Volume 10 Number 4 1982 Nucleic Acids Research

Gene shuttling: moving of cloned DNA into and out of eukaryotic cells

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Received 24 November 1981; Revised and Accepted 25 January 1982

ABSTRACT

Successful shuttling of cloned DNA in eukaryotic cells should allow isolation of expressed genes. We tested the utility of cosmids for moving DNA into and out of eukaryotic cells. The unique cleavage of DNA at the cos site by the terminase function of lambda was exploited to maintain the linkage between the vector and inserted gene sequences, a prerequisite for successful rescue of the transforming DNA from high molecular weight DNA of the eukaryotic transformant. A cosmid recombinant containing the HSV thymidine kinase gene and a lambda recombinant containing the chicken thymidine kinase gene were used to test the feasability of this method. It was found that these recombinants can be rescued with high efficiency from DNA of HAT-resistant cells.

INTRODUCTION

Eukaryotic cells can be transformed to a new phenotype by virtue of the stable integration and expression of DNA introduced into the cell (1 for review). These observations suggested that it might be possible to isolate the transforming DNA by rescue via E.coli, if a suitable cloning vector would be linked to the transforming DNA. Plasmid rescue has been successfully used as a method to isolate genes from eukaryotic cells after homologous (2) and heterologous (3) integration. There are, however, several disadvantages inherent in the plasmid rescue method: 1) It is difficult to find a restriction enzyme that cuts the functional gene out of the genome, since the restriction pattern of the gene of interest is usually not known. Such information is hard to obtain if the gene in question does not code for a function which can easily be identified e.g. by selection. 2) Since the size of a gene is not known it would be preferable to shuttle large DNA fragments. This is, however, difficult to achieve with

0305-1048/82/1004-1243$2.00/0

plasmids. 3) It has been observed (4) that the transformation efficiency of plasmid DNA into E.coli after passage through eukaryotic cells is markedly reduced. High efficiency of transformation of the rescued DNA is necessary for successful isolation. 4) The most serious disadvantage of plasmid rescue is, however, that the linkage between the gene of interest and the vector molecule is easily lost, when the DNA of the transformed eukaryotic cells is cleaved with an restriction enzyme in order to rescue the DNA by transformation into E.coli. We felt that these disadvantages might be overcome when DNA cloned in cosmid vectors is used to transform the eukaryotic recipient cell.

Cosmids are plasmids that contain the cohesive end site of λ-phage, which allows packaging of DNA into λ-particles. The presence of two of these sites in the same orientation at a distance of approximately 35 to 50 kb has been reported to be the obligatory requirement for the DNA substrate during packaging (5). During packaging of the DNA concatenates or circles are uniquely cleaved at the cos site. In vivo packaging of cosmid DNA allows the isolation of preparative amounts of linearized DNA. Oligomerization of linear cosmid DNA by ligation of the cohesive ends before transfection into eukaryotic cells should lead to integration products where the transforming DNA is flanked by two cosmid molecules. After identification of the eukaryotic transformant with the desired phenotype the cosmid carrying the gene of interest can be rescued by in vitro packaging of high molecular weight DNA of the transformed cell. Using a recombinant cosmid containing the HSV-thymidine kinase gene and a λ-phage recombinant containing the chicken thymidine kinase gene as models we show that DNA can be moved into and out of eukaryotic cells following this scheme.

MATERIAL AND METHODS

1. Construction of pHC79-2cos and pHC79-2cos/tk

pHC79 was cut by BglII and the fragments were isolated from a preparative agarose gel. The smaller fragment of 1.65 Kb that contains the cos site was ligated under conditions where mainly 2 to 3 molecules were ligated. The resulting oligomers were separated by agarose gel electrophoresis. The DNA in the band cor-

responding to dimers of the 1.65 Kb fragment was isolated and ligated to the large BglII fragment of pHC79 which had previously been treated with alkaline phosphatase. The DNA after ligation was used for transformation of HB101 and several transformants screened for the presence of a dimer of the 1.65 Kb BglII fragment. Presence of the dimeric BglII fragment was confirmed by restricting plasmid DNA from Ap^R, Tc^R clones with BglII and EcaI (unpublished results). BglII digestion gives the same fragments as pHC79, the 1.65 Kb band is, however, present in twice the amount. EcaI cleaves twice in pHC79-2cos producing a fragment of the same size as the small BglII fragment liberated from the tandem cos site-bearing fragments. All the enzymes that can be used for cloning with pHC79 (6) can also be used with pHC-2cos, except EcaI.

The cosmid pHC79-2cos/tk was constructed by ligating the 3.6 Kb BamHI fragment carrying the HSV thymidine kinase gene into the BamHI site of pHC79-2cos. The restriction map of pHC79-2cos/tk is shown in Fig. 1.

2. Construction of gene pools

a) E.coli 5K-DNA was digested partially with Sau 3A to yield fragments with an average length of about 40 Kb. This DNA was ligated to a six fold molar excess of BamHI cleaved pHC79 and pHC79-2cos respectively. Total DNA concentration during ligation was about 300 µg/ml.

b) High molecular weight DNA was isolated from mouse liver and partially restricted with TaqI. After phenol extraction and ethanol precipitation the DNA was ligated to ClaI digested pHC79-2cos/tk under similar conditions.

In vitro packaging of ligated DNA and transduction of cosmids in HB101 was done as described (6). Packaging efficiency was $2x10^4$ - $2x10^5$ transducing particles per µg foreign DNA. Transductions into λ-lysogens were carried out at 30°C.

3. In vivo packaging of cosmid recombinants

E.coli 1400, λBAM was obtained from P. Kourilsky (7), E.coli W3110, λ cIts857 Sam 7 from J. Collins, BHB3064 (HB101, λi^4cIts red 3 b2 Sam 7) was kindly provided by B. Hohn. λ-lysogenic strains harboring cosmids were grown to about 1 x 10^8 cells/ml at 30°C. Induction was by heating to 45°C for 30 min and pack-

aging was allowed to continue for 3 h at 37°C. Packaged cosmids were isolated as described (8).

4. Transformation of Ltk$^-$-cells

Recombinant cosmid DNA of the clone pCostk-511 was isolated after in vivo packaging (8). The linear pCostk-511 DNA and λchtk-1DNA was ligated in vitro via the cohesive ends. The efficiency of ligation was analyzed by 0.2 % agarose gel electrophoresis. Approximately one half of the DNA was present in concatenates of 3 or more molecules. In one experiment (Exp. 5 & 6, Table III) the ligated λchtk-1DNA consisting of at least 3 molecules was isolated before transfection into Ltk$^-$cells. Ltk$^-$cells were transformed following the protocol of M. Wigler (9). Southern analysis and nick translation of probe DNA was performed as previously described (10).

5. Rescue of cosmid- and λ-sequences

High molecular weight DNA ($\simeq$150Kb) was isolated from tk$^+$ colonies (9) and used for rescue of the transforming DNA by in vitro packaging. About 300 ng of high molecular weight DNA was added per packaging mix (6). Ampicillin resistant colonies were isolated after transduction of rescued cosmids into HB101. λ clones were isolated after infection of LE392.

RESULTS

1. Utility of pHC79-2cos containing two cos sites as a cloning vector.

In order to cleave the recombinant cosmid molecules at a unique site we chose to use in vivo packaging of cosmid molecules. This can most easily be achieved by induction of a lambda lysogen. During the packaging process the recombinant cosmids should be opened uniquely at the cos site. Since it was expected (11) that circular molecules containing only one cos site are packaged with low efficiency we constructed cosmids containing two cos sites (Fig. 1). To test the utility of the pHC79-2cos for the purpose described we constructed E.coli gene pools. We tested: a) whether a representative library can be made b) the efficiency of in vivo packaging of recombinant cosmids c) whether the representation of a specific gene remains unaltered by amplification and in vivo packaging.

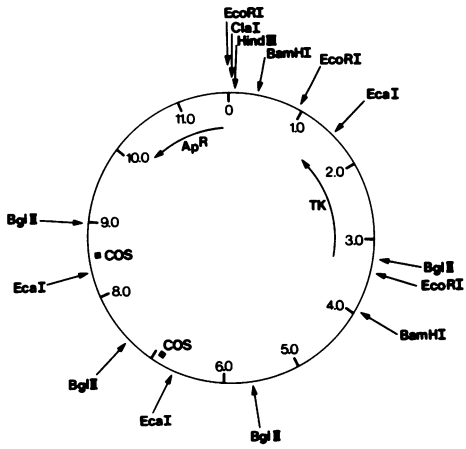

Figure 1. Physical map of pHC79-2cos/tk. Numbers indicate the distance from the EcoRI site in kilobasepairs (Kb). The selectable markers are the thymidine kinase gene (TK) of Herpes simplex virus and ampicillin resistance (Ap^R). •cos marks the position of the λcohesive end sites (6). In pHC79-2cos the BamHI fragment that contains the tk-gene is missing.

a) Construction of E.coli cosmid gene pools

High molecular weight chromosomal DNA of E.coli 5K was partially digested with Sau 3A to yield fragments with an average size of approximately 40 Kb. This Sau 3A digested chromosomal DNA was ligated to BamHI-cleaved pHC79 and pHC79-2cos respectively. After in vitro packaging of the ligated DNAs the packaged cosmids were transduced into HB101 or the lysogenic derivative BHB3064. Ampicillin resistant colonies were selected. About 80 % of the clones were tetracycline sensitive indicating true recombinants without oligomeric cosmids. Average length of hybrid cosmids was 40-50 Kb. Representation of specific genes within gene pools was analysed by testing the frequency with which the pro- and leu- mutations of HB101 and BHB3064 are complemented in the hybrid clones (5,12). Three of the four pools (Table I) gave values expected for random distribution of cloned sequences. Restriction analysis of cosmid-DNA from individual pro^+- and leu^+-colonies showed that these clones contain a whole series of overlapping chromosomal fragments. About 70 % of the clones derived from pHC79-2cos had retained the tandem cos-structure (data not shown). This is in agreement with results derived from packaging of λ-chromosomes with multiple cos-sites (13).

b) In vivo packaging

In vivo packaging of cosmids had been reported (8, 14). In initial experiments with E.coli 1400 we found relative low effi-

Table I: Gene representation in E.coli gene pools.

No of gene pool	Vector	Before Induction		After in vivo packaging and transduction	
		No pro$^+$/ No tested	No leu$^+$/ No tested	No pro$^+$/ No tested	No leu$^+$/ No tested
1	pHC79	10/ 770	8/ 770	9/ 800	12/ 800
2	pHC79	2/ 530	3/ 530	4/ 530	3/ 530
3	pHC79-2cos	1/1500	40/1500	2/1200	28/1200
4	pHC79-2cos	4/ 960	2/ 960	3/1000	5/1000

ciencies for cosmid packaging, very low phage background and no instability of the cloned sequences (unpublished observations). On the other hand using W3110 (λcIts857 Sam 7) we achieved packaging of up to 8 x 10^9 Ap^R-transducing particles per ml, but as expected packaging of the endogenous λ-phage was also very high. Furthermore we observed instability of hybrid cosmids, probably because cellular and phage recombination systems are functioning. Finally we used BHB3064 [HB101(λi^4 cIts red3 B2 Sam 7)], kindly provided by B. Hohn, for further experiments.

To test the influence of the cos-site duplication on in vivo packaging efficiencies we used two pairs of cosmids from the proline prototrophic clones. The Eca I-digests of each pair looked identical except for the presence of the 1.65 Kb band (Fig.1) that indicates the tandem BglII fragments (data not shown). In vivo packaging of tandem cos-structures can lead to deletion of the DNA between the cos-site (13), therefore cosmid DNA isolated from transducing particles consists of a mixture of linearized cosmids with one or two cos-sites.

E.coli 1400 and BHB 3064 harboring cosmids with one (pCos 31, pCos 41) or two (pCos 34, pCos 44) cos-sites were grown to about 5 x 10^7 cells/ml at 30°C. The λ-lysogen was induced by heating to 45°C for 30 minutes and packaging was allowed for 3 hours at 37°C. The number of packaged cosmids was determined by transduction into HB101 (Table II). Up to 30 or 80 transducing particles per induced cell were found for cosmids with one or two cos-sites, respectively. Table II also shows, that cosmids with two

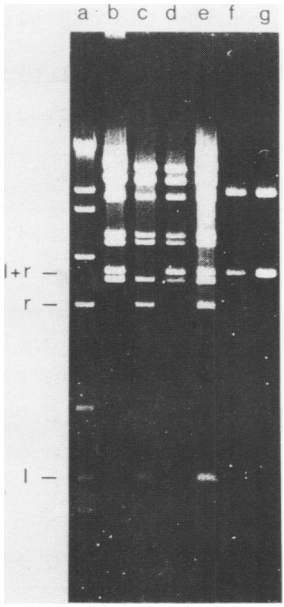

Figure 2. Linearization of cosmid DNA by in vivo packaging. Agarose gel electrophoresis of BglII digested DNA of a) λ Charon 4A b) pCos 31 isolated as ccc-DNA c) pCos 31 isolated from transducing particles d) pCos 34 ccc-DNA e) pCos 34 from transducing particles f) pHC79 g) pHC79-2cos. Samples were heated to 65°C for 10 minutes to melt the λsticky ends before loading onto the gel. l and r indicate the left and right hand BglII fragments of λCharon 4A.

tandem cos-sites gave about 2-4 times more transducing particles than the corresponding cosmids with one cos-site.

This finding was somewhat unexpected, because it had been reported, that λ-circles with a single cos-site were much poorer packaging substrates than circles with cos-duplication both in vivo and in vitro (11, 15). The number of transducing particles per induced cell dropped with increasing cell density, but the overall yield of packaged cosmids is highest at cell densities of 1-2 x 10^8/ml. For cosmids with 2 cos-sites titers of up to 10^{10} transducing particles/ml can be achieved in BHB 3064. This allowed preparative isolation of transducing particles (8). The packaged DNA was extracted from the particles and digested with BglII. Fig. 2 shows that the 1.65 Kb cos-fragment is missing in packaged pCos 31-DNA and that two new bands 1.3 and 0.35 Kb in length are found. This is in agreement with the values determined for the right and left end BglII fragments of λ Charon 4A (16), where the cos-fragment is derived from (6). In the BglII digest of packaged pCos 34-DNA these fragments are also produced, but the 1.65 Kb band is still present, even though not in molar amounts.

Table II: In vivo packaging of cosmids with one or two cos sites

Host strain	Cosmid	Cell density at time of induction cfu/mlx10^{-7}	Transducing particles/ ml x 10^{-7}	Transducing particles/ cell	Relative packaging efficiency
BHB 3064	pCos 31	5.3	172	32.4	0.41
		4.9	61	12.4	0.16
	pCos 34	5.0	391	78.2	1.00*
		5.3	334	68.0	0.81
	pCos 41	4.8	143	33.2	0.42
	pCos 44	4.0	301	75.2	0.96
1400	pCos 31	5.0	4.6	0.92	0.012
	pCos 34	4.0	21.4	5.35	0.068
	pCos 41	4.0	2.9	0.725	0.009
	pCos 44	5.8	17.0	2.93	0.037

*arbitrarily chosen as 1.00

c) Gene representation after in vivo packaging

λ -packaging is dependent on the presence of two cos-regions in the same orientation separated by DNA of 75-105 % of the length of the λ -genome, but not on the internal sequences (11). Thus cosmid pools fullfilling these requirements should be packaged with about equal efficiencies and no gross alteration of gene representation should occur. To test this point we used the E.coli gene pools (Table I). The ampicillin resistant colonies were washed from the plates, diluted to about 10^7 cells/ml and grown to about 10^8 cells/ml. Dilutions of these cultures were used to determine the gene representation before in vivo packaging. After in vivo packaging the packaged cosmids were used to infect HB101. Ampicillin resistant transductants were screened for proline- and leucine-prototrophy. No gross alteration in the relative abundance of pro- and leu-prototrophs was detected (Table I).

2. Transformation of eukaryotic cells by linearized cosmid DNA

For DNA mediated gene transfer we introduced into pHC79-2cos the 3.6 Kb BamHI fragment of M2 (17), that contains the HSV-thymidine kinase gene, a selective marker in Ltk^- cells. pHC79-

2cos/tk (Fig. 1) was cut at the unique Cla I site and ligated to mouse liver DNA which had been partially digested with Tag I. After in vitro packaging and transduction into BHB 3064 one clone (pCostk 511) was isolated from the mouse gene pool and used for further experiments. Linear pCostk-511-DNA was isolated after in vivo packaging into transducing particles (Fig. 3). The isolated linear pCostk-511-DNA was ligated with a 20-fold excess of λ-DNA to protect the sticky ends against possible nuclease attack during the transformation procedure and to reconstruct functional cos-sites. The ligation mixture was used for transformation of Ltk^- cells. Around 1000 colonies per µg of pCostk-511-DNA were found after HAT-selection (18).

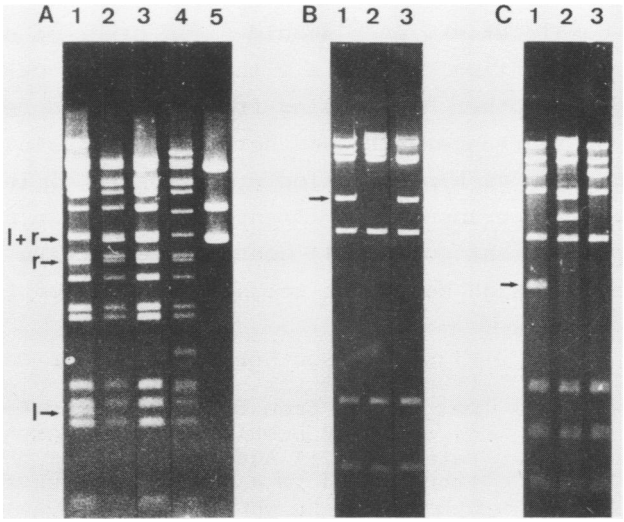

Figure 3. Restriction analysis of cosmids rescued from high molecular weight DNA of transformed Ltk^- cells. DNA fragments were separated using horizontal agarose slab-gel electrophoresis. A) BglII digests of 1) linear pCostk-511-DNA isolated from transducing particles 2) pCostk-511/42 3) pCostk-511/23 4) pCostk-511/41 and 5) pHC 79-2cos/tk. l and r indicate the position of the left and right arm BglII fragment of λ Charon 4A. The top band in lane 1 is most probably due to endogenous λ DNA. B) BamHI digests of 1) pCostk-511/42 2) pCostk-511/41 and 3) pCostk-511/23. The arrow marks the 3.6 Kb fragment that contains the thymidine kinase gene. C) Eca I digests of 1) pCostk-511/42, 2) pCostk-511/41 and 3) pCostk-511/23. The arrow marks the 1.65 Kb fragment that indicates the presence of two tandem cos-fragments in pCostk-511/42.

3. Rescue of cosmids from high molecular weight DNA of transformed Ltk$^-$ cells

Mixtures of about ten HAT-resistant transformants were grown into mass cultures. High molecular wheight DNA was isolated from these cultures, and analysed by Southern blotting. Presence of the ligation products was investigated by hybridization of nick translated pHC79-2cos/tk DNA to EcoRI digested DNA of HAT-resistant mouse cells. In addition to internal fragments from the tk region (0.9 and 2.4 Kb) hybridising bands of about 6.7, 8.4 and $\geq$ 20 Kb can be seen (Fig. 4). Fragments of these sizes are expected from the ligation of the λ sticky ends. Hybridising bands larger than 20 Kb are formed when the λ left arm fragment 21.7 Kb is ligated to λ or cosmid right end fragments. Ligation of the λ right end fragment to cosmid left end fragments or re-ligation of cosmid sticky ends should yield bands of about 6.7 and 8.4 Kb representing fragments with one or two cos-sites respectively. The other hybridising fragments could be due to integration or rearrangements. Southern analysis using pCostk-511 or rescued cosmids as hybridisation probe showed at least 15 hybridising bands superimposed on intensive background hybridisation indicating that pCostk-511 contains repetitive mouse DNA (data not shown). High molecular weight DNA was used for rescue

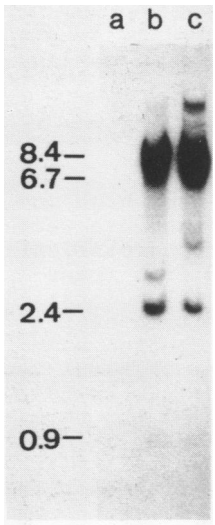

Figure 4. Southern analysis of DNA from transformed Ltk$^-$-cells. High molecular weight DNA from transformed mouse cells was digested by EcoRI and the fragments separated on a 1% Agarose gel. The DNA was transferred to a nitrocellulose filter and hybridized to pHC79-2cos/tk DNA ^{32}P-labelled by nick translation. a) Ltk -λchtk-250.1 b) Ltk-pCostk-511-17 and c) Ltk-pCostk-511-19. Sizes are given in Kb.

of cosmid DNA by in vitro packaging and subsequent transduction into E.coli HB101. In two experiments approximately 50 ampicillin resistant colonies per µg of DNA were isolated (Table III). Cosmid-DNA was prepared from these clones and characterized by restriction enzyme analysis (Fig. 3). Three types of cosmids were found: two of them (pCostk-511/23 and pCostk-511/42) are identical to pCostk-511 with one and two cos-sites respectively, the third cosmid type (pCostk-511/41), has suffered some rearrangement, most probably due to recombination with adjacent λ-sequences (unpublished observations). This type was rescued more efficiently, probably because of its larger size (46 Kb). It represented 80 % of all the rescued cosmids. pCostk-511 is only about 38 Kb long, a size near the lower boundary for efficient packaging (11).

4. Shuttling of λchtk-1

Rescue of transferred DNA by in vitro packaging should also be possible for λ -sequences. Therefore we used DNA of a λ -hybrid clone (λchtk-1) (3), that contains the chicken thymidine kinase gene as selectable marker for DNA mediated gene transfer into Ltk$^-$cells. Before transformation the λchtk-1DNA was oligomerized to retain the cos-sites. DNA of HAT resistant cells was

Table III: Rescue of cosmid- and λ-DNA from high molecular weight DNA of transformed mouse cells.

Experiment	DNA used for DNA-mediated gene transfer	DNA used for in vitro packaging	No of colonies or plaques rescued per µg of DNA
1	pCostk-511 ligated with λ-DNA	mixture from 10 clones	56
2	- " -	- " -	50
3	λchtk-1 ligated	mixture from ~ 100 clones	10
4	- " -	single clone	3
5	λchtk-1 ligated, isolated oligomers	mixture from ~300 clones	630
6	- " -	single subclone	570

isolated from clone mixtures or subclones and used for Southern hybridisation and in vitro packaging (Table III). λchtk-1 phage were identified by hybridization to a plasmid containing the chicken thymidine kinase gene (3). Restriction pattern and Southern hybridisation of four plaque purified phage are as expected for λ chtk-1 (Fig. 5).

DISCUSSION

These studies were initiated to find ways for moving DNA into and out of eukaryotic cells. Such shuttling of DNA should be valuable for the isolation of eukaryotic genes. This might most easily be achieved in the following way: a genomic library is constructed and suitable eukaryotic recipient cells are transformed with DNA of the entire gene library. From the DNA of the eukaryotic transformant which expresses the function of interest, the gene responsible for this function is recovered by using the

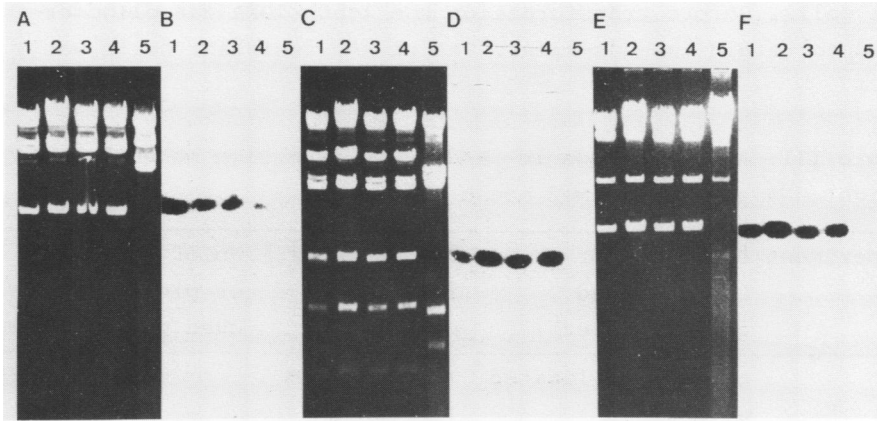

Figure 5. Analysis of λ-chtk-phage rescued from high molecular weight DNA of transformed mouse cells by in vitro packaging. DNA of four rescued phage was digested with EcoRI (A,B), EcoRI and HindIII (C,D) and HindIII (E,F). The fragments were separated by Agarose gel electrophoresis, blotted to nitrocellulose filters and hybridized to nick translated pchtk-2 DNA (3). A,C and E show the Ethidiumbromide stained gel, B,D, and F the corresponding autoradiograms. 1) λ chtk-8/1 2) λ chtk-250.1/1 3) λ chtk-250.1/3 4) λchtk-250.1/3 and 5) λ Charon 4A. λchtk-8/1 was rescued from DNA of a single HAT resistant clone (Table III, Exp. 4), λchtk-250.1/1,3 and 4 from DNA of a mixture of about 100 clones (Table III, Exp. 3).

original cloning vehicle molecule as a rescuant vector. We reasoned that cosmids would obviate many of the disadvantages experienced during plasmid rescue (see Introduction).

The most serious problem, the loss of linkage experienced in plasmid rescue is obviated, since the cosmid recombinant should preserve its original structure during shuttling. The ability of the lambda ter protein to cleave DNA uniquelly at the cos-site (12 bp sticky end sequence) is exploited in the method at several steps: a) when the cosmid recombinant is constructed, b) when the recombinant cosmid DNA is uniquely opened at the cos-site during in vivo packaging, c) when the cosmid recombinant DNA is excised from the DNA of the eukaryotic transformant.

In vivo packaging also provides a convenient way of amplification and storage of cosmid gene banks.

Since up to 45 Kb of DNA can be cloned in a cosmid vector, it is possible to clone large genes or multiple genes lying adjacent. It should also allow to clone genes and their regulatory region(s), even if they lie far apart.

To test the feasability of this approach we asked whether cosmid and lambda recombinants containing the thymidine kinase gene can be introduced into Ltk^- cells in such a form that rescue is possible. The experiments show that this can be done with efficiencies that should allow isolation of a gene from a genomic library.

Since the packaging reaction is very efficient it is necessary to ensure that the rescued cosmids are not resulting from packaging of DNA not introduced into the cell but contaminating the cell culture. This is, however, very unlikely, since similar efficiencies are observed when transformant DNA of mass cultures or of a clone is used as substrate for rescue. Furthermore the efficient rescue of one type of rearranged cosmids indicates that passage through eukaryotic cells had occured. The cos site in pCostk-511 had been reconstructed by ligation to λ-DNA before transformation of Ltk^--cells. The rearranged cosmids contain sequences from the right arm of λ (unpublished results). Work is now in progress to isolate a selectable gene from a genomic library using this method.

ACKNOWLEDGEMENT

We thank Dr. M. Wigler for the recombinants containing the chicken thymidine kinase gene, Prof. C. Weissmann for plasmid M2, Dr. B. Hohn for strain BHB 3064 and Dr. J. Collins for cosmid pHC79 and helpful discussions. We thank S. Jeep, A. Scott and R. Bonewald for expert assistance and B. Seeger-Kunth for typing of the manuscript. Some of the experiments were initiated while one of us (G.S.) was visiting the laboratories of M. Wigler and D. Kurtz at the Cold Spring Harbor Laboratory.

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