Volume 14 Number 14 1986 Nucleic Acids Research

Part of the human ribosomal RNA locus stabilizes a plasmid in yeast

J.Whittaker, J.Lang, P.R.Cook, S.Aspinall*, S.J.McCready* and B.S.Cox*

Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE and *Department of Plant Sciences, South Parks Road, Oxford OX1 3RA, UK

Received 14 May 1986; Revised and Accepted 30 June 1986

ABSTRACT

Most yeast plasmids - particularly those containing chromosomal replicators (ARS) - are unstable and do not segregate equally to mother and daughter cells unless they contain centromeric sequences. We have screened a fraction of the human genome for sequences that stabilize YRp7, a plasmid containing ARS1. We selected a fraction which we hoped would be enriched in human centromeric sequences - the DNA attached to the nucleoskeleton. We obtained one human sequence that partially stabilized a yeast plasmid and, surprisingly, it contained sequences homologous to those coding for the 3' end of 18s rRNA, the transcribed spacer and 5' end of 28s rRNA. This sequence did not show any ARS activity nor did it increase the copy number of the plasmid and so probably improved partition of the plasmid between mother and daughter cells. It had no homology to yeast centromeres.

INTRODUCTION

Linear nuclear DNA of higher eukaryotes is organized into a series of loops by attachment to a nucleoskeleton. (For a review see ref. 1). These points of attachment are of considerable interest not only because they maintain chromosome structure but also because they may be the sites of replication, transcription and the repair of damaged DNA. (For a review, see ref. 2). Therefore it is of interest to isolate attached sequences and to investigate their functional properties.

We might expect a sub-class of these sequences to be involved in the segregation of chromosomes to daughter cells (i.e. to be centromeric sequences) and we have attempted to devise a functional assay for such sequences. Recently, sequences with similar functions have been isolated from yeast cells. Yeast centromeric sequences (CENs) were isolated using plasmids which contain a chromosomal replicator (yeast replicating plasmids) and that were lost from rapidly-dividing yeast cells: insertion of the CEN sequence into the plasmid enabled the plasmid to segregate more efficiently to the daughters so ensuring that the majority of cells in the growing population contained the plasmid in spite of a reduced copy number (for a

review see ref. 3). We might expect that human centromeres might also improve partitioning of yeast plasmids. However, their concentration in total DNA is probably too low to allow direct screening and so we selected a DNA fraction which we hoped would be enriched in centromeres. Therefore, sequences at the point of attachment to the nucleoskeleton were prepared by lysing a population of mitotic HeLa cells in a non-ionic detergent and 2M NaCl to release the nuclear matrix or cage with attached DNA. Next the bulk of the DNA was detached using the restriction endonuclease BamHI. Then the cages were pelleted free of detached fragments and the two per cent of the total DNA that cosedimented with them purified. This fraction of the total DNA, which we hope might be enriched in sequences involved in chromosome segregation, was then redigested with BamHI to ensure complete digestion and inserted into the appropriate vector. After transforming bacteria and amplification, the resulting library was screened for sequences that affected the stability of the vector in yeast cells. One such sequence has been found and characterized.

MATERIALS AND METHODS

Cage-associated DNA

HeLa cells were grown and synchronized in mitosis (> 92% mitotic) using the nitrous oxide technique of Rao (4) as described by Warren and Cook (5). Mitotic cells were lysed in 2M NaCl and Triton to release nucleoids, then these nucleoids were treated with BamHI to detach all but two percent of the DNA and the cages and any associated DNA pelleted by centrifugation. The DNA in the pellet was then purified, and recut with BamHI (6).

Recombinant DNA techniques

The yeast vector YRp7 containing ARS1 (7) was cut with BamHI, treated with phosphatase, then 200ng ligated with 1000ng of the cage-associated DNA and the ligation mixture used to transform bacteria. 20000 colonies were obtained, pooled and amplified. At least 70 percent of the resulting plasmid DNA molecules contained inserts of more than 200bp. All techniques are described in (8).

Yeast Cells

Yeast cells [strain YNN27 (ura3.52, trp1] were grown in YEPD [2% glucose, 1% Bactopeptone (Difco), 1% yeast extract (Difco)] or YNB medium [1% glucose, 0.17% yeast nitrogen base without ammonium sulphate (Difco), 0.5% $(NH_4)_2$ SO_4, 1.8% purified agar (Oxoid)] supplemented with uracil and tryptophan (50μg/ml) when necessary. Yeast cells were transformed (9) using

0.2M CsCl to treat the cells and 5-10 microgram DNA in 15-30 microlitres for each transformation. Transformation frequencies of up to 1200 transformants per microgram DNA were obtained.

Screening

40,000 yeast transformants were subjected to 3 successive screens. 4 days after transformation with the library, colonies were replica-plated on to YEPD medium, incubated for 3 days and then replica-plated on to selective medium (i.e. lacking tryptophan) and incubated for 24 hours. Isolates showing heavier growth were plated on YEPD medium to give single colonies, incubated for 3 days and then replica-plated on to selective medium and incubated for a further 24 hours. For the third screen, 67 transformants showing fewer non-growing sectors than YRp7-transformed controls were inoculated into liquid YEPD medium and grown to stationary phase (3 days). Next, the cultures were plated on to YEPD to give 100-200 colonies per plate, incubated for 3 days and then replica-plated on to selective and non-selective media. The number of colonies on selective medium divided by the number on the non-selective medium gave the percentage stability.

Plasmids were rescued from yeast using the method of Nasmyth and Reed (10).

Estimation of Plasmid Copy Number

Yeast DNA was extracted (11) using caesium chloride gradients, in which ethidium bromide was replaced by a similar concentration of Hoechst 33258 (12). In these conditions, plasmid DNA and chromosomal DNA co-sediment. The purified DNA was restricted with *HindIII* and analysed by gel electrophoresis. Southern transfers were probed with ^{32}P-labelled YIp5 or Scp25 DNA. The former carries the yeast *URA3* gene cloned in pBR322 and hybridises to the pBR322 portion of the plasmids under study and to the single copy of the *URA3* gene in chromosome V. The latter plasmid, Scp25, contains the 2.6kb *HindIII* fragment of the rDNA repeat (13). The autoradiograms were scanned using a Joyce-Loebl densitometer, and the relative amounts of DNA in the bands containing the pBR322 and chromosomal genes estimated by weighing the peaks from the traces. Average copy-number was calculated using: weight of plasmid peak x Size (r) x N(r)/weight of reference peak x Size (p), where Size is the number of kilobase pairs of regions of homology between the reference gene (r) or the plasmid (p) and the probe and N(r) is the copy-number of the reference gene in the yeast genome. This was taken to be one for *URA3* and 100 for rDNA (14). A range of exposures was used to ensure that the densities of both bands being

measured fell into a linear range of responses for the densitometer. In a second experiment, DNA was extracted by a slightly different protocol and different restriction enzymes (BamHI + BglII) and probes were used (YRp7). In this case the reference gene was TRP1, which also appears in the plasmid, but on a different BamHI/BglII fragment.

A sample of each culture was taken before extracting the DNA and the fraction of the cell population containing plasmids was estimated by plating on selective and non-selective medium (YNB with or without tryptophan).

Blot hybridization

All procedures are described by Cook and Brazell (6).

RESULTS

The Library

The vector chosen for the construction of the library was YRp7 (7). This contains the yeast ARS1 and TRP1 genes and transforms trp1 yeast strains very efficiently because it replicates autonomously within them. However, it segregates poorly to daughter cells and is lost rapidly from populations grown on non-selective media. Insertion of a functional yeast centromere enables the plasmid to segregate more efficiently so that most transformants retain the plasmid even after extensive culture under non-selective conditions (15). The mitotic stabilization induced by the centromeric DNA enabled the direct selection of centromeric sequences from genomic libraries constructed using YRp7 (3).

Human sequences for the library were obtained by detaching all but 2% of nuclear DNA from mitotic HeLa nucleoids with BamHI (6). This DNA was recut with BamHI and inserted into the vector. After transforming bacteria, the resulting library containing human cage-associated sequences was used to transform trp1 yeast cells to tryptophan independence and the resulting transformants subjected to 3 successive screens to see how stable their new phenotype was. 67 colonies that survived the screen were grown in liquid culture containing tryptophan and then samples plated on selective and nonselective media. The number of colonies on the selective plates expressed as a percentage relative to those on nonselective plates gives an estimate of the stability of the plasmid (Table 1). As expected, the vector is rapidly lost from the liquid cultures so that only 1.6% of the colonies retain it. Insertion of the authentic yeast centromere from chromosome III to give plasmid pMA9 (A.J. Kingsman, personal communication) increases the stability to 62%. These two extreme values provide references with which to

TABLE I. The stabilities of transformants containing various plasmids. The size of BamH1 fragments of some of the plasmids is given in kilobase pairs.

Plasmid	% stability	Fragment sizes
YRp7	1.6	5.7
pMA9	62	
207	13	8.0
213	15	5.3, 5.7
1113	20	1.0, 3.0
1619	20	3.0, 9.0
3906	37	3.0, 4.0
3/p213	18	
21/p213	10	
16/p213L	17	
19/p213L	10	
20/p213S	2	
24/p213S	2	

judge the stabilities of the various isolates (Table II). The phenotype of the majority of isolates was completely stable and as all attempts to rescue plasmids from these failed, they were assumed to contain vector sequences integrated into the chromosome or to be revertants. Of the 12 isolates with intermediate stability (6-37%) seven had no detectable inserts. Fragment sizes of the remaining 5 isolates obtained after digestion with BamHI are given in Table I. Since isolate 213 was the only one which gave the expected restriction pattern of vector plus insert, it was characterized further.

Characterization of p213

To be sure that the increased stability was due to the presence of the plasmids and not because of a change in the host cell, the plasmid contained in isolate 213 (i.e. p213) was purified and used to retransformtrp1 yeast to tryptophan independence. The resulting transformants were as stable as isolate 213 (results not shown). Since a number of the stable isolates contained rearranged vector sequences (i.e. 1113, 1619, 3906: Table I), it was important to demonstrate that undetected rearrangements in the vector sequences in p213 were not responsible for the increased stability. Therefore the 5.3kb BamHI was excised from p213 and reinserted into the BamHI site of a new batch of YRp7: two of the resulting plasmids (i.e. 3/p213 and 21/p213) were tested and possessed the same stability as p213

TABLE II. The stabilities of various isolates

Number of isolates	% stability
40	100
1	37
11	6-23
15	0-5

(Table I). Since addition of a second autonomously replicating sequence (ARS) into plasmids like YRp7 can stabilise them (16), the BamHI insert was tested for its ability to promote autonomous replication and hence increase the transformation frequency of an integrating plasmid, YIp5 (17), but was found to have no effect (results not shown).

Fig. 1 illustrates a partial map of the restriction enzyme sites in the 5.3kb insert in p213. Both BamHI-XhoI fragments were inserted between the BamHI and SalI sites of YRp7 and the stabilities of the resulting plasmids showed that the stabilizing sequence resided in the larger BamHI-XhoI fragment [i.e. isolates 16/p213L and 19/p213L which contained plasmids with the large fragment were stable unlike 20/p213S and 24/p213S which contained the small fragment (Table I)].

The stabilizing sequence is part of the human ribosomal locus

We next showed that the insert contained sequences homologous to human DNA by radio-labelling p213 DNA by "nick-translation" and then hybridizing this with a "Southern" blot of human DNA that had been cut with EcoR1, HindIII or BamHI. The intensity of hybridization suggested that the homologous human sequence was repeated several hundred times in the human genome: furthermore, the pattern of hybridization was identical to that given by other probes known to contain ribosomal sequences (18-20). One example of the pattern given by a probe, pxlr101, containing ribosomal sequences from Xenopus laevis (21) - which cross hybridize with the human

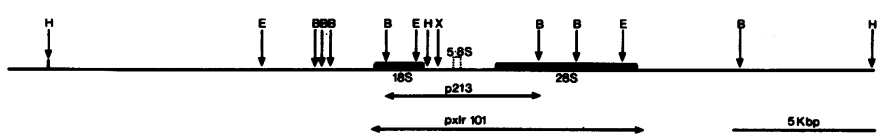

Fig. 1. A partial restriction enzyme map of the human ribosomal locus (Erickson et al., 1981). The thick black boxes give the positions of sequences coding for the 18S and 28S rRNA molecules and the dashed box that of the 5.8S molecule. Restriction sites are indicated by vertical arrows: EcoRI(E), HindIII (H), BamHI (B), XhoI (X). The homologous regions in p213 and pxlr101 are indicated.

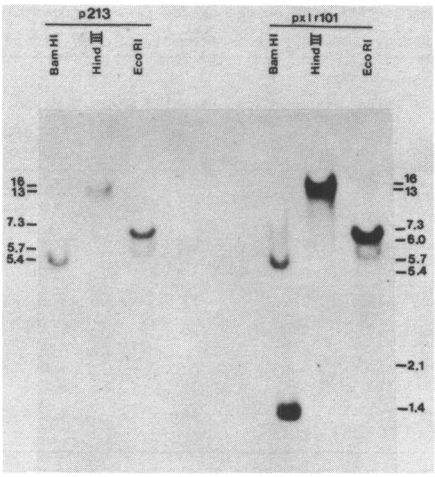

Fig. 2. Blot hybridization analysis of DNA from HeLa cells. HeLa DNA was cleaved with BamHI, HindIII orEcoRI, blotted, the filter hybridized with radio-labelled DNA from p213 or pxlr101 and an autoradiograph prepared. The sizes of the fragments are indicated in kilobase pairs.

locus - is illustrated in Fig. 2. Mapping the restriction sites in the insert in p213 and in the human ribosomal locus confirmed their similarity (Fig. 1).

Partition or Replication?

A plasmid might persist in a population because it replicates to a high copy-number, thereby increasing the likelihood of segregation to both mother and daughter during cell division or because a partition function improves the likelihood of even segregation regardless of copy-number (22, 23). We therefore compared the amount of plasmid in cultures of YNN27 transformed with YRp7 and p213 and grown under selection for tryptophan independence. The relative amounts of chromosomal and plasmid DNA from each culture were measured by comparing the hybridization of probe to a chromosomal marker and to plasmid bands on "Southern" blots of suitably restricted samples.

There was a difference in the copy-numbers determined using the multicopy (i.e. rDNA) and single-copy (i.e. URA3) internal references. [Note that accurate determinations of copy numbers are notoriously difficult]. We regard the comparisons using more nearly matched band densities as being closer to reality. In any case, the results (Table III) using either single-copy (URA3) or multicopy (rDNA) internal markers (in 3 separate experiments) showed that the average copy number of p213 never exceeded that of the parent plasmid, YRp7. However the proportion of plasmid-containing cells is different in each culture, indicating that the same number of p213 plasmids are more evenly distributed in the cell population. Considering only plasmid-containing cells, a "real" copy-number per cell is arrived at

TABLE III. The amount of plasmid DNA relative to a marker DNA, the percentage of plasmid-containing cells and estimates of average and "real" copy-numbers in transformants of YRp7 and p213

DNA from	Weight of Plasmid Peak	Weight of Reference Peak*	Size (r)	N(r)	Size (p)	Average Copy No.	% Cells with plasmid (TRP+)	Copy No./ TRP+ cell
YRp7[a]	23.2	47.25 (rDNA)	2.6	100	4.3	30	12	250
p213[a]	23.5	89.9 (rDNA)	2.6	100	4.3	16	60	26
YRp7[b]	116	6 (URA3)	1.2	1	4.3	5.4	12	45
p213[b]	63	6 (URA3)	1.2	1	4.3	3	60	5
YRp7(Bam/Bgl)[c]	34	4 (URA3)	0.8	1	1.2	6	14	43
p213(Bam/Bgl)[c]	46	9 (URA3)	0.8	1	1.2	3	74	4

See Materials and methods for definitions. *The reference gene is given in brackets. Supercripts a, b and c represent different experiments.

of between 4 and 26 for p213 in 60% of cells. This is clearly less than the 43-250 copies of YRp7 in 11.8% of cells. We conclude that the effect of the cloned fragment of human rDNA is to improve partition of YRp7 rather than to increase the copy number.

Lack of homology of p213 and centromeric DNA

10 of the 16 yeast centromeres have been cloned and sequenced (24). They share 3 common elements, an 8bp perfect homology (element I), a 78-86bp sequence containing >90% A + T (element II) and a 25bp sequence with a 9bp dyad symmetrical unit (element III). Some centromeres differ by up to 10bp from the consensus sequence for element III. We searched the EMBL and GENBANK data bases - which contain sequences for the yeast 2μ circle and the ribosomal DNA from a number of different species but not a complete human sequence - for homology to element I (UTCACUTG, where U is either purine) and element III ($TGTTT^{T}/_{A}TG$. TTTCCGAAA....AAA) but none was found except for yeast centromeres. [Note that the human ribosomal locus is heterogeneous (25)]. 37 sequences had complete homology to element I alone, but none were ribosomal or were adjacent to AT rich regions. 26 sequences had homology to element III if we allowed 4 mismatches, but none of these had adjacent AT rich regions and only two were ribosomal (i.e. Paramecia mitochondrial rDNA). Although the transcribed rDNA spacer of higher eukaryotes is AT rich, it seems unlikely that we have fortuitously selected from the human genome a sequence homologous to a yeast centromere.

DISCUSSION

Most yeast plasmids are unstable. The single exception is dsRNA which is present in very high copy-numbers. Low copy-number does not account for the instability of any yeast plasmid (24). After growth in selection medium the real copy-number of YRp7, one of the most unstable of them, has been variously estimated at between 20 and 250 (26: this study). [We do not know what gives rise to the different estimates, but it may result from a differential extraction of rDNA and/or plasmid DNA]. With a copy-number of 10 at the time of division, the probability of non-segregation is 2^{-10}, assuming every plasmid has an equal probability of partition to mother or daughter cells. The observed probability of non-segregation of YRp7 to one of the products of cell division is 0.9 (Table III). Clearly, the partition of circular yeast DNA plasmids is severely impaired. This is true even of the native 2μm circle, which has a copy-number of about 70 (27), and does apparently code for functions which improve partition (23, 28).

We have found a human sequence that partially stabilizes YRp7. The stabilizing sequence turned out to be part of the ribosomal locus containing sequences homologous to those coding for the 3' end of 18s rRNA, the transcribed spacer and 5' end of 28S rRNA. [We have shown previously that this region of the ribosomal locus is enriched in the fraction that resisted detachment from cages (29)]. In principle this sequence might stabilize the plasmid by increasing its copy-number or by improving its segregation. However, although other parts of the ribosomal locus in other species can act as an ARS (30), it lacks ARS activity and it does not increase copy-number (Table III) so must improve segregation. However, it has little homology with yeast centromeres. As discussed above, yeast plasmids are defective in partition and are not randomly distributed in the nucleus or cytoplasm at the time of division. It may be that release from attachment to a non-segregating structure is the critical event in improving plasmid segregation - and hence stability - in yeast.

Finally, it is interesting to note that ribosomal genes can exist as extrachromosomal circular plasmids in many unicellular eukaryotes (31) and during oogenesis of some higher eukaryotes (e.g. amphibians, fishes and insects; (32). Whether or not the sequence that we have isolated can stabilize plasmids in higher cells remains to be established and clearly it would be of great interest if it did.

ACKNOWLEDGEMENTS

We thank the Cancer Research Campaign for support.

REFERENCES

1. Hancock, R. (1982). Biol. Cell. 46, 105-122.
2. Jackson,D.A., McCready, S.J. and Cook, P.R. (1984). J.Cell.Sci. Suppl. 1, 59-79.
3. Carbon, J. (1984). Cell 37, 351-353.
4. Rao, P.N. (1968). Science N.Y. 160, 774-776.
5. Warren, A.C. and Cook, P.R. (1978). J. Cell Sci. 30, 210-226.
6. Cook, P.R. and Brazell, I.A. (1980). Nucl. Acids Res. 8, 2895-2906.
7. Stinchcomb, D.T., Struhl, K. and Davis, R.W. (1979). Nature 282, 39-43.
8. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). Molecular cloning: a laboratory manual. Cold Spring Harbour Laboratory.
9. Ito, H., Fukada, Y., Murata, K. and Kimura, A. (1983). J. Bact.153, 163-168.
10. Nasmyth, K.A. and Reed, S.I. (1980). Proc. Natl. Acad. Sci. U.S.A. 77, 2119-2123.
11. Cryer, D.R., Eccleshhall, R. and Marmur, J. (1975). In Methods in Cell Biology XII, 39-44 ed. Prescott, D.M., Academic Press, London.
12. Williamson, D.H. and Fennell, D.J. (1975). In Methods in Cell Biology XII, 335-351, ed. Prescott, D.M. Academic Press, London.
13. Larionov, V.L. and Grishin, A.V. (1981). Molekulyarnaya Biologiya 14, 1262-1266.
14. Petes, T.D. and Botstein, D. (1977). Proc. Natl. Acad. Sci. U.S.A. 74, 5091-5095.
15. Clarke, L. and Carbon, J. (1980). Nature 287, 504-509.
16. Kouprina, N.Y. and Larionov, V.L. (1983). Curr. Gen. 7, 433-438.
17. Struhl, K., Stinchcomb, D.T., Scherer, S. and Davis, R.W. (1979). Proc. Natl. Acad. Sci. U.S.A. 1035-1039.
18. Arnheim, N. and Southern, E.M. (1977). Cell 11, 363-370.
19. Erickson, J.M., Rushford, C.L., Dorney, D.J., Wilson, G.N. and Schmickel, R.D. (1981). Gene 16, 1-9.
20. Miesfield, R. and Arnheim, N. (1982). Nucl. Acids. Res. 10, 3933-3949.
21. Trendelenburg, R. and Gurdon, J. (1978). Nature 276, 292-294.
22. Futcher, A.B. and Cox, B.S. (1984). J. Bact. 157, 283-290.
23. Murray, A.W. and Szostak, J.W. (1983). Cell 34, 961-970.
24. Hieter, P., Pridmore, D., Hegemann, J.H., Thomas, M., Davis, R.W. and Philippson, P. (1985). Cell 42, 913-921.
25. Gonzalez, I.L., Gorski, J.L., Campen, T.J., Dorney, D.J., Erickson, J.M., Sylvester, J.E. and Schmickel, R.D. (1981). Proc. Natl. Acad. Sci. U.S.A. 82, 7666-7670.
26. Hyman, B.C., Cramer, J.H. and Round, R.H. (1982). Proc. Natl. Acad. Sci. U.S.A. 75, 1578-1582.
27. Gerbaud, C. and Guerineau, M. (1980). Curr. Genet. 1, 219-228.
28. Futcher, A.B. and Cox, B.S. (1983). J. Bact. 154, 612-622.
29. Jackson, D.A., Cook, P.R. and Patel, S.B. (1984). Nucl. Acids Res. 12, 6709-6726.
30. Amin, A.A. and Pearlman, R.E. (1986). Nucl. Acids Res. 14, 2749-2762.
31. Ravel-Chapuis, P., Nicholas, P., Nigon, V., Negret, O. and Freyssinet, G. (1985). Nucl. Acids. Res. 13, 7529-7537.
32. Long, E.O. and David, I.B. (1980). Ann. Rev. Biochem. 49, 727-764.