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PROTOCOLS D'ENGINYERIA GENÈTICA

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1. Objective

The aim of this course is to introduce you to current techniques used to construct recombinant DNA clones containing specific genes and to screen a large number of *E. coli* colonies for recombinant plasmids carrying a specified DNA sequence.

Two approaches can be used to construct recombinant DNA clones containing specific gene sequences; these are direct cloning of genomic DNA fragments or cloning of a double-stranded cDNA made from the mRNA. In this course we will construct a collection of recombinant DNA molecules which will permit the cloning of a large sample of DNA sequences complementary to rat liver mRNA species ("Construction of a cDNA library": Part I).

The cDNA library will be screened for inserts complementary to rat albumin mRNA by *in situ* colony hybridization using a ^{32}P-labelled rat albumin cDNA probe ("Screening for rat albumin cDNA sequences": Part II).

The recombinant plasmids bearing albumin cDNA sequences will be characterized by restriction endonuclease cleavage, electrophoretic analysis in agarose gels and "Southern blotting" techniques ("Characterization of albumin cDNA containing plasmids": Part III).

2. Construction of a cDNA library of messenger RNA species from rat liver

2.1. *Introduction*

The DNA copy of an mRNA molecule synthesized by the avian myeloblastosis virus (AMV) reverse transcriptase is termed "complementary DNA" (generally abbreviated to "cDNA"). The term cDNA clone is now used to describe a bacterial cell transformed by a plasmid containing the DNA copy of an RNA molecule. A "cDNA clone bank" or "cDNA library" from a given cell or tissue is a population of bacterial transformants, each containing a plasmid with a single cDNA insert, complementary to one of the cells mRNA molecules. A *complete* cDNA library will contain a sufficiently large number of individual transformants such that every mRNA species is represented at least once in the bacterial population.

The preparation of a cDNA library can be carried out by various procedures which differ in the enzymatic steps used to prepare recombinant plasmid DNA for the transformation of competent *E. coli* cells. The procedure we will use in this course is summarized in Figure 1. The first step is the synthesis of DNA copies of the mRNA molecules present in the polyadenylated RNA preparation, using reverse transcriptase. Following alkaline hydrolysis of the RNA, the single-stranded cDNA will serve both as template and primer for the synthesis of the second strand which is covalently linked to the first. The loop of the hairpin molecule formed can be specifically cleaved with single-strand-specific S_1 nuclease. The double-stranded cDNA (ds-cDNA) will be inserted into the

bacterial plasmid pBR322. This vector carries genes coding for resistance to ampicillin and to tetracycline. Insertion of a foreign DNA molecule into one of these genes will destroy the resistance capacity to the antibiotic. This offers a convenient first hand screening procedure for identifying recombinant clones.

To construct hybrid molecules, the pBR322 is linearized by the restriction endonuclease PstI which cleaves the DNA only once, and is tailed with oligo(dG) by the terminal deoxynucleotidyl transferase. The ds-cDNA is similarly tailed with oligo(dC) and annealed to the plasmid DNA. Following transformation in *E. coli* competent cells (MC1061), the gaps are completed in the bacterial cell and in this process, the PstI sites are reconstructed. The resistance to ampicillin is however lost. This cloning procedure which allow excision of the inserted cDNA sequence by restricting the recombinant clone with PstI, is described in detail below.

2.2. *Synthesis of complementary DNA to total polyadenylated polysomal RNA from rat liver*

The reaction mixture contains the following:

- 50 mM Tris HCl, pH 8.4.
- 10 mM $MgCl_2$.
- 60 mM NaCl.
- 10 mM DTT.
- 50 μg/ml oligo$(dT)_{12\text{-}18}$.
- 100 μg/ml actinomycin D.
- 1 mM of each dATP, dTTP, dGTP.
- 50 μM (^{32}P)-dCTP (65 mCi/mmole).
- 100 μg/ml bovine serum albumine.
- 1 mg/ml of rat liver polyadenylated polysomal RNA.
- 1.200 units/ml of AMV reverse transcriptase.

Reactions are carried out in sterile tubes in a final volume of 250 μl. After incubation at 43°C for 60 min the reaction is terminated by the addition of 10 μl of SDS 10 % and 10 μl of EDTA 0.5 mM. The template RNA is then hydrolyzed making the mixture 0.3 M in NaOH and incubating at 70°C for 60 min. The solution is neutralized, an aliquot is removed for TCA precipitation and unincorporated nucleotides are removed by Sephadex G-100 chromatography. The excluded fractions are monitored by Cerenkov counting and are pooled. The cDNA is then precipitated with 2 volumes of ethanol at —20°C overnight.

2.3. *Double-stranded cDNA synthesis*

The precipitated single-stranded cDNA is collected by centrifugation (10,000 xg for 90 min), washed with ethanol, dried and dissolved in 50 μl of EDTA 0.5 mM. An aliquot (1 μl) is removed for scintillation counting.

Second-strand synthesis is carried out in sterile tubes in a final volume of 125 μl with reverse transcriptase. Reaction conditions are the same as those used for the first-strand except that this time oligo (dT) and actinomycin D are omitted and ^{3}H-labelled dCTP (100 μM; 5 Ci/mmole) is used. The reaction is stopped as indicated above with EDTA (10 μl EDTA 0.5 M) and SDS (5 μl SDS 10 %). An aliquot is removed for TCA precipitation and unincorporated nucleotides are removed by Sephadex G-100 chromatography. The excluded fractions are monitored by scintillation counting and are pooled. The ds-cDNA is then precipitated with 2 volumes of ethanol at —20°C overnight.

2.4. *Cleavage of the hairpin loop and removal of single-stranded sequences*

The ethanol precipitate is collected by centrifugation (10,000 xg for 90 min),

washed twice with ethanol, dried and dissolved in 202 μl of EDTA 0.5 mM. A 2 μl aliquot is removed for scintillation counting and to the remainder the following are added: 2.5 μl of 4 M Na Acetate buffer pH 4.6: 34 μl of 2 M NaCl; and 12.5 μl of 100 mM $ZnSO_4$. The mixture is then incubated with 1500 units of S_1 nuclease (Miles Research Products Ltd.) at 25°C for 60 min. The reaction is stopped with 25 μl of 1 M Tris-HCl, pH 8.4, and an aliquot is TCA precipitated. The remainder is extracted with one volume of phenol: chloroform (1:1) and the aqueous phase is precipitated with 2 volumes of ethanol.

2.5. *Addition of homopolymer dC tracts to the 3'-ends of double-stranded cDNA*

The terminal addition of dCTP to the ds-cDNA by terminal deoxynucleotidyl + transferase is carried out in the presence of Co^{2+} instead of Mg^{2+} ions, which allows the enzyme to accept double-stranded DNA as a primer instead of single-stranded DNA, as under the usual assay conditions (Roychoudhury *et al., Nucleic Acid Res. 3:*101-116, 1976).

The double-stranded cDNA is collected by centrifugation, washed twice with ethanol and dissolved in a small volume of EDTA 0.1 mM. The reaction is carried out in an Eppendorf tube and contains in a final volume of 25 μl:

- 140 mM potassium cacodylate.
- 30 mM Tris base pH 6.9.
- 0.1 mM dithiothreitol.
- 1 mM $CoCl_2$.
- 0.1 mM (^{32}P)-dCTP 5 Ci/mmole.
- 100 μg/ml double-stranded cDNA.
- 25 units of terminal transferase (PL Biochemicals).

The reaction mixture is heated at 37°C prior to the addition of the enzyme. Add $CoCl_2$ last, just before the enzyme. The reaction is followed by incorporation of labeled dCTP into TCA-insoluble material. After 5 min incubation chill the entire reaction mixture on ice water. Remove 1 μl aliquot, TCA precipitate, count and calculate the number of bases added per end knowing the molarity in ends. Aim to add 15-20 nucleotides per 3'-end of DNA. If necessary the reaction can be restarted at 37°C without the addition of new enzyme to increase the length of the tails.

When the required number of nucleotides has been obtained the reaction is stopped by the addition of EDTA to 10 mM, followed by extraction with an equal volume of phenol: chloroform mixture. The aqueous phase is then passed over a smalll Sephadex G-50 column to separate unincorporated reaction components from tailed ds-cDNA, using 10 mM Tris-HCl pH 7.4, 0.1 M NaCl and 0.2 mM EDTA as the running buffer. The void volume is collected and stored at 4.°C.

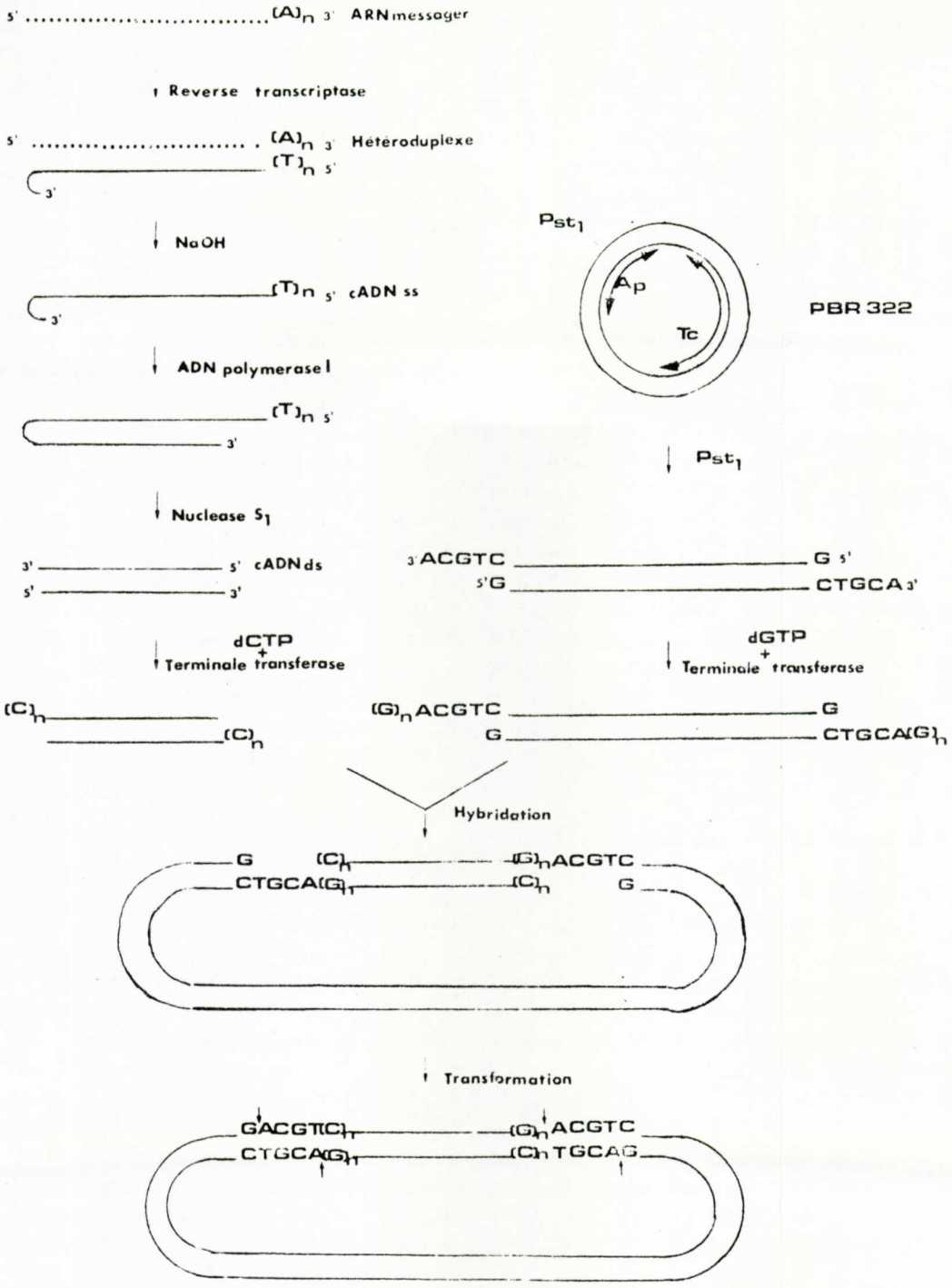
2.6. *Preparation of pBR322 plasmid vector: Restriction cleavage by PstI and terminal addition of dGTP*

In order to construct a hybrid plasmid, pBR322 is first linearized by restriction cleavage using the endonuclease PstI and then tailed with dGTP.

Mix in a plastic Eppendorf tube:

- 10 μl of 10 x restriction buffer (60 mM Tris-HCl, pH 7.4; 50 mM NaCl, 60 mM $MgCl_2$; 60 mM 2-mercaptoethanol).
- 10 μg of pBR322 DNA.
- 50 units of PstI.
- Distilled water up to a final volume of 100 μl.

Incubate at 37°C for 1 hr. Add another 50 units of restriction endonuclease PstI and continue the incubation for a second hour. The DNA is then extracted with phenol-chloroform and reprecipitated with NaCl and ethanol.


5' ..... (A)n 3' ARN messager
Reverse transcriptase
5' ..... (A)n 3' Heteroduplexe
(T)n 5'
3'
NaOH
(T)n 5' cADN ss
3'
ADN polymerase I
(T)n 5'
3'
Nuclease S1
3' 5' cADN ds
5' 3'
Pst1
Ap
Tc
PBR 322
Pst1
3' ACGTC G 5'
5' G CTGCA 3'
dCTP
+
Terminale transferase
dGTP
+
Terminale transferase
(C)n
(C)n
(G)n ACGTC G
G CTGCA (G)n
Hybridation
G (C)n (G)n ACGTC
CTGCA (G)n (C)n G
Transformation
GACGTC (C)n (G)n ACGTC
CTGCA (G)n (C)n TGCAG

Five μg of PstI restricted pBR322 are then tailed with dGTP residues in a 50 μl reaction volume composed of 140 mM potassium cacodylate, 30 mM Tris base (pH 6.9), 0.1 mM dithiothreitol, 1 mM $CoCl_2$, 0.2 mM ^{3}H-dGTP (5-10 Ci/mmole), and 50 units of terminal transferase. The reaction is carried out at 37°C for the terminal addition of dCTP to the 3'-ends of double-stranded cDNA. 10-15 nucleotides should be added per 3'-end of pBR322 DNA.

2.7. *Annealing to form hybrid plasmid DNA*

The molecular weight of pBR322 is about 2.6×10^6. If we estimate the average molecular weight of the ds-cDNA preparation to be about 4.4×10^5, we will need a mass ratio of approximately 1:6 for equimolar amounts. 17 ng of double-stranded cDNA tailed with poly(dC) will be annealed with 100 ng of pBR322 tailed with poly(dG) in 25 μl of 10 mM Tris-HCl pH 7.4, 0.1 M NaCl and 0.2 mM EDTA. The mixture is treated to 63°C for 3 min, transferred to a 43°C water bath for 2-3 hr, then cooled slowly to room temperature.

2.8. *Preparation of competent* E. coli *cells*

— Streak out *E. coli* HB 101 (thr$^-$, leu$^-$, pro$^-$, recA$^-$, hsdR$^-$, hsdM$^-$) or MC 1061 (galU$^-$, galK$^-$, recA$^-$, hsr$^-$, hsm$^+$) on LB agar plate on day preceeding experiment.

— Inoculate a single colony into 50 ml of L broth and incubate, with shaking, at 37°C until the A_{660} reaches 0.5-0.6 (about 5×10^7 cells/ml).

— Centrifuge 2×10^8 cells (4 ml) in a 15 ml Corex tube at 8000 g for 10 min at 4°C. Poor off supernatant and resuspend pellet in 2 ml of 10 mM MOPS pH 7.0, 10 mM RbCl. Cells should be resuspended as gently as possible.

— Centrifuge immediately at 8000 g for 10 min at 4°C. Resuspend the pellet in 2 ml of ice-cold 100 mM MOPS, pH 6.5, 50 mM$CaCl_2$, 10 mM RbCl. Hold cells on ice for 30 min. Treat cells as gently as possible after this step.

— Centrifuge for 10 min at 8000 g. Drain tubes throughly on absorbent paper (Kimwipes). Resuspend the pellet gently in 0.4 ml of the above indicated 100 mM MOPS buffer solution. The competent cells are then used for transformation.

2.9. *Transformation of* E. coli *competent cells*

Transformation is carried out by mixing 0.2 ml of competent cells with 3 μl of DMSO and 20 μl of the annealed mixture (about 100 ng of plasmid DNA). The mixture is hold for 30 min on ice without shaking then incubated at 43.5°C for 30 sec (heat shock). After dilution at room temperature with 2 ml of Z broth, tubes are hold for 60 min at 37°C without shaking. 100 μl of the suspension of transformed cells is spread on 9 cm Petri dishes containing LB medium supplemented with tetracycline. A control experiment is performed using 100 ng of unannealed dG-tailed plasmid vector as donor DNA to determine the degree of background clones arising from intact pBR322 molecules still remaining in the preparation.

To assess the efficiency of transformation another control experimental is carried out using 100 ng of intact pBR322 as donor DNA. In this case a series of dilutions (from 10^{-1} to 10^{-6}) are to be made from the 2 ml Z broth before plating. Then 100 μl of the 10^{-4}, 10^{-5} and 10^{-6} suspensions of transformed cells are spread on Petri dishes containing LB medium. 100 μl aliquots of the 10^{-1}, 10^{-2} and 10^{-3} suspensions are equally spread on LB plates supplemented with tetracycline or ampicillin.

2.10. *Selection of antibiotic resistant clones*

pBR322 carriers markers for ampicillin and tetracycline resistance. Insertion of foreign DNA into the PstI site of this plasmid causes inactivation of the beta-lactamase gene and allows identification of hybrid plasmids carrying foreign DNA.

Transformed cells which have taken up either pBR322 or hybrid plasmid DNA will be Tc^r and will grow on tetracycline containing plates whereas non transformed *E. coli* cells will not. From these clones, Ap^s cells (containing recombinant plasmids) are selected by replica plating. Individual clones are transferred using the tip of a sterile toothpick first to a ampicillin containing plate and then to a tetracycline containing one. A template is used to align the position of the transformed cells on the two plates. The plates are incubated for 18 hours at 37°C. Then by comparing the two plates colonies which grow on the Tc containing place but not Ap containing plates are identified.

3. Screening for clones containing specific DNA sequences: application to rat albumin cDNA sequences

3.1. *Introduction*

The preparation of a cDNA bank allows for the obtention of any particular cDNA clone for which we have a method of selection. The term "screening" is normally applied to describe any procedure designed to identify and isolate a particular clone from the bank.

Eucaryotic mRNA sequences are present at widely varying abundances in different cell types, and in general, the frequency of occurrence of a particular clone in a bank is proportional to its abundance. We have prepared a cDNA bank from rat liver polyadenylated polysomal RNA. Since albumin mRNA is the most abundant mRNA species in rat liver, albumin cDNA sequences should be present in a high proportion of the clones (around 5 %).

We will screen a moderate number of cDNA clones from the bank with an *in vitro* labeled probe derived from purified rat albumin mRNA. To illustrate the specificity of the *in situ* colony hybridization procedure of Grunstein and Hogness (*Proc. Natl. Acad. Sci. 72:*3961-3965, 1975) we shall prepare two different radioactive DNA probes: a) A ^{32}P-labeled cDNA made to purified rat albumin mRNA by using the AMV reverse transcriptase, and b) a ^{32}P-labeled probe made by nick-translation of pBR322 DNA. The cDNA wil allow us to select for clones containing an inserted albumin cDNA sequence, whereas the nick-translated pBR322 DNA should hybridize to all transformed *E. coli* colonies.

3.2. *Preparation of radioactive DNA probes*

a. *Albumin cDNA synthesis*

The reaction mixture contains the following:

- 50 mM Tris-HCl, pH 8.4.
- 10 mM $MgCl_2$.
- 50 mM NaCl.
- 10 mM DTT.
- 50 μg/ml oligo$(dT)_{12\text{-}18}$.
- 150 μg/ml actinomycin D.
- 500 μM of each dATP, dTT, dGTP.
- 50 μM ^{32}p-dCTP (250 Ci/mmole).
- 100 μg/ml bovine serum albumin.
- 20 μg/ml of purified albumin mRNA.
- 1200 units/ml of AMV reverse transcriptase.

Reactions are caried out in sterile Eppendorf tubes in a final volume of 20 μl. After incubation at 43°C for 60 min the reaction is terminated by the addition of 5 μl of SDS 2 % and 5 μl of 0.2 M EDTA. Micrococcal DNA carrier (30 μg) is added and the template RNA is then hydrolyzed making the mix-

ture 0.3 % in NaOH and incubating at 70°C for 60 min. The solution is neutralized, an aliquot is removed for TCA precipitation and unincorporated nucleotides are removed by Sephadex G-50 chromatography. The excluded fractions are monitored by Cerenkov counting and are pooled. The cDNA is then precipitated with 2 volumes of absolute ethanol at —20°C overnight.

b. *Labeling of pBR322 by nick-translation*

The labeling of DNA by nick-translation is based upon the observation that *E. coli* DNA polymerase I binds at a nick and, in the presence of the required deoxynucleoside triphosphates, extends the primer terminus. Nicks in DNA are introduced by limited DNase I action. Addition of DNA polymerase I catalyses then the nick-translation reaction by the simultaneous operation of the polymerase activity (which adds nucleotide residues at the 3'-hydroxyl terminus) and the 5'-3' exonuclease activity (which removes 5'-phosphate residues from the 5'-terminus).

The reaction is carried out in an stelire Eppendorf tube containing:

- 50 mM Tris-HCl pH 7.8.
- 5 mM $MgCl_2$.
- 5 mM DTT.
- 10 μM of each dTTP, dGTP.
- 5 μM of each ^{32}P-dATP, ^{32}P-dCTP (500 Ci/mmole).
- 50 μg/ml bovine serum albumine.
- 1 μg of DNA.

After addition of 3 μl of 1 ng/ml of DNase I (freshly diluted from a stock 1 mg/ml DNase solution) the mixture is incubated at 37°C for 10 min and immediately cooled in ice. 3 μl of *E. coli* polymerase I (5 units/ml) are then added and the mixture is incubated at 15°C for 2 h. The reaction is terminated by the addition of 20 μl of 50 mM Tris-HCl pH 8.0 containing 25 mM EDTA and 0.5 % SDS. The solution is then made 0.3 M in NaOH and boiled for 2 min, followed by immediate cooling in ice. Micrococcal DNA carrier (20 μg) is added and the mixture is neutralized with 1 M acetic acid. A small aliquot (1 μl) is removed for TCA precipitation before chromatography on a Sephadex G-50 column equilibrated in 10 mM Tris-HCl pH 8.0, 0.1 M NaCl and 0.5 mM EDTA. Excluded fractions are monitored by Cerenkov counting and pooled.

3.3. *Colony hybridization*

The procedure developped by Grunstein and Hogness (1975) has made possible to screen a large number of colonies of *E. coli* carrying different hybrid plasmids in order to determine which plasmids contain a specified DNA sequence. The colonies to be screened are formed on nitrocellulose filters by replica plating of a reference set of these colonies. After lysing the colonies, their DNA is denatured and fixed to the filter *in situ*. The resulting DNA prints of the colonies are then hybridized to a radioactive RNA or DNA probe that defines the sequence of interest, and the result of this hybridization is assayed by autoradiography.

a. *Transfer of colonies to nitrocellulose filters*

Following transformation of *E. coli* competent cells a first screening of recombinant plasmids is carried out by selecting appropriate antibiotic resistant clones as described in section 2.10. We will then screen 400 recombinant clones (from 4 master LB plates supplemented with tetracycline) for hybrid plasmids containing albumin cDNA sequences. The colonies obtained from the experiment described in Section 2.10 will be transferred to nitrocellulose filters.

The experimental procedure is a follows:

Step 1. Wash nitrocellulose filters (0.45 μm pores, Schleicher and Schüll,

BA 85) three times in boiling water for 1 min per wash.

Step 2. Place washed filters between sheets of 3 MM Whatman paper, autoclave at 120°C for 10 min and dry for 10 min in the autoclave.

Step 3. Orientate the filters with a pencil mark and layer on top of the LB master plates. The bacteria are transferred by lifting the filter once wet from the dish.

Step 4. Place the filters on blotting paper (Whatman 3 MM) saturated with the following solutions:

- 0.5 M NaOH during 7 min following by:
- 1 M Tris-HCl pH 7.5 for 2 min followed by:
- 1 M Tris HCl pH 7.5 for 2 min followed by:
- 0.5 M Tris-HCl pH 7.5, 1.5 M NaCl for 5 min.

Step 5. Transfer the filters to a sintered-glass funnel and suck dry.

Step 6. Wash filters twice with 100 ml of 95 % ethanol and suck dry.

Step 7. Bake filters under vacuum at 80°C for 2 hours.

b. *Preparation of nitrocellulose filters for hybridization*

In order to avoid non-specific binding of the labeled probe to the nitrocellulose paper, the filters are prehybridized at 66°C for at least 6 hours in 3 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M trisodium citrate), 10 x Denhardt's solution (Denhardt's solution is 0.002 % each of Ficoll, polyvinylpyrolidone and bovine serum albumin), 0.1 % SDS, 150 μg/ml sonicated and denatured *E. coli* DNA and 500 μg/ml of sonicated and denatured salmon sperm DNA. Prehybridation is carried out in a sealed plastig bag after wetting the nitrocellulose filter in 3 x SSC.

c. *Hybridization and detection of colonies containing specific DNA sequences*

Following prehybridization, the nitrocellulose filters are hybridized at 66°C for at least 24 hours in 10 ml of hybridization solution (3 x SSC, 10 x Denardt's, 0.1 % SDS, 250 μg/ml polyA, 150 μg/ml sonicated and denatured *E. coli* DNA). The radioactive DNA probe to be used is denatured together with *E. coli* DNA by boiling for 5 min. After immediate cooling in ice-water the DNA probe is added to the plastic bag. Use $> 10^6$ cpm of labeled DNA per filter.

To remove all radioactive probe other than the specifically hybridized one, filters are washed extensively as follows:

3 times at 63°C for 30 min each time with 100 ml of 2 x SSC, 0.1 % SDS and 0.1 % Na pyrophosphate.

4. Characterization of recombinant plasmids containing albumin cDNA sequences by restriction endonuclease cleavage

4.1. *Introduction*

Plasmid DNA isolated from the positive clones selected by the *in situ* colony hybridization screening will be characterized by restriction enzyme analysis. The size of the insert will be deduced by comparing the lenght of the recombinant plasmid DNA, linearized by EcoRI (which does not cut in the rat albumin cDNA sequence and which cuts pBR322 only once) to the length of the linear pBR322 DNA on agarose gels. Since the oligo(dG)-oligo(dC) tailing method used to generate hybrid plasmids allows reconstitution of the PstI site, PstI restriction cleavage of the recombinant plasmid DNA should excise the inserted sequence. This is an alternative method to determine the size of the insert, but several bands might be obtained due to the presence of several PstI sites in the albu-

min cDNA sequence. HindIII restriction cleavage of recombinant plasmids will also be performed; this endonuclease cuts pBR322 only once but cleaves the rat albumin cDNA in 3 distant positions. This should allow to orientate the inserted sequences by reference to the known restriction map of rat albumin cDNA.

The detection of specific sequences in DNA restriction fragments can be done using the methodology developed by Southern (*J. Mol. Biol. 98:*503-517, 1975). The double-stranded DNA fragments are separated by size by electrophoresis in an agarose gel, and the DNA then made single-stranded by soaking the gel in alkali. The gel is placed flat onto a "wick" of filter paper that connects with a trough containing concentrated salt solution. A single sheet of cellulose nitrate filter is then placed on top of the gel and a large stack of dry absorbent paper towels laid flat on top of this. The salt solution will be drawn up by the absorbent paper towels, passing through the gel and cellulose nitrate sheet. As the liquid passes through the gel the single-stranded DNA will be swept out of the gel and pass onto the membrane filter. Cellulose nitrate has the property of binding single-stranded DNA and so all the DNA will become attached to this sheet. The final result of this procedure will be a perfect replica of the DNA from the original agarose gel, but the DNA single-stranded and immobilized on a cellulose nitrate filter sheet. The DNA size pattern, from the original agarose gel, is faithfully preserved. Single-stranded DNA bound to a cellulose nitrate filter is still accessible to hybridization reactions.

We will apply the Southern hybridization technique to the detection of albumin cDNA sequences in the EcoRI, PstI and HindIII restriction fragments of the selected recombinant plasmids. For this purpose the nitrocellulose filters will be hybridized to the ^{32}P-labeled albumin cDNA probe or to the nick- translated pBR322 DNA.

4.2. *Rapid preparation of plasmid DNA from the positive clones*

Restriction enzymchanalysis of the recombinant plasmids does not require highly purified plasmid DNA, such as is obtained by lenghty re-banding in caesium chloride-ethidium bromide gradients. A number of suitable rapid purification procedures are now available. The method recently described by Holmes and Quigley (*Analyt. Biochem. 114:*193-197, 1981) can be used.

Inoculate single colonies of the positive recombinant clones into sterile test tubes containing 5 ml of L broth supplemented with 15 μg/ml tetracycline. Grow overnight at 37°C with agitation. Bacteria are collected by centrifugation in 15 ml Corex tubes and resuspended in 0.35 ml of 8 % sucrose, 5 % triton X-100, 50 mM EDTA, 50 mM Tris-HCl pH 8.0 and 25 μl of a 10 mg/ml stock of freshly prepared lysozyme. The solution is then brought to a boil as rapidly as possible over a naked flame and maintaned at boiling point for about 30 to 45 sec in a boiling water bath. The floculated material is pelleted by centrifugation at 12,000 g for 10 min at room temperature. The supernatant is transferred to an Eppendorf tube and precipitated by an equal volume of isopropanol at —20°C for 30 min. The precipitate is pelleted by centrifugation in a microfuge at 12,000 g for 10 min and resuspended in 100 μl of 10 mM Tris-HCl pH 8.0, 1 mM EDTA. Precipitation is repeated twice to obtain clean material for restriction analysis.

4.3. *Restriction endonuclease digestions*

The pBR322 and recombinant plasmid DNAs will be digested by the restriction nucleases EcoRI, HindIII and PstI.

Restriction endonuclease buffers:

— EcoRI: 100 mM Tris-HCl, pH 7.5; 50 mM NaCl, 5 mM $MgCl_2$.

— HindIII: 10 mM Tris-HCl, pH 7.4;

60 mM NaCl; 7 mM $MgCl_2$.
— PstI: 6 mM Tris-HCl pH 7.4: 50 mM NaCl; 6 mM $MgCl_2$; 6 mM 2-mercaptoethanol.

Mix in a plastic Eppendorf tube:
— 10 µl 10 x restriction buffer.
— 5 µg of plasmid DNA.
— 10 units of restriction enzyme.
— Distilled water up to a final volume of 50 µl.

Incubate at 37°C for 1 hour. Stop the reaction by adding 2 µl of 0.5 m EDTA.

4.4 *Agarose gel electrophoresis*

Restriction fragments are fractionated on 0.8 % or 1.2 % neutral agarose gels. Electrophoresis buffer is 10 mM Tris, 5 mM Na acetate, 1 mM EDTA to a final pH 7.9.

Solutions to be prepared:
— 10 x Electrophoresis buffer: 96.91 g Tris base; 8.2 g Na acetate (anhydrous); 7.44 Na_2 EDTA.

Make up to 2 l with distilled water. Adjust to pH 7.9 with glacial acetic acid.
— 10 mg/ml ethidium bromide in H_2O.
— Agarose 1.2 % in 1 x electrophoresis buffer + 5 µg/100 ml of ethidium bromide.
— Reservoir buffer.
1 x electrophoresis buffer containing 10 µg/ml of ethidium bromide.

Sample preparation: After addition of EDTA, suplement samples with 10 µl of 10 x electrophoresis buffer, *plus* 20 µl of a mixture containing 20 % Ficoll and 0.3 % SDS, *plus* 20 µl of a 0.1 % mixture of each bromophenol blue and xylene cyanol FF. Load 20 µl (1 µg of DNA) on gel.

Running the gel:
1. Pour in melted agarose.
2. Allow to harden for at least an hour. Gels can be stored in cold overnight.
3. Run at 50 mA for 3-4 hours. Under these conditions the bromophenol blue behaves like a 400 bp fragment, approximately.
4. Observe under U.V. light.
5. Take picture with Polaroid under U.V. light.

4.5. *Transfer of DNA from gels ("Southern blots")*

Method of Southern (1975) on nitrocellulose sheets.

Step 1. Prepare the transfer apparatus. This consists of a tray filled with 10 x SSC (1 x SSC is 0.15 m NaCl, 0.015 M Na citrate pH 7.0), a glass plate supported on two sides of the traye, and a thick pad of Whatman 3 MM filter paper soaked in 10 x SSC drapper over the glass plate wih two ends dipping into the solution in the tray.

Step 2. Place gel on a tray containing 250 ml of a solution of 1.5 M NaCl and 0.5 N NaOH for 15 min, rocking the tray gently. Decant solution and add new solution repeating operation for another 15 min.

Step 3. Carefully decant the alkaline solution, or draw it off at a water pump, and rinse the gel with water to remove residual NaOH.

Step 4. Soak the gel in a neutralizing solution (250 ml of 1 M Tris-HCl pH 7.0 containing 1.5 M NaCl) for 20 min. Check pH of solution, if pH > 7.5 repeat treatment once more for another 20 min.

Step. 5. Slide the gel carefully from the plate on to the pad of filter paper, taking care to avoid trapping air beneith it.

Step 6. Put plastic frame around the gel. This prevents the absorbent paper, which may sag down, from becoming saturared.

Step 7. Squeeze excess liquid form the surface of gel.

Step 8. Take a sheet of nitrocellulose paper (Schleicher and Schüll BA 85) and wet it first in water and then in 10 x SSC. Handle the cellulose nitrate sheet with care, wear gloves.

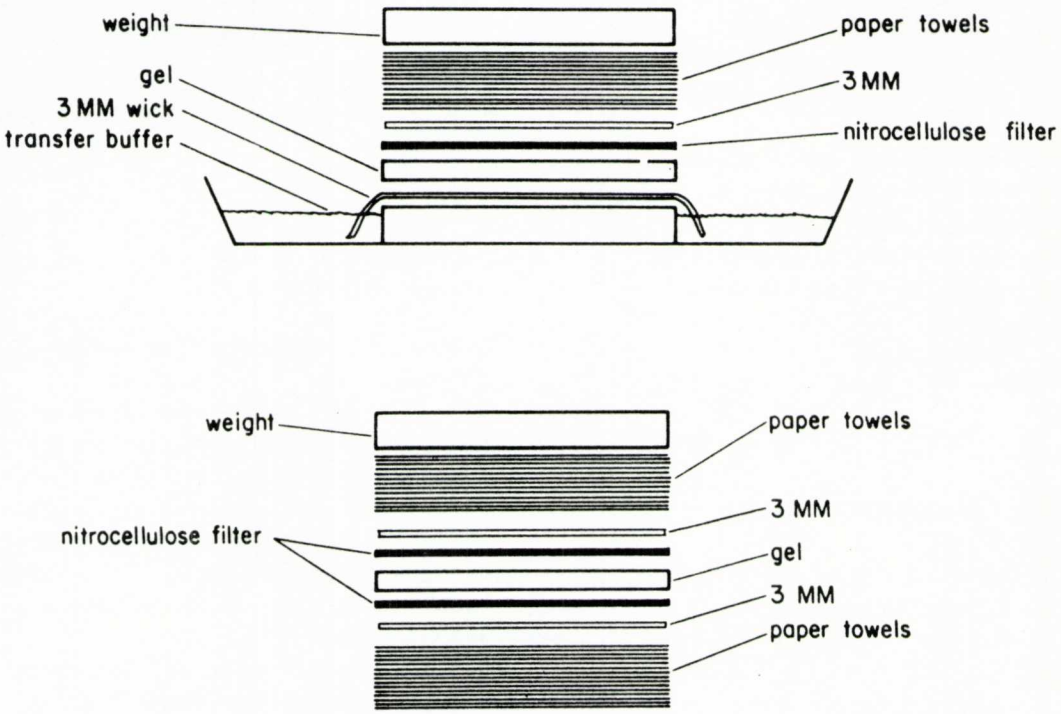


Fig. 3. Transfer of DNA from flat gels to cellulose nitrate paper. (a) Stack of paper towels weighted with a glass plate. (b) Cellulose nitrate paper. (c) Gel surrounded by plastic strips which support the edges of the cellulose paper and the towels. (d) Wad of thick filter paper that dips into the ray of 10 x SSC. (e) Tray of 10 x SSC with a glass plate to support the wad of wet filter paper.

Step 9. Lay the nitrocellulose sheet on the gel, taking care not to trap air beneith it. The edges of the sheet should be supported by the plastic frame.

Step 10. Soak a piece of Whatman 3 MM paper in 10 x SSC and lay it on top of the cellulose nitrate, taking care to avoid trapping air beneith it.

Step 11. Stack adsorbent paper on top of the 3 MM filter paper and weight it down lightly with a glass plate. Leave the transfer overnight (12 h to 24 h).

Step 12. After transfer, carefully remove absorbent paper and top 3 MM paper. Mark the position and orientation of the gel on the cellulose nitrate sheet with a pencil.

Step 13. Rinse nitrocellulose sheet thoroughly for 5 to 10 min with 3 x SSC to remove residual agarose particles clinging the gel. This is important as baked agarose will later lead to important background.

Step 14. Bake nitrocellulose sheet at 80°C in a vacuum over for 2 hr. The sheet can be stored for many months after baking.

Step 15. Restain gel in ethidium bromide 1 μg/ml and observe under U.V. light to ensure efficient DNA transfer.

4.6. *Blot hybridization to rat albumin ^{32}P-cDNA*

Before hybridization wash the nitrocellulose sheet with 5 x SSC at 65°C for 30 min. Then wash for another 30 min at 65°C with

5 x SSC, 10 x Denhardt's solution (0.2 % w/v of each Ficoll M.W. 400,000, polyvinylpyrollidone M.W. 360,000 and bovine serum albumin).

After washing prehybridize the sheet at 67°C for a minimum of 4 hr in a solution containing: 5 x SSC; 10 x Denhardt's solution; 50 mM Tris-HCl, pH 7.5; 1 mM EDTA; 0.1 % SDS and 100 μg/ml of sonicated and denatured salmon sperm DNA. Finally hybridize at 67°C for 24 to 48 h in 10 ml of the same solution to which the hybridization probe is added after denaturation.

After hybridization, the nitrocellulose sheet is washed step-wise as follow:

— 30 min at 67°C with 250 ml of 5 x SSC; 10 x Denhardt's solution; 0.1 % SDS and 0.1 % Na pyrophosphate.
— 30 min at 65°C with 250 ml of 3 x SSC; 10 x Denhardt's solution; 0.1 % SDS and 0.1 % Na pyrophosphate.
— 4 x 30 min at 63°C with 250 ml of 2 x SSC; 1 x Denhardt's solution; 0.1 % SDS and 0.1 % Na pyrophosphate.
— 2 x 30 min at 63°C with 250 ml of 1 x SSC; 1 x Denhardt's solution; 0.1 % SDS and 0.1 % Na pyrophospate.
— 20 min at 63°C with 250 ml of 0.7 x SSC; 0.1 % SDS and 0.1 % Na pyrophosphate.

After washing the filter it is blotted dry with Whatman 3 MM paper and exposed to an X-ray film (Kodak X-omat) with intensifying screens (Dupon Cronex Lightning Plus) at —70°C.

5. Addendum

5.1. *Buffers*

Column buffer cDNA synthesis:

— 20 mM Na acetate pH 5.5.
— 0.2 M LiCl.
— 1 mM EDTA.

Column buffer Nick-translation:

— 10 mM Tris-HCl pH 8.0.
— 0.1 M NaCl.
— 0.5 mM EDTA.

Tailing buffer: 10 x

— 1.4 M Na cacodylate.
— 0.3 M Tris.
— 1 mM DTT.

The pH should be 7.6, but will drop down to 6.9 upon dilution.

Annealing buffer:

— 10 mM Tris-HCl pH 8.1.
— 0.1 M NaCl.
— 0.2 mM EDTA.

Restriction endonucleases buffers:

EcoRI: 100 mM Tris-HCl pH 7.5; 50 mM NaCl; 5 mM $MgCl_2$.

HindIII: 10 mM Tris-HCl, pH 7.4, 60 mM NaCl; 7 mM $MgCl_2$.

PstI: 6 mM Tris-HCl, pH 7.4; 50 mM NaCl; 6 mM $MgCl_2$ 6 mM 2-mercaptoethanol.

Prepare 10 x concentrated for digestions.

5.2. *Culture media*

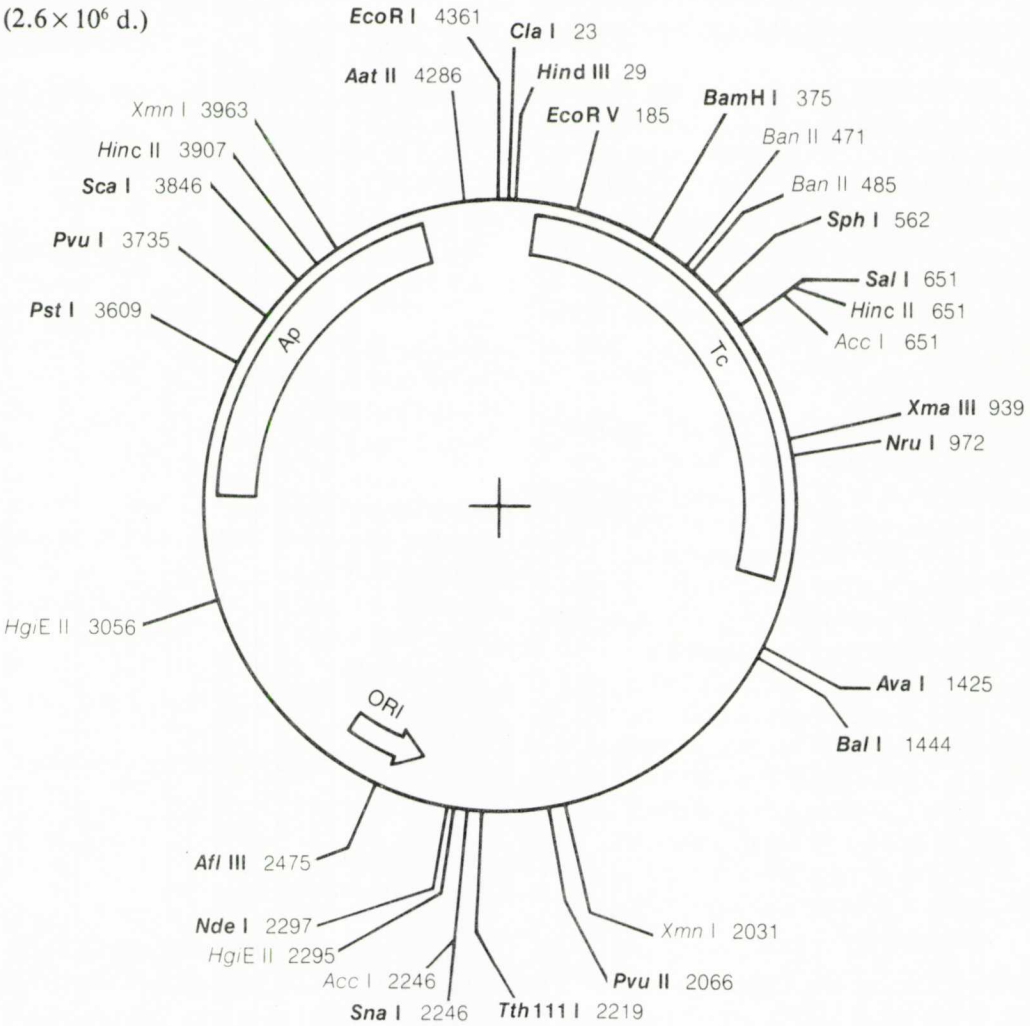
L Broth contains NaCl (10 g), Difco Yeast-Extract (5 g), Difco Bacto Tryptone (10 g) and distilled water in a total volume of 1 l. Dissolve, Adjust pH to 7.0 with 2 M NaOH. Autoclave at 120°C for 20 min.

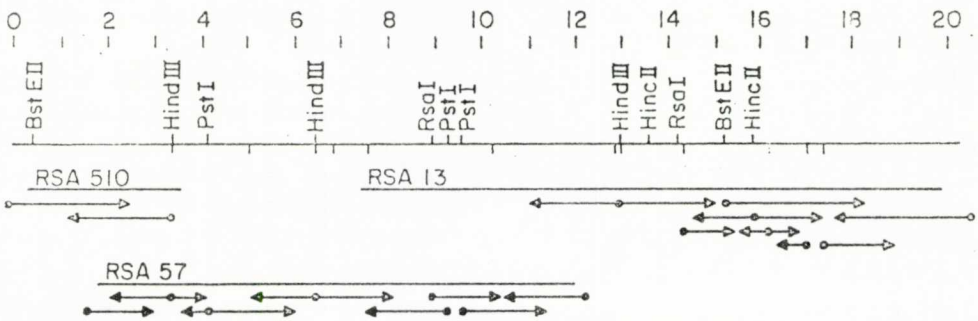
LB medium for plates contains 1 l of L broth and 15 g of Difco Bacto Agar added before autoclaving.

Antibiotic containing media: L broth of LB medium for plates is cooled to 60°C after autoclaving and supplemented with tetracycline (15 μg/ml) or ampicillin (10 μg/ml) from stock solutions (2 mg/ml for tetracycline and 20 mg/ml for ampicillin) sterilized by Millipore filtration.

Z broth contains 1.6 g Nutrient broth, 1 g

5.3 *Restriction map of pBR322 DNA*





peptone, 0.2 g glucose and 100 ml H_2O. Dissolve. Adjust to pH 7.5 with 2 M NaOH before autoclaving.

5.4. *Safety considerations: physical containment*

Although the genetic engineering experiments that will be performed are of null potential risk, special attention has to be paid in order to avoid that the *E. coli* recombinant clones obtained escape to the external environment. Work with this material will be carried out in a limited area and all cultures will be *immediately autoclaved after use.*

6. BIBLIOGRAPHY

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