GENETIC SELECTIONS AND THE CLONING OF PROKARYOTIC AND EUKARYOTIC GENES

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ABSTRACT.   We have constructed pools of viable hybrids of bacteriophage λ and DNA sequences from the enteric bacteria Klebsiella aerogenes, Klebsiella pneumoniae, and Escherichia coli, and the eukaryote Saccharomyces cerevisiae (yeast). Various genetic selections depending upon functional genetic selection of the foreign DNA were developed to isolate specific hybrids from the pools.   The genes for DNA polymerase I were isolated from both species of Klebsiella and several genes in the aromatic biosynthetic pathway were isolated from these three bacteria.   In all cases, these bacterial genes are functionally expressed in E. coli.
       We have also isolated a segment of DNA from yeast which, when integrated into the chromosome of an E. coli histidine auxotroph, allows this bacterium to grow in the absence of histidine.   From genetic experiments, we conclude that genetic expression of the segment of yeast DNA results in the production of a diffusible substance, and that transcription necessary for the complementation is most likely initiated from the segment of eukaryotic DNA.

INTRODUCTION

We are interested in the functional genetic expression of eukaryotic DNA in E. coli . The approach has been to clone segments of the yeast genome in bacteriophage λ and to select for a hybrid phage which can complement an auxotroph of E. coli . The intention in cloning a defined eukaryotic gene is to specifically probe gene expression in eukaryotes. We have chosen yeast as a model eukaryote because of the availability of many mutants including regulatory mutants, and because of the power of yeast genetics. Though yeast is a relatively simple organism, its

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molecular biology is very similar to that of higher organ-
isms and quite different from prokaryotes. In this paper,
we describe the following:
1) The system for cloning foreign DNA in bacteriophage λ.
2) Genetic selections to isolate specific fragments of
     interest.
3) Isolation and characterization of bacterial genes from
     E. coli, K. aerogenes, and K. pneumoniae.
4) Functional genetic expression of yeast DNA in E. coli.

RESULTS AND DISCUSSION

Cloning foreign DNA in bacteriophage λ; construction of hybrid pools.

The cloning vector for these experiments was
developed by Thomas, Cameron, and Davis (1) and Murray
and Murray (2). A diagram of the vector λgt-λB and the
cloning method is shown in figure 1. There are two sites
in λgt-λB which are recognized by EcoRI endonuclease. No
genes essential for λ growth are in the middle EcoRI frag-
ment. To form hybrid molecules, one separately cleaves
the vector and the foreign DNA with EcoRI, mixes, and
seals with DNA ligase via the cohesive ends generated by
EcoRI endonuclease (3). These molecules are transfected
for plaques into an E. coli strain which does not restrict
foreign DNA (rK^-) by the method of Mandel and Higa (4).
Various kinds of molecules are generated by this procedure.
A molecule consisting solely of the left and right ends of
λgt, while containing all the essential genes for λ growth,
is too short to be packaged and is not viable. A viable
molecule (one that results in a plaque) must contain an
EcoRI fragment inserted between the λgt ends. The
viable phage are therefore either hybrids containing the
foreign DNA or the original vector λgt-λB. By using
appropriate conditions (1), greater than 90% of the plaques
are hybrids. Following transfection, the plaques are
scraped to form a hybrid pool which is then passaged once
on a $rK^- mK^+$ strain to form a high titer stock which is K
modified. Phage in such a pool can infect any strain of
E. coli which is sensitive to λ infection.

This system for cloning DNA is not general since
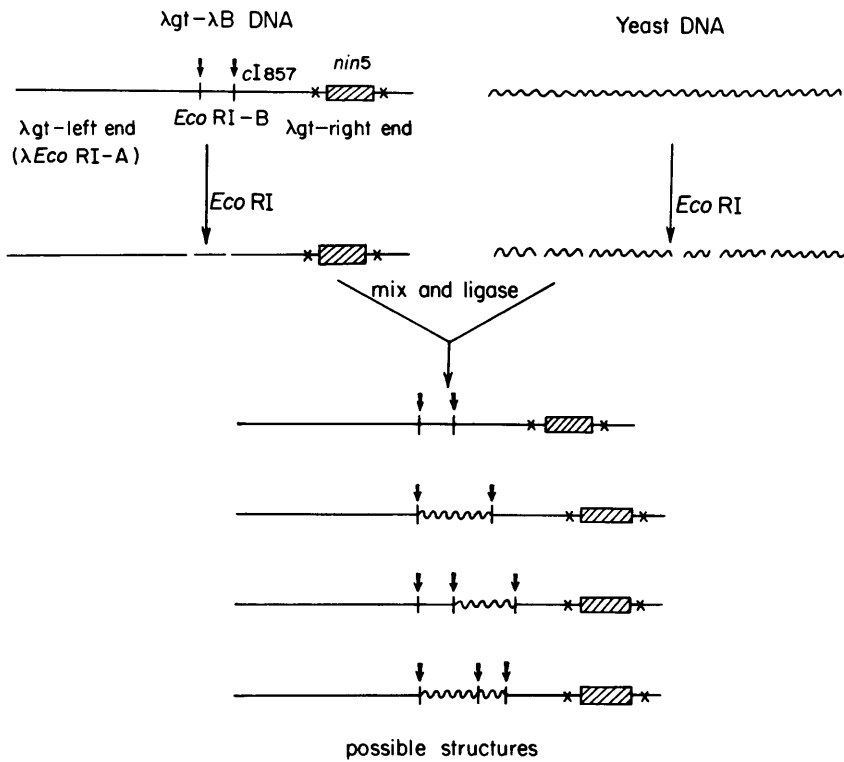only EcoRI fragments between the size of 1 and 15 kilobase

possible structures

Figure 1

Construction of hybrids. λgt-λB and yeast DNA's were separately cleaved to completion with EcoRI endonuclease (partial cleavage products of yeast DNA were used in some cases). They were then mixed at equal concentrations of vector and yeast DNA for covalent joining of the EcoRI cohesive ends by E. coli DNA ligase. This was carried out at 10°C for 18 hours. Four possible resulting structures of viable phage DNA are shown.

(kb) pairs can be cloned. In addition, large fragments will render the viable phage unstable, and small fragments will be inserted at a lower frequency. A more general approach would be the cloning of randomly sheared foreign DNA by the dA:dT homopolymer extension technique (5). To obtain viable hybrids at a reasonable efficiency with this technique, the vector DNA must be isolated as a closed covalent circle. To obtain large quantities of supercoiled λ DNA, we cloned the plasmids pMB 9 (6) and pSC 101 (7) in λgt. These double vectors have 2 origins of DNA replication and can be propagated as phage or as plasmids (selecting for tetra- cycline resistance in these cases). Isolation of DNA from cells containing the double vector replicating as a plasmid yields large quantities of supercoiled DNA.

Genetic selection techniques

λ, being a temperate phage can replicate actively as a lytic virus or passively when integrated into the E. coli chromosome as a stable lysogen. Selections can be per- formed when λ is in either the lytic or lysogenic state. In both cases, a hybrid pool from a given organism is put through a selective process in order to isolate a specific hybrid of interest. The essential feature of such genetic selections is that the inserted DNA be functionally expressed in the E. coli cell.

Direct lytic selections can be applied to the K modified λgt pools. For example, red⁻ derivatives of phage λ are unable to grow on hosts deficient in DNA ligase or DNA polymerase I activity. The vector λgt-λB and all hybrids are deleted for the red genes, and therefore can not grow on strains lacking either of these activities. A hybrid containing an expressed ligase or polymerase gene, however should be able to grow on the host lacking the respective activity. Hybrids containing the ligase gene from E. coli and the polymerase I genes from K. aerogenes and K. pneumoniae have been directly selected by this technique (8, 9).

A selection technique depending on altered host range of a specific hybrid, such as the one described above, is a relative rarity. Since many strains are nutritional auxotrophs, a method for the direct lytic selection of a

phage which supplies a function essential for cell growth
would be useful. A modification of a technique developed
by Naomi Franklin to detect λtrp circumvents the problem
of such a phage killing the auxotrophic host. λ can not grow
on hosts which are not growing. A phage carrying a func-
tion required for cell growth should be capable of growth
and can make a plaque without a lawn. Plaques without
lawns can be easily visulalized by spraying the plate with
ethidium bromide and viewing with ultraviolet light. The
fluorescent spots are due to released DNA in the plaques
from phage which supply an essential function to the cell.
An example of this is the cloning of the tryptophan operon
from E. coli in λgt (figure 2).

We have also developed a selection technique to
select for a hybrid phage which when integrated into the
chromosome of an E. coli auxotroph allows the cell to grow
in the absence of the auxotrophic requirement. All hybrid
phage are deleted for the integration gene (int) and the
phage attachment site (att). However, the hybrids can be
integrated into the E. coli chromosome by co-infection
with an integration helper phage which is int^+att^+ or by
infection of a pre-existing lysogen. This double lysogen
formation is mediated by bacterial general recombination
and occurs at a frequency of 1% (10). This selection tech-
nique requires that the inserted piece of DNA contain a
transcription initiation site since the λ promoters in λgt
are deleted or strongly repressed. This restriction is not
true of lytic infections since the inserted fragment is likely
to be transcribed from one of the λ antiterminated
promoters.

Isolation and characterization of the genes for DNA polym-
erase I from K. aerogenes and K. pneumoniae

Before attempting to clone eukaryotic genes, we
tested the cloning and selection systems described above
using pools made with non-E. coli bacterial DNA. The
rationale was that genetic expression of Klebsiella DNA in
E. coli was predicted and that recombination was not. A
hybrid from both the K. aerogenes and the K. pneumoniae
pool containing the gene for DNA polymerase I was selected
by plating the pools on a host deficient in pol I activity (9).
As expected, these hybrids did not plate on a host deficient
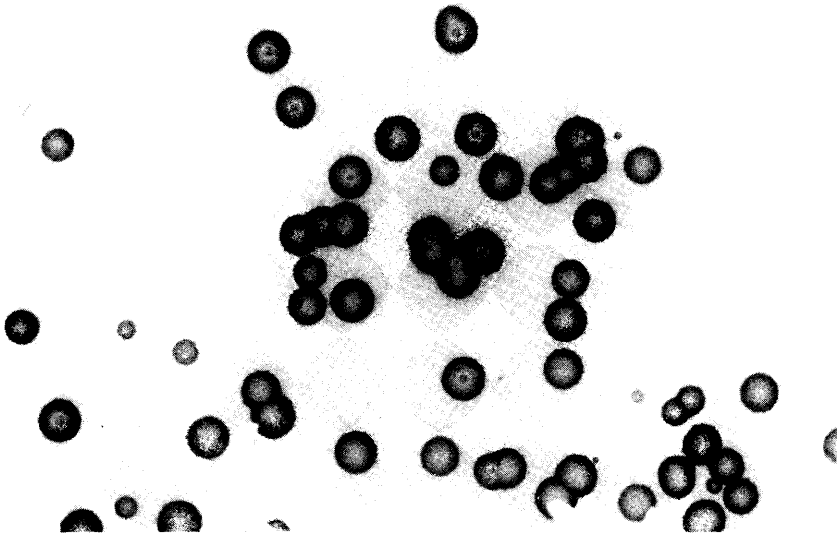
Figure 2

Visualization of plaques without visible lawns by
staining with ethidium bromide. Cells of the tryptophan
auxotroph, trp A 33, were grown overnight in maltose
minimal medium supplemented with tryptophan (trp). The
culture was washed twice with 10mM $MgSO_4$ and starved
for trp for 30 minutes. Approximately 10^8 cells were in-
fected with λgt-λB (10^7) and λgt-Ec trp (500) and plated on
glucose minimal plates at 37^oC. After 2 days, plates were
sprayed with 0. 1 mg/ml ethidium bromide and visualized
by fluorescence with ultraviolet light.

in DNA ligase activity. Agarose gel electrophoresis of DNA from the hybrid phage cleaved with EcoRI endonuclease gave the expected pattern for a λgt hybrid. The hybrid from the K. aerogenes pool has an inserted fragment of 6. 5 kb and the hybrid from the K. pneumoniae pool has an insert of 10 kb.

The hybrids upon infection into E. coli produce significant pol I activity. The pol I activity produced by the phage behaves identically to purified polymerase I since it is resistant to N-ethyl maleimide and sensitive to anti-pol I antibody. It has been shown that the hybrids do not suppress the pol I lesion but rather contain the pol I genes (9). Since this experiment was done in lytic infection, it is unclear whether transcription is from the normal pol I promoter or from read-through from the λ promoter. Both hybrids, however, contain the promoter for pol I (9). Heteroduplex analysis between the Aerogenes and Pneumoniae hybrids reveals a strong region of homology in the inserted segments. At the DNA level, this gene seems to be strongly conserved in these two organisms.

Selection of bacterial genes employing the lysogenic state of λ.

Hybrid pools containing E. coli, K. aerogenes, and K. pneumoniae were co-infected with an integration helper to form double lysogens as stated in a previous section. The gene for 3-enolpyruvylshikimate 5-P synthetase (aro A) was isolated from K. aerogenes and K. pneumoniae, and the gene for chorismate synthetase (aro C) was isolated from E. coli by complementation of an appropriate auxotroph. The frequency of colonies able to grow without any of the products of the aromatic biosynthetic pathway (aro$^+$) was 10^{-5}. When the complementing phage was purified away from the integration helper, the frequency of aro$^+$ increased to 10^{-2} which is the expected frequency of double lysogen formation. This represents an enrichment of 1000 fold from the original hybrid pool.

Expression of yeast DNA in E. coli

At this stage, we decided to employ the lysogenic selection procedure on hybrid pools made with yeast DNA. The mutant used was a histidine auxotroph of E. coli

(his B463). It was chosen primarily because it reverts with a frequency of less than 10^{-11}. His B codes for a product which is a bifunctional enzyme. It catalyzes both imidazole glycerol phosphate (IGP) dehydratase and histidinol phosphate phosphatase. His B463 by enzymological and complementation criteria lacks only the dehydratase activity.

When either of 2 yeast hybrid pools were co-infected with an integration helper into his B463, his$^+$ colonies were found at a frequency of 10^{-8} after 3 days. These are not revertants since no revertants of his B463 have ever been found even after treatment with strong mutagens.

One can imagine many possible artifacts besides the trivial case of bacterial revertants in a preliminary complementation result of this kind. The following experiments were done to rule out as many of these as possible (11). The his$^+$ colonies contain λ prophages. If the prophages are cured from the his$^+$ colonies, the resulting strains are unable to grow without histidine and are indistinguishable from the original parent his B463. The double lysogens formed were normal by two criteria. First, induction of them resulted in equal quantities of two phage. Second, after passaging the his$^+$ colonies once in non-selective medium, 5% of the colonies become his$^-$. This segregation is expected since it occurs by the reverse of the rec mediated formation of double lysogens.

Following induction of a culture of a his$^+$ colony, the presumptive complementing phage was purified away from the helper. This phage is called λgt-Sc his. When λgt-Sc his was integrated into the chromosome of his B463 with the integration helper, his$^+$ colonies were obtained at a frequency of 10^{-4}. This represents an enrichment of 10,000 fold from the yeast hybrid pool. This is the approximate frequency one would expect to find λgt-Sc his in the pool. Therefore, we have isolated a phage capable of complementing his B463. The complementation is not dependent on the double lysogen technique. It is possible to integrate λgt-Sc his into his B463 without the integration helper and obtain his$^+$ colonies. This occurs by abnormal integration (that is, not at the bacterial attachment site) and at very low frequency. Phage can be induced from these single lysogens. The excision is inefficient as ex-

pected but the viable phage induced are indistinguishable from λgt-Sc his by functional and heteroduplex criteria.

Complementation by λgt-Sc his is extremely specific. It complements the non-reverting his B463 and another his B auxotroph lacking only IGP dehydratase activity (his B 2404) which is revertible. It does not complement any other histidine auxotroph including his B mutants which lack both the dehydratase and phosphatase activities. This is consistent with the fact that these two activities are separate enzymes in yeast, but are part of the same poly-peptide in E. coli.

The specific complementing phage λgt-Sc his is indeed a λgt hybrid. DNA made from the phage when cleaved with EcoRI endonuclease reveals an EcoRI fragment of 10.3 kb inserted between the λgt ends.

One important consideration in work of this kind is whether the DNA in the insert comes from its stated source. Contamination of eukaryotic DNA preparations with bac-terial DNA is a particular worry. In addition, the hybrid phage pool has been passaged through essentially wild type E. coli . If the hybrid indeed contains yeast DNA, ^{32}P labelled complementary RNA made using λgt- Sc his DNA as a template should hybridize to an EcoRI fragment of yeast DNA which is 10.3 kb in length. Accordingly, cRNA made to λgt-Sc his was hybridized across a gel of total yeast DNA cut with EcoRI endonuclease by a technique developed by Southern (12). A new preparation of total yeast DNA made in a different manner, from a different strain, and in a different laboratory was used to minimize contamination. Figure 3 shows an autoradiograph from such an experiment. A single band of grains is clearly evident at a position corresponding to a length of 10-11 kb. When cRNA was made to the λgt-λB vector DNA, no hybrid-ization was detected. Therefore, not only does λgt-Sc his DNA contain a fragment of yeast DNA corresponding to a similar sized EcoRI fragment in total yeast DNA, but in addition, the fragment has probably not been rearranged with cloning and propagation in E. coli.

Two conclusions can be drawn from the nature of the selection system. First, the yeast DNA must code for a diffusible product. There can be no specific site effects on
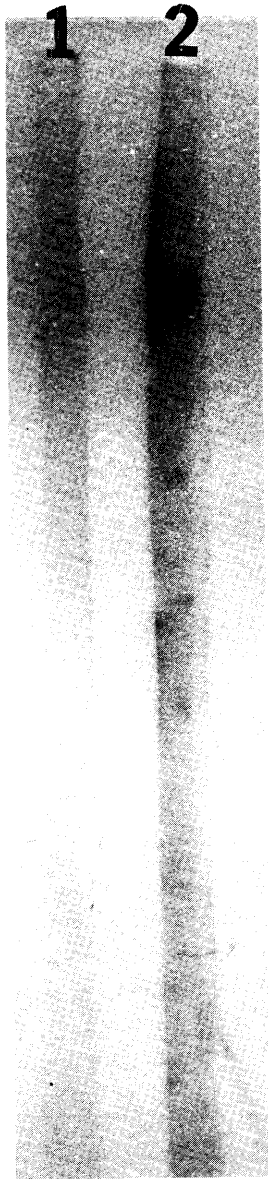
Figure 3
        Autoradiography. 1) Autoradiograph of cRNA to
λgt-λB DNA hybridized to total EcoRI cleaved yeast DNA,
2) Autoradiograph of cRNA to λgt-Sc his DNA hybridized
to total EcoRI cleaved yeast DNA.

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the E. coli histidine operon because only a very specific
class of histidine auxotrophs is complemented and because
attachment site for λ prophages does not map near the his-
tidine operon.   Secondly, transcription necessary for the
complementation is probably initiated in the segment of
yeast DNA.   Since the complementation occurs in a λ
lysogen, λ promoters are strongly repressed.   Strong sup-
port for these conclusions comes from the fact that the
complementation is independent of the orientation of the
fragment of yeast DNA with respect to λ.   λgt-Sc his was
cleaved with EcoRI   endonuclease and resealed with DNA
ligase.   A phage was isolated which contained the yeast
fragment in an inverted orientation as determined by
heteroduplex analysis.   The inversion λgt-Sc his'   com-
plements his B463 with an equal efficiency as the original
isolated λgt-Sc his.   Therefore, unless there are two as of
yet unknown λ promoters, transcription must be initiated in
the yeast fragment.

      Expression of this fragment of yeast DNA is not
dependent on a λ vector.   The fragment has been cloned in
pMB 9 and pSC 101.   When these hybrid plasmids are trans-
formed into his B463, all tetracycline resistant colonies
tested were his$^+$ at 30 and 37°.   At 42° however, they were
his$^-$ indicating that expression of the yeast DNA is tem-
perature sensitive.   This complementation has been re-
cently confirmed by Barry Ratzkin and John Carbon at
Santa Barbara.   Using their pool of col-El -yeast hybrids
which were generated in a different manner, and a selection
system depending on F' mediated transfer of the col El
factor, they have isolated a hybrid which complements
his B463.

      We believe that there is functional genetic expression
of eukaryotic DNA in E. coli.   The question is whether the
yeast DNA in λgt-Sc his   actually codes for the yeast
structural gene for IGP dehydratase (his 3).   It is still
formally possible that the product from the Sc his fragment
suppresses the his B   lesion in some manner.   We think
that the possibility of suppression is extremely unlikely.
First, it is difficult to conceive of how the product of a
segment of yeast DNA could suppress a mutation which is
non-suppressible by any E. coli   mechanism even after

treatment with strong mutagens. Second, a suppressor would have to complement two different IGP dehydratase-less mutants, one non-revertible and one revertible, yet not complement any other histidine auxotrophs including his B mutants lacking both activities. And third, tRNA suppression has been ruled out since λgt-Sc his DNA does not detectably hybridize to yeast transfer RNA (T. St. John and K. Struhl, unpublished). We believe that the most likely explanation for the data is that the yeast structural gene for IGP dehydratase is being transcribed with sufficient fidelity to produce a yeast protein which can complement the his B463 lesion. In any event, we have shown that there is functional genetic expression of eukaryotic DNA in E. coli.

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