A NEW SPECIFIC ENDONUCLEASE FROM *ANABAENA VARIABILIS*

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1. Introduction

A large number of site-specific endonucleases have been isolated from prokaryotes. Two such enzymes have been isolated from *Anabaena variabilis* [1] and we now describe a third from this organism, *Ava* III, which cuts SV40 DNA three times. From the positions of these breaks on the physical map of SV40, the DNA sequence recognised by the enzyme can be deduced. It has not yet been possible to separate this enzyme from *Ava* I.

2. Materials and methods

2.1. *Culture of the organism*

The *Anabaena variabilis* strain was obtained from the laboratory of Professor K. Murray (Dept. Molecular Biology, University of Edinburgh). Two different culture media were used: that in [2] or in [3]. The cultures were illuminated with normal fluorescent light and aerated with a mixture of CO_2 + air (5%, 95%). In order to obtain cultures uncontaminated by bacteria, the algae were also grown in Fernbach flasks without aeration. In these conditions, the algae grew fairly well and 1 g pellet was harvested by centrifugation from 250 ml after 10 days culture. Each flask was checked for bacterial contamination.

2.2. *DNAs and restriction endonucleases*

In order to determine the specificities of the endonucleases prepared from *A. variabilis*, SV40 DNAs obtained from strains 776 (a gift from W. Fiers, State University of Ghent), SP [4], and VA 45-54 (a gift from P. Tegtmeyer, State University of New York), λ plac DNA (a gift from A. Spassky, Institut Pasteur, Paris), Ad2 DNA (a gift from E. Lukanidin, CSH Laboratory, USA), and ΦX174 DNA (a gift from J. Fiddes, MRC Laboratory of Molecular Biology, Cambridge) were used.

Hae III, *Eco* RII and *Hin*dIII were used to obtain markers of known lengths and the electrophoreses were performed in agarose slab gels [5] or in polyacrylamide slab gel [6].

The gels were stained for 30 min in a solution of 0.4 μg/ml of ethidium bromide, illuminated with short-wavelength ultraviolet light and photographed on Polaroïd Type 665 film.

3. Results

3.1. *Detection of a new site-specific endonuclease from* A. variabilis

While preparing *Ava* I by the procedure developed [1], a new activity was separated during the phosphocellulose chromatography step. When the fractions containing *Ava* I activity obtained after DEAE-cellulose chromatography were loaded onto the phosphocellulose column, an extra activity was recovered in the flow through, while *Ava* I remained partly bound to the phosphocellulose and eluted at ~0.2 M NaCl in a linear gradient from 0–0.5 M in phosphocellulose buffer.

The comparison between the products of hydrolysis of SV40 obtained with these two fractions left no

Nomenclature: R. Roberts, W. Szybalski and the authors have decided to call this enzyme *Ava* III rather than *Ava* X which is the name used in previous papers

doubt that the flow through activity had a different specificity than *Ava* I (fig.1). It was not the same as *Ava* II since the patterns obtained were also different (fig.2).

In order to ensure that this specific endonucleolytic activity originated from the algae, the bacteria which were occasionally found at relatively low titers ($<10^{-7}$–10^{-3} bacteria/ml pellet) were isolated, identified and grown. None of them gave any activity comparable to that of the phosphocellulose flow

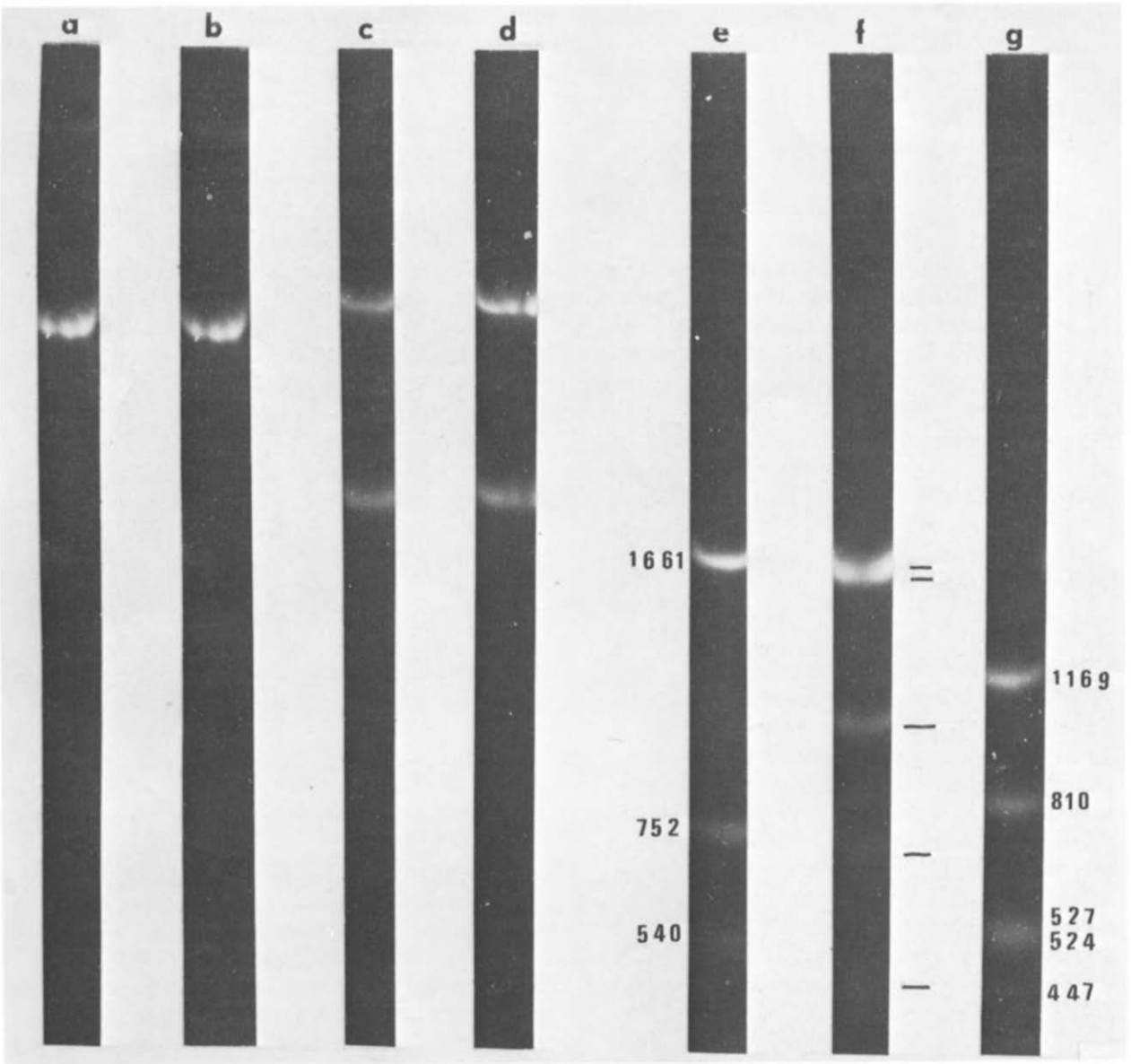


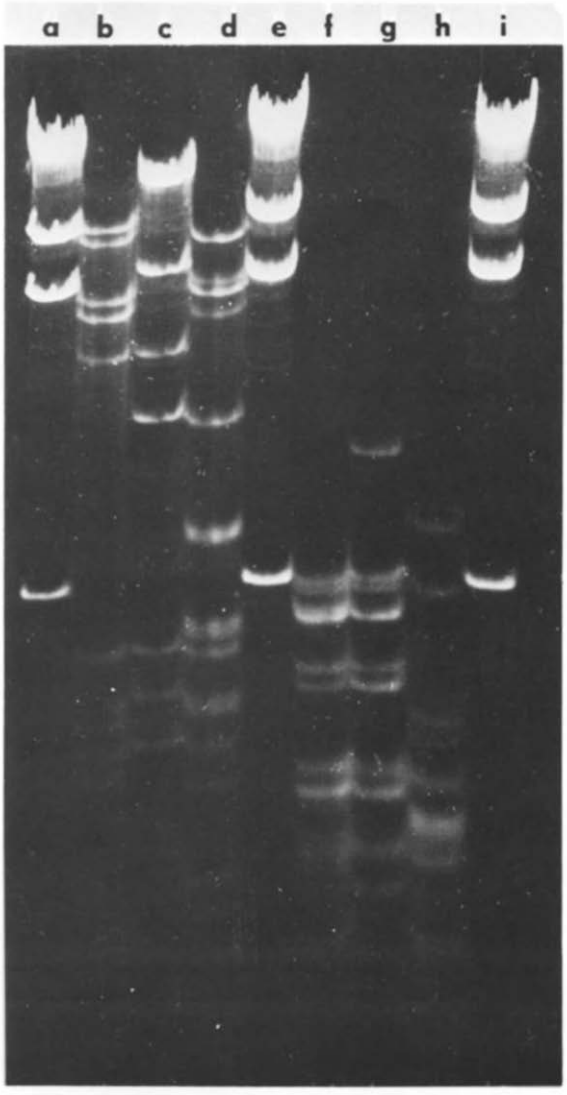
Fig.1. Hydrolysis of SV40 DNA by the *A. variabilis* specific endonucleases. Complete digestion of SV40 DNA (0.5–1 μg) was carried out at 37°C in 40 mM Tris (pH 7.5), 50 mM NaCl, 10 mM β-mercaptoethanol, 10 mM $MgCl_2$, with: (a) no enzyme; (b) *Ava* I; (c) the extra activity; (d) the extra activity plus *Ava* I. The lengths, in nucleotide pairs, of the largest fragment product by *Hae* III (e) and *Eco* RII (g) are shown. The fragments obtained with *Ava* II (f) are also shown. A third fragment in lanes (c,d) has run off the 1.4% agarose gel used for the electrophoresis.

through after the DEAE-cellulose chromatography step of the *Ava* I preparations. Furthermore, when the algae used for the enzyme extraction were grown in strictly sterile conditions as above, the same activity was still detectable. We concluded, therefore, that this specific activity, called *Ava* III, originates from *A. variabilis*.

3.2. *Comparison of the digestion products of* λ *plac and Ad2 DNAs by* Ava *I,* Ava *II and* Ava *III*

The fragments obtained by digestion of λ plac DNA and Ad2 DNA with *Ava* I, *Ava* II and the new site-specific endonuclease are shown in fig.2. Double digests of these DNAs with this new activity and *Ava* I gave patterns which were identical to those obtained with the new activity alone (results not shown). It was therefore concluded that the new activity was in fact a mixture of *Ava* I and *Ava* III.

In spite of many attempts using aminopentyl–Sepharose, DEAE-cellulose, phosphocellulose, DNA–agarose and CM-cellulose chromatography, it has not yet been possible to separate *Ava* III from *Ava* I.



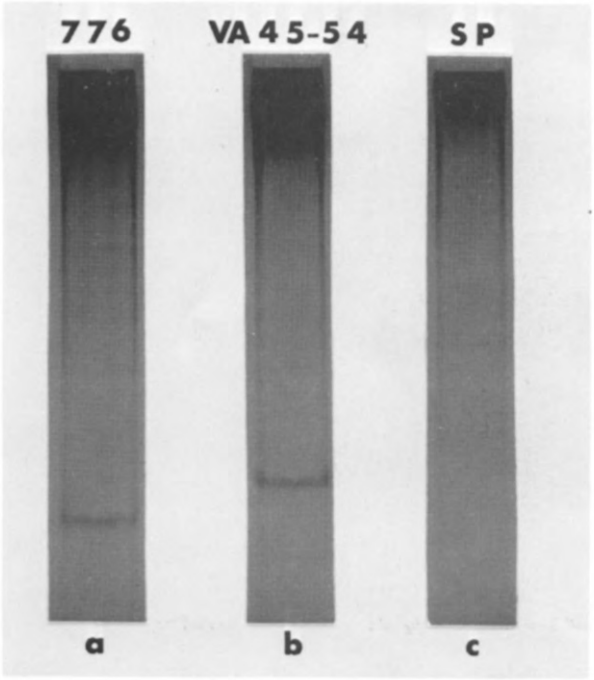


Fig.3. Hydrolysis of SV40 DNA by *Ava* III. SV40 DNA (15 μg) was incubated in the same conditions as in fig.1 with *Ava* III: (a) strain 776; (b) strain VA 45-54; (c) strain SP. The different samples were separated by electrophoresis on a 6.5% polyacrylamide slab gel. (In independent experiments it has been shown that 30 basepairs is the limit length of the fragments which would be detected on such a gel.)

Fig.2. Hydrolysis of λ plac and Ad2 DNAs by the various *A. variabilis* specific endonucleases. λ plac or Ad2 DNA (~1 μg) were incubated in the same conditions as in fig.1. In lanes (a,e,i) are markers obtained by digestion of λ plac DNA with *Hin*dIII. In lanes (b,c,d), λ plac DNA hydrolysed, respectively, by the extra activity, *Ava* I and *Ava* II and in lanes (f,g,h) Ad2 DNA hydrolysed, respectively, by the same enzymes.

3.3. *Location of the* Ava *III sites on the physical map of SV40 DNA*

Ava III digestion of SV40 DNA, strain 776, produces three fragments. The two larger fragments *Ava* III A and *Ava* III B can be clearly separated on 1.4% agarose gels (fig.1c). The very small fragment, *Ava* III C, can be detected on 6.5% polyacrylamide gels (fig.3a). The approximate lengths of these three fragments are, respectively, 3300, 1800 and 50 basepairs.

We therefore conclude that the *Ava* III enzyme recognizes at least three sites on SV40 DNA, two of which are separated by only 50 basepairs in strain 776.

Gel electrophoresis on 5% polyacrylamide gels of double digests of SV40 DNAs from strains 776, VA 45-54 and SP by *Hin*dII + III and *Ava* III produce the band patterns shown in fig.4b,d,f. Comparison of these patterns with those obtained after digestion with *Hin*dII + III alone (fig.4a,c,e) clearly shows that the only two *Hin*dII + III fragments which are affected by *Ava* III digestion are fragments *Hin*d C and *Hin*d I. Identification of fragment *Hin*d C as one of the fragments containing at least one *Ava* III site was facilitated by the fact that the *Hin*d C fragments derived from strains 776, VA 45-54 and SP are of different lengths. In particular *Hin*d C from Va 45-54

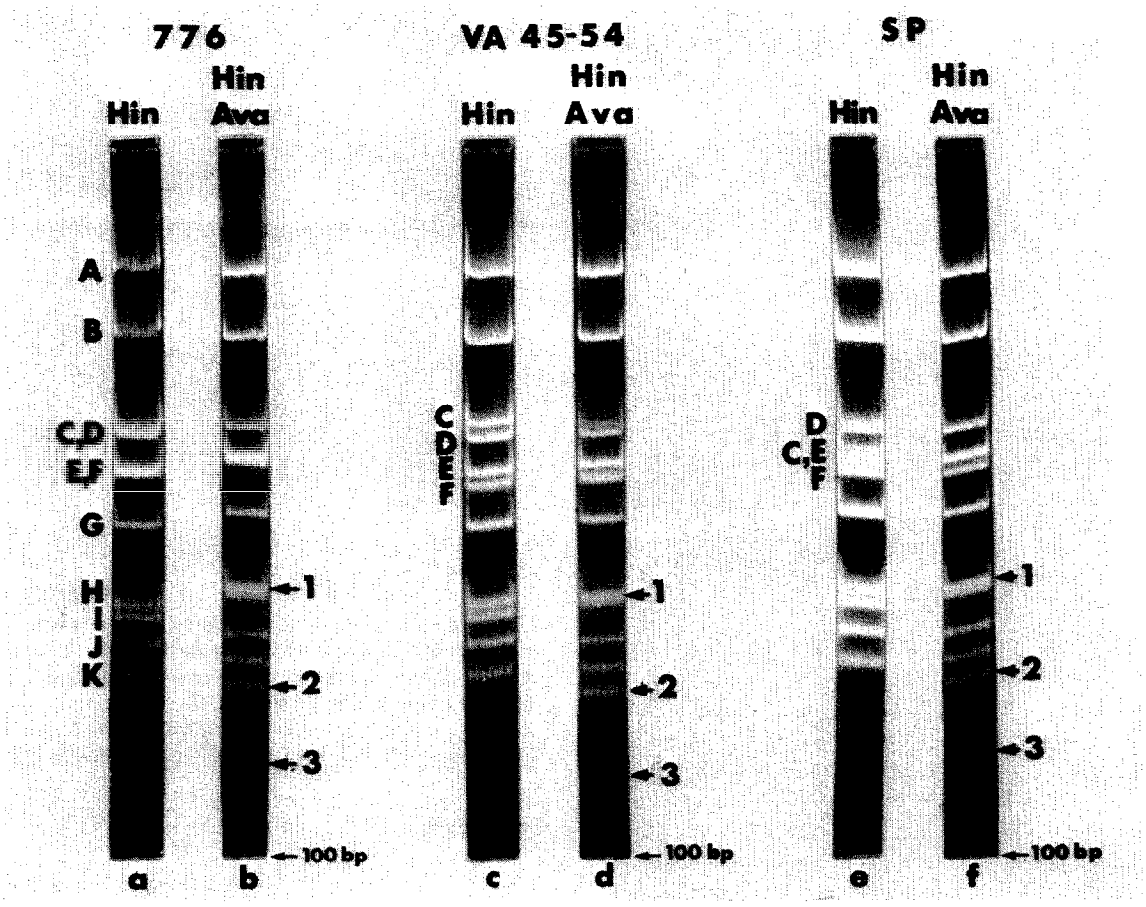


Fig.4. SV40 DNA (4 μg) was incubated at 37°C for 1 h in 6.6 mM Tris–HCl (pH 7.4), 6.6 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol with *Hin*dII + III. Half of each mixture was then supplemented with Tris–HCl (pH 7.4) (40 mM final) and treated with *Ava* III for 3 h. Lanes (a,c,e) show the *Hin*dII + III pattern of the DNAs of the SV40 strains 776, VA 45-54 and SP, respectively. Lanes (b,d,f) show the results of the double digestion with *Hin*dII + III and *Ava* III of the same viral DNA. The different samples were analysed on a 5% polyacrylamide slab gel. The nomenclature proposed [12] has been used for specific fragment identification (letters). The subfragments obtained by double digest have been entitled 1, 2, 3 (see text).

B is longer than *Hin*d C from 776 and therefore clearly separates from *Hin*d D (fig.4c). Notice on the contrary that fragment *Hin*d I has the same length in all three strains.

The small *Ava* III C fragment itself is of variable length, as shown in fig.3a–c. *Ava* III C from the VA 45-54 strain is longer than the *Ava* III C fragment from strain 776. No *Ava* III C fragment was detected on 6.5% polyacrylamide gels in the *Ava* III digest of strain SP, which has the shortest *Hin*d C fragment (fig.4e).

If an *Ava* III C fragment is produced at all from strain SP, its length must be <30 basepairs. In other words, the *Ava* III C fragment lengths derived from strains VA 45-54, 776 and SP vary in the same order as the *Hin*d C fragment lengths. We conclude that the two *Ava* III sites, which are separated by 50 basepairs in strain 776, are located inside fragment *Hin*d C. Independent experiments (J. Le Boucher, P.-C. N., unpublished observations) have shown that the insertion or deletion in the DNA which distinguish strain VA 45-54 or SP from strain 776 are located around position 0.69 on the standard 776 map. The two *Ava* III sites in *Hin*d C must themselves bracket position 0.69.

In the *Hin*dII + III–*Ava* III double digests, new fragments, which do not occur in *Hin*dII + III digests, can be detected (fig.4b,d,f). One of them, designated *Ava–Hin*d 1, which is slightly longer than fragment *Hin*d H (271 basepairs), has a length of ~275 basepairs. Fragment *Hin*d I itself is only 256 basepairs long. Therefore *Ava–Hin*d 1 must originate from *Hin*d C through *Ava* III digestion. This consideration, plus the approximate locations of the two *Ava* III sites in *Hin*d C mentioned above, leads to the unambiguous location of the *Ava* III sites in *Hin*d C which is shown in fig.5.

Ava III degradation of *Hin*d C (520 basepairs) from strain 776 must produce three fragments measuring around 275, 50 and 195 basepairs, respectively. A 195 basepair fragment is indeed detected in *Hin*dII + III–*Ava* III double digests (*Ava–Hin*d 2 in fig.4b,d,f). Notice that the *Ava–Hin*d 1 and *Ava–Hin*d 2 fragments have the same length irrespective of the strain, as expected from the locations of the *Ava* III sites and of the insertion or deletion in strains VA 45-54 or SP.

A third fragment *Ava–Hin*d 3 (fig.4b,d,f), with a length of 150 basepairs is clearly visible in *Hin*dII + III–*Ava* III double digests. Therefore the unique *Ava* III site located inside fragment *Hin*d I probably cuts this fragment into two unequal subfragments which are 150 and 100 basepairs long, respectively. The 100 basepair fragment can barely be seen at the very end of the gels shown in fig.4b,d,f, because 100 basepairs is precisely the limit length of the fragments which would still be detected on such gels. An unambiguous location of the unique *Ava* III site present in fragment *Hin*d I cannot be deduced from

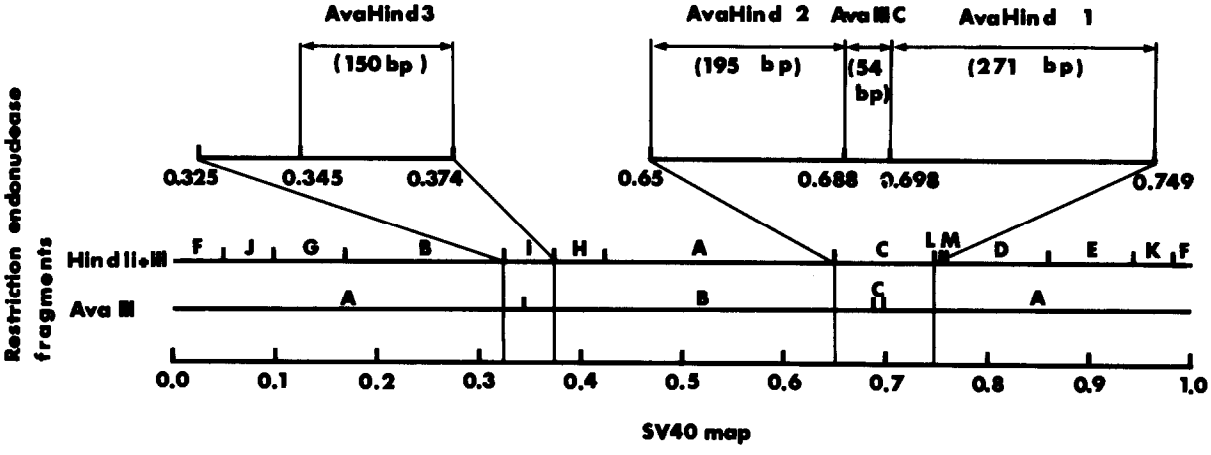


Fig.5. *Ava* III physical map of SV40 DNA. Each reported localization and size of the fragments was calculated according to the SV40 DNA sequence in [7]. The results (see text) are in good agreement with this figure.

the digestion data shown here. However examination of the nucleotide sequences in fragments *Hin*d C and *Hin*d I permits an exact location, as will be seen below.

3.4. *The DNA sequence recognised by* Ava *III*

On examination of the complete SV40 DNA sequence in [7,8], it can be seen that the hexanucleotide 5′ ATGCAT 3′ is present three times, at positions 0.345, 0.688 and 0.698. Positions 0.688 and 0.698 are located in fragment *Hin*d C in the area where we have located two of the *Ava* III sites. The third copy of the hexanucleotide sequence is located inside fragment *Hin*d I (at position 0.345), which is compatible with the fragment derived from SV40 DNA through digestion with *Ava* III. Furthermore, the hexanucleotide sequence is not present in ΦX174 [9] and the only break introduced by the *Ava* I + *Ava* III mixture corresponds to the unique *Ava* I site present in this DNA [10]. We, therefore, conclude that *Ava* III recognises the hexanucleotide 5′ ATGCAT 3′.

4. Conclusion

Although it was not possible to separate *Ava* III from *Ava* I by the chromatographic procedures used, *Ava* III was shown to be present because it can introduce breaks in SV40 DNA while *Ava* I cannot. The nucleotide sequence recognised was determined by mapping the recognition sites on SV40 DNA and was found to be different from any of those already known [11]. However, as the exact restriction site has not been determined it is not possible to say if *Ava* III is a type I or type II enzyme. The results we present do not rule out the possibility that *Ava* III recognises other sequences. However, computer analysis of the SV40 and ΦX174 DNA sequences shows that seven out of the nine palindromic sequences which can be derived from 5′ ATGCAT 3′ would lead to extrabreaks in either one or both these DNAs and can, therefore, be eliminated.

Acknowledgements

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