MICROBIOLOGICAL REVIEWS, Dec. 1995, p. 673–685 Vol. 59, No. 4
0146-0749/95/$04.00+0

Natural Plasmids of Filamentous Fungi

A. J. F. GRIFFITHS*

Botany Department, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z4

INTRODUCTION

Plasmids are small extragenomic DNA molecules that can reproduce inside living cells. They replicate separately from the genome, but some can integrate covalently into the genome and replicate as part of genomic DNA. Plasmids were originally discovered in bacteria, but later analogous molecules were found in eukaryotes. Many examples have been recorded in fungi, few have been found in plants, and none have been found in animals. This review concerns the natural plasmids of filamentous fungi. Yeast plasmids are excluded. The 2μm plasmid of the yeast *Saccharomyces cerevisiae* (12) has been important in the development of the molecular technology of yeasts. Also, the killer plasmids of the yeast *Kluyveromyces lactis* have been intensively studied at the molecular level (37). However, these yeast plasmids are quite different from the plasmids of filamentous fungi. One notable difference is that they are nuclear and cytoplasmic, respectively, whereas virtually all the plasmids discovered so far in filamentous fungi are mitochondrial. Henceforth, the term "fungi" is used to mean filamentous fungi. Also excluded from this review are plasmid-like elements that are derived from regions of mitochondrial DNA (mtDNA) in several different fungi (28, 30), for example, the senDNA plasmid-like elements found in senescent strains of *Podospora anserina* (86). Other reviews describing fungal plasmids have also been written over the past 10 years (23, 24, 48, 64, 70).

Fungal plasmids are found in cultures isolated directly from natural populations. The standard wild-type laboratory strains that have long been used for genetic analysis of fungi such as *Neurospora crassa* are inbred stocks that contain no plasmids. Even though attempts have been made to use natural plasmids as cloning vectors for fungal recombinant DNA technology (88), such attempts have so far met with only limited success. The cloning vectors used to transform filamentous fungi are generally bacterial plasmids into which fungal genes have been inserted to act as selectable markers. Such recombinant plasmids do not reproduce inside fungal cells and must integrate into the nuclear genome to achieve stable transformation.

This review will center on the natural biology of plasmids. This is interesting for three reasons. First, since eukaryotic plasmids are mostly fungal, the plasmids represent a special area of mycology that merits study in its own right. Such investigation can illuminate not only the properties of the plasmids but also those of their fungal hosts. In particular, the plasmids represent useful markers for studying fungi at the population level. Second, as with bacterial plasmids, the study of fungal plasmids can uncover a wealth of diverse molecular processes that are relevant not only to the plasmids themselves but also to the properties of DNA in general. In particular, the intimate association of plasmids with mitochondria can shed light on the molecular biology of mtDNA. Third, plasmids represent easily studied examples of a mysterious class of DNA known as parasitic or selfish DNA, a type of DNA that seems to exist only for the purpose of existing. This class of DNA shows a spectrum of DNA "life-styles" including plasmids, introns, transposons, and viruses. Analysis of plasmids has shown possible areas of connection between these different types, leading to new insights into their evolution. Most DNA—even genomic DNA—could be considered selfish, so the study of plasmids can provide clues about primitive genomes and how they were able to establish themselves in the face of the forces of disorder.

INCIDENCE AND DISTRIBUTION

Plasmids have been found in many different fungi, including *Absidia glauca* (38), *Agaricus* spp. (77), *Ascobolus immersus* (63b), *Ascosphaeria apis* (76a), *Alternaria alternata* (85), *Claviceps purpurea* (90), *Epichloe typhina* (67), *Erisyphe graminis* (27a), *Fusarium solani* (81), *Fusarium oxysporum* (45), *Leptosphaeria maculans* (59), *Morchella conica* (63a), *Nectria haematococca* (81), *Neurospora* spp. (30), *Podospora anserina* (42), *Pythium* spp. (62a), *Rhizoctonia solani* (65), *Tilletia* spp. (54), and *Trichoderma viride* (64a). Other examples can be found in reviews by Meinhardt et al. (64) and Kempken (48). All of the plasmids found in these fungi are mitochondrial with the pos-

* Mailing address: Botany Department, University of British Columbia, 6270 University Blvd., Vancouver, B. C., Canada V6T 1Z4. Phone: (604) 822-5629. Fax: (604) 822-9179. Electronic mail address: agriff@unixg.ubc.ca.

sible exception of the *Alternaria* plasmids. Plasmids are of two types, circular and linear. In *Neurospora* spp., both circular and linear types are found. The plasmids of *Absidia glauca* are circular, and all the remaining examples are linear.

In most fungi, plasmids were discovered initially through chance encounters, but systematic searches were made after the initial discoveries. In *Neurospora* species, the first discoveries were of circular plasmids. Three nonhomologous plasmids were found by chance and named after the collection sites of their host strains. They were the Mauriceville plasmid of *N. crassa* (17) and the LaBelle and Fiji plasmids of *N. intermedia* (87). Subsequently, plasmid surveys have been carried out on collections of natural isolates of *Neurospora* species. Natvig et al. (73) found circular plasmids in several different *Neurospora* species. Approximately half of the 24 strains examined had plasmids. The plasmids fell into the three homology groups Fiji, Mauriceville, and LaBelle. LaBelle was found in only one isolate of *N. intermedia*, from Louisiana. Fiji was found in *N. intermedia* from Fiji and *N. tetrasperma* from Hawaii. Mauriceville was found in *N. intermedia* from India and *N. crassa* from Texas. Both *N. crassa* and *N. intermedia* are heterothallic species, whereas *N. tetrasperma* is pseudohomothallic. Hence, it appeared that individual plasmids can have a wide distribution, sometimes crossing species boundaries. Reasons for the incidence of homologous plasmids in different species are discussed below.

A large collection of *Neurospora* isolates from the Hawaiian Islands has been tested for the presence of a linear plasmid called kalilo and a circular plasmid called Hanalei-2, which had been found together initially in one Hawaiian strain (10). It was shown that in the Hawaiian population, each plasmid is present in a majority of isolates but the two plasmids are not necessarily found together (21, 28). Hence, in one geographical area, plasmid-containing strains can be the rule rather than the exception.

A large worldwide survey of 171 isolates of *N. intermedia* and *N. crassa* investigated both linear and circular plasmids (98). The survey was based partly on Southern hybridizations with plasmids from previously known groups as probes and partly on a general search for unknown plasmids. Plasmids hybridizing to the linear plasmid maranhar (originally isolated from *N. crassa* in Aarey, India) were found in isolates of *N. intermedia* from New Guinea, the United States, Brazil, and China. The linear plasmid kalilo was found only in its original host, *N. intermedia*, in Hawaii. A new circular plasmid, Harbin-1, a member of the LaBelle group, was found to be widely distributed across the world, in both *N. crassa* and *N. intermedia*. A new circular plasmid, Harbin-2, was thought possibly to represent a new homology group. Many other examples of previously undescribed circular and linear plasmids were found; some of these have been named (99), but no cross-hybridization studies were done to try to group them. One set of three high-copy-number linear plasmids of 7, 8, and 9 kb in a strain from Harbin, China, have been called Harbin-3, -4, and -5 (99) or zhisi plasmids (33, 34). The two largest zhisi plasmids contain a small region from the maranhar plasmid, but the remaining part of their sequence might represent a different homology group. The smallest zhisi plasmid seems to be almost identical to maranhar (97). Many strains were found to contain several distinct plasmid types, up to a maximum of 11 plasmids. Mixtures of linear and circular plasmids were common.

A later study (6) confirmed the widespread distribution and diversity of plasmids in natural populations of *Neurospora* species. This survey encompassed 241 isolates of 10 *Neurospora* species and related fungi. Probes were used from the previously established groups LaBelle, Mauriceville, Fiji, and kalilo.

TABLE 1. Plasmid homology groups defined to date in *Neurospora* species

Plasmid type	Homology group
Circular	Mauriceville, Fiji, LaBelle, Java, MB1, VS, Harbin-2
Linear	Kalilo, Maranhar, Moorea, Zhisi

Three new groups were identified, Java and MB1 (both circular) and Moorea (linear). All plasmids were found in not less than three different species. Plasmids hybridizing to the kalilo plasmid were found in 41 isolates of four different species, all distributed throughout the world, thereby extending the range of this element considerably. Five *Neurospora* species (*N. crassa*, *N. discreta*, *N. intermedia*, *N. sitophila*, and *N. tetrasperma*) contained one or more plasmids, giving an overall frequency of 51% plasmid-bearing strains in these species. The remaining five *Neurospora* species (*N. africana*, *N. galapagosensis*, *N. lineolata*, *N. sublineolata*, and *N. terricola*) contained no detectable plasmids; however, only one representative of each was tested. In addition, 18 isolates of related fungi (*Gelasinospora*, *Aspergillus*, *Fusarium*, and *Sordaria* species) contained no detectable plasmids. Frequencies were calculated for all the homology groups except maranhar, and it was found that Fiji and LaBelle were the most common (26 and 20%, respectively) and MB1 was the least common (2%). *N. discreta* and *N. intermedia* showed the highest plasmid frequencies (approximately 70%), whereas *N. crassa* showed the lowest (36%). Different plasmids were found to be distributed in patterns that were statistically independent, suggesting that the plasmids are freely mobile and can take up any association. In a separate study, Marcinko-Kuehn et al. (62) found a kalilo group plasmid in a Louisiana population of *N. tetrasperma*. The plasmid groups of *Neurospora* species are summarized in Table 1.

A survey of 61 field isolates of *Fusarium oxysporum* from Japan (45) revealed six linear plasmids, pFO-A, pFO-B, pFO-C, pFO-L, pFO-M, and pFO-R, each unique to six formae speciales (fungal races defined through host range). Some formae speciales contained no plasmids, and three of those that did show plasmids also showed plasmidless strains.

Most strains of the ergot fungus *Claviceps purpurea* from different parts of Europe and collected on different hosts contained linear plasmids (22, 90). These plasmids had different sizes and restriction maps, but there were clear patterns of relatedness.

A survey of 114 isolates of *Rhizoctonia solani* from a wide range of plant hosts revealed that 48 strains contained one to three linear plasmids (65). Sequence homology was common between plasmids from the same fungal vegetative incompatibility group.

Judging from these examples, it appears that plasmids might be the rule rather than the exception in fungal populations. (It is worth noting that no plasmids have been found in the well-studied fungal genus *Aspergillus*, although no systematic surveys have been done.) Together, the above studies seem to have sampled reasonably well the overall global populations of *Neurospora* species, so although more homology groups will undoubtedly be found, the number may not be large. It also seems likely that similar sorts of plasmid distributions will be found in other genera when they are surveyed rigorously. The discovery of a kalilo plasmid in a Louisiana population of *Gelasinospora* species (102) suggests that there may be transgeneric distributions of other plasmids too.

PHENOTYPES

For virtually all plasmids, their impact on the host phenotype is still unclear. In most cases in which growth measurements have been made, no obvious effect on the growth rate or growth pattern of the host fungus has been found. However, there have been no rigorous attempts to try to find subtle differences in growth, respiration, or fecundity. If there really is no negative effect, it means that the host mycelium is capable of carrying a genetic burden of plasmids without detriment to itself. This is surprising since plasmids often can rise to a high copy number far in excess of that of mtDNA. An alternative view is that plasmids might confer some selective advantage on the host, perhaps in some aspect of mitochondrial metabolism or division. However, once again, no measurable effect of this type has been observed yet.

Many of the plasmid examples listed above are from parasitic fungi. In four of these cases, the hypothesis has been entertained that plasmids are responsible for the specific pathogenic properties of plasmid-containing strains in comparison with strains carrying no plasmid or a different plasmid. However, the hypothesis has been invalidated in three cases: *Fusarium oxysporum* (45, 68), *Rhizoctonia solani* (47, 66), and *Claviceps purpurea* (27). For example, in *C. purpurea*, plasmids were transferred from strain to strain by protoplast fusion without transferring pathogenic properties. The pathogenicity hypothesis has not been tested in the case of *Leptosphaeria maculans* (59).

The first fungal phenotype shown to be produced by an extragenomic plasmid is senescence in *Neurospora* species (reviewed by Griffiths [28]). The two linear plasmids kalilo and maranhar aggressively insert into mtDNA, acting as insertional mutagens leading to abnormal mitochondrial physiology and ultimately to death of the culture (32). Also, the circular Mauriceville plasmid of *Neurospora* species occasionally recombines with mtDNA in a variety of ways, leading to senescence (2). The mechanisms of plasmid integration and other interactions with mtDNA during senescence are discussed below. Note that the rare cases of senescence in *Neurospora* species and other fungi are not always associated with extragenomic plasmids (28).

In the zygomycete *Absidia glauca*, a small protein found only on the cell surface of mating-type-positive strains was shown to be encoded by a 1.25-kb circular plasmid (38). Neither the plasmid nor the protein is found in mating-type-negative cells.

All wild-type strains of *Podospora anserina* senesce and die through a well-studied but still poorly understood process involving the excision and amplification of one of several mtDNA regions (summarized by Griffiths [28]). The linear plasmid pAL2-1 is found only in a long-lived strain, AL2, of this normally rapidly senescing fungus. In crosses with AL2 as the maternal parent, some progeny show no plasmid, and these all experience rapid death. It was concluded that somehow the plasmid is the cause of the increased life span (40). Hence, this life-prolonging effect can be considered to be another example of phenotypic expression of a fungal plasmid.

STRUCTURE

Most commonly, the natural plasmids encountered in fungi are of the linear type. Although various kinds of circular aberrations of the mtDNA have been encountered in several different fungi, for example in *Cochliobolus* (25) and *Claviceps* (86) species, the natural circular type seems to have been found almost exclusively in *Neurospora* (30). An exception is the mating-type protein-coding plasmid in *Absidia glauca* (38).

Linear plasmids usually announce their presence after one-dimensional electrophoresis of DNA from mitochondrial preparations (see, for example, reference 10). However, pulsed-field gel electrophoresis has also been used to separate linear plasmids (59) and their derivatives (41). Success in visualizing a plasmid as a discrete band on an electrophoretic gel is dependent on prior treatment of the DNA sample with proteinase K, which removes the terminal protein from the plasmids (see below), allowing the DNA to move into the gel. Bands are sometimes seen in samples that have not been deproteinized, but they are generally faint. Another useful diagnostic tool for a linear plasmid is differential sensitivity to 5′ and 3′ exonucleases. Most linear plasmids have a protein covalently bound to the 5′ nucleotide at each end (see below), and this protects against digestion, whereas 3′ digestion still takes place.

The detection of circular plasmids is more complicated. Undigested circular plasmids do not show a discrete band corresponding to the intact circle but a complex ladder of forms that are assumed to represent different relaxation states of head-to-tail concatamers of the basic circle (reference 98 contains good examples). This ladder is generally not visible on an ethidium bromide-stained gel but is well visualized by a suitable probe. In the absence of a probe, the presence of a circular plasmid is sometimes revealed by the appearance of extra bands in a restriction enzyme-cut preparation of DNA from the mitochondrial compartment. These new bands are often brighter than the mtDNA bands, reflecting a higher copy number of the plasmid (6, 17). The extra bands do not hybridize to a probe composed of labelled mtDNA from a standard plasmid-free strain.

The well-studied natural plasmids of *Neurospora* species have provided protypes for fungi. The best characterized of the *Neurospora* plasmids are represented in Fig. 1, which is based on complete DNA sequences for all these plasmids (1, 14, 18, 58, 71, 76, 82). The overall structure of linear plasmids kalilo and maranhar are typical of most other linear plasmids discovered to date. The general type of structure has been termed an invertron (79). The characteristics are as follows. First, there is a terminal inverted repeat, whose size is characteristic of the individual plasmid. Second, at the terminus at each end there is a protein bound to the 5′ nucleotide. The presence of this protein was suggested by resistance to 5′ exonuclease digestion, but the protein can also be visualized in electron micrographs (93). Third, starting within the terminal repeats, there are two large nonoverlapping open reading frames (ORFs) running on opposite strands toward the middle of the plasmid. These reading frames are open only if mitochondrial codon usage is assumed. Smaller ORFs are present in other frames, but these are probably insignificant. Most fungal linear plasmids also show two comparable ORFs, but some show just one major ORF. Some variations on this basic ORF arrangement in other fungi are diagrammed in reference 49. The presumptive amino acid sequences of the ORFs suggest in one case a virus-type DNA polymerase and in the other case an RNA polymerase similar to those of bacteriophages and yeast mitochondria (14, 18). These ORFs are both transcribed (18a, 91), as discussed in the section on transcription below.

The origin of the 5′ proteins is not known, but there has been speculation that they are encoded by part of the DNA polymerase sequence. The presumptive DNA polymerases of the *Neurospora* linear senescence plasmids and of the *Podospora* plasmid pAL2-1 all show the spaced amino acid sequence S-Y-K-N. This sequence, with the same spacing, is also found in the terminal proteins that some viruses use as replication primers (14, 42). Even though in viruses this protein is part of a distinct gene, the possibility remains that a domain of the

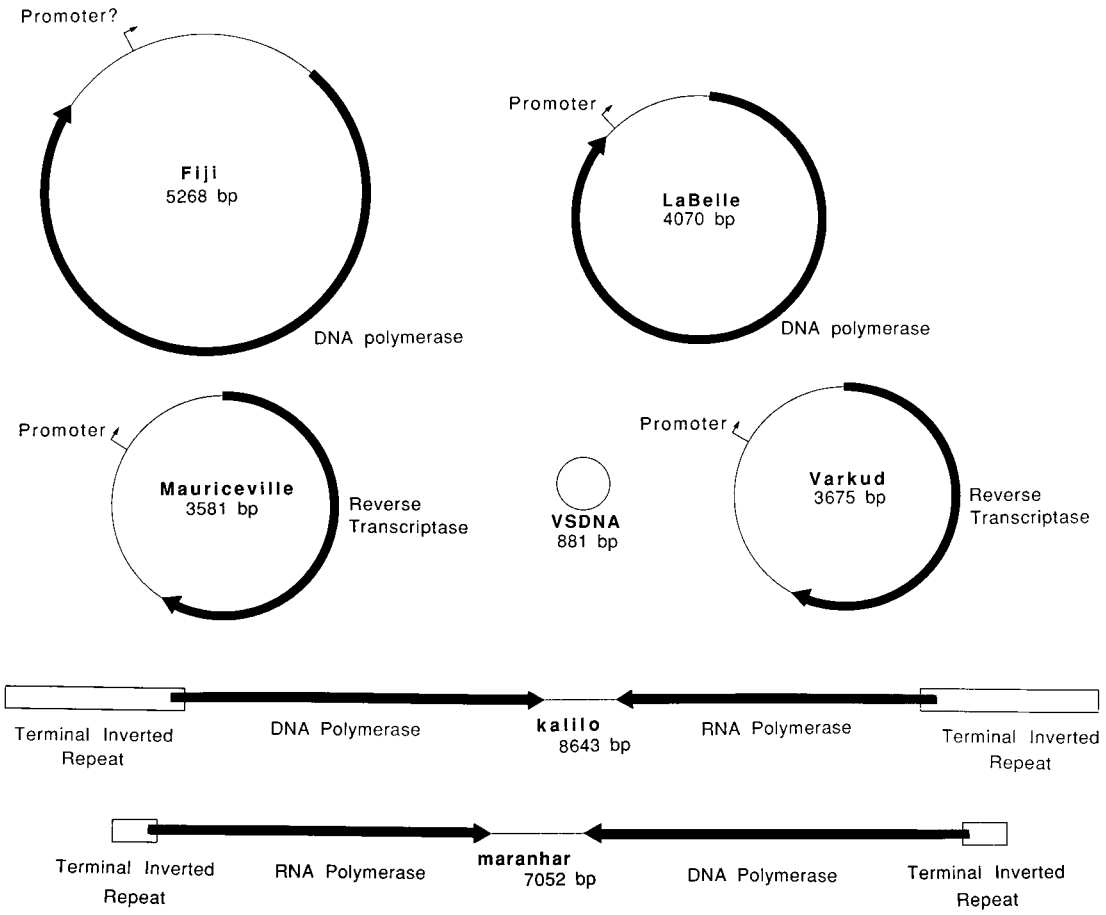

FIG. 1. Sizes and main topographic features of the seven sequenced *Neurospora* plasmids. ORFs are shown as heavy lines, with the transcriptional direction indicated by arrows. Reprinted from reference 30 with permission of the publisher.

plasmid polymerases serves this function. Hermanns and Osiewacz (42) further suggest from amino acid sequence analysis that the polymerase of pAL2-1 could code for three separate domains, the terminal protein, a 3′ → 5′ exonuclease, and a 5′ → 3′ DNA polymerase.

Fourth, there is an "intergenic region" between the ORFs. No function has been proposed for this region, although it presumably contains transcription termination signals. The intergenic region in the *Gelasinospora* kalilo plasmid is larger than the one in the *N. intermedia* kalilo plasmid and has been rearranged (102).

An interesting but atypical situation is the pair of linear plasmids pLm9 and pLm10 detected in pathologically aggressive isolates of *Leptosphaeria maculans* (58). Plasmid pLm9 hybridizes to probes from the RNA polymerase ORF of the *Podospora* linear plasmid pAL2-1, and pLm10 hybridizes to a probe from the DNA polymerase ORF of the same plasmid. The situation is reminiscent of that found in the S1 and S2 plasmids of maize (60).

Three linear plasmids of *Rhizoctonia solani* are atypical in several ways. First, their ends appear to be closed hairpin loop structures (47, 66). Such plasmids show two dimeric forms that are probably head-to-head and head-to-tail fusions produced during replication. Second, partial sequencing has revealed no ORF larger than 91 amino acids (47).

The circular *Neurospora* plasmids all generate full-length RNA transcripts, most abundantly in the cases of the Mauriceville and Varkud elements. In the Mauriceville plasmid, transcription starts 260 bp upstream of the normally detected end of the transcript. Apparently, the RNA polymerase circles the plasmid for more than one complete revolution and then this longer product is cleaved at the normally found end to give the unit-length molecule (53). The Fiji and LaBelle plasmids each contain a gene that codes for a DNA-dependent DNA polymerase (58, 76, 84). Even though the Fiji and LaBelle plasmids do not hybridize at the DNA level, they seem to be related elements, because their polymerases show 50% similarity. Analysis of the Mauriceville gene product has led to interesting speculation on the relationship between plasmids, introns, and viruses, as discussed below.

The VS plasmid, found only in strains containing the Varkud plasmid, has no ORFs or protein-coding functions of its own and presumably relies on functions provided by the Varkud plasmid. However, VS transcripts are found in vivo, and studies performed in vitro have revealed an interesting molecular process described in the section on functions, below.

INHERITANCE

Mitochondrial plasmids generally are transmitted in the same manner as mitochondria and mtDNA. In sexual crosses, the plasmids of a maternal parent are transmitted to most or all of the progeny (see, for example, reference 29). One exception is plasmid pAL2-1, found in the long-lived *Podospora*

anserina strain AL2. When AL2 is used as the maternal parent in a cross, some of the progeny are plasmid free (40).

With few exceptions, the plasmids of a paternal parent are not transmitted to progeny. Two determined attempts have been made to find possible cases of paternally transmitted plasmids. Using natural isolates of *Neurospora crassa* from Louisiana, May and Taylor (63) screened 130 progeny from matings of a circular Fiji plasmid-containing paternal strain and a plasmid-free maternal strain. A total of eight plasmid-containing progeny were found. The mtDNA of these eight strains was of the maternal parent type. Therefore, it appeared that plasmids from the male had somehow entered the ascogenous tissue without bringing with them the paternal mtDNA. Appropriate controls ensured that the observed paternal transmission was not an artifact. The finding is significant from the point of view of plasmid dissemination. Strict maternal transmission ensures that plasmids (and mitochondria) will be trapped in a vertical lineage (44); however, some paternal leakage provides an avenue for dispersal into other cytoplasmic lineages.

The second study of paternal leakage used the linear plasmid kalilo and the circular plasmid Hanalei-2 of *Neurospora* species (99). In this case, paternal transmission was demonstrated for both plasmids, with Hanalei-2 being transmitted more often. The transmission frequencies were generally comparable to those of May and Taylor (63), but there was variation between crosses, and this was thought to reflect genetic background. In some cases, the paternal mtDNA was also transmitted along with the paternal plasmids, suggesting that at least some cases of paternal plasmid transmission might be due to the plasmids being carried inside an invading mitochondrion. There was even evidence of recombination between the two parental mtDNAs in some progeny.

The obvious avenue of paternal transmission of paternal cytoplasmic elements is through the trichogyne (sexual hypha), the avenue followed by the paternal nucleus. However, this is not necessarily the case; another avenue might be through fusion of conidia from the strain used as the paternal parent with mycelium from the maternal parent followed by entry into the perithecium via a cytoplasmic "back door." Preliminary trials (21) suggest that the back-door method is used. The back-door route presumably is nothing more than a type of heterokaryosis.

It is possible that normal maternal inheritance represents a process of active elimination of paternal cytoplasm as a device to prevent explosive spread of parasitic elements such as plasmids. Hosts have additional ways of combating plasmids, and one of these, plasmid suppression, affects transmission efficiency from generation to generation. Griffiths et al. (35) demonstrated that some laboratory *Neurospora* strains carry suppressor alleles that eliminate the kalilo plasmid. In crosses heterozygous for such an allele, half of the progeny will not inherit a maternal plasmid. The study was extended to natural isolates (100). This showed that in natural strains, there is considerable variation in suppressive ability. However, there are at least two types of suppression. The first type is similar to that found in the laboratory strains, eliminating plasmids from half of the progeny. One case of this type of suppressor was found; it was highly specific and eliminated the Varkud circular plasmid but had no effect on two other circular plasmids, Hanalei-2 and Harbin-2, present in the same maternal strain. The other type of suppression seems to work through a complex pattern of gene interaction; when both parents contribute the appropriate interacting genotypes to the ascogenous tissue, plasmids are eliminated from all the progeny. This suppressive action is also plasmid specific; in a cross involving a maternal parent with several different plasmids, some of these plasmids can be eliminated whereas others are found in all the progeny.

The studies on global distribution of *Neurospora* plasmids (6, 98) showed that some plasmids are widely dispersed both within and between species. Furthermore, Arganoza et al. (6) showed that plasmid distribution is apparently random. In a study of Hawaiian strains of *N. intermedia*, Debets et al. (21) showed that kalilo and Hanalei-2 plasmids are also independently distributed in that population. These observations suggest that plasmids are freely mobile in natural fungal populations. Mobility of plasmids between different vertical lineages within a species might be achieved through paternal transmission in crosses (see above), but another possibility is horizontal transmission between somatic mycelia. Laboratory experiments have shown that horizontal transmission can occur within a species and between species. Griffiths et al. (31) showed that kalilo and maranhar plasmids are effectively transmitted from strain to strain of *N. crassa* by heterokaryosis. Furthermore, kalilo plasmids of a *N. intermedia* strain could invade a plasmid-free strain of *N. crassa* if the two strains are mixed in what must be a partial or transient heterokaryon. The kalilo plasmids take up residence in association with *N. crassa* mtDNA, so either the plasmids can cross cytosolic space or there is temporary mitochondrial fusion followed by segregation.

In a related experiment, Collins and Saville (16) demonstrated that circular plasmids of the Varkud group could be transferred to a heterokaryotically incompatible strain at high frequency. The donor plasmids became associated with the mtDNA of the "recipient" strain. In these experiments, the small VS plasmid was also present in the donor. In most cases, both were transmitted together, but in one case, the VS plasmid was not found. These results suggested that the plasmids could be transmitted independently of each other and of the mtDNA.

Similar experiments have been performed with other fungi. In *Claviceps purpurea* (27), natural linear plasmids have been transferred from one strain to another by protoplast fusion. Furthermore, in a dramatic demonstration of horizontal transmission, Kempken (49) has shown that a linear plasmid from *Ascobolus immersus* can been transferred to *Podospora anserina* by cytoplasmic contact. However, in its new host, the plasmid is unstable and gradually disappears.

In the plasmid transfer experiments described above, various special markers were used to promote and detect fusion. In an experiment designed to simulate better the situation in a natural population, Debets et al. (20) examined transfer of the kalilo and Hanalei-2 plasmids between wild-type prototrophic *Neurospora* strains. They found that in compatible pairings of strains, the plasmids are transferred aggressively, rapidly spreading through the "population." In incompatible pairings, plasmid transfer was arrested in all tests. In pairings heterozygous for heterokaryon incompatibility gene *hetD* or *hetE*, after a lengthy delay the plasmids eventually found their way into the recipient strain, but heterozygosity for *hetC* prevented all transmission within the time frame of the experiment. Caten (13) proposed that a possible reason for the unexpectedly high level of *het* gene heterozygosity in natural fungal populations is to check the spread of infectious elements such as viruses or virulent plasmids. *het* genes do indeed seem to check the spread, but they do not necessarily prevent it.

There is only one case of intergeneric distribution of plasmids in fungi, and that is the kalilo plasmid, which is found in *Neurospora* and *Gelasinospora* species (102). Sequence information is available for the originally discovered *N. intermedia* form (14), an *N. tetrasperma* form (62), and the *Gelasinospora*

form. The two *Neurospora* sequences are almost identical, but the *Gelasinospora* sequence shows many length and point mutations. Taken at face value, this suggests that if the plasmids transferred horizontally between genera, this could not have occurred recently because of the considerable divergence that is observed at the DNA level. However, the data do not rule out the hypothesis that the plasmids were all derived from a common ancestor, with subsequent vertical descent.

In routine propagation of plasmid-containing strains, subcultures generally all show the plasmid in more or less constant amounts. (Senescence plasmids are an exception to this rule.) In the few cases in which asexual cells have been examined by isolating single conidial isolates, the majority of these isolates shows the plasmid, but occasionally a plasmid-free isolate will be found that might represent some kind of suppressor mutation or an accidental loss. For example, in culturing kalilo *Neurospora* strains, occasional nonsenescent conidia are produced (29). Also, occasional ascospores lack plasmids, but tests on these have shown no evidence of suppressors (101), so some type of cytoplasmic segregation is inferred. Overall, these types of observations, which seem to be common to most plasmid systems, show that fungal plasmids are in a relatively stable numerical relationship with mitochondria and are effectively transferred from one cell and mitochondrial generation to the next. If plasmid replication were out of control, it seems likely that there would be more cases of cell death from excessive plasmid burdens. Also, if partitioning of plasmids between daughter mitochondria and daughter cells were random, more plasmid-free cells might be expected (11). However, no studies on regulation of plasmid copy number have been attempted.

FUNCTION

Transcription

In the linear senescence plasmids of *Neurospora* species, transcripts have been identified that correspond to the major ORFs. Court and Bertrand (18a) mapped the 5′ start site of both ORFs of maranhar very close to the ends of the plasmid within the repeats, at around position 50. Upstream of the start site, a 23-bp "promoter" region showed 10 matches to the comparable region of the *Claviceps* plasmid pClK1 (26). The kalilo ORFs also have a common start site close to the end of the plasmid, at around position 101 (91). However, the kalilo presumptive promoter region did not resemble that of maranhar. The 3′ ends of both kalilo and maranhar map to the intergenic region. Court and Bertrand (18a) detected two proteins in maranhar strains which were absent in non-maranhar strains and could represent the translational products of the ORFs. The arrangement whereby transcripts and ORFs start in the terminal inverted repeat has also been found in several linear plasmids in other fungi (for example, in *Claviceps* [26] and *Podospora* [43] species), and it is a curious one, for which there is no adequate explanation. The proteins encoded by the two ORFs must share a short polypeptide sequence, but it would seem to be an easy evolutionary step to move the ORFs to wholly within the central unique region. The complete identity between the terminal repeats means that there must be some active mechanism for matching them. In adenoviruses, which also have linear genomes, this correction is achieved in the "racquet handle" formed by single-stranded replication intermediates. It is possible that the formation of such a racquet handle in linear plasmids, regularly engulfs the beginning of the ORFs, which then must become part of the repeat after correction. It is interesting that copies of the *Podospora* plasmid pAL2-1 integrated into mtDNA can undergo subsequent mutation, and when this is in the inverted repeat, the mutation becomes copied into both repeats (40). Therefore, the matching process must be possible in the integrated state too, perhaps also by racquet handle formation.

Although translational products have not been definitely identified in the *Neurospora* linear plasmids, both transcriptional and translational products have been identified for the RNA and DNA polymerase ORFs of *Claviceps purpurea* linear plasmid pClK1 (22, 26). Also, Hongo et al. (46) found transcripts of the *Rhizoctonia solani* plasmid pRS64 and used immunological methods to detect a protein product.

In cultures bearing circular plasmids, full-length plasmid transcripts are found, but these are cut from larger transcripts. Since these plasmids have no coding region for an RNA polymerase, the mtRNA polymerase must be used. The Mauriceville and Varkud plasmids replicate by reverse transcription, so transcription and replication are intimately linked and will be discussed in the section on replication below.

Like other circular plasmids, VS exists as multimeric concatamers. From these, multimeric transcripts are produced. In vitro, monomers form by self-splicing, and these then circularize, all in the absence of any protein (83). These functions are characteristic of ribozymes, but the VS sequence shows no similarity to other ribozymes and so must have evolved independently.

Replication

Linear plasmids. Little is known about the replication of linear fungal plasmids. Because of the omnipresent 5′-terminal protein, it is assumed that plasmid replication is protein primed, as it is in adenovirus and phage ϕ29, which also have a 5′ protein and a terminal inverted repeat (79, 80). A precursor of the protein attaches noncovalently to the end of the virus and provides an $-$OH group to begin polymerization with the $3' \rightarrow 5'$ strand as the template. This generates one complete new double-stranded virus and one displaced single strand. The single strand circularizes by pairing of the terminal inverted repeats, and the double-stranded DNA formed by the inverted repeat acts as the start site for another round of replication. Evidence for plasmid replication in this manner was obtained by Kempken et al. (51) with the pAI2 plasmid of *Ascobolus immersus*. It was found that radioactive label was incorporated preferentially at the ends of the plasmid and into the appropriate strand. Indirect evidence in support of the model has been obtained from studies on deletion derivatives of the kalilo plasmid (see the section on mutation, below).

Linear plasmids of *Rhizoctonia solani* are exceptional in that they are resistant to 3′ exonuclease and 5′ exonuclease (47, 66). One explanation is that they have closed hairpin loops at both termini, with no exposed 5′ or 3′ ends anywhere in the molecule (66). This structure is also found in certain viruses. The model proposed for replication is based on a viral mechanism involving nicking the DNA close to the base of the loops at each end (66). Variant types of nicking can account for the duplex structures observed in these cultures.

The replication of the circular plasmids of *Neurospora* species has been the subject of intensive research. A mechanism that suggests itself is the rolling circle, used in the replication of circular elements in prokaryotes. Indeed, Maleszka (61) reported that for LaBelle and Mauriceville, over 90% of plasmid DNA is in a concatenated linear form with single-stranded regions, suggesting rolling-circle intermediates. However, the rolling-circle mechanism, which uses DNA polymerase, seems incompatible with the fact that the Mauriceville ORF codes for a reverse transcriptase. Indeed, a great deal of research on the

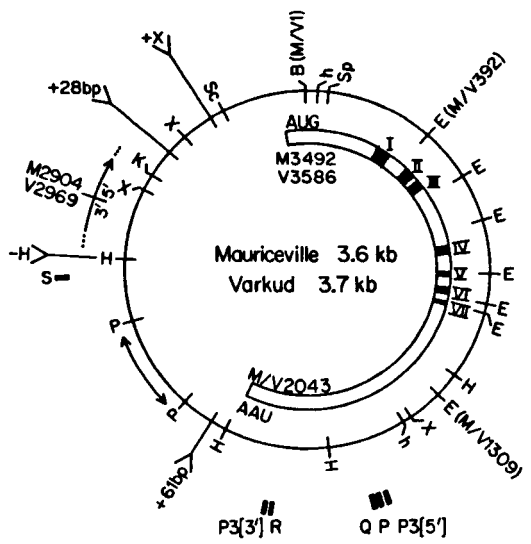

FIG. 2. Map of the *Neurospora* Mauriceville and Varkud plasmids, showing ORF, restriction sites, and transcript ends. The roman numerals show the sizes and positions of seven blocks of homology to reverse transcriptase. The antennae show positions and sizes of the differences between these plasmids. Bars S, P, Q, and R in the outer arc show positions of elements characteristic of group I introns. P-P is the region containing Pst-I palindromes. Reprinted from reference 4 with permission of the publisher.

Mauriceville plasmid points to an interesting type of reverse transcriptase-based replication, so it is worth considering this in some detail.

Mauriceville. The Mauriceville plasmid was first described by Collins et al. (17), who established the monomer length and demonstrated the presence of circular concatamers of up to six repeats. They also showed that the plasmid produced an approximately full-length transcript. Nargang et al. (71) sequenced the plasmid and found structural features reminiscent of introns. First, the ORF showed a codon usage similar to that of fungal mtDNA introns. Second, there were DNA sequences characteristic of the conserved elements E, P, Q, R, E′, and S, which interact to promote splicing in group I mtDNA introns. These were all in correct position and alignment. This suggested that Mauriceville is an intron progenitor, that it is an excised intron that can replicate independently, or that it has recombined with part of an intron. The intron connection grew more curious when Michel and Lang (64b) showed that the Mauriceville ORF contained seven conserved blocks of amino acids that resembled those of reverse transcriptases (Fig. 2), a property that is shared with four different group II introns. These observations, together with the existence of a full-length transcript, gave rise to the idea that the plasmid might be a mobile intron, capable of insertion by reverse transcription, a property also shared by retrotransposons (57).

To test this idea, Akins et al. (2, 3) reasoned that if the plasmid could act like a transposon and occasionally integrate into mtDNA, this rare event might be detectable, because it had previously been shown (10, 19, 29) that integration of the kalilo and maranhar plasmids into mtDNA results in senescence and death of the host strain. After repeated subculturing, such senescent strains were indeed found, and they proved to contain a variety of interesting molecules. The plasmids in these strains were found to have acquired mtDNA additions, most commonly a mitochondrial tRNA at or near their 5′ ends, suggesting that the hybrid had formed via RNA intermediates (Fig. 3). Most senescent strains also contained defective mtDNAs, some showing deletions and some showing insertions of a portion of the plasmid DNA into the mtDNA. Three independently arisen inserts were studied, and in all three the mtDNA-plasmid junction corresponded exactly to the 5′ end of the plasmid transcript. The 3′ junction was not characterized. These findings suggest insertion through an RNA intermediate. (It was noted that the splice junctions were not those expected for either group I or group II introns, so the plasmid inserts were not acting as introns.) Akins et al. (1) found an additional transcript of the Varkud plasmid that had at its 5′ end a 1.2-kb fragment derived from the 5′ end of the mitochondrial small rRNA, also pointing to a joining ability of reverse transcriptase.

Reverse transcriptase activity was detected in ribonucleoprotein preparations in nonsenescent Mauriceville strains. The product was a full-length minus-strand cDNA (55). No tRNA primer was detected as is used in other reverse transcriptases, but it was noted that the 3′ end of the RNA transcript has a potential tRNA-like cloverleaf secondary structure. This raised the possibility that the plasmid reverse transcriptase is an evolutionary forerunner of other reverse transcriptases which sequester tRNAs for the same purpose. The actual reverse transcriptase protein was identified by Kuiper et al. (56). Wang et al. (94) purified the enzyme away from the ribonucleoprotein and showed that in vitro the cDNA strand synthesis uses short noncomplementary DNA primers, beginning synthesis exactly opposite the 3′ end of the RNA template. The primers remain attached. A mitochondrial RNase is used to remove the RNA template (95), in contrast to other reverse transcriptases, which carry their own RNase activity.

In the absence of any DNA primer, the reverse transcriptase can still operate, but it initiates from the penultimate nucleotide (53, 96). This process requires some way of regenerating the terminal nucleotide. Such unprimed polymerization requires only the recognition of the 3′ tRNA-like structure. The addition of a real tRNA molecule to the cDNA strand in some growth mutants might result from the unprimed reverse transcriptase "mistaking" the tRNA for the tRNA-like end of the transcript and then jumping to the transcript to form a double

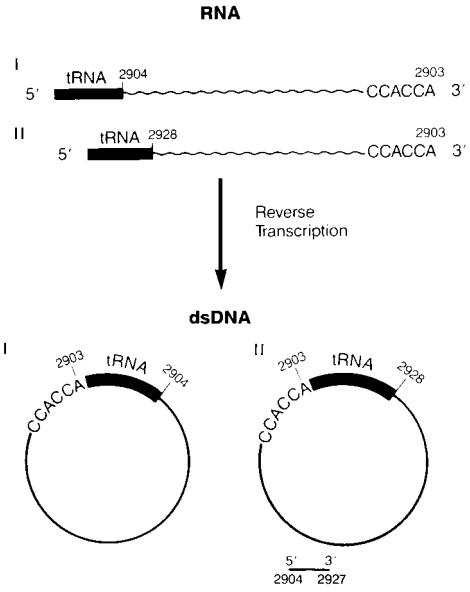

FIG. 3. Model for how the Varkud plasmid acquires mitochondrial tRNA sequence. Reprinted from reference 2 with permission of the publisher.

cloverleaf on the end of the DNA (53). However, other types of primers can be used in vivo: Kennel et al. (53) found that the 3′ end of another negative-strand cDNA molecule can prime DNA synthesis at the cloverleaf.

On the basis of these and other observations, Wang and Lambowitz (96) suggested that evolution of DNA polymerases began in the RNA world with RNA replicases using a 3′ recognition cloverleaf (much like modern bacterial and plant RNA viruses). The crucial step of transition to a DNA world was the evolution of a reverse transcriptase, such as the Mauriceville type, which also only needs the cloverleaf as a recognition signal. From this would spring modern reverse transcriptases, which use an additional tRNA as the primer, and eventually fully primed DNA polymerases would arise.

The 861-bp VS plasmid, which is always found associated with the larger Varkud plasmid, also replicates via reverse transcriptase (52). The VS DNA is transcribed by using the mitochondrial RNA polymerase, which is then reverse transcribed to a negative DNA strand with the Varkud reverse transcriptase enzyme. This is a unique mode of replication for a satellite molecule and seems to be possible because the reverse transcriptase does not require a specific primer to initiate DNA synthesis.

LaBelle. The LaBelle plasmid was first described by Stohl et al. (87). Subsequently, it was sequenced by Pande et al. (76), who found an ORF of 1,151 amino acids. The ORF contains six blocks of similarity to the yeast nucleus-encoded RNA polymerase plus seven blocks of homology to reverse transcriptase. No reverse transcriptase activity has been reported. Moreover, Schulte and Lambowitz (84) found a DNA polymerase activity associated with this plasmid that is not present in plasmid-free strains. This DNA polymerase showed biochemical properties (such as NaCl and Mg^{2+} optima) different from those of the mitochondrial DNA polymerase. Because of the regions of homology to reverse transcriptase, it was argued that this enzyme might represent a "missing link" in the evolution of DNA polymerase from reverse transcriptase.

However, the situation became complicated by a finding resulting from the sequencing of another *Neurospora* circular plasmid, the Fiji plasmid (58). The 1,278-amino-acid ORF of the Fiji plasmid showed 49% similarity to the ORF of LaBelle, so the plasmids seem to be related, even though they do not cross-hybridize at the DNA level. Both plasmid ORFs were shown to contain three amino acid motifs characteristic of DNA polymerases. In the third motif, the Fiji and LaBelle ORFs both showed the amino acid sequence Thr-Thr-Asp whereas other DNA polymerases in the comparison all showed Asp-Thr-Asp, which is presumed to be an important functional unit in the protein. The common possession of such a deviant sequence strongly suggests that Fiji and LaBelle are related. The Fiji plasmid does not contain the strong blocks of reverse transcriptase homology shown by the LaBelle plasmid, so Li and Nargang (58) argued that the reverse transcriptase blocks in LaBelle are probably not significant. Nevertheless the missing-link idea is so appealing that it seems premature to discard it at present. There is no reason why descendents of the evolutionary missing link should have all inherited reverse transcriptase homology. It seems safe to assume, however, that LaBelle and Fiji plasmids replicate by the action of the DNA polymerase demonstrated.

Integration into mtDNA

The prototype for plasmid integration was provided by the senescence plasmids kalilo from *N. intermedia* and maranhar from *N. crassa*. These plasmids were originally found in senescent strains (7, 19, 29). Most wild-type *Neurospora* strains do not senesce, but grow continuously when inoculated in long growth tubes. The senescent phenotype is an aberration. It was shown that the presence of the plasmids is strongly correlated with the ability of a strain to senesce, that the senescent phenotype is transferred from strain to strain along with plasmid transfer (31), and that the onset of senescence from a presenescent (or "juvenile") culture is correlated with the appearance of mtDNA molecules into which plasmids have integrated (summarized by Griffiths [28]). Eventually, at or just before death, virtually all of the mtDNA molecules are of the inserted type but the free plasmids are still present in the culture. The free plasmids were named AR-kalDNA and AR-marDNA, and the inserted forms were named IS-kalDNA and IS-marDNA. The precise ways in which the plasmids kill are not understood in detail, but it is generally assumed that the integration of the plasmid has the effect of an insertional mutagen, disrupting gene function and leading to the abnormalities of mitochondrial physiology which are observed in senescent strains (28). In a senescent culture at any given time, generally one inserted type of molecule predominates, not a random array of insertions at different sites (69). Therefore, the specific IS-kalDNA or IS-marDNA species found in any one culture seems to be a molecular clone, amplified from an original single insert. This behavior of aberrant mtDNA is not unique to the senescence process; many mitochondrial mutations, whether base pair substitutions or rearrangements, will amplify at the expense of the wild-type molecule (8). The property is called suppressivity. One possible explanation for suppressivity of aberrant mtDNA molecules, including those containing IS forms of plasmids, is that the mitochondrion recognizes some aspect of the physiological impediment and this triggers mitochondrial replication, perhaps as an attempt to overcome the impediment (9).

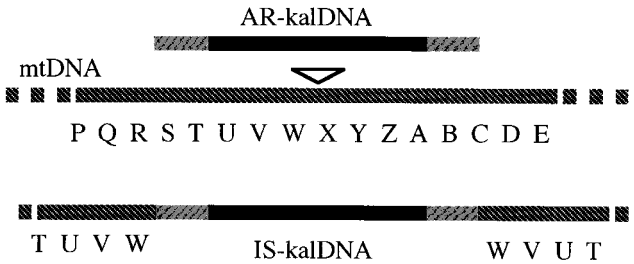

FIG. 4. Insertion of kalilo plasmid into mtDNA. The letters are arbitrary labels for regions of mtDNA. AR, autonomously replicating kalilo plasmid; IS, inserted-sequence kalilo plasmid.

The mode of kalilo and maranhar insertion is quite novel, having never previously been observed in any other system, eukaryotic or prokaryotic. Both IS-kalDNA and IS-marDNA are found flanked by long inverted repeats of the mtDNA, formed from the DNA to one side of the insertion point, as shown in Fig. 4 (14, 19). The reciprocal of this product (containing the other flanking sequence) has not been demonstrated. The insertion mechanism must be different in the two plasmids. AR-kalDNA inserts by matching 5 bp from anywhere within the last 20 bp or so at its terminus with an identical quintet in the mtDNA (14). Presumably, some kind of crossover event then forms a recombinant molecule. This crossover might occur between mtDNA and a single-stranded kalilo replication intermediate, which has assumed the shape of a racquet by pairing of its terminal inverted repeats. Crossing-over leads to an almost full-length kalilo molecule flanked by the

mtDNA to one side of the recombination point (19). Maranhar integrates as a full-length copy, so this crossover mechanism seems unlikely.

The plasmid pAL2-l found in an abnormally long-lived *Podospora* strain also inserts into mtDNA and apparently also generates inverted repeats of mtDNA (41). One difference from the *Neurospora* inserts is that 15-bp AT-rich sequences of unknown origin are added between the plasmid and the mtDNA at each end. Two different inserts (pAL2-1I1 and pAL2-1I2) were found, both inserted into the apocytochrome *b* gene. The "recombinant" molecules are 50 and 70 kb in size, so that they evidently do not include the entire mtDNA molecule, which is 100 kb in size. Another difference is that neither is suppressive, because the frequency of the inserted molecules varies between 10 and 50% but is never higher. The mtDNA of strain AL2 contains a homoplasmic 3.6-kb deletion, but this is at a different site from the plasmid insertions.

Full-length insertion of the *Neurospora* circular plasmid Varkud has also been detected. Some Varkud subcultures contain a 4.9-kb hybrid RNA composed of the 5′ 1.2 kb of the mitochondrial small rRNA plus the full-length 3.7-kb Varkud transcript (1). In such cultures, there are low levels of an inserted mtDNA species that contains the Varkud plasmid flanked by a small rRNA sequence, all at the small rRNA locus (15). A likely mechanism for the origin of this insertion is that the Varkud reverse transcriptase switched templates after completing a full-length Varkud cDNA to an internal region of the small rRNA. The double-stranded form of this hybrid DNA circularized and underwent homologous recombination with the small rRNA gene, resulting in the observed integration. Other examples of this process were also reported.

In addition to the above examples of full-length or almost full-length insertions, there are several cases of partial insertions. Most strains of *Claviceps purpurea* that contain plasmids also show regions of plasmid homology within the mtDNA (74, 90). In three sequenced cases, it was found that it is the right-hand terminal repeat plus some adjacent sequence which is inserted; within the plasmid-homologous inserted region, there are additional nonhomologous regions that complicate the picture (74).

The mtDNAs of all wild-type *Neurospora* species examined contain a 1.6-kb segment that is clearly homologous to the LaBelle plasmid (72, 87). This region contains the end of the LaBelle ORF and the plasmid promoter region. The insert is into an intergenic region of the mtDNA. Nargang et al. (72) argued that it was most likely that the mtDNA had acquired this sequence from the plasmid and not the other way around. The segment concerned is not found in the mtDNA of other fungi examined to date, so it seems to have been specifically acquired by some ancestral *Neurospora* strain. Furthermore, the region contains DNA for what is presumed to be part of a functional DNA polymerase, and it seems unlikely that a nonfunctional segment could have been incorporated into the plasmid polymerase. Another observation that is relevant to this point is that the ORF section of the LaBelle insert contains several insertions that interrupt the reading frame, and it is not clear how these could have been lost upon incorporation into the plasmid. If the model is correct, the insertion event must have occurred in an ancestral form, and then the plasmid must have been secondarily lost in most *Neurospora* species to account for the disjunct distribution presently observed for LaBelle and its homolog Harbin-1 (98).

A situation similar to that of the LaBelle insert has been found in *Agaricus* species (77). A section of the RNA polymerase ORF of the linear plasmid pEM, originally found in *Agaricus bitorquis* Ag4, was found to be incorporated into the mtDNA of that strain. The insert is also found in strains of *A. bitorquis* that do not have the pEM plasmid and in a different species, *A. bisporus*, which also bears no pEM plasmid. The insert in *A. bisporus* has been sequenced, and it shows 80% identity to the native plasmid. The differences are caused by substitutions and by small deletions and additions. As in the case of the LaBelle insert, it seems as though the insertion was an ancient one in an ancestor common to both *A. bitorquis* and *A. bisporus.*

A third example of ancestral insertion into mtDNA has been found in *Podospora anserina* (43). In the mtDNA of the wild-type strain A, which contains no extragenomic plasmids, three unassigned ORFs are clearly derived from plasmid pAL2-1 or a related plasmid. As in the *Neurospora* and *Agaricus* examples, the plasmid must have been subsequently lost. It would be interesting to examine more wild-type *Podospora* strains for the presence of these molecular fossils.

MUTATIONS AND REARRANGEMENTS

The above discussion on plasmid inserts into mtDNA showed that the insert can diverge from the free plasmid by base pair substitutions and short mutations. Different forms of free plasmids also show divergence based on mutations. Comparisons of the circular Fiji plasmid found in *Neurospora crassa*, *N. tetrasperma*, and *N. intermedia* showed a general similarity of plasmid size and of restriction map, but there were several differences in individual restriction sites (89). Such differences are most probably caused by point mutations or mutations of very short length. Again, the complete DNA sequence of the circular plasmids LaBelle (58) and Harbin-1 (33) showed clear homology and overall similarity in size but many sequence differences caused by point mutations. The closely related Mauriceville and Varkud plasmids also show a combination of substitutions and variously sized length mutations (Fig. 2).

Because there are few examples of homologous linear plasmids, only limited sequence comparisons can been made on this type of plasmid. One comparison involves the kalilo plasmids of *N. intermedia* (14), *N. tetrasperma* (39, 62), and *Gelasinospora* species (102). These studies have shown that the *N. tetrasperma* form (LA-kalDNA) is almost identical to the *N. intermedia* form (kalDNA), differing only by a 60-bp deletion and a 13-bp insertion of unknown origin at the same position in the center of the inverted repeats. This modification might account for the lack of insertion of the LA-kalDNA plasmid into mtDNA in *N. tetrasperma*. The *Gelasinospora* plasmid (Gel-kalDNA) is clearly homologous to the other two. Its ORFs are virtually the same as those in the *Neurospora* plasmids; although there are small mutations, the continuity of the reading frames is preserved. However, the intergenic region and the terminal repeats show numerous small and large mutations, which might prevent its insertion.

Changes in plasmid sequence have been observed in culture. Vierula and Bertrand (92) observed two related deletion derivatives of the 8.6-kb plasmid in kalilo cultures treated with chloramphenicol. These deletions had lost most of the material between the inverted repeats. The 2.8-kb deleted form was found to contain two terminal inverted repeats consisting of the usual kalilo terminal inverted repeats, each extended by a 30-bp adjacent region of the RNA polymerase ORF, and centered on a 32-bp adjacent region flanked by a pair of repeats of the sequence GAAAC. (The kalilo DNA normally contains the 32-bp sequence flanked by the 5-bp repeat in this region.) This structure suggested several mechanisms of origin. The most satisfactory mechanism was that two plasmids aligned with a

head-to-head partial overlap and that a crossover event occurred at one of the GAAAC regions. The other deletion derivative was a 1.4-kb hairpin structure that seemed to be a foldback version of a single strand of the 2.8-kb form. This is an interesting finding, because it is likely that this 1.4-kb form represents the displaced single-stranded DNA produced by protein-primed replication of the 2.8-kb form (see the section on replication, above). Therefore, this finding constitutes indirect evidence for protein-primed replication of normal kalilo plasmids.

Among the senescent derivatives of Mauriceville and Varkud strains, two deleted plasmid forms were found (4). Both deletions were in an area that contains a 160-bp direct repeat and five Pst-1 palindromes. Such Pst-1 palindromes are common in mtDNA and are presumed to be mobile elements; indeed, that is how they may have been acquired by these circular plasmids. The Varkud deletion was 0.35 kb. Its breakpoints were near the bases of long hairpin-like secondary structures built around Pst-1 palindromes. The 0.5-kb Mauriceville deletion also showed breakpoints in possible hairpin structures. The deletions may have arisen by aberrant recombination based on the hairpins or perhaps by the reverse transcriptase skipping over the deleted region.

A variety of spontaneous structural changes has been observed in the kalilo plasmid, all of unknown origin (98). These are similar-sized "sibling" plasmids, giant circular and linear concatamers, and a complex series of internal deletions. These all arise de novo in somatic cultures and so presumably represent some of the ways in which the propagation cycle of the DNA of this plasmid can go awry.

Although many natural *Neurospora* isolates bear several different types of plasmids, there is only one case of genetic interaction between heterologous types giving rise to recombinant molecules (34). This case was observed in a strain of *N. intermedia* from Harbin, China. This strain contained three linear plasmids (the zhisi plasmids) and two circular plasmids, Harbin-1 and Harbin-2. During subculture, a strain spontaneously arose that had lost the zhisi plasmids and Harbin-1 but had gained a new linear plasmid, Harbin-L, and a new small circular plasmid, Harbin-0.9. Sequencing analysis (34) revealed that both of the new plasmids probably arose from a double crossover between one of the linear zhisi plasmids and the circular plasmid Harbin-1. The double crossover had the net effect of replacing the presumptive RNA polymerase region of the linear plasmid with a segment of the circular plasmid. The Harbin-0.9 plasmid seemed to be a derivative of the reciprocal product. Both the crossover points were 7-bp regions of identity between the recombining molecules. Such small regions of identity have acted as substrates for crossing over in other mitochondrial systems (5, 36). Heterologous recombination may have been important in the evolution of the currently observed heterogeneous range of fungal plasmids. An illustration of this evolutionary process is the structure of the two largest zhisi plasmids, which contain small inserts that are clearly derived from maranhar (34).

EVOLUTION

Various aspects of the evolution of circular plasmids have already been considered. Perhaps most significant is that these plasmids seem to show intermediate steps in the transition from an RNA world to a DNA world. Like many "living fossils," these relic forms survive only in a specialized environment, in this case the mitochondrion. Despite the romantic nature of this idea, it is still difficult to believe that such forms have persisted since the time of the RNA world, perhaps for more than 2 billion years. Given the demonstrated ability of host genomes to suppress plasmids, it is not clear why all plasmids have not been eliminated during the 2 billion years available for this to be accomplished.

There are clear cases of relatedness between circular plasmids, of which the best examples are the two highly similar pairs Mauriceville-Varkud and LaBelle–Harbin-1. Nevertheless, other less obvious cases of relatedness are emerging from careful analysis of sequence data, such as the LaBelle-Fiji pair.

The number of related circular plasmids sequenced is currently too small to attempt to draw phylogenetic trees based on sequence comparisons. However, Taylor et al. (89) constructed a tree by using restriction site differences of a Fiji group plasmid found in *N. crassa*, *N. tetrasperma*, and *N. intermedia*. They found that the only two completely identical plasmid forms were from *N. crassa* and *N. tetrasperma* and that the other forms differed at one or more sites. This pattern does not match the general phylogenetic pattern of these species, so it was suggested that the plasmids might be transmitted horizontally independent of their mitochondria.

This analysis introduces the general principle that can be used to distinguish horizontal transmission from ancestry based on vertical lineages. If a plasmid tree can be superimposed on the tree of the hosts, based either on genomic or mtDNA sequence or on conventional taxonomy, then it is unnecessary to postulate horizontal transmission. If the trees do not match (in other words, if the pattern of divergence in plasmids does not correspond to the pattern of divergence of genomes), horizontal transmission can be invoked.

It was shown above that the small kalilo plasmid family in *Neurospora* and *Gelasinospora* species conforms to the "superimposition rule," a finding compatible with a vertical lineage of kalilo inheritance.

Full DNA sequences are available for several linear plasmids from fungi, and five of these have been used to make evolutionary trees (50, 64, 78a). These five were compared with each other and also with other linear plasmids and with viruses and phages. Both DNA and RNA polymerase ORFs were used, focusing on the area in each of these ORFs that shows the greatest conservation of amino acid sequence. The sequences fell into five general groups: adenoviruses, prokaryotic linear phages, other viruses and phages, cytoplasmic linear plasmids (from yeasts), and the mitochondrial linear plasmids. Within the mitochondrial group, the maize plasmid S2 was the outlier in both ORF trees. The remaining mitochondrial elements were all from filamentous fungi, and of these, the single basidiomycete plasmid pEM (of *Agaricus* species) was the outlier. This pattern is generally in agreement with the idea that the plasmids have evolved in diverging lineages because there is a general overlap with taxonomic phylogeny. However, there is a curious anomaly connected with the two *Neurospora* senescence plasmids: although these two are closely grouped in the RNA polymerase tree, kalilo lies closest to the *Ascobolus* plasmid and maranhar is closest to the *Morchella* plasmid in the DNA polymerase tree. This inconsistency is difficult to explain, because both ORFs are part of the same element today and the ORFs could not have evolved separately as an attached structure. The observation might point to separate origins of the two ORFs.

The group that emerges closest to the mitochondrial linear plasmids on the DNA polymerase tree is that containing the phages with terminal protein-primed replication (ϕ29, M2, and PRD1). In the RNA polymerase tree, the closest group contains phages T3 and T7 and the nucleus-encoded mtRNA polymerase of yeasts. Several authors (for example, Oeser and Tudzynski [75], Sakaguchi [79], Chan et al. [14], Rohe et al.

[78], and Hermanns and Osiewacz [43]) have used these observations to suggest that fungal plasmids have a viral origin. The endosymbiosis theory of the origin of eukaryotes proposes that mitochondria began as bacteria, which invaded another prokaryotic cell type. If this is true, modern mitochondrial plasmids may have evolved from the phage complement of the original invading bacteria. This attractive idea must be viewed warily. It is possible that at least some of the identity between polymerases is due to convergence. For example, all DNA polymerases must bind to DNA; indeed, the DNA binding region is part of the highly conserved region used for comparison in the phylogenetic trees. There might well be fundamental constraints on the types of amino acid motifs that can do the job of a DNA polymerase. There could also be horizontal transmission superimposed on top of a vertical lineage. Although the existence of a phylogenetic correlation argues that convergence or horizontal transmission cannot be the whole explanation for the patterns of identity, until the phylogenetic correlation is firmly established for a wider selection of plasmids, it is too soon to use it for speculation on such fundamental issues.

The relationship between circular and linear plasmids is still unclear. Griffiths and Yang (34) showed that circular and linear plasmids may recombine, so there is no intrinsic reason why they should not show related sequences. The DNA polymerases of the circular plasmids LaBelle and Fiji share three motifs with each other and with those of the linear plasmids kalilo (*Neurospora* species), pAI2 (*Ascobolus* species), and pClK1 (*Claviceps* species) (58), which could indicate relatedness. More surprisingly, a partial sequence of an ORF of the linear plasmid Et2.OL from *Epichloe typhina* revealed a 24% identity (across 258 amino acids in two blocks) with the reverse transcriptase of the circular Mauriceville and Varkud plasmids (67). As more plasmids are characterized, these relationships should fall into place.

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