Filter replicas and permanent collections of recombinant DNA plasmids

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ABSTRACT

    A permanent, ordered collection of 23,000 recombinant DNA plasmids containing <u>Drosophila</u> <u>melanogaster</u> DNA has been established. Simple and practical methods for storing and manipulating this collection were developed. In addition, an improved, simple and inexpensive method for making paper filter replicas of such an ordered collection and of a high density (10,000 colonies/petri dish) unordered collection was developed. These filter replicas are suitable for nucleic acid hybridization screens of recombinant DNA colonies and each filter replica can be used for many ($\geq$5) successive screens. The kinetics of this hybridization reaction were examined and allow design of experiments that detect colony complementarity to a nucleic acid that is 0.5% of the hybridization probe.

INTRODUCTION

    Recent advances in recombinant DNA technology have led to the isolation of a large number of genes and other DNA segments of interest. Most of these isolations have required the combination of an ability to obtain large numbers of bacterial colonies (or phage plaques) each containing different in vitro DNA recombinants and an ability to screen these colonies with nucleic acid hybridization techniques. In this general approach, usually called the shotgun strategy, the hybridization screen is used to identify colonies containing sequences homologous to an RNA or DNA preparation (the probe). In the case of gene isolation, for example, the probe is usually a partially purified mRNA preparation. The most common version of the shotgun strategy requires hybridization to a large unordered collection of colonies, selection of colonies with DNA complementary to the probe and then destruction of the remaining colonies. This version provides a disposable and, frequently, a rapid path to a particular gene. However, if it is used to

isolate one of the many interesting eukaryotic genes that code for nonabundant mRNAs, it can be laborious or non-productive because almost all attempts to purify particular nonabundant mRNAs lead to preparations that hybridize to unwanted colonies. In eukaryotes, for example, contaminating rRNA, 5S RNA and abundant mRNAs will identify many unwanted colonies because they are complementary to repeated DNAs.

In this paper we will describe a number of techniques that permit an alternative version of the shotgun strategy. This alternative avoids a substantial part of the probe contamination problem by accumulating information from successive screens of a single permanent and ordered collection of recombinants. Thus, recombinants complementary to common contaminants need to be identified only once. One additional advantage of this accumulated information is that it identifies recombinants that happen to contain DNA segments homologous to more than one probe.

One technique that makes successive screens of an ordered collection practical is a very simple and rapid procedure that we have devised for making filter replicas of recombinant colonies. The filter replicas produced by this method can be used in a colony filter hybridization screening method like that described by Grunstein and Hogness.[1] However, this new method produces filter replicas by simple batch procedures and yields filters that are reusable. The reusable feature makes them particularly useful for repeated screening of a permanent collection.

We have investigated the kinetics of the colony filter hybridization reaction and our results allow detection of colonies that are complementary to 0.5% of the probe. This allows partially purified, nonabundant mRNAs to be used to identify candidate recombinants. The particular candidate that contains the gene can then be identified by other techniques.[2,3,4]

We have also devised and adopted methods which several years of experience have proven useful in storing, recovering and screening tens of thousands of individual colonies. These methods make permanent, ordered colony collections practical for small to medium sized genomes, that is, genomes the size of bacteria, yeast or Drosophila.

Using these methods we have established a collection of 23,000 Drosophila melanogaster DNA recombinants and have accumulated information about it through repeated screens. In this paper we describe these methods

in detail and report preliminary information acquired from several screens of the collection.

METHODS

DNA

D. melanogaster (Oregon R) DNA was purified from nuclei of 0-12 hr embryos by the method of Schachat and Hogness (1974)[5] with the exception that nuclei were sedimented through 43% (w/w) sucrose in 1 mM $CaCl_2$, 10 mM cyclohexyl-aminopropane sulfonic acid (CAPS, Calbiochem), pH 9.2, 0.2 M 2-methyl 2, 4-pentanediol (Matheson, Coleman and Bell). The sucrose concentration was increased in order to reduce contamination of the nuclear pellet by the less dense mitochondria. The nuclear DNA was sedimented to equilibrium in CsCl and then extracted five times with H_2O saturated, redistilled phenol and twice with chloroform. It was then extensively dialyzed against 10 mM Tris, 1 mM EDTA (TE) pH 8.1. This DNA was sheared at a concentration of 100 µg per ml in 0.25 M NaCl, TE pH 8.1 by five passages through a 21 GA syringe needle under moderate thumb pressure applied to a 1 ml syringe. The shearing gave DNA with an average length of 20 kilobases (kb) as determined by electron microscopy. It was fractionated by size on a 5-20% sucrose gradient in 25 mM NaCl, TE pH 8.1. DNA calculated by relative sedimentation rate[6] to be greater than 22 kb was pooled and precipitated with ethanol.

E. coli plasmids were purified from a cleared detergent lysate[7] of bacteria by centrifugation in ethidium bromide/cesium chloride density gradients.[8] Ethidium bromide was extracted with n-butanol and low molecular weight RNA was removed by gel filtration chromatography with Biogel A-15M, 100-200 mesh (Biorad) in TE pH 8.1.

The plasmid pKB7 is the 17 kb EcoRI rDNA fragment from pDM103[29] inserted into the EcoRI site of pWR77 (R. White and M. Rosbash, personal communication) by K. Beckingham and R. White.

Construction of recombinants

The in vitro recombinants were formed by the poly dA : poly dT tailing method essentially as described by Wensink, et al. (1974).[9] EcoRI (New England Biolabs) restricted pMB9[10] and sheared D. melanogaster DNA were digested with lambda exonuclease[11] to remove an average of 30 bases from the 5' end of each strand. Homopolymer (dA) tails of about 90 nucleotides were

added with calf thymus terminal deoxynucleotidyl transferase[12] (a generous gift from R.L. Ratliff) to D. melanogaster DNA and (dT) tails of the same length to pMB9.

The homopolymer tails of these molecules were annealed and used to transform the HB101 strain[13] of E. coli K12 as described previously.[9] Following transformation, these cells were incubated 30 min at 37°C in L broth[14] to allow cells to recover from $CaCl_2$ treatment and were then plated by the soft agar method onto L broth agar containing 10 µg/ml tetracycline. The transformants were selected by their resistance to tetracycline which is due to pMB9 genes. The tetracycline concentration was selected because a model experiment suggested that increased concentration of tetracycline might select smaller recombinants from the heterogeneous population. In the model experiment, recombinants that had different lengths of DNA inserted into the EcoRI site of pMB9 were used to transform HB101 cells and were plated on LB agar with different concentrations of tetracycline (7.5 to 45 µg/ml). Transformation efficiency diminished over this range and the effect was larger for plasmids with large inserts (approximately 20 fold) than for those with small or no inserts (approximately 3 fold).

All bacterial containing recombinants were treated under P2 conditions in accordance with the NIH Guidelines for recombinant DNA research.

Storage and replication of the colony collection

Recombinant colonies were grown and stored in 144 well polystyrene microtiter plates (Linbro Scientific). The microtiter plates were sterilized by fifteen minute exposure to the light from three G30T8 Sylvania Germicidal lamps. The lamps were spaced 6 1/2 inches apart and were 8 inches above the plates. Both sides of the plates were sterilized so that one plate could serve as the lid for another, thereby allowing growth and storage in stacks.

Medium was delivered simultaneously to all wells of a plate using a multipipette device.[15] The medium in the wells (150 µl/well) allowed growth and also freeze storage and recovery of viable cells without further additions. The medium contained L broth with 25 µg/ml tetracycline and the following compounds (grams/liter) : K_2HPO_4 (6.3), KH_2PO_4 (1.8), NaCitrate (0.45), $MgSO_4 \cdot 7H_2O$ (0.09), $(NH_4)_2SO_4$ (0.9), glycerol (44).[16] Individual colonies were transferred from agar plates to microtiter

plate wells using a radial inoculator.[17] It was necessary to roll the 12 spoke inoculator back and forth along a row of 12 wells in order to obtain efficient transfer of cells to all wells. For small scale work toothpicks were used.

Following transfer the small cultures were grown to saturation without evaporation by placing a stack of microtiter plates on top of damp paper towels, enclosing the stack in a plastic bag and incubating at 37°C. Stacks of plates were stored in plastic bags at -80°C.

A replica of the original collection was made. The cell cultures from all 144 wells of a microtiter plate were simultaneously replicated into another plate using a metal device with 144 stainless steel prongs arranged like the wells. The pronged device was sterilized with flaming ethanol. The medium in the wells had to be gently stirred with the pronged device before replication because the cells pelleted under force of gravity. Replicas of the original collection were used for day to day operations in order to minimize contamination of the original collection. This strategy was also necessary because each replication removed approximately 20 µl from each well.

Filter replicas of the collection

Filter replicas of the collection were made from LB agar replicas of the microtiter plate colonies. Commercial baking sheets (2.5 x 26 x 38 cm) covered with aluminum lids were sterilized and then filled with 500 ml of sterile agar medium (L broth, 1.5% Bacto-agar, 25 µg/ml tetracycline). Pools of condensed moisture on the agar surface were removed with sterile paper towels. A simple mechanical jig (constructed by K. Bostian) allowed the 144 prong replicator to print the colony arrays from two microtiter plates slightly offset from each other. Six of these nested pairs, a total of 1728 colonies, were printed onto one baking sheet agar surface. The offset printing reduced by 1/2 the consumption of agar, filter papers, hybridization probes and film.

Colonies were grown to a diameter of 3 to 4 mm (18 hrs at 37°C) before being transferred to Whatman 541 filter paper. Transfer of the colonies to paper was accomplished by placing the paper over the colonies and incubating an additional two hours at 37°C. The 11 x 11 cm squares of Whatman 541 filter paper were UV sterilized before the transfer, and then were laid over

a nested set of 288 colonies. Air bubbles trapped between the agar surface
and the filter paper prevented transfer. After transfer, the plasmid DNA of
the filter bound colonies was amplified by transferring the filter, colony
side down, onto LB agar containing 250 μg/ml chloramphenicol, and incubating
an additional 24 hrs at 37°C.

These colonies were lysed and their DNA denatured and immobilized on
the filters by washing the filters twice with agitation for five min in each
of the following solutions: 0.5 $\underline{M}$ NaOH, 0.5 $\underline{M}$ Tris pH 7.4, 2 X SSC (1 X SSC
= 0.15 $\underline{M}$ NaCl, 0.015 $\underline{M}$ Na Citrate) pH 7. The filters were then washed
briefly in 95% ethanol and dried in air. Since the filters have very high
wet strength and the DNA is immobilized very rapidly the filters were
treated together as a batch and with no special care. The filters were
labelled with black ink "Sharpie" pens (Sanford's). This label survived all
of the treatments we describe, although substantial amounts of ink were
removed during the first wash with ethanol. In the drying step that followed
this ethanol wash, the labels were kept from contact with other filters in
order to prevent the transfer of ink from one filter to another.

Between hybridization experiments the colony filter replicas were
passed through the entire washing procedure just described. Filters washed
in this manner were autoradiographed to test the extent of signal removal.
More than 95% of the hybridized DNA probe and 100% of the RNA probe was
removed by this procedure.

Labelling of nucleic acids

RNA was labelled $\underline{in}$ $\underline{vitro}$ with polynucleotide kinase (New England Bio-
labs). The RNA was hydrolyzed to an average length of 50 nucleotides by
incubation at 90°C for 30 min in 5 m$\underline{M}$ glycine, 10μ$\underline{M}$ EDTA, 100 μ$\underline{M}$ spermidine,
pH 9.5. 90 μl containing 5-25 μg of hydrolyzed RNA was added to dried γ-
^{32}P-ATP (about 1000 curies/mmole)[18]. The γ-^{32}P-ATP was in molar excess to
the number of 5' ends and was never less than 0.1 nmoles (or 1 μ$\underline{M}$). A ten
times concentrated reaction buffer was added to a final concentration of 10
m$\underline{M}$ MgCl$_2$, 5 m$\underline{M}$ dithiothreotol, 50 m$\underline{M}$ Tris, pH 9.5. T4 polynucleotide kinase
(New England Biolabs) was added to a final concentration of two units (New
England Biolabs) /100 μl and the reaction was incubated at 37°C for 30 min.
Specific activities of 10^7 cpm/μg were routinely achieved. tRNA was not
reproducibly labelled by this technique, and was instead digested with

bacterial alkaline phosphatase (Worthington, BAPF). This enzyme was stored at 0.5 units/ml in a stock solution of 0.1 $\underline{M}$ Tris, pH 8.3 at 4°C for greater than 5 months with little loss of activity. 5 x 10^{-3} units of enzyme/µg of tRNA removes 95% of the terminal 5' phosphate in 30 min at 60°C in 0.1 $\underline{M}$ Tris, pH 8.3. The reaction mixture was extracted 3 times with H_2O saturated redistilled phenol, 3 times with H_2O saturated chloroform and then precipitated with ethanol. After this procedure, the molecular weight distribution of this tRNA as visualized in acrylamide gels was not appreciably altered from the undigested tRNA. However, the small hydrolysis products are preferentially labelled due to their large number.

The DNA was radiolabelled either by the method of Maxam and Gilbert[19] or by the nick translation method[20] as described by Maniatis et al.[21]

For hybridization to colony DNA bound to Whatman 541 paper it is necessary to remove all unincorporated label from the probes before hybridization. Labelled ATP or phosphate binds at random sites on the filter and gives a characteristic speckling which, although distinct from label hybridized to colony DNA, can obscure the hybridization signal. Chromatography with either ion exchange resin DE52[22] (Whatman) or filtration gel G-100 (Sephadex) is adequate for this removal.

Hybridization to colony DNA on filters

Hybridization was done in a solution of 50% formamide, 5 x SSC, pH 7.5, 250 µg/ml carrier tRNA or DNA in a volume of 10 ml/filter. This solution was gently agitated at 37°C during hybridization. After hybridization, the filters were washed 4 times in 2 x SSC, 25°C. The filters hybridized to labelled RNA were digested with 10 µg/ml RNAse A from bovine pancreas (Calbiochem) in 2 x SSC, for 30 min at 25°C to reduce the amount of randomly bound label. RNAse treatment at a higher temperature reduced the signal from RNA that was end-labelled. The filters may be reused after re-washing in NaOH, Tris and 2 x SSC as described above. Treatment with 0.05% diethyl pyrocarbonate for 30 min at 25°C removes residual RNAse activity.

Detection of hybridization

The washed, air dried filters were used to expose either Kodak NS-5T X-Ray film or for increased sensitivity, Kodak RP50 X-Ray film used with Dupont Kronex Lightning plus XG intensifying screens. Colony positions on the autoradiogram can be determined either by previous knowledge of the

positions of colonies complementary to contaminating rRNA or by staining
the filters with 1 µg/ml ethidium bromide which allows the colonies to be
located by fluorescence when irradiated with UV light (see Fig. 1).

RESULTS AND DISCUSSION

Construction and storage of a permanent collection of recombinant plasmids

A collection of plasmids containing random segments of Drosophila
melanogaster DNA was constructed and stored in a way that allowed copies of
each plasmid to be recovered many times. The plasmid molecules were con-
structed by inserting molecules from one size fraction of sheared Drosophi-
la DNA into the EcoRI site of the E. coli plasmid pMB9 by the in vitro
poly(dA):poly(dT) tailing method. These recombinant molecules were used to
transform E. coli cells and more than 23,000 of the resulting transformants
were individually picked and transferred to separate wells of microtiter
plates. The growth media in these wells allowed recovery of viable cells
from all wells even after several years of storage and after repeated (>15)
cycles of freezing and thawing. Many procedures were adopted or devised to
simplify the picking of transformants and the storage and replication of the
entire collection of recombinant DNA clones. These procedures are detailed
in the Methods section.

The fraction of the D. melanogaster genome that is included in this
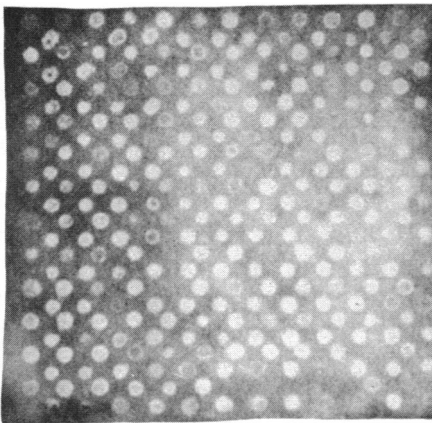collection was estimated from the sizes of plasmids isolated from the col-

Figure 1. A filter copy of DNA from
bacterial colonies. The colonies
from two microtiter plates of the
permanent collection were printed on
Whatman 541 filter paper in a nested
array as described in the Methods
section. The filter bound DNA was
stained with ethidium bromide and
then photographed while illuminated
by uv light.

lection. Agarose gel electrophoresis[23] of detergent lysates[24] from each of
45 randomly selected clones indicated that the average plasmid has 8 kb
(kilobases) of Drosophila DNA. Thus the entire collection contains about
1.8×10^5 kb of Drosophila DNA. It is likely that almost all of this DNA is
from the nonsatellite portion of the genome because simple sequence satel-
lite DNAs are difficult to clone[25] by the methods we have used. For this
reason we assume that only nonsatellite DNA is present in the collection.
Since there is about 1.2×10^5 kb[26,27,28] of nonsatellite DNA in the Droso-
phila genome, we calculate that the average nonsatellite DNA sequence is
represented 1.54 times in the collection.   If each nonsatellite DNA se-
quence has an equal chance of being in the collection, then by the Poisson
distribution equation, the probability of any nonrepeated segment being
present "r" times is $P(r) = m^r e^{-m}/r!$, where m is the mean representation
frequency, 1.54. Thus the probability that a given nonrepeated DNA segment
or any given copy of a repeated but nonsatellite sequence is absent (r=0) is
0.21 and the probability that it is present in the collection is 0.8 or 80%.

Reusable colony filter replicas

A permanent collection of genome segments has several advantages that
result from an accumulation of information about the homology between each
cloned segment and every radiolabelled nucleic acid probe used to screen the
collection. In order to accelerate the accumulation of this information we
have developed a simple and inexpensive version of the colony filter hybrid-
ization method.[1] This version uses Whatman 541 filter paper and is de-
scribed in Methods. One of the most useful features of this version is that
the filter replicas retain DNA under harsh mechanical and chemical treat-
ment. For this reason filter replicas can be made and washed using batch
procedures. More importantly, each filter can be used for many hybridiza-
tion experiments with little loss of sensitivity. The latter feature of
Whatman 541 colony replicas is illustrated in Figure 2 which demonstrates
that replicas that had been used in 2 or in 4 previous filter hybridization
experiments gave signal intensities that were almost identical to those
given by an unused filter.

Filter replicas of unordered clone collections

The Whatman 541 filter replica methodology can also be used to screen
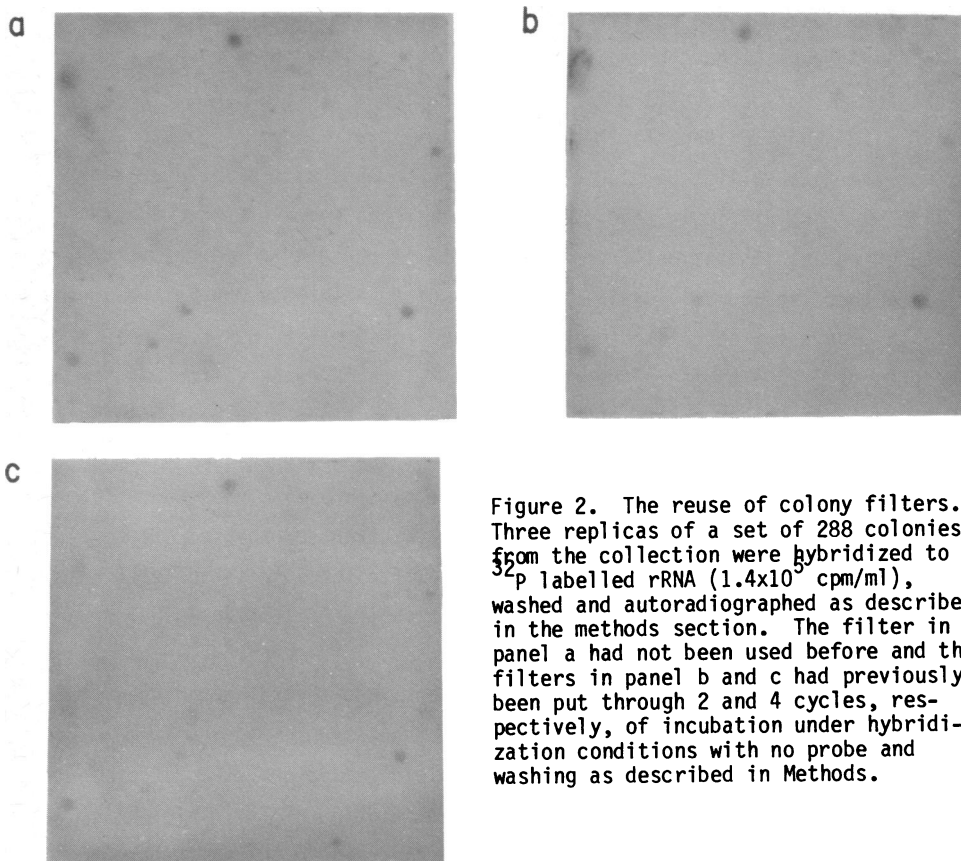large numbers of unordered colonies that have been grown on hard agar sur-

a

b

c

Figure 2. The reuse of colony filters.
Three replicas of a set of 288 colonies
from the collection were hybridized to
^{32}P labelled rRNA (1.4×10^5 cpm/ml),
washed and autoradiographed as described
in the methods section. The filter in
panel a had not been used before and the
filters in panel b and c had previously
been put through 2 and 4 cycles, res-
pectively, of incubation under hybridi-
zation conditions with no probe and
washing as described in Methods.

faces. As a test of the colony densities that can be screened by this
methodology, several model clone searches were conducted. A fixed number of
bacteria containing a plasmid (pKB7) with an rRNA gene was mixed with dif-
ferent number of bacteria containing plasmids that did not have this gene.
These mixtures were grown on standard 10 cm diameter petri dishes for 18
hours. Filter replicas were made and then were hybridized to radiolabelled
rRNA, washed and autoradiographed (Fig. 3). Under these conditions signals
from single colonies could be easily detected on plates that had a total of
approximately 10^4 colonies. At this density a detected colony could
be purified only by screening dilutions of bacteria from the plate region
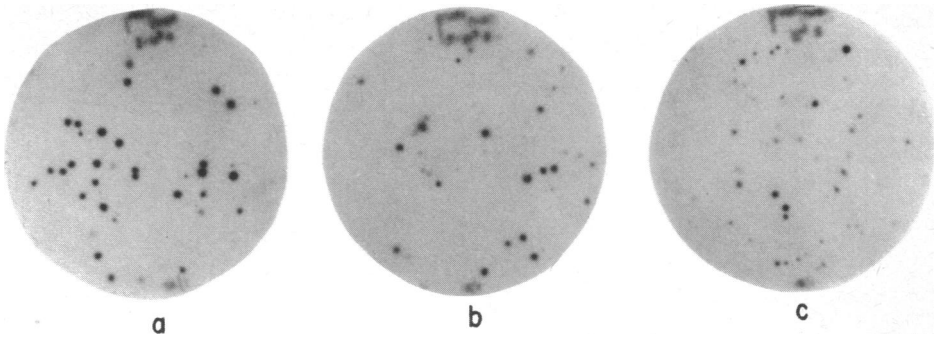that gave the original hybridization signal.

Figure 3. Screening large unordered colony collections. Bacterial dilutions containing approximately 50 bacterial cells with the rDNA plasmid pKB7 and either a) 2 x 10³, b) 5 x 10³ or c) 10⁴ bacteria with the pMB9 plasmid that does not have rDNA were plated on 10 cm diameter petri dishes that contain L-broth with 25 µg/ml tetracycline. Whatman 451 filter replicas were made as described in Methods. The DNA on these filters was hybridized to ³²P labelled rRNA, washed and autoradiographed as detailed in Methods.

Details that affect the sensitivity of colony filter hybridization

We have investigated a number of variables that affect the sensitivity in detecting homology between a probe and colony DNA bound to Whatman 541 paper. A major factor influencing sensitivity is the radioactive noise caused by non-specific binding between the probe and the filter paper. This binding depends on both the concentration of radiolabelled probe and the incubation time. This was demonstrated by autoradiography of filters that had been incubated for different lengths of time with varying concentrations of radiolabelled nucleic acid. Densitometry of these autoradiograms (Fig. 4) showed that the noise level increased with both the concentration of radiolabelled probe and the length of incubation time.

A number of treatments reduce the noise level but do not affect its dependence on hybridization time and radiolabel concentration. For example, increasing the concentration of unlabelled DNA or tRNA in the hybridization solution caused substantial reduction in the noise level. Although higher concentrations of carrier nucleic acid were more effective we found that a concentration of 250 µg/ml was practical in our experiments. Also, the noise was reduced and made more uniform by increasing the hybridization
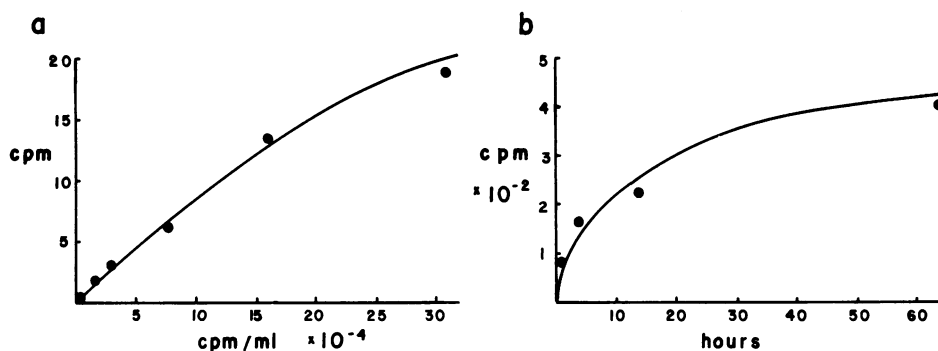
Figure 4. Nonspecific binding of nucleic acid to the filter paper. Whatman 541 filter paper was incubated with ^{32}P labelled rRNA under hybridization conditions. After incubation the filters were washed and autoradiographed. The cpm bound to an area equal to the area of an average colony ($\sim$20mm^2) is shown as a function of the concentration of radiolabel at 1 hr incubation (panel a) and as a function of hours of incubation at 5x10^5 cpm/ml(panel b). The data for panel a and b were obtained from densitometer tracings of the autoradiograms from the rate experiments described in Figure 7 and from the 20% point of the experiment described in Figure 8, respectively. The data was converted to cpm bound by comparison to densitometer scans of simultaneously exposed samples of known radioactivity. Each data point is the average of five colony sized areas.

solution volume three-fold above the minimum necessary to wet the filters. Moreover when radiolabelled RNA was used as probe, RNAse A treatment reduced background two to three-fold.

Several of the treatments that have proven effective in reducing noise when radiolabelled DNA is hybridized to DNA bound to nitrocellulose paper were ineffective when Whatman 541 paper was used. Filter pretreatment with and hybridization in, Denhardt's solution[30] and/or 0.1% sodium dodecyl sulfate were not helpful. In addition exhaustive washing under hybridization conditions was no more effective in reducing noise than were four washes for 30 min in 2 x SSC at 25°C.

In addition to the level of radioactive noise, a second factor influencing the sensitivity of this technique is the amount of colony plasmid DNA available for hybridization. Additional DNA increases the amount of probe that can hybridize to a colony and reduces the hybridization time necessary to obtain a given signal. This factor is particularly important since the experiments just described show that the background increases with the

length of hybridization time. Since the bacterial plasmid used to construct
our collection is under relaxed control of DNA replication it seemed likely
that, as in liquid culture[31], the number of plasmid copies per cell could be
increased by specifically halting protein synthesis. Using colonies con-
taining a relaxed control plasmid that includes the rDNA genes of Drosophila
we tested the effect of such treatment on the sensitivity of colony filter
hybridization. These colonies were grown on a nutrient agar surface, trans-
ferred to Whatman 541 paper and then some of the papers were treated with
chloramphenicol (see Methods). Since previous liquid culture studies of
this amplification process[31] demonstrated that the physiological state of
the bacteria had an important influence on the amount of amplification, we
varied the length of time colonies were grown before application of 541
paper (8 to 24 hrs) and the length of the chloramphenicol treatment (12 to
24 hrs). The extent of amplification was then assayed by autoradiography
after the colonies had been hybridized with ^{32}P labelled rRNA. The signal
was greatest when colonies were grown 24 hours and then exposed to the drug
for 24 hours. Densitometry of the autoradiogram indicated that this signal
was 9 times greater than that from colonies not exposed to the drug (Fig. 5).

Kinetics of filter hybridization

A major use of a permanent colony collection is to identify cloned
plasmids that are homologous to a particular low abundance mRNA. Since
these mRNAs cannot be purified to homogeneity and rarely can be enriched to
greater than a few percent of an mRNA preparation, a two step colony identi-
fication procedure may be used. The first step would identify clonies that
are homologous to more than a few tenths of a percent of the mRNA prepara-
tion. Most of these colonies would be complementary to rRNA and other abun-
dant mRNAs and could be discarded on the basis of previous results. The
second step of this procedure would use a method such as translation assays
of hybridization[2,3,4] to identify the colonies that contain DNA with homolo-
gy to a particular mRNA. The first step in this procedure would require
some knowledge of the kinetics of filter hybridization so that hybridization
conditions could be designed to allow sufficient hybridization of the minor
RNA species yet would minimize the hybridization time and thereby minimize
the noise. The kinetics could also be used to distinguish between colonies
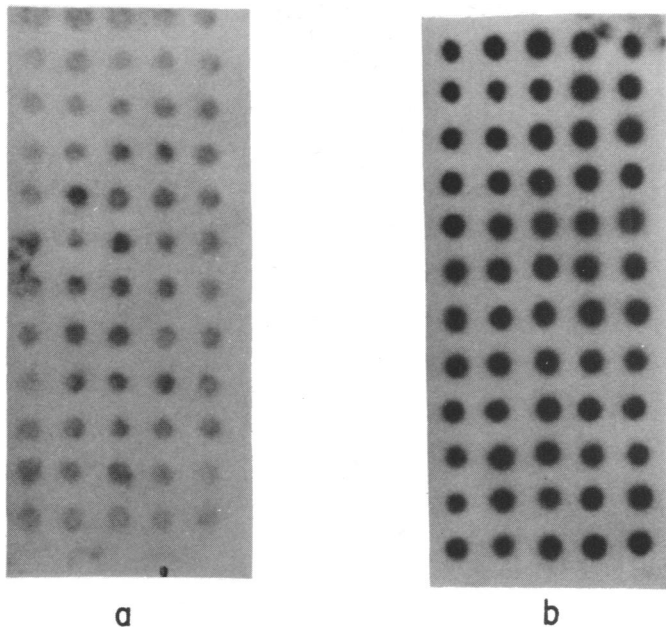that are complementary to RNAs of different abundance.

a   b

Figure 5. Chloramphenicol amplification of signal. Two arrays of pKB7 colonies were printed from the same microtiter dish on LB agar containing 25 µg/ml of tetracycline. After growth overnight at 37°C filter replicas were made by laying strips of Whatman 541 paper across the colonies and incubating for 2 hours at 37°C. One filter strip was then exposed to chloramphenicol for 18 hours at 37°C. After processing, the two filter strips were hybridized with the same rRNA probe and then washed. Autoradiograms of a) the unamplified pKB7 filter and b) the chloramphenicol treated filter are shown.

In order to obtain a simple and useful rate constant we determined several characteristics of the hybridization reaction. We first determined that preincubation under hybridization conditions did not reduce the amount of filter bound DNA available for hybridization. Colony filters that had been preincubated for varying lengths of time were hybridized and autoradiographed together (Fig. 6). Densitometry of the autoradiogram revealed no significant difference between the signals obtained with and without pre-hybridization. Thus our kinetic treatment assumes that filter bound DNA is available until it has hybridized with a nucleic acid from the solution.

We next determined both the initial rate of hybridization and the total amount of colony plasmid DNA available for hybridization. Colony filter
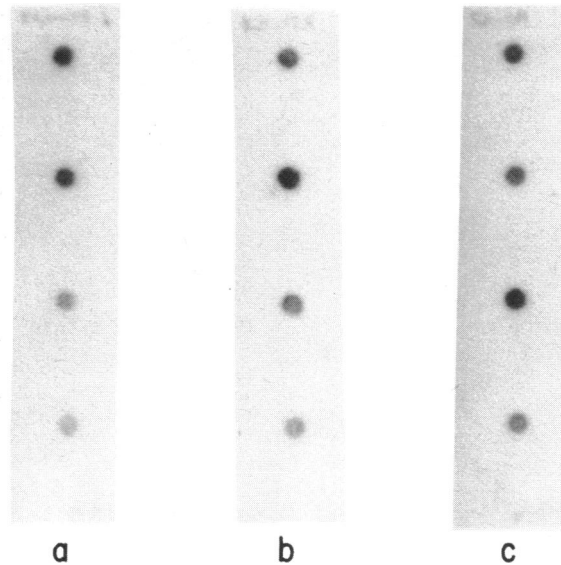
Figure 6. Preincubation of colony filters. Three colony filters with bound plasmid DNA from pKB7 colonies were made. These were preincubated under hybridization conditions in the absence of any probe for a) 0, b) 12 and c) 24 hours before being hybridized with an rRNA probe, washed, and autoradiographed together.

replicas of the pKB7 rDNA clone were hybridized to various concentrations of radiolabelled rRNA for the same length of time (Fig. 7). These filters were autoradiographed and the exposure densities were measured with a densitometer. The mass of rRNA hybridized to each colony was then determined by comparing these exposure densities to those of serial dilutions of the radiolabelled rRNA. The data is plotted in Figure 7B. The hybridization solution had a great deal more rRNA than was hybridized to the colonies so it is likely that the process can be described by the pseudo-first order rate equation, $dD/dt = -kDR_o$, where D is the amount of available, unhybridized DNA, t is the time of reaction, k is the rate constant and R_o is the initial concentration of RNA. This equation can be rearranged and integrated to give: $\ln (D/D_o) = - kR_ot$, where D_o is the amount of DNA available for hybridization at t=0. No good fit to this equation can be generated using all of the data, however if the two data with R_ot values greater than
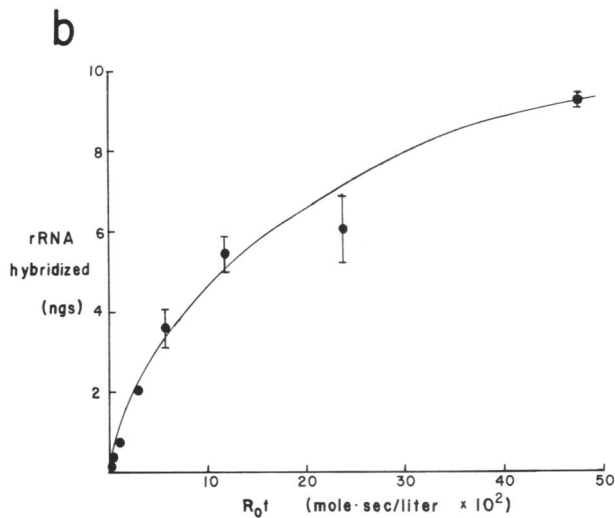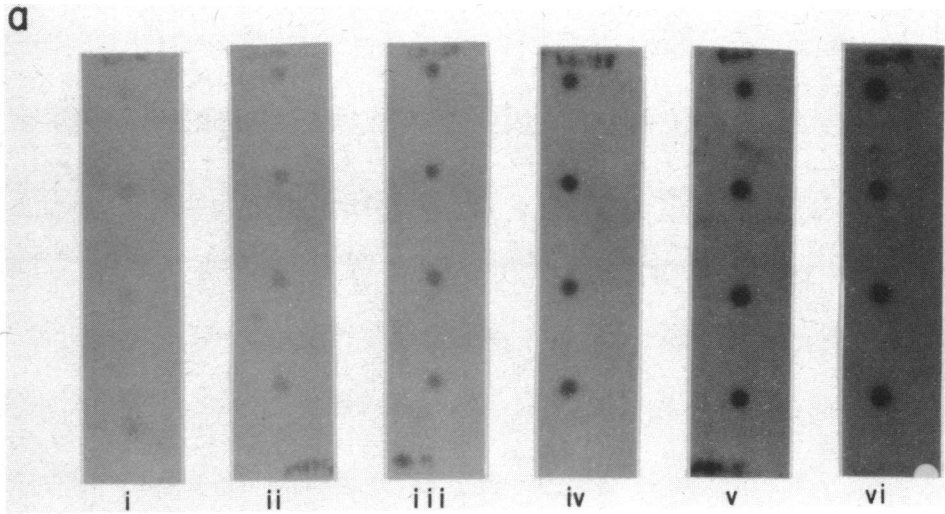
Figure 7. The initial rate of hybridization. a) Six identical colony filter strips containing DNA from pKB7 colonies were hybridized with increasing amounts of radiolabelled 18S + 25S rRNA. The concentrations of rRNA (3.1 x 10^4 cpm/μg) are i) 0.1 μg/ml, ii) 0.5 μg/ml, iii) 1.0 μg/ml, iv) 2.5 μg/ml, v) 5.0 μg/ml and vi) 10.0 μg/ml. Each hybridization was done in 5 mls of 50% Formamide, 5 x SSC, 50 μg/ml salmon sperm DNA, pH 7.5 for 1 hour at 37°C. The filter strips were then washed and autoradiographed together.

b) Amounts of rRNA hybridized to colony filter strips in a) were quanti-tated by densitometry of the autoradiograms. Calibration was done by com-paring the densities with those obtained from autoradiograms of serial dilutions of the probe. The data from two experiments is shown in the graph.

0.2 mole-sec/liter are excluded a good fit can be generated. Since in this experiment the time of hybridization was constant and R_0 was varied, we interpret this result as an indication that the kinetics are non-ideal at high concentrations of probe (>10 μg/ml). Most filter hybridization experiments are done at substantially lower concentrations of probe and should follow a rate constant calculated using data with $R_0 t$ values less than 0.2 mole-sec/liter. In an inverse plot these data extrapolate to a saturation value of 12.4 nanograms of rRNA. Since the plasmid used in this experiment contains 5.4 kb of rRNA complementary DNA,[41] we calculate that there are 2.4 nanograms of hybridizable DNA for every kilobase pair of complementary plasmid DNA. When the data are fit to the pseudo-first order rate equation using the least squares method, k is 5.8 liter/mole-sec. This rate is eighteen times slower than for hybridization to nitrocellulose bound DNA and six times slower than for hybridization in situ to polytene chromosomes.[32] These observations suggest that diffusion through either the Whatman 541 paper or the bacterial debris is rate limiting.

Theoretical and practical limits to detecting colony hybridization signals

The kinetic parameters we have established can be used to estimate the result of any colony filter hybridization experiment and the theoretical limit to detection of hybridization signals. In our experience the defined shape and position of the colonies allows detection of a signal that is only 5% of the background noise intensity. Thus it can be calculated that only 0.1% of a probe need be complementary to colony DNA in order to produce a detectable hybridization signal.[33] This sets a theoretical limit on detection, but this limit is an approximation because our treatment assumes that all plasmid DNA is bound to the filter with the same efficiency and that all colonies have the same number of plasmid copies.

In order to demonstrate the experimental limit in detecting hybridization to filter bound colony DNA we performed a model clone search. Filter replicas of colonies that contain pKB7 (rDNA) or pMB9 (vector) were hybridized to probes that had different ratios of rRNA and tRNA. Both RNAs were radiolabelled to the same specific activity and all hybridization mixes had the same concentration of labelled RNA. Autoradiograms of these filters are shown in Fig. 8 which demonstrates that the signal does depend on the fraction of probe that is complementary to colony DNA. The complementary col-
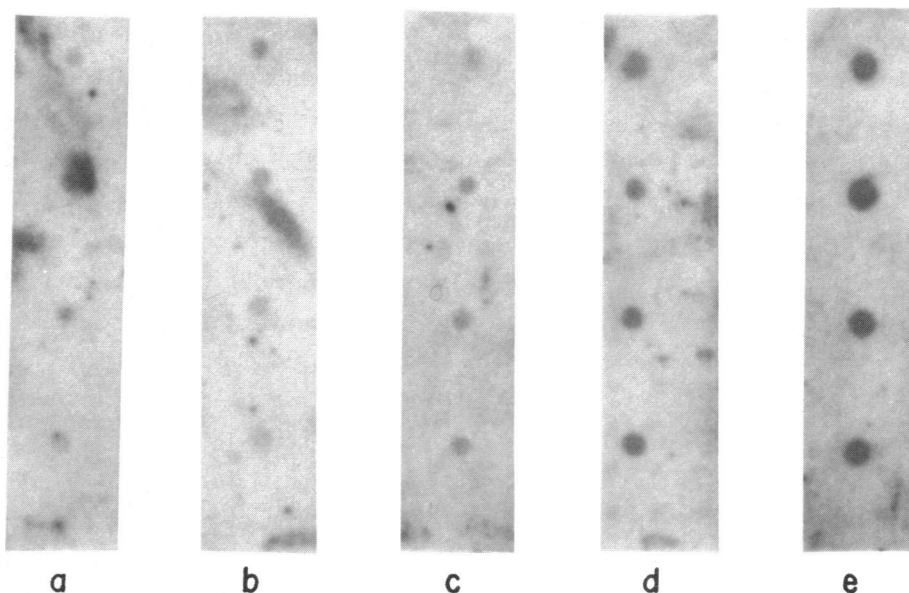
Figure 8. Experimental limit of detection. Radiolabelled rRNA (2.7 x 10^8 cpm/μg) was mixed with yeast tRNA^{phe} (2.7 x 10^6 cpm/μg) to give five hybridization solutions with the same volume and radiolabel concentration, but with differing percentages of label in rRNA. These were hybridized to filter strips containing DNA from pKB7 and pMB9 clones. After a 16 hour incubation the strips were washed and autoradiographed. The rRNA was a) 0.1%, b) 0.5%, c) 1.0%, d) 5% and e) 20% of the label in the hybridization mixtures.

onies are clearly detectable when 0.5% of the probe is complementary. When 0.1% of the probe is rRNA the complementary pKB7 colonies give a weak, barely detectable signal that is not significantly greater than the background. The result indicates a signal detection limit that is in the range of the calculated limit.

The pattern of homologies to the Drosophila colony collection

In general all hybridization screens of our permanent collection of Drosophila recombinants have given results (Table I) that are in accord with current knowledge of the D. melanogaster genome. The exception to this generalization is an approximately five fold over-representation of sequences that are complementary to DNA from the RNA complementary region of cDM412. This over-representation may well be due to the difference between

TABLE I

Probe	Number of complementary colonies	
	expected	found
18S+28S rRNA gene repeat unit[a]	550[g]	593
5S rRNA gene repeat unit[b]	15[g]	13
356 telomere repeat unit[c]	25-55[g]	67
412[d]	60[h]	338
copia[e]	90[h]	92
297[f]	45[h]	113

a. The 11kb EcoRI fragment originally cloned in DmrY22.[34]
b. The 0.38 kb repeat unit isolated from the plasmid 12D1.[37]
c. The 3 kb repeat unit from cDm356.[38]
d. The Hind III restriction fragment that includes the terminal repeat and about 4 kb of the 412 element.[39] It was isolated from cDm 626 by G. Rubin (personal communication).
e. The Hha I fragment that includes the terminal repeat and about 4 kb of the element.[39] It was isolated from cDm 351[40] by G. Rubin.
f. The 2.3 kb EcoRI restriction fragment from pPW303. This fragment is homologous to and is the same size as an EcoRI restriction fragment from the RNA complementary region of pPW297.[40]
g. For these tandemly repeated units the expected number was calculated by multiplying the number of genome masses in the collection (1.54) by the number of gene copies per genome, (360,[35] 210,[35] 30-60 (G. Rubin, personal communication) for rDNA, 5SDNA and cDm356, respectively) and by the size of the repeat unit (an average of 14.5 kb,[36] 0.38 kb,[37] and 3 kb[38] for rDNA, 5SDNA and cDm356, respectively) and dividing by the size (8 kb) of the average insert in the plasmid collection.
h. The expected number was calculated by multiplying the number of genome masses in the collection (1.54) by the number of gene copies per genome (40, 60, 30 for cDm412, copia and pPW297, respectively).[40]

the Drosophila strains used because the abundance of this element is known to differ in the genomes of closely related Drosophila species and to differ even between single flies in the same laboratory stock.[40]

A byproduct of accumulated homology data is the identification of colonies that are complementary to more than one probe. Examination of plasmids from these colonies may reveal that genes or other DNA sequences are close neighbors or that there is sequence homology between different probes. As might be expected, the first of these coincidentally homologous colonies that were detected in our collection have been those containing moderately repetitive DNA. These colonies have been helpful in determining several characteristics of the organization of moderately repetitive DNA (Wensink, Tabata and Pachl, 1979).

SUMMARY

This paper has described methods that simplify the storage and manipulation of large ordered collections of recombinant DNA containing colonies. It has also described a rapid and inexpensive method for making filter replicas of such ordered colony collections and of the unordered collections commonly used in the shotgun strategy of gene isolation. These filter replicas are useful for nucleic acid hybridization screens of colony collections and are reusable, thus simplifying repeated screens of a single collection. The kinetics of hybridization to DNA bound on these filters was investigated and they provide a useful guide for the design of hybridization reactions.

This methodology has proven useful in accumulating information from many screens of an ordered and permanent D. melanogaster recombinant DNA collection. As we have pointed out in the introduction, such an accumulation of information is likely to aid the purification of genes that are complementary to nonabundant mRNAs.

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33. In this calculation it is assumed that the gene of interest is 1 kb long, therefore the rate constant, k, for the complementary RNA is 32 l/mole-sec, and the saturating amount of plasmid DNA, D_o, is 2.28 ngs. Typical hybridization conditions will use a probe with a specific

activity of 10^6 cpm/µg at a concentration of 10^5 cpm/ml. From Figure 4 one estimates that in 48 hours of hybridization this concentration of radiolabel will lead to a background binding of approximately 30 cpm in a colony sized area. Since the limit of detection is a colony with 5% of this amount of radiolabel bound, then in order to detect a positive clone 1.5 pgs of RNA at 10^6 cpm/ug must hybridize. This represents 0.07% of colony saturation. This can be obtained in 48 hours only if the concentration of the RNA of interest, $R_0 \geq 4\times10^{-5}$ µg/ml. The probe is at 0.1 µg/ml, therefore the RNA of interest must be at least 0.04% of this complex probe in order to detect a complementary colony. If this calculation is done for probes varying in specific activity from 10^4 cpm/ug to 10^8 cpm/ug approximately the same result is obtained as long as the concentration of radiolabel does not exceed 10^6 cpm/ml. Above 10^7 cpm/ml the background level of radiolabel binding vastly exceeds obtainable levels of specific hybridization.

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