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# A new recombinant DNA strategy for the molecular cloning of rare membrane proteins

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We have constructed a cDNA library in the plasmid expression vector pUEX enriched in sequences encoding membrane proteins. The procedure involved positive selection of sequences common to two different rat tissues (thus excluding tissue-specific mRNA) followed by positive selection between this material and RNA extracted from membrane bound polysomes (thus excluding cytoplasmic proteins). The resultant library prepared from rat kidney cDNA hybridized with rat liver poly(A)$^+$ RNA, contained 30000 clones and was shown to be enriched in cDNAs encoding membrane proteins. Seventeen clones selected because they encode large fusion proteins were shown to be single copy in the library, and not present in nucleotide data banks. Thus the strategy is particularly suitable for cloning low abundance cDNAs encoding membrane proteins.

## INTRODUCTION

Several disciplines in biological research now demand sequence data for groups of rare cDNA molecules. Additional biological information would be obtained if such rare cDNA clones could first be sorted into groups, for example those encoding membrane rather than cytoplasmic proteins. A particular application of cDNA libraries encoding membrane proteins is the elucidation of the molecular targetting and retention signals which determine their final intracellular location. The availability of cDNAs for these proteins would permit the dissection of molecular address signals by sequence comparison and molecular cut and paste experiments. In addition it is important to study cDNAs encoding rare membrane proteins since it is possible that they achieve their intracellular location by a different mechanism to abundant membrane proteins.

We have recently described a strategy for obtaining organelle-specific cDNA molecules in which clones corresponding to individual antibodies in a polyclonal anti-(organelle membrane) serum are immune-selected into different groups using immuno-affinity purification of the polyclonal serum on expressed fusion proteins from individual clones (Luzio *et al.*, 1990). However, in the course of these experiments it was shown that polyclonal antisera raised against a membrane fraction may only detect a small percentage of the membrane proteins on a Western blot and when an anti(-Golgi membrane) serum was used for expression cloning more than half of the cDNAs obtained were of high abundance (1 in 3000 to 1 in 10000). Additional low abundance clones would be relatively difficult to obtain using this procedure because many high abundance clones would be obtained in screening before a new low abundance clone was encountered. Very immunogenic, highly glycosylated species of membrane proteins may not even be genuinely resident in a particular organelle. Thus, for instance, the most abundant clone obtained using this screening procedure with an anti-Golgi serum was shown by sequence homology to be the rat pIg receptor (Banting *et al.*, 1989) which, although a known immunogenic component of the Golgi complex (Howell & Sztul, 1982), is not regarded as a resident Golgi protein.

In this paper we describe an approach in which the selection for housekeeping membrane proteins is made during construction of the cDNA library, and identification of the membrane of origin of each cDNA encoded protein is made subsequently using antibodies against cloned epitopes (ACEs) raised from the expressed fusion proteins. Using this strategy the clones obtained should be randomly distributed between intracellular membranes, making it possible to identify markers for organelles and maybe previously undescribed membranous structures in the cell. The approach used employs hydroxyapatite columns to isolate cDNA–RNA hybrids formed between a single-stranded cDNA from one tissue and mRNA from a different tissue or subcellular fraction (Davis, 1986). By selecting the hybrid peak from the column, a library enriched in cDNAs coding for proteins found in both tissues can be constructed. We have used two such positive selection steps, first between cDNA from rat kidney and mRNA from rat liver, followed by a second selection between the remaining cDNA and mRNA from membrane-bound polysomes of rat liver. The cDNA library should be enriched in molecules present in all of these fractions, namely cDNA encoding common membrane proteins and common secretory proteins. Since most secretory products are tissue-specific and of relatively high abundance they are depleted in the library, leaving cDNA molecules encoding common membrane proteins many of which will be of low abundance.

In the present study the selected cDNA library was cloned in the bacterial plasmid expression vector pUEX (Bressan & Stanley, 1987), since this allows well-controlled expression of fusion proteins at high levels (about 30% of total protein) and an efficient adaptor strategy for library construction is available (Haymerle *et al.*, 1986).

Abbreviations used: LRP, LDL receptor-like protein; ORF, open reading frame.

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## MATERIALS AND METHODS

### RNA preparation

RNA was prepared by using the method of Chomczynski & Sacchi (1987). The preparation of mRNA from membrane bound polysomes was modified from Mechler & Rabbitts (1981) using a vertical rotor to separate membrane material in a post-mitochondrial supernatant by floatation in a discontinous sucrose gradient. Rat liver ribosomes were prepared as described by Martin *et al.* (1971).

### cDNA first strand

First strand cDNA was synthesized in a volume of 50 μl from 15 μg of kidney RNA which had been twice purified by oligo(dT)-cellulose affinity chromatography, using 1.5 μg of random primer pd$(N)_6$ (Pharmacia) as described by Haymerle *et al.* (1986). After 2 h incubation at 42 °C, approx. 6 μg of cDNA had been synthesized. NaOH was added to a final concentration of 0.2 M and the RNA strand was hydrolysed for 20 min at 68 °C. The reaction mix was then neutralized by addition of HCl to 0.2 M. First strand cDNA was separated from free nucleotides by gel filtration over a 3 ml column of Sephadex G-50 in a 5 ml plastic pipette equilibrated in sterile water treated with Chelex-100 (Bio-Rad), and then lyophilized.

### Positive selection of first strand cDNA

The single-strand cDNA was dissolved in 6 μl of water to which sodium phosphate buffer, pH 7.0, was added to 0.25 M, dithiothreitol to 62.5 mM, SDS to 0.125% and EDTA to 6.25 mM in a final volume of 8 μl, essentially as described by Davis (1986). This material was fractionated on a 1 ml hydroxyapatite column (Bio-Rad) maintained at 68 °C as described by Davis (1986), to remove any hairpin-looped cDNA. The single-stranded material eluting in 120 mM-sodium phosphate buffer containing 0.1% SDS was again desalted on a G-50 column, lyophilized and then dissolved in water containing 20 μg of rat liver poly$(A)^+$ RNA. A hybridization mix containing 0.25 M-sodium phosphate, 0.125% SDS and 62.5 mM-dithiothreitol was made up as above, but in a final volume of 20 μl and hybridized for 22 h at 68 °C. The reaction mix was then passed over another hydroxyapatite column and the double-stranded peak eluting in 500 mM-sodium phosphate buffer containing 0.1% SDS was taken, desalted on a G-50 column and lyophilized. RNA was hydrolysed from the double-stranded material with NaOH and the reaction mix neutralized with HCl as described above. In a second round of hybridization 11 μg of RNA extracted from membrane-bound polysomes from rat liver was used in a hybridization mix of 16 μl. After 21 h hybridization at 68 °C a final hydroxyapatite column was run. The double-stranded material from this fractionation was desalted over a G-50 column, lyophilized and used directly for second strand synthesis by the method of Gubler & Hoffman (1983). Half (25 ng) of the resultant double-stranded cDNA was cloned into pUEX1 (Bressan & Stanley, 1987), as described by Haymerle *et al.* (1986) and used to transform competent cells made from the *Escherichia coli* strain DH5 (Hanahan, 1985) which led to the production of a library of 30000 clones.

### Open reading frame screening

Individual colonies were picked into 5 ml of L broth containing 50 μg of ampicillin/ml and grown overnight at 30 °C with agitation; 2 μl of this culture was diluted into 400 μl of medium in a sterile Eppendorf microfuge tube and grown for a further 2 h at 30 °C. The tubes were then transferred to a 42 °C incubator and rotated for another 2 h. The tubes were then cooled on ice, centrifuged for 5 min in an Eppendorf centrifuge and the pellet taken up in 25 μl of sample buffer (200 mM-Tris, pH 8.8) containing 4% SDS and 50 mM-dithiothreitol. After boiling for 5 min the DNA in the samples was sheared by passage through a Hamilton syringe and 5 μl was used to load a 5 cm × 2 cm 7%-polyacrylamide gel. After electrophoresis the gels were stained in Serva Brilliant Blue.

### Hybridization studies

A $^{32}$P-labelled first strand cDNA probe complementary to rat liver rRNA was synthesized using random primer pd$(N)_6$ (Haymerle *et al.*, 1986). cDNA clones for rat albumin and β-actin were obtained from their human and chicken counterparts respectively by screening a rat liver cDNA library. DNA fragments excised from plasmids and purified on NA45 paper (Schleicher und Schüll) were used for labelling with $^{32}$P nucleotides by the random primed extension method (Feinberg & Vogelstein, 1983). The frequency of clones in each parental cDNA library, i.e. total rat kidney or total rat liver (Haymerle *et al.*, 1986; Banting *et al.*, 1989), was estimated in the following way. Pools of 1000 colonies were scraped off large agar plates and used for preparation of mixed plasmid DNA. Then 10 μg of each pooled DNA was digested with *Bam*HI, separated on a 1% agarose gel and transfered to nitrocellulose filters. The filters were hybridized with each probe (Maniatis *et al.*, 1982) and the number of pools of 1000 colonies containing a hybridizing band recorded. cDNA inserts from open reading frame clones isolated from the selected library were screened in the same way using DNA extracted from pools of 5000, 10000 and 20000 colonies.

### Raising antisera against fusion proteins

L-broth (100 ml) containing 50 μg of ampicillin/ml was inoculated from fresh overnight cultures of colony purified clones and grown to early exponential phase ($5 \times 10^7$ cells/ml). The cultures were then transferred to a water bath at 42 °C and incubated with aeration for a further 2 h. After cooling the cells were harvested and inclusion bodies prepared by the method of Seth & Vande Woude (1985). Purified inclusion bodies were solubilized in PAGE sample buffer containing 4% SDS and electrophoresed on preparative 7% polyacrylamide gels. The position of the fusion protein band was determined by staining a parallel strip of gel and then the remainder was cut out and homogenized in phosphate-buffered saline. After incubation overnight at 4 °C the gel fragments were spun down and the supernatant emulsified with Freund's adjuvant for immunization of rabbits according to the protocol of Louvard *et al.* (1982).

### Characterization of antisera

Western blotting was performed according to Burnette (1981) omitting methanol from the transfer buffer. For blocking and antibody incubation, phosphate-buffered saline containing 3% (w/v) skimmed milk powder was used. Antibodies were used at a dilution of 1:1000 and visualized using horse-radish peroxidase- or alkaline phosphatase-conjugated second antibodies. Extraction of membrane proteins in Triton X-114 was performed as described by Bordier (1981). Immunofluorescence of NRK cells grown on glass coverslips in 35 mm culture dishes was performed as previously described (Luzio *et al.* 1990).

### Affinity purification of antibodies

Protein bound to nitrocellulose filters was incubated with antibodies as for Western blotting. After extensive washing, antibodies were eluted from the protein with ice-cold 200 mM-glycine, pH 2.5, containing 0.1% gelatin. After 4 min the eluted antibodies were transferred to a new tube and the pH immediately brought to approx. 7.0 by addition of 2 M-Tris base.

### Rat liver fractionation

A fresh liver (10 g) from a rat starved overnight was homogenized (10 strokes at 2000 rev./min in a Teflon/glass homogenizer) in 30 ml of ice-cold 0.25 M-sucrose containing 1 mM-$MgCl_2$ and 10 mM-Hepes, pH 7.4. A low speed ('nuclear') fraction, collected after sedimentation at 1000 **g** for 10 min, was resuspended in 15 ml of the homogenization medium and sedimented again at 1000 **g** for 10 min. The combined supernatant fractions were then sedimented at 10000 **g** for 10 min to give a 'mitochondrial fraction' which was also washed once by resuspension in 10 ml of homogenization medium and resedimentation. A third, 'microsomal' fraction was made by sedimentation of the remaining supernatant fraction at 100000 **g** for 60 min. The three membranous fractions were dialysed against phosphate-buffered saline to remove sucrose and diluted to about the same protein concentration (1.5 mg/ml). These were then extracted four times with 1% Triton X-114 as described (Bordier, 1981). The first three detergent phases were pooled, and the fourth one was discarded. Both fractions were precipitated with 5 vol. of acetone (−20 °C) and dissolved in the same volume giving a detergent and aqueous phase for each fraction. Equal volumes of these containing between 5 and 40 µg were loaded on 10–15% SDS/polyacrylamide gels.

## RESULTS

### Construction of a positively-selected cDNA library in pUEX

Fig. 1 shows the strategy used to obtain a library enriched in cDNA encoding membrane proteins. It was reasoned that many membrane proteins, being housekeeping genes, would be common between different tissues so that the bulk of cDNAs coding for tissue-specific secretory products could be removed by selecting double-stranded molecules after hybridizing cDNA from one tissue with mRNA from another. Kidney and liver from rat were chosen since RNA is easily prepared from these tissues and they are also available in bulk for subsequent biochemical experiments. The first strand cDNA was synthesized from kidney poly(A)$^+$ RNA. Random primers (Haymerle *et al.*, 1986) were used in order to increase the probability that all open reading frames in the kidney mRNA were reverse transcribed. Since the double stranded cDNA/RNA hybrids were to be selected, the background of double-stranded (presumably hairpin-looped) cDNA was first removed by hybridization of the cDNA with itself and separation on a hydroxyapatite column (Alt *et al.*, 1978; Davis, 1986). The fraction of cDNA removed in this procedure (16%, Fig. 1) must represent a discrete class of molecules, since repeating the procedure resulted in a further loss of only 4% of the radioactivity. Linear first strand kidney cDNA which was not retained on this first hydroxyapatite column was hybridized to poly(A)$^+$ RNA from rat liver to an $E_{cot}$ value of 1200 mol·s/l and again fractionated on hydroxyapatite (Davis, 1986). The double stranded fraction retained on the column was eluted in 0.5 M-phosphate and found to contain 48% of the loaded radioactivity. This observation suggests that kidney poly(A)$^+$ RNA contains a relatively low fraction of tissue-specific secreted message, and is in agreement with the analysis of lymphocyte and HeLa cell mRNA which has been shown to have 50–90% of the mRNA species in the low-abundance, presumably housekeeping, class (Hedrick *et al.*, 1984; Bishop *et al.*, 1974).

The resultant pool of common cDNA was subjected to a further round of hybridization with mRNA extracted from membrane-bound polysomes of rat liver. This step was designed to remove cDNA molecules encoding cytoplasmic proteins which would also be present in the common cDNA pool obtained from the first hybridization step. The second hybridization mixture was fractionated on an hydroxyapatite column at 68 °C and the double-stranded material eluted in 0.5 M-phosphate and saved as before. After this selection step 20% of the original labelled cDNA remained in the double-stranded hybrid molecules. Second strand cDNA was synthesized from this material and a library constructed in the bacterial expression vector pUEX1. Of 30000 colonies approx. 6% hybridized with a cDNA probe made from rRNA, suggesting that it might be of ribosomal origin. The percentage of clones without insert in the library was presumably low since the frequency of clones containing an expressed cDNA insert was close to the theoretical prediction of 1 in 6 (see Table 2). The average DNA insert size in 30 clones from the library was 1 kb.

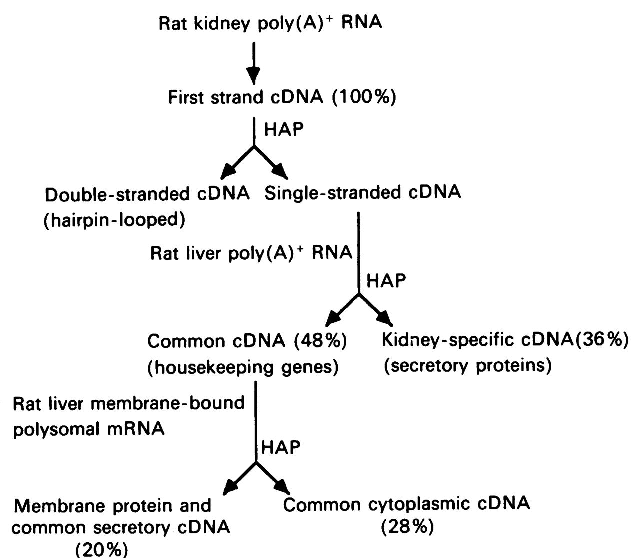


**Fig. 1. Positive selection of cDNA encoding membrane proteins**

Abbreviation: HAP, hydroxyapatite column chromatography. The double-stranded fraction is always shown on the left-hand side; percentages indicate recoveries of radioactive cDNA at each step.

### Characterization of the library

The selected cDNA library was characterized in terms of the contamination of cDNAs which should have been excluded by the selection procedure, the enrichment of cDNAs which should be present in the library and also in terms of the relative abundance of selected cDNA clones in the library with respect to total cDNA libraries made from the two parent tissues, rat liver and kidney. Serum albumin is the most abundant message in rat liver, accounting for about 10% of the total mRNA. Since the first strand cDNA was made from kidney, albumin and other liver-specific secretory messages should have been excluded from the library. In Table 1 it can be seen that in the selected library, albumin is present at less than 1/500th its concentration in a total liver library. This demonstrates that liver-specific genes are expressed at very low levels in kidney and are not present in the selected library. β-Actin was used as an example of a cytoplasmic protein. As expected, clones hybridizing with a β-actin probe were found in both liver and kidney cDNA libraries with a frequency of about 1 in 1000 (Table 1). In the selected library, however, the frequency was $< 1$ in 5000, indicating an enrichment of non-cytoplasmic messages of at least 5-fold after selection with poly(A)$^+$ RNA isolated from membrane-bound polysomes. β-Laminin was used as a probe for a potentially common secretory message. Hybridization of Southern filters made from

**Table 1. Enrichment of selected library for membrane proteins**

The selected library and libraries made from unselected poly(A)$^+$ RNA from the parent tissues (Haymerle *et al.*, 1986; Banting *et al.*, 1989), were analysed by hybridization of the cDNA probes with DNA preparations from pools of 1000, 5000 and 10000 clones digested with *Bam*HI and transfered to nitrocellulose filters. For albumin screening in rat liver individual colonies were streaked onto filters. For screening with LRP and clone 8 probes a library of 400000 colonies on ten 22 cm × 22 cm filters was screened. Values have been corrected to allow for the background of clones without insert in each library. N.D., not determined.

| cDNA probe | Total liver | Total kidney | Selected library |
|---|---|---|---|
| Rat albumin | 10:100 | 0:5000 | 0:5000 |
| Mouse $\beta$-laminin | 0:5000 | 3:5000 | 0:5000 |
| Rat $\beta$-actin | 3:5000 | 5:5000 | 0:5000 |
| Mouse LRP* | 4:400000 | 4:400000 | 1:20000 |
| Clone 8 | N.D. | 0:400000 | 1:20000 |

* A 2.2 kb fragment from mouse LRP corresponding to amino acids 657–1378 in the human LRP sequence (Herz *et al.*, 1988) was used as probe.

the libraries showed however that it was less abundant in liver than in kidney (Table 1) and presumably for this reason was not observed in 5000 colonies of the selected library.

For a direct measurement of the enrichment of membrane proteins in the selected library we used a cDNA probe encoding a 503 kDa plasma membrane protein, the LDL receptor-like protein (LRP) which probably functions as an apoE receptor (Beisiegel *et al.*, 1989; Soutar, 1989) and is present in most tissues (Herz *et al.*, 1988). The LRP cDNA was shown to be of similar abundance (about 1:100000) in both liver and kidney; however, it was detected in a screen of only 20000 clones from the selected library (Table 1). This is consistent with the 5-fold enrichment indicated by the recovery of radioactive cDNA in the selection procedure (Fig. 1). When clone 8 from the selected library was used to probe a total rat kidney library no positive clones in 400000 (Table 1) were found. This suggests an enrichment of at least 10-fold for this clone in the selected library.

### Selection of in-frame fusions

The probability of encountering a 600 bp ORF in the wrong reading frame or orientation of a cDNA fragment has been shown to be very small (Senapathy, 1986). We therefore screened a large number of cDNA clones picked at random from the selected library in order to isolate clones with in-frame fusions between the $\beta$-galactosidase of the pUEX vector and the cloned DNA insert. In Table 2 it can be seen that the frequency of clones

**Table 2. Screening of random clones by expression**

Cultures (5 ml) were inoculated with 1161 randomly picked colonies and processed for expression. The size of fusion protein was analysed on 7% SDS/polyacrylamide gels. The pUEX expression vector itself encodes a *cro*–$\beta$-galactosidase fusion protein of 120 kDa.

| Fusion protein size (kDa) | Number of colonies | Frequency (%) |
|---|---|---|
| 120 | 992 | 85.5 |
| 120–140 | 105 | 9.0 |
| 140–160 | 52 | 4.5 |
| > 160 | 12 | 1.0 |

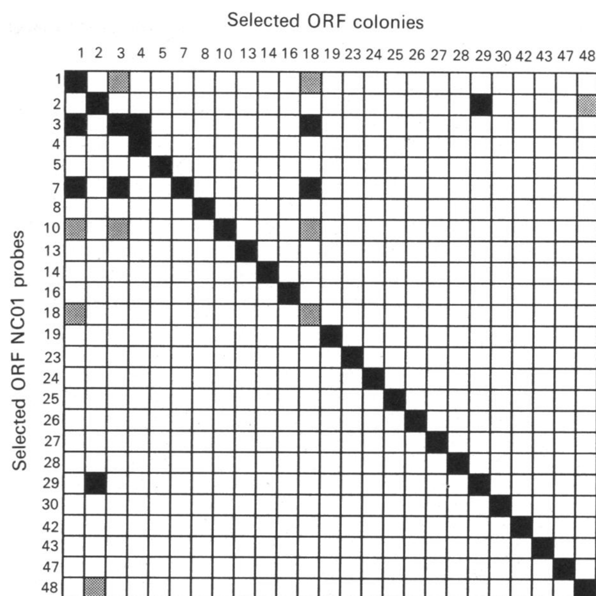


**Fig. 2. Cross-hybridization of selected ORF clones**

Radio-labelled probes were made from 25 selected open reading frame clones and used to probe filters streaked with all 25 colonies. Strongly hybridizing clones are marked with solid squares (■), those weakly hybridizing with shaded squares (▩). □, No hybridization.

having any detectable ORF (14.5%) is close to the expected frequency of in-frame fusions (1 in 6) while large ORF fusions (> 140 kDa) occur at about one-third this rate (5.5%), consistent with the average size of DNA insert in the library. Twenty-five clones with large ORFs were selected and analysed by hybridization against each other (Fig. 2). This showed that the majority of clones selected were unique, only hybridizing with themselves. One exception was clone 2, which was shown by DNA sequence analysis to be identical with clone 29. Clones 3 and 7 also cross-reacted with other clones, but in a non-reciprocal way. This may be due to the presence of repetitive sequences which are only detected when the smaller clone is labelled and used as a probe. Seventeen of the selected clones were also checked by hybridization back to the original library and with one exception, which hybridized with three other clones in the library, these were all present at one copy in 30000 clones. The nucleotide sequence at the 3′ and 5′ ends of the 25 clones shown in Fig. 2 was determined and used for comparison with each other and in a search of the EMBL nucleotide sequence data base (release 13, using the WORDSEARCH program from the University of Wisconsin genetics computer group). This search revealed that clones 2 and 29 were identical, as expected from the hybridization study, but that none of the clones have previously been sequenced and published.

### Subcellular location of cloned proteins

Nine of the ORF clones expressing particularly large fusion proteins were selected for raising polyclonal antibodies. Several different immunization schedules were used in an attempt to raise antibodies which might cross-react with native proteins. In each case the inclusion bodies from cultures of *E. coli* were first purified by the method of Lacal *et al.* (1984) and then either injected directly with Freunds adjuvant, or first run on SDS/polyacrylamide gels and the fusion protein isolated from the band by homogenization and incubation in phosphate-buffered saline at 4 °C overnight. We found that immunization with whole inclusion bodies, especially into guinea pigs, produced

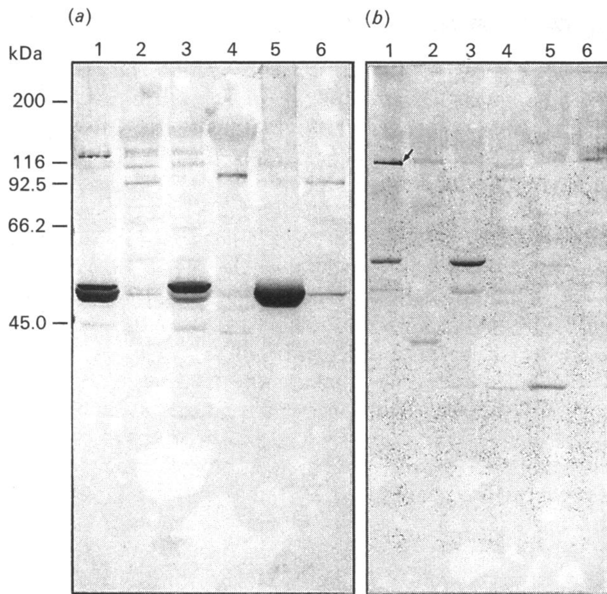


**Fig. 3. Anti-(fusion protein) antibodies recognize membrane proteins**

A Western blot of rat liver homogenate was fractionated by centrifugation at 1000 $g$ for 10 min (lanes 1 and 2), 10 000 $g$ for 10 min (lanes 3 and 4) and 100000 $g$ for 60 min (lanes 5 and 6). In each pair of lanes the left-hand lane shows proteins extracted into the detergent phase of Triton X-114, those on the right remain in the aqueous phase. The blots were probed with polyclonal rabbit antiserum raised against the fusion protein encoded by clone 8 (*a*) or clone 47 (*b*) The mobilities of molecular mass markers are shown in kDa. Arrow shows band used for affinity purification in Fig. 5.

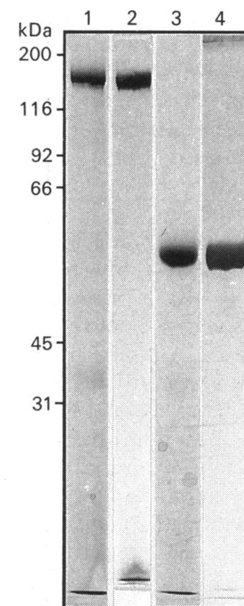


**Fig. 4. Separation of *E. coli* whole cell lysates by SDS/polyacrylamide-gel electrophoresis**

Tracks 1 and 2 are from cells harbouring the construct of clone 8 in pUEX, tracks 3 and 4 from cells harbouring the construct of clone 8 in pUBSEX. Tracks 1 and 3 are from a gel stained in Serva Brilliant Blue, tracks 2 and 4 from parallel strips of the same gel after transfer to nitrocellulose and immunoblotting with rabbit polyclonal antiserum raised against the fusion protein encoded by clone 8 in pUEX. All lysates are from cells incubated at 42 °C to induce fusion protein production (see the Materials and methods section). The mobilities of molecular mass markers are shown in kDa.

inconsistent results, possibly due to polyclonal activation by residual lipopolysaccharides present in the inclusion body preparation (Morrison & Ryan, 1979). Immunization of rabbits with proteins eluted from SDS gels consistently gave anti-$\beta$-galactosidase antibodies, although the titre against the expressed cDNA insert part of the fusion protein, determined by Western blotting liver membrane fractions, was often lower (1:200–1:500). In comparison to the ease of raising antisera against viral fusion proteins (Tooze & Stanley, 1986), the problems that we encountered, despite the large size and stability of the fusion proteins, suggest that the proteins selected were not very immunogenic.

The antibodies raised against protein bands cut out from SDS gels were tested on Western blots of different membranous fractions. A simple subcellular fractionation into 'nuclear', 'mitochondrial' and 'microsomal' fractions was used. Six out of nine of the antibodies raised against gel-purified fusion proteins stained predominantly Triton X-114 detergent phase proteins from these fractions. Two of these are shown in Fig. 3. Antibodies against clone 8 in Fig. 3(*a*) stained a single band of 50 kDa which was present in the Triton X-114 detergent phase of all three fractions of rat liver. Several of the other antibodies stained more than one band as shown for antibodies against clone 47 in Fig. 3(*b*). The antibody affinity-purified on the 116 kDa band (arrow,

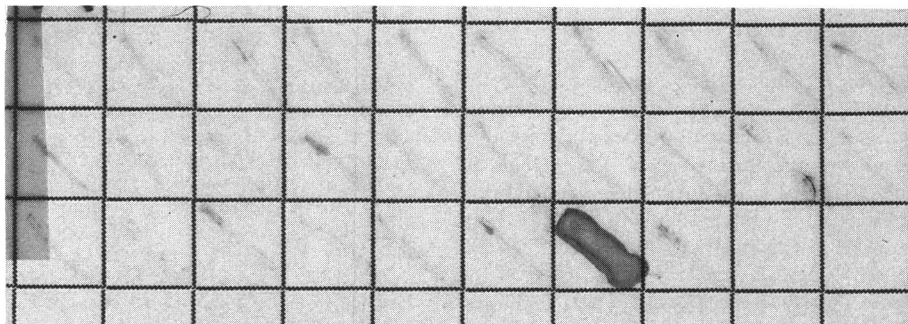

**Fig. 5. Affinity-purified antibodies bind to expressed fusion proteins**

Antibodies against clone 47 were eluted from a Western blot (arrow in Fig. 3) of a rat liver membrane fraction with 0.2 M-glycine, pH 2.5, and after neutralization bound back onto fusion protein made in *E. coli*. Of 28 different clones from the selected library containing large ORFs grown on nitrocellulose filters, only clone 47 reacted with the affinity-purified antibodies (from the upper left squares clones are designated nos. 1, 2, 3, 4, 5, 7, 8, 10, 13, 14, 15, 16, 18, 19, 23, 24, 25, 26, 27, 28, 29, 30, 41, 42, 43, 44, 47 and 48).

Fig. 3*b*) was, however, capable of binding to the 55 kDa band, showing that the multiple band pattern is caused by proteolytic degradation of the sample. In this example the stained bands are found predominantly in the 'nuclear fraction', suggesting a location in the plasma membrane or nuclear envelope. As expected, the antibodies raised against expressed kidney cDNAs also blotted liver proteins at high dilutions, indicating a conservation at protein as well as DNA level.

A particular problem when producing antibodies to fusion proteins is to determine if the serum is reacting with the foreign antigenic determinant or the β-galactosidase portion of the fusion protein, especially when no native protein is available. We therefore subcloned the DNA inserts from some of the clones into a modified pUEX vector (pUBSEX) in which the *lacZ* region of the pUEX vector has been deleted (Banting *et al*., 1990). Despite the fact that the fusion protein produced now only has nine amino acids derived from *cro* at the *N*-terminus of the protein, the level of expression was still high in three out of five cases determined (Fig. 4). Furthermore, it was possible to show that polyclonal antisera raised against the intact *lacZ*-encoded fusion proteins of clones 8 and 47 contained antibodies against the expressed insert (Fig. 4 for clone 8; results for clone 47 not shown). For clone 8 the crude antiserum was affinity purified subsequently on the fusion protein expressed in pUBSEX and eluted in low pH buffer. For clone 47 crude antiserum was affinity purified on a positive band from rat liver homogenate (arrow in Fig. 3*b*). In both cases the affinity-purified antibody was capable of binding back to colonies expressing the fusion protein, but not to colonies expressing fusion proteins from other clones (Fig. 5 for clone 47; results for clone 8 not shown).

## DISCUSSION

Studies on the targetting signals of membrane proteins located in intracellular organelles require antibody and cDNA probes for resident integral membrane proteins. We have described in this paper an approach based on positive selection of RNA/cDNA hybrids on hydroxyapatite columns. This approach generates a large number of membrane protein clones from which antibodies can be raised without bias due to the immunogenicity of individual proteins. The strategy we have presented here describes the first use to our knowledge of positive selection of cDNA clones. The selected library which we constructed and characterized contain predominantly low-abundance cDNAs and is enriched in cDNA molecules encoding membrane proteins. Seventeen selected clones were present as single copies in the library, in contrast to many selected libraries which contain multiple copies of individual clones. The lack of multiple copies is presumably a result of the selection for housekeeping genes by hybridization between tissues. By using housekeeping proteins for immunization the corresponding antibodies are more likely to recognize their antigen in different tissues and possibly different species. The average size of insert in this library is greater than in most selected libraries and may also be attributed to the positive selection since the cDNA is maintained in a double-stranded state during the long hybridization reactions. The modest 5-fold enrichment of cDNA in the final selected fraction (Fig. 1) is partly because the values are expressed relative to total cDNA made from kidney poly-$(A)^+$ RNA. Had liver been chosen as the starting material a larger purification factor would have been expected due to the larger mass of abundant tissue-specific secretory messages. The value also reflects the fact that many different mRNA molecules encoding membrane proteins are present in a cell, even though each is only present at low abundance. This is supported by the hybridization and sequence comparison studies, which showed that most selected clones are present as single copies in the library.

Analysis of a cDNA library by an immunological screening procedure requires an efficient means for raising and testing antibodies. Antibodies against bacterial fusion proteins can have a high level of specificity, since the antigen is clonally pure, and contaminants in the antigen preparation are of bacterial origin and unlikely therefore to evoke antibodies which can cross-react with mammalian cells (Tooze & Stanley, 1986). The fusion proteins taken from the selected library, however, were not found to be very immunogenic and frequently the high level of immune reaction against the β-galactosidase part of the fusion masked the low levels of specific antibodies produced. This is perhaps not surprising since endogenous membrane proteins might bear a number of signals involved in intracellular sorting, targetting and organelle identification which would be conserved in evolution. In order to help overcome this problem we designed a new expression vector system (pUBEX and pUBSEX; Banting *et al*., 1990) which allows both fusion protein and native protein to be expressed. In the present study we subcloned DNA inserts from a pUEX library directly into the *Bam*HI site of pUBSEX, but in future it will be possible to clone cDNA libraries directly into pUBEX by using a modified form of the adaptors described in Haymerle *et al*. (1986). The large size of the *lacZ* portion in this vector ensures a uniform level of expression of a β-galactosidase fusion protein, allowing easy identification of large open reading frames. By a single restriction enzyme cleavage and intramolecular ligation, individual clones expressing β-galactosidase fusions may then be converted into pUBSEX constructs in which only nine amino acids of *cro* are fused to the *N*-terminus of the DNA insert (Banting *et al*., 1990).

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