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NOTES

Cloning of Methylated Transforming DNA from *Neurospora crassa* in *Escherichia coli*

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An *arg-2* mutant of *Neurospora crassa* was transformed to prototrophy with a pBR322-*N. crassa* genomic DNA library. Repeated attempts to recover the integrated transforming DNA or segments thereof by digestion, ligation, and transformation of *Escherichia coli*, with selection for the plasmid marker ampicillin resistance, were unsuccessful. Analyses of a *N. crassa* transformant demonstrated that the introduced DNA was heavily methylated at cytosine residues. This methylation was shown to be responsible for our inability to recover transformants in standard strains of *E. coli*; transformants were readily obtained in a strain which is deficient in the two methylcytosine restriction systems. Restriction of methylated DNA in *E. coli* may explain the general failure to recover vector or transforming sequences from *N. crassa* transformants.

Many genes from *Neurospora crassa* have been cloned by sib selection procedures (1, 16). This method identifies individual clones which complement a mutant of interest and involves progressive reduction in the complexity of a genomic DNA library. Sib selection has been used with *N. crassa* because there is no shuttle vector for both *N. crassa* and *Escherichia coli* and because it has been difficult or impossible to reclone integrated complementing sequences from *N. crassa* transformants in *E. coli*. Transformant sequences should be recoverable by using selectable markers of the vector, since transforming and associated vector sequences are often integrated intact by nonhomologous integration (4).

Analysis of one *arg-2* transformant has provided an explanation for these difficulties. We have found that pBR322 and associated complementing sequences that are integrated together during *N. crassa* transformation are methylated at cytosine residues. We demonstrate here the successful recovery of pBR322 sequences from *N. crassa* at high frequency with *E. coli* K802 as a transformation recipient; this strain is deficient in two methylcytosine restriction systems, *mcrA* and *mcrB* (10).

N. crassa arg-2(CD80) *pyr-3*(DFC3) *A* was transformed to arginine prototrophy with a pBR322-based genomic DNA library. Repeated attempts to recover plasmid sequences from one *N. crassa arg-2*$^+$ transformant, designated T-AR2-1, and an *arg-2*$^+$ descendant strain, designated T-AR2-1-1, were unsuccessful. Recovery was attempted by digestion of total T-AR2-1 and T-AR2-1-1 DNA with appropriate restriction endonucleases, followed by ligation of the resulting DNA fragments and transformation into *E. coli* LE392 or HB101 (7). Southern analyses of T-AR2-1 and T-AR2-1-1 DNA indicated there were no gross rearrangements of the pBR322 sequences that would have prevented the beta-lactamase gene or the plasmid origin of replication from functioning in *E. coli*.

We proceeded to clone the plasmid sequences without requiring their functions from a library containing *Sau*3AI partial digests of the DNA of strain T-AR2-1-1 in phage λ J1 (8). Phage clones that hybridized to ^{32}P-labeled pBR322 (5) were isolated, and *arg-2*$^+$ sequences were demonstrated by complementation of a *N. crassa arg-2* mutant to be adjacent to the vector sequences. These clones were noticeably underrepresented in the library; they were present at a frequency less than 10% that of clones containing the beta-tubulin gene of *N. crassa* (9), even though Southern analyses indicated that pBR322 sequences were present in the transformant at one to three copies per genome (data not shown).

The plasmid sequences in the phage clones were tested for their ability to function in *E. coli* by digestion of phage DNA with restriction endonucleases, followed by ligation and transformation of *E. coli* LE392. Ampicillin-resistant plasmids were recovered at high frequency, which suggests that the transforming DNA was transiently modified in *N. crassa*, preventing its direct recovery in LE392, and that this modification was removed by replication as a segment of a λ clone vector in *E. coli*.

The recent demonstration of two methylcytosine restriction systems in *E. coli* (10) raised the possibility that these restriction systems were responsible for our inability to reclone pBR322 sequences directly from *N. crassa* transformants. If the plasmid sequences in these transformants contained methylated cytosine residues, the introduction of these sequences into most *E. coli* strains would result in their restriction by *mcrA*- or *mcrB*-endoced endonuclease. As a test for cytosine methylation, DNAs from transformant strains T-AR2-1 and T-AR2-1-1 were digested separately with *Sau*3AI and *Mbo*I endonucleases. Both enzymes recognize and cleave the sequence GATC, but *Sau*3AI will not cut this site if the C is methylated, while *Mbo*I will not cut this site if the A is methylated. Southern transfers of *Sau*3AI and *Mbo*I digests of T-AR2-1 and T-AR2-1-1 DNAs were prepared and probed with ^{32}P-labeled pBR322 (Fig. 1A) and *N. crassa arg-2* DNA (Fig. 1B). The *Sau*3AI digests contained many high-molecular-weight bands not present in the *Mbo*I digests. Comparison of the hybridization patterns of

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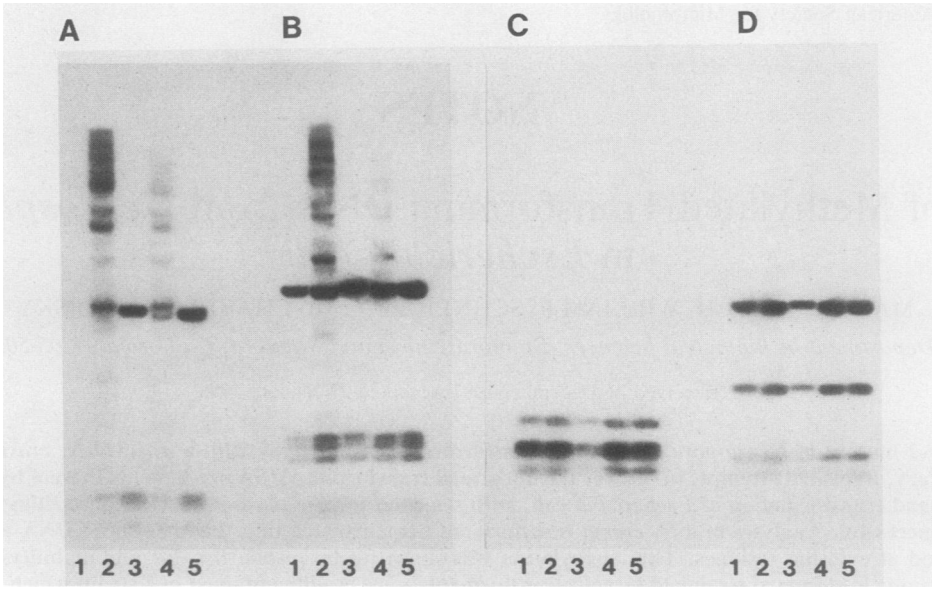

FIG. 1. Analysis of DNA methylation in an *arg-2* transformant. DNA from wild-type strain 74-OR23-1A (lane 1), the arg-2 transformant T-AR2-1 (lanes 2 and 3), or one of its *arg-2*$^+$ progeny, T-AR2-1-1 (lanes 4 and 5), was digested with either *Sau*3AI (lanes 1, 2, and 4) or *Mbo*I (lanes 3 and 5) endonucleases. DNA fragments were separated by electrophoresis on a 1.2% agarose gel in Tris-borate-EDTA buffer (7) and were transferred to a Nytran membrane (Schleicher & Schuell, Inc.). The membrane was probed with ^{32}P-labeled pBR322 DNA (A) or a restriction fragment of *N. crassa arg-2* (B). The probes were stripped from the filters, and the membranes were rehybridized with labeled fragments of *pyr-4* (C) (3) and *his-3* (D) (6) DNA.

T-AR2-1 and T-AR2-1-1 DNAs digested with *Sau*3AI and *Mbo*I and probed with pBR322 DNA revealed only faint hybridization in the *Sau*3AI lanes at positions corresponding to complete digestion products in the *Mbo*I lanes. This finding indicated that most of the *Sau*3AI sites in the transforming DNA were methylated. The same DNAs probed with *arg-2* DNA gave prominent bands in *Sau*3AI lanes, which comigrated with *Mbo*I bands and had extra *Sau*3AI bands. This finding suggests that two copies of the *arg-2* sequence are present, i.e., the introduced copy and the recipient (mutant) copy, with only one of these methylated. To demonstrate that digestion of T-AR2-1 and T-AR2-1-1 DNAs was complete, the 1A and 1B filters were reprobed with ^{32}P-labeled *pyr-4* and *his-3* DNA (Fig. 1C and D, respectively). *pyr-4* and *his-3* are present only at their normal locations in these transformants and thus should not be affected by the transformation event. The hybridization patterns obtained with *Sau*3AI and *Mbo*I digests with these probes were identical. The same pattern is seen with DNA of wild-type strain 74A digested with *Sau*3AI. We conclude, therefore, that the extra bands observed when pBR322 and *arg-2* DNAs are used as probes with *Sau*3AI digests of *arg-2*$^+$ transformant DNA are due to methylation of cytosine residues.

TABLE 1. Recovery of plasmid DNAs from *N. crassa arg-2*$^+$ transformant strain T-AR2-1-1

Expt and DNA[a]	Frequency of Amp^r colonies[b] with strain:	
	K802	LE392
1		
pBR322	1.8×10^7	3×10^6
T-AR2-1-1 (*Eco*RI)	139	0
2		
pBR322	3.2×10^7	1.6×10^6
T-AR2-1-1 (*Eco*RI)	85	0
T-AR2-1-1 (*Bam*HI)	68	0
T-AR2-1-1 (*Sal*I)	137	0
T-AR2-1-1 (*Bgl*II)	60	0

[a] DNA was either pBR322 supercoiled plasmid DNA or strain T-AR2-1-1 DNA, digested with the enzyme given in parentheses and ligated.
[b] Frequencies are the numbers of ampicillin-resistant transformants per microgram of DNA.

Successful direct recloning of these methylated sequences in *E. coli* was achieved with a recipient strain, K802, which is deficient in both the *mcrA* and *mcrB* restriction systems (10). DNA from *N. crassa* T-AR2-1-1 was digested with different restriction endonucleases, ligated, and introduced into either K802 or LE392 (*mcrB*$^+$) (Table 1). Ampicillin-resistant colonies were selected but were obtained only with K802. K802 gave higher transformation frequencies than LE392 with the control plasmid, pBR322, but these differences (6-fold in experiment 1 and 21-fold in experiment 2) do not explain the rescue of plasmids from *N. crassa* T-AR2-1-1 only in K802. These plasmids were shown to have sequences identical to those of the *arg-2*$^+$-pBR322 λ clones by restriction digest analysis and transformation of an *arg-2* mutant.

In most eucaryotes, DNA methylation is common; methylation is thought to influence gene expression (17). Recently, differential methylation of mouse chromosomal DNAs has been implicated as a means of marking the parental origin of each chromosome (11, 13). In *N. crassa*, the overall level of DNA methylation is low (12). However, DNA introduced into *N. crassa* by transformation has been shown here and by others (2, 14) to be subject to high-level cytosine methylation. Selker et al. (14, 15) have proposed that in this organism, methylation is used to mark duplicated sequences. We have shown that methylation prevents the cloning of transforming DNA in normal *E. coli* strains but that such DNA can be cloned by using an *E. coli* strain deficient in restriction systems that recognize methylated

DNA. The ability to recover transforming DNA from *N. crassa* should greatly facilitate the cloning of genes of interest and should aid in the analysis of transformation events.

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