Transformation in Fungi

JOHN R. S. FINCHAM

Department of Genetics, University of Cambridge, Cambridge CB2 3EH, United Kingdom

HISTORICAL INTRODUCTION ......................................................... 148

PROCEDURES FOR TRANSFORMATION ......................................... 149

Preparation of the Cells ................................................................. 149

Protoplast preparation ................................................................. 149

Alternatives to protoplasts ............................................................ 150

Conditions for Uptake of DNA ...................................................... 150

REGENERATION OF PROTOPLASTS AND SELECTION OF TRANSFORMANTS .......................................................... 151

Regeneration ............................................................................ 151

Dominant Selectable Markers ....................................................... 151

Two-Way Selection ..................................................................... 151

Visual Selection of Transformants ................................................ 151

Cotransformation ....................................................................... 151

Gene Purification of Transformants .............................................. 151

AUTONOMOUSLY REPLICATING VECTORS .................................. 152

Shuttle Vectors Based on the S. cerevisiae 2μm Plasmid ................. 152

Mitochondrial Plasmids ............................................................... 152

ARSs in S. cerevisiae ................................................................. 152

ARSs in Other Fungi ................................................................. 154

Rearrangements in ARS-Dependent Plasmids ................................ 155

Synthetic Chromosomes for S. cerevisiae ...................................... 155

TRANSFORMATION BY INTEGRATION OF DNA INTO THE CHROMOSOMES ............................................................. 155

Classification of Integration Events ............................................. 155

Filamentous fungi ...................................................................... 156

Multiple-Copy Integration .......................................................... 158

Mechanisms of Integration ........................................................ 158

USE OF TRANSFORMATION FOR ANALYSIS OF GENE FUNCTION ................................................................. 160

Cloning Genes by Complementation ............................................ 160

Gene Disruption ........................................................................ 161

S. cerevisiae .............................................................................. 161

Other fungi .............................................................................. 162

Premeiotic disruption—the RIP effect .......................................... 162

Gene Replacement ..................................................................... 163

S. cerevisiae .............................................................................. 163

Other fungi .............................................................................. 164

Titration of trans-Acting Gene Products ....................................... 164

APPLICATIONS TO BIOTECHNOLOGY ......................................... 165

ACKNOWLEDGMENTS ............................................................... 166

LITERATURE CITED ................................................................. 166

HISTORICAL INTRODUCTION

The first report of deoxyribonucleic acid (DNA)-mediated transformation of a fungal species came in 1973 from the laboratory of E. L. Tatum in Rockefeller University (81, 82). The species was Neurospora crassa, and the strain transformed was an inositol-requiring mutant (inl). DNA isolated from the wild type (inl') was supplied to growing cultures together with calcium, and from the conidia formed on such cultures it was possible to select inositol-independent strains. The use of inl' as the selectable marker was continued in the subsequent studies of Mishra (79) and Szabo et al. (127, 128). The inl mutant was thought to be particularly competent to take up DNA because of the supposedly greater porosity of its cell membranes when starved of inositol (see below).

These early results were received with some scepticism. There was at the time a rather widespread disposition to regard the transformation of eucaryotes as difficult if not impossible. The procedure was not readily reproducible, and an unsatisfactory feature of the inl mutant used was that it was spontaneously revertible to the wild type at a low but appreciable frequency. Although the transformation frequency was reported (79) to be up to 30 times the spontaneous reversion rate, some sceptics were more inclined to postulate some kind of selection of spontaneous revertants rather than accept that conversion by DNA was really taking place. Mishra (80) strengthened his case considerably with his demonstration that the transformation of inl with DNA from another mutant with a temperature-sensitive inl allele resulted in temperature-sensitive inl allele that could not be explained as being due to spontaneous reverse
mutation. However, it was not until fungal transformation was extended to other genes and other species that it became widely accepted as a working technique.

The first breakthrough came with Saccharomyces cerevisiae. Hutchinson and Hartwell (58) had devised a way of preparing S. cerevisiae protoplasts (or spheroplasts—the names are interchangeable) by dissolving the cell walls with a commercial gluconase preparation (Glusulase) and stabilizing the resulting protoplasts with 1 M sorbitol. Their object was to use the protoplasts for studies on macromolecular synthesis. Hinnen et al. (53) found that protoplasts prepared in this way from a len2 mutant could be readily transformed to leucine independence by treatment with wild-type DNA in the presence of calcium chloride. Very soon afterwards, Beggs (12) reported on the construction and use of the first S. cerevisiae-Escherichia coli shuttle vector. This was a chimeric plasmid with two replication origins, one from the E. coli plasmid ColE1 and one from the S. cerevisiae 2μm plasmid (17). It also contained the S. cerevisiae LEU2 gene for selection in S. cerevisiae and a tetracycline resistance gene for selection in E. coli. Random S. cerevisiae DNA sequences, cloned into this vector, could be selected for the ability to complement S. cerevisiae mutants and then transferred to E. coli for amplification. The vector was also found to replicate well in Schizosaccharomyces pombe (fission yeast) (11).

The use of protoplasts for transformation was extended to the filamentous members of the class Ascomycetes N. crassa and Aspergillus nidulans by Case et al. in 1979 (26) and Tilburn et al. in 1983 (134), respectively, and to several other species over the next few years (Table 1). The original protocols have been varied and improved in detail (see below), but have not been fundamentally changed, except for the adoption by some groups (31, 37) of the use of high concentrations of lithium ions as a means of rendering cell walls permeable to DNA without forming protoplasts: a procedure devised for S. cerevisiae (59, 61).

**PROCEDURES FOR TRANSFORMATION**

**Preparation of the Cells**

**Protoplast preparation.** For preparation of transformable protoplasts, the choice of enzyme for digesting the cell walls is crucial. In their pioneering work, Beggs (12) and Hinnen et al. (53) used commercial snail stomach preparations (Helix-case or Glusulase), and, a little later, Hsiao and Carbon (55) obtained good results with an enzyme concentrate of microbiological origin called Zymolyase. Beach and Nurse (11) prepared protoplasts from Schizosaccharomyces pombe with an enzyme preparation from the fungus Trichoderma viride, marketed under the name of Novoyme 234. These various preparations all contain a complex mixture of hydrolytic enzymes, notably 1.3-glucanases and chitinases.

The earlier successes with transformation of N. crassa protoplasts were obtained with Glusulase (26). Anecdotal evidence suggests that the particular batch of enzyme used was of great importance. Kinsey et al. (68, 69) had great success with Glusulase, obtaining up to 10^4 transformants per μg of DNA when selecting for the am4 (glutamate dehydrogenase) gene, a frequency that was not then matched in other laboratories. With a new batch of Glusulase, however, the frequency slumped 100-fold (J. A. Kinsey, personal communication). Akins and Lambowitz (1) identified Novoyme 234 of a particular batch number as particularly suitable for preparation of receptive Neospora protoplasts, and this and other batches of this product have been used in most subsequent experiments on the transformation of filamentous fungi. However, some workers have used better-defined enzyme mixtures; Binninger et al., for example (15), used a mixture of cellulase and chitinase for preparing protoplasts from Coprinus lagopus.

Filamentous fungi often offer a choice of cell types from which protoplasts may be prepared. In Neospora species, germinating macroconidia, which are predominantly multinucleate, are most commonly used; the unicellular microconidia will also yield transformable protoplasts (106) but are more troublesome to obtain. Another alternative is young mycelium (22); protoplasts released from the hyphae by enzyme treatment can be easily separated from the hyphal debris. In Aspergillus and Penicillium species, both germinating conidia and mycelium are used by different groups; one procedure is to grow the mycelium for protoplast formation on a cellophane membrane covering the surface of an agar plate (7). *Podospora anserina* (18) and *Ascobolus*

| Type of fungus | Reference |
|----------------|-----------|
| Ascomycetes    |           |
| Budding yeasts |           |
| Saccharomyces cerevisiae | 53 |
| Kluyveromyces lactis | 30 |
| Kluyveromyces fragilis | 31 |
| Yarrowia lipolytica | 32 |
| Hansenula polymorpha | 133 |
| Fission yeast  |           |
| Schizosaccharomyces pombe | 11 |
| Pyrenomycetes  |           |
| Neurospora crassa | 20 |
| Podospora anserina | 18 |
| Cochliobolus heterostrophus (pathogen on Zea mays) | 137 |
| Gaeumannomyces graminis (take-all fungus of wheat) | 51 |
| Glomerella cingulata (pathogen of beans) | 105 |
| Magnaporthe grisea (rice blast fungus) | 97 |
| Related Fungi Imperfecti |           |
| Colletotrichum trifolii (pathogen of alfalfa) | 38 |
| Fulvia falvum (leaf mold of tomato) | 87a |
| Discomycetes   |           |
| Ascomobolus immersus | Faugeron et al. in press |
| Plectomycetes  |           |
| Aspergillus nidulans | 134 |
| Related Fungi Imperfecti |           |
| Aspergillus niger | 46 |
| Aspergillus oryzae | 75 |
| Penicillium chrysogenum | 39 |
| Cephalosporium acremonium | 118 |
| Basidimyces     |           |
| Ustilaginales   |           |
| Ustilago maydis (smut of Zea mays) | 8, 126 |
| Agaricales     |           |
| Coprinus lagopus | 15 |
| Schizophyllum commune | 83 |
| Phycomycetes   |           |
| Mucorales      |           |
| Phycomyces blakesleeanus | 126 |
| Mucor circinelloides | 143 |
| Other Fungi Imperfecti |           |
| Fusarium oxysporum (wilt fungus of tomato etc.) | 70 |
| Septoria nodorum (leaf spot of wheat) | 28 |

**TABLE 1. Fungal species in which transformation has been achieved**

**Ascomycetes**

Budding yeasts

Saccharomyces cerevisiae ........................................ 53
Kluyveromyces lactis ................................................ 30
Kluyveromyces fragilis ............................................ 31
Yarrowia lipolytica .................................................. 32
Hansenula polymorpha ............................................ 133

Fission yeast

Schizosaccharomyces pombe ........................................ 11

Pyrenomycetes

Neurospora crassa ................................................. 20
Podospora anserina .................................................. 18
Cochliobolus heterostrophus (pathogen on Zea mays) ........ 137
Gaeumannomyces graminis (take-all fungus of wheat) ........ 51
Glomerella cingulata (pathogen of beans) ..................... 105
Magnaporthe grisea (rice blast fungus) ......................... 97

**Related Fungi Imperfecti**

Colletotrichum trifolii (pathogen of alfalfa) .................. 38
Fulvia falvum (leaf mold of tomato) ............................. 87a

**Discomycetes**

Ascosobolus immersus ............................................. Faugeron et al. in press

**Plectomycetes**

Aspergillus nidulans ............................................. 134

**Related Fungi Imperfecti**

Aspergillus niger .................................................. 46
Aspergillus oryzae .................................................. 75
Penicillium chrysogenum ........................................... 39
Cephalosporium acremonium ........................................ 118

**Basidimyces**

Ustilaginales

Ustilago maydis (smut of Zea mays) .............................. 8, 126

**Agaricales**

Coprinus lagopus ................................................... 15
Schizophyllum commune ............................................ 83

**Phycomycetes**

Mucorales

Phycomyces blakesleeanus ........................................ 126
Mucor circinelloides ............................................... 143

**Other Fungi Imperfecti**

Fusarium oxysporum (wilt fungus of tomato etc.) ............. 70
Septoria nodorum (leaf spot of wheat) ........................... 28
immersus (G. Faugeron, G. Goyon, and A. Grégoire, Gene, in press) do not produce conidia, but mycelium is a satisfactory source of protoplasts in these species. For the mushroomlike fungi (members of the class Basidiomycetes and the order Agaricales), it is possible to use either the basidiospores (the products of meiosis) or the dikaryotic mycelium, or, in some cases, the very small vegetatively produced oidia. A published procedure for Schizophyllum commune specifies germinating basidiospores (83), whereas, for Coprinus lagopus, either oidia or mycelium have been used (15). The choice of cell type in any fungus is a matter of convenience; there is little reason to think that one gives better results than another.

All protoplast preparations have to be protected by the presence of an osmotic stabilizer in the suspending medium. Sorbitol, at concentrations between 0.8 and 1.2 M, has been most commonly used and seems to be satisfactory for all fungi. Alternatives are mannitol, used at 0.8 M for Coprinus species (15), and sodium chloride at 0.6 or 0.7 M, which seems to be standard for Aspergillus and Penicillium species (7, 39, 99). Magnesium sulfate at 1.2 M has been used during Aspergillus protoplast preparation (134). Stabilized with sorbitol, Neurospora protoplasts have been found to remain viable indefinitely at -70°C, and so a single batch can be used for several successive transformation experiments (145).

**Alternatives to protoplasts.** Making protoplasts is not difficult, but it needs care. Cell wall digestion must be monitored, and the optimum timing has to be determined for each batch of enzyme. To avoid this trouble, researchers in several laboratories have explored ways to avoid making protoplasts. Three methods have been used by different groups.

The first is to use as the recipient for DNA a mutant strain that supposedly has more permeable membranes or cell walls. This was the rationale for the use of the int mutant in the early experiments on Neurospora transformation. More recent use has been made of a double mutant carrying both int and os, a mutation causing sensitivity to high osmotic pressure (J. C. Wootten, M. J. Fraser, and A. J. Baron, Neurospora NewsL. 27:33, 1980). Conidia of this strain, germinated in medium containing 20% sucrose, produced hyphae with swellings indicative of weak spots in their cell walls. These germinal were shown to be transformable, although at low frequency by comparison with more recent methods involving protoplasts.

A second and more widely adopted way to avoid making protoplasts is to use high concentrations of alkali metal ions to induce permeability to DNA in intact cells. Imura et al. (59) used 0.2 M calcium chloride to make yeast cells transformable with cloned DNA and obtained somewhat better transformation frequencies than when they used protoplasts. Ito et al. (61) tried a variety of cations and found that 0.1 M lithium, supplied as the acetate salt, gave the best results. Recently, essentially the same method has been used to transform Saccharomyces cells picked directly from colonies on plates (66). The lithium procedure has also been successful when applied to N. crassa (37) and C. lagopus (15). In each case, germinating spores were exposed to the transforming DNA in the presence of 0.1 M lithium acetate. Exactly how alkali metal cations assist the passage of DNA into cells does not seem to be well understood (but see references 48 and 136).

Very recently, Costanzo and Fox (29) have reported successful transformation of S. cerevisiae cells by suspending them in growth medium supplemented with 1 M sorbitol and DNA, adding glass beads, and agitating the mixture at the highest speed of a vortex mixer for 30 s. This violent treatment killed 80 to 90% of the cells, but transformed colonies were produced by a small proportion of the survivors following plating on selective medium containing 1 M sorbitol. The efficiency of transformation in terms of yield per microgram of DNA was relatively low (only of the order of 100μg when replicating plasmid DNA was used [see Table 2]), and was increased somewhat by the addition of carrier calf thymus DNA. Unlike all other transformation procedures in current use, this method does not require the use of alkali metal cations. Its simplicity is attractive.

### Conditions for Uptake of DNA

In fungi, as in animals cells and bacteria, the universal component of transformation mixtures, apart from the DNA itself, is calcium ion. The exceptions to this generalization are the non-protoplast-forming protocols involving the use of high concentrations of lithium, in which there is no additional need for calcium, and the mechanical disruption procedure described above (29). In transformation of protoplasts the concentration of calcium chloride most commonly used for S. cerevisiae is 10 mM, and the same applies to most of the protocols for Aspergillus species and other filamentous fungi, with the exception of Neurospora species, for which 50 mM has been more often used. The typical components of a transformation mixture are protoplasts at a density of about 10^6 to 10^7/ml DNA (different workers use different concentrations, but for cloned DNA, 5 μg/ml should be ample). 10 or 50 mM calcium chloride, and a buffer which may be 10 mM Tris hydrochloride (pH 7.5 or 8.0) or 10 mM morpholinepropanesulfonic acid (MOPS) (ca. pH 6). It is not clear whether variation of pH in the range 6 to 8 has much effect. Double-stranded DNA, either linear or circular, has usually been used, but single-stranded DNA has also been shown to be effective in Saccharomyces species (117) and Ascoyobus immersus (Goyon and Faugeron, unpublished).

The results of one of the few careful studies of the relation between DNA concentration and yield of transformants are shown in Fig. 1a. An incubation time of 15 to 30 min at room temperature has generally been found sufficient for DNA uptake.

**Neurospora** workers, following Case et al. (26), have generally added about 1% dimethyl sulfoxide to their transformation mixtures, and some of the most successful protocols have included 0.05 to 0.1 mg of heparin per ml (69) and sometimes 1 mM spermidine as well (145). These further additives to not seem to have been much used in transformation of fungi other than Neurospora species; whether this is a reason for the substantially lower yields of transformants in these other fungi is not clear (Table 2).

**Virtually all** fungal transformation protocols call for the addition of high concentration of polyethylene glycol (PEG) following the initial period of exposure to DNA. This is true of the procedures involving the use of lithium for nonprotoplast cells as well as those involving protoplasts. Up to 10 volumes of 40% PEG 4000 is commonly used, although a protocol for Schizophyllum commune specifies only a little over 1 volume of 44% PEG (83). The PEG is added along with calcium chloride and buffer, to maintain the previous concentrations of these components. The effect of PEG is to cause the treated cells to clump, and this may facilitate the trapping of DNA.

An alternative way of delivering DNA to the cells, and one that could in principle be more efficient, was devised by...
Radford et al. (101). They encapsulated the DNA in liposomes (artificially constructed lipid vesicles) and induced these to fuse with protoplasts. This procedure was effective but appears to offer insufficient advantage over the new routine procedures with free DNA to justify the extra work of preparing liposomes.

REGENERATION OF PROTOPLASTS AND SELECTION OF TRANSFORMANTS

Regeneration

The essential requirement for obtaining growing colonies from protoplasts is the maintenance of the osmotic stabilizer in the growth medium until the cell wall has been regenerated. The same stabilizer is generally used as for the protoplast preparation, i.e., 1.0 to 1.2 M sorbitol, 0.6 to 0.7 M sodium chloride, or 0.6 M sucrose (the last used for Podospora species [18]). For Coprinus species, however, 0.5 M mannitol has been used for protoplast preparation and 0.5 M sucrose has been used for regeneration (15).

Dominant Selectable Markers

DNA-treated cells are usually plated on agar medium that is selective for the desired type of transformant. Most experiments have been concerned with transforming auxotrophic mutants to prototrophy, and in that case, selection of transformants is usually straightforward. Certain wild-type genes capable of complementing auxotrophic mutants have been particularly useful as general-purpose selectable markers and have frequently been incorporated into cloning vectors (see below). A number of them are functional in several different species (Table 3).

Two-Way Selection

For some purposes, especially connected with gene replacement (see below), it is extremely useful to have a marker that can be both selected and counterselected. There are a number of markers in different fungi that fulfill this requirement. In both S. cerevisiae and N. crassa, mutations resulting in loss of orotidine-5'-phosphate decarboxylase (ara3 and psy-4, respectively) confer resistance to the normally inhibitory analog 5-fluoro-orotic acid (2, 39, 40). S. cerevisiae lys2 (2-amino-2-deoxyglucose deficient) mutants can grow (in the presence of lysine) on 2-amino-2-deoxyglucose as the sole nitrogen source, whereas wild-type S. cerevisiae cannot (9).

Visual Selection of Transformants

In some special cases, the expression of the transforming gene can be seen by eye on an indicator plate. For example, Reepen et al. (104) transformed S. cerevisiae with a DNA construction in which the E. coli β-lactamase gene (bla) was placed under the control of the S. cerevisiae ADHI (alcohol dehydrogenase) ethanol-inducible promoter. Transformants could be easily picked out on plates containing ethanol, penicillin, and iodine plus potassium iodide as an indicator of the penicillin–β-lactamase reaction. Van Gorcom et al. (141) provided a valuable new technique for the study of Aspergillus transformation by fusing the E. coli lacZ (β-galactosidase) gene in frame into the coding region of Aspergillus trpC. Aspergillus colonies transformed with this construction turned blue on medium containing the chromogenic β-galactosidase substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

Cotransformation

In cases in which a transforming gene cannot easily be directly selected for, one option is to look for its assimilation along with a more readily selectable marker. It appears that, when recipient cells are exposed to two different kinds of DNA simultaneously, there is a high probability that a cell that takes up one will also take up the other. Wernars et al. (150), working with Aspergillus nidulans, were among the first to exploit this phenomenon of cotransformation, which can be rationalized by supposing that not all protoplasts are equally prone to take up DNA and that those most competent to do so will tend to take up several molecules simultaneously. Figure 1b shows some of the results obtained with A. nidulans. One example of the usefulness of cotransformation concerns two genes with roles in the utilization of acetate as the sole carbon source in N. crassa. Such genes are in principle selectable on acetate growth medium, but since there was no established protocol for regenerating protoplasts on this medium, the cloned genes were introduced by cotransformation with am′ (encoding glutamate dehydrogenase), the recipient cells being am′ as well as acetate non-utilizing (132).

Genetic Purification of Transformants

When the cells being transformed are multinucleate conidia, as in N. crassa, or protoplasts made from mycelial compartments which are also multinucleate, the initial transformant colonies are likely to be heterokaryons, with some nuclei transformed and some not, or with different nuclei transformed in different ways. When the fungus forms uninucleate conidia, e.g., Aspergillus species, a heterokaryon can be resolved into its components very simply by plating conidia and isolating single colonies. When, as in N. crassa, the conidia are multinucleate, genetic purification can be achieved more laboriously by several successive rounds of reisolation of the transformed phenotype from single-conidial colonies; after three rounds the probability of stochastic loss of one or other nuclear component is high. When, as in Podospora species, there are no conidia, the obvious way to obtain pure transformants is by crossing the primary transformant cultures with an untransformed strain and reisolating them from the meiotic products (ascospores in the relevant examples). Although it is generally possible to obtain at least some purified transformants in this way, their yield can be very low, far lower than the likely frequency of transformed nuclei in the original transformed culture. The recently discovered so-called RIP phenomenon (113) (see below) provides a likely explanation of these difficulties, at least as far as Ascomycetes fungi are concerned. Faugeron et
TRANSFORMING DNA can be supplied to fungal cells either without a fungal replication origin, in which case the maintenance of transformation depends on integration of the DNA into the chromosomes, or ligated into a plasmid capable of autonomous replication in the recipient species.

Shuttle Vectors Based on the S. cerevisiae 2μm Plasmid

Beggs (12) constructed the first S. cerevisiae-E. coli shuttle vectors. These included, within a single closed-circular plasmid, the E. coli CoIE1 plasmid replication origin, a gene for tetracycline resistance for selection in E. coli, the replication origin of the S. cerevisiae 2μm plasmid (17), and the S. cerevisiae LEU2 gene for selection in S. cerevisiae leu2 mutant cells. The hybrid plasmids were able, under the conditions summarized in Table 2, to transform leu2 to LEU2 at frequencies of about 10³/μg of DNA. The transforming DNA was present in the transformed cells predominantly in the form of the original hybrid plasmid, although, in one transformant, some plasmid sequences appeared to have undergone rearrangement, yielding restriction fragments of novel sizes. DNA from transformed clones was used to transform E. coli to tetracycline resistance, and the original shuttle vector was recovered unchanged from most of the E. coli transformants that were analyzed. Shuttle vectors have subsequently been extensively used for cloning S. cerevisiae genes; a sequence from an S. cerevisiae DNA bank can be selected for function in S. cerevisiae and amplified in E. coli. The S. cerevisiae 2μm plasmid will also replicate well in Schizosaccharomyces pombe, and so similar kinds of shuttle vectors can also be used for cloning and analysis of genes in this organism. It has not been found to replicate in filamentous fungi.

Mitochondrial Plasmids

The great usefulness of the S. cerevisiae 2μm plasmid prompted attempts to obtain comparable vectors for use in filamentous fungi. No nuclear plasmids are known in fungi other than Saccharomyces species, but a number of mitochondrial plasmids have been discovered in various species. Stohl and Lambowitz (123) inserted a 4.1-kilobase-pair (kb) mitochondrial plasmid derived from the Labelle strain of Neurospora intermedia together with the Neurospora selectable marker qa-2¹ into the E. coli CoIE1-based vector pBR325 to make plasmid pALS1. The transformation efficiency of this hybrid plasmid was at least 5 to 10 times higher than was given by the pBR325-qa-2 construct without the Labelle sequence. This enhanced ability to transform was at first attributed to the replication origin or the mitochondrial plasmid. However, a derivative of pALS1 which had undergone a spontaneous deletion of virtually the whole of the Labelle sequence functioned nearly as well in transformation (122). Consequently, the relatively good performance of pALS1 as a vector has to be attributed to a replication origin(s), either in the qa-2¹ sequence or in pBR325, which may have been in some way modified during the construction of the vector.

ARSs in S. cerevisiae

At the same time as shuttle vectors based on the 2μm plasmid were being developed, Stinchcomb et al. discovered a Saccharomyces chromosomal DNA sequence that, when
| Species                  | Protoplast formation procedure | DNA uptake procedure | Yield per μg of DNA | Reference |
|-------------------------|--------------------------------|----------------------|---------------------|-----------|
| *N. crassu*             | Germinating conidia, 50 mM Tris (pH 8.0), 1.4 mg of Novozyme 234/ml, 1 M sorbitol, 1 h at 30°C | (i) 1 to 2 μg of DNA in 5 μl of heparin (5 mg/ml), 5 μl of protoplasts (ca. 10^7) in 100 μl of 50 mM Tris–1 M sorbitol–50 mM CaCl₂ (TSC), 10% PEG 4000, 1.2% dimethyl sulfoxide, 30 min on ice (ii) Add 1 ml of 40% PEG 4000 in TSC, 20 min at room temp | (1–2) × 10^4 | 145 |
| *Aspergillus nidulans*  | Germinating conidia, 10 mM sodium phosphate (pH 5.8), 4 mg of Novozyme 234/ml + β-glucuronidase, 1 mg of serum albumen/ml, 90 min at 30°C | (i) 1–10 μg of DNA, ca. 10^7 protoplasts in 100 μl of TSC, 25 min at room temp (ii) Successive additions of 200, 200, and 850 μl of 60% PEG 4000 in TSC, 20 min at room temp | 10–50 | 70 |
| *Aspergillus nidulans*  | Mycelium on cellophane membrane, 5 mg of Novozyme 234/ml, 0.6 M KCl, 90 min at 30°C | (i) 1 μg of DNA, 10^7–10^8 protoplasts in 50 μl of 50 mM CaCl₂–0.6 M KCl plus 12.5 μl of 25% PEG 600 in 50 mM KCl–10 mM Tris (pH 7.5), 20 min on ice (ii) Add 8 vol of 25% PEG–KCl–Tris, 5 min at room temp | 50–100 | 6, 7 |
| *Podospora anserina*     | Mycelium, 25 mM potassium phosphate (pH 6.0), 10 mg of Novozyme 234/ml, 0.6 M sucrose, 1 h at 37°C | (i) 10 μg of DNA + protoplasts in 200 μl of 10 mM Tris (pH 7.6)–10 mM CaCl₂, 15 min at room temp (ii) Add 2 ml of 60% PEG 4000, 10 min at room temp | 10–20 | 18 |
| *Coprinus lagopus*       | No protoplasting; germinating conidia, 0.1 M lithium acetate, 30 min at 37°C | (i) 10–20 μg of DNA in 50 μl of 10 mM Tris (pH 7.5)–1 mM EDTA + Li-treated cells in 400 μl of 0.1 M lithium acetate, 30 min at 37°C (ii) 4 ml of 40% PEG 4000 in 0.1 M lithium acetate, 1 h at 37°C | ca. 10^3 | 15 |
| *S. cerevisiae*          | Log-phase cells; 10× concentrated culture (2 × 10^8 cells/ml), 1% glusulase, 1 M sorbitol, 1 h at 30°C | (i) ca. 10^7 viable protoplasts in 500 μl of TSC + 5–10 μg of DNA, 5 min at room temp (ii) Add 5 ml 40% PEG 4000 in TSC, 10 min at room temp | ca. 1–2× | 53 |
| *S. cerevisiae*          | No protoplasting; log-phase cells at 10^5/ml in 10 mM Tris (pH 8.0)–1 mM EDTA–0.1 M lithium acetate, 1 h at 30°C | (i) ca. 10^7 cells + 1 μg of DNA in 115 μl, 30 min at 30°C (ii) Add equal vol of 70% PEG 4000, 1 h at room temp | ca. (1–4) × 10^5 | 61 |
| *Schizosaccharomyces pombe* | Late log-phase cells, 20 mM citrate–phosphate (pH 5.6), 1.2 M sorbitol, 5 mg of Novozyme 234/ml, 1 h at 32°C | (i) 5 × 10^6 protoplasts + 10 μg of DNA/ml of TSC, 15 min at 25°C (ii) 10 vol of 20% PEG 4000, TSC, 15 min at 25°C | 10^6 | 11 |

* Higher frequency with *pse* sequence in transforming plasmid (see text).  
* A With nonreplicating plasmid; >10^3-fold higher with replicating plasmid.  
* With replicating plasmids.
integrated into a transforming plasmid, increased the frequency of transformation 1,000-fold (121). The plasmid, previously unable to replicate autonomously in *S. cerevisiae*, had acquired the ability to do so, and it was inferred that the newly inserted DNA segment contained an *S. cerevisiae* replication origin (autonomous replication sequence [ARS]). The sequence isolated by Stinchcomb et al. (121). ARS1, was the first of a number of *Saccharomyces* sequences of like kind to be uncovered (27, 56). They appear to be dispersed throughout the *Saccharomyces* genome at an average spacing of about 35 kb (27) and share an 11-base-pair (bp) adenine-plus-thymine-rich sequence which occurs also in the replication origin of the 2μm plasmid and, with some minor variations, in a wide range of other eucaryotic replication origins (16, 64, 74).

Plasmids able to replicate in *S. cerevisiae* by virtue of their possession of an ARS have been found to be more frequently lost during cell budding than those with the 2μm origin of replication. Murray and Szostak (85), by pedigree analysis, found that this instability was at least partly due to preferential segregation of the plasmids into the mother cell at budding. This property of maternal transmission was not found in 2μm plasmid derivatives. Plasmids dependent on ARSs of chromosomal origin for their replication have been found to be subject to loss even when carrying a selective marker under selective conditions; Panchal et al. (95) reported that, in one experiment, 40% of cells no longer carried a selectively favored plasmid after 100 cycles of budding following isolation of the clone. This loss occurred despite an initially high plasmid copy number. The reduction of growth rate that must follow, under selective conditions, from the formation of a proportion of inviable cells presumably imposes selection pressure in favor of integration of the essential gene into the chromosome.

**ARSs in Other Fungi**

Although ARSs do not in themselves provide a sufficient basis for stable maintenance of a plasmid, they undoubtedly do increase transformation frequencies, at least in *S. cerevisiae*. Unfortunately, *S. cerevisiae* ARSs have not been found to function well in cells of other species, not even in *Schizosaccharomyces pombe*, which is a good host for the 2μm plasmid. Searches for ARSs in the species other than *Saccharomyces* for which replicating vectors are desired have had variable success. It has been shown that in *Schizosaccharomyces pombe*, some DNA segments are able to confer replicating ability on otherwise nonreplicating plasmids, whereas others are not. Sakai et al. (111) obtained evidence that the former class, presumed to contain ARSs, helped the replication of the latter when the two were introduced together in different plasmids, apparently through the formation of mixed oligomers. From the proportion of cloned *Schizosaccharomyces pombe* sequences, averaging about 3 kb in size, that were able to promote replication of an otherwise nonreplicating vector, Maundrell et al. (76) calculated that ARSs were distributed through the genome at a mean spacing of about 19 kb. Many of the sequences with the property of ARS in *Schizosaccharomyces pombe* failed to replicate when introduced into *S. cerevisiae*. However, an ARS from *Kluyveromyces fragilis*, a budding yeast, did function in *S. cerevisiae*, as well as in *K. fragilis* and *K. lactis* (31).

Buxton and Radford (22) screened a bank of 700 cloned *N. crassa* sequences in the size range 1 to 7 kb for the ability to improve the *N. crassa* transformation frequency when inserted into a plasmid that also included *psr-A* or *psr-2p* as a selective marker. Only four were found to have a significant effect. Improvement of the transformation frequency is not a very sensitive criterion for an ARS in *N. crassa*, in which the frequency of transformation by nonhomologous integration is apt to be high (see below). However, there is evidence that suggests that many *N. crassa* genomic fragments do have replication origins that confer some limited autonomy on plasmids carrying them. *N. crassa* transformation experiments typically yield a great many colonies on the selective plates that, although apparently quite strong initially, are

| Marker | Species of origin | Phenotypes(s) | Genera in which marker was used | Reference |
|--------|-------------------|---------------|-------------------------------|-----------|
| Hyg1'  | *E. coli*         | Hygromycin B resistance | *Saccharomyces* | 63 |
|        |                   |               | *Celphadospiorum* | 119 |
|        |                   |               | *Cochliobolus* | 137 |
|        |                   |               | *Cottertorichum* | 38 |
|        |                   |               | Fusia | 87a |
|        |                   |               | Septoria | 28 |
|        |                   |               | N. usitigoi | 146 |
|        |                   |               | *Phycomyces* | 126 |
| Neo1'  | *E. coli*         | Kanamycin, G418 resistance | *Schizoplyllum* | 140 |
|        |                   |               | *Ustilago* | 8 |
| Ben1'  | *N. crassa*       | Benomyl resistance | *Penicillium* | 14 |
|        |                   |               | *Cochliobolus* | 137 |
|        |                   |               | *Cottertorichum* | 105 |
| oltC'  | *Aspergillus niger* | Oligomycin resistance | *Aspergillus* | 148 |
|        | *Aspergillus nidulans* | Acetamide utilization | *Penicillium* | 14 |
| 35S'   | *N. crassa*       | Pyrimidine synthesis | *Aspergillus* | 7 |
|        |                   |               | *Penicillium* | 38 |
|        | *Aspergillus nidulans* | Arginine synthesis | *Aspergillus* | 21 |
|        | *K. lactis*       | β-Lactamase | *Saccharomyces* | 10 |
| lacZ'  | *E. coli*         | β-Galactosidase | *Aspergillus* | 14 |

* Aspergillus niger.
* Aspergillus nidulans.
* *K. lactis*.

For visual selection.

TABLE 3. Selectable markers used across species
unable to maintain their transformed character on vegetative transfer; these are known as abortive transformants (22, 68). It seems likely that these are due to DNA able to replicate outside the chromosomes but subject to frequent loss during growth. It appears that in a very different fungus, the phycomycete fungus *Mucor circinelloides*, certain plasmids containing chromosomal sequences can replicate indefinitely under selective conditions (143).

A study by Grant et al. (47) on the stability of *Neurospora* transformants gave an apparently paradoxical result. These workers found that a 2.6-kb *Neurospora* fragment containing *am* ′, cloned into the *E. coli* plasmid pUC8, transformed a *Neurospora am* mutant with high efficiency. The transformants did not carry the *am* ′ sequence in plasmid DNA of the original size but rather in high-molecular-weight DNA that, on restriction digestion, yielded some fragments of sizes expected from the plasmid and some of novel sizes. The investigators were reluctant to postulate that the transforming DNA had been integrated into the chromosomes because it was not transmitted, or transmitted only at very low frequency, through sexual crosses. They proposed instead that it was present in the form of plasmid oligomers, with some deletions or other structural rearrangements to account for the novel restriction fragment sizes. Such an interpretation had been advanced in one example from *Schizosaccharomyces pombe* (110); in this case, sequences derived from the transforming DNA were isolated from transformants in 40- to 80-kb fragments that could be reduced to plasmid monomer size by restriction enzymes cutting once within the sequence of the transforming plasmid. It now seems likely that in the *Neurospora* example, *am* ′ really was integrated and the failure of sexual transmission was due to a general instability of duplicated sequences in meiotic cells (the so-called RIP effect [see below]). However, the general difficulty of distinguishing between integration and oligomer formation combined with some sequence rearrangement remains.

Another example of the difficulties of interpretation that can arise from experiments on hypothetically autonomously replicating plasmids is provided by the search for ARSs in *Aspergillus nidulans*, reported by Ballance and Turner (7). One hundred different *Aspergillus nidulans* DNA fragments, selected for ARS function in *S. cerevisiae*, were tested for the ability to improve transformation frequencies in *A. nidulans*. One sequence, designated *ansl*, had a very strong effect, enhancing the frequency of transformation of a *pyrG* mutant to pyrimidine independence by a factor of 50 to 100 when it was included in the *pyr* ″ transforming plasmid. However, the transforming DNA was not found in plasmid-sized molecules in transformants, but rather in high-molecular-weight DNA, presumably chromosomal. The authors interpreted these results as meaning that *ansl* was in some way enhancing the frequency of transformation by chromosomal integration rather than functioning as an ARS. Since the *ansl* sequence appears to be present in multiple copies in the *Aspergillus* genome, its inclusion in the transforming plasmid may provide more opportunities for homologous integration (see below).

**Rearrangements in ARS-Dependent Plasmids**

*S. cerevisiae* shuttle vectors based on the 2 μm origin of replication seem in general to maintain themselves without structural change, but there are several reports of structural instability of plasmid replication through chromosome-derived ARSs. For example, in *N. crassa* a transforming plasmid consisting of pu-2 ″ ligated into pBR322 could be recovered from transformant DNA by retransformation of *E. coli*, indicating that the pu-2 ″ -containing *N. crassa* segment might contain an *N. crassa* ARS. About 10% of the plasmids so recovered appeared to have suffered deletions within the pBR322 sequence (93). In another study (10), plasmids recovered from *Aspergillus* species not only had undergone structural rearrangements but also had apparently acquired sequences derived from the chromosomes of the transformant strain. A similar conclusion was drawn from an investigation of some unstable transformants in *Schizosaccharomyces pombe* (152). It may be that rather frequent interaction with the chromosomes is a common feature of unstably maintained plasmids in fungi and that this may contribute to their maintenance, although the molecular mechanisms involved are not understood.

**Synthetic Chromosomes for *S. cerevisiae***

*S. cerevisiae* clones transformed with hybrid plasmids can be easily maintained by selection, but when the selection pressure is lifted, subclones lacking the selective marker tend to segregate out at inconveniently high frequencies. Consequently, great efforts have been made to obtain more stable cloning vehicles and, in particular, to fabricate artificial minichromosomes.

To create somal groups with the replicational and transmission properties of a chromosome, it is necessary to include (i) at least one ARS, (ii) telomere sequences to form the termini of linear DNA (56, 87), and (iii) a centromere sequence that will attach to the fibers of the division spindle and ensure regular distribution at mitosis (86). The best constructions so far show fair but not complete stability. It appears that a minimum overall size is needed before the construction can approximate to a normal chromosome in its properties (84, 155). The main potential of synthetic chromosomes is as carriers of very large genomic fragments. Further discussion of their development is beyond the scope of the present review.

**TRANSFORMATION BY INTEGRATION OF DNA INTO THE CHROMOSOMES**

**Classification of Integration Events**

In their pioneering paper, Hinnen et al. (53) demonstrated the transformation of a *leu2* mutant strain of *S. cerevisiae* with a plasmid containing the *LEU2* gene and the *E. coli* ColEl replication origin but no *S. cerevisiae* ARS. The frequency of transformation was very much lower than in the parallel experiments of Beggs (12), who used autonomously replicating shuttle vectors. Hinnen et al. were unable to find evidence of free plasmid DNA in their transformants and concluded that the transforming DNA had all been chromosomally integrated. An apparently different result was obtained by Hsiao and Carbon (55), who used a ColEl-based plasmid with *ARG4* as the selective marker and were able to recover the plasmid by transforming *E. coli*. It seems likely that the *ARG4*-containing DNA included an ARS.

Struhl et al. (125), who used a shuttle vector, found both free vector and integrated DNA in their transformants.

By restriction fragment analysis of transformant DNA, Hinnen et al. (53) distinguished three kinds of integration of transforming sequences (Fig. 2). Type I transformants contained both *LEU2* and the recipient mutant allele *leu2*, tandemly arranged with the whole of the vector sequence between them. This was exactly what one would expect from homologous crossing over between the resident *leu2*
and the incoming LEU2. This type of event may be called homologous additive integration.

Type 2 transformants had no sequence modification in the vicinity of the resident leu2, but each had LEU2 and its plasmid vector integrated somewhere else in the genome. The site of integration varied from one transformant to another. These were attributed to single crossovers between the plasmid and various nonhomologous chromosomal sites. It is now thought, however, that the type 2 (ectopic) transformants identified by Hinnen et al. (53) were probably due to homologous recombination involving a sequence in the transforming DNA that was also present in dispersed repeated copies in the chromosomes. It is known (3) that immediately upstream of LEU2 there is a copy of the 0.25-kb terminal repeat (6) of the S. cerevisiae Ty1 transposon and also a leucine transfer ribonucleic acid (tRNA) gene. The δ sequence is present in the genome in about 100 dispersed copies (23), and the tRNA gene is present in several copies also. It seems that virtually all integration of DNA into S. cerevisiae chromosomes is by homology. According to one report (120), even a small amount of sequence divergence—in the case investigated, between two ribosomal DNA (rDNA) repeats differing in only three single base-pair substitutions and four single-base-pair insertions/deletions—can have a statistically significant effect on the frequency of integration.

The type 3 events of Hinnen et al. (53) were those that left no trace on the genome visible as changes in restriction fragments or acquisition of plasmid sequences but simply replaced leu2 by LEU2. They could not be explained by spontaneous reverse mutation, since no LEU2 colonies appeared unless the protoplasts were treated with LEU2 DNA. They were attributed to homologous interaction between chromosomal and plasmid resident gentransformation (leu2 to LEU2) but not crossing over. That gene conversion was a process that might or might not be accompanied by crossing over was a familiar idea following the very extensive analysis of the fine structure of recombination in S. cerevisiae and other ascomycete fungi and the unifying general recombination model of Meselson and Radding (77).

The light was shed on the possible origins of the homologous type 1 (addition) and type 3 (replacement) events by the experiments of Orr-Weaver et al. (89, 92). They first showed that the frequency of type 1 transformants could be increased some 50-fold by cleaving the nonreplicating transforming plasmid within the LEU2 gene that was being used as the selective marker. The transformation frequency was not reduced when the double-stranded nick was extended to a gap by cutting at two restriction sites within LEU2. It is important, however, to note that the gap did not remove the wild-type sequence corresponding to the mutational lesion in the leu2 allele. The transformants were found to contain single copies of both leu2 and LEU2 tandemly arranged with the remainder of the cloning plasmid between them (Fig. 3). The LEU2 allele must have been reconstructed through repair of the gap by copying from the corresponding nonmutant sequence of leu2, the repair process being accompanied by a crossover integrating the repaired transforming plasmid into the chromosome. This experiment led to the important concept of double-strand break repair as a possibly general mode of recombination (91, 129).

The stimulatory effect of double-strand nicking or gapping provided a means of targeting integration to specific chromosomal loci. When the plasmid carried both LEU2 and HIS3 and was cut within either marker, integration was found predominantly at the chromosome locus homologous to the cut ends of the plasmid (92). That double-strand break repair could also account for replacement events was suggested by a further experiment of Orr-Weaver and Szostak (89), in which transformation was effected by a replicating plasmid carrying LEU2 or HIS3 or both. When the plasmid was gapped within the selected gene, two kinds of transformants were recovered in approximately equal numbers. In one kind the plasmid was integrated, and in the other it was extrachromosomal and autonomously replicating. It was inferred that gap repair was associated with crossing over in about 50% of cases (Fig. 3). If double-strand break repair in the general mode of interaction between a transforming wild-type gene and its mutant chromosomal counterpart, presumably the type 3 (replacement) transformants of Hinnen et al. (53) must arise from a break in the chromosome (the recipient of information) rather than in the plasmid donor, with no crossing over accompanying the repair process. Type 1 (additive) events, on the other hand, could arise from a break either in the chromosome or in the plasmid, with repair accompanied by crossing over: in both cases the result would be tandemly arranged genes (one wild type and one mutant unless gap repair has caused conversion of one to the other) with a vector sequence between them. Whether all events in S. cerevisiae is due to double-strand break or gap repair, or whether at least some of it arises from single-strand nicking and transfer, as in the widely favored model of Meselson and Radding (77), is a moot point. Both models provide for information transfer either with or without crossing over (Fig. 4).

Filamentous fungi. The linkage or nonlinkage of integrated transforming DNA to the homologous chromosome locus can be determined by analysis of the progeny of a cross between the transformant and the true wild type. Close linkage of the integrated DNA to the homologous locus is indicated by the absence or extremely low frequency of the untransformed mutant type among the products of the cross. An unlinked transformant is expected to give 25% mutants from such a cross. Wernars et al. (149) carried out a formal linkage analysis on a series of Aspergillus nidulans strains transformed with respect to adh2. They mapped the transforming adh2′ gene to several different chromosomes in different transformants. Kinsey and Rambosk (69), transforming an N. crassa adh deletion mutant with an adh′ clone with about 1 kb of homology on each side of the deletion, found approximately equal numbers of integrations of adh′ very closely linked to (perhaps inseparable from) the normal locus and unlinked (ectopic) integrations. Rather similar proportions of linked and unlinked events have been found in N. crassa transformed with qa-2′ (26, 36) and tri-1′ (67).

Comparable studies have been made on the related ascomycete member Podospora anserina, for which the analysis is helped by the availability of ascospore color mutants. Brygoo and Debuchy (18) used a recipient strain which carried suppressible chain termination mutations in two genes, one necessary for leucine biosynthesis and one for spore pigmentation. The cloned DNA that they used for transformation carried a suppressor (su-8, presumably a mutant tRNA gene) of the effects of both of these mutations. Transformants were selected through their leucine-independer phenotype, and the site of integration of su-8 was mapped by using the spore color difference as a marker in test crosses. Four of seven transformants analyzed had the suppressor integrated at or very close to the normal su-8
VOL.

FIG. 2. Three modes of integration of transforming DNA in S. cerevisiae (53). S. cerevisiae leu2 mutant cells were transformed with a nonreplicating CoIE1-based plasmid carrying S. cerevisiae LEU2. Type 1 is homologous additive integration by homologous crossing over, leading to tandemly arranged LEU2 and leu2 alleles with the plasmid sequence between them. Type 2 is ectopic integration of LEU2 and plasmid sequences, leaving the original leu2 locus undisturbed. b indicates a terminal repeat of the movable Ty1 element; copies of b are dispersed through the genome. Type 3 is replacement of leu2 by LEU2 without integration of plasmid sequence. Modified from reference 53.

locus. In another study of Podospora transformation, in which selection was made for ura4⁻, 21 of 32 stable transformants had the marker integrated at or very close to its normal locus (99).

Genetic analysis of transformants in members of the class Ascomycetes is likely to be complicated by the RIP effect (see below), which tends to inflate the number of mutant phenotypes and so exaggerate the frequency of apparent recombination between the transforming sequence and the normal gene locus. More definitive results can be obtained from direct examination of the DNA by Southern blot analysis of restriction fragments.

Using DNA analysis, Kim and Marzluf (67) investigated the effect of cutting their trp-1⁻-bearing plasmid within the region of its homology with the chromosomal locus, either within or outside the coding sequence. When the cut was within the trp-1 coding region, all trp-1⁻ transformants analyzed had the transforming sequence integrated at the homologous locus; this was not surprising, since recombination with the chromosomal homolog may be the only way of repairing the break within the coding sequence. However, when the cut was made within the region of homology with the chromosome but outside the coding region, there was no greater tendency to homologous over nonhomologous integration than there was with the uncult circular plasmid. There was, however, a surprising difference between the results obtained from two different recipient strains. One gave 31 homologous (or at least closely linked) integrations and 6 unlinked, whereas the other gave 13 and 17, respectively.

Molecular analysis of Neurospora transformants has led to the identification, with various degrees of precision, of all three patterns of integration recognized by Hinnen al. for S. cerevisiae (53). Both Case et al. (26) and Kim and Marzluf (67) found what appeared to be replacement events, with restriction fragment patterns indistinguishable from those of the wild type. Ectopic integration, with different integration sites from one transformant to another, appears generally to be the commonest mode of transformation. It is formally possible to misclassify replacements as ectopic events if, as well as gene replacement at the normal locus, there is a functionally ineffective gene fragment integrated elsewhere. This interpretation is ruled out in cases in which the recipient strain is a deletion mutant with no homology with the transforming gene (47) and can be tested in all cases by genetic analysis (which has not usually been done). Demonstrations of complete homologous addition (type 1 event of Hinnen et al. (53) are few in N. crassa, but there is at least one clear example (113), and other observations (26, 93) of integration at the homologous locus together with vector sequence are at least consistent with it.

Rather more information is available about modes of integration in Aspergillus nidulans. Tilburn et al. (134), selecting for umdS⁻, obtained evidence consistent with additive integration either into the partially deleted umdS recipient locus or (when rDNA was incorporated into the vector) into rDNA genes. Yelton et al. (134), selecting for trpC⁻, analyzed 10 transformants obtained with a circular transforming plasmid and 5 obtained when the plasmid was linearized by a single cut within the selective marker. From the first set, seven transformants appeared to be due to homologous events (five additions and two replacements) and three were due to ectopic integration. In the second set the numbers were four homologous (all additions) and one ectopic. The overall transformation frequency was not affected by linearizing the plasmid. Six of the nine homologous addition events evidently involved something more complex than a simple crossover integrating the entire plasmid into
the chromosome. The restriction fragments indicative of such an event were indeed present, but there were also other sequences derived from the transforming DNA that could be due to integration of further plasmid copies by recombination with the first one, or to nonhomologous insertions at other loci. For the first of these alternatives to be true, some of the resulting tandemly integrated copies would have to have suffered deletions or other rearrangements in order to account for restriction fragments of sizes not obtained from the original plasmid. The second possibility is perhaps more likely. One of the more thorough investigations of integrative transformation in _A. nidulans_ was made by De Graaff et al. (34), who selected for the gene encoding pyruvate kinase. Of 13 transformants analyzed, 10 were homologous (4 additions and 6 replacements) and 3 were ectopic.

Results with _Penicillium chrysogenum_ are not very different from those with _A. nidulans_. Bull et al. (19) found, in a sample of 10 transformants, 7 homologous (3 additions and 4 replacements) and 2 ectopic transformants. One of the homologous addition types had two complete copies of the transforming plasmid integrated at the _oli_ locus.

In their recent demonstration of transformation in _Asco- bullas immersus_, Goyon and Faugeron (unpublished) trans- formed a _met-2_ (methionine-requiring) mutant with _met-2_ cloned in bacteriophage M13 and an alternative forms: closed-circular double-stranded DNA, closed- circular single-stranded DNA, and double-stranded DNA linearized by cutting within the _met-2_ coding sequence. The circular double-stranded DNA gave predominantly (17 of 18) ectopic integrations. Interestingly, the circular single- stranded DNA, with 8 of 12 homologous integrations, seemed more attracted to the chromosomal _me-2_ locus than was its double-stranded counterpart. The linearized double- stranded DNA transformed predominantly by homology: 11 of the 13 transformants analyzed showed homologous integra- tion; 2 were of the replacement type. 7 were additions of single copies of the transforming plasmid, and 2 each had three plasmid copies integrated in tandem array. It is some- thing of a puzzle that two transformants had regenerated a functional _met-2_ gene from the _met-2_ coding sequence without the benefit of homologous integration. It seems that in these cases the plasmid must have been recircularized within the cell without any erosion of the cut ends.

For the very different fungus _Coprinus lagopus_, the avail- able evidence points to ectopic integration as the predomi- nant mode of stable transformation by plasmids not cut within the transforming gene (15). Of 92 _trp-1_ transformants that were analyzed by Southern blotting, 88 (96%) had the sequence at ectopic sites; in at least 3 cases, more than one copy had been integrated. The remaining four appeared to be due to additive integration at the _trp1_ locus; in three of them, two tandemly arranged plasmid copies were present. No examples were found of simple gene replacement.

**Multiple-Copy Integration**

Even though transforming DNA in _Saccharomyces_ spe- cies is usually integrated by interaction with single-copy chromosomal sequences, it is itself often present in transform- ants in multiple copies, usually linked in tandem orienta- tion. There are two obvious alternative mechanisms through which the repeat copies might originate. Either circular plasmids might first undergo homologous recombination with each other to form circular oligomers, which could then integrate by homology with the chromosomal single copy, or the primary integration might be of a monomeric plasmid and the tandem repeats may then arise through secondary integration of further plasmid copies by homology with the first one. In an ingenious experiment, Orr-Weaver and Szostak (90) ob- tained strong evidence for the latter alternative. They trans- formed _S. cerevisiae_ _his3_ cells with a mixture of two largely homologous but distinguishable _HIS3_ plasmids, both gapped in the _HIS3_ gene. Most of the transformants had multiple integrated copies of _HIS3_, about two-thirds of them with a mixture of the two plasmids in tandem array. All copies had the gap in _HIS3_ repaired. Since both kinds of plasmids started with the same gap in _HIS3_, they could not have been repaired by recombination with each other but only by sequential integration into the chromosome.

As mentioned above, there are several examples of integra- tion of transforming genes in tandemly repeated copies in filamentous fungi. In both _Aspergillus nidulans_ (153) and _Asco- bullas immersus_ (Goyon and Faugeron, unpublished), transformants have been found which can be explained as the result of successive rounds of homologous integration either into the homologous chromosomal locus or into plasmid copies already integrated. There is also the likelihood that transformants in filamentous fungi will have multiple copies of transforming genes as a result of nonhomologous integration. For example, Wernars et al. (151) obtained _mne-2_ transformants _linearized_ in three and _mne-2_ copies inserted in tandem arrays at _mne-2_ loci; presumably, nonhomologous primary integra- tion events were followed by successive rounds of homol- ogous recombination between free plasmids and the plasmids already integrated.

Transformants with single integrated gene copies are presumably more likely to be obtained when lower concentra- tions of DNA are used. However, the available data (150) on the relationship between DNA concentration and the relative frequencies of cotransformation and single-marker transformation (Fig. 1b) suggest that the protoplasts most receptive to DNA will assimilate and integrate multiple copies even from rather low external concentrations. The larger number of protoplasts that take up DNA less avidly (Fig. 1a) probably accounts for most of the single-copy transformants that are found in fair abundance in most experiments.

**Mechanisms of Integration**

The three types of integration originally distinguished by Hinne n et al. (53) (Fig. 2) still provide a good framework for discussion, even though there are occasional complications such as deletions in the transforming DNA and multiple integrations at the same or different loci. These can be regarded, at least as a working hypothesis, as being due to additional events superimposed on the primary mechanisms.

The data presently available are not sufficiently extensive for an accurate estimate of the relative frequencies of homologous additive, homologous replacement, and ectopic events in any fungus, although it is clear that ectopic integration is much less frequent in _S. cerevisiae_ than in the other fungi investigated; when it does occur in _S. cerevisiae_, it may always be by virtue of homology with dispersed repetitive sequences. As far as homologous integration is concerned, there is no satisfactory hypothesis that would predict what any set of transformants would do.

With a mechanism similar to that postulated by Meselson and Radding (77), the homologous interaction between donor DNA and recipient chromosome would lead to single- strand exchange and heteroduplex formation, giving the
FIG. 4. Possible mechanisms of homology-dependent transformation. The donor DNA is supposed to be a circular plasmid with a selectable marker (+), as distinct from the corresponding (-) site in the recipient chromosome. The region of homology between plasmid and chromosome is indicated by the thicker line. Note that for sequence replacement, the plasmid does not have to be circular. (a) This panel is modified from the general recombination model of Meselson and Radding (77). Two cases are considered. (i) In the first the hybrid DNA does not cover the +/- site. Resolution of the Holliday junction by cutting and rejoining the noncrossing strands (β) leads to crossing over and plasmid integration. (ii) In the second the hybrid DNA does cover the +/- site. With crossing over (β resolution of the Holliday junction), transformation by plasmid integration results, provided that the +/- mismatch is not corrected to -/-; if no correction to +/- occurs, transformation is not complete until after a further round of DNA replication. With resolution of the Holliday junction by cutting and rejoining of the crossing strands (α), transformation by sequence replacement will result, provided that the +/- mismatch is not corrected to -/-; if no correction to +/- occurs, transformation will be completed only after one further round of DNA replication. Note that in case (i) but not in case (ii) the same consequences follow if the chromosome rather than the plasmid acts as a single-strand donor. (b) This panel is modified from the double-strand break repair general recombination model of Szostak et al. (129). Three cases are considered. (i) The first is a break in the plasmid not leading to removal of the + site. Crossing over, following αβ or βα resolution of the two Holliday junctions, leads to plasmid integration. (ii) The second is a break in the chromosome not leading to removal of the + site. Crossing over (αβ or βα resolution) again leads to plasmid integration. (iii) The third is a break in the chromosome with enlargement to a gap removing the + site. Repair without crossing over (αα or ββ resolution) leads to sequence replacement. Repair with crossing over (αβ or βα resolution) leads to plasmid integration and two tandemly integrated + gene copies.

opportunity for gene conversion and a 50% chance of crossing over. In this case, we might expect a majority of addition (crossover) events, since all crossovers would result in transformation, except for those that were accompanied by conversion of the donor marker to its counterpart in the recipient chromosome. In cases when crossing over did not occur, transformation would follow only when the recipient was converted by the donor. The proportions of the two outcomes would depend on the proportion of cases in which heteroduplex formation in the region of homology was followed by conversion covering the particular site or segment in which the donor and recipient differed. This, in turn, would depend on the lengths of conversion tracts in comparison with the lengths of the heteroduplex. In fact, the 50% relationship between conversion and crossing over is an oversimplification even for meiotic recombination, and there is little reason for expecting it to apply to transformation in vegetative cells. For conversions between homologous genes at different chromosome loci in S. cerevisiae, the association with crossing over may be close to zero (71). To the extent that the same applied to homologous transformation, one would expect replacements rather than additions. The different possibilities are sketched in Fig. 4a.

If the mechanism underlying homologous transformation is double-strand break repair (129), the type of integration will depend on whether the break is in the donor plasmid or the recipient chromosome. A break in the donor would lead to transformation only when the repair was accompanied by crossing over and only when any gapping in the plasmid left the critical part of the selective marker intact. A break in the recipient chromosome will lead to replacement-type transformation if there is no crossover, provided that the break is
enlarged to a gap so that recipient-to-donor conversion can occur. A break in the recipient with crossing over will give additive transformation, with the complication that if conversion occurs as well, both of the tandemly arranged gene copies will be of the donor type (Fig. 4b). This discussion has been based on the assumption that transformation is being effected with a circular plasmid. If the transforming DNA is linear, as, for example, when the selective marker has been cloned in a lambda vector (68), presumably either the vector will have to become circularized after uptake or the break will have to be in the recipient chromosome, since a break in a linear transforming sequence would leave it unable to be integrated in one piece.

There is no shortage of more or less plausible models for homologous integration, but we lack means of discriminating among them. If accurate data could be obtained about the relative frequencies of addition and replacement events, they might help to set limits on the various possibilities.

Only one analysis seems to have been made at the DNA sequence level on the nature of ectopic integration in filamentous fungi. Razanamparany and Bégueret (V. Razanamparany and J. Bégueret, Gene, in press) obtained the DNA sequences spanning the integration junctions in three *Podospora anserina* transformants with ectopic copies of *ara*5*. Three conclusions emerged. First, the originally circular transforming plasmid was gapped prior to integration, in one case to the extent of only 6 bp and in the other two cases much more extensively. Second, the recipient site was gapped to very different extents in different transformants. In two cases there was no loss of chromosomal sequence detectable by changes in restriction fragment sizes, but in the third about 10 kb had been lost at the site of insertion (Fig. 5). Third, there was no clear homology between the chromosomal sequences flanking the insertions and the sequences that they replaced in the transforming plasmid. There was, however, a hint of rather more matching than would be expected on a random basis; in one case there was a match of 6 bp on one side of the integration site and 2 bp on the other. It seems that extensive matching of sequences is not required for ectopic integration, but perhaps even a small amount of chance sequence similarity can increase the probability of integration at a particular site. The mechanism of integration remains obscure. In particular, the nature of the complex that presumably holds together the free ends of a gapped plasmid and a gapped chromosome is hard to imagine in the absence of knowledge of the protein components of the system. The mechanism at work here may be the same as that responsible for apparently random rejoining of broken chromosomes following X-irradiation, the classical way of obtaining chromosomal segmental interchanges and inversions.

Very little is known about the genes and enzymes needed for integrative transformation. In *S. cerevisiae*, *RAD52* is necessary both for homology-dependent repair of double-strand breaks and for targeted plasmid integration (91, 92), and that seems to be the extent of our present knowledge.

**USE OF TRANSFORMATION FOR ANALYSIS OF GENE FUNCTION**

Cloning Genes by Complementation

The availability of shuttle vectors based on the *S. cerevisiae* 2 μm plasmid makes it comparatively simple to clone any *S. cerevisiae* gene capable of mutating to an auxotrophic or other conditionally lethal phenotype. A bank of *S. cerevisiae* DNA sequences is made in one of the several available shuttle vectors, and the mutant is transformed with the entire bank. The transformed colonies that grow up under selective conditions will have acquired a plasmid carrying the gene required to complement the mutant. The plasmid is then extracted and transferred by transformation to *E. coli* for amplification and purification. This is now a standard procedure, and examples of successful use of the method are too numerous to itemize.

Some of the shuttle vectors are provided with *S. cerevisiae* promoter sequences adjacent to the cloning site, making it possible to select for protein-encoding sequences even when they are separated from their own promoters. Generally speaking, however, it is better to screen for genes still attached to their normal upstream flanking sequences, including promoters and upstream transcriptional activators, since these are often even more interesting than the coding sequences. It is also possible to search in the genome for effective promoters and upstream activators by including a potentially selectable but promoterless gene in the cloning plasmid. When random sequences are cloned into such a plasmid just upstream of the promoterless gene and selection is made for transformants exhibiting the gene activity, the clones selected should be a rich source of transcription-promoting sequences. This promoter trap strategy has been recently used by Turgeon et al. (138) to obtain such sequences from *Cochliobolus heterostrophus*.

The lack of satisfactory shuttle vectors makes it somewhat more difficult to clone genes by complementation in filamentous fungi, but in some cases bacterial plasmids can be recovered intact from transformants. Thus plasmids carrying *qii-2* have been obtained from *Neurospora* transformants by extracting DNA, using it to transform *E. coli*, and selecting for a plasmid antibiotic resistance marker (123). As already noted, plasmids recovered from filamentous fungi are quite often rearranged and/or partially deleted, but in a majority of cases they are still carrying the genes for which they were originally selected.

In cases in which there is no detectable free plasmid remaining, a transforming sequence can still sometimes be recovered by cleaving the transformant DNA with a restriction enzyme that cuts once, but no more than once, within the sequence duplicated as a result of type 1 integration. The fragments so generated are circularized with ligase, and the reconstituted plasmid is selected by transformation of *E. coli* (153). To take one example, the *Aspergillus* gene encoding isocitrate lyase has been cloned in this way (139). Such procedures can be expected to work, but they are sometimes laborious and inefficient.

A more efficient way of cloning complementing *Aspergillus nidulans* genes was developed by Yelton et al. (154). They constructed a cosmid vector—a plasmid with bacteriophage λ cos (packaging) sequences—including *trpC* for selection in *Aspergillus nidulans*, ampicillin and chloramphenicol resistance genes for selection in *E. coli*, and a BamHI cloning site which would accept fragments cut with the “four-cutting” endonuclease *MboI*. A bank of cosmids carrying *A. nidulans* genomic fragments of 35 to 40 kb, generated by partial *MboI* digestion, was used to transform a *trpC*Δ (yellow-spored) double-mutant strain. The initial selection was for *trpC*Δ, and then green-spored (Δ+) colonies were searched for by eye; 3 were found in an initial sample of 10,000. DNA isolated from these transformants was treated with to an in vitro lambda packaging system, and the cosmids so packaged were recovered by infection into *E. coli*, which was selected for ampicillin resistance. The se-
sequences recovered had in common an open reading frame that was evidently the coding sequence of \( yA \). This general method was subsequently used for the isolation of other developmentally regulated Aspergillus genes. The problem of locating the relevant gene within the 35 to 40 kb cloned in the cosmid was solved after the demonstration (135) that cosmid subfragments excised from a preparative electrophoretic gel were effective in transformation.

An alternative to plasmid or cosmid recovery from transformants was devised by Akins and Lambowitz for \( N.\ crassa \) (1) and applied on a larger scale by Vollmer and Yanofsky (145). This is the method of sib selection. A bank of plasmid (1) or cosmid (145) clones is combined into a number of pools, and each pool is tested for the ability to transform a particular mutant to the wild type. The clones present in the successful pool are then combined into smaller pools, and these subpools are tested, and so on until the transforming ability is assignable to a single clone. The cosmid bank used by Vollmer and Yanofsky (145) contained randomly cut genomic fragments in the range of 35 to 45 kb. The 3,072 items in the bank were expected to include any given sequence with 99% probability. In the initial screen, they were combined into 32 pools each of 96 clones. In the next round the successful 96 were combined into 12 pools of 8, and the desired clone was then obtained in one more round of selection. About 50 \( N.\ crassa \) genes were cloned in a short time by this method, not all of them easily obtainable by direct selection. Perhaps the most notable capture was one of the two allelles of the \( N.\ crassa \) mating-type system. Here, the fortunate close linkage to an uncharacterized temperature-conditional lethal mutation enabled the mating type allele to be recovered in the cosmid clone that was selected as complementing this mutant (45). The only limitation of this very powerful method is that, as it turns out, the cosmid bank has more items missing than calculation had predicted. Some may have been lost selectively during the initial amplification of the cosmid bank.

**Gene Disruption**

\( S.\ cerevisiae \). It often happens that a cloned DNA sequence looks like a functional gene in that it is transcribed, contains an open reading frame, and perhaps has some interesting similarities to known genes in other organisms, but it cannot be assigned a function because no mutations in it have been identified. A good first step in such cases is to use the clone to disrupt the equivalent sequence in the genome to create a null mutant.

One way of doing this is to construct a plasmid containing both a selective marker and a cut-down copy of the gene one wishes to disrupt. Shortle et al. (115) used \( URA3 \) to transform a diploid \( ura3 \) homozygous mutant strain and included in the vector a presumptive actin-encoding gene that had been truncated at both ends. Uridine-independent transformants had the plasmid integrated in some cases into \( ura3 \) and in others into the actin gene. The effect of the latter mode of integration was to generate two nonfunctional actin gene derivatives, one deleted at the 5' and the other deleted at the 3' end (Fig. 6a). The latter class were all haploid inviable, as shown by 2:2 segregation of a lethal phenotype when the diploid transformant was induced to sporulate. This confirmed that the cloned gene did indeed have an essential function.

The one-step disruption procedure described by Rothstein (107) consists of inserting a copy of a selectable marker (\( HIS3 \) in the example he first described) into the cloned gene under investigation. This construction is then used to transform a mutant, in this case \( his3 \), selecting for the disrupting marker. Screening transformants for the presence or absence of the vector sequence will discriminate between
additive and replacement modes of integration: the latter class will have the target gene disrupted and almost certainly nonfunctional (Fig. 6b). If the recipient strain is not a deletion mutant, some of the transformants, perhaps about half, will be due to integration into his3, and these will have to be distinguished from the desired class by Southern transfer analysis of the state of the his3 locus.

A refinement of the disruption procedure that allows for several successive rounds of disruption of different genes, using the same selective marker was devised by Alani et al. (2). They used URA3 as the disrupting selective marker and introduced it into their transforming plasmid flanked by tandem repeats, which, in their demonstration, were derived from E. coli. The special advantage of URA3 in this context is that it can be counterselected by growth on medium containing 5-fluorouracil. After the first one-step disruption, selection against URA3 was imposed; 5-fluorouracil-resistant clones arose at reasonably high frequency as a result of spontaneous excision of the URA3 sequence by crossing over between the flanking repeats (Fig. 6c). The target gene remained disrupted, since one of the flanking repeats was still present following URA3 excision.

There are many examples of successful use of gene disruption in S. cerevisiae. It is especially useful for genes whose mutant phenotypes are not well known. The two genes for the subunits of phosphofructokinase provide a good example (49). Another, particularly noteworthy, is that of the duplicate ras oncogene-related sequences. Disruption of either one of these had no effect on the phenotype, but the double disruption, obtained from the cross between the two singles, was lethal (130). A recent tour de force was the construction of an S. cerevisiae strain simultaneously deficient in six different genes for certain kinds of small nuclear RNA molecules: surprisingly, the strain was viable (96).

Other fungi. One-step gene disruption has been found to work well in fission yeast, Schizosaccharomyces pombe (109), and there is no apparent reason why the same should not apply to filamentous fungi. Its efficiency is bound to be reduced by the high frequency of ectopic integration in species other than Saccharomyces, but it should always be possible to sort out the desired homologous transformants. There are, nevertheless, only a few examples of successful gene disruption in N. crassa and A. nidulans.

Paietta and Marzluf (94) inserted qa-2' into the cloned am' gene and used the construction to transform a qa-2 am' strain, selecting for qa-2'. The initial transformants were all am' in phenotype; this is not surprising, since they had been obtained by transforming multinucleate conidial protoplasts and were expected to be heterokaryotic. Homokaryotic qa-2' derivatives were recovered from crosses of the primary transformants to qa-2 (although not all of the transformants transmitted qa-2' through a cross, presumably because of the RIP effect [see below]), and 10 of 117 of these were null with respect to am'. Southern blot analysis showed that these derivatives had the am' locus disrupted. In one strain that was analyzed in detail, the pattern of restriction fragments was consistent with a gene replacement event, except that the observed restriction fragment containing the disrupted gene was 4 kb longer than expected, perhaps because of duplication of some of the am' sequence. Unexpected rearrangements were also found in other transformants with a disrupted am' sequence. Therefore, although the disruption worked, the mechanism seemed more complicated than in S. cerevisiae.

Miller et al. (78) were able to disrupt argB in Aspergillus nidulans by inserting trpC' into it and selecting for transformation of a trpC mutant. About 30% of the trpC transformants (all homokaryotic because of the unicellular nature of the conidial protoplasts in this species) were arginine dependent. Four of the latter class were analyzed at the DNA level; two gave results consistent with simple gene replacement, and two showed more complicated rearrangements, reminiscent of the result obtained with N. crassa (94).

Premeiotic disruption—the RIP effect. There is now another possibility for targeted disruption that can be applied to any N. crassa gene that has been cloned. Selker et al. (113) discovered that when a normally single-copy sequence was duplicated by transformation and the duplication strain was crossed with any other strain, a proportion of the meiotic products (ascusspores) had both duplicated copies (but not the corresponding single-copy sequence from the normal parent) extensively changed. The changes took the form of both heavy methylation and numerous nucleotide sequence changes, as evidenced by the appearance and disappearance of restriction sites. Nearly identical changes were seen in two of the four members of a meiotic tetrad, and different ascis from the same fruiting body generally showed different patterns of disruption; these observations placed the disruptive events before premeiotic DNA replication (which occurs immediately before meiosis) but after the initial association of a single pair of nuclei of complementary mating types at fruiting body initiation. The phenomenon was called the RIP effect (rearrangements induced premeiotically), although it now seems (J. R. S. Fincham, unpublished data; E. U. Selker, personal communication) that the changes are usually within the duplicated sequences without rearrangement of their positions in the genome. The base pair changes are found to be exclusively guanine-cytosine to adenine-thymine transitions (Selker, personal communication), and their frequency is extremely high (of the order of 50% of guanine-cytosine base pairs may be affected).

The proportion of meiotic tetrads that undergo the RIP effect seems to be a function of the degree of proximity of the duplicated sequences. In the original experiment, they were almost adjacent in tandem, and virtually all the meiotic tetrads showed the effect (113). With unlinked sequences (for example, a normally placed gene and an unlinked ectopic copy introduced by transformation), the frequency may be of the order of 50% or less (113; Fincham, unpublished).

The mechanism and function (if any) of the RIP effect are by no means clear. One may speculate that sequences anomalously duplicated in cells that are almost ready to enter meiosis may be prone to premature synopsis and that this, for reasons that are quite obscure, triggers methylation of a high proportion of the cytosine residues within the synapsed region. N-Methylcytosine may then be deaminated to give thymine, presumably enzymatically, since the process seems much more rapid than could be accounted for by chemical instability. The effect, in any case, is likely to be to destroy the function of any gene present within the duplication.

It is important to note that the RIP effect is not selective between a normally located wild-type gene and an ectopic copy added by transformation; both copies are inactivated. It follows that any cloned sequence of unknown function can, if introduced as an ectopic duplication by cotransformation, be used to disrupt the resident wild-type homolog and eliminate its function if it has one.
It appears highly probable that the RIP effect occurs in all 
Ascomycetes with a dikaryotic phase in their life history. 
The methylation aspect of the phenomenon has been 
demonstrated for Aspergillus niger by Goyon and 
Faucover (unpublished). It very probably accounts for a reported case 
of two-copy lethality in Podospora anserina (33) and is 
almost certainly responsible for the repeated finding that in 
A. nidulans (134, 153) and P. anserina (99, 103), as well as in 
N. crassa (127), many transformants contained the transformed 
character through crosses. The RIP phenomenon 
also accounts for the observation of Case (25) that 
transforming qa-2' sequences whose activity had been lost 
during outcrossing appeared to still be present in the genome 
as DNA recognized by a qa-2 probe.

The difference between transformants that will transmit 
through meiosis and those that will not is very probably that 
the members of the latter class have the transforming 
sequence in two or more closely linked copies, whereas 
the members of the former class are either single copy with 
respect to the sequence (in the case when the recipient strain 
was a deletion mutant) or with a single-copy ectopic se-
quence unlinked to the normal gene locus.

**Gene Replacement**

*S. cerevisiae*. In gene replacement, as opposed to gene 
disruption, the purpose is to retain gene activity but to 
modify its product or its mode of regulation. The first 
procedure for replacement was described for *S. cerevisiae* 
by Scherer and Davies (112). Their method was to transform 
with a plasmid containing both a modified form of the target 
gene and a separate selectable marker. Transformants with 
the plasmid integrated by homology into the target gene had 
tandemly arranged copies of both the target gene and the 
modified version that was to replace it, with the rest of 
the plasmid including the selective marker between them. 
They then screened, after about 10 cycles of budding, for 
sb-clones that had lost the marker by crossing over between 
the tandem gene copies (these will, of course, have the greater 
part of their sequence in common). Depending on where the 
crossover occurs—to the right or to the left of the sequence 
distinguishing the natural and modified gene copies—there 
will be either restoration of the natural gene or its replace-
ment. An example of the application of this general method 
to Aspergillus nidulans is illustrated in Fig. 7a.

A disadvantage of this method is that if the effect of the 
modification that one wants to introduce is recessive in the 
presence of the normal gene, it is not possible to screen for 
that effect in the primary transformants. Shortle et al. (116) 
replaced the *S. cerevisiae* actin gene with recessive alleles 
conferring temperature sensitivity by a procedure that they 
called integrative disruption/replacement. Their transform-
ing plasmid carried, as well as *URA3* as a selectable marker, 
a truncated copy of the actin gene that had been subjected to 
chemical mutagenesis. *URA3* transformants with the plas-
mid integrated by homology at the actin gene locus had only 
one functional actin gene copy, which was likely to have 
undergone mutation. Three of the few thousand that were 
screened were temperature sensitive for growth because of 
actin modification (Fig. 7b).

After excision of *URA3* by crossing over between the repeats (——), 
one copy of the repeat remains, so that *TRP1* is still disrupted. 
Modified from reference 2.
The procedures just described take advantage of type 1 integration, which permits the use of a selective marker in the plasmid carrying the replacement gene. By using a counterselective method it is possible to make use of type 3 integration, which gives simple gene replacement in one step. Struhl (128) disrupted HIS3 by inserting into it a copy of CYH2, the gene that encodes ribosomal protein L29. When inserted at the his3 locus in a cycloheximide-resistant (cyh2') S. cerevisiae strain, the effect of CYH2 was to restore cycloheximide sensitivity. Selection could then be made for replacement of the HIS3-CYH2 compound by any other his3 allele by plating the transformants on medium containing cycloheximide. In this way a his3 mutant allele, his3-25, with an upstream 31-bp deletion, was inserted in place of the wild-type gene; it showed altered regulation, without the normal derepression in response to amino acid starvation (Fig. 8a).

A drawback of the Struhl procedure was that there was no positive selection for the initial gene disruption. Furthermore, the subsequent selection against CYH2 was not as efficient as might have been wished. Rudolph et al. (108) used cotransformation for gene replacement. They first disrupted the S. cerevisiae gene PHOS (encoding acid phosphatase) by inserting URA5 into it and substituted the disrupted for the normal gene by transformation of a strain with both ura3 and leu2 mutations. They then transformed the strain again with a mixture of two plasmids, one bearing LEU2 and one bearing a pho5 derivative that had suffered an intron deletion in its promoter region. Among the leucine-independent transformants, about 1/5, easily identified by replica plating, were pyrimidine auxotrophs, having had the pho5-URA3 compound replaced by the new pho5 allele.

**Other fungi.** There is great potential for application of essentially the same procedures of gene replacement to filamentous fungi. Miller et al. (78), working with Aspergillus nidulans and following essentially the same plan as Scherer and Davis (112), replaced the spoCIC gene of the spoCI (sporulation-specific) gene cluster with a partially deleted derivative. A plasmid carrying the modified spoCIC sequence together with trpC+ as a selectable marker was used to transform a trpC mutant strain. A transformant with trpC+ integrated by crossing over with spoCIC showed altered regulation, without the spoC sequences. Five of eight tryptophan-requiring derivatives had the partial deletion in spoCIC (Fig. 7a). Wernars et al. (150) used cotransformation to replace Aspergillus nidulans amdS+ with a construction that had E. coli lacZ fused into the trpC reading frame. Here the cotransforming plasmids carried amdS+ and the trpC-lacZ construct, separately. About 75% of transformants showed P-galactosidase activity, but the great majority of these were still trpC+, presumably because the lacZ construction had been integrated elsewhere than at trpC. However, screening of a large population of transformants did reveal some in which trpC+ had been replaced by trpC-lacZ (Fig. 7b).

One of the most fruitful applications of gene replacement techniques is likely to be the analysis of upstream regulatory sequences. Using the disruption-replacement principle, Frederick et al. (G. D. Frederick, D. K. Asch, and J. A. Kinsey, Mol. Gen. Genet., in press) replaced the normal upstream nontranscribed region of the Neurospora am gene with various deletion derivatives. By transforming an am-negative strain, with a point mutation near the 5' end of the coding sequence, with a linearized plasmid carrying a modified upstream region and a truncation at the 3' end of the gene, they ensured that only homologous DNA replacement would generate a functional am gene (Fig. 9).

**Titration of trans-Acting Gene Products**

The introduction by transformation of multiple copies of a cis-acting sequence that binds to the protein product of a trans-acting regulatory gene can give valuable information...
FIG. 8. Direct selection for gene replacement. (a) Replacement of \textit{S. cerevisiae} HIS3 by an in vitro-generated derivative with a 31-bp upstream deletion (Δ). First the chromosomal copy of HIS3 in a cycloheximide-resistant strain (\textit{cyh}2), modified ribosomal protein L29 was disrupted by insertion of \textit{CYH2}, the wild-type gene which confers dominant cycloheximide sensitivity. Then transformation was carried out with a plasmid carrying the deletion derivative of HIS3, and selection was made for cycloheximide resistance and elimination of \textit{CYH2}. The mutant \textit{HIS3}, which turned out to be nonrepressible in response to amino acid deprivation, replaced the \textit{HIS3}/\textit{CYH2} construct. Modified from reference 124. (b) The use of cotransformation to replace \textit{Aspergillus nidulans} trpC- by a \textit{trpC}lacZ fusion. A mixture of two plasmids, one carrying the fusion gene and one carrying \textit{amds}+, was used in transformation, and \textit{amds}+ was selected for. Screening of a large number of \textit{amds}+ transformants revealed some in which \textit{lacZ} was expressed. Modified from reference 150.

more, these functions could be restored by the addition, by transformation, of multiple copies of \textit{amdR}.

APPLICATIONS TO BIOTECHNOLOGY

The development of methods for transforming fungi with DNA opens up many possibilities of engineering suitable species for commercial production of enzymes and other proteins. The general plan is to construct a transforming plasmid in which the gene encoding the desired protein is linked to an efficient promoter and an enhancer or upstream transcriptional activator, each capable of functioning in the species chosen as the producer. A highly desirable feature that it may be necessary to build into the protein code is a signal sequence to enable the protein to be secreted through the cell membrane into the growth medium, from which a protein can be purified far more easily than from a cell extract.

One can assume that far more work is going on in this area than is being published. Most of what has appeared in print has been concerned with \textit{S. cerevisiae}, but the same general strategy could presumably be applied to any species that can be easily grown on a large scale in liquid culture without noxious by-products.

There is considerable current interest in fungal genes encoding polysaccharide-degrading enzymes because of their potential uses in the food industry and for the disposal of plant waste (114, 131). Innis et al. (60) inserted the coding sequence of the \textit{Aspergillus awamori} gene for glucoamylase between the promoter and transcription termination sequences of the strongly expressed \textit{S. cerevisiae} enolase gene. When introduced by transformation into \textit{S. cerevisiae}, this construction resulted in the production of substantial amounts of \textit{Aspergillus}-type glucoamylase which, moreover, was correctly glycosylated and secreted to the extent of more than 90% into the growth medium. It is noteworthy that the \textit{S. cerevisiae} cell was able to recognize the glycosylation and secretion signals in the \textit{Aspergillus} primary
FIG. 10. Plasmid designed for synthesis and secretion of human interleukin-1β by S. cerevisiae. The plasmid contains origins of replication for E. coli and S. cerevisiae (ORI and ORI-2α respectively), URA3 for selection in S. cerevisiae, ampicillin resistance (AmpR) for selection in E. coli, and the GAL upstream activator sequence (UAS-GAL) coupled to the CYC1 (cytochrome e gene) promoter (P-CYC) and an initiation codon leading into a synthetic coding sequence for a 16-amino-acid hydrophobic peptide (kl) to act as a secretion signal. cDNA coding for interleukin-1β (CILβ) was inserted into the plasmid adjacent to and in frame with the kl sequence. Transcription initiation/termination signals: >■■ translation initiation/termination signals. Modified from reference 5.

A translation product. It is unlikely, however, that the normal four introns of the Aspergillus gene could have been dealt with by the S. cerevisiae splicing machinery, and these were removed from the gene before insertion into the transformation vector. A somewhat more sophisticated expression system was used by Baldari et al. (5) for obtaining production and secretion of human interleukin-1β by S. cerevisiae. They constructed their expression-and-secretion vector from a plasmid with a 2μm replication origin. Through a series of manipulations, they introduced into this plasmid, in 5’ to 3’ order, a galactose-inducible transcriptional activator sequence (UAS-GAL), the strong promoter of the S. cerevisiae CYC1 (cytochrome c) gene, and a synthetic oligonucleotide with an initiation codon followed by an open reading frame coding for an 18-amino-acid secretion signal sequence. The last sequence was taken from another yeast species, Kluyveromyces lactis, in which it is part of a gene encoding a secreted toxin active on other yeast strains. Downstream of this array, and in frame with the signal sequence, they placed the coding sequence of human interleukin-1β (supplied as cDNA to avoid difficulties with introns). The construction is diagrammed in Fig. 10. When S. cerevisiae carrying this plasmid was grown with galactose as the carbon source, it secreted interleukin-1β into the medium to a concentration of 1 to 2 mg/liter virtually unaccompanied by other proteins.

Methods such as this will undoubtedly be important for the pharmaceutical industry. There is no evident reason why some of the fast-growing species of filamentous fungi should not be just as suitable as S. cerevisiae for this kind of technology; indeed, they might have the advantage in being easily separable from the growth medium by simple filtration.

ACKNOWLEDGMENTS

I am indebted to R. H. Davis and J. A. Kinsey for helpful comments on the manuscript of this review and to J. A. Kinsey, G. Faugeron, and J. Bégueret for letting me see drafts of papers prior to publication.

LITERATURE CITED

1. Akins, R. A., and A. M. Lambowitz. 1985. A general method for cloning Neurospora crassa nuclear genes by complementation of mutants. Mol. Cell. Biol. 5:2272-2278.
2. Alani, E., L. Cao, and N. Kleckner. 1987. A method for gene disruption that allows repeated use of URA3 selection in the construction of multiply disrupted yeast strains. Genetics 16:541-545.
3. Andreadis, A., Y.-P. Hsu, G. B. Kohlhaw, and P. Schimmel. 1982. Nucleotide sequence of yeast LEU2 shows 5’-noncoding region has sequences cognate to leucine. Cell 31:319-325.
4. Andrianopoulos, A., and M. J. Hynes. 1988. Cloning and analysis of the positively acting regulatory gene undR from Aspergillus nidulans. Mol. Cell. Biol. 8:3532-3541.
5. Baldari, C., J. A. H. Murray, P. Ghiara, G. Cesareni, and C. L. Galeotti. 1987. A novel leader peptide which allows efficient secretion of a fragment of human interleukin 1 in Saccharomyces cerevisiae. EMBO J. 6:229-234.
6. Ballance, D. J., F. P. Buxton, and G. Turner. 1983. Transformation of Aspergillus nidulans by the orotidine-5-phosphate decarboxylase gene of Neurospora crassa. Biochem. Biophys. Res. Commun. 112:284-289.
7. Ballance, D. J., and G. Turner. 1985. Development of a high-frequency transformation vector for Aspergillus nidulans. Gene 36:321-331.
8. Banks, G. R. 1983. Transformation of Ustilago maydis by a plasmid containing yeast 2-micron DNA. Curr. Genet. 7:73-77.
9. Barnes, D. A., and J. Thorner. 1986. Genetic manipulation of Saccharomyces cerevisiae by the use of the LYS2 gene. Biol. 6:2828-2838.
10. Barnes, D. E., and D. W. MacDonald. 1986. Behaviour of recombinant plasmid in Aspergillus nidulans: structure and stability. Curr. Genet. 10:767-776.
11. Beach, D., and P. Nurse. 1981. High-frequency transformation of the fission yeast Schizosaccharomyces pombe. Nature (London) 290:140-142.
12. Beggs, J. D. 1978. Transformation of yeast by a replicating hybrid plasmid. Nature (London) 275:104-109.
13. Bégueret, J. V., Razanamparany, M. Perrot, and C. Barreau. 1984. Cloning gene undR for the orotidylate acid pyrophospho- rylase of the filamentous fungus Podoporus uncinatus: transformation of protoplasts. Gene 32:487-492.
14. Beri, R. K., and G. Turner. 1987. Transformation of Penicillium chrysogenum using the Aspergillus nidulans undS gene as a dominant selective marker. Curr. Genet. 11:639-641.
15. Binninger, D. M., C. Skrzynia, P. J. Pukkila, and L. A. Casselton. 1987. DNA-mediated transformation of the basidiomycete Coprinus cinereus. EMBO J. 6:835-840.
16. Bouton, A. H., and M. M. Smith. 1986. Fine-structure analysis of the DNA sequence requirements for autonomous replication of Saccharomyces cerevisiae plasmids. Mol. Cell. Biol. 6:2354-2363.
17. Broach, J. R. 1981. The yeast plasmid 2 μ circle, p. 445-476. In J. N. Strathern, E. W. Jones, and J. R. Broach (ed.). The molecular biology of the yeast Saccharomyces: life cycle and inheritance. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. Brygoso, Y., and R. Debuchy. 1985. Transformation by integration in Podoporus uncinatus. I. Methodology and phenomenology. Mol. Gen. Genet. 200:128-131.
19. Bull, J. H., D. J. Smith, and G. Turner. 1988. Transformation of Penicillium chrysogenum with a dominant selectable marker. Curr. Genet. 13:375-382.
20. Bull, J. H., and J. C. Wootton. 1984. Heavily methylated amplified DNA in transformants of Neurospora crassa. Nature (London) 310:701-704.
acting regulatory proteins. Curr. Genet. 12:21–31.
66. Keszenman-Pereya, D., and K. Heida. 1988. A colony procedure for transformation of Saccharomyces cerevisiae. Curr. Genet. 13:21–23.
67. Kim, S. Y., and G. A. Marzluf. 1988. Transformation of Neurospora crassa with the trp-1 gene and the effect of the host strain upon the fate of the transforming DNA. Curr. Genet. 13:85–70.
68. Kinnaird, J. H., M. A. Keighren, J. A. Kinsey, M. Eaton, and J. R. S. Fincham. 1982. Cloning of the am (glutamate dehydrogenase) gene of Neurospora crassa through the use of a synthetic DNA probe. Gene 20:387–396.
69. Kinsey, J. A., and J. A. Rambousek. 1984. Transformation of Neurospora crassa with the cloned am (glutamate dehydrogenase) gene. Mol. Cell. Biol. 4:117–122.
70. Köstler, H. C., and U. K. Benny. 1988. Genetic transformation of the fungal plant with pathogen Fusarium oxysporum. Curr. Genet. 14:154–147.
71. Klein, H. L. 1984. Lack of association between intrachromosomal gene exchange and reciprocal exchange. Nature (London) 310:740–753.
72. Kuiper, M. T. R., and H. de Vries. 1985. A recombinant plasmid carrying the mitochondrial plasmid sequence of Neurospora intermedia LaBelle yields new plasmid derivatives in Neurospora crassa transformants. Curr. Genet. 9:471–477.
73. Kunes, J., D. Botstein, and M. S. Fox. 1985. Transformation of yeast with linearized dimers and recombinant plasmid products. J. Mol. Biol. 184:375–387.
74. Marunouchi, T., Y.-J. Matsumoto, H. Hosoya, and K. Okabashii. 1987. In addition to the ARS core, the ARS box is necessary for autonomously replicating sequences in yeast. Mol. Gen. Genet. 206:60–65.
75. Mattern, I. E., S. Unkles, J. R. Kinghorn, P. H. Pouwels, and C. A. M. J. J. van den Houdt. 1987. Transformation of Aspergillus oryzae using the A. niger pyr4 gene. Mol. Gen. Genet. 210:460–461.
76. Maundrell, K., A. P. H. Wright, M. Piper, and S. Shall. 1985. Evaluation of heterologous ARS activity in Saccharomyces cerevisiae using cloned DNA from S. pombe. Nucleic Acids Res. 13:3711–3722.
77. Meselson, M., and C. M. Radding. 1975. A general model for recombination. Proc. Natl. Acad. Sci. USA 72:2358–361.
78. Miller, B. L., K. Y. Miller, and W. E. Timberlake. 1985. Direct and indirect gene replacements in Aspergillus nidulans. Mol. Cell. Biol. 5:1714–1721.
79. Mishra, N. C. 1977. Characterization of the new osmotic mutants (os) which originated during genetic transformation in Neurospora crassa. Genet. Res. 29:9–19.
80. Mishra, N. C. 1979. DNA-mediated genetic changes in Neurospora crassa. J. Gen. Microbiol. 113:255–259.
81. Mishra, N. C., G. Szabo, and E. L. Tatum. 1973. Nuclear acid induced genetic changes in Neurospora, p. 259–268. In M. C. Niu and S. J. Segal (ed.), The role of RNA in reproduction and development. Elsevier/North Holland Publishing Co., Amsterdam.
82. Mishra, N. C., and E. L. Tatum. 1973. Non-mendelian inheritance of DNA-induced inositol independence in Neurospora. Proc. Natl. Acad. Sci. USA 70:3875–3879.
83. Munoz-Rivas, A., C. A. Specht, B. J. Drummond, E. Froeliger, C. P. Novotny, and R. C. Ulrich. 1986. Transformation of the basidiomycete Schizophyllum commune. Mol. Gen. Genet. 205:103–106.
84. Murray, A. W., N. P. Schultes, and J. W. Szostak. 1986. Chromosome length controls mitotic chromosome segregation in yeast. Cell 45:529–536.
85. Murray, A. W., and J. W. Szostak. 1983. Pedigree analysis of plasmid segregation in yeast. Cell 34:961–970.
86. Murray, A. W., and J. W. Szostak. 1986. Construction of artificial chromosomes in yeast. Nature (London) 305:189–193.
87. Murray, A. W., and J. W. Szostak. 1986. Construction and behavior of circularly permuted and telocentric chromosomes in Saccharomyces cerevisiae. Mol. Cell. Biol. 6:3166–3172.
88. Orbach, M. J., E. B. Porro, and C. Yanofsky. 1986. Cloning and characterization of the gene for β-tubulin from a benomyl-resistant mutant of Neurospora crassa and its use as a dominant selectable marker. Mol. Cell. Biol. 6:2452–2461.
89. Orr-Weaver, T. L., and J. W. Szostak. 1983. Yeast recombination: the association between double-strand gap repair and crossing-over. Proc. Natl. Acad. Sci. USA 80:4417–4421.
90. Orr-Weaver, T. L., and J. W. Szostak. 1983. Multiple tandem plasmid integration in Saccharomyces cerevisiae. Mol. Cell. Biol. 3:747–749.
91. Orr-Weaver, T. L., and J. W. Szostak. 1985. Fungal recombination. Microbiol. Rev. 49:33–58.
92. Orr-Weaver, T. L., J. W. Szostak, and R. J. Rothstein. 1981. Yeast transformation: a model system for the study of recombination. Proc. Natl. Acad. Sci. USA 78:6354–6358.
93. Paetia, J., and G. A. Marzluf. 1985. Plasmid recovery from transformants and the isolation of chromosomal DNA segments improving plasmid replication in Neurospora crassa. Curr. Genet. 9:383–388.
94. Paetia, J. V., and G. A. Marzluf. 1985. Gene disruption by transformation in Neurospora crassa. Mol. Cell. Biol. 5:1554–1557.
95. Panchal, C. J., L. Bast, T. Dowhanick, J. Johnstone, and G. P. Stewart. 1987. Studies on stability of miniplasmids comprised of only yeast DNA. Curr. Genet. 12:15–20.
96. Parker, R., T. Simmons, E. O. Schuster, P. E. Siliciano, and C. Gantt. 1988. Genetic analysis of small nuclear RNAs in Saccharomyces cerevisiae: viable sestuple mutant. Mol. Cell. Biol. 8:3150–3159.
97. Parsons, K. A., F. G. Chumley, and B. Valient. 1987. Genetic transformation of the fungal pathogen responsible for rice blast disease. Proc. Natl. Acad. Sci. USA 84:4161–4165.
98. Pichard, M., R. Debuchi, J. Julien, and Y. Brygoo. 1987. Transformation by integration in Podospora anserina. II. Targeting to the resident locus with cosmids and instability of the transformants. Mol. Gen. Genet. 210:129–134.
99. Picknett, T. M., G. Saunders, P. Ford, and G. Holl. 1987. Development of a gene transfer system for Penicillium chrysogenum. Curr. Genet. 12:449–455.
100. Radford, A. S., Pope, A. Sazci, M. J. Fraser, and J. H. Parish. 1981. Lipoamide-mediated genetic transformation of Neurospora crassa. Mol. Gen. Genet. 184:567–569.
101. Rambousek, J. A., and J. Leach. 1987. Recombinant DNA in filamentous fungi: progress and prospects. Rev. Biotechnol. 6:357–373.
102. Kanazanaparany, V., and J. Begueret. 1986. Positive screening and transformation of IRL mutants in the fungus Podospora anserina: characterization of the transformants. Curr. Genet. 10:811–817.
103. Reipen, G., E. Erhart, K. D. Breunig, and C. P. Hohlenberg. 1982. Nonselective transformation of Saccharomyces cerevisiae. Curr. Genet. 6:189–193.
104. Rodriguez, R. J., and O. C. Yoder. 1987. Selectable genes for transformation of the fungal plant pathogen Glomerella cingulata f. sp. phaseoli (Colletotrichum lindemuthianum). Gene 54:73–80.
105. Rossier, C., A. Pugin, and G. Turian. 1985. Genetic analysis of transformation in a micro-coordinating strain of Neurospora crassa. Curr. Genet. 10:315–320.
106. Rothstein, R. J. 1983. One-step gene disruption in yeast. Methods Enzymol. 101:202–211.
109. Rudolph, H., J. Koenig-Rauseo, and A. Hinnebus. 1985. One-step gene replacement in yeast by co-transformation. Gene 36:87–95.
110. Russell, P., and P. Nurse. 1987. The mitotic inducer nim* functions in a regulatory network of protein kinase homologues controlling the initiation of mitosis. Cell 49:560–576.
111. Sakaguchi, J., and M. Yamamoto. 1982. Cloned oral locus of Schizosaccharomyces pombe propagates autonomously in this yeast assuming a polygenic form. Proc. Natl. Acad. Sci. USA 79:7819–7823.
132. Thomas, G. H., I. F. Connerton, and J. R. S. Fincham. 1988. Molecular cloning, identification and transcriptional analysis of genes involved in acetate utilization in Neurospora crassa. Mol. Microbiol. 2:599-606.

133. Tikhomirovaa, L. P., R. N. Ikonomova, and E. N. Kuznetsova. 1986. Evidence for autonomous replication and stabilization of recombinant plasmids in the transformants of yeast Hansenula polymorpha. Curr. Genet. 10:741-747.

134. Tilburn, J. C., C. Scarzecchio, G. G. Taylor, J. H. Zabicky-Zissima, R. A. Lockington, and R. W. Davis. 1983. Transformation by integration in Aspergillus nidulans. Gene 26:205-221.

135. Timberlake, W. E., M. T. Boylan, M. B. Cooley, P. M. Mira-bito, E. B. O’Hare, and C. E. Willet. 1985. Rapid identification of mutation-complementing restriction fragments from Aspergillus nidulans cosmids. Exp. Mycol. 9:351-355.

136. Tsuchiya, E., S. Shaktuto, T. Miyakawa, and S. Fukui. 1988. Characterization of a DNA uptake reaction through the nuclear membrane of isolated yeast nuclei. J. Bacteriol. 170:547-551.

137. Turgeon, B. G., R. C. Garber, and O. C. Yoder. 1986. Transformation of the fungal maize pathogen Cochliobolus heterosperme using the Aspergillus nidulans amds gene. Mol. Gen. Genet. 204:450-453.

138. Turgeon, B. G., R. C. Garber, and O. C. Yoder. 1987. Development of a fungal transformation system based on selection of sequences with promoter activity. Mol. Cell. Biol. 7:3397-3405.

139. Turner, G., and D. J. Ballance. 1985. Cloning and transformation in Aspergillus. p. 259-278. In J. W. Bennett and L. L. Lasure (ed.). Gene Manipulation in fungi. Academic Press, Inc., New York.

140. Ulrich, R. C., C. P. Novotny, C. A. Specht, E. H. Froehlinger, and A. M. Munoz-Rivas. 1985. Transforming Basidiomycetes, p. 39-57. In W. E. Timberlake (ed.). Molecular genetics of filamentous fungi. Alan R. Liss, Inc., New York.

141. Van Gorcom, R. F. M., P. H. Poulwels, T. Goosen, J. Visser, H. W. J. van den Broed, J. F. Hamer, W. E. Timberlake, and C. A. M. J. van den Hondel. 1985. Expression of an Exechrichia coli β-galactosidase fusion gene in Aspergillus nidulans. Genet. 40:99-106.

142. van Hartingsved, W., L. E. Mattern, C. M. J. van Zeij, P. H. Powlows, and C. A. M. J. van den Hondel. 1987. Development of a homologous transformation system for Aspergillus niger based on the pyrG gene. Mol. Gen. Genet. 206:71-75.

143. Van Heeswijk, R. 1986. Autonomous replication of plasmids in Mucor transformants. Carlsberg Res. Commun. 51:433-443.

144. Van Heeswijk, R., and M. I. G. Roncero. 1984. High-frequency transformation of Mucor with recombinant plasmid DNA. Carlsberg Res. Commun. 49:691-702.

145. Vollmer, S. J., and C. Yanofsky. 1986. Efficient cloning of genes of Neurospora crassa. Proc. Natl. Acad. Sci. USA 83:4867-4873.

146. Wang, J., D. W. Holden, and S. A. Leong. 1988. Gene transfer system for the phytopathogenic fungus Ustilago maydis. Proc. Natl. Acad. Sci. USA 85:865-868.

147. Ward, M., B. Wilkinson, and G. Turner. 1986. Transformation of Aspergillus nidulans with a cloned, oligomycin-resistant ATP synthase subunit 9 gene. Mol. Gen. Genet. 202:265-270.

148. Ward, M., J. F. Wilson, C. L. Carmore, and G. Turner. 1988. The olcC gene of Aspergillus niger: isolation, sequence and use as a selectable marker for transformation. Curr. Genet. 14:37-42.

149. Wernars, K., T. Goosen, K. Sewart, and H. W. J. van den Broek. 1986. Genetic analysis of the genes involved in acetate utilization in Neurospora crassa. Mol. Microbiol. 2:599-606.

150. Wernars, K., T. Goosen, B. M. J. Wennekes, K. Sewart, C. A. M. J. van den Hondel, and H. W. J. van den Broek. 1987. Cotransformation of Aspergillus nidulans: a tool for replacing fungal genes. Mol. Gen. Genet. 209:71-77.

151. Wernars, K., T. Goosen, B. M. J. Wennekes, J. Visser, C. J. Bos, H. W. J. van den Broek, R. F. M. van Gorcom, C. A. M. J. van den Hondel, and P. H. Poulwels. 1985. Gene amplification in Aspergillus nidulans by transformation with vectors containing the amds gene. Curr. Genet. 9:361-368.

152. Wright, A. P. H., K. Maundrell, and S. Shall. 1986. Transfor-
formation of *Schizosaccharomyces pombe* by non-homologous, unstable integration of plasmids in the genome. *Curr. Genet.* 10:503–508.

153. Yelton, M. M., J. E. Hamer, and W. E. Timberlake. 1984. Transformation of *Aspergillus nidulans* by using a *trpC* plasmid. *Proc. Natl. Acad. Sci. USA* 81:1470–1474.

154. Yelton, M. M., W. E. Timberlake, and C. A. M. J. J. van den Hondel. 1985. A cosmid for selecting genes by complementation in *Aspergillus nidulans*: selection of the developmentally regulated yA locus. *Proc. Natl. Acad. Sci. USA* 82:834–838.

155. Zakian, V. A., H. M. Blanton, L. Wetzel, and G. M. Dani. 1986. Size threshold for *Saccharomyces cerevisiae* chromosomes: generation of telocentric chromosome from an unstable minichromosome. *Mol. Cell. Biol.* 6:925–932.