arations was carried out with standards on agarose gels. It is known that alpha and beta globins are coded by monocistronic mRNA's. The mRNA used for cloning was cytoplasmic in origin (obtained by release from purified polysomes), and selected for size. Therefore, it can be inferred with confidence that it does not contain any hazardous gene sequence. By contrast, a clone made from nuclear DNA or RNA and shown to contain the beta globin sequence might still be hazardous since it could contain neighboring nucleotide sequences which might include an unknown hazardous gene sequence.

Even considering the degeneracy of the genetic code, the chance that a nucleotide sequence 30 or more nucleotides in length will have a fortuitous fit with an amino acid sequence the size of the globin chains is less than 1 in $10^9$. Consequently, when inserts are made by the cDNA technique, the amino acid sequence is known, the mRNA is known to be monocistronic, and the known protein product is deemed harmless, then determination of a sequence of 30 or more nucleotides within the structural gene can provide an effective approach for establishing that a plasmid does not contain a harmful sequence.

We have used this approach and other characterization data as the basis for petitioning for the reduction of the biocontainment requirements from P3 plus EK2 to P2 plus EK1 for pHb23 and other alpha and beta globin plasmids derived from rabbit and chicken. This is the procedure specified in the recombinant DNA guidelines (*10*) for those clones of DNA experiments recombinants derived from shotgun (cloning with DNA preparations less than 99 percent pure) which have been thus characterized.

Thus far we have emphasized the use of our cloned mRNA sequences as a means for determining mRNA sequences. Now that several of these recombinant globin plasmids have been extensively characterized we can use them as hybridization probes for gene enrichment to permit cloning of chromosomal DNA fragments containing the very interesting regions adjacent to the globin structural genes.

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8. Thymidine- and cytidine- specific cleavages are begun by depyrimidization in 15*M* hydrazine (Eastman) with 20 μg of salmon sperm carrier DNA for 50 minutes at 20°C. Cytidine specific depyrimidization is done with 15*M* hydrazine, 1*M* NaCl, and 20 μg of salmon sperm DNA for 100 minutes at 20°C. Strand scission was accomplished by heating the samples for 30 minutes at 90°C in 0.5*M* aqueous piperidine. The samples were then dried and suspended in 0.1*M* NaOH. An equal volume of a solution containing 10*M* urea, 0.02 percent xylene cyanol FF, and 0.02 percent bromophenol blue was added to each sample. Purine specific cleavage was achieved by first methylating the bases by incubating samples in a solution of 50 m*M* sodium cacodylate (*p*H 8.0), 10 m*M* $MgCl_2$, 0.1 m*M* EDTA, 20 μg of salmon sperm DNA, and a 1/250 dilution of dimethyl sulfate (Eastman) for 60 minutes at 20°C. The reaction was stopped by the additon of a 1/10 volume of a solution containing 2.5*M* 2-mercaptoethanol, 3*M* sodium acetate (*p*H 7.5), 0.1*M* magnesium acetate, and tRNA (1 mg/ml). Base release of guanine residues was accomplished by heating at 90°C for 15 minutes. Base release of adenine residues was carried out at 0°C for 60 minutes in 0.2*N* HCl. Strand scission for the purines was accomplished by heating for 15 minutes at 90°C at an alkaline *p*H. The samples were then mixed with an equal volume of the urea dye solution. One-half of each fraction was layered on a gel (0.2 by 20 by 40 cm) consisting of 20 percent acrylamide, 0.67 percent bisacrylamide, and 7*M* urea. The gel buffer and the running buffer consisted of 50 m*M* trisborate (*p*H 8.3) and 1 m*M* EDTA. The samples were placed on the gel and subjected to electrophoresis until the bromophenol blue dye marker had migrated 36 cm. The second half of each sample was then layered on in a second set of tracks, and electrophoresis was continued until the bromophenol blue dye marker of the second set had migrated approximately 22 to 23 cm. The gel was visualized by autoradiography.
9. Abbreviations for the amino acid residues are Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Cys, cysteine; Glu, glutamic acid; Gln, glutamine; Gly, glycine; His, histidine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Try, tryptophan; Tyr, tyrosine; and Val, valine. Other abbreviations are A, adenine; C, cytosine; U, uracil; and G, guanine.
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11. We thank A. Maxam and W. Gilbert for supplying details of their technique in advance of publication and we thank S. Weissman and B. Forget for the sequences they determined for human globin mRNA. This research was supported in part by PHS grants GM18586 and CA15940. J.B. was supported by PHS molecular biology training grant GM-1351. G.V.P. was supported by a Helen Hay Whitney Fellowship. A.L. and H.C.H. were supported by National Cancer Institute predoctoral training grant 4-783825-32923-7.

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## Novel Screening Procedure for Recombinant Plasmids

Abstract. *Lysed bacterial colonies containing potential recombinant plasmids were mixed with molten agar and sealed into slots of an agarose gel. After electrophoresis overnight, the gel was stained with ethidium bromide, which clearly reveals recombinant plasmids.* Xenopus laevis *ribosomal DNA and histone DNA of* Psammechinus miliaris *were ligated into pCRI plasmids and screened by this method.*

DNA sequences of particular interest are often recognized to be associated with restriction fragments of defined length (*1*). In these cases, the construction of recombinants is greatly facilitated provided that screening methods are employed which depend on recognizing particular DNA size classes.

Histone DNA (hDNA) was purified from sperm DNA of *Psammechinus miliaris* by actinomycin $C_1$/CsCl centrifugation (*2*). The DNA was then restricted with HindIII restriction endonuclease, which cleaves the hDNA cluster once every 6 kb (1 kb = $10^3$ base pairs) repeat (*1*). The plasmid pCRI has only one HindIII site, which resides in the gene or control region conferring kanamycin resistance (*3, 4*). Thus, integration of foreign DNA at this site prevents expression of kanamycin resistance, but the resistance to colicin is unaffected. This property was used to distinguish between ligated pCRI molecules and pCRI recombinants. The 6-kb fragments of cellular histone DNA or fragments reclaimed from the recombinant λSam7h22 (*5*) were ligated with HindIII restricted pCRI, and the products were used to transfect $CaCl_2$-treated HB101. Selection was performed on L plates containing colicin EI (*6*). Colicin-resistant colonies were picked onto L plates containing kanamycin (100 μg/ml), and colonies unable to grow were tested by the method described below.

*Xenopus laevis* ribosomal DNA (rDNA) was prepared from total blood DNA (*7*) by two runs on CsCl gradients containing actinomycin (*2*) and after removal of the actinomycin on a third normal CsCl gradient. The peak fractions

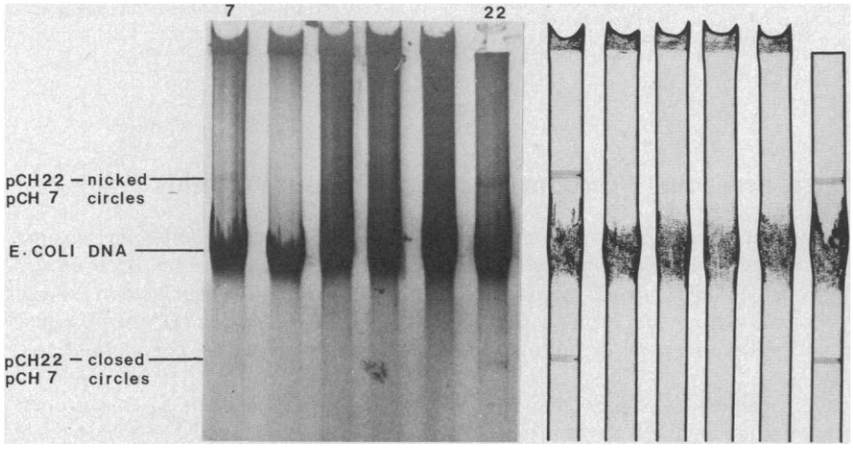
were collected, and after enzyme restriction Eco RI showed the normal pattern (*8*) with a homogeneous 28*S* containing fragment at 3.0 Md (1 Md = $10^6$ daltons) and a range of 18*S* containing fragments up to 7.5 Md. The conditions for partial and total Eco RI restriction were established, and the products from these restrictions were ligated into the Eco RI–restricted pCRI plasmid (resistant to Col El and kanamycin) (*9*). These ligated plasmids were used to transfect $CaCl_2$-treated *Escherichia coli* HB101 (*10*), and selection was made on L plates with kanamycin (100 μg/ml). Colonies were analyzed by the following gel screening method.

The method takes advantage of the fact that bacteria containing Col EI type plasmids have about 24 copies per cell under normal growth conditions (*11*). The kanamycin-resistant colonies from the rDNA experiment and the kanamycin-sensitive colonies from the hDNA experiment were picked onto master plates. Individual colonies were transferred from the plates into 100 μl of Loening's E buffer (*12*) and to this suspension we added 100 μl of E buffer containing 10 μg of proteinase k and 1 percent sodium dodecyl sulfate; the mixture was lysed (Vortex) and then incubated at 60°C for 30 minutes; then 200 μl of 0.6 percent molten agarose (50°C) containing bromphenol blue was added. Portions (200 μl) of the lysis mixture were then applied to slots 8 by 5 mm of an 0.8 percent agarose gel (14 by 16.5 cm). The gel in the slots was allowed to set, and was then sealed with 0.8 percent agarose. The DNA was subjected to electrophoresis overnight at 2 volt/cm in Loening's E buffer, and then stained in a solution of ethidium bromide (1 μg/ml) for ½ to 1 hour. Examples of these gels are shown in Figs. 1 and 2.

DNA from *E. coli* runs as a broad band, whereas the relaxed (single-stranded, nicked) plasmids migrate slower and the supercoiled plasmids migrate faster. Recombinant plasmids were detected (by photography on Ilford FP4 film, not Polaroid type exposures) by their different migration rates, which were different from those of the pCRI plasmid. In the rDNA experiments, colonies either contained pCRI or rDNA recombinant plasmids. Moreover, for the hDNA experiment, the kanamycin-sensitive colonies either contained recombinant plasmids or were Col E1–resistant mutants. However, in one experiment a large number of Col E1 mutants were obtained, and, in order to reduce this high proportion of resistant mutants, the total population of kanamycin-sensitive clones were grown in L broth and then treated with chloramphenicol (170 μg/ml). Supercoil plasmids were isolated by centrifugation on a CsCl–ethidium bromide gradient. Consequently, the DNA was no longer limiting, and very high ratios of DNA to $CaCl_2$-treated HB101 could be used; this greatly reduced the percentage of transfectants caused by the colicin-resistant mutants.

Positive colonies, for both rDNA and hDNA were characterized by hybridization and restriction mapping.

This screening method affords certain advantages which should be mentioned. Because the individual colonies were transferred (via a loop) off the master plates and immediately lysed, this method allows simple containment and minimal handling hazards. If chloramphenicol amplification is thought to be required, growth in l-ml liquid cultures is adequate and thereby eliminates the necessity of large batch production. (Spraying of the master plates with chloramphenicol causes no apparent improvement.) The method does not require hybridization for the screening (*13*) and consequent loss of valuable labeled RNA's is avoided. The actual screening process is rapid (2 days, from picking transfectants until final visualization on gels) and gives a clear answer as to whether integration has taken place and to its efficiency. As plasmid markers of known size can be run, the size of the recombinant plasmids can easily be evaluated from the gels (see Fig. 2).



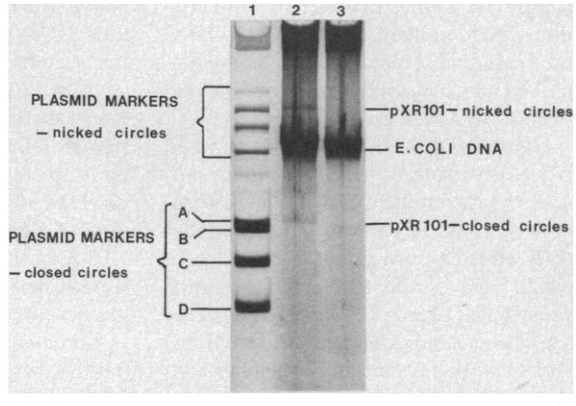




Fig. 1 (above). Electrophoresis of potential hDNA recombinants. The photograph shows the presence of two recombinants, pCH7 and pCH22 on the outermost slots of the gel. Fig. 2 (right). Electrophoresis of potential rDNA recombinants. (slot 1) Plasmid marker A was pXL108 (pCRI plus the 18*S* fragment from pCD4) and equal to 12.7 Md. Plasmid marker B was pXL212 (pCRI plus the 28*S* fragment from pCD4) and was equal to 11.5 Md. Plasmid marker C, pCD30, was 9.6 Md. Plasmid marker D was pCRI and equal to 8.5 Md. (slot 2) Recombinant rDNA plasmid pXR101 (pCRI plus the 18*S* fragment and equal to 4.2 Md) was 12.7 Md. (slot 3) Negative colony containing only pCRI plasmid (faint band). Plasmids pCD4 and pCD30 were constructed by Morrow *et al.* (*8*).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## Plasmid Detection and Sizing in Single Colony Lysates

Abstract. *A simple and contained procedure for the rapid assay of the presence and size of plasmids similar to Col E1 is described. Bacteria are picked from an agar plate with a toothpick, lysed with dodecyl sulfate and heat, and placed directly on an agarose gel for electrophoresis.*

Bacteria often contain extrachromosomal genetic elements in the form of small circles of DNA called plasmids. One of the two classes of DNA cloning vehicle used in genetic engineering is the plasmid; the other class is phage or virus. The most useful plasmids for DNA cloning are those with the Col E1 replicon, since these plasmids are present in a relatively high number of copies (20 to 100) per cell, and they can be further amplified (increased in number) in the presence of chloramphenicol until they constitute 45 percent of the cellular DNA (*1*–*3*). In manipulating DNA on plasmids it is often desirable or necessary to determine the size of the plasmids. Potentially dangerous plasmids should not be cultured or manipulated more than necessary while their properties are being determined. I have devised a procedure which can determine the presence and size of the Col E1 class of DNA plasmids by analyzing a small amount of bacteria which are picked from an agar plate, and then immediately killed and lysed by heating with detergent.

Bacteria containing various plasmids were grown as small (0.5 $cm^2$) streaks on an agar plate with any suitable growth medium (legend to Fig. 1). About 1 $mm^3$ of cells was picked up from each streak or large colony with the flat end of a small toothpick and suspended in 0.2 ml of electrophoresis buffer (0.16*M* tris-acetate, *p*H 8.3, 0.08*M* sodium acetate, 8 m*M* $Na_2EDTA$) by mixing with a Vortex stirrer. One drop (30 to 50 μl) of lysis mixture (5 percent sodium dodecyl sulfate, 50 percent glycerol, 0.1*M* $Na_2EDTA$, 0.2 percent xylene cyanol) was then added with mixing. After heating at 70°C for 10 minutes, the lysate was sheared slightly by taking it up in a drawn capillary, and 40 μl was then placed directly from the capillary into the well of a 1 percent agarose slab gel. Electrophoresis was carried out slowly (1 to 2 volt/cm) for 9 hours; after this time the bromphenol blue dye in a control slot had migrated 5 cm (*4*). Agarose gels were run in the horizontal conformation described by Shinnick *et al.* (*5*). Two parallel rows of 15 samples could be conveniently analyzed on a slab gel (20 by 20 cm). High-quality photographs were necessary for visualization of the plasmid DNA bands (see legend to Fig. 1).

An example toothpick assay is shown in Fig. 1. Several classes of stable nucleic acid are partially resolved on the gel, including transfer RNA (tRNA) and 16*S* and 23*S* ribosomal RNA (rRNA). The RNA bands were overexposed in order to visualize the faint plasmid bands. Host chromosomal DNA just entered the gel and fluorescing material of unknown composition remained at the origin. The plasmids migrated as faint bands between the rRNA and bacterial DNA.

Plasmids with the Col E1 replicon (mini E1, Col E1, and pMB9) have 20 to 30 copies per cell (*1*) and can be readily visualized in this crude assay. Plasmid pSC201 [pSC101 with a temperature-sensitive replicon (*6*)], with one to two copies per cell (*7*) can just be visualized at the limit of detection with this technique, but not reproducibly. It is not visible in Fig. 1. Plasmids pWB91, -2, -3, and -4 are mini E1 plasmids carrying a portion of the histidine operon from *Salmonella typhimurium* (*8*). They are reproducibly more easily detected than the parent plasmid in this assay. Evidently their replication is in some way activated (*8*).

The predominant plasmid band for each sample (except the lysed Col E1 strain) is the supercoiled form of the plasmid. In some of the toothpick assays, and in the slots heavily loaded with purified DNA, the nicked (relaxed) form of the DNA circles can be seen as a band with less mobility than the supercoils. The distance traveled by the supercoils (measured from Fig. 1) is proportional to the log of their size (*9*) at least over the range 3,300 to 18,000 base pairs.

The toothpick lysate of a Col E1–carrying strain which is $thy^-$ shows a background which may be due to DNA dam-

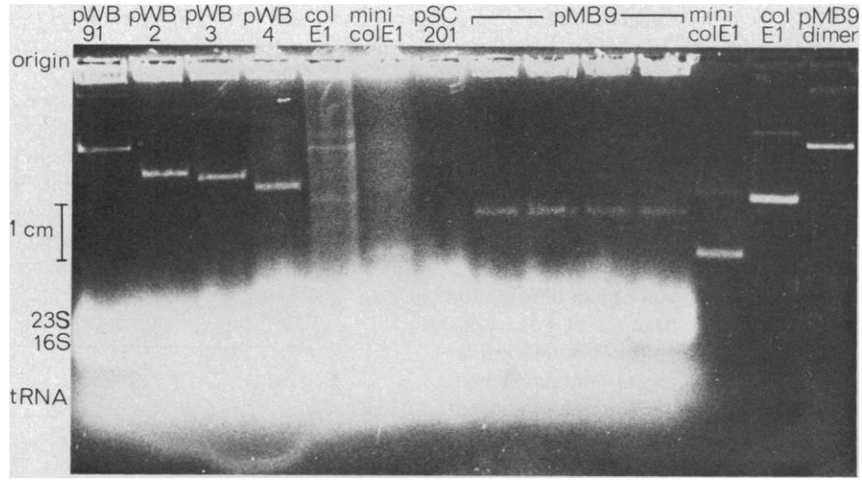


Fig. 1. Sample toothpick assays. The lysis and assay were performed as described. After electrophoresis, the gel was stained by soaking in ethidium bromide (0.5 μg/ml) and then placed on an exposed (black) sheet of x-ray film as a photographic background. The gel was photographed under shortwave ultraviolet illumination through an orange filter and with Tri-X 35-mm film. Polaroid type 57 (ASA 3000) film was not suitable. The *Escherichia coli* background for mini Col E1, pWB91, pWB2, pWB3, and pWB4 was TA2043 (*$sm^r$*, *his-gnd* deletion) (*11*), the background for Col E1 was JC411 ($thy^-$) (*12*), and pMB9 (*13*) was in W3110 (*14*). Bacteria were grown in rich agar with added thymidine. Tetracycline (20 μg/ml) was added to the medium for pMB9 and pSC201. In the right-hand three samples there were 50 ng of purified plasmid DNA prepared as described (*15*).