2 protein was approximately 1–2%. The differences in levels is probably due to differences in their respective stabilities.

**DISCUSSION**

We have developed a novel method for isolating baculovirus recombinants that overcomes many of the difficulties and time consuming aspects of existing methods. The recombinants are constructed by homologous recombination in yeast rather than in transfected insect cells. As a result it is considerably more rapid and efficient than currently used methods and in addition, is devoid of any background of parental, non-recombinant virus. Final purification of recombinants by one or more rounds of plaque isolation is therefore completely unnecessary. In common with all methods, the initial step involves the insertion of the foreign sequence into an appropriate transfer vector. Once this has been done, using the method we have described, it is possible to obtain a stock of pure recombinant virus within 10–12 days. We envisage that one of the biggest advantages of this method is that several different recombinants can be very easily isolated simultaneously. This should prove useful if, for example, a number of mutant forms of a protein need to be overexpressed for *in vitro* studies.

Although the method involves the use of yeast as an intermediate host it should be stressed that the yeast manipulations involved are very basic and could be easily adopted by a non-yeast laboratory. In addition, certain modifications could be used to simplify the procedure even further. Other yeast transformation methods have been described which are both simpler and quicker than spheroplast transformation. Lithium acetate transformation is widely used especially when high efficiency transformation is not essential. Improvements to this method have been described which result in higher transformation frequencies, approaching those obtained using spheroplasts (36,37). Therefore it should be possible to use this procedure for the gene replacement step of the protocol. Likewise, high efficiency yeast transformation has been described that utilises electroporation (38). It may also be possible to modify the procedure for introducing yeast DNA into the insect cells. As described above, although successful transfer of viral DNA into Sf9 cells can be accomplished by transfection or lipofection with crude, total yeast DNA preparations, we recommend that the DNA is first fractionated on a sucrose gradient. Transfer with such fractionated DNA is substantially more consistent and reproducible. The reason for this is not clear but may involve the removal of substances within the crude preparation that decrease or interfere with the transfection or lipofection procedure. Other methods for introducing DNA into cells, such as electroporation, may not be affected in the same way and therefore may eliminate the requirement for partial purification. A second possibility involves the direct transfer of recombinant DNA by fusion of yeast spheroplasts to S9 cells. Such yeast-mammalian cell fusion has been used to transfer yeast artificial chromosomes into a variety of different mammalian cell types (39,40). This procedure would eliminate altogether the requirement for isolating viral DNA from yeast.

We have demonstrated the efficacy of this system by the isolation of recombinants which directly the synthesis of two different members of the CREB/ATF family of DNA binding proteins. In both cases expression was detected although the accumulated levels of each varied significantly. It should be possible to readily isolate the proteins in a pure form by specific DNA affinity chromatography. Given the degree of overexpression as compared to the level of endogenous insect cell proteins that have the same binding specificity, the purified preparations should be highly homogenous. This has the obvious advantage over purifying CREB/ATF binding activity from mammalian cells where the binding activity is much lower and often very heterogenous. The proteins should therefore prove valuable for *in vitro* analysis and physical characterisation.
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