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Baculovirus cDNA libraries for expression cloning of genes encoding cell-surface antigens

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Abstract

We describe a method for the production of baculovirus-based cDNA libraries. By staining with monoclonal antibodies, single positive cells can be sorted and the virus encoding for the surface epitope can be isolated by limiting dilution. We have used this method to isolate cDNAs encoding several cell-surface antigens.

Keywords: Expression cloning; Baculovirus; Surface antigen

1. Introduction

Expression cloning entails the isolation of cDNAs encoding proteins which render a specific phenotype to cells in which they are expressed. Over the last decade, several approaches have been developed to enhance this powerful method of gene identification. Initially, the technique was based on the production of cDNA libraries in vectors able to replicate in particular eukaryotic cell types. For example, transient transfection of cDNA libraries produced in vectors containing the simian virus 40 origin of replication, are amplified in transfected cells expressing the SV40 Large T antigen (e.g., COS-transformed African green monkey kidney cell line) (Gluzman, 1981). Cells transfected with pools of these libraries can then be assayed for the expression of the particular protein using various screening techniques, such as growth factor/cytokine assays (Lee et al., 1985; Wong et al., 1985; Yang et al., 1986), capture on antibody coated plates (Aruffo and Seed, 1987; Seed and Aruffo, 1987; Marken et al., 1992; Simmons et al., 1992), cell sorting or binding of labeled ligands (Sims et al., 1988; Gearing et al., 1989; Mathews and Vale, 1991; Wang et al., 1991; Lin et al., 1992). By gradually reducing the pool size, pools containing the clone of interest can be rescreened and the clone eventually isolated. This strategy has been successfully used to isolate cDNAs encoding several different classes of proteins, such

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as cell-surface antigens, cytokines, cytokine receptors and transcription factors. This technique, however, is very labor intensive, since it requires multiple rounds of recovery and plasmid amplification.

Recently, several groups have reported the development of retroviral based cDNA expression libraries which offer several advantages over the previous system (Mathews and Vale, 1991; Kitamura et al., 1995; Zannettino et al., 1996). Among such advantages are: (1) the higher efficiency of gene transfer; (2) the wider range of host cell type; (3) the high titers and representativity of the libraries; and (4) the possibility of obtaining stable transfectants facilitating the screening assay.

We are interested in identifying and characterising cell-surface molecules (CSM) for which antibodies are available and whose expression pattern is limited to specific cell types. Mainly due to the limitations of the COS cell-based method described above, we sought to increase the efficiency of gene transfer, the ease of screening and the levels of expressed proteins. We describe here a method for the production of baculovirus-based cDNA expression libraries which we have successfully used to isolate cDNAs encoding CSMs using antibodies to those antigens combined with FACS.

2. Materials and Methods

2.1. cDNA synthesis

Total RNA was extracted from human placental tissue using guanidinium thiocyanate (Chomczynski and Sacchi, 1987). PolyA$^+$ mRNA was isolated using an Oligotex-dT mRNA maxi kit (Qiagen, Chatsworth, CA). Unidirectional (5′-*Eco*RI–3′-*Xho*I) double-stranded cDNA was produced using a ZAP-cDNA Synthesis Kit (Stratagene, La Jolla, CA), following the manufacturer's instructions, with minor modifications. In a total volume of 20 μl of water, 5 μg of human placental polyA$^+$ RNA and 2.8 μg of a synthetic oligo-dT-*Xho*I oligonucleotide linker-primer [5′-$(GA)_{10}$ACTAGTCTCGAG$(T)_{18}$-3′], were incubated at 70°C for 10 min and immediately cooled at 0°C for 5 min. First-strand synthesis was then performed at 42°C for 2 h in a total reaction volume of 50 μl containing first-strand buffer, 0.6 mM each of dATP, dGTP, dTTP and 0.3 mM 5-methyl dCTP, 0.8 U/ml RNase block ribonuclease inhibitor and 250 U of Stratascript RNase H$^-$ reverse transcriptase (Stratagene). Second-strand synthesis was carried out by adding to the first-strand reaction (on ice) 20 μl of second strand buffer, 6 μl of second strand nucleotide mix (10 mM each of dATP, dGTP, dTTP and 26 mM of dCTP), 3.5 μl *Escherichia coli* RNase H (0.9 U/μl) and 10 μl of *E. coli* DNA polymerase I (10 U/μl) and then incubated at 16°C for 2.5 h. The cDNA was then blunt ended by the addition to the second-strand reaction of 23 μl of blunting dNTP mix (2.5 mM each of dATP, dCTP, dGTP and dTTP) and 2.5 μl of cloned *Pfu* DNA polymerase (2.5 U/μl), and incubation for 30 min at 72°C. Following phenol extraction and ethanol precipitation, the blunted double-stranded cDNA was resuspended in 7 μl of *Eco*RI adaptors (0.4 μg/μl), which consist of an annealed pair of synthetic oligonucleotides, having the following sequences: 5′-AATTCGGCACGAG-3′ and 5′-CTCGTGCCG-3′, the latter being 5′-phosphorylated. Ligation of the adaptors to the cDNA, was then carried out in a total volume of 10 μl by the addition of 1 μl of 10 × ligation buffer (1 × ligation buffer: 50 mM Tris-HCl, pH 7.5, 7 mM $MgCl_2$, 1 mM DTT), 1 μl of 10 mM ATP and 1 μl of T4 DNA ligase (4 U/μl) at 8°C for 24 h. The ligation reaction was then heat-inactivated for 30 min at 70°C, then cooled on ice. The cDNA was subsequently phosphorylated by the addition to the ligase reaction of 1 μl of 10 × ligation buffer, 2 μl of 10 mM ATP, water to 19.3 μl and 0.7 μl of T4 polynucleotide kinase (10 U/μl), followed by incubation at 37°C for 30 min. The kinase reaction was heat-inactivated at 70°C for 30 min and then placed on ice. The cDNA was then digested with *Xho*I (40 U/μl), followed by incubation at 37°C for 1.5 h. The cDNA was fractionated on 1% NuSieve GTG Agarose (FMC BioProducts Rockland, ME) in 1 × TAE buffer. Two fractions containing cDNA sizes, from 1 to 3 kb and > 3 kb (up to ~ 12 kb), were recovered from the gel by phenol extractions of melted agarose followed by ethanol precipitation. The resulting pellet was resuspended in 20 μl of 1 × TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA). cDNA was quantitated using a microfluorimeter.

2.2. Construction of cDNA libraries in pBacPAK9 transfer vector

To allow the insertion of the cDNA, 10 μg of the baculovirus transfer vector pBacPAK9 (Clontech, Palo Alto, CA) were digested in a total reaction volume of 100 μl consisting of 1 × *Eco*RI buffer (50 mM NaCl, 100 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.025 Triton X-100) (New England Biolabs, Beverly, MA), 80 U each of *Eco*RI and *Xho*I (both enzymes at 20 U/μl) (New England Biolabs) and incubated at 37°C for 2 h. The digest was phenol extracted, ethanol precipitated and resuspended in 89 μl of water, 10 μl of 10 × CIP buffer (10 × buffer: 500 mM Tris-HCl, pH 8.5, 1 mM EDTA) (Boehringer-Mannheim, Germany) and 10 U of calf intestinal phosphatase (20 U/μl) (Boehringer-Mannheim). The reaction was incubated at 37°C for 1 h. The vector was then separated from the MCS stuffer fragment by fractionation on 0.8% NuSieve GTG Agarose (FMC BioProducts Rockland, ME, USA) in 1 × TAE buffer. β-Agarase I (1 U/μl) (New England Biolabs) was then used to recover the vector from melted gel. The cDNA was then directionally cloned into the digested vector using a ligation express kit (Clontech). Test ligations using approximately 100 ng of vector and 50–100 ng of size selected cDNA were performed to find out the optimal conditions. Ligation reactions were made up to a volume of 100 μl with 1 × TE, phenol extracted and ethanol precipitated in the presence of 20 μg of glycogen. The pellet was resuspended in 4 μl of water for electroporation into 40 μl of *E. coli* ElectroMAX D10HB cells (Gibco, Gathersburg, MD). The electroporation was performed using 0.1 cm cuvettes (BioRad, Richmond, CA) with a BioRad electroporation apparatus set at 1.6 kV, 25 μF and 200 Ω. Immediately following transformation, 1 ml of SOC medium was added to the cells which were then incubated for 1 h at 37°C in an orbital shaker (200 rpm). Titers of the libraries were established by dilution plating of the transformations on LB agar plates containing 100 μg/ml ampicillin (Sigma, St. Louis, MO) overnight at 37°C. Several colonies were picked and analyzed for the presence of cDNA inserts. Once the optimal ligation conditions were established, the ligation reactions were scaled up in order to amplify the library. The library was amplified by the growth overnight at 37°C on 150-mm LB agar plates containing 100 μg/ml ampicillin. Cells were harvested from the plates by scraping in LB medium and combined for extraction of plasmid DNA using a Maxi Plasmid Kit (Qiagen). The resulting plasmid DNA from each size-selected cDNA fraction was pooled.

2.3. Production of baculovirus libraries

Sf9 (ATCC) cells were maintained at 27°C in TC100 medium (Gibco) supplemented with 5% (v/v) heat-inactivated fetal calf serum, 1 × penicillin/streptomycin (Gibco) and 0.1% Pluronic-F68 (Gibco) (complete TC100). For cotransfection, Sf9 cells were plated at 4.5×10^6 cells per 60-mm dish in complete TC100 medium and allowed to attach for 1 h. The cells were then washed 3 times with 6 ml of TC100 medium (10 min between washes). The final wash was replaced with 3 ml of TC100 medium for the cotransfection. The 1–3 kb and > 3 kb cDNA libraries were cotransfected separately with linearized baculoviral DNA to create two size-selected baculovirus libraries. For cotransfection, 5 μg of cDNA library plasmid DNA was mixed with 5 μl of BacPAK6/*Bsu*36I digest baculovirus DNA (Clontech) in a total volume of 150 μl of TC100. The DNA mixture was then combined with 300 μl of TC100 medium containing 30 μl of Lipofectin Reagent (Gibco) and allowed to stand for 15 min at room temperature. The lipofectin/DNA mixture was then overlayed onto the washed cells and incubated at 27°C for 20 h, at which time the transfection medium was removed and replaced with 3 ml of complete TC100 medium and incubated for a further 4 days. Titers of the viral supernatants were determined by plaque assay as described. To create large stocks of high titer libraries, the viral supernatants were amplified in two rounds of low multiplicity of infection (m.o.i. approximately 1) of Sf9 cells for 4–5 days.

2.4. Infection of Sf9 cells

4×10^6 Sf9 cells in 15 ml complete TC100, were seeded into 100-mm tissue culture dishes and incubated at 27°C for 30 min. The supernatant was removed and the adherent cells overlaid with an

appropriate amount of virus in ~ 3 ml of medium to obtain the desired level of multiplicity. The plates were gently rocked at room temperature for 60 min, supplemented with 12 ml complete TC100 and incubated for an additional 48 h at 27°C.

2.5. Screening of baculovirus expression libraries

In a typical experiment, 5×10^6 Sf9 cells were seeded into a 100-mm Petri dish for 1 h. The supernatant was removed and 3 ml of an appropriate dilution of the library was added to obtain an m.o.i. of 20. The cells were incubated at room temperature on a rocking platform. After 1 h, 12 ml of complete medium were added and the cells were incubated at 27°C. After 48 h, the cells were washed twice in TC100 and incubated for 20 min with 20% human serum to saturate Fc receptors. Specific mouse monoclonal antibodies were then added for 30 min on ice. The cells were washed twice with TC100, incubated for 20 min with 20% human serum and stained with a Phycoerythrin (PE)-labeled goat anti-mouse antibody (SBA, Birmingham, AL) on ice. The cells were finally washed once and sorted on a FACSvantage (Becton Dickinson). Dead cells were gated out with propidium iodide (PI). The positive cells were seeded into 96-well plates containing 1×10^4 uninfected Sf9 cells per well in order to have a distribution of ~ 0.5 infected cells per well. After 6 days, half of the cells from each well were stained with the original monoclonal antibody as described above.

2.6. Cloning of baculovirus by limiting dilution

The viruses present in the supernatant of positive wells were cloned by limiting dilution. Several tubes containing 2×10^6 Sf9 cells in 10 ml complete medium, were infected with different dilutions of viral supernatant (from 10^{-5} to 10^{-7}). After incubation at room temperature for 2 h with slow rotation, the cells were washed twice in TC100 to remove excess virus, resuspended in 40 ml complete medium and seeded in two 96-well flat-bottom plates. After 12 days, cultures containing cloned viruses were identified visually for the presence of lysed cells. A sample of the viral supernatant was used in a short-term infection (48 h) to identify the clones encoding the correct specificity.

2.7. Recovery of inserts by PCR and sequencing

Viral DNA was extracted from supernatants of infected cells using 20% polyethylene glycol 8000 (PEG) and 1 M NaCl as described (Sambrook et al., 1989). The inserted cDNA present in recombinant viruses, was amplified by polymerase chain reaction (PCR). The reaction was performed in 100 μl with 100 ng template, 0.2 mM dNTPs, 5 U Taq extender additive (Stratagene), 5 U AmpliTaq DNA polymerase (Roche), 1 × Taq extender buffer (Stratagene) and 100 pm of the primers listed below.

Forward: 5′-ACCATCTCGCAAATAAATAAG3-′
Reverse: 5′-ACAACGCACAGAATCTAGCG-3′

The cycle conditions were: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 3 min for 40 cycles and a final extension at 72°C for 10 min. The amplified samples were resolved on a 1% agarose gel and purified using a QIAEX Gel Extraction Kit (Qiagen). The purified cDNA was cloned into the pCRII vector using a TA cloning kit following the manufacturer's instructions (Invitrogen, San Diego, CA). Cloned inserts were sequenced with Sequenase version 2.0 (United States Biochemical, Cleveland, OH). The sequences were analyzed using the FastA program (Genetics Computer groups, University Research Park, Madison, WI) (Pearson and Lipman, 1995).

3. Results

3.1. Production of a baculovirus library from human placenta

The method for the production of baculovirus expression libraries is outlined in Fig. 1. PolyA$^+$ RNA from human placenta was reverse transcribed into cDNA. The double-stranded cDNA obtained was inserted into the pBacPAK9 vector and used to transform *E. coli* cells. The baculovirus transfer vector pBacPAK9 has a multiple cloning site (MCS) for the introduction of cDNAs under the control of the baculoviral polyhedrin promoter. The MCS and polyhedrin gene promoter are flanked by regions of the viral genome which allow the homologous recombination of the transfer vector with linearized viral DNA. As the viral DNA contains a lethal

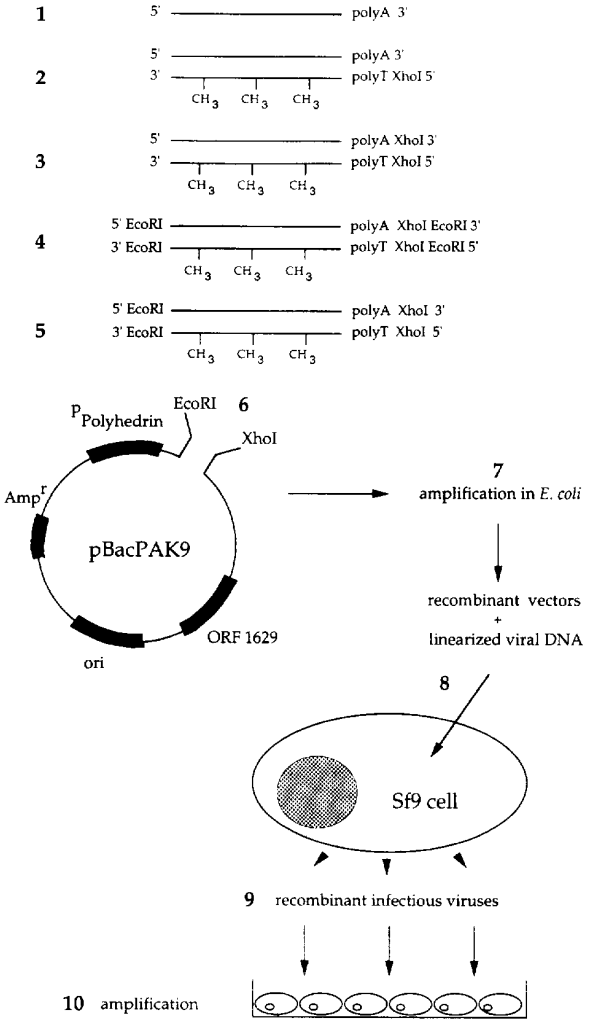

Fig. 1. Production of cDNA expression libraries in baculovirus. (*1*) Isolation of mRNA. (*2*) Synthesis of sscDNA. (*3*) Synthesis of dscDNA. (*4*) Ligation of *Eco*RI adaptors. (*5*) Digestion with *Xho*I. (*6*) Cloning into pBacPAK9 transfer vector. (*7*) Amplification in *E. coli*. (*8*) Cotransfection of Sf9 cells with viral DNA and recombinant pBacPAK9. (*9*) Production of recombinant infectious viruses. (*10*) Amplification of viral stocks.

deletion, the ORF 1629 gene, it has to be complemented by recombination with an appropriate transfer plasmid (as pBacPAK9) so that only recombinant viruses should form plaques. The library was estimated to have 6×10^5 independent clones. The recombinant vectors containing the placental cDNAs were cotransfected with defective baculovirus DNA into Sf9 cells and the recombinant baculoviruses formed by homologous recombination were recovered from the culture supernatants. The library was amplified and stocks with a titer of $\sim 8 \times 10^7$ PFU/ml were obtained.

3.2. Screening with mAb and isolation of monoclonal recombinant virus by a two-step procedure

Sf9 cells were infected with the placental library using a multiplicity of infection (m.o.i.) of 20 to

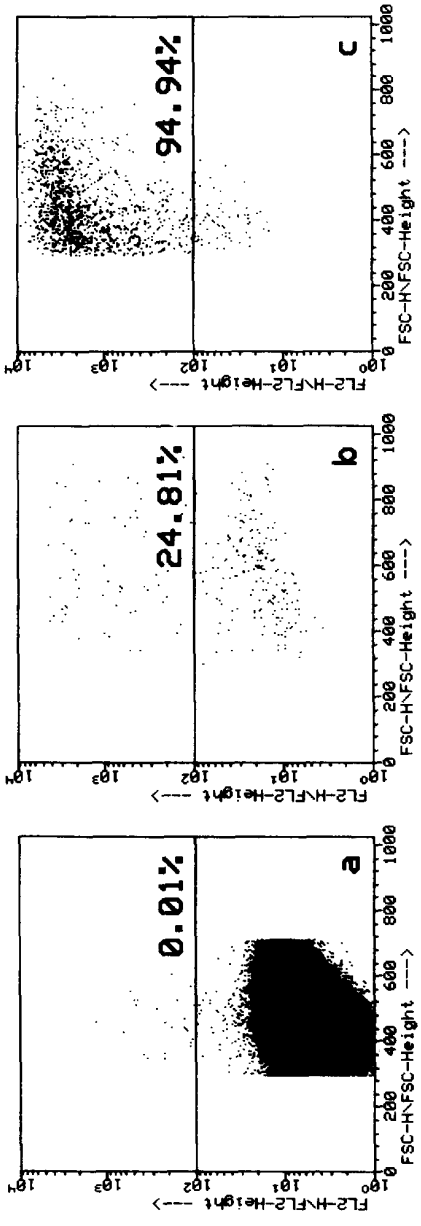

Fig. 2. Cloning of the gene encoding the epitope recognized by mAb D1093. Sf9 cells infected with the library were stained with mAb D1093. 38 positive cells were sorted and cultured in limiting dilution in the presence of uninfected Sf9 cells. After 6 days, all cultures were tested with the same mAb and the virus from positive cultures was cloned by limiting dilution. After 12 days, the viral clones were identified by their cytopathic effects and tested for their capacity to encode the D1093 epitope. (*a*) Staining with mAb D1093 and sorting gate. (*b*) Staining of one out of 8 positive cultures on day 6. (*c*) Staining of cells infected with a cloned virus.

ensure expression of at least one virus in each cell. Since the cDNA was under the control of the polyhedrin promoter, which is active in the very late phase of infection, the screening was performed after 48 h. Fig. 2 shows a typical experiment. The infected cells were stained with a monoclonal antibody of unknown specificity. To avoid non-specific binding of Ig to Fc-receptors, the latter were blocked by incubation with human serum. Dead cells were excluded by staining with PI. The positive cells stained by the monoclonal antibody (Fig. 2a) were sorted and plated in 96-well culture plates containing non-infected Sf9 cells in order to have less than one infected cell per well. After 6 days (the time required for spreading of the virus to the whole culture), all the wells were stained with the same antibody to identify those containing the recombinant virus (Fig. 2b). Due to the high m.o.i., the positive cultures contain a mixed population of viruses among which the one of interest can be present in different proportions. For this reason, it was necessary to clone viruses from, at least, two independent supernatants by limiting dilution.

For the subcloning of a single recombinant baculovirus, target cells were pulsed with different dilutions of viral supernatant to synchronize the infection. The cells were then plated in 96-well plates. After 12 days, the cultures infected by a monoclonal virus were identified by cytopathic effects and aliquots of the supernatants were tested for the presence of the virus of interest (Fig. 2c). Fig. 3 shows Sf9 cells infected with monoclonal viruses stained with the relevant, as well as irrelevant, monoclonal antibodies. The viral DNA of the positive cultures

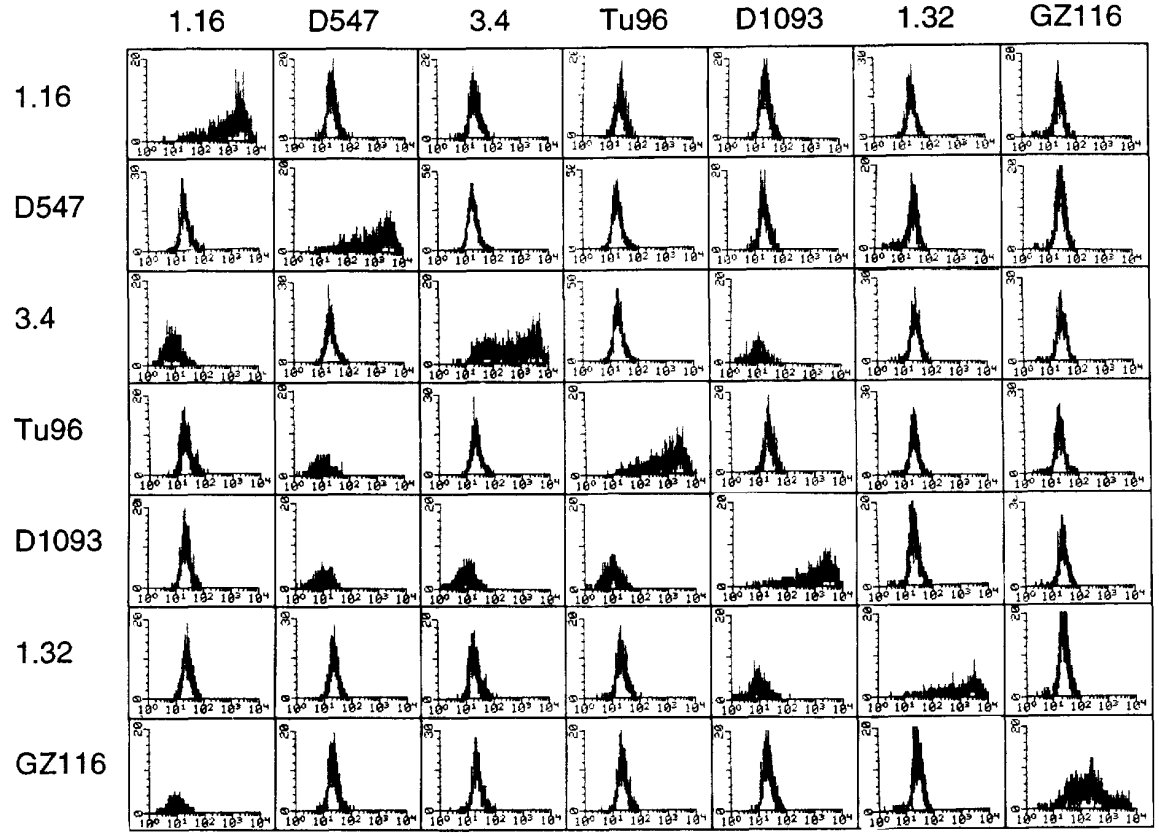

Fig. 3. Specificity of cloned viruses detected by monoclonal antibody staining. Sf9 cells were infected with various cloned viruses and stained with the relevant antibody used for isolation of the virus as well as with other irrelevant antibodies.

was isolated and the cDNA harbored by the virus was amplified by PCR using primers within the vector and then sequenced.

3.3. Identification of genes encoding CSMs

The placental expression library was used to identify the genes encoding the epitope recognized by 10 monoclonal antibodies for which the specificity was not known (Table 1).

All the genes identified have previously been described. In most cases the cDNA insert was found to be full length, even when the size was > 4 kb, as for example was the case for the transferrin receptor (CD71). In 4 cases, the identification of the genes was confirmed by staining of the infected cells with specific monoclonal antibodies against the gene product.

It is interesting that in two cases, genes that encode for molecules that are expressed as heterodimers (the β-chain of CD29 and the large subunit of 4F2), could be identified in this system in the absence of their human counterpart. It is possible that, either the molecules dimerize with a partner expressed in Sf9 cells or, simply, that the expression system is more permissive, thus allowing expression of single chains.

Table 1
Identification of the genes encoding for the recognized epitopes

mAb	Gene	Size (bp)
Tu96 [e]	CD39	1818
M1.32 [b]	CD14 [a]	1500
D1093 [b]	CD71 [a]	5010
GZ101 [c]	EGF-receptor	5532
MOON 4A11 [d]	CD29	3614
Tu143 [e]	4F2 heavy chain [a]	1861
CB43 [d]	M6	1638
M3.4 [b]	CD9	1192
Tu157 [e]	DAF	2101
M2.13 [b]	CD14 [a]	1500

[a] Confirmed by staining with other specific mAbs.
[b] From our laboratory.
[c] From Gottfried Dohr's laboratory (University of Graz, Austria).
[d] From Fabio Malavasi's laboratory (University of Turin, Italy).
[e] From Andraes Ziegler's laboratory (University of Berlin, Germany).

4. Discussion

We were interested in identifying and characterising CSMs involved in cell-type-specific expression or function. The production and screening of cDNA libraries based on a baculoviral expression vector described above represents a new and rapid approach for the isolation of cDNAs encoding CSMs.

Many CSMs and their corresponding cDNAs, have been identified in the past using mAbs in conjunction with transient cDNA library expression in eukaryotic cell lines following the method originally described by Aruffo and Seed (Aruffo and Seed, 1987; Seed and Aruffo, 1987). This method, however, has the disadvantage that several rounds of screening, and hence a great deal of time and effort, are required to isolate the cDNA of interest. In contrast, the baculovirus libraries allow rapid isolation of specific viral clones (20 days) using only two rounds of screening and does not require the isolation of the vector during the screening.

A pool of 10 mAbs directed against unknown CSMs was used to screen a placental cDNA library produced in the baculovirus system and expressed in Sf9 cells. A baculoviral clone harboring a cDNA encoding a previously described CSM was isolated corresponding to each mAb used. In 4 instances, the identity of the cDNAs in the baculoviral clones was confirmed using a second specific mAb. The size of the cDNA inserts isolated (up to 5 kb) demonstrates that full-length cDNAs can be easily cloned using this system. The primary titer and hence the representativity of these libraries is high, although we cannot rule out the possibility that recombinants may have replicated during the course of the initial transfection.

We have demonstrated that this method may be adapted to the cloning of growth factor receptors when a ligand is in hand and its cognate receptor remains unidentified. Using a biotinylated endothelin probe and streptavidin-coated magnetic beads, we were able to isolate a baculovirus harboring the endothelin B receptor from Sf9 cells infected with an adult mouse brain baculoviral cDNA library (P.N. and H.H.R., data not shown).

Advantages over the traditional COS cell-based transient expression system have recently been reported by several groups who have produced retrovi-

ral based cDNA libraries. Two limiting factors of the COS cell-based system are its restriction to specific cell types and its transient nature. Retroviral based libraries negate these drawbacks by being able to stably infect all cell types, thus allowing for screening of cDNAs by function using such assays as factor independency and transformation. Hence, this has enabled the cloning by function of cDNAs encoding cytokines (Mathews and Vale, 1991), cytokine receptors (Kitamura et al., 1995) and molecules by expression (Kitamura et al., 1995; Zannettino et al., 1996).

Thus, when assessing the best method to use for a particular expression cloning project there is now clearly a great deal of choice. That choice depending on the specific characteristics of the molecule being sought and the assay or probe being used in the screening procedure. We have demonstrated that the baculovirus based cDNA library system described here is an extremely efficient and powerful method for the cloning of cDNAs encoding CSMs using mAbs as probes.

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