# *Aha*III: A restriction endonuclease with a recognition sequence containing only A:T basepairs

Philip R. Whitehead and Nigel L. Brown

*Department of Biochemistry, University of Bristol, University Walk, Bristol BS8 1TD, England*

Received 19 May 1982

*Cyanobacteria* *Aphanothece* *DNA-sequencing* *Site-specific endonuclease* *A:T-rich DNA*

## 1. INTRODUCTION

Over the last decade a large number of site-specific endodeoxyribonucleases has been isolated from a variety of bacterial genera [1]. These enzymes are widely used in the characterization and restructuring of DNA molecules and they are being increasingly used as model systems to study DNA–protein interactions.

Here, we describe the identification of a class II restriction endonuclease, *Aha*III, from the cyanobacterium, *Aphanothece halophytica*, and the determination of its recognition sequence. This is the symmetrical hexanucleotide sequence:

$$5'\text{–T–T–T}\overset{\downarrow}{}\text{A–A–A–}3'$$

$$3'\text{–A–A–A}\underset{\uparrow}{}\text{T–T–T–}5'$$

This is the first restriction endonuclease described that contains only A:T basepairs in its recognition sequence.

## 2. MATERIALS

### 2.1. *Enzymes*

*Aha*III was isolated from packed cells of *Aphanothece halophytica* kindly provided by Professor A.E. Walsby. These had been grown at 32°C in ASN-III medium [2] modified to include 12.5% (w/v) NaCl, 1% (w/v) $MgCl_2 \cdot 6\ H_2O$ and 0.25% (w/v) KCl, under low light intensity ($\sim$ 500 lux). *Aha*III was partially purified until free of contaminating nuclease activities by chromatography on Sepharose 6B, phosphocellulose P11, heparin–Sepharose and DEAE-cellulose by standard methods [3]. *Aha*III activity eluted from phosphocellulose at 0.3–0.4 M KCl, from heparin–Sepharose over 0.1–0.45 M KCl, and from DEAE-cellulose over 0.35–0.6 M KCl. Full details of the purification of *Aha*III and of other restriction endonucleases in *A. halophytica* will be presented elsewhere, as they are currently being improved. In two separate preparations, the yield of *Aha*III varied from 25–200 units/l original culture.

*Hgi*AI was prepared as in [3]. *Hpa*II and *Sau*3AI were purchased from New England Biolabs Inc. DNA polymerase I fragment A (Klenow enzyme) was purchased from Boehringer Corp. and T4 DNA polymerase was obtained from PL Lab.

### 2.2. *DNA and reagents*

Plasmid DNAs (pBR322, pA03) were isolated from *Escherichia coli* C600 strains after chloramphenicol-enrichment. Bacteriophage M13*mp*7 RFI DNA and $\phi$X174–*am3cs*70 RFI DNA were prepared from infected *E. coli* cells. All covalently-closed circular DNAs were purified by CsCl–ethidium bromide isopycnic centrifugation of cleared lysates. Bacteriophage $\lambda$*c*I857*Sam*7 DNA was prepared as in [3]. Adenovirus-2 DNA was a kind gift of Dr Janet Arrand.

Deoxyadenosine [$\alpha$-$^{32}$P]triphosphate (>600 Ci/nmol) was obtained from New England Nuclear GmbH. All other chemicals were obtained from the suppliers in [3].

*Published by Elsevier Biomedical Press*

## 3. METHODS AND RESULTS

### 3.1. *Optimal conditions for* Aha*III activity*

The temperature and ionic strength optima for *Aha*III were assayed as described for other enzymes [3], using 1 μg bacteriophage λ DNA as substrate in 20 μl buffer containing 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 10 mM Tris–HCl, (pH 7.9, adjusted at 20°C). Maximal *Aha*III activity occurred at 37–40°C in a buffer containing 80 mM NaCl, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 10 mM Tris–HCl (pH 7.9) (A3 buffer; data not shown).

### 3.2. *Preliminary mapping of the cleavage sites*

All *Aha*III digests were performed in A3 buffer at 37°C. Digestions with *Hgi*AI were done in the same buffer, and in *Hgi*AI/*Aha*III double digests both enzymes were present simultaneously. Digestion of DNA with *Sau*3AI was performed in the buffer recommended by the suppliers. *Sau*3AI/*Aha*III double digests were performed by treating the DNA separately with each enzyme in the appropriate buffer, the DNA being ethanol-precipitated between each digest.

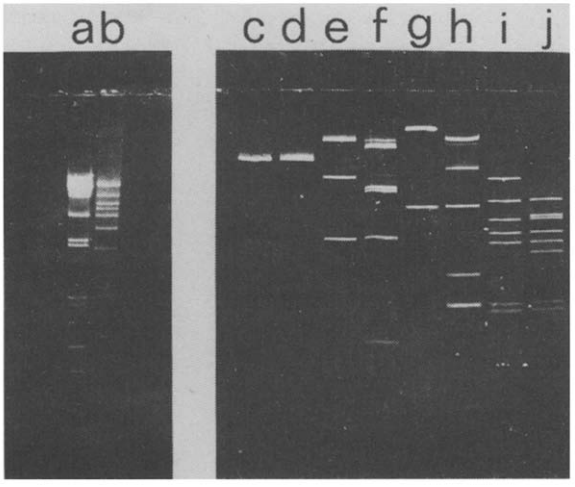


Fig.1. Gel electrophoretic separation of the products of digestion of: (a) bacteriophage λ DNA with *Aha*III; (b) adenovirus-2 DNA with *Aha*III, on a 1% agarose slab gel; of (c,d) pAO3 DNA, (e,f) φX174 RFI DNA, (g,l) M13*mp*7 RFI DNA, (i,j) pBR322 DNA, with *Hgi*AI (c,e,g,i) or *Hgi*AI and *Aha*III (d,f,h,j) on a 5% acrylamide slab gel. The gels were run, stained and photographed by standard methods [3].

Fig.1 shows the *Aha*III cleavage patterns of a number of DNAs. These data show that *Aha*III has ~ 13 sites on bacteriophage λ DNA, and > 16 sites on adenovirus-2 DNA. Double-digest experiments with *Hgi*AI show that the minimum number of sites on the following, fully sequenced DNAs are: pAO3 DNA, no sites; φX174 RFI DNA 2 sites; M13*mp*7 RFI DNA, 5 sites (one band in fig.1 h is double intensity and therefore contains two fragments); pBR322 DNA, 1 site.

Further mapping on pBR322 DNA indicates that there is >1 site on this plasmid (fig.2). *Sau*3AI

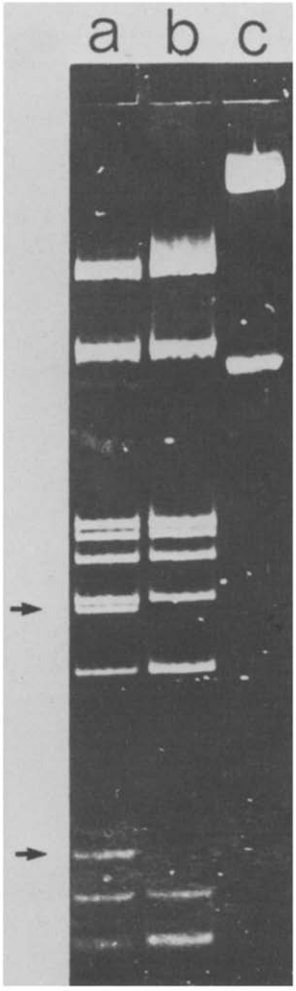


Fig.2. Gel electrophoretic separation of the products of digestion of pBR322 DNA with (a) *Sau*3AI, (b) *Sau*3AI and *Aha*III, (c) *Aha*III, on a 5% acrylamide slab gel.

fragment 7 (258 basepairs) is cleaved to give a fragment ~ 210 basepairs long; and *Sau*3AI fragment 9 (105 basepairs) is also cleaved. Digestion of pBR322 with *Aha*III alone yields a fragment of ~690 basepairs (the size of this fragment was determined on agarose gels; it has an anomalous mobility on acrylamide gels).

### 3.3. *Determination of the cleavage specificity of* Aha*III*

The determination of a complete map of sites for a new restriction enzyme is not a prerequisite for the characterization of the recognition and cleavage specificities of the enzyme [4]. The data in fig.2 demonstrate that the *Aha*III sites in pBR322 *Sau*3AI fragments 7 and 9 lie within 55 nucleotides of one end of each fragment.

*Sau*3AI fragments 7 and 9 were separately excised from a gel similar to that shown in fig.2(a), and were eluted by soaking in 4 gel vol. of 500 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% (w/v) SDS at 37°C for 5 h. Gel fragments were removed by filtration; the DNA was precipitated with ethanol and resuspended in water at ~ 0.01 pmol/μl. A ligation mixture (10 μl) containing 0.02 pmol fragment, 20ng *Bam*HI-cut M13*mp*7 RFI DNA, 10 nmol ATP, 100 nmol $MgCl_2$, 500 nmol NaCl, 10 nmol DTT, 100 nmol Tris–HCl (pH 7.5), 1 μg bovine serum albumin (nuclease-free) and 0.1 unit T4 DNA ligase was incubated at 14°C for 6 h. Competent *E.coli* 71–18 cells [5] were prepared by the $CaCl_2$ technique [6], and 200 μl aliquots of cells were transfected with ⅓rd of each ligation mixture. Plaques of transfected

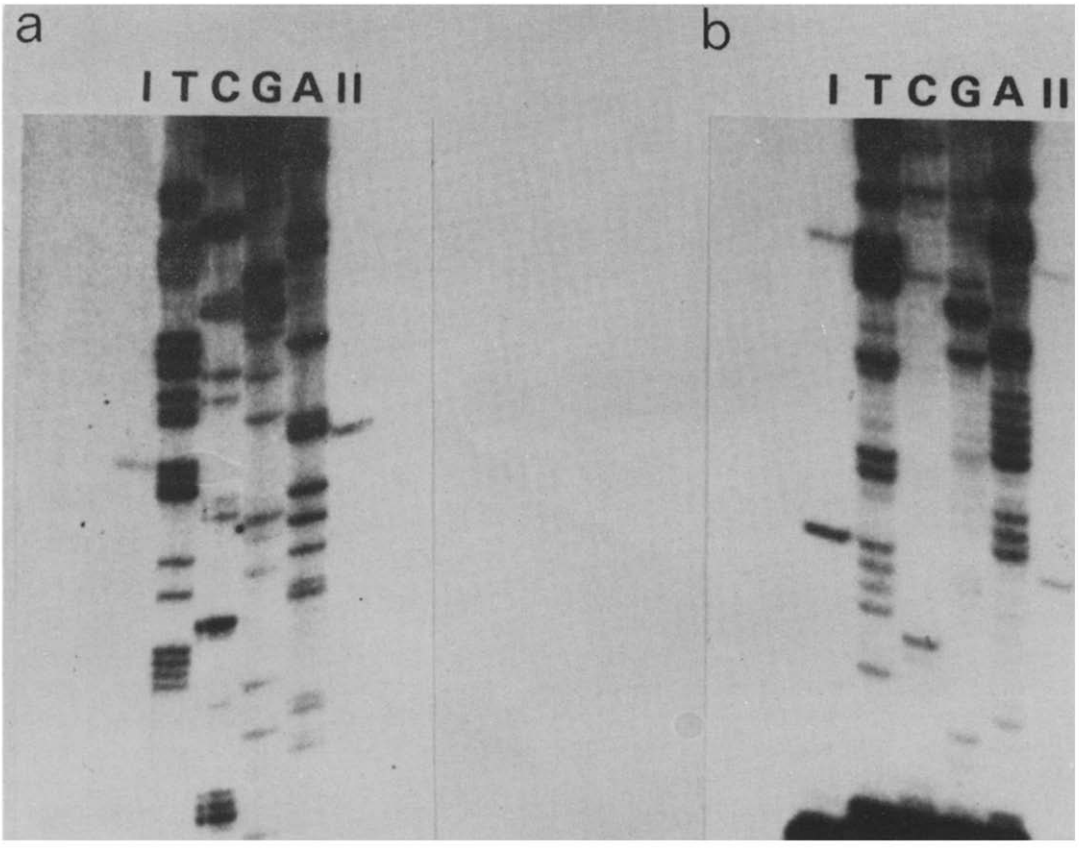


Fig.3. Autoradiographs of sequencing gels locating the *Aha*III cleavage sites in pBR322 DNA: (a) *Sau*3AI fragment 7; (b) *Sau*3AI fragment 9. Channel I locates the phosphodiester bond cleaved in the sequenced strand; channel II locates that in the complementary (template) strand.

(a) 5'-G-A-G-C-A-C-T-T-T-T-A-A-A-G-T-T-C-T-G-C-3'
3'-C-T-C-G-T-G-A-A-A-A-T-T-T-C-A-A-G-A-C-G-5'

(b) 5'-A-G-A-T-C-C-T-T-T-T-A-A-A-T-T-A-A-A-A-A-3'
3'-T-C-T-A-G-G-A-A-A-A-T-T-T-A-A-T-T-T-T-T-5'

(c) 5'-A-T-G-A-A-G-T-T-T-T-A-A-A-T-C-A-A-T-C-T-3'
3'-T-A-C-T-T-C-A-A-A-A-T-T-T-A-G-T-T-A-G-A-5'

Fig.4. DNA sequences around the *Aha*III cleavage sites in pBR322 DNA. The top strand in each case shows the sequence read from the autoradiographs in fig.3: (a) *Sau*3AI fragment 7 site; (b) *Sau*3AI fragment 9, primer-proximal site; (c) *Sau*3AI fragment 9, primer- distal site.

cells containing recombinant M13*mp*7 DNA carrying a cloned pBR322 DNA fragment identified by their *lac*$^-$ phenotype [5,7]. Single stranded recombinant DNA from each plaque was prepared as in [7].

The cloned pBR322 fragments were sequenced by the chain-termination method [8], using a 27-basepair primer (the *Eco*RI–*Eco*RI primer in [9]. The sites of cleavage of *Aha*III in both strands of DNA were determined as in [10]. The results of these site-location experiments are shown in fig.3.

The site-location experiment for the recombinant containing pBR322 *Sau*3AI fragment 9 (fig.3b) was performed under conditions which gave only partial digestion of DNA at *Aha*III cleavage sites. This allowed a second, primer-distal *Aha*III site to be demonstrated. This second site had been suspected from the results of an initial experiment (not shown) which identified the primer-proximal *Aha*III site, but showed an identical octanucleotide sequence 19 basepairs away. The DNA sequences around the 3 *Aha*III sites are shown in fig.4.

## 4. DISCUSSION

From the data in fig. 4 the recognition and cleavage site of *Aha*III was predicted to be:

5′–T–T–T↓A–A–A–3′

3′–A–A–A–T↑–T–T–5′

The 3 sites also show a further homologous nucleotide – a T preceding the hexanucleotide. This was not included in the predicted recognition site, as the majority of type II restriction endonucleases recognise 2-fold rotationally-symmetrical sites, and cleave these symmetrically. No other DNA sequence within 20 nucleotides either side of the 3 *Aha*III cleavage sites on pBR322 DNA is found at all 3 sites.

The predicted recognition sequence was tested by comparing the predicted and actual numbers and sizes of DNA fragments produced by *Aha*III on a variety of fully-sequenced DNA molecules [11–16]. The predictions were made using a modification of the SEARCH program in [17], and they match with the data shown in fig.1,2. The predicted number of sites for *Aha*III are: bacteriophage λ DNA, 13 sites; pAO3 DNA, no sites; φX174 RFI DNA, 2 sites; M13*mp*7 RFI DNA, 5 sites) pBR322 DNA, 3 sites; SV40 DNA, 12 sites.

The method used to determine the recognition and cleavage specificity of *Aha*III was very rapid, and offers 3 advantages over computer methods which fit the cleavage pattern to the DNA sequence in order to predict the recognition site [18]:

(i) The site of cleavage is determined, so the types of fragment termini generated by the enzyme are known. This information is important in the potential applications of the enzyme to genetic engineering methods, for example.

(ii) The method can be applied to enzymes which recognise families of related sequences (e.g., *Hgi*AI [3] or which cut away from their recognition sites and may give non-stoichiometric products (e.g., *Mbo*II [19]).

(iii) The method can be applied to any piece of DNA, and can be used with enzymes which fail to cut DNAs of known sequence sufficiently often.

*Aha*III will be of use in the analysis of A:T-rich DNA, and in generating specific large fragments of G:C-rich DNA. Poly(dA) 'tailing' of *Aha*III-generated DNA fragments, and subsequent cloning into dT-tailed vectors will regenerate the *Aha*III sites at both ends of the fragment.

This is the first restriction endonuclease reported that has only A:T basepairs in its recognition sequence, whereas those containing only G:C basepairs are quite common [1]. The reasons for this are not known, and there is no obvious relationship

between the (G + C) content of a bacterial DNA and the recognition specificity of its restriction endonuclease. Apart from some reported reduction in the specificity of the *Eco*RI restriction enzyme [20], the only other site-specific nuclease known have an all A:T basepair recognition sequence is the *tnpR* gene product of the transposon Tn3 [21]. Other restriction enzymes with all A:T recognition sites will certainly be discovered.

## ACKNOWLEDGEMENTS

We are grateful to Professor A.E. Walsby and Annette Bees for supplying cells, and to Dr Janet Arrand for adenovirus-2 DNA. This work was supported by a Medical Research Council grant (G. 978/1047/CB) to N.L.B. who is a Royal Society EPA Cephalosporin Fund Senior Research Fellow.

## REFERENCES

[1] Roberts, R.J. (1982) Nucleic Acids Res. 10, r117–r144.
[2] Rippka, R., Deruelles, J., Waterburg, J.B., Herdman, M. and Stanier, R.Y. (1979) J. Gen. Microbiol. 111, 1–61.
[3] Brown, N.L., McClelland, M. and Whitehead, P.R. (1980) Gene 9, 49–68.
[4] Brown, N.L. (1980) Biochem. Soc. Trans. 8, 398–399.
[5] Messing, J., Gronenborn, B., Muller-Hill, B. and Hofschneider, P. (1977) Proc. Natl. Acad. Sci. USA 74, 3642–3646.
[6] Dagert, M. and Ehrlich, S.D. (1979) Gene 6, 23-28.
[7] Sanger, F., Coulson, A.R., Barrell, B.G., Smith, A.J.H. and Roe, B.A. (1980) J. Mol. Biol. 143, 161–178.
[8] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[9] Anderson, S., Gait, M.J., Mayol, L. and Young, I.G. (1980) Nucleic Acids Res. 8, 1731–1745.
[10] Brown, N.L. and Smith, M. Methods Enzymol. 65, 391–404.
[11] Sanger, F., Coulson, A.R. and Hong, G.F. (1982) personal communication.
[12] Oka, A., Nomura, N., Monta, M., Sugisaki, H., Sugimoto, K. and Takanami, M. (1979) Mol. Gen. Genet. 172, 151–159.
[13] Sanger, F., Coulson, A.R., Friedmann, T., Air, G.M., Barrell, B.G., Brown, N.L., Fiddes, J.C., Hutchison, C.A. iii, Slocombe, P.M. and Smith, M. (1978) J. Mol. Biol. 125, 225–246.
[14] Messing, J. (1982) personal communication.
[15] Sutcliffe, J.G. (1979) Cold Spring Harb. Symp. Quant. Biol. 43, 77–90.
[16] Fiers, W., Contreras, R., Haegeman, G., Rogiers, R., Van de Voorde, A., Van Heuverswyn, H., Van Herreweghe, J., Volckaert, G. and Ysebaert, M. (1978) Nature 273, 113–120.
[17] Staden, R. (1977) Nucleic Acids Res. 4, 4037–4051.
[18] Gingeras, T.R., Milazzo, J.P. and Roberts, R.J. (1978) Nucleic Acids Res. 5, 4105–4127.
[19] Brown, N.L., Hutchison, C.A. iii and Smith, M. (1980) J. Mol. Biol. 140, 143–148.
[20] Woodbury, C.P. jr, Hagenbuchle, O. and Von Hippel, P.H. (1980) J. Biol. Chem. 255, 11534–11546.
[21] Reed, R.R. and Grindley, N.D.F. (1981) Cell 25, 721–728.