Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 834–838, February 1985
Genetics

A cosmid for selecting genes by complementation in *Aspergillus nidulans*: Selection of the developmentally regulated *yA* locus

(shuttle vector/gene library/gene transfer/fungi)

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Communicated by E. Peter Geiduschek, October 5, 1984

ABSTRACT **We constructed a 9.9-kilobase cloning vector, designated pKBY2, for isolating genes by complementation of mutations in *Aspergillus nidulans*. pKBY2 contains the bacteriophage λ *cos* site, to permit *in vitro* assembly of phage particles; a bacterial origin of replication and genes for resistance to ampicillin and chloramphenicol, to permit propagation in *Escherichia coli*; the *A. nidulans trpC*$^+$ gene, to permit selection in *Aspergillus*; and a unique *Bam*HI restriction site, to permit insertion of DNA fragments produced by digestion with restriction endonucleases *Bam*HI, *Bgl* II, *Mbo* I, or *Sau*3A. We used this cosmid to form a quasirandom recombinant DNA library containing 35- to 40-kilobase DNA fragments from a wild-type strain of *A. nidulans*. DNA from this library transformed a yellow-spored (*yA*$^-$) *pabaA*$^-$ *trpC*$^-$ *Aspergillus* strain (FGSC237) to *trpC*$^+$ at frequencies of approximately 10 transformants per μg of DNA. Three of approximately 1000 *trpC*$^+$ *pabaA*$^-$ colonies obtained were putative *yA*$^+$ transformants, because they produced wild-type (green) spores. DNA from each of the green-spored transformants contained pKBY2 sequences and DNA from two transformants transduced *E. coli* to ampicillin resistance following treatment *in vitro* with a λ packaging extract. The cosmids recovered in *E. coli* had similar restriction patterns and both yielded *trpC*$^+$ transformants of *A. nidulans* FGSC237, 85% of which produced green spores. Several lines of evidence indicate that the recovered cosmids contain a wild-type copy of the *yA* gene.**

Complementation of mutations in *Saccharomyces cerevisiae* (yeast) and in animal cells by transformation with recombinant DNA libraries provides a convenient and valuable method for the isolation of specific eukaryotic genes (1–4). Plasmid "shuttle" vectors for this purpose typically include genes for selection in both *Escherichia coli* and the eukaryotic host and one or more unique restriction sites for insertion of foreign DNA fragments. In addition, these vectors must transform the eukaryotic host at a frequency sufficient to allow screening of entire genomes and should provide a simple way to recover the complementing DNA in the prokaryotic host. Shuttle vectors for *S. cerevisiae* frequently contain sequences mediating autonomous replication in yeast to enhance the frequency of transformation and to allow recovery of plasmids by direct transformation of *E. coli* with total DNA isolated from yeast cells (5–9). Generally, analogous vectors have been constructed for use with cultured mammalian cells (10, 11). However, because of restrictions in the size of DNA fragments that can be introduced into these plasmids, it is difficult to screen entire animal genomes. Some shuttle vectors for mammalian cells have been constructed that contain one or two bacteriophage λ *cos* sites to allow *in vitro* assembly of phage particles containing large (30–40 kilobase) cloned inserts, in order to reduce this problem (3, 4).

Aspergillus nidulans is an Ascomycetous fungus related to *S. cerevisiae* and, like *S. cerevisiae*, it has been used extensively to investigate eukaryotic gene structure and regulation (12–15). It is a multicellular organism having the ability to produce a variety of differentiated cell types that are organized into higher order structures (16, 17). For this reason, and because of specific attributes of its genetic system, we and others have been using *A. nidulans* to investigate mechanisms controlling gene expression during eukaryotic development (15, 18–22). Recently, several plasmids and procedures have been developed for integrative transformation of *Aspergillus* and for plasmid recovery from transformants to facilitate molecular genetic investigations of this organism (23–25). The properties of the *Aspergillus* transformation system are similar to those reported for integrative transformation of yeast (see, e.g., ref. 26). However, the relatively low transformation frequencies characteristic of currently available vectors and the absence of appropriate, unique restriction sites have made them unsatisfactory for screening complete gene libraries by complementation of mutations in *Aspergillus*.

To overcome this problem, we made a cosmid vector for *A. nidulans*, structurally analogous to one that has been used for isolation of the human thymidine kinase gene by transformation of cultured mammalian cells (3), and used it to construct a complete library of *Aspergillus* nuclear DNA. In this paper, we report the use of this library to isolate a gene, designated *yA*, encoding a biochemically well-characterized developmentally regulated enzyme called conidial laccase.

MATERIALS AND METHODS

Materials. Bacteriophage λ packaging extracts and [α-^{32}P]dNTPs were purchased from Amersham; calf intestinal alkaline phosphatase was from Boehringer Mannheim; restriction endonucleases (except for *Nru* I), T4 DNA ligase, polynucleotide kinase, *E. coli* DNA polymerase I, and *Bam*HI linkers were from Bethesda Research Laboratories; *Nru* I was from New England Biolabs; and GeneScreen membrane was from New England Nuclear.

Bacterial and Fungal Strains. *E. coli* K-12 strain HB101 (27) was used for routine propagation of plasmids and strain MC1066 (28) was used for complementation tests of plasmids containing the *A. nidulans trpC* gene. *A. nidulans* strains FGSC4 (Glasgow wild type) and FGSC237 (*pabaA1 yA2 trpC801*) were obtained from the Fungal Genetic Stock Center (Humboldt State University Foundation, Arcata, CA).

Construction of pKBY2. Standard recombinant DNA techniques (29) were used to construct pKBY2. A 2.1-kilobase *Hin*dIII/*Sal* I fragment from pJB8 (30), containing the λ *cos*

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site, was ligated to *Hin*dIII/*Sal* I-digested pBR329 (31) DNA to produce pKBY0.1. A 4.1-kilobase *Xho* I fragment containing a wild-type copy of the *A. nidulans trpC* gene (25) was inserted into the *Sal* I site of pKBY0.1 to produce pKBY0.2. A *Bam*HI linker was ligated into the *Nru* I site of the pBR329 component of pKBY0.2 to produce pKBY1. Finally, a 1.5-kilobase *Xba* I fragment was excised from the *trpC* region of pKBY1 and replaced with a similar fragment from which a *Bam*HI site had been removed by sodium bisulfite mutagenesis (32) to produce pKBY2 (see Fig. 1).

Preparation of the *Aspergillus*/pKBY2 Library. *A. nidulans* DNA fragments were prepared by treating five 40-μg samples of nuclear DNA with various amounts of *Mbo* I to yield digestion products ranging in size from 0.5 to about 70 kilobases. The reaction products were combined and subjected to sucrose density gradient ultracentrifugation as described by Maniatis *et al.* (33). Fractions containing 30- to 50-kilobase fragments, as determined by agarose gel electrophoresis, were precipitated by addition of 2.5 vol of 95% ethanol, collected by centrifugation, washed twice with 70% ethanol, dried and suspended in TE buffer (10 mM Tris·HCl, pH 7.5/1 mM EDTA). The DNA was then reprecipitated, washed, and resuspended in TE buffer at a concentration of 400 μg/ml.

Mbo I fragments (4 μg) were mixed with 1 μg of *Bam*HI-digested dephosphorylated pKBY2 DNA in 20 μl (final volume) of ligation buffer (29) and ligated for 18 hr at 15°C. Samples of the ligation reaction (4 μl) were treated with a bacteriophage λ *in vitro*-packaging extract by using the procedures recommended by the supplier. The reaction mixtures were combined, serially diluted, and used to transduce *E. coli* HB101 to ampicillin resistance. The results indicated that 1.6×10^5 colony-forming units were produced by using this procedure.

Preparation of DNA. Approximately 4000 ampicillin-resistant transductants were grown in 10-cm Petri dishes. A heavy suspension of cells was prepared in L broth containing ampicillin at 100 μg/ml and used to inoculate 1 liter of the same medium (OD_{600} = 0.1). The cells were grown at 37°C to an OD_{600} of 0.6, spectinomycin was added to 50 μg/ml, and the culture was incubated for an additional 12 hr. Cosmid DNA was isolated by using standard procedures (29, 34). *A. nidulans* nuclear and total DNAs were isolated as described (25, 35).

Transformation of *Aspergillus*. Procedures for preparation and transformation of *Aspergillus* protoplasts were as described (25).

Genetic Techniques. Standard *Aspergillus* genetic techniques were used (12, 36).

Gel Blotting and DNA Labeling. Partially depurinated DNA was transferred to GeneScreen membrane by using the procedures recommended by the manufacturer. DNA was labeled by nick-translation (37, 38) to a specific radioactivity of $2–10 \times 10^7$ cpm/μg.

RESULTS

Characteristics of pKBY2. Cosmid pKBY2 (Fig. 1) is 9.9 kilobases long and contains functional genes for chloramphenicol and ampicillin resistance derived from pBR329 (31), the bacteriophage λ *cos* site derived from pJB8 (30), and a *trpC*$^+$ gene derived from *A. nidulans* (25). The single *Bam*HI site occurring near the 3′ terminus of the *trpC* gene was eliminated by *in vitro* mutagenesis and a synthetic *Bam*HI linker was inserted into the *Nru* I site of the pBR329 component of the plasmid to provide a unique cloning site for restriction fragments produced by digestion with *Bam*HI, *Bgl* II, *Mbo* I, or *Sau*3A. pKBY2 transformed *E. coli* MC1066 and *A. nidulans* FGSC237 to tryptophan prototrophy at frequencies similar to those reported for the *A. nidulans trpC* plasmid pHY201 (25).

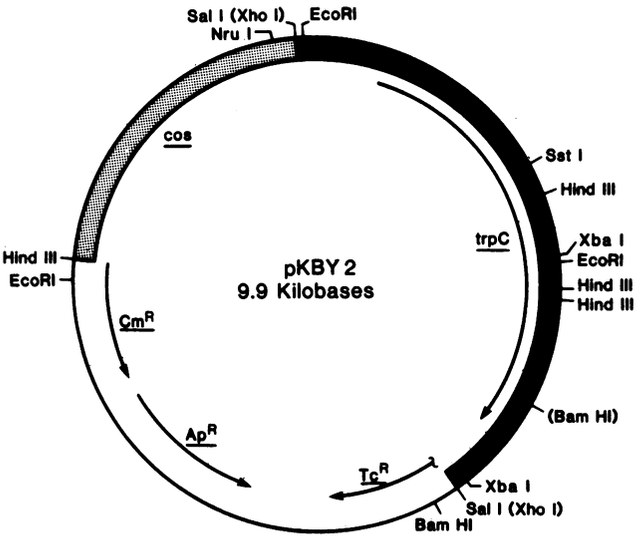

FIG. 1. Partial restriction map of pKBY2. The plasmid was constructed as described in *Materials and Methods* by using DNA fragments from pBR329 (—), pJB8 (▭), and pHY101 (■), a plasmid containing a complete wild-type copy of the *A. nidulans trpC* gene. The *Bam*HI site enclosed in parentheses was removed by sodium bisulfite mutagenesis.

Characteristics of the *Aspergillus*/pKBY2 Gene Bank. We used pKBY2 to construct a library of *A. nidulans* FGSC4 nuclear DNA. Restriction analysis of cosmid DNA from 15 randomly selected ampicillin-resistant bacterial colonies showed that each of the cosmids contained a different insert. The average size of the cloned fragments, estimated by electrophoretic analysis of *Eco*RI-digested DNA, was 35–40 kilobases. Thus, approximately 800 cosmids from the library are equivalent to the *A. nidulans* genome (genome size = 2.6×10^7 base pairs; refs. 35 and 39) and about 3,000 clones must be screened to have a 0.98 probability of obtaining a particular sequence (40).

The *Eco*RI restriction patterns of DNA from an amplified sample of the library and from *A. nidulans* nuclei are compared in Fig. 2. Although the cloned DNA contained a wide size range of *Eco*RI restriction fragments, the digestion pattern differed noticeably from that of nuclear DNA. In a separate experiment, we isolated cosmid DNA from bacterial cultures after only two doublings and without treatment of the cells with spectinomycin. The restriction pattern produced by *Eco*RI digestion of this DNA more closely resembled that of nuclear DNA (data not shown). Thus, the nonrandom representation of fragments shown in Fig. 2 was probably the result of a preferential reduction in the population of some clones during growth or amplification or both.

Complementation of the *yA2* Allele by Cosmid DNA. We transformed the yellow-spored *A. nidulans* strain FGSC237 (*yA2 pabaA1 trpC801*) to *trpC*$^+$ by using DNA from the cosmid library. In this experiment, 10 samples of protoplasts were treated with 10 μg of DNA under standard conditions (25) and plated separately. Each plate yielded approximately 100 *trpC*$^+$ transformants, and three plates produced single, green-spored colonies (putative *yA*$^+$ transformants). A control transformation with 10 μg of pKBY2 DNA yielded a similar number of *trpC*$^+$ colonies, all of which were yellow-spored. We have never observed spontaneous reversion of the *yA2* allele, either in standard reversion tests or in many transformation experiments with *trpC*$^+$ plasmids that do not contain additional *A. nidulans* DNA sequences. All three of the green-spored colonies retained the *pabaA1* marker of the recipient strain. These transformants, designated Gn I, II,

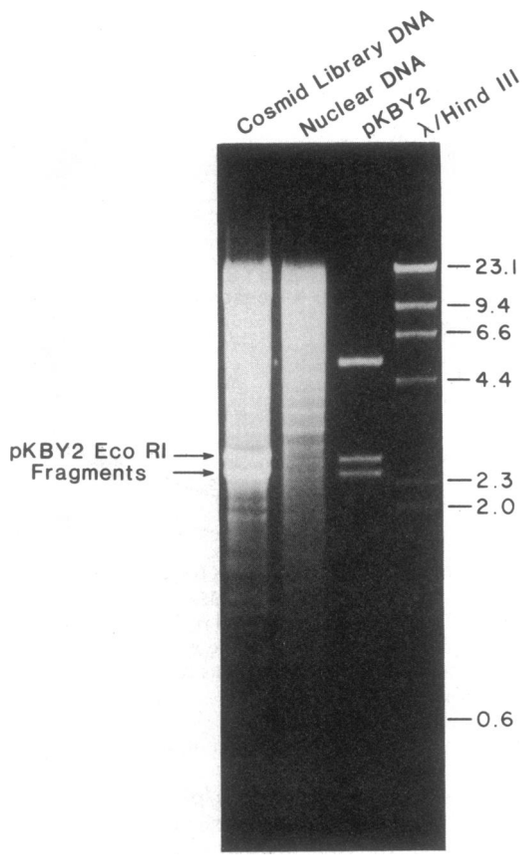

FIG. 2. Comparison of DNA from the pKBY2 library and nuclear DNA. A pool of approximately 4000 pKBY2 clones containing 35- to 40-kilobase inserts of *A. nidulans* nuclear DNA was grown as described in *Materials and Methods* and cosmid DNA was isolated. Samples of cosmid, nuclear, and pKBY2 DNA were digested to completion with *Eco*RI and fractionated by electrophoresis in a 1% agarose gel. Sizes of molecular weight standards are given in kilobases.

and III, were purified by two cycles of single-spore isolation on medium lacking L-tryptophan before further analyzing them.

Analysis of Green-Spored Transformants. The three green-spored transformants were grown in medium lacking L-tryptophan, and total DNA was isolated and subjected to hybridization analysis. We hybridized gel blots of undigested and *Xho* I-digested DNA with either radiolabeled pBR329 DNA or a probe (λAn trpC12; ref. 25) that hybridizes with the 4.1-kilobase *Xho* I fragment containing the *A. nidulans trpC* gene as well as with two adjacent *Xho* I fragments, 6.3 and 7.9 kilobases long. As shown in Fig. 3, each transformant contained sequences complementary to pBR329 DNA. With undigested DNA, hybridization with pBR329 occurred at the same position as chromosomal DNA. *Xho* I digestion of DNA from each of the transformants produced a single, large fragment (>10 kilobases) that hybridized with pBR329. With strains Gn I and Gn II, the fragments had similar or identical sizes, whereas with strain Gn III the fragment was smaller. DNA from untransformed *A. nidulans* FGSC237 contained no sequences that hybridized with pBR329 at the hybridization criterion used (25). Radiolabeled λAn trpC12 DNA hybridized with the expected 4.1-, 6.3-, and 7.9-kilobase *Xho* I fragments of the recipient strain and with fragments having the same molecular weights as those that hybridized with pBR329. The presence of an unaltered 4.1-kilobase *Xho* I fragment indicates that the transforming DNA had not integrated into the resident *trpC* gene (25). The intensities of the novel *Xho* I fragments relative to those of the other fragments that hybridized with λAn trpC12 DNA suggest that the transformants contain only one additional copy of the *trpC* gene per haploid genome.

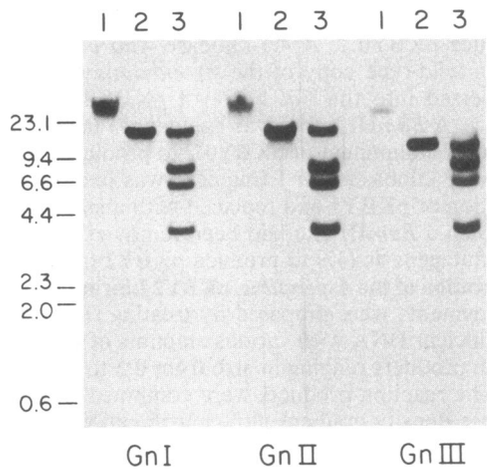

FIG. 3. Hybridization analysis of DNA from green-spored transformants. Samples of DNA from three green-spored *Aspergillus* transformants were fractionated by electrophoresis in a 1% agarose gel either undigested (lanes 1) or after digestion with *Xho* I (lanes 2 and 3). Gel blots were then hybridized with radiolabeled pBR329 DNA (lanes 1 and 2) or λAn trpC12 DNA (lanes 3). Sizes of molecular weight standards are given in kilobases.

We also tested the green-spored transformants for production of the protein product of the *yA* gene, conidial laccase. Protein extracts were prepared from spores and tested for enzymatic activity and for immunologically crossreacting material with a laccase antiserum by fused rocket immunoelectrophoresis with wild-type proteins, as described (41). Reactions of identity were observed in all cases.

Recovery and Characterization of Cosmids from the Gn I and II Transformants. We subjected 0.2–0.5 μg of total DNA from each green-spored transformant to *in vitro* λ packaging. Samples of the packaging reaction were then used to transduce *E. coli* HB101 to ampicillin resistance. Packaged DNA from the Gn I and II transformants yielded 2400 and 80 ampicillin-resistant colonies, respectively. By contrast, DNA from the Gn III strain yielded only a single slow-growing colony that was not included in the analyses described below.

Several transductants derived from the Gn I and II transformants were colony purified, and cosmid DNA was isolated and subjected to restriction analysis. Cosmids recovered from each class yielded indistinguishable electrophoretic patterns following digestion with *Eco*RI or with *Sst* I. One cosmid from each class was chosen and designated Cos yAI and Cos yAII, corresponding to *Aspergillus* transformants Gn I and II. The *Eco*RI and *Sst* I restriction patterns of Cos yAI and Cos yAII DNAs are shown in Fig. 4. The restriction patterns demonstrated that the two cosmids contained highly related but distinguishable inserts, indicating that they were obtained as a result of positive selection. The size of each insert was approximately 35 kilobases. Hybridization of gel blots of *Eco*RI- or *Sst* I-digested *A. nidulans* nuclear DNA with radiolabeled clone DNA produced patterns consistent with those shown in Fig. 4 (data not shown), demonstrating that the inserts were derived from *Aspergillus* and that the sequences were unique in the genome at the hybridization criterion used (0.036 M Na^+, 68°C). We further determined that both Cos yAI and Cos yAII transformed *A. nidulans* FGSC237 to tryptophan prototrophy at a frequency similar to that obtained with pKBY2. Of 75 *trpC*$^+$ transformants obtained, 64 (85%) produced green conidia, demonstrating that the cosmids were capable of complementing the *yA2* mutation in a majority of cases.

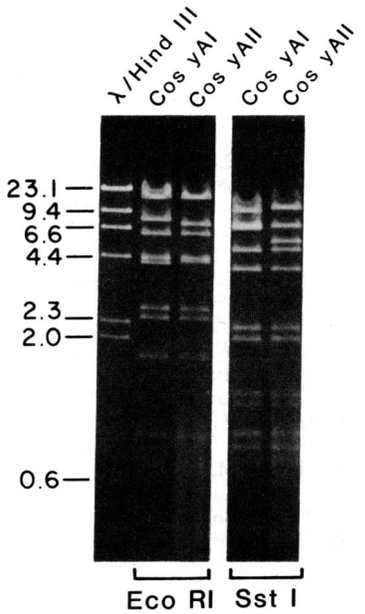

FIG. 4. Comparison of Cos yAI and Cos yAII. Cosmids recovered from two green-spored *Aspergillus* transformants were digested with either *Eco*RI or *Sst* I and fractionated by electrophoresis in a 1% agarose gel. Sizes of molecular weight standards are given in kilobases.

Complementation of the *pabaA1* Allele by Cosmid DNA. We made a suspension of spores from the $trpC^+$ colonies obtained from transformation of *A. nidulans* FGSC237 with cosmid library DNA (see above) and selected for $pabaA^+$ strains. The *pabaA2* allele reverts at a frequency of $<4 \times 10^{-7}$. Four $pabaA^+$ *yA2* $trpC^+$ colonies were selected and purified by two cycles of single-spore isolation, and total DNA was prepared. Analysis of gel blots showed that each isolate contained pKBY2 DNA sequences and an unaltered 4.1-kilobase *Xho* I fragment containing the *trpC801* allele, as was the case with the green-spored transformants. DNA from two of these strains was subjected to *in vitro* λ packaging, and samples were used to transduce *E. coli* HB101 to ampicillin resistance. Cosmids were recovered in both cases and had indistinguishable electrophoretic patterns following digestion with *Eco*RI or *Sst* I, indicating that they were obtained as a result of positive selection.

DISCUSSION

The data presented in this paper show that the cosmid cloning vector pKBY2 is useful for constructing libraries of eukaryotic DNA that can be conveniently introduced into *E. coli*. Nearly random populations of cosmids can be isolated from bacteria and used to transform $trpC^-$ strains of *A. nidulans* to $trpC^+$ at frequencies that allow screening of entire fungal genomes. The data further show that a pKBY2-*A. nidulans* wild-type DNA library introduced into a yA^- (yellow-spored) $trpC^-$ mutant strain is capable of complementation of the yA^- allele, giving rise to green-spored $trpC^+$ transformants. These transformants occurred at a frequency consistent with that predicted for a unique *Aspergillus* gene by the Poisson distribution [$p(3;1000) = 0.1$]. Related cosmids were recovered from two of the green-spored transformants by direct packaging of total DNA and transduction of *E. coli*. These procedures permit identification and isolation of complementing cosmids in about 2 weeks.

Complementation of the *yA2* mutation might have resulted from expression of a wild-type copy of *yA* or from suppression. Although we have not proven that the Cos yAI and Cos yAII clones contain the yA^+ gene, several lines of evidence suggest that they do. First, the two cosmids recovered had nearly identical restriction patterns, indicating that they were obtained as a result of the selection applied (production of green spores). Immunological tests showed that both transformants produce the product of the *yA* gene, conidial laccase. Second, both of the recovered cosmids are capable of simultaneously transforming a yellow-spored $trpC^-$ *Aspergillus* strain (FGSC237) to the $trpC^+$ green-spored phenotype at high frequencies. Third, Cos yAI and Cos yAII were obtained from a library prepared by using DNA from a wild-type *Aspergillus* strain (FGSC4). The observation that 3 of approximately 1000 $trpC^+$ transformants also produced green spores indicates that they did not arise by reversion, because the *yA2* allele does not revert at a detectable frequency. Yellow-spored and green-spored progeny from crosses of FGSC4 and *yA2* strains occur at a ratio of 1:1, indicating that the wild-type strain does not contain an unlinked suppressor of *yA2*.

Finally, two types of genetic evidence support the conclusion that the Cos yAI and Cos yAII clones contain the yA^+ gene. Chromosomal integration of transforming DNA in *Aspergillus* most frequently occurs by homologous recombination. We therefore crossed the Gn I and Gn II strains with a $pabaA^+$ *yA2 biA1* strain and observed segregation of markers in progeny to test for linkage of the transforming activity to the *pabaA* and *biA* genes flanking the *yA* gene (*paba* $A\text{–}\overset{15\text{ cM}}{\text{—}}yA\text{–}\overset{7\text{ cM}}{\text{—}}biA$) (cM, centimorgan). The observed frequencies of the segregation classes differed significantly from those expected if the transforming DNA were unlinked to either *pabaA* or *biA* and were consistent with linkage to both genes. However, if it is assumed that green-spored transformants resulted from homologous recombination of cosmid insert DNA at or near the *yA* locus, then an unexpectedly high proportion (9%) of the progeny were in the double recombination class $pabaA^-$ yA^- biA^+ and an unexpectedly low proportion (22%) were in the parental class $pabaA^-$ yA^+ biA^+. The reason(s) for this segregation distortion is unknown but might be related to the size of the inserted DNA fragment or to its instability during meiosis (see below). It is also possible that the transforming DNA is maintained as an extrachromosomal element.

If the Gn I and Gn II strains arose from integration of a single cosmid by homologous recombination, as indicated by the results of the crosses described above and the DNA blot analyses shown in Fig. 3, then the transformants should contain a tandem duplication of the *yA* region separated by pKBY2 DNA. Tandemly duplicated chromosomal sequences and vector DNA introduced into the *Aspergillus* genome by transformation are frequently lost during selfing, probably because of intrachromosomal recombination (ref. 25; unpublished results). We therefore isolated $trpC^-$ colonies following selfing of the Gn II strain, several of which remained green-spored. Blot analysis of DNA from these strains showed that they had lost the integrated pKBY2 DNA. These strains continued to produce laccase antigen and enzyme activity. Thus, if they arose by resolution of a tandem duplication, then the introduced DNA sequences from the cosmid library almost certainly contain the yA^+ structural gene. Formal proof that the Cos yA clones contain the yA^+ gene will require additional experimentation.

The mechanisms responsible for *in vitro* assembly of phage particles from *Aspergillus* DNA are unknown. The Gn I, Gn II, and $pabaA^+$ transformants all appeared to have single copies of integrated cosmid DNA and would therefore not be expected to provide the two or more *cos* sites needed for packaging (42, 43). It is possible that a low proportion of nuclei contain cosmid concatemers and that these provide the substrate for packaging. Whatever the mechanisms, cosmids can be readily recovered from DNA prepared by using

a rapid isolation procedure (25), without extensive additional manipulation.

Our ability to recover cosmids that complement the *yA* and *pabaA* mutations indicates that pKBY2 may be useful for isolating many other *A. nidulans* genes. The fact that only a few thousand *Aspergillus* transformants need to be screened means that the procedures described in this paper should be useful even when used to complement mutations that revert at relatively high frequencies. The high cloning capacity of pKBY2 may allow direct isolation of very large genes or gene clusters and will facilitate the study of extensive chromosomal regions. We have recently demonstrated that the *Neurospora crassa trp-1* and *Aspergillus niger trpC* genes can be used to complement an *A. nidulans trpC* mutation (unpublished results). Ballance *et al.* (23) have similarly shown that the *N. crassa pyr-4* gene can be used to complement an *A. nidulans pyrG* mutation. It is thus possible that many genes from other filamentous Ascomycetes will function in *A. nidulans*, permitting heterologous libraries prepared in pKBY2 to be used for selection of genes from economically important species.

We thank Drs. Bruce Miller and Margaret Boylan for critical reviews of the manuscript, Mr. Jim Giovannoni for assistance with some of the experiments, and Ms. Linda Arnold for assistance in preparing the manuscript. This work was supported by Grant 82-CRCR-1-1145 from the U.S. Department of Agriculture (to W.E.T.). M.M.Y. was a Predoctoral Trainee supported by a training grant from the McKnight Foundation.

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