Polyoma and hamster papovavirus large T antigen-mediated replication of expression shuttle vectors in Chinese hamster ovary cells

Michael Heffernan and James W.Dennis*
Samuel Lunenfeld Research Institute, Mt Sinai Hospital, 600 University Avenue, Toronto, Ontario M5G 1X5 and Department of Medical Genetics, University of Toronto, Toronto, Ontario, Canada

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ABSTRACT

Eukaryotic expression vectors have been used successfully in viral LT-expressing cell lines (ie. COS) to clone cDNAs encoding proteins that can be detected through their bio-activity or reactivity with specific antibodies. Since Chinese hamster ovary cells (CHO) have been used extensively for the isolation and characterization of somatic cell mutants, we felt it would be an advantage to develop an expression cloning system in CHO cells.

We have modified the eukaryotic expression vector CDM8 by replacing the polyoma and SV40 origins of replication with the 427bp non-coding region of the Syrian hamster papovavirus. Wild-type CHO cells and the CHO glycosylation-mutant Lec4A were transfected with plasmids bearing the early genes of either polyoma virus or hamster papovavirus in order to establish stable, LT antigen-expressing cell lines designated CHOP or CHOH, respectively. CHOP cell lines expressing polyoma LT antigen supported efficient replication of CDM8, but replicated pMH poorly. Conversely, CHOH cells expressing the hamster papovavirus LT antigen supported replication of pMH, and at a lower efficiency, CDM8. Replication of CDM8 and pMH vectors were equally efficient in selected CHOP and CHOH cell lines, respectively and comparable to that of CDM8 replication in COS-1 cells. A bacterial β-galactosidase fusion gene inserted into the multiple cloning site of a CDM8 derivative was efficiently expressed when transiently transfected into CHOP and CHOH cells but not CHO cells since only the former supports autonomous plasmid replication. These results show that expression-cloning in CHO cells expressing either polyoma virus or hamster papovavirus LT antigens is possible using either the CDM8 or the pMH vectors, respectively.

INTRODUCTION

The Chinese hamster ovary cell line (CHO) has been utilized by many laboratories for studies on somatic cell genetics (1). CHO mutants have been selected for defects in cellular metabolism, loss of cell surface molecules, resistance to cytoxic drugs, and for defects in glycoprotein glycosylation reviewed in (2,3). Although most of the available CHO mutants have been characterized biochemically, many of the genes have not been identified and cloned. Of particular interest to us are mutations affecting glycoprotein glycosylation. These mutants have been grouped into more than 28 genetic complementation classes, and together, affect points spanning most of the biochemical pathway of N-linked oligosaccharide biosynthesis and processing (3). Only a few of the hundreds of enzymes involved in oligosaccharide biosynthesis have been cloned and most of these were based on a knowledge of peptide sequence obtained from the purified proteins (4). Bovine β1−4Gal transferase cDNA was isolated from a λgt11 library using an antibody raised against the enzyme purified from milk (5). A similar approach was used to clone rat liver α2−6SA transferase (6), murine α1−3Gal transferase (7), and the allelic N-acetylgalactosaminyltransferase- and galactosyl-transferases required for A,B blood group antigen synthesis (8). It is possible that homologies between different transferases might offer a means of cloning related enzymes, however, recent observations suggest that this may not be the case. For example, no sequence homology between α1−3Gal and β1−4Gal transferases have been observed although the enzymes transfer the same sugar (7). Since purification of transferases can be a difficult and protracted process, it may be more efficient to clone cDNAs encoding many of the glycosylation processing enzymes by expression cloning.

Transfection of human genomic DNA into Lec1, a CHO mutant deficient in GlcNAc transferase I activity, has resulted in the isolation a 5Kb genomic fragment which corrects the Lec1 mutation (9). Similarly, stable expression of blood group H determinants and α1−2 fucosyltransferase in mouse L cells has been achieved by transfection with human DNA (10). However, there are several potential problems with expression-cloning by genomic transfer. For large genes, the coding region may be fragmented in the transfected DNA and therefore be nonfunctional. The identification and recovery of the relevant transfected DNA based on proximity to species-specific sequences (ie. association with alu sequences) may also be difficult. These

* To whom correspondence should be addressed

problems are avoided by using a shuttle vector in which a cDNA library can be transfected into cells and transiently expressed from episomal plasmid (11). Cells expressing the desired protein can be isolated with specific antibodies or detected through the bioactivity of the desired protein and the plasmids are recovered and amplified in E. coli. The transfection and selection procedure is repeated several times to enrich for the desired cDNA. Expression-cloning using cDNA libraries has lead to the isolation of cDNA coding for such molecules as CD2 (12), CD28 (11), human Fc receptors (13), interleukin-6 receptor (14), ICAM-2 (15) and endothelial leukocyte adhesion molecule 1 (16). Murine $\alpha 1-3$ galactosyltransferase has also been isolated from a CDM8 cDNA library which was transfected into COS cells followed by panning for adherent cells on plates coated with antibodies specific for Gal$\alpha 1-3$Gal (17).

The basic features of the CDM8 shuttle vector include a strong CMV/T7 promoter for high level expression of cDNA sequences inserted at the stuffer region, a poly adenylation site following the cDNA for mRNA stability in the mammalian cell and, SV40 and polyoma virus origins of replication for autonomous plasmid replication in permissive cells (11) (figure 1). COS cells, a simian endothelial cell line transfected with an SV40 origin-defective mutant which expresses the viral early genes (18), is the permissive host that has been used most often in cloning efforts with CDM8. In addition, the polyoma origin of replication allows CDM8 to replicate in murine cells expressing polyoma LT antigen, such as the WOP cell line, which was used to clone LFA-3 (19). The ability of the CDM8 plasmid to replicate in cells expressing LT antigen in combination with efficient transcription of cDNA inserts, results in high levels of protein expression in transfected cells and greatly increases the chance of isolating cells with the desired phenotype during the selection procedure (11).

Our aim was to adapt the CDM8 expression-cloning system for use in CHO cells and its glycosylation mutants in an effort to clone cDNAs encoding Golgi enzymes involved in oligosaccharide biosynthesis. This would facilitate the identification and characterization of cDNAs encoding proteins which either correct recessive Lec mutations or induce dominant LEC mutant phenotypes in wild type cells. To test the feasibility of this approach, we have established CHO and Lec4A cell lines expressing both the polyoma virus LT antigen and the recently cloned Syrian hamster papovavirus LT antigen. The cell lines were tested for replication competence of plasmids CDM8 and pMH. The latter is a modified CDM8 vector containing the hamster papovavirus origin of replication. The results confirm that the papovavirus ori, which was previously identified on the basis of sequence homology to the polyoma ori, does indeed function as the hamster origin of replication. More importantly, either the CDM8 vector containing the polyoma ori in polyoma large T expressing CHO cells (CHOP), or the pMH vector containing the hamster papovavirus ori in hamster large T expressing CHO cells (CHOH) allows efficient amplification of the vectors and can be used for expression-cloning in CHO cells.

MATERIALS AND METHODS

Cell Culture

Wild type CHO cells (gat$^-$) and the glycosylation mutant Lec4A were kindly provided by Dr. P. Stanley (3). FOP, a polyoma LT-expressing mammary adenocarcinoma cell line was a generous gift of Dr. J. Hassel (McMaster University, Canada). COS is a monkey kidney cell line expressing SV40 T antigens (18). The cell lines were propagated in αMEM plus 10% FCS.

Plasmids

The plasmid pSVE1-B1a containing the polyoma early genes was generously provided by Dr.J. Hassel (20). The Syrian hamster papovavirus genome inserted into pUC13 and the expression plasmid pGEM-Ha24 containing the hamster papovavirus early genes were gifts of Dr.J. Feunteun (Institut Gustave-Roussy, France) (21). The CDM8 expression vector, its derivative pcDNA I, and their E. coli host MC1061/P3 were purchased from Invitrogen (La Jolla, California). The vector pSV2neo, containing the selectable neomycin gene, was used in cotransfection studies (22).

The expression plasmid pMH was constructed in the following way. CDM8 plasmid was double digested with Bam HI and Sfi I and the 3427 bp fragment lacking both SV40 and polyoma origins of replication was gel purified. The 5′ and 3′ overhangs were filled in with the enzymes DNA polymerase I and T4 DNA polymerase, respectively. The blunt-ended fragment was subcloned into the Sma I site of pBluescript KS (Stratagene, La Jolla, California). The CDM8 portion was then removed with the flanking Eco RV and Bam HI sites from the pBluescript vector and ligated to the putitive hamster papovavirus origin of replication. The ori was prepared as a 427bp Eco RI fragment of the Syrian hamster papovavirus genome and inserted into the Eco RI site of pBluescript KS. The ori was then removed following digestion with the flanking enzymes Eco RV and Bam HI within the pBluescript multiple cloning site. Following ligation of the hamster papovavirus ori and the ori-minus fragment of CDM8 recombinants were isolated and identified as CDM8 containing the HaPv origin of replication. The plasmid was named pMH.

The vector pBFZ which contains a promoterless c-fps/lacZ fusion gene inserted into pBR322 was kindly provided by T. Pawson (University of Toronto, Canada). Flanking Bam HI and Hind III sites were used to subclone the c-fps/lacZ fusion gene into the multiple cloning site of the CDM8 derivative, pcDNA I, to create the plasmid pCDlacZ.

Isolation of CHOP cell lines

The wild type and Lec4A LT-expressing CHO cell lines were established by cotransfecting cell monolayers with pSV2neo and either pSVE1-B1a or pGEM-Ha24 (fig 1). The cells were plated at a density of 3×10^5 in 100 mm diameter tissue culture dishes (Falcon) and were cotransfected with 10 μg of supercoiled LT-encoding plasmid and 1 μg of pSV2neo per plate, using a calcium phosphate technique (Pharmacia) which was optimized for CHO cells. After 48 hours the medium was replaced with G418 containing medium at a concentration of 400 μg/ml and was changed every 4 days until foci appeared (approx. 14 days). Only one foci was isolated from each dish and these were cloned by limiting dilution. Clones with the most transformed appearance were analyzed for replication competence. Established CHOP and CHOH cell lines were propagated in αMEM containing 10% FCS and 200 μg/ml of G418 (Gibco).

DNA replication assay

Plasmid DNA was transiently transfected into cell lines using a modification of the DEAE-dextran procedure (23). Cells were plated at a density of 3×10^5 in 60 mm diameter tissue culture dishes the day before transfection. On the day of transfection, cells were washed with 5 ml of αMEM and then incubated in 2 ml of αMEM containing 350 μg/ml of DEAE-dextran, 50 mM

Tris-HCl pH 7.3, 10% NuSerum (Collaborative Research), and 2 μg of supercoiled DNA at 37°C for 2 hours in a humidified CO_2 incubator. The cells were then washed twice with 5 ml of αMEM before shocking the cells with 2 ml of a 10% DMSO solution in phosphate-buffer saline (PBS) for 1 min. Cells were washed twice in 5 ml of PBS before incubating for 48−72 hours in 5 ml of αMEM containing 10% FCS and 200 μg/ml of G418. Low molecular weight plasmid DNA was then isolated from the cells using the Hirt procedure (24). Plasmid DNA was resuspended in 50 μl of TE buffer and 10 μl of the sample was digested sequentially with Bam HI and either Mbo I or Dpn I (Boehringer Mannheim). Digested plasmid was electrophoresed through a 0.8% agarose gel and transferred to Genescreen (NEN) according to the manufacturer's recommendations. Southerns were incubated with radioactive ^{32}P probes synthesized using a random priming kit (Boehringer Mannheim). Following washes the filters were exposed to Kodak XAR-5 film from 2 hr−24 hr.

Northern analysis

Total RNA was isolated from celi lines using the method of Chomczynski and Sacchi (25). RNA was electrophoresed through a formaldehyde-agarose gel and transferred overnight onto a Genescreen membrane (NEN) according to the conditions specified by the manufacturer. Random primed probes (Boehringer Mannheim) constituting exclusively the polyoma LT coding sequence or the entire Syrian hamster papovavirus early region were used to probe the membranes.

Western analysis

Cells were transiently transfected with pCDlacZ or pBFZ using the DEAE-dextran procedure and after 48 h, cells were removed from the tissue culture plates with a sterile rubber policeman and washed twice in PBS. Cell pellets were solubilized in 10 mM Tris-HCl pH7.4, 0.15 M NaCl, 1% Triton X-100, 1 mM PMSF, 0.24 TIU/ml aprotinin and were lysed for 10 min. on ice. The suspension was centrifuged at 1000 g and the supernatant containing solubilized protein was retained for subsequent analysis. 60 μg of protein per sample was separated by a 12% sodium dodecyl sulfate-polyacrylamide gel and transferred electrophoretically onto nitrocellulose in a 25 mM Tris-HCl, 0.2 M glycine, 20% methanol buffer overnight at 4 C. Following transfer the membrane was blocked with 20% FCS in TBS. The filters were incubated with a 1/5000 dilution of anti-β galactosidase monoclonal antibody (Promega) overnight, and the alkaline detection system (Biorad) was used to detect expression of the lacZ fusion gene.

Histochemical staining

β-galactosidase activity was detected histochemically using the procedure of Sanes et al. (26). Monolayers of cells were washed once with phosphate-buffered saline (PBS) and fixed with 0.2% glutaraldehyde for 5 min. at 4°C. Cells were washed twice with PBS and stained overnight at 37°C. The staining solution contained 400 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), 1 mM $MgCl_2$, 0.45 mM potassium ferrocyanide, 0.45 mM potassium ferricyanide in PBS.

RESULTS

CDM8 plasmid replication in CHO cells

In order to establish CHO and Lec4A cell lines which express functional LT antigen (i.e. CHOP and CHOP4A), the cell lines were co-transfected with pSVE1-B1a containing the polyoma virus early genes (sT, mT, and LT) driven by the SV40 early promoter, and with the plasmid pSV2neo (fig. 1). Stable transfectants were selected in medium containing G418 and individual colonies were sub-cloned by limiting dilution. To determine whether the G418 resistant cell lines also expressed functional polyoma LT antigen, CDM8 was transiently transfected into each cell line and allowed to replicate for 48−72 hours. Low molecular weight plasmid DNA was then isolated from transfected cells, linearized with Bam HI and digested with the frequent cutters Mbo I and Dpn I, the actions of which are dependent upon the methylation-status of adenine residues at the common recognition site 'GATC'. Plasmid DNA used for transient transfections was isolated from a dam methylase-positive bacteria, E.coli MC1061/P3, and is therefore sensitive to Dpn I digestion and resistant to Mbo I. Mammalian cells do not possess dam methylase and therefore plasmid replicated in the eukaryotic host cells are expected to be resistant to Dpn I and sensitive to Mbo I digestion. Figure 2 illustrates the results of such a replication assay performed with COS-1 and CHO cells; only the former cell line expresses LT antigen (ie SV40) and was able to support CDM8 replication. CDM8 replication was observed in 4 of 10 and 2 of 24 G418 resistant clones of CHO and Lec4A, respectively. Plasmid recovered from CHOP-C2, C4, C7, CHOP4A-T24 and T19 cells was resistant to Dpn I digestion and co-migrated with the linearized CDM8 marker, indicative of replication (fig 3A and B). The low molecular weight bands present in the DPN I digests represent the bacterial input DNA transfected into the cells. The lack of replication in CHOP-C6, C17 and in other G418- resistant cell lines (data not shown), may be due to integration events of pSVE1-B1a which disrupts the LT antigen coding sequence or prohibits expression of the protein since Southern analysis of genomic DNA indicated that integrated vector sequences were present (data not shown). These results demonstrate that polyoma LT antigen is compatible with the replication machinery in selected CHO cell lines and supports replication of the CDM8 plasmid bearing a polyoma virus origin of replication.

CHOP cell lines which supported CDM8 replication did so with unequal efficiency. Whether this is a feature intrinsic to the particular transfected cell clone or due to differences in LT antigen transcript expression was explored by Northern analysis using a probe made with a portion of the polyoma early genome coding for LT antigen. The replicative efficiencies of the clones examined did not correlate with message levels (figure 4). Indeed, the CHOP cell line with the greatest replicative capacity, CHOP-C4, expresses the least amount of LT antigen message. Southern analysis of genomic DNA indicated that each CHOP cell line had approximately the same number of integrated copies of pSVE1-B1a (data not shown). Therefore, it would appear, that intrinsic differences in CHOP cell lines rather than expression levels of LT antigen may account for the observed differences in plasmid replication between the cell lines.

Hamster papovavirus ori and LT antigen for plasmid replication in CHO cells

The murine-specific replicative components of polyoma in the context of a hamster cell may be less than fully compatible for DNA replication and as such, higher levels of plasmid replication in CHO cells might be achieved by using the constituents of a hamster DNA virus. To examine this possibility, a 427bp non-coding ECO RI fragment of the hamster papovavirus genome was inserted into CDM8 in place of a 926bp portion constituting the SV40 and polyoma virus origins of replication. The SV40

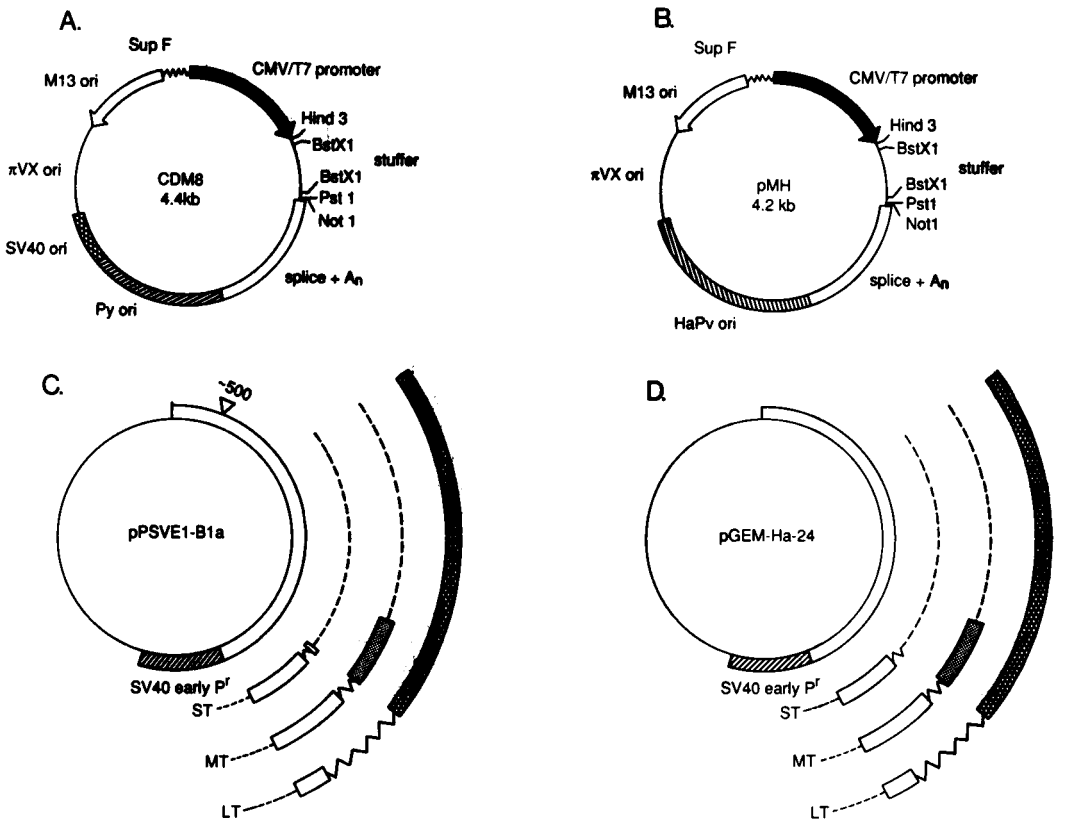


Figure 1. Schematic map of plasmids: (A), CDM8; (B), its derivative pMH; (C), pSVE1-B1a for stable expression of the polyoma virus early genes: and (D), pGEM-Ha24, for stable expression of the Syrian hamster papovavirus early genes.

and polyoma viral origins were removed from CDM8 to prevent any possible interfering effects arising from three closely spaced viral origins. The inserted 427bp sequence is the putative Syrian hamster papovavirus origin of replication based on sequence homology with polyoma, SV40, and bovine papilloma viruses (21). The resulting expression vector has been designated pMH (figure 1).

In order to use complementary hamster LT antigen, CHO cells were co-transfected with pSV2neo and the vector pGEM-HA24 which contains the SV40 early promoter driving the Syrian hamster papovavirus early genes (fig. 1). As shown in figure 5, the modified cloning vector pMH replicated to high levels in 6 of 18 G418 resistant clones. The CHOH clones showed varying degrees of replicative capacity with CHOH-H11 displaying the highest efficiency. Northern blot analysis on total RNA from CHOH cell lines showed that LT antigen was expressed in three representative cell lines that supported pMH replication (fig 4). Southern blot analysis of genomic DNA from CHOH cells showed that pGEM-HA24 integrations into the genome were comparable in number to the LT antigen-expressing cell lines examined in figure 2 (data not shown). These results demonstrate that the 427bp ECO RI non-coding fragment of the Syrian hamster papovavirus contains all the necessary sequence elements to permit DNA replication in the presence of hamster LT antigen. Furthermore, it appears that utilization of the hamster papovavirus ori and LT antigen in CHO cells (ie CHOH) did not increase the efficiency of plasmid replication (ie pMH) when compared to that of CDM8 vector in CHO cell lines expressing polyoma virus LT antigen (ie CHOP). In addition, plasmid replication in

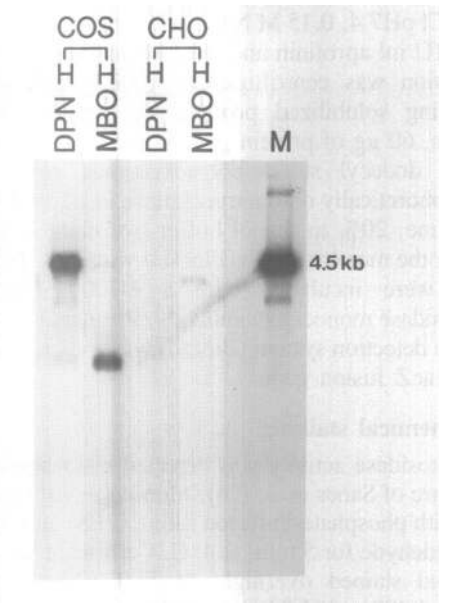
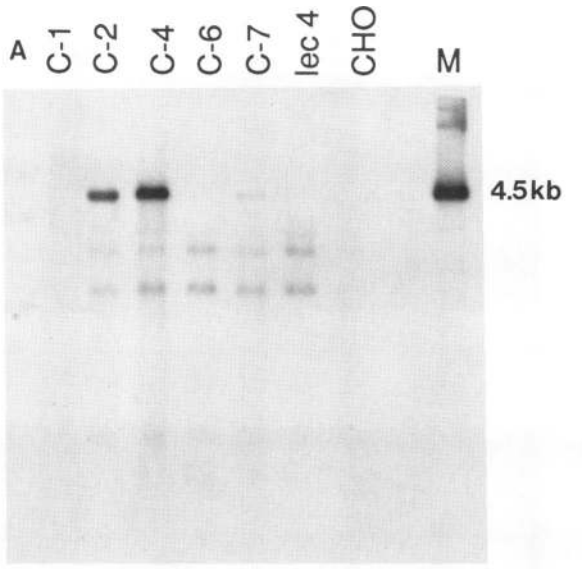


Figure 2. Replication of the CDM8 plasmid in COS-1 and CHO cells. A 2μg sample of CDM8 was transiently transfected into 3×10^5 cells and after 48 h, low molecular weight DNA was prepared from the cells. The plasmid DNA was linearized with BAM HI, and digesting with DPN I or MBO I and separated by electrophoresis, blotted and probed with ^{32}P-labelled CDM8. DNA replicated in the eukaryotic host cells is resistant to DPN I digestion and therefore comigrates in the gel with linear CDM8 DNA (M), while input CDM8 is sensitive to DPN I digestion. Conversely, replicated DNA is sensitive to MBO I digestion while input DNA is resistant.



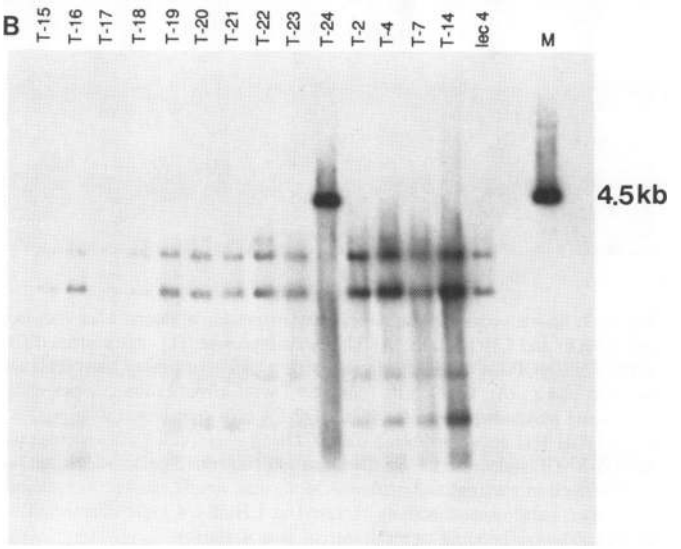


Figure 3. Replication of CDM8 in CHOP cell lines. CHOP cell lines were derived by cotransfecting CHO cells with the plasmids pSV2neo and pSVE1-B1a followed by selection in G418. CDM8 was transiently transfected into CHOP cells derived from (A), wild-type CHO cells and from (B), the CHO glycosylation mutant Lec4A. Plasmid DNA recovered from the cells after 48 hours, linearized with BAM HI, digested with DPN I and processed as described in materials and methods. Wild type CHO transfectants CHOP-C2, C4, and C7 as well as Lec4A cells lines CHOP4A-T19 and CHOP4A-T24 supported autonomous CDM8 plasmid replication.

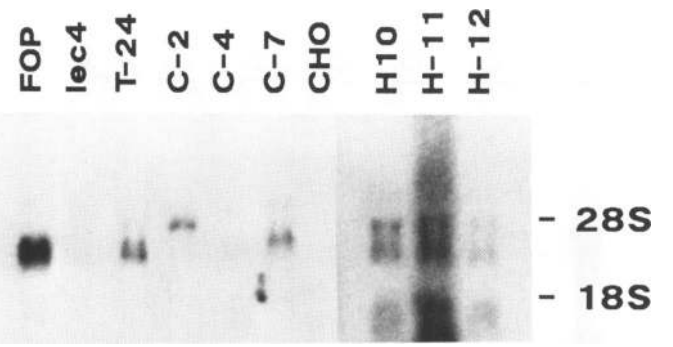


Figure 4. Detection of polyoma and hamster papovavirus LT antigen messages in replication-competent CHOP and CHOH cell lines by northern analysis. Total RNA samples from CHOP cell lines were probed with a portion of the polyoma virus early genome encoding only LT antigen. The entire hamster papovavirus early gene segment (ST, MT, LT) was used to probe total RNA from CHOH cell lines. The murine cell line FOP expresses LT antigen from stably integrated pSVE1-B1a, and serves as a positive control.

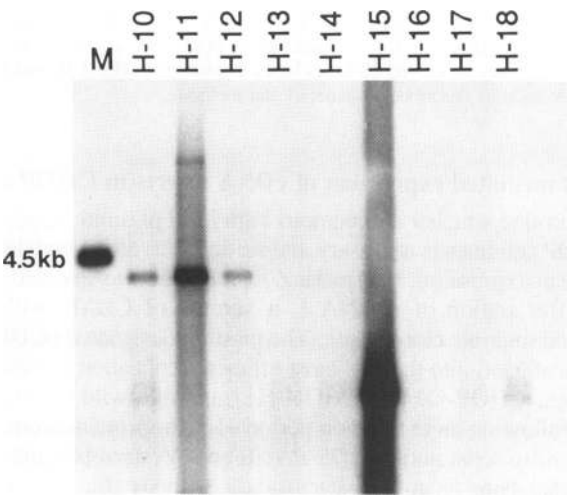


Figure 5. Replication of pMH in CHOH cell lines. The CHOH cell lines were derived by cotransfecting CHO cells with the plasmids pSV2neo and pGEM-Ha24 followed by selection in G418. The modified CDM8 expression vector, pMH, bearing the hamster papovavirus origin of replication, was transiently transfected into CHOH cell lines and plasmid DNA recovered 48 hours later, linearized with BAM HI, subjected to DPN I digestion and analyzed as described in materials and methods. The undigested pMH plasmid is smaller than CDM8 and therefore migrates in the gels slightly faster than the linear CDM8 marker.

CHOP-C4, CHOP4A-T24 and CHOP-H11 cell lines was comparable to that observed for CDM8 replication in COS-1 cells (figure 2). This comparison is based on the intensity of the linearized CDM8 marker applied to each gel.

Compatibility of polyoma virus and hamster papovavirus oris and LT antigens

To determine whether the viral replicative components (ie ori and LT antigen) of the two papovaviruses, polyoma and hamster papovavirus, can be interchanged, CHOP and CHOH cell lines (ie. CHOP-C2, C4, C7, CHOP4A-T24 and CHOH-H10, H11, H12) were compared for their abilities to replicate both the CDM8 and pMH vectors (fig. 6). CHOP cell lines supported replication of CDM8 more efficiently than pMH. However, upon longer film exposures of the southern blots, it is clear that the polyoma LT antigen is able to support replication of pMH but at a level 5−10×lower than that achieved for CDM8 in the same cell lines. The CHOH series of cell lines expressing hamster papovavirus LT antigen supported replication of both vectors, however, pMH replicated more efficiently (3−5×) than CDM8 in these cells. These data indicate that complementarity between ori and LT antigens for polyoma virus and hamster papovavirus allows for efficient plasmid replication in selected LT-antigen expressing CHO cell lines.

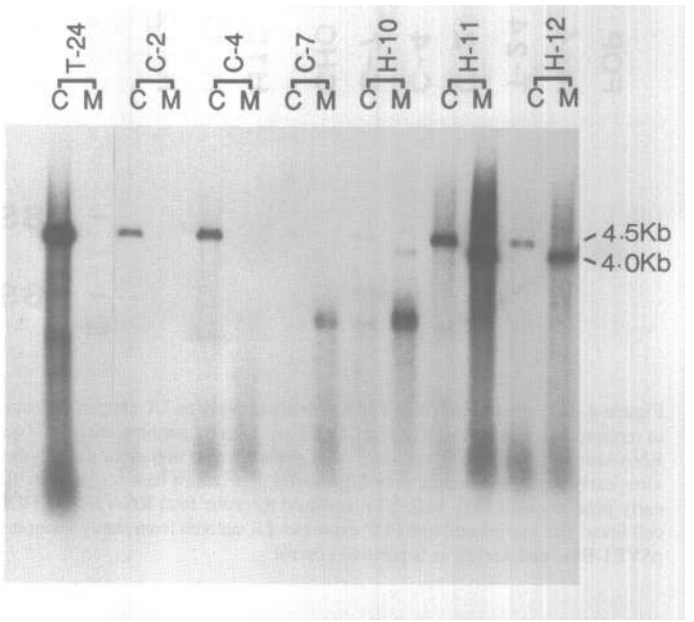


Figure 6. Comparison of CDM8 and pMH plasmid replication in selected CHOP and CHOH cell lines. CDM8 (C) and pMH (M) were each transfected into CHOP4A-T24, CHOP-C2, C4, C7, and CHOH-H10, H11, and H12 cell lines. Plasmid DNA was recovered 48 hours later, digested with BAM HI and DPN I and processed as described in material and methods.

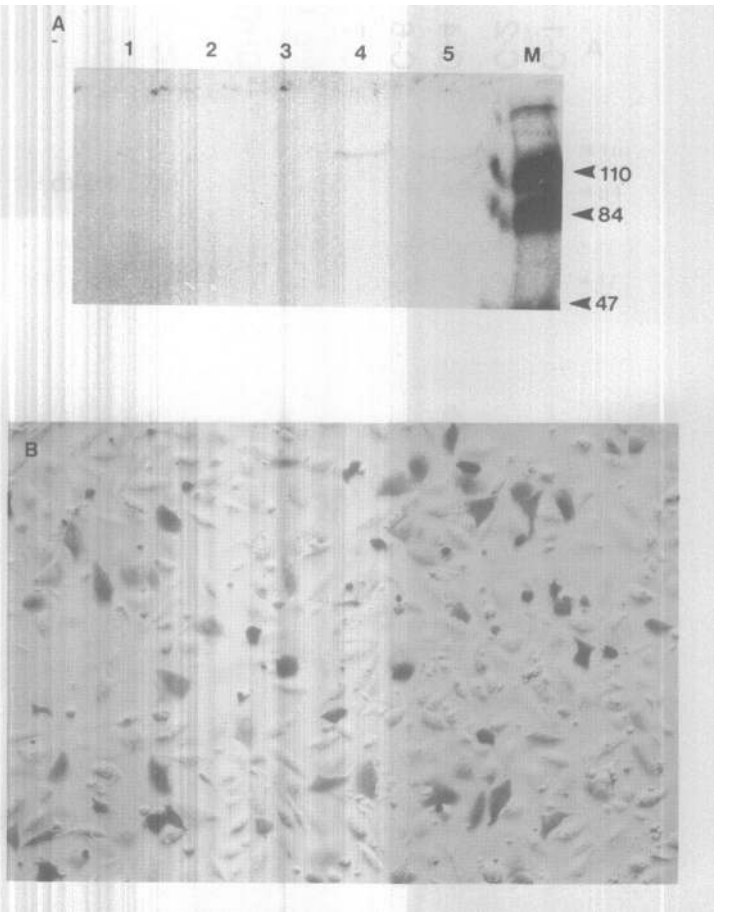


Figure 7. Shuttle vector replication enhances expression of inserts when transfected into CHOP and CHOH cells. A) The lanes represent: (1), untransfected CHO cells; (2),CHOP-C4 cells transfected with a lacZ containing, non-replicating plasmid pBFZ; (3), CHO cells transfected with a replication competent lacZ containing plasmid, pCDlacZ; (4), CHOP-C4 cells transfected with pCDlacZ; (5), CHOH-H11 cells transfected with pCDlacZ. Total cell lysates were applied to SDS-PAGE and western blotted with an anti-bacterial β-galactosidase antibody as described in material and methods. Molecular weight markers are indicated (M). B) β-galactosidase activity detected in CHOP-C4 cells transfected with pCDlacZ (corresponding to the lysate of lane 4 above).

CDM8 mediated expression of cDNA inserts in CHOP cells

To determine whether autonomous high level plasmid replication in CHOP cell lines is necessary and sufficient to direct high levels of protein expression, a c-fps/lacZ fusion gene was inserted into the stuffer region of pcDNA I, a version of CDM8 with an enhanced multiple cloning site. The plasmid designated pCDlacZ was transfected into the two most efficient replication competent cell lines, CHOP-C4 and CHOH-H11 and into wild type CHO cells. Following an expression period of 72 h, proteins from cell lysates were separated by SDS-PAGE and Western-blotting was performed using an anti β-galactosidase antibody (fig. 7A). Only in those situations where the fusion gene was expressed from an autonomously replicating plasmid, within a LT-expressing cell line, was the protein product detectable. In addition, the difference in protein expression between CHOH-H11 and CHOP-C4 cells is consistent with the less efficient replication observed for CDM8 in the CHOH cell lines (fig 6). As expected, the fusion gene product was not detected when CHOP-C4 cells were transfected with a non-replicating, promoterless construct containing the lacZ fusion gene (pBFZ). Finally, fixation and staining of pCDlacZ transfected CHOP cell monolayers for β-galactosidase activity indicated that 15−20% of the cells were expressing the enzyme (fig. 7B) while less than 0.01% of transfected CHO cells show enzyme activity (data not shown). These results demonstrate that the CDM8 vector is capable of directing expression of inserted cDNA efficiently in CHO cell lines expressing polyoma and hamster papovavirus LT antigen, and that autonomous plasmid replication clearly enhances protein expression levels.

DISCUSSION

In this report, we have established that the CDM8 expression cloning vector can replicate efficiently in CHO cell lines which express polyoma LT antigen. This implies that the replicative components of polyoma can act in a cooperative manner with the necessary host cell factors (ie. DNA polymerase I:α primase complex) (27) of Chinese hamster ovary cells to elicit replication. Furthermore, plasmid replication in CHOP cells greatly enhanced protein expression directed by a β-galactosidase fusion gene inserted into a CDM8 derivative. The plasmid pMH, a modified version of CDM8, containing the origin of replication from the Syrian hamster papovavirus in place of the polyoma and SV40 origins of replication also replicated efficiently in CHO cell lines which express the hamster papovavirus LT antigen. These results demonstrate that expression cloning of cDNA using either the CDM8 or pMH plasmids in wild type or mutant CHO cell lines expressing the appropriate viral LT antigen, is feasible.

Since the polyoma virus is murine specific (28), it was not known whether efficient polyoma-directed replication would be attained in CHO cells. Interestingly, the replication of CDM8 in CHOP cells was comparable to that of CDM8 in the SV40 LT-expressing COS-1 cells (fig. 2) and in the polyoma LT-expressing murine FOP cell line (data not shown). However, previous studies of polyoma viral replication of the entire viral genome in CHO cells showed weaker replication as compared to that obtained in a permissive murine cell line, NIH 3T3 (29). Our success in achieving similar and high level CDM8 replication

in CHO and FOP cell lines may be due, in part, to the use of the pSVE1-B1a vector. Polyoma virus is known to autoregulate its production of LT antigen during its lytic cycle (30). In order to avoid transcriptional repression of LT expression as a result of this feedback loop, pSVE1-B1a was constructed such that the 5′ upstream LT-binding autoregulatory sites were removed and fused to the SV40 early promoter (20) Removal of the regulatory binding sites for LT production, and substitution of the viral promoter and enhancer elements with the SV40 early promoter may be contributing factors to the higher levels of plasmid replication we observed.

To determine whether hamster-specific viral components might allow even more efficiently vector replication in hamster cells, the putitive origin of replication from Syrian hamster papovavirus (21) was inserted into an originless form of CDM8 to produce pMH. The inserted 427 bp fragment encompasses the majority of the hamster papovavirus non-coding sequence. The fragment contains two consecutive near-perfect palindromic sequences with sequence similarity to the polyoma virus and bovine papilloma virus origins of replication, respectively. In addition, this fragment contains a consensus SV40 enhancer and two adenovirus-like enhancers located on the late side of the ori in keeping with enhancer-dependent origin function seen in polyoma virus (31). Apart from sequence similarity, one other piece of evidence also indicated that this fragment would possess origin function. The Syrian hamster papovavirus induces lymphomas and leukemias when injected subcutaneously into newborn hamsters. Large amounts of extrachromosomal viral DNA was found in the tumour cells which were subsequently shown to possess deletions extending into the coding sequence of the late proteins thereby preventing viral lysis. However, in all cases the non-coding sequence from the SV40-like enhancer element to the viral early region was preserved (32). The present results using the pMH vector in CHOH cell lines confirms that this 427bp fragment possesses all the necessary sequence elements to function as an origin of replication. However, the level of pMH replication was equivalent to that of CDM8 in polyoma LT antigen-expressing CHOP cells. Replication reflects the ability of viral components and host machinery to function together (27,33,34). Since the species specificities of these two viruses are quite different this supports the notion that host-range can be restricted by elements of the virus life cycle other than those associated with DNA replication (29).

Although comparable replicative capacities were obtained in CHO cell lines employing either viral replicative system, interchanging the viral components reduced the replicative ability of the plasmids. Polyoma LT antigen was between 5 and 10 times less efficient at directing pMH replication compared to CDM8. However, hamster papovavirus LT antigen directed replication of both pMH and CDM8, with only a two fold advantage for the former. This indicates that the hamster papovavirus LT has a more relaxed specificity for the polyoma ori as compared to the polyoma LT antigen which directs little replication from the hamster papovavirus ori.

The importance of cloning by expression has been highlighted in recent years by the success of cloning cDNAs inserted into the vector CDM8, or one of its derivatives, and transiently expressing such inserts in COS-1 or WOP cells (11−16). Crucial to the success of this system is the requirement that COS-1 cells fail to naturally express the protein whose cDNA is being pursued. In situations where this condition cannot be met, other host cell lines would obviously be desirable. Our results show that CHO cell lines expressing polyoma LT antigen may be used in place of COS-1 and WOP cells for expression-cloning studies using CDM8 . In addition, existing or newly generated somatic cell mutants of CHO cells can be readily transfected with an expression vector for LT antigen and used to clone cDNA corresponding to the mutated genes. A similarly approach may be taken with Syrian baby hamster kidney cells (BHK). However CDM8 is unlikely to be useful in this cell line for expression cloning studies since polyoma virus is unable to infect or replicate in BHK cells (35). With the present demonstration that the Syrian hamster papovavirus LT antigen and ori are functional in CHO cells, it is probable that pMH will replicate efficiently in Syrian hamster derived BHK cells. The pMH vector, therefore, may be useful in expression cloning efforts where BHK cells is the host of choice.

Several strategies may be employed to clone cDNAs based upon their expression in appropriate eukaryotic cells. Numerous cDNAs have been cloned by transiently expressing cDNA libraries within COS cells followed by selection of those cells expressing the desired cDNA based upon bio-activity (36,37) or reactivity to antibodies specific for the gene product (11−17). With this approach in mind, we have established Lec4A polyoma LT-expressing cell lines (CHOP4A) in order to clone the glycosylation processing enzyme GlcNAc transferase V which initiates the β1−6 linked antennae of complex-type N-linked oligosaccharides. The plant lectin leukoagglutinin or L-PHA binds to β1−6 branched structures (38) and has been used to select CHO glycosylation mutants which are either deficient in GlcNAc transferase V activity (Lec4) (39) or which show inappropriate localization of the enzyme (Lec4A) (40). Following transfection of a cDNA library into CHOP4A, the lectin L-PHA has been used to recover phenotypically wild type cells. Subsequent isolation of a cDNA encoding GlcNAc transferase V is currently underway. Alternatively, in situations where a counter-selecting agent is available, it can be used to eliminate the host mutant cells while rescuing cells that have acquired plasmids with the desired cDNA. Continuous selective pressure in the growth medium should result in the outgrowth of cell clones in 2−3 weeks that have maintained episomal plasmid bearing cDNAs which confer resistance to the selective agent. In this regard, COS-1 cells transiently transfected by a gpt containing plasmid have been shown to maintain multiple copies of the plasmid episomally for an extended period of time in the presence of a biochemical selection (41). Finally, cDNAs conferring dominant mutant phenotypes may be cloned in a similar manner by synthesizing cDNA libraries in CDM8 from mutant cells and introducing the library into wild-type CHOP cells followed by panning or the addition of a selective agent.

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