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N-acetoxy-N-acetyl-2-aminofluorene binding sites on [phi] X174 and SV40

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ABSTRACT

N-Acetoxy-N-Acetyl-2-Aminofluorene BINDING SITES ON ΦX174 AND SV40

by

Marta L. Bascoy

Restriction enzyme inhibition and lambda exonuclease studies indicate that carcinogen N-acetoxy-N-acetyl-2 aminofluorene (AAAF) binds to sequences on ΦX174 RF and SV40 plasmids DNA that are similar to the eight preferred binding sites previously located on pBR 322.

Both DNAs were digested with enzyme Hinf I and resultant fragments ^{32}P end-labeled. Labeled fragments were reacted with the carcinogen to give one to sixteen bound moieties per DNA. Fragments were isolated and restriccion enzyme and lambda exonuclease inhibition assays were performed.

Inhibition detected occurred at selected sites and was not specific for a certain enzyme or certain size of recognition sequence.

Results of these assays allow mapping of the location of high affinity binding sites of the carcinogen on both DNAs.

All sites have common sequence elements: the presence of either the sequence T(G/C)TT(G/C) or the sequence T(G/C) CTT(G/C).

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Marta L. Bascoy

A thesis submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

DEPARTMENT OF CHEMISTRY

at

FLORIDA INTERNATIONAL UNIVERSITY

Committee in charge:

Professor Stephen Winkle (Chairperson)

Professor Jeffrey Joens

Professor Ramón López de la Vega

February 1991

To Professors Stephen Winkle, Jeffrey Joens and Ramon Lopez de la Vega

This thesis, having been approved in respect to form and mechanical execution, is referred to you for judgement upon its substantial merit.

Dean-Professor Arthur Herriott
College of Arts and Sciences

The thesis of Marta L. Bascoy is approved.

Professor Stephen Winkle

Professor Jeffrey Joens

Professor Ramón López de la Vega

Date of examination: February 8th, 1991

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PROPOSAL FOR RESEARCH THESIS

MASTER OF SCIENCE DEGREE

FLORIDA INTERNATIONAL UNIVERSITY

COLLEGE OF ARTS AND SCIENCES

DEPARTMENT OF CHEMISTRY

Marta L. Bascoy

I propose to the Guidance Committee a study of the following topic to be conducted in partial fulfillment of the requirements for the degree of Master of Science: N-Acetyl-N-Acetoxy-2-Aminofluorene BINDING ON ϕX174 AND SV40.

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DEDICATION

To my husband Elizardo and my children Soraya and Alejandro.

To my mother Carmenza and my brother Gustavo.

To the memories of my father and my uncle.

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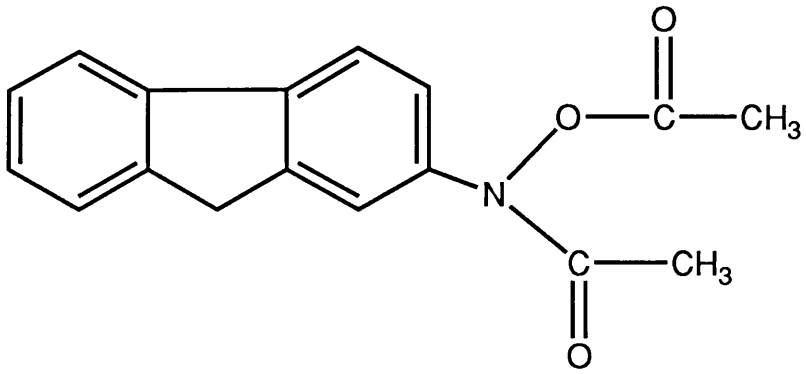
INTRODUCTION

The present research intends to locate carcinogen N-acetoxy-N-acetyl-2-aminofluorene (AAAF) binding sites on bacteriophage ϕX-174 and monkey tumor virus SV-40 DNAs, using restriction enzyme inhibition and lambda exonuclease inhibition studies. The carcinogen used for this study was chosen because its structure is known (fig.1), it is reasonably stable and there have been preliminary studies done on how it attacks DNA. (Singer & Grunberger, 1983; Evans et al, 1980; Grunberger & Weinstein, 1978; Hogan et al, 1980; Santella et al, 1980; Hingerty & Broyde, 1983).

AAAF is an intercalative binder with DNA that binds covalently to guanine bases (Krick & Westra,1979; Hemminki, K.,1983; Beland et al,1980). It has been shown by unwinding supercoiled circular DNA (Drinkwater,N., et al,1978) and circular dichroism (Fuchs & Daune,1972) that the planar ring of AAF stacks between the base pairs. Circular dichroism suggested that the guanine is displaced outside of the helix when carcinogen is inserted into it. Linear dichroism suggested that the AAF chromophore sits 80° to the helical axis (Fuchs R., et al, 1976).

Formaldehyde unwinding, which measures helix stability showed that the adducts increase the rate of unwinding and destabilize the helix (Fuch & Daune,1974).

It is also evident that the G residue displaced by AAF in the double helix can not base pair with the C residue on



Carcinogen N-Acetoxy-NAcetyl-2-Amino-Fluorene

FIGURE 1

the complimentary strand. During the process of replication or transcription no base pairing at this position can occur.

The structural change that results from the presence of the adduct is described as an insertion-deletion model (fig.2) (Fuchs, R., et al, 1976; Grunberberger & Weinstein, 1978). The first feature of this model is that the attachment of the AAF residue to the 8 position of G is associated with a glycosyl change in conformation from anti to syn (Evans et al,1978; Leng et,1980). Evidence of this is observed in a study of molecular models of AAF-G that indicated severe steric hindrance between AAF and the deoxyribose of the nucleoside unless G base is rotated about the glycosyl bond.

The second major feature of this model is that there is a stacking interaction between AAF and a base adjacent to the substituted G residue (Nelson et al,1971; Santella et al,1980; Leng et al,1980; Lipkowitz et al,1982; Hingerty & Broyde,1982).

Thermal denaturation studies are in good agreement with The Base Displacement Model since AAF modification decreases the melting point temperature of DNA. For each 1% of the modified bases there is an approximate 1.5 °C decrease in melting point. A marked decrease of the intrinsic viscosity of the DNA supports the idea that AAF binding causes localized regions of distortion (Fuchs, R.,1975).

Since attachment of AAF to residues requires rotation of the base about the glycosyl bond and there is less

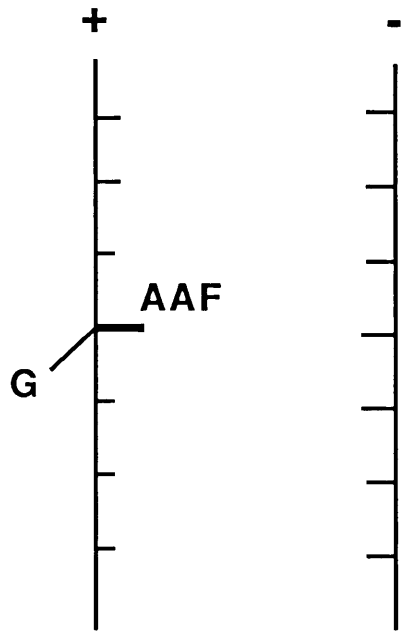


FIGURE 2. Base Displacement Model

Schematic model in which G residue has been displaced by AAF in the double helix.

hindrance to the rotation of bases in single stranded DNA than in double stranded one, it follows that single stranded regions of nucleic acid are more susceptible to AAF modification than double stranded ones. Regardless of the specifics of the model it is clear that AAF binding distorts it.

The interaction mechanism between a restriction endonuclease enzyme and the specific DNA sequence is an initial binding to non specific sites and locating and recognizing the specific sites by random diffusion along the DNA helix, in particular, results obtained with EcoRI enzyme suggest that the average distance scanned by the random walk mechanism is about 1000 b.p.per DNA binding event under the conditions employed (Richter & Eigen,1974; Schranner,1978).

Restriction inhibition enzyme has been used to specifically locate the structural sequence features on the DNA. Type II restriction endonucleases are sequence specific enzymes which cleave double stranded DNA. Their relative structural simplicity has rendered them attractive for study of sequence-specific protein DNA-interaction (Modrich & Roberts,1982). Type II recognition sites vary from 4 to 6 base pairs in length corresponding to expected frequencies of sequence of one per 256 pairs to one per 4096 base pairs respectively (Roberts,R.J.,1976; Roberts,R.J.,1980). With few exceptions these sequences exhibit twofold rotational symmetry and sites of DNA strand are symmetrically disposed relative to the twofold axis.

It has been shown that drugs like cis-dichlorodiamine platinum II, inhibit the activity of a specific enzyme, in this case enzyme BamH I, blocking at the recognition sequence, causing inhibition by preventing access to the DNA by the enzyme. (Weinstein & Grunberger,1974; Coffman & Gaubatz,1982).

Lambda exo is a DNA exonuclease which cleaves one base at a time from the 5' end. It is highly processive and will stop when it encounters a block or a nick within the double helix or will slow down when a single stranded region is encountered. The processive nature of lambda exonuclease may be explained by the enzyme having two active sites (Thomas & Olivera,1978). It is believed that the enzyme is inhibited at nicks because it remains bound to the last base pair before the nick. (Carter & Radding,1971).

It has been shown that an alteration in the DNA at the recognition sequence of a restriction enzyme causes inhibition of the restriction enzyme (Mallamaci,1988). Since AAAF modification has been shown to cause DNA denaturation about its binding site, any restriction endonuclease or lambda-exonuclease which has a recognition sequence near the AAAF binding site should also be inhibited.

By systematic digestion of both plasmids under study at a large number of restriction sites throughout them, it was possible to map their location. Sites that showed inhibition toward fragments which contained bound AAF and those sites not inhibited were located.

For this purpose, the plasmids were divided into fragments of sizes between 100 to 700 base pairs in the case of OX 174 and fragments from 20 to 1800 base pairs long in SV 40's. Hinf I, used to generate a pool of fragments, was chosen because it does not show detectable inhibition in the presence of bound AAF moieties. (Mallamaci,1988)

Isolated fragments were then attacked by various restriction enzymes that preferably cut only once at specific sites within each fragment. The reason to choose enzymes that cut once is because after the second enzymatic digestion, fragments should be large enough to isolate and visualize. They were run on an electrophoresis gel, placed on a piece of X-ray film and analized.

MATERIALS AND METHODS

MATERIALS

PLASMID: SV 40 and ϕX 174 RF plasmids used in this study were obtained from Bethesda Research Laboratories or International Biotechnologies Incorporated.

ENZYMES: Restriction enzymes as well as lambda exonuclease were obtained either from Bethesda Research Laboratories or International Biotechnologies Incorporated. All enzymes were used in the buffers provided by the manufacturer (see table of enzymatic conditions in appendix 1).

CARCINOGEN: The N-acetoxy-N-acetyl-2-amino-fluorene used in the present research was prepared by acetylation of the hydroxy-AAF with acetic anhydride in pyridine and purified on an alumina column with ethyl acetate.

METHODS

PREPARATION OF DOUBLE-END ^{32}P LABELED HINF I FRAGMENTS: For SV 40 DNA, 10 ug of SV 40 DNA was

digested in a volume of 20 ul with 2 units of Hinf I restriction endonuclease in reactant buffer #2 for 2 hours at 37 °C.

The restriction fragments were labeled by the addition of ^{32}PdATP, along with 2 ul of reactant buffer #2, 8 ul of water and Klenow fragment. The mixture was incubated for 20 minutes at room temperature.

Fragments were precipitated by the addition of 2 volumes of 95% ethanol followed by an incubation of 15 minutes at -70 °C in a dry ice bath. The DNA was pelleted by centrifugation at 13000 rpm, washed with 70% ethanol, and vacuum dried.

The same procedure was used for ϕX174 using 20 uL of DNA, 2uL of the enzyme (Hinf I) and 2 uL of Reactant #2.

REACTION OF LABELED FRAGMENTS WITH ACETOXY-AAF:The fragments obtained above were resuspended in TE buffer (0.01 M tris, 0.001 M EDTA, ph 8.0) and divided into equal portions. To these samples were added increasing amounts of acetoxy-AAF as appropriate to give the desired amounts of bound carcinogen moieties per DNA.

ISOLATION OF FRAGMENTS: After reaction with AAAF samples were electrophoresed on 6% polyacryla-

mide. For this purpose 6% polyacrylamide gel and stock solutions were prepared according to appendix 2. The procedure followed for the 6% polyacrylamide electrophoresis is described in appendix 3.

When the gel was finished, bands were visualized by staining the gel with ethidium bromide (0.1 g/ml) and transilluminated with a short wave U.V. light box. DNA bands were excised and gel slices soaked for 48 hours in 0.5 ammonium acetate (pH 7.9).

Fragments were removed from ammonium acetate by precipitation with 95% ethanol followed by incubation in a -70 °C dry ice/ethanol bath for 15 minutes and washed. The DNA was pelleted by centrifugation at 13000 rpm for 15 minutes and washed with 70% ethanol. Finally the DNA pellets were vacuum dried and stored at 4 °C.

At this point 5 sets of Hinf I digested SV 40 or ϕX 174RF fragments, each with a different amount of bound AAAF,were available for inhibition essays.

REACTION OF HINF I FRAGMENTS SETS WITH RESTRICTION ENZYMES: The following procedure was used in all assays wich involved an enzyme possessing only one cleavage site within the test DNA fragment. To each sample was added 4 uL of the restriction enzyme being tested. The enzyme was allowed to

incubate with DNA in its manufacturer's recommended buffer (see appendix for list) for one hour in a 37 °C water bath. The enzyme reaction was determined by freezing the samples.

Reacted samples were placed on a 6% polyacrylamide gel and electrophoresed at 300 V for two hours. For a single marker for partial digestion products, where necessary, ^{32}P labeled ϕX 174 or SV 40 DNA were also run.

Once the electrophoresis was finished, gel was removed from the apparatus and wrapped in plastic film. The gel was placed in the film holder and in the dark a piece of X-ray film was placed directly onto the gel.

After exposure of the film to the radioactive gel for an appropiate amount of time, the film was developed.

The film was removed from the holder and placed into a tray containing developer and agitated for four minutes. Development was stopped by washing the film in cold water for one minute. The film was placed into a tray containing fixer and agitated for four minutes. The developed film was washed in cold water five minutes and then air dried.

The bands were then identified and analyzed qualitatively on the radiograms by visible observa-

tion. (This analysis are in the chapter of Results).

LAMBDA EXONUCLEASE INHIBITION ASSAYS: Digestion of the labeled fragment sets with lambda-exonuclease was carried out in 67 mM glycine-KOH (pH 9.4), 2.5 mM magnesium chloride at 37 °C. To each sample 0.01 ug salmon sperm DNA was added prior to digestion.

For each digestion 0.05 units of lambda-exonuclease per base pair was employed. Digestion time was 30 minutes. The amount of exonuclease required to digest non acetoxy-AAAF containing fragments was determined experimentally. Digestion was stopped by ethanol precipitation.

The dried precipitated fragments were taken up into deionized formamide (containig 0.001% Xylene cyanol cis trach's dye) and heated in boiling water for one to two minutes to denature the DNA.

The fragments were electrophoresed in 8 M urea gel on 8% polyacrylamide gels. (See Appendix 2).

RESULTS

I. SV 40 DNA INHIBITION USING RESTRICTION ENZYMES

After obtaining double-end ^{32}P labeled Hinf I fragments, the general design of these inhibition assays was to isolate restriction enzyme activity within a fragment.

The gels were interpreted qualitatively as follows: Figure 3 shows any set Hinf I fragment, cut once by a restriction enzyme. The set of fragments has increasing amounts of carcinogen, starting with no carcinogen concentration up to 16 moieties of AAAF per DNA molecule.

If the action of the restriction enzyme is inhibited by the carcinogen, what is observed on the gel is: (fig.4). The intensity of the band, uncut fragment A + B with no carcinogen is almost imperceptible because in this case the restriction enzyme was not inhibited and most of the fragment has been split in two sub-fragments A and B. When the carcinogen reacts with DNA, the action of the enzyme starts to be inhibited, so the fragment cannot be cut. Therefore the intensity of the bands is observed to increase with the increasing amount of carcinogen.

Since the restriction enzyme cuts the fragment into sub-fragments A and B; then when the carcinogen is

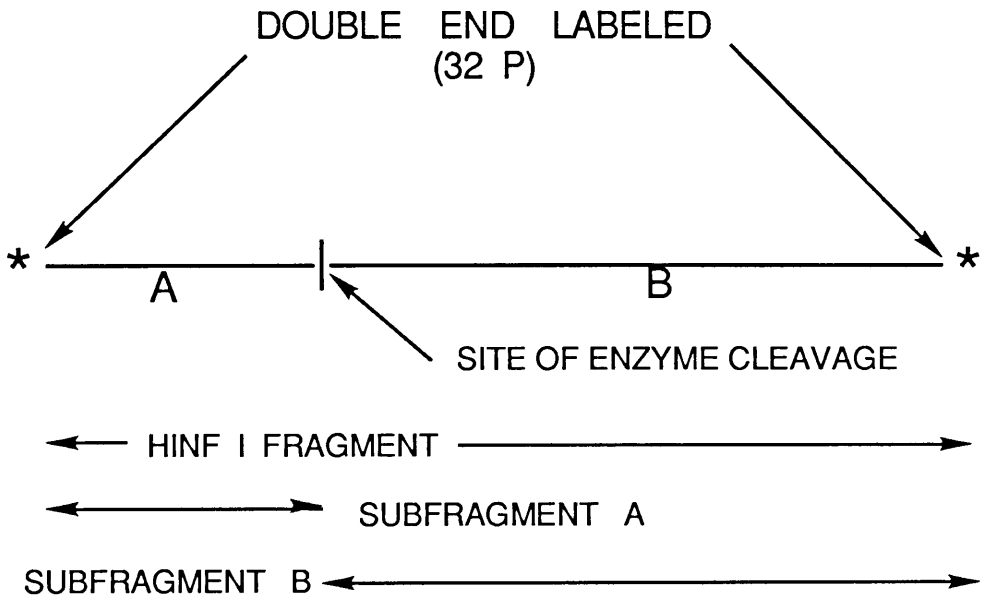


FIGURE 3

Schematic reaction of Double-end ^{32}P labeled Hinf I fragment.
The Hinf I fragment is divided into two sub-fragments A and B when the enzyme cuts it.

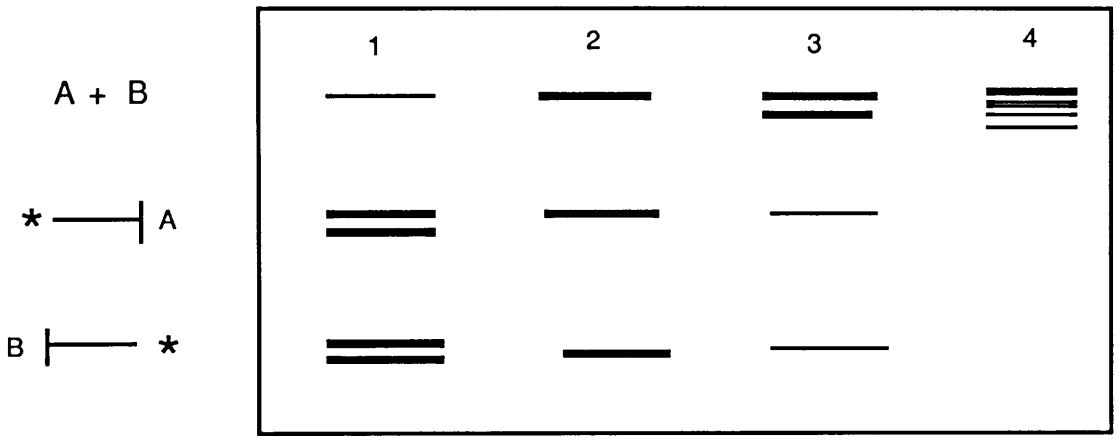


FIGURE 4

Qualitative interpretation of gels when Hinf I fragment is cut by a restriction enzyme.
A+B is the uncut fragment.
Numbers represent a proportional amount of carcinogen.
Lines represent band intensity.

not present, the band of any of these sub-fragments is intense, but the intensity gradually disappear with higher concentration of carcinogen.

If a restriction enzyme not inhibited by the carcinogen is used, (fig. 5) bands of equal intensity are observed because action of carcinogen does not inhibit the restriction enzyme.

Figure 6 shows when a enzyme cuts any set of Hinf I fragment more than once (twice in this case) generating sub-fragments A, B and C and inhibition occurs in A. The set of this fragment is attacked by increasing amounts of carcinogen concentration up to 16 moieties of AAAF/DNA.

For uncut fragment A+B+C (fig. 6), the intensity of the band is almost imperceptible (when no AAAF is added), because the enzyme tends to cut the fragment. When the carcinogen reacts with DNA, the action of the enzyme is inhibited as the concentration of the carcinogen increases.

For sub-fragments A + B, with no carcinogen, no bands appear on the gel, because enzyme has not been inhibited and it cuts twice, and sub-fragment B which is not labeled is impossible to see. With increasing concentration of carcinogen a more intense band is seen.

For sub-fragment A, when no carcinogen is present,

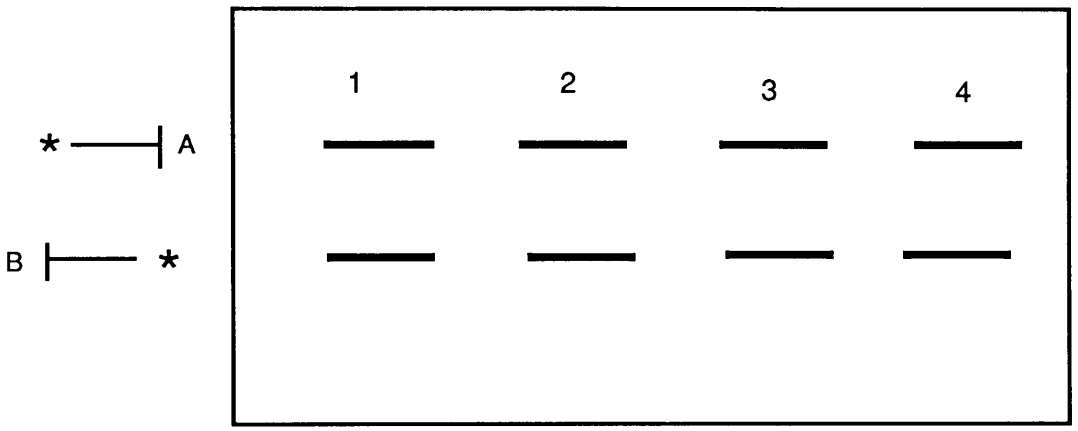


FIGURE 5

Qualitative interpretation of gels when Hinf I fragment is cut once by the restriction enzyme, but this inhibited by the carcinogen.

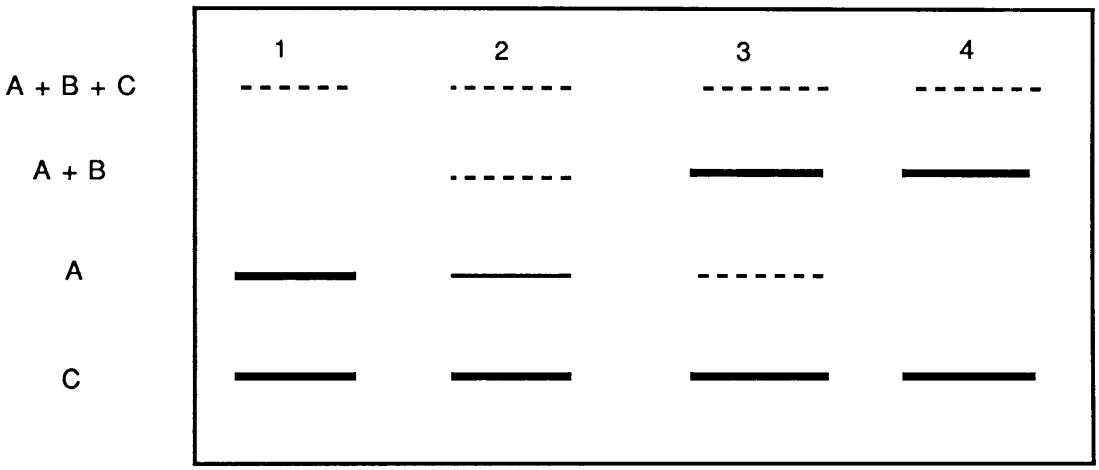


FIGURE 6

Qualitative interpretation of gels when Hinf I fragment is cut by the restriction enzyme more than once.

an intense band is observed because action of the enzyme is not inhibited. Increasing the concentration of the carcinogen causes the band become less intense and finally to disappear. For sub-fragment C when no carcinogen is present an intense band is observed, for the same reason explained for fragment A, but in this case the bands have almost the same intensity when concentration of AAAF increases, because it was not inhibited. For most of the analysis enzymes cutting once were preferentially used.

To verify visual observations bands were cut from gels, placed into vials and ^{32}P counted using Beckmann LS5000 scintillation counter.

The counts per minute obtained from the total of both sub-fragments A and B and the uncut fragment (A+B) were added to obtain a total CPM level for each bound AAF sample. This total is proportional to the number of DNA ends. To determine the fraction of fragment digested, CPM of the sub-fragments A and B are added and divided by the total CPM of the sample and multiplied by 100.

$$\% \text{ digested} = \frac{A+B}{\text{total}} \times 100$$

Where total = A+B+(A+B)

These data are plotted on a percent digested vs. bound

AAF graph.

Graphs yielding a zero slope indicates no apparent inhibition while graphs with a negative slope indicate the presence of enzyme inhibition. (Fig. 7 and 8)

Table 1 provides information about enzymes used for inhibition enzymes on SV 40 DNA.

Once restriction inhibition enzymes assays as described in Materials and Methods, reaction of the Hinf I fragment set with restriction enzymes and further qualitative analysis of the autoradiography, it was possible to know which enzymes were inhibited by the carcinogen and which were not. Results are reported in table 2.

There was one restriction enzyme which possessed a single site within the Hinf I 1847 parent fragment. The EcoRV site at base 771 appeared not inhibited. One restriction enzyme with 2 sites, the Hha I at base pair 346 which was not inhibited and 836 which was inhibited. The Hinf I 1085 bp fragment has 3 enzymes with one restriction site. The enzymatic activities of Ava II site at base pair 2014 and Pst I site at base pair 1993 were not inhibited. Same fragment was cut by Hae III between 2261 ad 2801 bp, being first one inhibited and the other not inhibited.

Two enzymes on fragment Hinf I 525 were checked. Sau 96 I at base pair 3171 which was inhibited and Rsa I at bp 3074 , non inhibited.

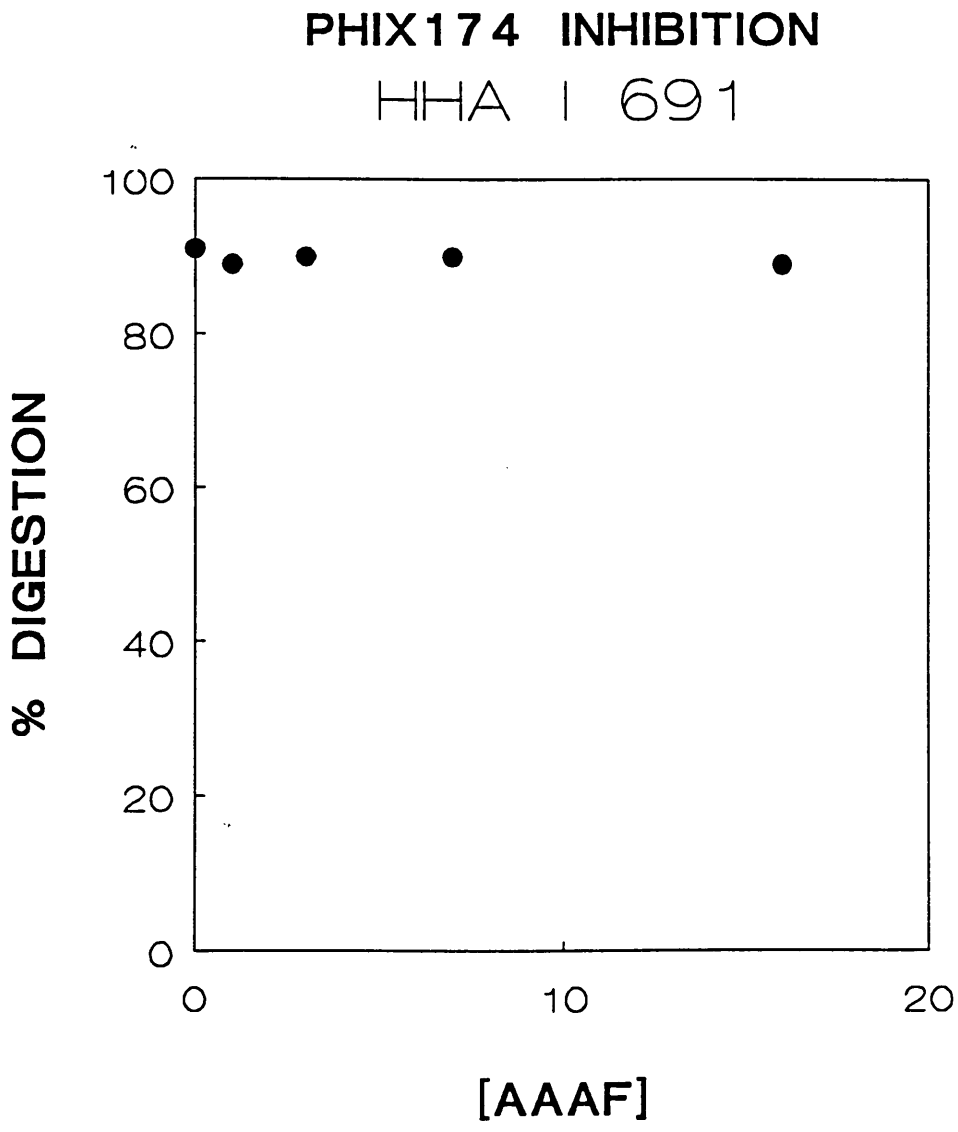


FIGURE 7

Percentage Inhibition vs. AAAF bound for digestion of Hinf I base pair fragment of OX 174 RF with Hha I.

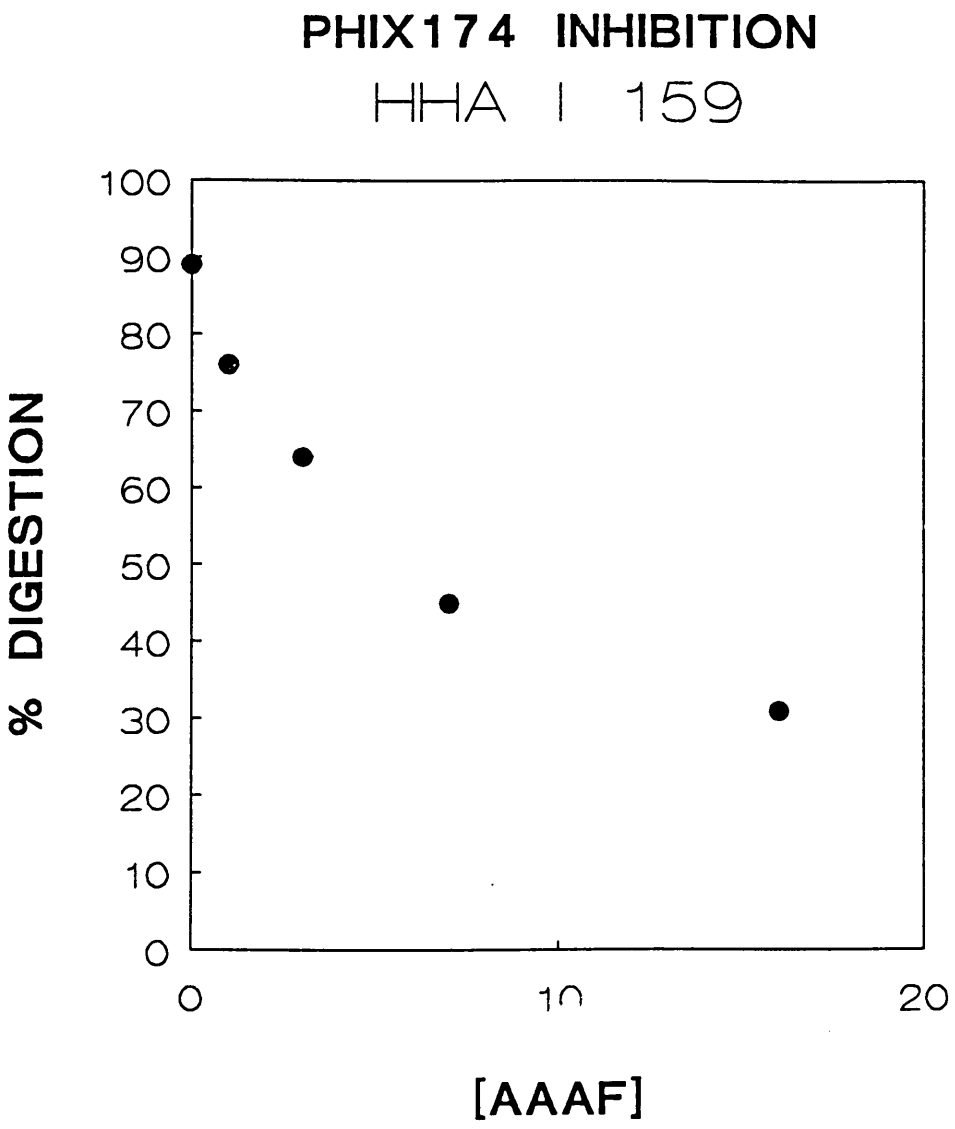


FIGURE 8

Percentage Inhibition vs. AAAF bound for digestion of Hinf I 1847 base pair fragment of SV 40 with Hha I.

Table 1

Inhibition Assays for SV 40 DNA
Hinf I Parent Fragment

Fragment Size (1)	Restriction Sites (2)	Enzyme Used (3)	Location (4)
1847	5136-1740	Hha I	346,836
		EcoRV	771
1085	1740-2825	Hae III	2261-2800
		Ava II	2014
		Pst I	1993
766	3611-4377	Dra I	3910
		Mbo I	3776-4100
525	2849-3374	Sau 96 I	3172
		Rsa I	3074-3227
543	4593-5136	Taq I	4740
		Hae III	4864
		Ava II	5119
		Sau 96 I	5119
		Mbo I	4770
237	3374-3611	Sau 96 I	3539
		Rsa I	3578
		Ava II	3539
109	4460-4569	Dde I	4500
83	4377-4460	Dde I	4388

(1) The numbers given are the sizes (in base pairs) Hinf I digested SV 40 fragments.
(2) The fragment size is contained between these two sites.
(3) Restriction enzyme used for inhibition assay.It should cut within the range of restriction sites.
(4) Location of enzyme cleavage.

Table 2

SV 40 Inhibition

	0-1K(2)	1-2K	2-3K	3-4K	4-5K	5-6K
INHIBITED (1)						
Dde I	--	--	--	--	4500	--
Hae III	--	--	2261	--	--	--
Hha I	836	--	--	--	--	--
Rsa I	--	--	--	3578	--	--
Sau 96I	--	--	--	3172	--	--
NON-INHIBITED						
Ava II	--	--	2014	3539	--	5119
Dde I	--	--	--	--	4388	--
Dra I	--	--	--	3910	--	--
Eco RV	771	--	--	--	--	--
Hae III	--	--	2801	--	4864	--
Hha I	346	--	--	--	--	--
Mbo I	--	--	--	3716	4100 4770	--
Pst I	--	1993	--	--	--	--
Rsa I	--	--	--	3074	--	--
Sau 96 I	--	--	--	3539	--	5119
Taq I	--	--	--	--	4740	--

For fragment Hinf I 543, five enzymes were used Taq I, Hae III, Ava II, Sau 96 I and Mbo I with enzymatic activities at 4740, 4864, 5119 (Ava II and Sau 96 I) and 4770 bp respectively. All of them were not inhibited. Sau 96 I, Rsa I and Ava II were the enzymes with a single site within the Hinf I 237 fragment at 3539,3578 and 3539 bp respectively. First and third one were not inhibited and 3578 bp was inhibited.

Dde I base pair 4500 was the enzyme assayed to see its enzymatic activity on the Hinf I fragment 109. It was inhibited by the carcinogen. Finally the same enzyme possessed only one site within the Hinf I fragment 83 at bp 4388 which was not inhibited.

II. INHIBITION ASSAYS FOR ϕX 174 DNA USING RESTRICTION ENZYMES.

The same procedure and analysis was followed for this DNA as for SV 40 one.

Table 3 summarizes the enzymes used for inhibition assays on ϕX 174 DNA.

Qualitative analysis from autoradiography gave the following results:

Four inhibition assays were performed on fragment 726 bp. Two of the 4 enzymes used have one site, Alu I cuts at 447 bp and Sau 96 I at bp 979. Both of them

Table 3

Inhibition Assays for ϕX 174 RF DNA
Hinf I Parent Fragment

Fragment Size	Restriction Sites	Enzyme Used	Location
726	393-1119	Alu I	447
		Sau 96 I	979
		Hha I	1023,691
		Hae III	980
713	1185-1898	Hae III	1777
		Rsa I	1433
		Alu I	1454,1593
		Hha I	1415
553	4149-4702	Hae III	4208-4489
		Dde I	4253
		Taq I	9181
500	2878-3378	Hha I	2980
		Alu I	3166
		Hae III	3130
		Hpa II	3020
		Rsa I	3189
427	4702-5129	Ava II	5043
		Sau 96 I	5043
417	3732-4149	Taq I	4040
		Alu I	3883,4137
413	2265-2678	Hha I	2366
311	5129-54	Alu I	5211
		Taq I	5356
		Pst I	1
249	2016-2265	Dde I	2014
		Rsa I	2152
		Tha I	2149
151	54-205	Hha I	159
		Xho I	163
		Alu I	171
118	1898-2016	Rsa I	1905
66	1119-1185	Hae III	1174
66	3378-3444	Alu I	3390

Table 4

PHI X 174 Inhibition

	0-1K	1-2K	2-3K	3-4K	4-5K	5-6K
INHIBITED						
Alu I	171	--	--	3166 3312	--	--
Hae III	--	1777	--	3130	4208	--
Hha I	159	1023	--	--	--	--
Hpa II	--	--	--	3020	--	--
Rsa I	--	--	--	3189	--	--
Taq I	--	--	--	--	4181	--
Xho I	163	--	--	--	--	--
NON-INHIBITED						
Alu I	447	1454 1594	--	3390	--	5211
Ava II	--	--	--	--	--	5043
Dde I	--	--	2041	--	4253	--
Hae III	980	1174	--	--	4489	--
Hha I	691	1415	2366 2980	--	--	--
Pst I	1	--	--	--	--	--
Rsa I	--	1433 1905	2152	--	--	--
Sau 96 I	979	--	--	--	--	5043
Tha I	--	--	2149	--	--	--
Taq I	--	--	--	--	4040	5356

appeared to be inhibited. Although Hae III possessed 3 cleavage sites within this fragment (436, 670 and 980 base pairs) a label only on 980 end would enable visualization in an autoradiogram. This site is not inhibited by the carcinogen.

When Hha I cuts this fragment, it generates other sub-fragments from which two of them were end-labeled as described previously. One sub-fragment is from base pair 150 to pb 691 and a second one from bp 1023 to bp 1146. Site 691 showed no inhibition and site 1023 was inhibited.

Fragment 713 was checked by four different enzymes Hae III, Rsa I, Alu I and Hha I.

The first two enzymes possessed one cleavage site each on this fragment: Hae III activity showed inhibition at 1777 bp but the activity of Rsa I was not inhibited at bp 1433.

Alu I has 3 cleavage sites within this fragment, at 1454, 1538 and 1593 but labeling at 1454 and 1593 ends would enable visualization on the radiograms. Both sites showed non inhibition.

Hha I cuts 3 times within this fragment: at 1415, 1720 and 1865 bp; but only the one at 1415 was labeled and the action of the enzyme was not inhibited.

On fragment 553, three inhibition assays were performed. Enzyme Dde I had only one site at 4253 pb which was not inhibited.

Hae III cuts twice within this fragment at bp 4208 and 4489 respectively. First one was inhibited and second one showed no inhibition.

Taq I cuts once at bp 4181 which showed inhibition. Five inhibition assays were tested on fragment 500 bp. Enzymes used were Hha I, Hae III, Rsa I, Alu I and Hpa II. Hha I, Hae III and Rsa I have one site within fragment. Hha I activity at 2980 bp was not inhibited, Hae III activity at 3130 bp was inhibited and Rsa I at 3189 bp, was inhibited.

Alu I cuts the fragment twice at 3166 and 3313 bp. Both sites were inhibited.

Although Hpa II has two sites within this fragment, 3020 bp and 3368 bp, only the first one was end labeled which appeared inhibited by the action of the carcinogen.

Ava II and Sau 96 I were the enzymes assayed on fragment 427. Both of them have enzymatic activities at 5043 bp site and were not inhibited.

Two inhibition assays were performed on 417 parent Hinf I fragment.

Taq I only possessed one site on this fragment at 4040 bp which was non inhibited. Alu has three cleavage sites within the same fragment, at 3841 bp, 3883 bp and at 4137 bp. It was labeled on 3883 and 4137 ends. After qualitative observation it was concluded that none of these sites were inhibited.

On fragment 413, just one inhibition assay was done, using Hha I which cuts at 2366 bp. This site was not inhibited.

Three restriction enzymes which possess a single site within the Hinf I 311 bp parent fragment. The Alu I site at bp 5211, the Taq I site at bp 5356 and the Pst I at bp 1. They were not inhibited.

On fragment 249, three restriction enzymes were observed, Dde I, Rsa I and Tha I, at bp 2041, bp 2152, and at bp 2149 respectively. They were not inhibited by the carcinogen.

Three restriction enzymes were used to test inhibition on fragment 151, Hha I, Xho I and Alu I.

Hha I and Xho I, at bp 159 and at bp 163, respectively, were inhibited. For Alu I, which cuts twice within this fragment (at bp 162 and at bp 171), only the 171 bp end was labeled for observation in the autoradiogram which showed inhibition.

For the Hinf I 118 parent fragment one inhibition assay was done with the enzyme Rsa , which cuts once within this fragment, at 1905 bp and was not inhibited by the carcinogen.

Finally, there are 2 Hinf I parent fragments with the same size of 66 bp. One has restriction sites between 1119 bp and 1185 bp and the second one between 3378 bp 3378 and 3444 bp. For the first one, Hae III was the enzyme checked which cuts at 1174 bp. For the

second fragment, also one inhibition assay was done. Alu I was the restruction enzyme chosen, that cuts this fragment at 3390 bp. In both cases, activities of the enzymes were inhibited.

III. LAMBDA EXONUCLEASE INHIBITION ASSAYS:

The results of the lambda-exo digests are summarized in table 5. This information is obtained from the sequencing gels. In all the autoradiograms the size of the fragments was determined using the ϕX marker. Analysis of the digest patterns are based on the differences observed between the control and experimental lanes. By comparing the lambda exo lanes to the marker and counting down from the top of the parent fragment, the size of the inhibition can be determined.

Results from digestion of 4 fragments of SV 40 and 3 of ϕX174 DNA are reported . The inhibition bands for which locations are given are those appearing at the lowest carcinogen concentration used, i.e., 1-3 carcinogen bound/DNA molecule. At higher concentrations additional bands appeared in the vecinities of the given bands.

Observing table 5 on fragment 525 (SV 40 DNA), lambda exonuclease was inhibited between 3079 and 3089 bp. When restriction enzymes were analyzed on the same

Table 5

Carcinogen Induced Lambda Exonuclease Inhibition

Fragment (1)	Fragment Location (2)	Location of Inhibition (3)
SV 40		
Hinf I 525	2849-3374	3079-3089
" 237	3374-3611	3554,3574
" 766	3611-4377	None
" 109	4460-4569	4489,4500
ΦX 174		
Hinf I 151	54-205	144-150,170
" 249	2016-2265	None
" 311	5129-54	None

fragment, inhibition of restriction enzyme Sau 96 I around that region also was observed. (3172 bp).

On Hinf I fragment 237 the exonuclease was inhibited at 3554,3574 bp. Restriction enzyme Rsa I at 3578 bp also showed inhibition.

When fragment 766 was examined, no inhibition was observed, which agrees with the studies of restriction enzymes. None of them (Dra I or Mbo I) were inhibited.

On fragment 109, lambda exonuclease presented inhibition at 4489,4500 bp. Action of the restriction enzyme Dde I over the same fragment at 4500 bp. was also inhibited. For ϕX 174 RF DNA, same analysis was done.

On fragment 151, exonuclease is inhibited in the region of 144-150 bp and 170 bp.

Restriction enzymes used to cleavage the same fragment: Hha I, Xho I and Alu I showed inhibition around the region of 159,163 and 171 bp, respectively.

On the other side, on fragments 249 and 311, neither lambda exonuclease nor the restriction enzymes Rsa I, Tha I, Alu I and Pst I were inhibited.

The above analysis supports the idea about the possibility of inhibition of any restriction enzyme or lambda exonuclease as a consequence of the presence of the carcinogen AAAF whose binding site is close to the reconition site of the given enzyme, which was suggested in the introduction of the present study.

DISCUSSION

The purpose of these experiments was to identify and localize any potential high affinity AAAF binding site within SV 40 and ϕX 174 DNAs plasmids. As discussed earlier, the main concern was to detect any DNA alteration created by the presence of the bound carcinogen. This alteration can cause some form of inhibition to the restriction enzyme if it is near its cleavage site.

The first region along the plasmid SV 40 in a clockwise direction from the Bgl I, Sfi I site noted as base pair 0 up to base pair 1740 found to contain an AAAF high affinity binding sites. From the three sites tested, Hha I at bp 346, Eco RV at pb 771 and Hha I at bp 836, only the last one was inhibited. Since an AAF adduct is known to alter the helix up to 20 bases away, a single adduct bound between bp 816 and 856 could be responsible for the inhibition observed in this region but far enough away to cause inhibition at the non inhibited sites.

The Hinf I 1085 bp fragment contains another high affinity AAAF binding site. Within this fragment, Hae III whit a restriction site at bp 2261 was inhibited. The sites at bp 1993 for enzyme Pst I, bp 2014 for enzyme Ava II and bp 2301 for Hae III were not affected by bound AAF so the carcinogen bound between bp 2241 and 2281 bp could be responsible for this inhibition. Two inhibition assays were performed on the Hinf I fragment 525 bp extending from bp

2849 to 3374. The one at bp 3172 showed evidence of inhibition for the enzyme Sau 96 I, but the one at bp 3074 for Rsa I did not. So the possible location for carcinogen binding site is between 3152 and 3192 bp. The next fragment in a clockwise direction around the plasmid was the 237 bp fragment, which extends from bp 3374 to 361. Inhibition was observed within this fragment at the Rsa I site located at bp 3578. However, sites at bp 3539 for Ava II and Sau 96 I reflected no signs of inhibition. Bound carcinogen between 3558 bp and 3598 bp could be responsible for this inhibition. Within the next fragment exists another high affinity binding site. Assays on this 766 bp fragment at restriction site 4500 bp for Dde I, observed enzyme inhibition. The rest of the inhibition assays performed on other fragments with enzymes Mbo I at 4100 and 4770 bp, Dde I at 4388 bp, Taq I at 4740 bp, Hae III at 4864 bp and Ava II and Sau 96 I, both at 5119 bp did not show inhibition.

For the ϕX 174 RF DNA, the results of inhibition studies are the following: In the first region along the plasmid in a clockwise direction fom the bp 0 to bp 54, only one site was tested, Pst I at bp 1 which did not show inhibition. The Hinf I 151 bp fragment was checked by three enzymes, Hha I at bp 159, Alu I at bp 171 and Xho I at 163. All three enzymes were inhibited indicating that the carcinogen bound in this region affected all three sites at the same time. The next fragment was the Hinf I 726 bp, which extends from 393 to 1119. Inhibition was observed in

this fragment at the Hha I bp 1023. The sites at bp 447 for Alu I, 979 for Sau 96 I, 691 for Hha I and 980 for Hae III reflected no signs of inhibition. No inhibition was observed within the fragment 66 bp, extending from 1119 to 1185 bp when Hae III was tested at bp 1174 site. Within the 713 bp fragment between 1185 and 1898 there exists another high affinity binding site for Hae III at bp 1777. Three other inhibition assays were performed on this fragment, at bp 1433 for Rsa I, at bp 1454 and bp 1594 for Alu I. None of these sites showed signs of inhibition. One inhibition assay was performed on the Hinf I 118 bp fragment extending from 1898 to 2016, which did not show inhibition at bp 1905 for Rsa I restriction enzyme. The next fragment in a clockwise direction around the plasmid was the 249 bp fragment which extends from bp 2016 to bp 2265. No inhibition was found at any of the sites checked, which were at bp 2041 for Dde I and 2152 for Rsa I. For fragment Hinf I 413 bp only one inhibition assay was done. No inhibition was observed at the Hha I site located at bp 2366. Within the next region the fragment 500 bp, five inhibition assays were peformed. Only the one at site 2089 bp for Hha I did not show inhibition. The rest of sites at bp 3166 for Alu I, 3130 for Hae III, 3020 for Hpa II and 3189 for Rsa I were inhibited. Within these high affinity binding sites of carcinogen, there are three possible sites where the carcinogen could affect. One covered the sites 3166 and 3189, a second was at site 3020 and the last one was at 3130. Over the next

fragment of 66 bp, extending from 3378 to 3444 only one inhibition assay was done, bp 3390 for Alu I, which did not show inhibition. One site of high affinity binding site of carcinogen exists within the Hinf I 417 bp fragment. This fragment goes from bp 3732 to bp 4149. It was located at bp 4137 for Alu I. No inhibition was observed for the same enzyme at bp 3883 or for the Taq I site at 4040 bp. The next region in the clockwise direction along the plasmid is 4702 bp. Three inhibition assays were performed on it, activity of Hae III at bp 4208 and at bp 4489 and bp 4253 for Dde I were tested. Both assays for Hae III showed inhibition while the site for Dde I did not. None of the inhibition assays checked on fragment 427 bp between bp 4702 and 5129 showed inhibition. Those included sites at bp 5043 for Ava II and for Sau 96 I.

Based on the above analysis, tables 6 and 7 were constructed; these are segments of sequence close to the inhibition site.

From these segments of sequence for all the enzymes inhibited on both DNAs, a common sequence was observed which appears in the vicinities of the inhibition site. This common sequence is underlined on each segment of sequence.

For SV 40 DNA, five sites of high affinity for binding carcinogen were found, while seven were found on ϕX 174. It is clear from the results summarized in tables 6 and 7 that restriction enzyme inhibition does not occur randomly along the plasmids.

Table 6

Carcinogen Binding Sequences SV 40

	*841 (2)
G T G T G A G C G C T G T T G C T A A G T G	(1)
	(3)
	*2258
A T G A G C A G G G T G T T G G G C C C T T	
	*4499
A G A T G G C A T T T C T T C T G A G C A A	
	*3576
T T T T T A G G A A T G T T G T A C A C C A	
	*3088
T T T A A A T A A T C C T T G G C C C T A A	

(1) Segment of total nucleotide SV 40 DNA sequence.
(2) *- Reference number to locate the common sequence.
(3) Underlined common seuqence.

Table 7

Carcinogen Binding Sequences ϕX174RF

	*154
A C C T A	T C C T T G C G C A G C T C
	*4095
C C C A A	T G C T T G G C T T C C A T
	*3337
G C T G A	T G C T T C C T C T G C T G
	*3036
G T G A G	T G C T T G C T A C C G A T
	*4213
A A G G C	T G C T T C T G A C G T T C
	*1054
T T C C C A	T C T T G G C T T C C T T
	*4173
T C A A G C	T C T T G G A A G A G A T
	*3174
A G G A C T	T C T T G A A G G T A G G

Table 8

Carcinogen Binding Sequences pBR 322

													*962						
G	C	T	G	G	G	C	T	A	C	G	T	C	T	T	G	C	T	G	G	C	G	T
														*3040					
G	A	G	T	T	G	G	T	A	G	C	T	C	T	T	G	A	T	C	C	G	G	C
														*692					
C	C	C	A	G	T	C	A	G	C	T	C	C	T	T	C	C	G	G	T	G	G	G
														*2394					
C	T	G	C	G	C	T	C	G	G	T	C	G	T	T	C	G	G	C	T	G	C	G
														*3725					
A	G	C	G	G	T	T	A	G	C	T	C	C	T	T	C	G	G	T	C	C	T	C
														*2194					
G	C	G	T	C	A	G	C	G	G	G	T	G	T	T	G	G	C	G	G	G	T	G
														*2356					
G	C	A	T	C	A	G	G	C	G	C	T	C	T	T	C	C	G	G	T	T	C	C
														*1113					
T	C	G	C	T	C	G	C	G	G	C	T	C	T	T	A	C	C	A	G	C	C	T

On SV 40 DNA, four of the segments showed the common sequence T(G/C)TTG and one was TCCTTG. For ϕX 174, five of the segments showed the common sequence T(G/C)CTT(G/C) and three were TCTTG.

Generalizing over the elements of these common sequences, it was possible to assign a general common sequence.

For four sites on SV 40 DNA and three on ϕX 174 DNA :

T(G/C)TT(G/C)

For one site on SV 40 and five sites on ϕX 174 :

T(G/C)CTT(G/C)

It should also be pointed out that the same enzyme found inhibited at one site, should to be not inhibited at other site. This suggests that the inhibition detected in these assays was not specific for a certain enzyme.

In contrast with this, other researchers have found that an alteration in the DNA at recognition sequence for a specific enzyme causes inhibition in its activity (Vardimon, L. and Rich, A.,1984). Agents like cis-dichlorodiamino-platinum II block the enzyme BamH I at its recognition sequence, causing inhibition by preventing access to the DNA for the enzyme. (Weinstein, I.B., Grunberger, 1974; Coffmann, G.L., Gaubatz, J.W.,1982). Additionally daunomycin (Malcolm, A.D., Moffati, J.R.,1981) as well as anthramycin (Sumner, W., Bennett, G.N. 1981) which are non-covalent DNA binding agents cause specific restriction enzyme inhibition. The size of the recognition sequence did not seem to be a

factor since enzymes with 5 base pairs all possess sites on SV 40 and OX 174, where they were inhibited in the presence of the carcinogen.

Experiments performed on pBR 322 (Mallamaci, M.A.,1988) have shown that locations of observed lambda exonuclease as well as restriction endonuclease inhibition are the same than those on the DNAs examined in the present study.

Comparison of the sequences at the sites of inhibition on pBR 322 indicate the presence of the same common sequence elements on SV 40 and ϕX 174. (see table 8)

This fact is providing strong evidence that the carcinogen AAAF targets for a family of specific DNA sequences which are found on DNAs from a variety of sources.

There are two models describing the conformational details of AAF-modified nucleic acids. The base displacement model has the bound AAF moiety displacing the guanine (to which is attached) from the helix. (Weinstein, I.B., Grunberger, D. 1974; Evans, F.E., Miller, D.W.,1980; Leng, M.,Ptak, M.,Rio, P.,1980; Hingerty, B. and Broyde, S.,1982; Sage, E. and Leng M.,1980). The insertion denaturation model has the bound AAAF disrupting the helix by placement in a groove of the DNA. (Fuchs, R. and Daune, M.1971,1972,1973, 1974,1975,1976; Box, H.C, Lilga, K.T. et al,1984).

Regardless of differences in structural detail, both models invoke alterations in DNA structure resulting from carcinogen binding.

Other researchers suggest that for certain sequences a

Z- like DNA structure has been determined with the fluorene ring residing on the outside of the helix.(Santella, R., Grunberger, D., Hingerty, B., and Broyde S.,1981; Standford and Krugh, 1985; Fuchs, Schwartz, Daune,1983).

However, in either case the presence of the adduct would present a blockage for the enzyme to overcome which would result in enzyme inhibition.

It is clear that the complex sequence found within every high affinity AAAF binding site strongly suggest the binding is sequence specific.

The process by which an AAAF molecule goes to specific sites is still unknown; however some aspects make the sequence likely target for attack by the carcinogen : the presence of adjacent "GC" bases preceeded by adjacent "TT" bases and sometimes preceeded by a third "T" base. It has been shown by Fuchs (1983), that the carcinogen binds preferentially to a G adjacent to a C. Also the presence of flanking thymines may help to ease denaturation because AT base pair have a lower melting point than GC base pairs.

Other studies have proposed that AAAF binding sites occur either in the middle or terminal regions of resistance genes or within the origin of replication. (Mallamaci,M.A., 1988) (see table 9 and figures 9 and 10) (Reddy,V.B., Thimmappaya, B., et al, 1978; Sanger, F.,Air,G.M., et al, 1978; Fiers W., Contreras, R., et al,1978; Sanger, F., Coulson, A.R., et al, 1978). Since none of these binding sites occur within a non-coding region, that is suggesting

that the processes of replication or transcription do not take place in the presence of carcinogen.

Table 9

	Gene code for struct.protein	Site of init.(bp)	Site of terminat.(bp)
SV 40	A	2220	3396
	B	888	1698
	C	3396	3908
	D	3970	4493
	E	4493	4963
	F	5146	5224
	G	512	888
	H	1943	2220
	I	1698	1943
	J	261	512
	K	4963	5146
	L	3907	3944
	M	3944	3970
OX 174	A	3973	123
	B	5064	38
	C	134	391
	D	390	846
	E	568	840
	F	1001	2276
	G	2387	2912
	H	2923	3907

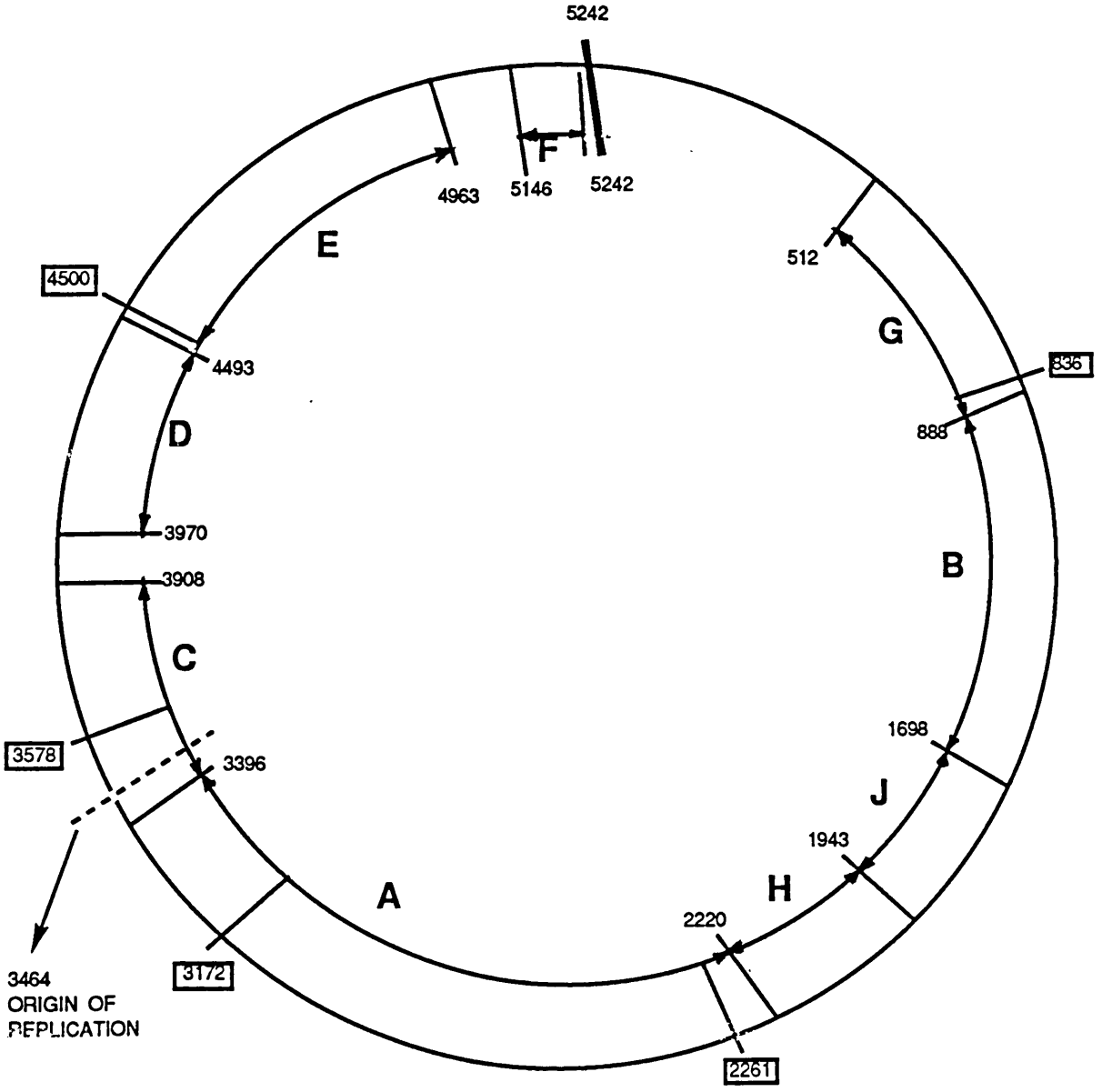


FIGURE 9: SV 40 DNA
MAP FOR INHIBITION SITES AND GENES CODING
STRUCTURAL PROTEINS

Inner circle shows location of proteins and origin of replication along the DNA in terms of base pairs. Outer circle shows carcinogen sites location.

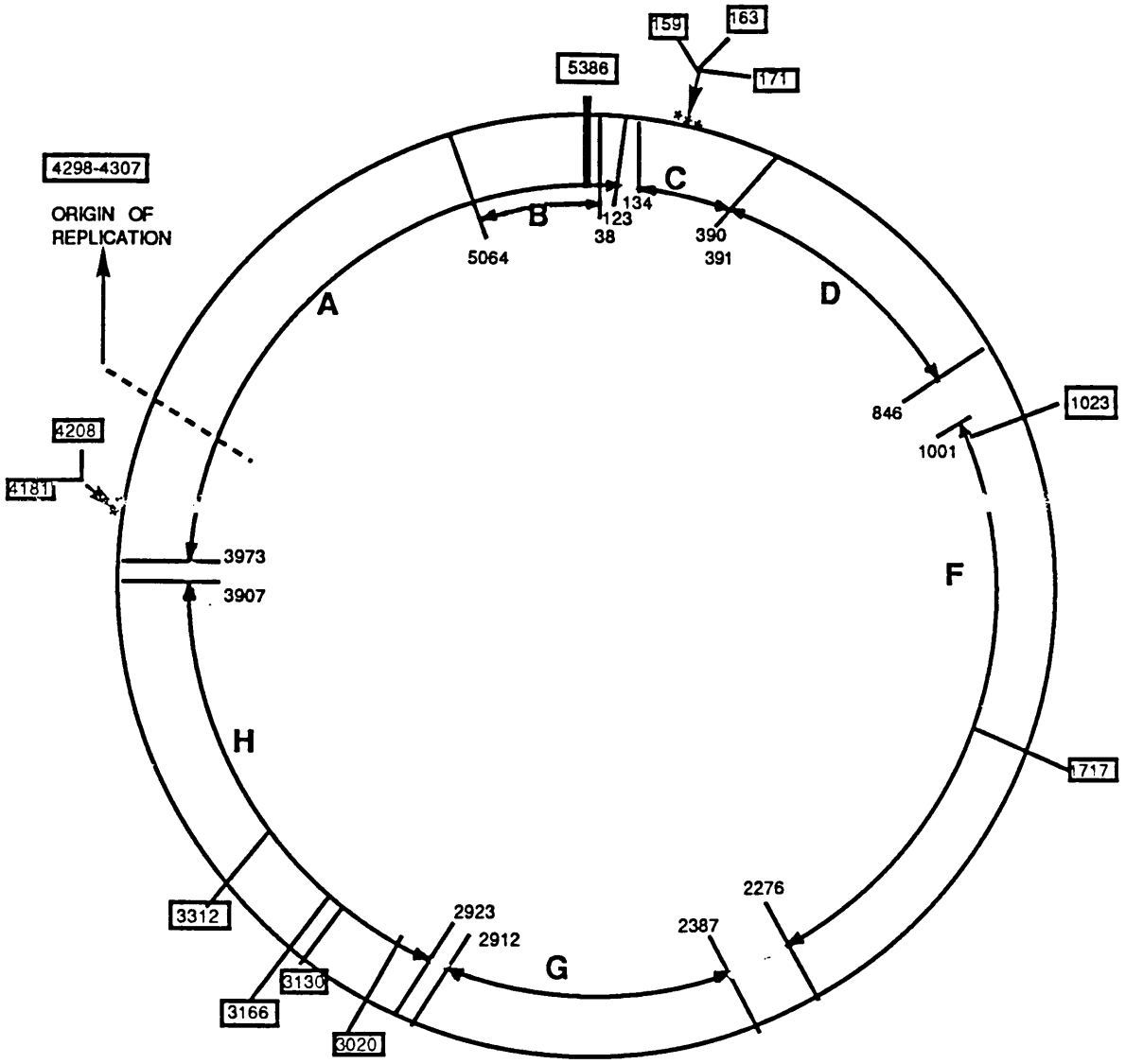


FIGURE 10: ΦX 174 RF
MAP FOR INHIBITION SITES AND GENES CODING
STRUCTURAL PROTEINS

CONCLUSIONS

As shown by the results of restriction enzyme inhibition and lambda exonuclease inhibition assays, these studies allowed to map the locations of high affinity binding sites of the carcinogen N-acetoxy-N-acety1-2-aminofluorene (AAAF) on the plasmids SV 40 and ϕX 174 RF DNAs.

For both, enzymes types inhibition increased as bound carcinogen concentration increased; this was basically determined qualitatively and in some cases verified quantitatively.

Inhibition detected is not specific for a certain enzyme or certain size of recognition sequence.

The locations of observed lambda exonuclease inhibition are the same, in all cases, as the locations of observed restriction enzyme inhibition.

With the number of inhibition assays performed, it was found eight sites on ϕX 174 and five on SV 40 of inhibition of the enzymatic activity by the carcinogen.

All sites have common sequence elements. The presence of either the sequence T(G/C)TT(G/C) or the sequence T(G/C)C TT(G/C). It is also clear that inhibition enzyme occurred at selected sites.

Appendix 1

Restriction Endonucleases Used and Their Enzymatic Conditions

Restriction Enzyme	Reaction conditions
Alu I	37 °C- React #1
Ava II	37 °C- React #2
Dde I	37 °C- React #2
Dra I	37 °C- React #1
Eco RV	37 °C- React #2
Hae III	37 °C- React #2
Hha I	37 °C- React #2
Hpa II	37 °C- React #8
Mbo I	37 °C- React #2
Pst I	37 °C- React #2
Rsa I	37 °C- React #1
Sau 96 I	37 °C- React #11
Taq I	65 °C- React #2
Tha I	60 °C- React #1
Xho I	37 °C- React #2

(Taken from Bethesda Research Laboratories catalogue and reference guide)

Appendix 2

6% Polyacrylamide gel

Reagents	Volume (mL)
30% acrylamide	6
10X TBE Buffer	3
Water	21
Ammonium persulfate	

30% Acrylamide

Acrylamide	29 g
Bis	1 g
Complete to 100 mL with water	

10X TBE Buffer

Tris (basic)	121 g
Boric acid	55 g
EDTA	7.44 g

8M Urea gel in 8% acrylamide

Urea	14.4 g
10 TBE	3 mL
Acrylamide	8 mL
Water to 30 mL	
Ammonium persulfate	

Appendix 3

6% polyacrylamide electrophoresis

For pouring the gel, glass plates (2) were used. They were washed with ethyl acetate to remove grease and set aside to dry. The larger outer plate was laying on a bench and two space bars were placed along the sides with vaseline to help keeping the spacer bars in position during next steps. The inner plate was laying in position resting on the space bars and four clamps were placed to compress and form a seal between plates. Four mls from the total volume solution of 30 mls of 6% polyacrylamide were taken and a proportional amount of TEMED (30 ml of TEMED/100 ml of polyacrylamide) was added and mixed with the solution to help polymerization. This solution was poured with a pasteur pipette into the space between plates at the foot to make the lower position of the gel. When acrylamide polymerized, the rest of the gel (26 ml) was poured, previous addition of TEMED filling to the top.

Inmediately an appropriate comb was inserted between plates in such a way that the tops of the teeth were slightly higher tan the top of the glass plate and being careful not to allow air bubbles under the teeth. Acrylamide was allowed to polymerize at room temperature for 20 minutes. When polymerization was complete, Schlieren pattern appeared just beneath the teeth.

Comb was removed and gel was attached to the electrophoresis tank, leaving the inner plate against the tank with bulldog clips. Then, reservoirs were filled with the ten times diluted 10X buffer, flushing out the wells. Electrodes were connected to a power pack (positive out let connected to the bottom reservoir) and gel was run for 30 minutes at 250 volts.

For addition of DNA samples, 10 ul of marker dye (bromo phenol blue 0,1%) and they were loaded on patterns of 6% polyacrylamide gel and electrophoresed for 2 to 3 hours.

Abbreviations

AAAF.....	Carcinogen N-acetoxy-N-acetyl-2-aminofluorene
DNA.....	Deoxyribonucleic acid
G.....	Guanine base
C.....	Cytosine base
bp.....	Base pair(s)
ug.....	Microgram
ul.....	Microliter
rpm.....	Revolutions per minute
TE buffer	0.01 M tris, 0.001 M EDTA, pH 8 buffer
U.V.....	Ultraviolet
V.....	Volts
mM.....	Milimolar
CPM.....	Counts per minute
EDTA.....	Ethylendiaminotetraacetic acid
TBE.....	Tris, boric acid, EDTA, buffer
TEMED....	N,N,N',N'-Tetramethylethylenediamine

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VITA

N-acetoxy-N-Acetyl-2-Aminofluorene Binding Sites on ΦX174 and SV40

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DNA sequences which are attacked by the carcinogen N-Acetyl-N-Acetoxy-2-Aminofluorene. Biophysical Journal vol 55.1989

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