Volume 15 Number 22 1987 Nucleic Acids Research

Sequence and centromere proximal location of a transformation enhancing fragment *ans1* from *Aspergillus nidulans*

D.Cullen*, L.J.Wilson, G.L.Grey, D.J.Henner[1], G.Turner[2] and D.J.Ballance[2]+

Genencor Inc., [1]Genentech Inc., South San Francisco, CA 94080, USA and [2]Department of Microbiology, University of Bristol, Bristol, UK

Received September 25, 1987; Revised October 16, 1987; Accepted October 27, 1987 Accession no. Y00493

ABSTRACT

The Aspergillus nidulans sequence ans1, previously known to enhance transformation frequencies of pyr4-based vectors, was shown to enhance the efficiency of argB and trpC-based vectors. Increased efficiencies could be obtained by constructing vectors containing argB and ans1 or by cotransforming selectable plasmids (containing argB, trpC or pyr4) with the non-selectable ans1 sequence. The preponderance of evidence suggests that the mechanism of ans1 activity does not involve homologous recombination events, in spite of the presence of multiple regions of homology in the A. nidulans genome. Genetic mapping localized ans1 to the vicinity of the centromere of linkage group I. The nucleotide sequence of a 1.8 Kb functional subclone of ans1 was determined and found to be highly A+T rich (81%).

INTRODUCTION

One of the first transformation systems developed for Aspergillus nidulans involved complementation of pyrG auxotrophs with the corresponding Neurospora crassa gene, pyr4 (1). Initially, transformation frequencies obtained with pyr4-based vectors were extremely low (i.e. <10 transformants per µg DNA) and many 'abortive' transformants were observed. Incorporating an A. nidulans DNA sequence ans1, into pyr4-based plasmids dramatically increased transformation efficiency (3). The efficiency of Penicillium chrysogenum transformation is also improved by employing ans1-bearing plasmids (4).

Two possible mechanisms of ans1 enhancement of transformation seem possible. The multiple regions of homology might provide 'hot spots' for integration or the ans1 sequence might promote autonomous replication of the plasmid. If correct, the latter possibility must involve low levels of free plasmid

and/or early plasmid replication followed by integration because Southern hybridization of undigested DNA from transformants failed to detect free plasmid (2, unpublished observations).

In this, study we investigate further the nature of ans1.

MATERIALS AND METHODS

Strains and Plasmids

Plasmids were amplified in E. coli strains 294 (ATCC 31446) or DH1. Aspergillus nidulans recipients were G191 (pabaA1, pyrG89; uaY9, fwA1), WI (argB2; methH2; biA1), and FGSC237 (pabaA1, yA2, trpC801). Aspergillus nidulans strain M1343 (FGSC375) (suA1adE20, adE20, biA1; acrA1; sA4; pyroA4; pA2; lacA1; nicB8; riboB2) was used for mitotic haploidization (6) and strain HB16 (proA1, hisB98, biA1; chaA2, kindly provided by Dr. S. Martinelli) and AU213 (galD5, suA1, riboA1, anA1, proA94, lysF88, pabaA1, ya2, adE20, kindly provided by Dr. J. Croft) were used for sexual analysis of 612-ΔE-9.

The argB-containing plasmid pBB116 has been described (7), as have the pyr4-containing plasmids pDJB1 and pDJB3 (2, 3). A genomic library of wild-type A. nidulans in cosmid pKBY2 (8) was provided by W.E. Timberlake.

Plasmid pAA1 was constructed by combining the Pst1/BamHI fragment containing argB from pBB116, the EcoRI/Pst1 fragment containing ans1 from pDJB3, and pUC18. pA1 was derived from pAA1 by deleting the EcoRI/Pst1 fragment, filling the ends with Klenow fragment of DNA polymerase I, and religating (Figure 1). Plasmid pDH4 contains the 1.8 Kb PvuII/Pst1 fragment of ans1 in pUC18.

Plasmid pMW3 was constructed by inserting an EcoRI-HindIII fragment containing the oligomycin resistance allele, oliC31, into pDJB4, a derivative of pDJB2 (2; D.J. Ballance, unpublished, M. Ward, unpublished). The single EcoRI site was then removed by Klenow-filling the cohesive ends, followed by blunt end ligation to produce the plasmid pMW3ΔE (see Fig. 3).

Aspergillus Transformation

Three transformation protocols were employed. The first was as previously described (2). The second involved modifications whereby protoplasts were prepared from 12 hr germlings harvested by centrifugation from yeast-glucose medium (0.5% yeast extract,

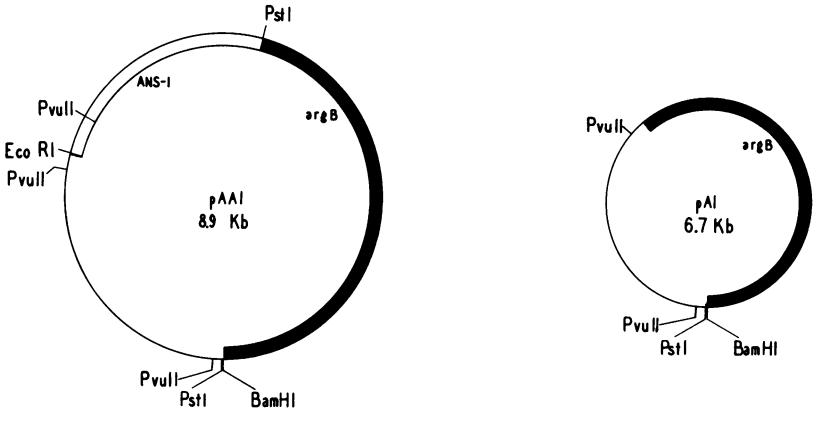


FIGURE 1
Partial restriction map of pA1 and pAA1. Plasmids were constructed as described in Materials and Methods from pUC18 (thin line), a PstI-BamHI fragment of pBB116 containing the entire A. nidulans argB gene (thick black lines) and the ans1 fragment from pDJB3 (open area).

2% glucose). A further modification included addition of 0.6M KCl to the 25% (v/v) polyethylene glycol (PEG) solution.

The third method employed frozen protoplasts similar to the procedure described by Vollmer and Yanofsky (9). For freezing, the protoplasts were prepared from germlings, washed in 0.6M KCl, and resuspended in sorbitol buffer (1 M sorbitol, 50 mM Tris-HCl, 50 mM $CaCl_2$) yielding a final concentration of 2 x 10^8 protoplasts ml^{-1}. 40% PEG4000, 50 mM Tris-HCl pH 8.0, 50 mM $CaCl_2$, 5% DMSO (0.25 vol) were added and 200 µl aliquots transferred to 1.5 ml eppendorf tubes and immediately frozen (-80°C). Plasmids were preincubated in a heparin-containing buffer before PEG- induced fusion with thawed protoplasts (9).

In a typical transformation experiment, 5 µg of vector DNA was incubated with ca. 5 x 10^7 protoplasts. For cotransformation experiments, varying amounts of pDH4 were mixed with 5 µg of the selectable plasmid. Controls included protoplasts incubated with TE (10 mM Tris-HCl, 0.5 mM Na_2 EDTA, pH 8.0) instead of vectors, and, in cotransformation experiments, pUC18 instead of pDH4.

Throughout, only those colonies showing continuous growth on selective media were scored as transformants. Abortive

transformants were identified as slow growing, minute colonies which, after several days, ceased growth. Transformation frequencies differed between protoplast batches and the protocols employed. To minimize variation, comparisons presented in our result section represent a single experiment using one batch of protoplasts and one of the above mentioned protocols. Frozen protoplasts yielded the most reproducible results. Throughout the transformation experiments, spontaneous revertants were never observed on any control plates.

Sequencing and DNA Manipulations

The sequence of ans1 was determined by the dideoxy method (10). Fungal DNA was extracted and purified as previously described (1). All enzymes were used in accordance with manufacturer's recommendations.

RESULTS

Transformation Experiments

Inclusion of the ans1 fragment in an argB vector increased transformation frequency approximately 4 fold. Plasmids pA1 and pAA1 were identical, except pA1 lacked the ans1 sequence. In an experiment using frozen protoplasts, pA1 yielded 105 transformants μg^{-1} DNA whereas pAA1 yielded 484. This enhancement was reproducible regardless of the protocol used. Inclusion of ans1 also reduced the number of abortive transformants as a percentage of total colonies, i.e. from 96% with pA1 to 50% with pAA1.

Cotransformation with a plasmid containing the ans1 sequence (pDH4) also increased transformation efficiencies for pA1 (Figure 2). This cotransformation effect was also evident in similar experiments using pyr4 as the selectable marker: a vector consisting of pyr4 in pUC8 (unpublished) produced approximately 10 transformants μg^{-1} on its own and 1000 transformants μg^{-1} when cotransformed with 1 µg of an ans1-containing vector. A two-fold stimulation was also observed for the pKBY2 library DNA.

Genomic Location of ans1

When genomic blots of A. nidulans DNA were probed with the ans1 sequence, in addition to hybridization to ans1 itself, evidence of homology to other regions of the genome was also

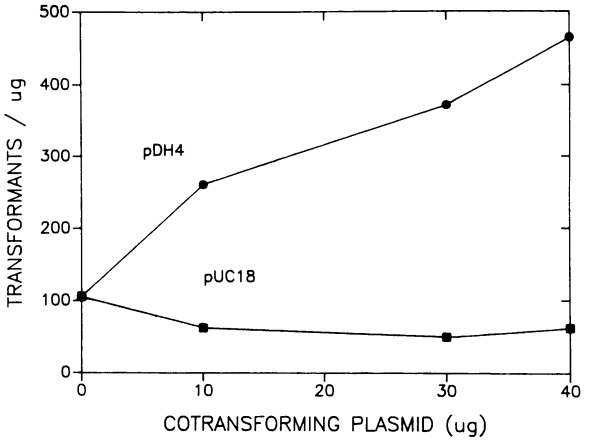


FIGURE 2
Effect of cotransforming plasmid concentrations on transformation frequency of pA1. Transformation protocol is described in Materials and Methods. In each case, protoplasts were incubated with 5 µg pA1 and varying amounts of pDH4 or pUC18. Frequency is expressed as the number of transformants per µg of pA1.

observed (2). To determine whether these homologous sequences were contributing directly to enhancement of transformation efficiency, analysis of transformants obtained with pMW3 E was undertaken. This plasmid consists of ans1, pyr4 and oliC31 in pBR325 and contains no sites for EcoRI (Fig. 3). Aspergillus nidulans G191 was transformed with this plasmid, selecting for Pyr prototrophy, and the transformants were purified twice on selective medium.

Integration of the plasmid containing oliC31 at the oliC locus by a type I recombination event (11) leads to an intermediate level of resistance to oligomycin due to the presence of both sensitive and resistant copies of the gene. However, if such transformants are plated on oligomycin, they rapidly sector to full resistance as a result of recombination between the two alleles (12). Integration of such a plasmid at sites other than the oliC locus leads to stable semi-resistance. The pMW3ΔE transformants were stabbed out onto oligomycin-containing medium and scored for their propensity to sector to full resistance: only 5 out of 112 transformants gave rise to

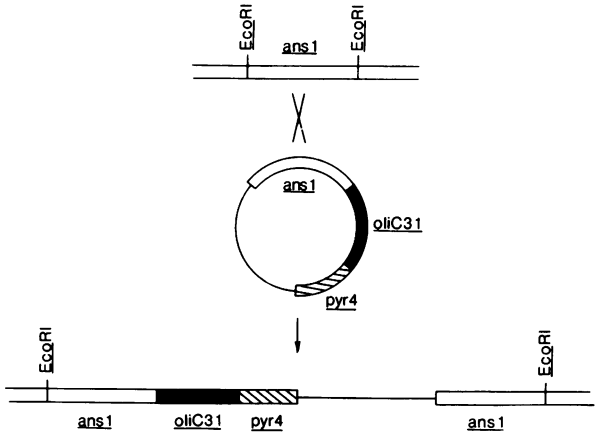


FIGURE 3
Integration of pMW3ΔE at the ans1 locus. The vector contains no EcoRI sites. Disruption of the native ans1 band by type I integration would generate a new EcoRI band of 17.1 Kb.

fully resistant sectors and were therefore assumed to contain insertions at the oliC locus.

Eleven transformants which failed to produce fully resistant sectors, suggesting that integration at the oliC locus had not occurred, were selected for further study. DNA was prepared from these transformants and EcoRI digests were blotted onto nitrocellulose and hybridized with the ans1 fragment (Figure 4). Integration at the ans1 locus by recombination via the ans1 sequence should be detectable by a decrease in the mobility of the ans1-containing fragment (Figure 3). Integration of the plasmid at any of the sites of homology to ans1 should be similarly discernible.

Transformant 612-Δ E-9 appears to have resulted from integration at the ans1 locus since the ans1 band has been replaced by a larger band of the expected size (17kb). In none of the other transformants is there evidence of disruption of ans1 or its homologues.

Most of the transformants obtained in this experiment were found to be unstable, the Pyr$^+$ phenotype being detectable in <20% of conidia from a selective plate. Transformants 612-ΔE-1 through 612-ΔE-6 are of this type, and grew slowly in selective

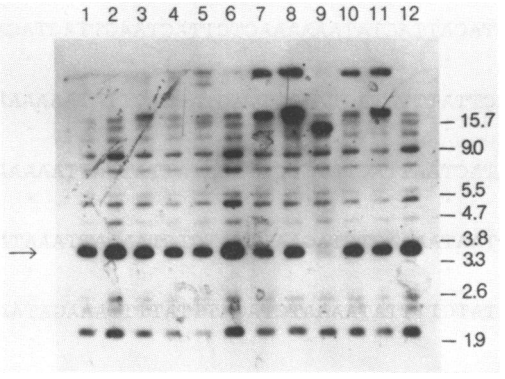


FIGURE 4
Analysis of pMW3ΔE transformants. Total transformant DNA (approx. 3 µg) was digested with EcoRI and subjected to agarose gel electrophoresis, blotted onto nitrocellulose, and probed with ^{32}P-labelled ans1 fragment. Lanes 1-11, transformant strains 612-ΔE-1 through 612-ΔE-11, lane 12, untransformed control. The gel was calibrated with a lambda digest (Kb). An arrow marks the position of the native ans1 EcoRI fragment.

medium. Others, such as 612-ΔE-7, 8 and 10, apparently stabilized during the subculturing process. Additional bands hybridizing to ans1 are evident in the latter transformants. The instability of transformants 612-ΔE-1 through 612- E-6 may explain our difficulty detecting transforming DNA using either ans1 or pBR322 probes. Weakly hybridizing DNA is, however, evident just below the origin in 612-ΔE-5.

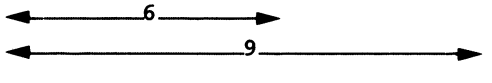
Transformant 612-ΔE-9 was found to be perfectly stable through meiosis and was therefore used to assign the genomic location of ans1 by mitotic haploidization and sexual crosses using oligomycin resistance as a marker. This showed that ans1 is located on linkage group I between proA1 and hisB98:

proA — 3 — (oliC31) — 5 — hisB

The integrated plasmid also mapped between a different proA mutation, proA94, and lysF88.

Mapping data suggest that the centromere of linage group I is situated between proA and lysF, in the order:

proA ——— lysF ——— hisB



PvuII
CAGCTGACTTCATAATTACATTACTATAAAAAACTCTTACTAAGTTACTAGATAAGAACT
. 60

ScaI
TTATTTATATTAGTTACTTATTTATAGTAACTTTAGTACTACTTATAAAAAAGCTAGATA
. 120

GAGATTTATAGTTCTATACTAATTACTATGCCTTAAATATTATTATTAAAAAAGATTATT
. 180

ATCCCCTTTCTCTAATTAATAAAACTCTAGAATTTATCTCTAAAATTAAATAATTTATAA
. 240

AACTTAATATTATTACTATCTTTTATAAAATCTAGATTTATTTTAAAGATAAATAGAAGA
. 300

TAGCTTTCAGGATATATTTTAGGTTATATAAATAGTTAATTACTTTTTTTAAGCTAGTAA
. 360

BglII
ATATTCTAAGTATATTCTAATATTATATTAACTAGATCTTACATAACTTTCTAGATAAAT
. 420

TTGCCTTTATATACTTAGATAATATATTAATTTTTACTAATAGCTTATTAAAAAAGTATT
. 480

ATAATTATATATAATTAATTTTCTAGAAACTAGGCAAAGCAGGACTATAGCTTAATATTA
. 540

ATAAATATAATTTTAAAATTAACTAAGTCTTTAAGATTTATTATTAATATAAAGAAGGGT
. 600

ATATAAATAGATCCTAAGAAAATAAGCTATCCTTAAATAATAAATCCTTATATTAGTTAA
. 660

AAAAAATATACTTCTTTTTAAGATTTATAAATTTTTATTAATTTATATAAAACTTCTTAG
. 720

ACCTGATATACCTACTTACTATTCTAATATAAAAAATTATTAAATTTTTATAGATTAAGA
. 780

AccI
AGTATACCTAGTTTAAATAGCTAAATTAAATATTTATAATAACTTCTATTTTTATATAAT
. 840

TTAATCCTAATTAAGAAATAATTTTTAAAGCTCTTATATTAAACTAGACTACTAGAAAAA
. 900

ScaI
TATTATTATAATATAATAATAATAAAGTACTATATTTTTATATTTATTATTTAAGAACTC
. 960

TCCCTGCAAAATATAATTATAAAATTTATAATAAAAATTATTAGTAATTATTAATAGCTT
. 1020

AAAAGTTTAGAAATTAGAGCTTATAAGTTTAAAAAATTTTATTATTATTATAAATTATAA
. 1080

AAACCTACTATACTTTACTATAATTAGATATTTTAATAAATAGTAAATATATTAGACAGA
. 1140

TATACTTAATTATTTTAATTTTATTTTAAATATTAACTAGGTAAGCTAGCAGCTTTGCCT
. 1200

```
                                                AccI
AGATATATTATTATACTATAAATAAGATATACTAGCCAGGGTAGACAATAATTAACTTAA
          .         .         .         .         .         .  1260

AATATATAAAAAATAGCTACTAAAACTGAATAATATTAAAGAATAGTATTTATCTAAGTA
          .         .         .         .         .         .  1320
                                                            H
TAACTTAAATCTTAGTAATACTAAAGAATTTAATTAATTAATTAAAATATATAAATATAG
          .         .         .         .         .         .  1380
indII
TTGACCTTGAAACTGTTACTAAACTTTATTATAATATTATTAATTATAATAACTAAGCTA
          .         .         .         .         .         .  1440

AGAATAAATTACTTAAGAAACTATAAAAAATAGTCTAGGAGAATAATAAAGCTCTATAAA
          .         .         .         .         .         .  1500

AAATTATTAAGATTATTAAGAAGAATAGATAGAGCTTCCTAAGAGATTAGAAATATATAT
          .         .         .         .         .         .  1560

CTTATTTTCTAAATATTTAATATTATCTAGAGATAATTTTTTTTATTCTTAATTTTAGGT
          .         .         .         .         .         .  1620
                                      BglII
CCTGGGAAGTAAACCTTTAAAAACCTAAATTATATAGATCTGGATTAACTAGCTACCCTG
          .         .         .         .         .         .  1680

GGCAAAACAGCCTATATATTATATATATTAATTCTTAATAATCTAGTAGTATATCTTTTT
          .         .         .         .         .         .  1740

TACCTATTATAGATCAAGAGATTAAAACTAGCTAGGGCTAATATATAATAAAATTTGTTT
          .         .         .         .         .         .  1800
                                                          Ps
TTACTTAGTTACTTATTAGTTTGTCAATCCGCACCGCAACCCGCAGCGGGTCACCACACT
          .         .         .         .         .         .  1860
tI
GCAG
```

FIGURE 5
Nucleotide sequence of functional PvuII - PstI subclone of ans1. An 11 bp consensus to S. cerevisiae ARS sequence is located at 935. The sequence TTCCTAA at 1535 contains a single base mismatch to S. cerevisiae CDEIII, TTCCGAA.

and the indications are that proA is the closest marker to the centromere (13). We cannot be certain of the actual physical distance between ans1 and the centromere or of the effect of integration of pMW3ΔE at the ans1 locus on the apparent genetic distance. It would appear, however, that the closest genetic marker to ans1 is proA.

Sequence analysis

A 1,8 Kb fragment of ans1, previously shown functional in A. nidulans (2), was sequenced (Figure 5). The A + T composition is 81%. A typical S. cerevisiae ARS sequence, TTTTATATTTA, is

located at position 935 and may be responsible for the demonstrated ARS activity. However, subcloning experiments have indicated that several other regions of the sequence are able to confer such activity, albeit with varying efficiencies. Indeed, several imperfect matches to the consensus ARS can be found.

In attempts to identify the smallest functional subunit of ans1, several deletions were previously tested (2). Three additional subclones, i.e. a 831 bp ScaI fragment (bp 97 - bp 928 in Fig. 5), a 783 bp PvuII - AccI fragment (1-783), and a 1382 bp PvuII - HincII fragment (1-1382), were found to be inactive.

DISCUSSION

Vectors containing the genomic sequence ans1 show increased transformation frequencies over analogous vectors lacking the sequence (2; unpublished results; 4).

This effect is most striking in the case of vectors containing the N. crassa pyr4 gene as a selectable marker, but is also evident with argB, as demonstrated here. The especially marked effect with pyr4 could be related to the low degree of homology between pyr4 and the A. nidulans genome (2) or be due to efficient expression of the gene at many different sites in the genome. We describe here an extension of these observations to cotransformation experiments wherein the ans1 sequence is on a different plasmid to the selectable marker. This approach presents a convenient method for improving transformation efficiencies without necessarily reconstructing vectors and it may be particularly useful for low-frequency cosmids such as pKBY2.

The mechanism by which transformation frequency is increased by cotransformation with a plasmid containing ans1 is not known. One possibility is a direct interaction between homologous regions of the two molecules. This might involve providing sites for homologous integration by initial single or multiple integration of the ans1 plasmid, or recombination between free molecules resulting in plasmids containing both ans1 and the selectable marker, which would then behave as the in vitro constructs used previously. In view of the frequency of non-homologous integration (Type II) and the failure of the ribosomal

gene repeat unit to enhance transformation (14), the latter alternative seems more likely. However, Orr-Weaver and Szostak (15) showed in yeast that integration of one plasmid created a hot-spot for integration of a second plasmid at that site though this has yet to be shown to be a mechanism for tandem integration in Aspergillus.

There are a number of possibilities for the mechanisms of action of ans1, including: 1) The sequence represents a hot-spot for recombination; 2) The sequence provides a large number of possible sites for homologous recombination; or 3) The sequence directs or permits transient or persistent replication of the plasmid as a free molecule.

Alternatives 1 and 2 are effectively ruled out by the experiments described here which failed to show any evidence for preferential integration of pMW3ΔE at ans1 or ans1-homologous sites. There remains the possibility, however, that ans1 promotes integration at unrelated sites.

We have previously failed to demonstrate the presence of freely replicating plasmids in transformants. Detection of transient replication which might enhance the chances of stable integration is very difficult to achieve. In most instances, where integration of ans1-containing vectors has occurred, there is evidence of rearrangement of the DNA, particularly in the ans1 region of the vector (2): transformant 612-ΔE-9 is, to date the only example of a single Type I integration event. The latter transformant has enabled the genomic location of ans1 to be determined, this being between the centromere flanking markers on linkage group I, proA and lysF. Whether the proximity to the centromere is purely coincidental or whether ans1 is a part of the functional centromere requires further investigation.

The DNA sequence failed to shed any light on the question of the mode of action of ans1. The minimum sequence required (PvuII to PstI) is highly A + T rich though this characteristic does extend beyond this fragment (D.J. Ballance, unpublished). A + T richness per se is probably not responsible for the activity since most fragments of the A + T-rich mitochondrial genome do not enhance transformation frequencies (R.K. Beri and G. Turner, unpublished). Centromeric DNA from S. cerevisiae has been well-

characterized and consists of three conserved elements; CDEI (CTCACATG), CDEII (an 88-89 bp domain of >90% A + T and CDEIII (TTCCGAA) (16). The ans1 sequence is very A + T rich and contains the sequence TTCCTAA at position 1535 (Fig. 5). No convincing CDEI homologue is evident.

In contrast to S. cerevisiae, centromeres of the fission yeast Schizosaccharomyces pombe appear to be complex, containing large repeated units present in one or more copies in the centromere of each chromosome (17, 18, 19). The repeat units dg and dh are A + T rich; dgI is 67% A + T overall and 70% A + T from nucleotides 1-1900 while dhII is also 67% A + T overall and 72% A + T from nucleotides 1-2200. These units also contain numerous short direct repeats (17, 18). The ans1 sequence contains many short direct repeats but these tend to be dispersed and consist almost exclusively of A + T (not shown). Such repeats would be expected in a sequence of 1.8 kb wherein 81% of the bases are either A or T.

The apparent proximity of ans1 to the centromere of chromosome I and the demonstrated homology between the centromeres of the three S. pombe chromosomes (17, 18, 19) raises interesting questions regarding the ans1-homologus regions seen on Southerns. We are currently engaged in screening gene libraries in an attempt both to isolate more of the ans1 region and to clone DNA from the homologous regions of the genome. It will then be possible to address the question of the relationship between ans1 and the centromere of chromosome I and to map the genetic location of the homologues. It would also be interesting to determine the effect of these sequences on the efficiency of transformation.

ACKNOWLEDGEMENTS

We are indebted to Marlies Chrzanowski for typing this manuscript. D.J.B. was supported by an SERC grant to G.T.

+To whom correspondence should be addressed at: Delta Biotechnology Ltd, Castle Court, Castle Boulevard, Nottingham NG7 1FD, UK

*Present address: USDA Forest Products Laboratory, Madison, WI, USA

REFERENCES

1. Ballance, D.J., Buxton, F.P. and Turner, G. (1983). Biochem., Biophys. Res. Comm. 112, 284-289.
2. Ballance, D.J. and Turner, G. (1985). Gene 36, 321-331.
3. Ballance, D.J. and Turner, G. (1986). Mol. Gen. Genet. 202, 271-275.
4. Cantoral, J.M., Diez, B., Barredo, J.L., Alvarex, E. and Martin J.F. (1987). Bio/Technology 5, 494-497.
5. Barnes, D.E. and MacDonald, D.W. (1986). Curr. Genet. 10, 767-775.
6. McCully, K.S. and Forbes, E. (1965). Genet. Res. 6, 352-359.
7. Berse, B., Dmochowska, A., Skrzypek, M., Weglenski, P., Bates, M.A. and Weiss, R.L. (1983). Gene 25, 109-117.
8. Yelton, M.M., Timberlake, W.E. and van den Hondel, C.A.M.J.J. (1985). Proc. Natl. Acad. Sci. USA 82, 834-838.
9. Vollmer, S. and Yanofsky, C. (1986). Proc. Natl. Acad. Sci. USA 83, 4869-4873.
10. Sanger, F., Nicklen, S. and Coulson, A.R. (1977). Proc. Natl. Acad. Sci. USA 74, 5463-5467.
11. Hinnen, A., Hicks, J.B. and Fink, G.R. (1978). Proc. Natl. Acad. Sci. USA 75, 1929-1933.
12. Ward, M., Wilkinson, B. and Turner, G. (986). 202, 265-270.
13. Käfer (1977). Adv. Genet. 19, 33-131.
14. Tilburn, J., Scazzocchio, C., Taylor, G.G., Zabicky-Zissman, J.H., Lockington, R.A. and Davies, R.W. (1983). Gene 26, 205-221.
15. Orr-Weaver, T.L. and Szostak, J.W. (1983). Mol. Cell. Biol. 3, 747-749.
16. Hieter, P., Pridmore, D., Hegermann, J.H., Thomas, M., Davis, R.W. and Philippsen, P. (1985). Cell 42, 913-921.
17. Nakaseko, Y., Adachi, Y., Funahashi, S., Niwa, O. and Yanagida, M. (1986). EMBO J. 5, 1011-1021.
18. Nakaseko, Y., Kinsohita, N. and Yanagida, M. (1987). Nucl. Acid Res. 15, 4705-4715.
19. Clarke, L., Amstutz, H., Fishel, B. and Carbon, J. (1986). Proc. Natl. Acad. Sci. USA 83, 8253-8257.