Characterization of deletion derivatives of an autonomously replicating *Neurospora* plasmid

Lori L. Stohl, Robert A. Akins and Alan M. Lambowitz*

E.A. Doisy Department of Biochemistry, Saint Louis University School of Medicine, St. Louis, MO 63104, USA

Received 26 March 1984; Revised and Accepted 29 June 1984

ABSTRACT

We previously described two plasmids that replicate autonomously in both *Neurospora* and *E. coli* (Stohl and Lambowitz, Proc. Natl. Acad. Sci., U.S.A. **80**, 1058-1062, 1983). One plasmid, pALS1, consists of the *Neurospora* *ga-2*⁺ gene (3 kb Hind III-fragment), the mitochondrial plasmid from *N. intermedia* strain P405-Labelle, and *E. coli* plasmid pBR325. The other, pALS2, is a putative deletion derivative of pALS1 that lacks most or all of the Labelle insert and that was repeatedly recovered from *Neurospora* transformants. We have now sequenced the region encompassing the deletion in five pALS2 plasmids isolated independently in two different laboratories. All five plasmids are identical in this region and completely lack the Labelle insert. We have also characterized an additional deletion derivative that retains a small (approximately 0.5 kb) segment of the Labelle insert. The results for pALS2 suggest that pBR325 plus the *ga-2*⁺ segment constitute a *Neurospora* replicon.

INTRODUCTION

There has been considerable interest in the development of genetic engineering techniques for filamentous fungi, an important group of organisms in both basic and applied research. We have recently reported that recombinant plasmid, pALS1 (13.1 kb), can replicate autonomously in both *Neurospora* and *E. coli* (1). This plasmid consists of the mitochondrial plasmid from *Neurospora intermedia* strain P405-Labelle, the *Neurospora* *ga-2*⁺ gene (3 kb Hind III-fragment), and *E. coli* plasmid pBR325. When *E. coli* was transformed with DNAs from *Neurospora* transformants, we recovered not only pALS1, but also altered derivatives, the most prevalent being a smaller plasmid, pALS2 (9 kb; ref. 1 and unpublished data). Restriction enzyme mapping and Southern hybridization analysis showed that pALS2 lacks most and possibly all of the Labelle plasmid sequences, as well as the Pst I-site in pBR325 in which Labelle was inserted (1).

In the present work, we have sequenced the region encompassing the deletion in five pALS2 plasmids isolated independently in two different laboratories. We find that all these plasmids completely lack the Labelle insert. We have also characterized an additional plasmid that retains a small (approximately 0.5 kb) segment of the Labelle insert. The results for pALS2 suggest that pBR325 plus the ga-2⁺ segment constitute a Neurospora replicon.

MATERIALS AND METHODS

Recombinant Plasmids

Fig. 1 shows restriction enzyme cleavage maps of pALS1, pALS2, and pALS1-(MC). pALS1 consists of E. coli plasmid pBR325 with the 4.1 kb mitochondrial plasmid from N. intermedia strain P405-Labelle (FGSC #1940) inserted in the Pst I-site, and the N. crassa ga-2⁺ gene (3 kb Hind III-fragment) inserted in the Hind III-site (1). pALS1-1 and pALS1-2 indicate pALS1 recovered after one or two passages through Neurospora transformants (1). pALS2 is a putative deletion derivative of pALS1 that was recovered from Neurospora strains transformed with pALS1 or with passaged derivatives of pALS1 (1). pALS1-(MC) is another deletion derivative recovered from a pALS1-1 transformant by Dr. Mary Case (University of Georgia, Athens, GA). pLSHQ2, a plasmid used in the construction of pALS1 (ref. 1), consists of pBR325 plus the ga-2⁺ gene (3 kb Hind III-fragment) inserted in the Hind III-site. Plasmids were maintained in E. coli strain HB101 and isolated from spectinomycin-amplified cultures by centrifugation in CsCl-ethidium bromide gradients (1,2).

Restriction Enzyme Analysis and Southern Hybridization

Restriction enzyme analysis and Southern hybridizations were as described (3,4). Recombinant plasmids used as probes in hybridization experiments were ³²P-labeled by nick translation (5).

DNA Sequencing

Most of the DNA sequencing was carried out by the dideoxy method using oligonucleotide primers constructed for sequencing inserts in the Pst I-site of pBR322, essentially as described by Wallace et al. (6). The plasmid to be sequenced was digested

with Hind III which linearizes the plasmid, but does not cut the Labelle insert. After digestion, the DNA was phenol-extracted and resuspended in distilled water. Oligonucleotides used as primers were Pst I-clockwise and Pst I-counterclockwise (#'s 1206 and 1207; New England Biolabs, Beverly, MA) or Pst I Primer PII (P-L Biochemicals, Milwaukee, WI). Oligonucleotide primer (approx. 6 to 10 pmoles) and the linearized plasmid DNA were mixed, adjusted to 50 mM NaCl, 10 mM MgCl₂, 10 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, sealed in a capillary tube, heated to 100°C (5 min), and immediately cooled in ice water. The primer-template mixture was then used for dideoxy sequencing, essentially as described by Sanger *et al.* (7). Sequencing reactions were analyzed on gels containing 6 or 8% polyacrylamide (20:1 acrylamide:bisacrylamide), 8.3 M urea, 90 mM Tris-Borate, and 2.5 mM EDTA. The pALS1 sequence was confirmed by the method of Maxam and Gilbert (8).

RESULTS AND DISCUSSION

Fig. 1A,B show restriction enzyme maps of pALS1 and its putative deletion derivative, pALS2. The region encompassing the deletion in pALS2 was sequenced by the dideoxy method using primers constructed for sequencing inserts in the Pst I-site in pBR322. Fig. 2 shows a representative sequencing gel, and Fig. 3 summarizes sequences determined for the different plasmids.

The sequence determined for pALS2 is identical to that of pBR325, except that the four internal nucleotides of the Pst I-site are missing (Figs. 2 and 3A). There is no trace of the Labelle insert that was originally present in pALS1. The same sequence was obtained for five different pALS2 plasmids: three isolated from independent transformants in our initial experiments (ref. 1), and two others recovered by Mr. Marc Orbach and Dr. Charles Yanofsky (Stanford University, Stanford, CA) from Neurospora strains transformed with an independently constructed version of pALS1. The published pBR325 sequence (9,10) was confirmed using pLSHQ2, the pBR325-derivative from which pALS1 was constructed (see ref. 1).

We also characterized an additional plasmid recovered by Dr. Mary Case (University of Georgia, Athens, GA) from a

Figure 1. Restriction enzyme cleavage maps of **A**, pALS1; **B**, pALS2, and **C**, pALS1-(MC). The maps of pALS1 and pALS2 were constructed previously (1). The map of pALS1-(MC) was constructed from results of single and double digestions. Southern hybridization experiments showed that pALS1-(MC) hybridizes to the 363 base pair Hinc II/Pst I fragment and to the 1065 base pair Hinc II-fragment of native Labelle plasmid (4). We infer, therefore, that the deletion breakpoint occurs within the 1065 base pair Hinc II-fragment. DNA sequencing of the Labelle insert from the Pst I-site in pALS1-(MC) showed that the first 100 nucleotides are identical to pALS1 (not shown).

Neurospora strain transformed with pALS1-1. Restriction mapping showed that this plasmid retains a small (approximately 0.5 kb) segment of the Labelle insert and that the deletion includes both Labelle and pBR325 sequences (Fig. 1). The sequencing showed that one of the Labelle-pBR325 boundaries is intact (see Fig. 1; data not shown). The other Pst I-site and surrounding regions are missing, and no sequence could be obtained using the primer for this region.

We previously reported that pALS2 could replicate autonomously in Neurospora, as judged by the recovery of unmodified plasmid from Neurospora transformed with pALS2 (1). We have

Figure 2. DNA sequence analysis of pLSHQ2 and pALS2. The Pst I-site in pLSHQ2 is indicated in brackets. The four missing bases in pALS2 are shown in the boxed area. The sequence for pLSHQ2 is identical to the published sequence for pBR325 (ref. 9,10). The sequence shown was obtained using the Pst I-clockwise primer (see Materials and Methods).

since repeated the recovery of unmodified pALS2 from pALS2 transformants. Since we now find that there is no trace of the Labelle insert in pALS2, our results suggest that the remaining pBR325 sequences plus the *ga-2*⁺ segment constitute a *Neurospora* replicon. The replication origin may be in the *Neurospora* chromosomal DNA fragment or in the pBR325 portion of the plas-

A. pLSHQ2 and pALS2

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      10      20      30      40      50
5'  GTTTGGCGAA  CGTTGTTGCC  ATTGCTCCAG  GCATCGTGCT  GTCACGCTCG  3'
3'  CAAACGGCTT  GCAACAACGG  TAACGACCTC  CCTAGCACCA  CAGTCCGAGC  5'
                Pat I

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      60      70
5'  TCGTTTGTA  TGGCTTCATT
3'  AGCAAACCAT  ACGAAGTAA

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B. pALS1

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      10      20      30      40      50
5'  GTTTGGCGAA  CGTTGTTGCC  ATTGCTCCAG  CGTAAAGAC  GTTCTCATCC  3'
3'  CAAACGGCTT  GCAACAACGG  TAACGACCTC  GCATTTTCTG  CAAGAGTAGG  5'
                pBR325 ← Pat I → Labelle

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      60      70      80      90     100
5'  AAAGAAAAGT  TTTTATGATC  TTATCTATAA  GTTGATTGCT  AATTCTATAT  3'
3'  TTTCTTTTCA  AAAATACTAG  AATACATATT  CAACTAACCA  TTAAGATATA  5'

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     110     120     130     140     150
5'  ACGGTTTAGC  TTCTCAGGCT  ATTGGTGGTA  AAAAGACTTT  TGATATAAAA  3'
3'  TGCCAAATCG  AAGAGTCCCA  TAACCACCAT  TTTTCTGAAA  ACTATATTTT  5'

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     160     170     180
5'  ACCAAAA  Labelle  GGGTGTGAGT  ATTGAGTTGG  3'
3'  TGGTTTT  CCCACACTCA  TAAGTCAAGC  5'

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     190     200     210     220     230
5'  TAGCCTATG  GGTGTGAAA  TGACTGTAA  GAGTGGGGTG  ATGGTACCGT  3'
3'  ATCAGATAC  CCAACACTTT  ACTGACAATT  CTCACCCAC  TACCATGGCA  5'

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     240     250     260     270     280
5'  TTGTCAAAAG  ATCTAAAAGA  ATTGAAAAG  ATGCTCTGGA  TTATAAAAGT  3'
3'  AACAGTTTTT  TAGATTTTCT  TAATTTTTTC  TAGCAGACCT  AATATTTTCA  5'

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     290     300     310     320
5'  CCCTATAGAC  AAATAATGTC  AGATCTCCAG  GCATCG  3'
3'  GGGATATCTG  TTTATTACAG  TCTAGACCTC  CGTAGC  5'
                Labelle ← Pat I → pBR325

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Figure 3. Summary of DNA sequences. Sequences were determined by the dideoxy method, as described in Materials and Methods. The sequences for pLSHQ2 and pALS2 were determined from both directions using appropriate primers. The pALS1 sequence was completed and checked by the method of Maxam and Gilbert (8). For Maxam-Gilbert sequencing, pALS1 was digested with Pst I, 5' end-labeled, and redigested with Hinc II. Pst I/Hinc II-fragments (637 and 363 base pairs) were isolated from agarose gels and sequenced. Pst I-sites are underlined and the four base pairs missing in pALS2 are "boxed".

mid. We cannot completely exclude the possibility that critical replication sequences have been incorporated elsewhere in pALS2 during passage through *Neurospora*. However, no such alterations were detected by restriction enzyme analysis (1). We consider it unlikely that unmodified pALS2 recovered from *Neurospora*

transformants reflects a high frequency of excision of integrated plasmid from chromosomal DNA.

We have carried out additional experiments in which Neurospora was transformed with pLSHQ2 or with other plasmids consisting solely of pBR322, pBR325, or pBR329 plus the Neurospora ga-2 gene (3 kb Hind III fragment or some segment thereof). The situation is complex. Although we have been unable to recover pLSHQ2 from Neurospora transformants, we have been able to recover some of these other plasmids. Those plasmids which were recoverable were obtained from at least one third of Neurospora transformants. The recovered plasmids were generally modified, but the same modified form was often found in multiple E. coli transformants, suggesting that multiple copies were present in the original Neurospora DNA preparations. As they stand, the experiments are consistent with our conclusions, but provide only limited additional support since the recoveries were inefficient, since standard Neurospora DNA preparations have a propensity to contain inhibitors of E. coli transformation, and since the recovered plasmids were generally modified.

The repeated isolation of pALS2 from Neurospora transformants could be accounted for in several ways. One possibility is that the Labelle insert was repeatedly deleted in Neurospora, either by some specific recombination process or by random deletion followed by selection. The sequences at opposite ends of the Labelle insert cannot form a long inverted repeat, but they do contain short runs of A's and T's (Fig. 3B). The Neurospora recombination process may be one which shows some specificity for sequences in Labelle or for sequences around and including the Pst I-sites in which Labelle is cloned. It should be noted, however, that other plasmids recovered from Neurospora transformants have alterations in the ga-2 and E. coli plasmid segments (unpublished results). The frequent modification of plasmids in Neurospora could reflect the operation of some mechanism which protects the organism from foreign DNA. SV40-based shuttle vectors recovered from mammalian cells also show a high frequency of alterations, including deletions, duplications, point mutations, and insertions of host cell DNA (11,12).

An alternative possibility is that pALS2 was generated in

Figure 4. Southern blots of plasmid DNA preparations used to transform *Neurospora*. Plasmids DNAs were digested with Pst I and Hind III and separated in an 0.8% agarose gel. Digest patterns were transferred to nitrocellulose strips and hybridized with pALS1 that had been 32 P-labeled by nick translation. Two different pALS1 preparations are shown. pALS1-1 and pALS1-2 indicate pALS1 recovered after one or two passages through *Neurospora* (ref. 1). The pALS1, pALS1-1, and pALS1-2 DNA preparations were all used in *Neurospora* transformation experiments that subsequently yielded pALS2.

E. coli, that it was present at very low levels in the pALS1 DNA preparations, and that it was somehow selected during propagation in *Neurospora*. Although this possibility seems intuitively more likely, Southern blots of the pALS1 DNA preparations used to transform *Neurospora* showed no detectable pALS2 (Fig. 4). We are aware that the altered Pst I-site in pALS2 could be a cloning artifact resulting from cleavage at the Pst I-site, degradation of single stranded tails, and blunt end ligation. It would be imprudent to exclude this possibility. We note, however, that essentially the same pALS2 plasmids were recovered independently in two different laboratories, and that in our experiments, pALS2 was recovered from *Neurospora* transformed with passaged forms of pALS1 (i.e., pALS1-1 and pALS1-2) that were substantially distant from the original cloning mix.

In a paper published simultaneously with our original report on pALS1 and pALS2, Hughes *et al.* (13) reported that plas-

mid pDV1001, which consists of pBR322 + the Neurospora ga-2⁺ gene (3 kb Hind III-fragment), could also replicate autonomously in Neurospora. This plasmid seemed better behaved than ours since it did not undergo modification in Neurospora and since it could be recovered from Neurospora transformants at high frequency (400 to 6000 E. coli transformants per μ g of Neurospora transformant DNA; 13). In subsequent experiments, however, Hughes *et al.* (14) could not repeat the recovery of pDV1001 from Neurospora transformants and concluded that their original experiments were compromised by plasmid contamination (14).

The retraction of the data demonstrating efficient recovery of unmodified plasmid (14) necessitates a reevaluation of the potential utility of all these plasmids as shuttle vectors. The evidence for autonomous replication of pALS1 and pALS2 is two-fold: free plasmids can be detected in Southern blots of DNAs from Neurospora transformants, and unmodified plasmids can be recovered by retransformation of E. coli. However, both in our initial experiments and subsequently, the recovery of plasmids from Neurospora DNA preparations was relatively inefficient (a maximum of ten E. coli transformants per μ g DNA and usually less; ref. 1 and unpublished results). Hughes *et al.* noted in their retraction that the fact that pDV1001 also produces "abortive transformants" suggests that it carries an ars sequence (14). This statement seems consistent with our conclusions. However, the inefficient recovery suggests that the concentration of monomeric plasmid is relatively low. The frequent modification of plasmids and the propensity of some plasmids to form high molecular weight oligomers (Grant, Lambowitz, Rambosek and Kinsey, in press) are additional potential difficulties. Thus, further work will be required to determine if any of the presently available plasmids will be a useful shuttle vector for nuclear gene isolations. However, as long as the plasmids replicate autonomously, it may be possible to modify them to improve their copy number and stability.

In our original experiments, the Labelle mitochondrial plasmid appeared to be a beneficial addition, as judged by somewhat higher Neurospora transformation frequencies and by recovery of plasmid from Neurospora transformants. We now recognize that both characteristics may be influenced by a variety of

factors including the proportion of supercoiled plasmid DNA and impurities in the DNA preparations. Judged by Neurospora transformation frequency, Labelle appears to be a beneficial addition in some plasmids, but not others (unpublished data). In addition, we find considerable variability in transformation frequencies between different experiments and, in some experiments, pLSHQ2 and pALS2 gave higher transformation frequencies than pALS1. The present work supports the previous conclusion that the Labelle mitochondrial plasmid is not essential for autonomous replication in Neurospora (1).

ACKNOWLEDGEMENTS

We thank Mr. Marc Orbach and Dr. Charles Yanofsky (Stanford University, Stanford, CA) and Dr. Mary Case (University of Georgia, Athens, GA) for plasmids and for their comments on the work. This research was supported by NIH grant GM23961.

*To whom correspondence should be addressed

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