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Transformation in Fungi

JOHN R. S. FINCHAM

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

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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 transformants, a result 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 glucanase 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 *leu2* 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).

TABLE 1. Fungal species in which transformation has been achieved

Type of fungus	Reference
Ascomycetes	
Budding yeasts	
Saccharomyces cerevisiae	53
Kluveromyces lactis	30
Kluveromyces 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 fulvum (leaf mold of tomato)	87a
Discomycetes	
Ascobolus 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
Basidiomycetes	
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

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 (Helicase 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 Novozyme 234. These various preparations all contain a complex mixture of hydrolytic enzymes, notably 1,3-glucanases and chitinase.

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 *am*$^+$ (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 Novozyme 234 of a particular batch number as particularly suitable for preparation of receptive *Neurospora* 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 *Neurospora* species, germinating macroconidia, which are predominantly multinucleate, are most commonly used; the uninucleate 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*

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 *inl* mutant in the early experiments on *Neurospora* transformation. More recent use has been made of a double mutant carrying both *inl* and *os*, a mutation causing sensitivity to high osmotic pressure (J. C. Wootton, 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 germlings 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. Iimura 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 100/μg 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 10^8 to 10^9/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 *Ascobolus 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 a 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 on 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 now 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).

A sometimes inconvenient consequence of relying on selection against auxotrophy in isolation of transformants is that one must always have the appropriate auxotrophic mutation in the recipient strain. An alternative is to use a dominant mutant gene as the selectable marker. A number of mutations conferring drug resistance, some of bacterial origin, have proved to be suitable for this purpose. Some of these are also listed in Table 3. Of particular importance is the mutant β-tubulin gene (*Ben*^{r}) of *N. crassa*, which confers resistance to the drug benomyl (88). This mutant allele is incorporated into the cosmid vector which was used (145) to construct a supposedly near-comprehensive gene bank for *N. crassa*.

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 (*ura3* and *pyr-4*, respectively) confer resistance to the normally inhibitory analog 5-fluoro-orotic acid (2, 39, 40). *S. cerevisiae lys2* (2-aminoadipate reductase deficient) mutants can grow (in the presence of lysine) on 2-aminoadipate 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, Reipen 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 ADH1* (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

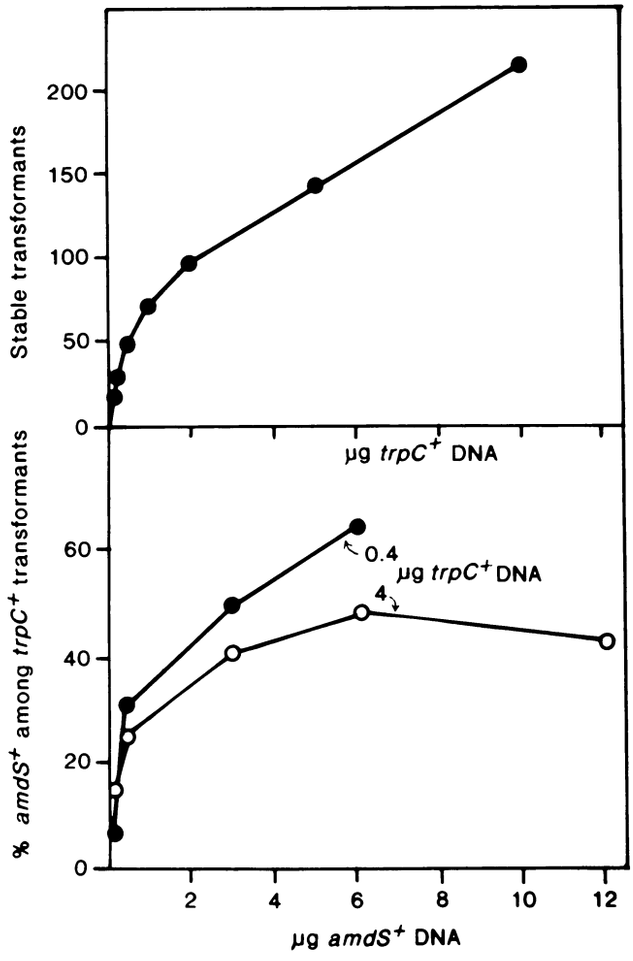

FIG. 1. (a) Relationship between transformation frequency and DNA concentration in *Aspergillus nidulans*. Approximately 2×10^6 viable *trpC* protoplasts were treated with various amounts of $trpC^+$ plasmid DNA in a final volume of 125 μl. The form of the curve suggests that a small minority of protoplasts (about 70) were transformed at DNA concentrations of less than 1 μg/125 μl and that the remainder of the population took up DNA much less readily to an extent proportional to the DNA concentration. The yield of transformants per microgram of DNA was about 10 times higher at very low than at high DNA concentrations. Modified from reference 153. (b) Relationship between cotransformation frequency and DNA concentration in *Aspergillus nidulans*. Either 0.4 or 4 μg of $trpC^+$ plasmid DNA was supplied to the protoplasts, along with various amounts of $amdS^+$ plasmid DNA. The conditions for transformation were essentially the same as in panel (a). Above 4 μg of $amdS^+$ DNA, the proportion of $trpC^+$ transformants that were also $amdS^+$ approached a maximum of 40 to 50% as if, at that concentration, the subpopulation of protoplasts likely to take up more than one DNA molecule was already saturated with DNA. The converse experiment, with a constant amount of $amdS^+$ DNA and varying concentrations of $trpC^+$ DNA, gave a similar result, except that cotransformation approached 90% at higher $trpC^+$ concentrations. Modified from reference 150.

al. (Faugeron et al., in press) largely avoided the problem of heterokaryotic transformants in *Ascobolus immersus*, another aconidial species, by fractionating the protoplasts by size; the fraction passing through a sintered glass filter included many protoplasts that were anucleate and inviable, but of those that were viable, 93% were uninucleate.

AUTONOMOUSLY REPLICATING VECTORS

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* ColE1 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 10^4/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 $qa\text{-}2^+$ into the *E. coli* ColE1-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 $qa\text{-}2^+$ 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

TABLE 2. Current procedures for transformation

Species	Protoplast formation procedure	DNA uptake procedure	Yield per μg of DNA	Reference
N. crassa	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 g 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 $CaCl_2$ (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	$(1–2) \times 10^4$	145
	No protoplasting; germinating conidia, 0.1 M lithium acetate, 30 min at 30°C	(i) 20 μg of DNA in 50 μl of 10 mM Tris (pH 7.5)–1 mM EDTA plus 5×10^7 cells in 400 μl of 0.1 M lithium acetate, 30 min at 30°C (ii) Add 10 vol 40% PEG 4000 in 0.1 M lithium acetate, 1 h at 30°C, 5 min at 37°C	10–50	37
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	70	153
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 $CaCl_2$–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[a]	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 $CaCl_2$, 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 oidia, 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. 10^8 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[b]	53
S. cerevisiae	No protoplasting; log-phase cells at 10^8/ml in 10 mM Tris (pH 8)–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. $(1–4) \times 10^2$[c]	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) 5×10^8 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	10^4[c]	11

[a] Higher frequency with *ans* sequence in transforming plasmid (see text).
[b] With nonreplicating plasmid; $>10^3$-fold higher with replicating plasmid.
[c] With replicating plasmids.

TABLE 3. Selectable markers used across species

Marker	Species of origin	Phenotype(s)	Genera in which marker was used	Reference
HygB^r	*E. coli*	Hygromycin B resistance	*Saccharomyces*	63
			Cephalosporium	119
			Cochliobolus	137
			Colletotrichum	38
			Fulvia	87a
			Septoria	28
			Ustilago	146
Neo^r	*E. coli*	Kanamycin, G418 resistance	*Schizophyllum*	140
			Ustilago	8
			Phycomyces	126
Ben^r	*N. crassa*	Benomyl resistance	*Colletotrichum*	38
			Gaeumannomyces	51
oliC	*Aspergillus niger*	Oligomycin resistance	*Aspergillus*[a]	148
amdS$^+$	*Aspergillus nidulans*	Acetamide utilization	*Penicillium*	14
			Cochliobolus	137
			Colletotrichum	105
pyr-4$^+$	*N. crassa*	Pyrimidine synthesis	*Aspergillus*	7
			Penicillium	38
argB$^+$	*Aspergillus nidulans*	Arginine synthesis	*Aspergillus*[b]	21
			Magnaporthe	97
bla[c]	*E. coli*	β-Lactamase	*Saccharomyces*	10
lacZ[c]	*E. coli*	β-Galactosidase	*Aspergillus*	14

[a] *Aspergillus nidulans*.
[b] *Aspergillus niger*.
[c] For visual selection.

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 *pyr-4*$^+$ or *qa-2*$^+$ 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

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 premeiotic 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 *ans1*, 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 *ans1* was in some way enhancing the frequency of transformation by chromosomal integration rather than functioning as an ARS. Since the *ans1* 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 $qa\text{-}2^+$ ligated into pBR322 could be recovered from transformant DNA by retransformation of *E. coli*, indicating that the $qa\text{-}2^+$-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 something with the replicational and transmissional 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* ColE1 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 ColE1-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 1 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 varying from one transformant to another. These were attributed to single crossovers between the plasmid and various nonhomologous chromosome 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 (δ) 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 chromosome and plasmid, resulting in gene conversion (*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).

Further 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 reconstituted 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 the vector sequence between them. Whether all transformation 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 this formal linkage analysis on a series of *Aspergillus nidulans* strains transformed with respect to *amdS*. They mapped the transforming *amdS*$^+$ gene to several different chromosomes in different transformants. Kinsey and Rambosek (69), transforming an *N. crassa am* deletion mutant with an *am*$^+$ clone with about 1 kb of homology on each side of the deletion, found approximately equal numbers of integrations of *am*$^+$ 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 *trp-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-independent 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*

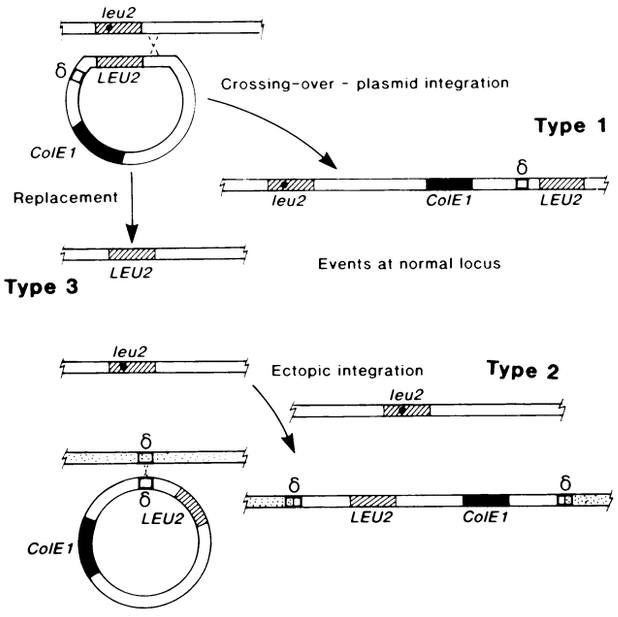

FIG. 2. Three modes of integration of transforming DNA in *S. cerevisiae* (53). *S. cerevisiae leu2* mutant cells were transformed with a nonreplicating ColE1-based plasmid carrying *S. cerevisiae LEU2*. Type 1 is homologous additive integration by homologous crossing over, leading to tandemly arranged *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. δ indicates a terminal repeat of the movable Ty1 element; copies of δ are dispersed through the genome. Type 3 is replacement of *leu2* by *LEU2* without integration of plasmid sequence. Modified from reference 53.

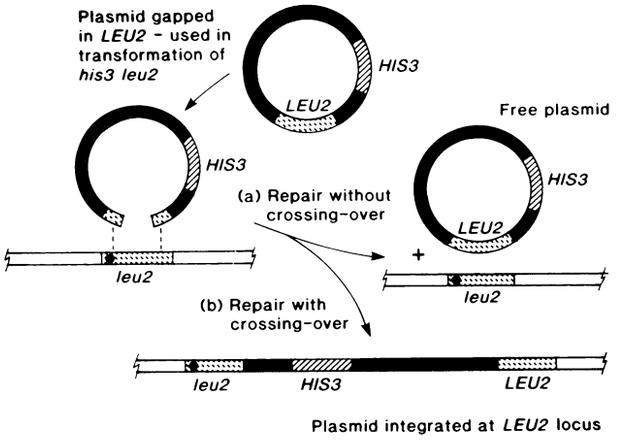

FIG. 3. Interaction in *S. cerevisiae* between a replicating plasmid gapped in the selectable marker *LEU2* and the homologous chromosome locus with a mutant *leu2* allele. The mutation distinguishing *leu2* from *LEU2* (●) is, in this example, not within the region deleted by gapping. There are two possible outcomes, occurring with approximately equal frequencies: (a) gap repair without crossing over, leaving free *LEU2* plasmid and an unstable transformant; and (b) gap repair with crossing over, leading to stable transformation with *LEU2* integrated in tandem with *leu2* and the rest of the plasmid sequence between them. When the plasmid is gapped not in *LEU2* but in the alternative selectable marker *HIS3*, the interaction is with the *his3* chromosomal locus. Modified from reference 89.

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 uncut 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 et 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 *amdS*$^+$, obtained evidence consistent with additive integration either into the partially deleted *amdS* recipient locus or (when rDNA was incorporated into the vector) into rDNA genes. Yelton et al. (154), 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 *Ascobolus immersus*, Goyon and Faugeron (unpublished) transformed a *met-2* (methionine-requiring) mutant with *met-2*$^+$ cloned in bacteriophage M13 and supplied in three alternative forms: closed-circular double-stranded DNA, closed-circular single-stranded DNA, and double-stranded DNA linearized by cutting within the *me-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 integration; 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 something of a puzzle that two transformants had regenerated a functional *me-2*$^+$ gene from the cut 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 available evidence points to ectopic integration as the predominant mode of stable transformation by plasmids not cut within the transforming gene (15). Of 92 *trp1*$^+$ 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* species is usually integrated by interaction with single-copy chromosomal sequences, it is itself often present in transformants in multiple copies, usually linked in tandem orientation. There are two obvious alternative mechanisms through which this might come about. 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) obtained strong evidence for the latter alternative. They transformed *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 integration of transforming genes in tandemly repeated copies in filamentous fungi. In both *Aspergillus nidulans* (153) and *Ascobolus 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 *amdS*$^+$ transformants in high copy number in *Aspergillus nidulans*. Most of the copies seemed to be in tandem arrays at ectopic loci; presumably, nonhomologous primary integration events were followed by successive rounds of homologous recombination between free plasmids and the plasmids already integrated.

Transformants with single integrated gene copies are presumably more likely to be obtained when lower concentrations 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 Hinnen 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 any set ratio of additions to replacements.

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

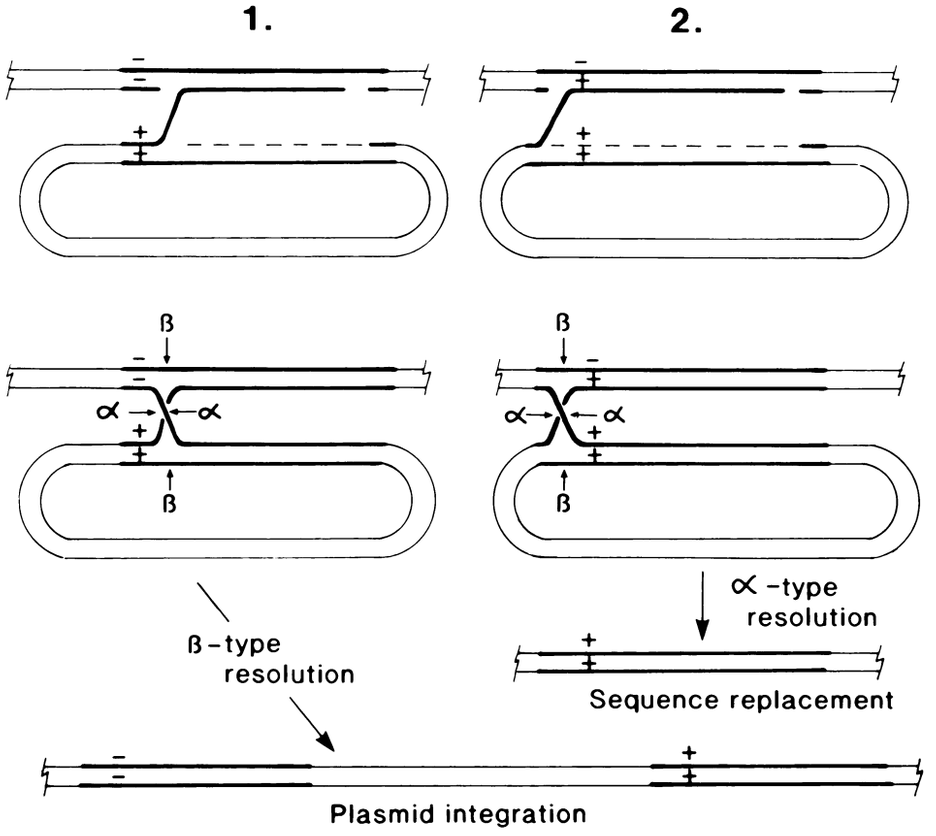

FIG. 4. Possible mechanisms of homology-dependent transformation. The donor DNA is supposed to be a circular plasmid with a selectable marker (+), as distinct from the corresponding (−) site in the recipient chromosome. The region of homology between plasmid and chromosome is indicated by the thicker 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 α/β or β/α 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 (α/β or β/α 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 (α/α or β/β resolution) leads to conversion by sequence replacement. Repair with crossing over (α/β or β/α 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 *ura5*$^+$. 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 chromosome 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 chromosome ends 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 *qa-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 *Bam*HI cloning site which would accept fragments cut with the "four-cutting" endonuclease *Mbo*I. A bank of cosmids carrying *A. nidulans* genomic fragments of 35 to 40 kb, generated by partial *Mbo*I digestion, was used to transform a *trpC yA* (yellow-spored) double-mutant strain. The initial selection was for *trpC*$^+$, and then green-spored (*yA*$^+$) 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-

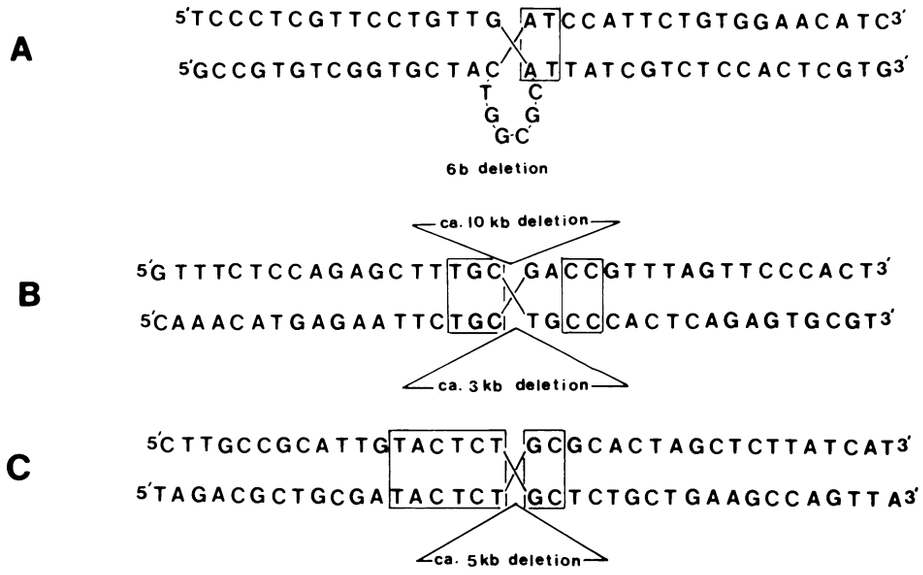

FIG. 5. Modes of nonhomologous integration of transforming DNA in *Podospora anserina*. Of three ectopic *ura5*$^+$ transformants analyzed, one (A) had all of the plasmid integrated except for a 6-bp gap at the site of integration and no detected loss of chromosomal DNA. Two others (B and C) each had extensive gapping of the plasmid to create the ends joined into the chromosome. One (C) had lost no detectable chromosomal DNA, and one (B) had a 10-kb deletion of the chromosome at the site of plasmid integration. In each diagram, the upper sequence is chromosomal and the lower sequence is plasmid, with the crossover point indicated. Only one strand of each DNA duplex is drawn. Short stretches of sequence similarity flanking the insertion sites are boxed. Modified from Razanamparany and Bégueret (Gene, in press).

quences 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 alleles 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 coding 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 uninucleate 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 (ascospores) 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 asci 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 karyogamy and 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 synapsis and that this, for reasons that are quite obscure, triggers methylation of a high proportion of the cytosine residues within the synapsed region. 5-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.

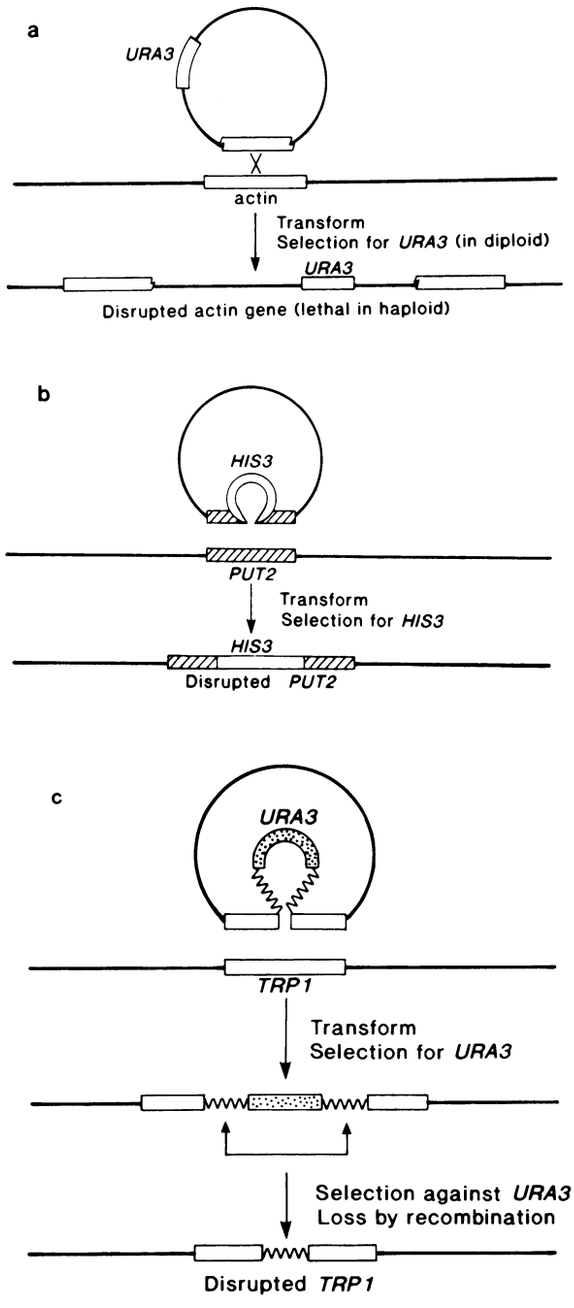

FIG. 6. Procedures for gene disruption. (a) The method of Shortle et al. (115) applied to the *S. cerevisiae* actin gene. The gene copy present in the transforming plasmid had deletions at both ends. Integration of the plasmid by actin gene homology split the chromosomal gene into two nonfunctional pieces. Modified from reference 115. (b) The one-step method of Rothstein (107) used to disrupt the *S. cerevisiae PUT2* (proline utilization) gene. The transforming plasmid carried a copy of *PUT2* with the selectable marker *HIS3* inserted into it. Type 3 *HIS3* transformants may have the selectable marker integrated either at the *his3* or at the *PUT2* locus; in the latter case, the chromosomal *PUT2* is disrupted. Modified from reference 107. (c) Procedure for removal of the selectable marker so that it can be used again for another round of disruption in *S. cerevisiae*. In the transforming plasmid the selectable marker *URA3*, flanked by 1.1-kb direct repeats of bacterial DNA, was inserted into *TRP1*. Following the primary disruption, removal of *URA3* was selected as conferring resistance to 5-fluoro-orotic acid. 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.

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 *Ascobolus immersus* by Goyon and Faugeron (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 fail to transmit 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 sequence 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 subclones 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 replacement. 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 transforming 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 plasmid 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).

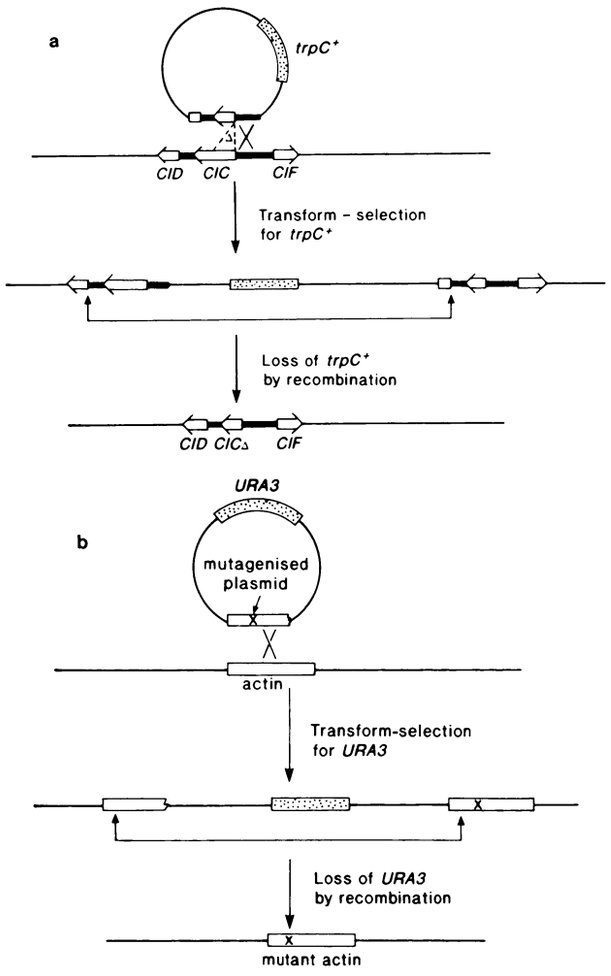

FIG. 7. Procedures for gene replacement. (a) Replacement of the *spoC1-C* gene of the *Aspergillus nidulans spoC1* (sporulation-specific) gene cluster (comprising *spoC1-C*, *spoC1-D*, and *spoC1-F*) with a partially deleted derivative by the two-step principle first applied by Scherer and Davis (112) to *S. cerevisiae*. The *spoC1* sequence is shown by thicker lines, with open arrows representing the genes. Selection was first made for $trpC^+$, the selectable marker in the plasmid carrying the partially deleted *spoC*, and then for loss of $trpC^+$. One class of crossover event removed $trpC^+$ together with the normal *spoC1-C*, leaving the partially deleted derivative. Modified from reference 78. (b) The method of disruption/replacement of Shortle et al. (116) applied to the creation of a temperature-sensitive actin mutant of *S. cerevisiae*. The donor plasmid included *URA3* as selectable marker and also a downstream-deleted derivative of the actin gene. The plasmid was chemically mutagenized before transformation; × represents a mutation. *URA3* transformants were screened for temperature sensitivity. The now irrelevant partially deleted actin gene was eliminated by selection for loss of *URA3* by crossing over between homologous sites in the direct repeats. Modified from reference 116.

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 ($cyh2^r$) *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 *PHO5* (encoding acid phosphatase) by inserting *URA3* 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 in vitro deletion in its promoter region. Among the leucine-independent transformants, about 1%, 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 *spoC1C* gene of the *spoC1* (sporulation-specific) gene cluster with a partly deleted derivative. A plasmid carrying the modified *spoC1* sequence together with $trpC^+$ as a selectable marker was used to transform a *trpC* mutant strain. A transformant with $trpC^+$ integrated by crossing over within *spo* tended to lose $trpC^+$ during vegetative growth by further crossing over between the flanking *spo* sequences. Five of eight tryptophan-requiring derivatives had the partial deletion in *spoC1C* (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 selected for $amdS^+$ showed β-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. 8b).

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

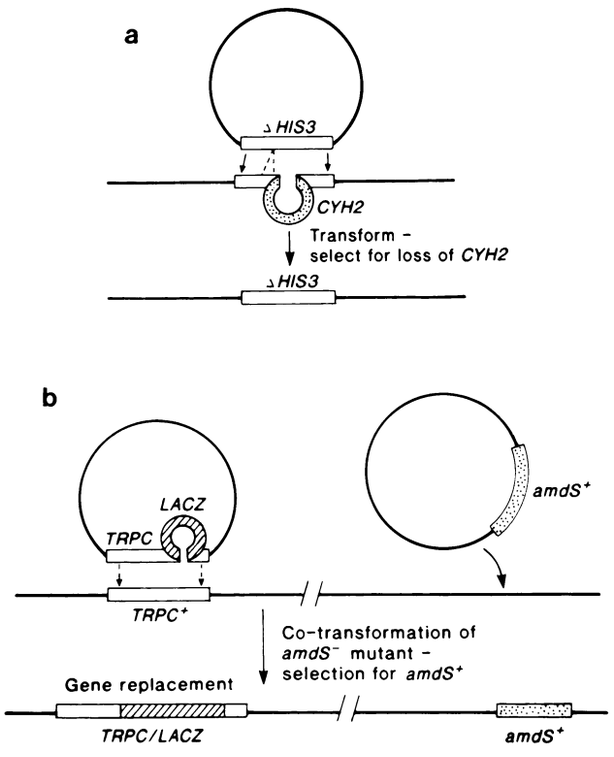

FIG. 8. Direct selection for gene replacement. (a) Replacement of *S. cerevisiae HIS3* by an in vitro-generated derivative with a 31-bp upstream deletion (Δ). First the chromosomal copy of *HIS3* in a cycloheximide-resistant strain (*cyh2*^{r}, modified ribosomal protein L29) was disrupted by insertion of *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 *CYH2*. The mutant *HIS3*, which turned out to be nonderepressible in response to amino acid deprivation, replaced the *HIS3/CYH2* construct. Modified from reference 124. (b) The use of cotransformation to replace *Aspergillus nidulans trpC*$^+$ by a *trpC/lacZ* fusion. A mixture of two plasmids, one carrying the fusion gene and one carrying *amdS*$^+$, was used in transformation, and *amdS*$^+$ was selected for. Screening of a large number of *amdS*$^+$ transformants revealed some in which *lacZ* was expressed. Modified from reference 150.

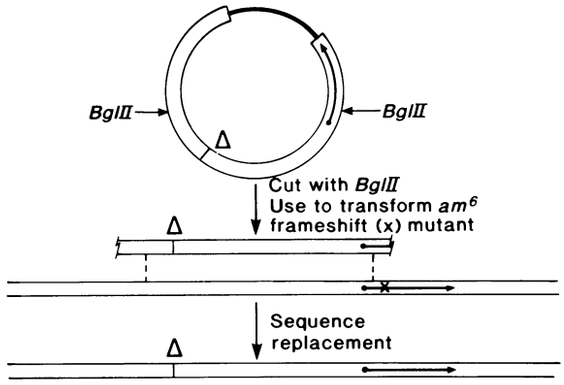

FIG. 9. Replacement of the upstream control region of the *N. crassa am* (glutamate dehydrogenase) gene. The transforming plasmid carried a copy of the *am* gene with a short deletion (Δ) in its upstream region. It was linearized before transformation by cutting with *Bgl*II, resulting in removal of all but the 5′ 367 base pairs of the protein-coding sequence. The chromosomal *am* copy had a frameshift mutation (shown as ×) just downstream of the initiation codon. Transformants with restored *am* function (although possibly with altered regulation) had usually acquired the upstream deletion. Transformation with the linear plasmid apparently occurred by replacement as indicated, without crossing over or integration of vector sequence. Modified from G. D. Frederick, D. K. Asch, and J. A. Kinsey (Mol. Gen. Genet., in press).

about the functions of that gene. A good example was provided by Kelly and Hynes (65), who obtained transformants of *A. nidulans* with multiple copies of *amdS*, a gene that is subject to multiple *trans*-acting controls. Genetic evidence had indicated that the *trans*-acting control genes that activated transcription of *amdS* under different conditions each acted also on a group of other genes. Thus, *areA* controls a wide range of ammonium-regulated genes, and *amdR* is necessary for efficient expression of genes encoding omega-transaminases and β-lactamase. A transformant with many copies of *amdS* grew relatively poorly on nitrogen sources dependent for their utilization on genes subject to *areA* or *amdR* control. This was interpreted as being due to titration of the *areA* and *amdR* protein products by binding sites upstream of each of the multiple copies of *amdS*. This interpretation was supported in the case of *amdR* by the demonstration (4) that the functions supposedly controlled by this gene were depressed following the introduction in multiple copies of an *amdS* upstream DNA fragment including the putative binding site for the *amdR* product. Furthermore, these functions could be restored by the addition, by transformation, of multiple copies of *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 *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 *Aspergillus awamori* gene for glucoamylase between the promoter and transcription termination sequences of the strongly expressed *S. cerevisiae* enolase gene. When introduced by transformation into *S. cerevisiae*, this construction resulted in the production of substantial amounts of *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 *S. cerevisiae* cell was able to recognize the glycosylation and secretion signals in the *Aspergillus* primary

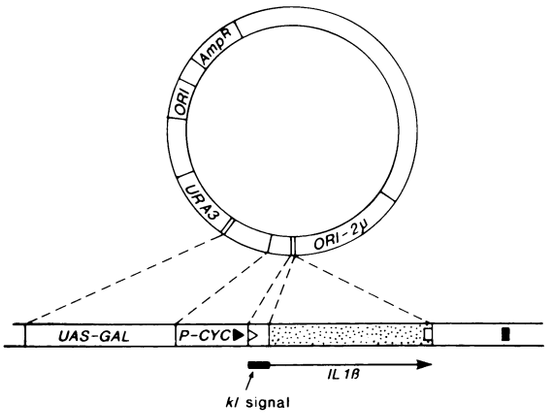

FIG. 10. Plasmid designed for synthesis and secretion of human interleukin-1β by *S. cerevisiae*. The plasmid contains origins of replication for *E. coli* and *S. cerevisiae* (*ORI* and *ORI*-2μ respectively), *URA3* for selection in *S. cerevisiae*, ampicillin resistance (Amp^R) for selection in *E. coli*, and the *GAL* upstream activator sequence (*UAS-GAL*) coupled to the *CYC1* (cytochrome *c* 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 (▭) 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.

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 uncontaminated 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.

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