Nucleic Acids Research, Vol. 19, No. 18 ***4861–4866***

Efficient cDNA cloning by direct phenotypic correction of a mutant human cell line (HPRT$^-$) using an Epstein – Barr virus-derived cDNA expression vector

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Received July 10, 1991; Revised and Accepted August 19, 1991

ABSTRACT

Human cells are, in general, poor recipients of foreign DNA, which has severely hampered the cloning of genes by direct phenotypic correction of deficient human cell lines after DNA mediated gene transfer. In this communication a methodology is presented which largely circumvents this problems. The method relies on the use of a recently developed episomal Epstein – Barr-virus-derived cDNA expression vector (Belt et al. (1989) *Gene 84*, 407 – 417). The cloning of hypoxanthine phosphoribosyltransferase (HPRT) cDNA, corresponding to a low abundant mRNA in wild type cells is used as a model system. Size fractionated poly (A)$^+$ RNA from wild type cells, which resulted in an approximately 10 fold enrichment in HPRT mRNA, was used to construct a cDNA library of 25,000 independent clones in the pECV25 vector. An HPRT deficient human cell line was transfected and subsequently selected with hygromycin B for DNA uptake. In a small scale experiment only 7000 hygromycin B^R transfectants were sufficient to isolate 2 independent HAT^R clones which were shown to replicate episomes harbouring HPRT cDNA. The first insert had a 5′ untranslated region (UTR) and a 3′ UTR perfectly in agreement with published data. The second cDNA clone harboured an unusually long 5′ UTR and a shorter 3′ UTR due to alternative polyadenylation of the HPRT transcript which has not been previously recognized.

INTRODUCTION

Cloning of mammalian genes by direct phenotypic correction through DNA-mediated gene transfer is a difficult and very laborious task especially in cases where little is known about the gene or protein of interest and only mutant cell lines of human origin are available. There are only a very few recent examples of successful correction of deficient human cell lines by cDNA or genomic DNA transfections leading to complementation and cloning of the correcting gene (1–3). Molecular cloning in this way is hampered by the—in general—poor transfectability and limited DNA uptake of human cells. It has been demonstrated that human cells are several orders of magnitude less efficient in gene cloning experiments than most commonly used rodent cells. (4–7). To circumvent this problem, a number of research groups have first generated mutant cell lines of non-human species (e.g. rodent cell lines, but lower eukaryotes or even bacteria have been used as well), which have a comparable phenotype to the human mutant, but are much easier to transfect (8–14 for examples). After the introduction of human genomic DNA or cloned cDNA into these cells the gene of interest is isolated and tested in the human or rodent mutant for complementation. However, this approach is still very laborious and depends greatly on the availability of homologous mutants of non-human origin as well as the evolutionary conservation of the studied genetic system. Hence, we have looked for a system that should facilitate cloning of human genes by direct complementation of the human mutant cell line.

Recently, we developed an EBV-derived cDNA expression vector with promising features in relation to the problem described above (15). Our results showed that in the presence of the EBV (*oriP*, EBNA-1) sequences: 1.) the transfection efficiency increases 5–10 times in comparison to vectors lacking these sequences, 2.) the copynumber is 5–10 fold higher in human cells and 3.) in a reconstruction experiment the isolation of a low-abundance sequence from a mixture of vectors is at least 100-fold more efficient then when conventionally used integrating vector systems are used. These characteristics predicted the suitability of this vector in cDNA cloning experiments.

In this report we have assessed the efficiency of the EBV vector system in cloning human cDNA from a library by direct

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phenotypic correction of a mutant human cell line. As a model system we used human cells deficient in hypoxanthine phosphoribosyltransferase (HPRT), a defect related to the Lesch-Nyhan syndrome. Our results suggest that episomal cDNA expression vectors constitute an efficient tool to isolate genes by direct complementation of human mutant cell lines.

MATERIALS AND METHODS

Isolation and Fractionation of poly(A)$^+$ RNA

Total RNA was prepared from a human chronic myelogenous leukemia cell line K562 as described by Auffray and Rougeon (16). Poly(A)$^+$ RNA was isolated by two passages over oligo dT cellulose. 400 μg of poly(A)$^+$ RNA was size-fractionated by sucrose gradient centrifugation essentially as described by Legerski et al. (17) with minor modifications.

Microinjection

170LAD primary human fibroblasts from a Lesch-Nyhan patient (18) were cultured in Ham's F10 medium supplemented with 10% fetal calf serum and antibiotics, (penicillin 100 U/ml and streptomycin 100 μg/ml). Microinjection of poly(A)$^+$ RNA fractions (2μg/μl RNA dissolved in 1mM Tris-HCl pH 7.5, 0.1mM EDTA) into Lesch-Nyhan cells was performed as described by de Jonge et al. (19). After an expression time of 0.5−4 h, cells were cultured in hypoxanthine-free Ham's F10 medium supplemented with 10% dialyzed fetal calf serum, antibiotics and ^{3}H-labeled hypoxanthine (5 μCi/ml; specific activity 6.7 Ci/mmole) for 20 hr. After Bouin fixation the preparations were processed for autoradiography (Ilford K2 tracking emulsion), exposed for 2−3 days at 4°C, developed and stained with Giemsa solution.

cDNA library construction and subcloning into pECV25

The poly(A)$^+$ RNA fraction correcting the HPRT$^-$ phenotype to the highest level was used to generate a cDNA library. The cDNA preparation was carried out essentially as described by D'Alessio et al.(20). Briefly, 3 μg of size-selected poly(A)$^+$ RNA was converted into cDNA using 200 U of murine reverse transcriptase (Bethesda Research Laboratories) and oligo(dT) as a primer. After second strand synthesis the cDNA was made blunt-ended with T4 DNA polymerase. The cDNA was cloned in the *Eco*RI site of λZAP (Stratagene) after methylation with *Eco*RI-methylase and ligation of *Eco*RI linkers as previously described (21). Total DNA was isolated from the λZAP library using standard techniques (22) and 16 μg was digested with *Not*I and *Sal*I for subcloning of the cDNA into the pECV25 vector (15, see figure 2). 4 μg of pECV25 plasmid DNA was digested with *Not*I, *Xho*I and *Pvu*II (to destroy the AGPT gene) and added to the *Not*I + *Sal*I digested cDNA (molar ratio vector:cDNA 1:1). Ligation was performed in a reaction volume of 250 μl with 50 U of T4 ligase (Pharmacia) for 16h at 14°C. The ligation mixture was used for transformation of *E.coli* strain MH1 (23).

Transfection and Isolation of HAT^R Clones

SV40-transformed XP12ROH$^-$ cells (an HPRT deficient mutant originated after 6-Thioguanine selection from an SV40-transformed primary fibroblast line of a xeroderma pigmentosum patient) were cultured in DMEM/F10 (1:1) supplemented with 10% fetal calf serum, penicillin and streptomycin. Transfection was performed as described earlier (15). Hygromycin B selection (75 μg/ml) was started 48 hr after transfection and continued for 11 days. Then HAT selection (24) was performed. HAT resistant clones were isolated and grown under continuous HAT selection pressure.

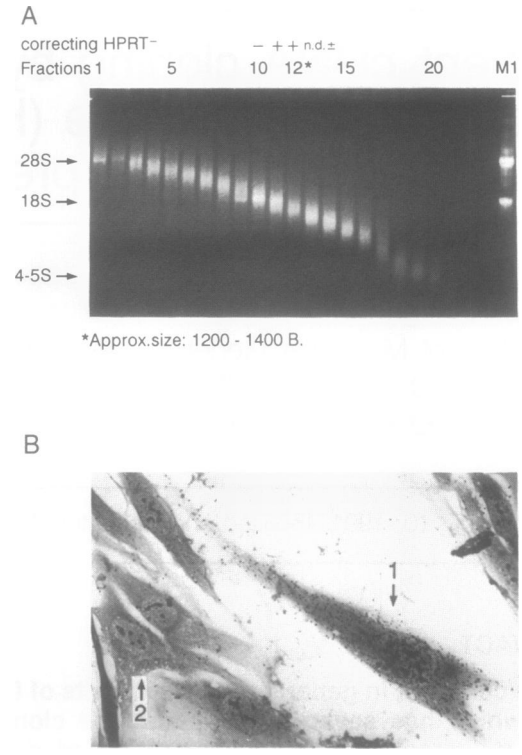

Figure 1. A. Sucrose gradient centrifugation of total poly(A)$^+$ RNA from K562 cells. Samples of collected fractions were loaded on a 1.2% agarose gel and stained with ethidium bromide after electrophoresis. M1: Ribosomal RNA markers. After microinjection of several fractions into Lesch-Nyhan cells screening was done for incorporation of tritium-labeled hypoxanthine. Results are indicated at the top panel. (n.d. = not done). **B.** Micrograph of an HPRT deficient 170LAD primary fibroblast of a Lesch-Nyhan patient, microinjected with size-fractionated poly(A)$^+$ RNA from K562 cells (fraction no. 12, fig. 1A) incubated in the presence of ^{3}H labeled hypoxanthine and processed for autoradiography. Autoradiographic grains indicate incorporation of ^{3}H labeled guanine into RNA. 1) Injected cell. 2) Non-injected cell.

Low Molecular Weight DNA isolation and sequencing

LMW DNA isolation was carried out as previously described (15). Isolated material was introduced into *Escherichia coli* strain MC1061 (23) by electroporation as detailed by Dower et al. (25) using a Biorad Gene Pulser set at 25 μF and 2.5 kV, and a Pulse Controller (Biorad) set at 200 Ω.

For sequencing of the 5′ and 3′ end of the isolated HPRT cDNA's, the inserts were subcloned into the *Eco*RI site of pUC19 (26). Double strand sequencing was performed with α-^{35}S-dATPS and T7 DNA polymerase (Pharmacia) according to the procedure specified by the enzyme supplier.

RESULTS

Generation of a cDNA library enriched in HPRT cDNA sequences

As a first step in the isolation of hypoxanthine phosphoribosyltransferase (HPRT) cDNA, K562 poly(A)$^+$ RNA was enriched in mRNA coding for HPRT through size-selection by sucrose-gradient centrifugation. Fractions were collected, microinjected

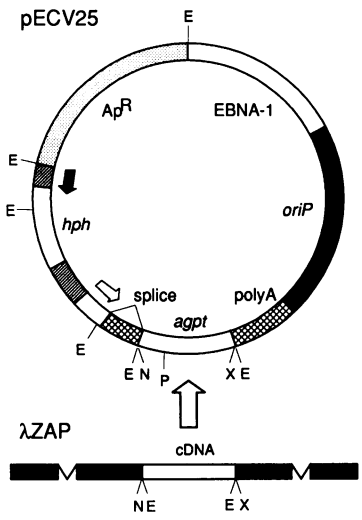

Figure 2. Subcloning of cDNA from the λZAP vector into the EBV-derived cDNA expression vector pECV25. Abbreviations: *agpt*: aminoglycoside 3'phosphotransferase gene conferring resistance to G418; Ap^R: *E. coli* β-lactamase gene conferring resistance to ampicillin; E: *Eco*RI site; EBNA-1: Epstein–Barr virus nuclear antigen 1; *hph*: hygromycin B phosphotransferase gene conferring resistance to hygromycin B; *oriP*: Epstein–Barr virus origin of replication; N: *Not*I site; P: *Pvu*II site; polyA: rabbit β-globin polyadenylation site; splice: rabbit β-globin second intron; X: *Xho*I site; Open arrow: RSV-LTR; Black arrow: HSV-1 *tk* promoter region.

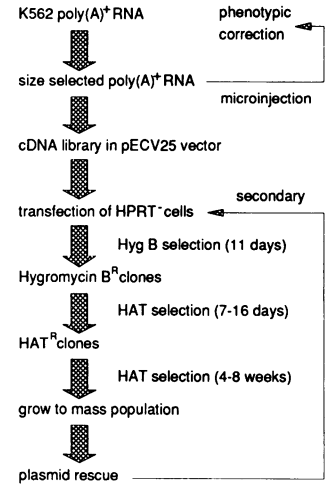

Figure 3. Scheme of the HPRT cDNA cloning procedure. (for further explanation see text.)

into 170LAD Lesch-Nyhan primary fibroblasts (HPRT deficient) and screened for the incorporation of tritium-labeled hypoxanthine (fig. 1). Fraction no. 12 with an approximate size of 1300–1400 b. on average was chosen for the preparation of a cDNA library in λZAP. This library, which had an original complexity of 25,000 independent clones and contains cDNA inserts in two possible orientations, was subcloned into the EBV-derived cDNA expression vector pECV25 (fig. 2) as described in MATERIALS and METHODS. Here approximately 200,000 independent Ap^R clones were generated. The pECVZAP1 library was used for transfection experiments with the HPRT deficient cell line $XP12ROH^-$ as a recipient. A schematic representation of the transfection and selection strategy is shown in figure 3.

Transfection of pECVZAP1 cDNA library to $HPRT^-$ cells

18 dishes of $XP12ROH^-$ cells (1×10^6 cells/dish) were transfected with 10 μg of pECVZAP1 per dish. As a control, each of 6 dishes of cells was transfected with 10 μg of pECV25 vector DNA. After 11 days of hygromycin B selection (75μg/ml) approximately 400 hyg B^R clones per dish were obtained from cells transfected with the library (transfection frequency: 4×10^{-4}), whereas 700 hyg B^R clones appeared on the control dishes (transfection frequency: 7×10^{-4}). The apparent lower transfection efficiency with pECVZAP1 may be due at least in part to overexpression of either sense or antisense orientated cDNA sequences that interfere with cell proliferation.

The hyg B^R clones were subsequently grown in HAT selection medium. One week later 3 HAT^R clones became visible on 3 separate dishes. 16 days after starting HAT selection 2 clones could be isolated and grown to mass culture. Clone no. 1 did not survive the isolation and the subsequent selection procedure. During growth of the remaining two clones we had some difficulties in passaging cells, especially from clone no. 2. This is probably due to accumulation of a population of cells loosing the HPRT cDNA but surviving selection by cross-feeding (metabolic cooperation) from cells still maintaining vectors with a HPRT insert. (27). To specifically enrich for the HPRT cDNA among other cDNA's within the HAT^R clone (one cell can replicate up to 50 different episomes, see 15), growth under HAT selection was continued for another 2 months. The rationale of such an approach is based on the fact that 2–5% of the cells per generation loose vector DNA in the absence of selecting agent (28). Thus when the specific selection is continued long enough one can expect to end up with cells harbouring a plasmid population enriched in the cDNA of interest. We have shown previously in reconstruction experiments that this is indeed the case (15).

Table 1. Efficiencies of the pCD system (33) and the pECV system (this study) in the generation of HAT^R clones.

cDNA library	Recipient cell line	Number of transfected cells	Number of transfectants	Number HAT^R clones
pCD-GM637	A9 (mouse)	10^7	10^5 ($G418^R$)	3
pECVZAP1	$XP12ROH^-$ (human)	1.8×10^7	7×10^3 (hyg B^R)	3

After two months of growing cells in HAT medium, plasmid material was rescued from both clones by low molecular weight extraction (see fig. 4). From clone no. 3 a total of 43 plasmids was rescued. Restriction analysis revealed that these 43 plasmids consisted of two classes of plasmids: one class represented by pECVH3-1 (33 out of 43) had an insert of approximately 750 bp. whereas the insert of the other class of plasmids (10 out of 43) represented by pECVH3-2 had a length of approximately 1350 bp.

From the other HAT^R clone no. 2 initially only two plasmids could be rescued. pECVH2-1 was deleted and contained only sequences for EBNA-1, ampicillin resistance, ori of pBR322 and the element *oriP*. Plasmid pECVH2-2 consisted of a tandem repeat of 3 vector copies (one incomplete copy) with 3 different cDNA inserts of approximately 700 bp., 1150 bp. and 1350 bp. (see fig. 4). To check which of these 4 classes of plasmids were

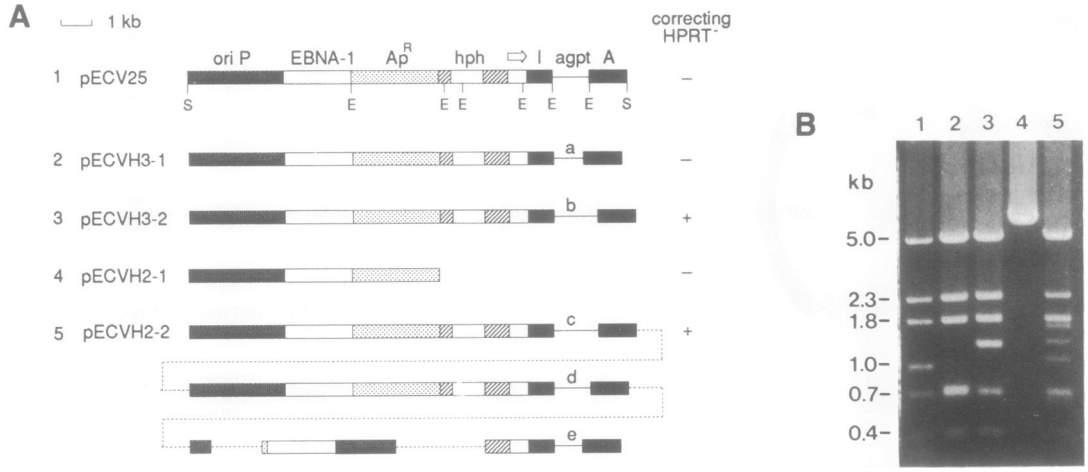

Figure 4. Plasmid DNA rescued from HAT^R clone 2 and 3. **A.** Schematic representation of plasmids pECVH3-1 and pECVH3-2 from clone 3 (no. 2 and 3) and of plasmids pECVH2-1 and pECVH2-2 from clone 2 (no. 4 and 5) as compared to pECV25 (no. 1). Results of the individual transfection of these plasmids into XP12ROH$^-$ cells are indicated on the right. **B.** *Eco*RI digest of rescued plasmids loaded on a 1% agarose gel, stained with ethidium bromide after electrophoresis. Numbers are as described under A. Note the complex structure of pECVH2-2 as indicated by the different intensities of the digested bands on the gel. Abbreviations as in figure 2. S: *Sal*I site; M: Phage λ DNA digested with *Eco*RI and *Hin*dIII; a–e: different cDNA inserts.

responsible for correcting the HPRT$^-$ phenotype, the rescued plasmids were introduced individually into XP12ROH$^-$ cells. First hygromycin B selection and then HAT selection was performed. Transfection of pECVH2-2 and pECVH3-2 resulted in the generation of HAT^R clones with a high efficiency, whereas transfection of pECVH2-1 and pECVH3-1 as well as the pECV25 control plasmid did not correct the HPRT$^-$ phenotype.

Analysis of the cDNA inserts of plasmids correcting the HPRT$^-$ phenotype

The 5′ and 3′ end of the HPRT cDNA insert from pECVH3-2 were sequenced and compared to the published HPRT cDNA sequence (29). Results are shown in figure 5. The 5′ end was only 7 bp. shorter then the cDNA cloned by Jolly et al. (29) and no difference was observed at the 3′ end of the cDNA. Since the structure of pECVH2-2 was more complicated (see above), we used pECVH3-2 as a probe and showed that the insert of approximately 1150 bp. harboured the HPRT cDNA. That this insert is indeed responsible for the acquired HAT^R phenotype is further substantiated by the following observation: A rescue of plasmid DNA from clone no. 2 performed after one extra month of culturing cells in HAT selective medium resulted in another 8 plasmids, all rearranged but originating from pECVH2-2. Out of 8 plasmids, 6 harboured the HPRT insert of 1150 bp. (data not shown). After subcloning the 1150 bp. cDNA insert into pUC19 the 5′ and 3′ end were sequenced. Comparison with the published sequence of Jolly et al. revealed that at the 5′ end the 1150 bp. cDNA is 44 bp. longer, which results in a 5′-untranslated region of 129 bp. This feature is in agreement with the reported heterogenous set of transcription initiation sites for HPRT, mapped in this region aswell (30, 31). At the 3′ end the pECVH2-2 cDNA is 322 bp. shorter as compared to the published sequence. Our results suggest that this shorter mRNA is the result of alternative polyadenylation. Indeed an additional polyadenylation signal AATAAA is present in HPRT and localized 21 bp. upstream of the poly(A) tail of the 1150 bp. cDNA (figure 5). These results are the first indications of differential polyadenylation of HPRT mRNA in human cells. Alternative polyadenylation of HPRT has also been observed in hamster cells (32), but at a different position compared to its human homolog.

DISCUSSION

In our previous work (15) an episomal (Epstein–Barr-virus-derived) cDNA expression vector was compared with conventional integrating vectors for several molecular genetic applications. A number of advantages of the episomal vector system were found. A higher transfection efficiency of human cells, a higher copynumber and the episomal state of the vector resulted in a 100 fold increase in the efficiency of isolating underrepresented inserts from a large mixture of vectors with different inserts. Therebye, selected episomes could easily be rescued from isolated human cell clones.

This communication shows that the same episomal vector system can be efficiently utilized in cDNA cloning. The isolation of 2 functional cDNA clones by direct correction of a human genetic defect in cultured cells in a small scale transfection experiment is presented. HPRT deficiency was used as a model system because many human mutant cell lines, either originating from patients with the Lesch-Nyhan syndrome or derived experimentally from cell lines, are available and efficient selection for a corrected HPRT$^+$ phenotype is possible in cell culture (24). Furthermore HPRT is a low abundant mRNA in wild type cells (2–5 mRNA copies per cell, 29). The procedure described here is highly efficient. Indeed, from 18 dishes (10^6 recipient cells per dish) transfected with the pECVZAP1 library (initial complexity 25,000 cDNA inserts cloned in two orientations), 3 independent HAT^R clones were obtained. In table 1 a comparison is made between the results obtained here and those obtained in the experiments of Okayama and Berg (33) who have isolated HAT^R clones after transfection of mouse L cells with the λNMT-pCD library (an expression library with cDNA inserted in an oriented fashion in the SV40-based pCD vector, which subsequently is incorporated in a bacteriophage lambda

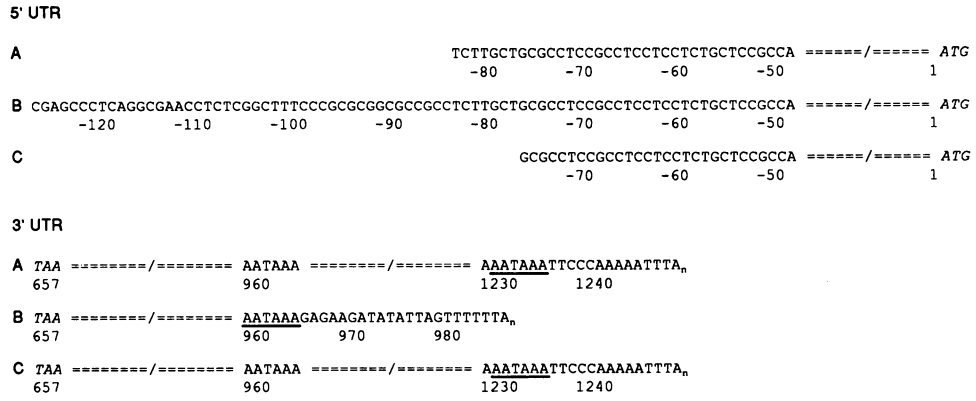

Figure 5. Sequences of the 5′ and 3′ untranslated region of the isolated HPRT cDNA's as compared to the sequence published by Jolly et al. (29). Polyadenylation signals responsible for the poly(A) tail are underlined. **A.** HPRT cDNA sequence reported by Jolly et al. (29); **B.** HPRT cDNA insert from pECVH2-2; **C.** HPRT cDNA insert from pECVH3-2. Numbers indicate the relative distance from the translational start site.

vector for efficient transduction of mammalian cells; the system is dependent on chromosomal integration for stable expression of the cDNA). Obviously an absolute comparison of both studies is not possible, as different experimental protocols were used including different cDNA libraries, recipient cells and transfection and selection procedures. However, a juxtaposition of both sets of data may nevertheless be useful to emphasize the specific advantages and limitations of each of the two systems. In the case of the λNMT-pCD experiment the high efficiency of isolating HAT^R clones is mainly due to the very high transfectability of the rodent acceptor cells which have been used (transfection frequency: 10^{-2}) whereas the frequency of selecting HAT^R clones from the pool of transfected cells is rather low (selection frequency: 3×10^{-5}). In the case of the episomal library transfected to human cells the transfection efficiency is relatively low (transfection frequency: 3.9×10^{-4}) as with many cells of human origin, however, the ability to select HAT^R clones from the pool of transfected cells (selection frequency: 4.3×10^{-4}) is clearly increased as compared to the integrating vector system. Due to this higher selection frequency the overall efficiencies of both systems are similar, suggesting that the use of the episomal expression system does compensate for the low transfectability inherent to the use of human acceptor cell lines. Of course one has to take into account that a size-fractionated mRNA population has been used for the construction of the pECVZAP1 library (from figure 1 we estimate an enrichment of approximately 10 fold in HPRT mRNA). However, since the cDNA is cloned in two possible orientations the gain in cloning efficiency obtained by mRNA enrichment is reduced to half this figure when compared with a total library cloned in an oriented fashion such as the λNMT-pCD library. Although the efficiency of the pECV/human system using a complete cDNA library might be 5−10 times less efficient as compared to the λNMT-pCD/mouse system, it is still much more efficient as what is expected by taking into account the differences in transfection efficiency and DNA uptake between both cell types. Apparently, the major advantage of using episomal cDNA expression vectors resides in the fact that most of the 50 episomes per cell (15) have the same ability to express their specific cDNA insert efficiently in contrast to conventional vectors where expression of each insert might be differently influenced by positional effects due to chromosomal integration.

Another important outcome of the present study is the fact that the use of an episomal cDNA library does significantly simplify the rescue of the cDNA clone of interest. In the case of integrating vector systems diluting-out of integrated vectors which do not harbour the cDNA of interest can only be performed by secondary or even tertiary transfections depending on the number of vector copies per cell originally present in the primary transformant. Once a transfectant has been obtained which contains only a few copies of plasmid DNA the cDNA of interest can be rescued by cosmid cloning or by PCR amplification. It should be clear that such experiments are very laborious and time consuming. In contrast, we have shown here that diluting-out of episomes which do not contain the cDNA insert is easily managed by simply culturing isolated clones obtained during the primary transfection procedure, under conditions which select specifically for the cDNA insert of interest for a period of 1−2 months. This feature is based on the fact that episomes which are not subjected to selection pressure are lost from the cells with a frequency of 2−5% per generation (28). The results presented here show that after a culture period of 2 months only a few different episomes could be isolated from HAT^R cells, a number which was small enough to allow the individual testing of each of the rescued plasmids.

At this moment we do not understand the nature of the rearranged plasmids rescued from clone no. 2. As a result of the ligation procedure during the subcloning of the cDNA into pECV25 concatamers might have been formed, which are further rearranged in the recipient cell. On the other hand they might have arisen in the transfectant itself. Several groups have reported on rearrangements or even integration of EBV-derived vectors (34−37). In contrast to our system however, in these cases repetitive sequences were present in the vector or insert used, which might have been the cause of the observed rearrangements. The data presented here show that the presence of rearranged episomes does not need to be a major disadvantage. Indeed, despite the rearrangements found in clone no. 2, rescue of plasmids harbouring full length HPRT cDNA was possible, even after 3 months of culture.

The cDNA library used in this study had a low original complexity (25,000) and was cloned in λZAP in two orientations, reducing the number of cDNA's that are properly expressed to half this figure. The use of cDNA libraries cloned in expression

vectors in an oriented manner would certainly further optimize the system described here, keeping in mind that apart from a 2 times enrichment in sequences, negative effects of highly expressed antisense products is circumvented as well. Such improvements might than make prefractionation of mRNA unnecessary if the gene of interest is constitutively expressed. Prefractionation of mRNA and libraries of higher complexity might still be necessary if genes which are expressed only at very low level are to be isolated.

During the last decades a large number of human biochemically mutant cell strains have been collected. Of special interest are cell lines which are hypersensitive to environmental agents, as selection for a corrected phenotype can be easily performed in cell culture. Unfortunately, the isolation of the genes involved has been hampered by the poor transfectability of human cell lines. We believe that episomal cDNA expression vectors constitute a new efficient tool which might greatly simplify the isolation of these human genes*.

ACKNOWLEDGEMENTS

We thank Drs. Claudio Schneider and Guido Manfioletti (EMBL, Heidelberg) for hospitality and excellent help in preparing cDNA libraries, Dr. D.Bootsma for stimulating interest and Henriëtte Grote Gansey for typing the manuscript. This work was financially supported by the Netherlands Organization of Advancement of Pure Research through the foundation of Medical Scientific Research (contract no. 900-501-091), the J.A.Cohen Institute for Radiopathology and Radiation Protection (IRS, project no. 4.2.8.) and EURATOM (contract BJ6-141-NL and BI6E-167-NL)

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* During the preparation of this manuscript Hayakawa and coworkers (1) succeeded in the isolation of the cDNA coding for O^6-methylguanine methyltransferase, making use of an EBV-derived vector system and by directly correcting the deficiency of a human cell line. Their system however was far from perfect due to the presence of the EBNA-1 gene on a separate plasmid, which resulted in the integration of the cDNA of interest.