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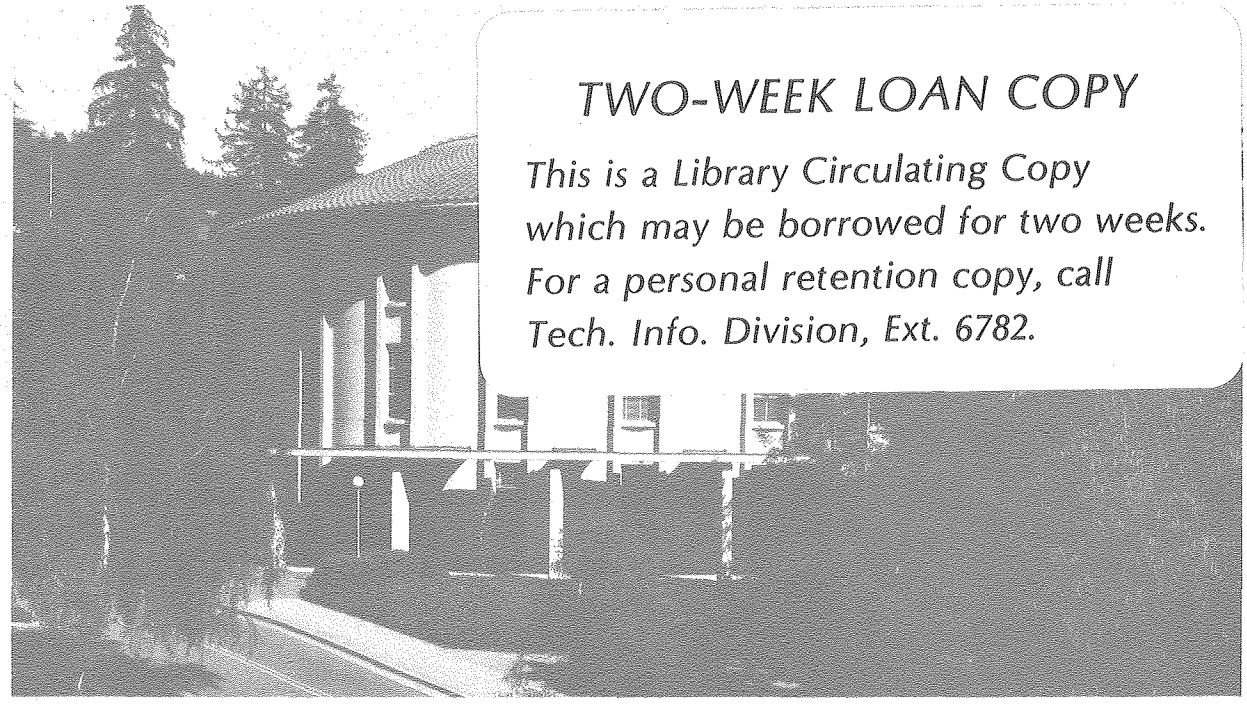

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Interaction of Benzo[a]pyrene Diol Epoxide with SV40 Minichromosomes

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ABSTRACT

SV40 minichromosomes were reacted with (+)7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BaP diol epoxide). Low levels of modification (< 5 DNA adducts/minichromosome) did not detectably alter the structure of the minichromosomes but high levels (> 200 DNA adducts/minichromosome) led to extensive fragmentation. Relative to naked SV40 DNA BaP diol epoxide induced alkylation and strand scission of minichromosomal DNA was reduced or enhanced by factors of 1.5 and 2.0, respectively. The reduction in covalent binding was attributed to the presence of histones, which competed with DNA for the hydrocarbon and reduced the probability of BaP diol epoxide intercalation by tightening the helix. The enhancement of strand scission was probably due to the catalytic effect of histones on the rate of β-elimination at apurinic sites, although an altered adduct profile or the presence of a repair endonuclease were not excluded. Staphylococcal nuclease digestion indicated that BaP diol epoxide randomly alkylated the minichromosomal DNA. This is in contrast to studies with cellular chromatin where internucleosomal DNA was preferentially modified. Differences in the minichromosomal protein complement were responsible for this altered susceptibility.

Benzo[a]pyrene (BaP) is a common pollutant and a potent carcinogen (1). In mammalian cells it is metabolically activated to the ultimate carcinogen (+)7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]-pyrene (BaP diol epoxide) (2). This reactive electrophile alkylates DNA at the N^2 position of guanine and to a lesser extent at the N^6 position of adenine (3 - 6). Furthermore, approximately 1% of the alkylation sites, which may represent N-7 guanine adducts, undergo depurination/depyrimidination strand scission (7 - 9). We have utilized superhelical SV40 DNA to probe the microenvironment of the alkylation sites (10) and to study the mechanism of the hydrocarbon induced strand scission (Gamper et al., unpublished experiments).

Within the nuclei of infected cells SV40 DNA is associated with cellular histones in a chromatin-like structure called the minichromosome (11 - 13). Viral chromatin closely resembles the more complex chromatin of mammalian cells and is replicated and transcribed by the same cellular enzymes (14). Given the physical and genetic simplicity of the SV40 minichromosome, it has proven to be a useful structural as well as functional model for eucaryotic chromatin (13). This paper describes an investigation of the integrity of BaP diol epoxide modified SV40 minichromosomes and the modulation of DNA alkylation and strand scission by minichromosomal histones.

MATERIALS AND METHODS

Cells and Virus Stocks. Confluent TC-7 African green monkey kidney cells were infected with wild type SV40 small plaque strain Rh911 at 10-20 plaque forming units/cell. The DNA was labeled by adding 1 mC_i of [^{32}P]-orthophosphate (ICN), 200 μC_i of [^{14}C]-thymidine (New England Nuclear), or 200 μC_i of [^{3}H]-thymidine (New England Nuclear) to 10^7 cells 18 hr after

infection. At the peak of SV40 replication, 42 hr after infection, viral DNA or chromatin was extracted from 10^8 cells.

Isolation of SV40 DNA. Viral DNA was isolated by a modification of the Hirt extraction essentially as described by Hallick et al (15).

Isolation of SV40 Chromatin. The procedure used here is similar to published methods (11, 16 - 18). Approximately 10^8 cells at 42 hr post-infection were rinsed with cold Tris-diluent (0.137 M NaCl-5 mM KCl-5 mM Na_2HPO_4-25 mM Tris base, pH 7.5) and then scraped into the same buffer. A loose cellular pellet was obtained by centrifuging the cell suspension (JA-20 rotor, 2 min, 2,000 rpm at 4°). The pellet was resuspended in 5.0 ml of cold Tris-diluent and gently mixed with 80 ml of cell lysis buffer (50 mM Tris-HCl, pH 7.5-5 mM $MgCl_2$-25 mM KCl-0.25 M sucrose [Schwarz/Mann; RNase free]-0.5% Nonidet P-40 detergent [Shell Oil Ltd., London]). No intact cells were detectable microscopically after several minutes at 4°. Nuclei were pelleted by centrifugation (JA-20 rotor, 5 min, 2,000 rpm at 4°). The pellet was suspended in 2.0 ml of nuclei lysis buffer (10 mM Tris-HCl, pH 8.1-10 mM EDTA-0.13 M NaCl-0.5 mM dithiothreitol-1.0 mM phenylmethylsulfonyl fluoride-0.25% Triton X-100 [Rohm & Haas]) and gently shaken 1-3 hr at 4° to maximize extraction of minichromosomes. Cellular chromatin was removed by centrifugation (JA-20 rotor, 5 min, 8,000 rpm at 4°). The supernatant, which contained viral chromatin, was layered onto two 5-40% sucrose gradients in 10 mM Tris-HCl, pH 7.8-1 mM EDTA-0.13 M NaCl and centrifuged in a Beckman SW 27 rotor for 5 hr at 25,000 rpm and 4°. The gradients were collected from the tube bottom in 1.0 ml fractions. DNA and protein concentrations were estimated from the optical density values at 260 nm and 280 nm using a nomograph (19).

BaP Diol Epoxide. Crystalline BaP diol epoxide was synthesized by K. Straub of this laboratory essentially using the method of McCaustland and Engel (20). [^{3}H]-BaP diol epoxide, 1.5 mg/ml in 19:1 tetrahydrofuran-triethylamine, was also synthesized by K. Straub according to published procedures (21) and had a specific activity of 1.23 C_i/mmole. The BaP diol epoxide stocks were stored at -70° and dilutions were made with dimethyl sulfoxide.

BaP Diol Epoxide Alkylation. SV40 form I DNA (ca. 10 µg/ml) was modified with BaP diol epoxide in 20 mM Tris-HCl, pH 8.0-0.5 mM EDTA-10% dimethyl sulfoxide at the indicated molar reaction ratios (BaP diol epoxide/DNA mononucleotide). Reaction mixtures were incubated in the dark at 37° for 2-24 hr. Aliquots were analyzed for strand scission directly after reaction by gel electrophoresis. Alkylation was quantified by radioactivity counting using SV40 [^{14}C]-DNA and [^{3}H]-BaP diol epoxide. The modified DNA was diluted to 1.0 ml with 20 mM Tris-HCl, pH 8.0-0.5 mM EDTA-0.5 M NaCl and extracted 3 times with 1.0 ml of ethyl acetate. After adding 30 µg of carrier calf thymus DNA, the DNA was precipitated from the aqueous phase by addition of 2.0 ml of NaCl-saturated ethanol and by storage overnight at -20°. The DNA was pelleted, washed, and resuspended in 1.0 ml of NaCl-saturated 70% ethanol. It was then collected on Millipore 0.45 µ HAWP filters, combusted, and counted. For ^{32}P-labeled DNA the filters were dried and counted in Permafluor I (Packard). Binding data was calculated assuming SV40 contained 5200 base pairs (22) and had a molecular weight of 3.6×10^6 daltons (14).

SV40 chromatin (ca. 10-25 µg/ml) was reacted with BaP diol epoxide in the sucrose gradient isolation buffer (5-40% sucrose in 10 mM Tris-HCl, pH 7.8-1 mM EDTA-0.13 M NaCl) or after dialysis into 20 mM Tris-HCl, pH

8.0-0.5 mM EDTA with or without 0.13 M NaCl. Reaction aliquots were analyzed directly as chromatin or were deproteinized and analyzed as DNA. The chromatin was deproteinized by incubation with 0.85 mg/ml pronase (Calbiochem; B grade, nuclease free) for 3 hr at 37° followed by 2 equal volume extractions with chloroform-phenol-isoamyl alcohol (24:25:1). The phases were clarified by centrifugation and the precipitated protein was discarded. Alkylation of ^{32}P or ^{14}C-labeled minichromosomal DNA was determined after deproteinization, ethyl acetate extraction, and ethanol precipitation as described above. For quantification of binding the minichromosomes were incubated 30 min at 37° with 90 ng/ml RNase (Calbiochem; A grade, 5X crystallized) immediately after alkylation.

Staphylococcal Nuclease Digestion. SV40 chromatin or DNA was digested with 1-10 E.U. staphylococcal nuclease (Worthington Biochemical)/µg viral DNA at 37° in 10-20 mM Tris-HCl, pH 7.8-0.5-1.0 mM EDTA-3.0 mM $CaCl_2$-3.0 mM $MgCl_2$. Most incubations also included 0.13 M NaCl and 20-30% sucrose. Nuclease activity was abolished by raising the EDTA concentration to 18 mM.

Gel Electrophoresis. Vertical slab gel electrophoresis was conducted in an apparatus constructed according to the design of Studier (23). Gel dimensions were 13.3 X 13.0 X 0.3-0.5 cm. SV40 DNA was electrophoresed at 50V for 12 hr on 1.4% agarose gels prepared and run in 40 mM Tris-HCl, pH 7.9-5 mM sodium acetate-1 mM EDTA. SV40 minichromosomes aggregate in the above buffer and so were electrophoresed 6 hr at 50 V on 1% agarose gels prepared in 10 mM Tris-HCl, pH 7.8-2 mM EDTA (17). Staphylococcal nuclease DNA digests were electrophoresed 2 hr at 150 V on 6% polyacrylamide gels containing 5.85% acrylamide and 0.15% N,N'-methylene-bisacrylamide. These gels were prepared and run in 89 mM Tris-89 mM boric acid, pH 8.3-2.8 mM EDTA.

Gels containing SV40 DNA or chromatin were stained for at least 1 hr at 4° in electrophoresis buffer containing 0.5 μg/ml ethidium bromide. The fluorescent DNA bands were illuminated with short wavelength uv (Ultra Violet Products) and photographed on Polaroid type 665 film through a yellow filter. For determination of radioactivity ^{14}C-labeled DNA bands were excised under uv illumination, dried overnight at 37° on adsorbent pads, combusted in a Packard automatic combustion apparatus, and counted. ^{32}P-labeled DNA bands were solubilized for counting. Agarose gel slices were dissolved in 1.0 ml of H_2O at 100°. Gel slices containing polyacrylamide were degraded in 1.0 ml of 30% H_2O_2 at 55°. Excess H_2O_2 was destroyed by adding 200 μl of 1 M NaOH and incubating an additional 2 hr at 55°. Chemiluminescence was minimized by acidifying with 1 drop of 12 N HCl. Solubilized gel solutions were mixed with 4.0 ml of Aquasol-2 (New England Nuclear) and counted. For autoradiography, gels containing ^{32}P-labeled DNA were dried on filter paper in a Hoefer model SE540 slab gel dryer and exposed for 60 hr to Kodak SB-5 X-ray film.

Ultracentrifugation. ^{3}H-labeled SV40 chromatin was analyzed by neutral sucrose density gradient centrifugation. The viral chromatin (1-3 μg in 100 μl) was layered on 5.0 ml 5-30% sucrose gradients prepared in 10 mM Tris-HCl, pH 7.7-1.0 mM EDTA-0.13 M NaCl and centrifuged in a Beckman SW 50.1 rotor for 90 min at 50,000 rpm and 4°. The gradients were fractionated from the bottom and counted. The sedimentation coefficient of chromatin was determined relative to form I SV40 DNA run on a separate gradient.

The buoyant density of ^{3}H-labeled SV40 chromatin fixed as described by Christiansen and Griffith (24) was determined by equilibrium centrifugation in 20 mM Tris-HCl, pH 8.0-0.5 mM EDTA-0.13 M NaCl containing 0.73 g/ml CsCl. After 48 hr at 35,000 rpm and 4° the gradient was collected from the bottom in 8 drop fractions. Refractive index values were converted to buoyant densities using an experimentally determined standard curve.

RESULTS

Characterization of Minichromosomes. The viral chromatin employed here was extracted with 0.25% Triton X-100 from nuclei of lytically infected cells at the peak of DNA synthesis. The yield of minichromosomes was maximized and the loss of histone H1 minimized by extracting the nuclei in the presence of 0.13 M NaCl (17). Enzymatic digestion of the liberated chromatin was inhibited by EDTA and phenylmethylsulfonyl fluoride. After pelleting the cellular chromatin, the nuclear extract was centrifuged through a 5-40% sucrose gradient. The radioactivity and absorbance profiles of a typical gradient are shown in Figure 1A. The peaks of optical density and radioactivity did not coincide, probably reflecting contamination of the viral chromatin with unlabeled ribonucleoprotein particles (25). When fractions in the vicinity of the two peaks were treated with SDS to remove proteins and analyzed by agarose gel electrophoresis (Figure 1B), the ethidium bromide staining bands were attributed to forms I and II SV40 DNA. The level of viral DNA correlated with the [^{3}H]-thymidine label. Typically, peak SV40 chromatin fractions were pooled and used within 72 hr.

The SV40 minichromosomes were characterized by standard techniques. In electron micrographs at low ionic strength they exhibited the characteristic beads-on-a-string morphology (A. Tung, unpublished results). SDS-

polyacrylamide gel electrophoresis of the minichromosomes gave 4 bands corresponding to the core histones H2A, H2B, H3, and H4 and a less intense band corresponding to histone H1 (S. Treon, unpublished results). In addition, slower moving bands with mobilities similar to the viral capsid proteins were also present. Digestion of the minichromosomes with staphylococcal nuclease liberated 50-60% of the DNA and left the remainder as 140 base pair fragments (see Figure 2).

The protein to DNA weight ratio of the viral chromatin was determined by CsCl density gradient centrifugation of a formaldehyde/glutaraldehyde fixed sample. The buoyant density of the minichromosomal complex, 1.41-1.43, was below literature values of 1.45-1.49 (24, 26, 27). A weight ratio of protein to DNA of 2.0-2.5 was calculated using equation 1 from buoyant density values of 1.701 (28), 1.295 (29), and 1.41-1.43 for DNA,

$$(1 + x)\,\rho_{chromatin} = \rho_{DNA} + \rho_{protein} \qquad (1)$$

histones, and chromatin, respectively. The high ratio obtained suggests that the minichromosomes contained nonhistone protein, a large fraction of which could be viral capsid protein.

The sedimentation coefficient of viral chromatin was 55S in 20 mM Tris-HCl buffer and 63S in the same buffer with 0.13 M NaCl. The minor increase in sedimentation implies only slight compaction of the minichromosomes at physiological ionic strength and is indicative of a histone H1 deficiency (13). It is conceivable that the minichromosomes isolated here are depleted in histone H1 and rich in nonhistone (possibly viral capsid) protein because a large fraction are derived from mature virons (26).

Reaction of BaP Diol Epoxide with SV40 Minichromosomes. We have found that a small fraction of the BaP diol epoxide adducts in SV40 DNA rearrange with strand scission (7) and that the remainder induce a strain dependent denaturation of the DNA helix (10). The SV40 minichromosome is an ideal substrate with which to study how such alterations affect the integrity of chromatin structure. However, since chromatin is by definition a histone-DNA complex, it is important to recognize that changes in minichromosomal structure could also arise from modification of protein. When chromatin is reacted in vitro with BaP diol epoxide, approximately 15% of total alkylation occurs on histone protein (A. Kootstra, personal communication).

Staphylococcal nuclease is a sensitive probe of chromatin structure. When chromatin is incubated with this enzyme, the deproteinized DNA gives a characteristic time dependent migration pattern in polyacrylamide gels (30). Brief digestion produces a series of discrete DNA bands which are multiples of a monomer 180-200 base pairs in length. The multimers are precursors of the monomer which upon further digestion gives rise to a homogeneous 140 base pair fragment. We have investigated the integrity of BaP diol epoxide modified SV40 minichromosomes with staphylococcal nuclease. The viral chromatin, labeled with ^{32}P, was deproteinized and analyzed as a function of digestion time in a 6% polyacrylamide gel. Figure 2 is an autoradiograph of that gel and shows the DNA fragmentation pattern for (A) control and (B) modified minichromosomes. The gel was calibrated with a Hind II digest of ϕX174 RF DNA, and the percent DNA rendered ethanol soluble is shown at the bottom of each track. A close examination reveals no differences between the two chromatin samples. In both the DNA monomer band had an initial length of circa 200 base pairs which decreased to 140 base pairs at 46-48% digestion. A high background

obscures the dimer and trimer bands. The band comigrating with the largest ϕX174 DNA fragment is unidentified. The identical digestion profiles imply that low level BaP diol epoxide binding (< 5 DNA adducts/minichromosome) does not disturb gross nucleosomal structure. A similar conclusion has been reached from chromatin reconstitution experiments employing BaP diol epoxide modified DNA and either untreated or modified histones (31; A. Kootstra, personal communication).

When more highly modified viral chromatin was analyzed by sucrose density gradient centrifugation, changes in the sedimentation pattern indicated structural alterations (Figure 3). The minichromosomes in this study were alkylated at molar reaction ratios of 0.2-3.0 (BaP diol epoxide/minichromosomal DNA mononucleotide) and are estimated to contain 35-450 DNA adducts (see Figure 5) and 5-10 fewer protein adducts. Their overall structure was not affected by alkylation at molar reaction ratios as high as 0.8. The increased sedimentation coefficient of these samples relative to an unreacted control is probably due to the added mass of physically and covalently bound hydrocarbon although a more compact hydrodynamic configuration cannot be ruled out. At higher molar reaction ratios the minichromosomes were converted to slowly sedimenting heterodisperse fragments; this is attributed to BaP diol epoxide induced DNA strand scission.

The modified minichromosomes were also characterized by agarose gel electrophoresis. After alkylation in 20 mM Tris-HCl buffer at molar reaction ratios of 0.05-3.0 the minichromosomes were divided into two sets. One was deproteinized and electrophoresed as DNA (Figure 4A). The other was electrophoresed at low ionic strength as a nucleoprotein complex (Figure 4B). Tracks from left to right corresponded to increasing modification;

track a was an unreacted control. The DNA banding pattern was very similar to that obtained with superhelical SV40 DNA (data not shown). At lower molar reaction ratios form I minichromosomal DNA, which exhibited a reduced electrophoretic mobility due to hydrocarbon induced unwinding, was converted to nicked circular DNA (10). At higher molar reaction ratios extensive strand scission led to full length linear DNA and smaller fragments which ran off the gel. This degradation was probably due to depurination strand scission at minor alkylation sites (Gamper et al., unpublished experiments).

In the corresponding nucleoprotein gel the minichromosomes electrophoresed as a fairly sharp band with a slight amount of high molecular weight trailing material. At intermediate reaction ratios, where the viral DNA contained up to several single strand nicks, the minichromosomes continued to migrate as a discrete band. Since the minichromosomes contain little or no torsional strain, occasional DNA nicks did not significantly alter their configuration. The slight decrease in band mobility at intermediate reaction ratios is probably equivalent to the enhancement of sedimentation seen in sucrose gradients. Taken together, these effects are consistent with a molecular weight increase of the modified minichromosomes due to the presence of physically and covalently bound hydrocarbon. At the highest molar reaction ratios, where the viral DNA was extensively fragmented, the minichromosomes were degraded and ran as a low molecular weight heterodisperse band.

Just as chromatin structure was altered by BaP diol epoxide adducts, DNA alkylation and strand scission by the hydrocarbon might be modulated by the repeat structure of the nucleoprotein complex. The question of modulation was studied by reacting ^{3}H-labeled BaP diol epoxide with ^{14}C-labeled

SV40 minichromosomes or superhelical DNA. The reactions were conducted in 20 mM Tris-HCl, pH 8.0, buffer containing 0.5 mM EDTA and 10% dimethyl sulfoxide. To ascertain the effect of minichromosome compaction on DNA alkylation and strand scission, a duplicate set of reactions were conducted in the same buffer containing 0.13 M NaCl. After 2-4 hr incubation at 37° the viral DNA was isolated and assayed for covalent binding. Separate samples were incubated 24 hr prior to analysis for strand scission. The average number of nicks per DNA molecule (μ) was calculated from the equation $U = e^{-\mu}$ (32) following electrophoretic determination of the fraction of form I DNA (U) remaining in the reaction mixture.

The results in Figure 5 demonstrate that DNA alkylation and nicking were modulated by the minichromosome structure. In low ionic strength buffer minichromosomal DNA exhibited enhanced nicking and reduced binding relative to superhelical DNA. In the presence of 0.13 M NaCl both nicking and binding were reduced 10-fold for the two DNA substrates. Although higher binding was still observed with superhelical DNA, both substrates exhibited comparable strand scission in physiological salt. The ratio of adducts per nick and the precent inhibition of binding and nicking by 0.13 M NaCl are summarized for the two DNAs in Table 1.

Alkylation of Nucleosomal Versus Internucleosomal DNA. With the recent availability of enzymes such as staphylococcal nuclease and pancreatic DNase (DNase I) which only partially digest the DNA in a nucleoprotein complex, it has become possible to map the distribution of alkylation sites within the repeat unit of chromatin. Staphylococcal nuclease preferentially attacks linker DNA while DNase I is less specific and attacks both inter- and intranucleosomal DNA stretches (30, 33, 34). Most carcinogens when investigated exhibit nonrandom binding to chromatin. Some

preferentially alkylate nucleosomal core DNA and are selectively released by DNase I while others preferentially alkylate internucleosomal spacer DNA and are selectively released by staphylococcal nuclease (35 - 41). Reduced binding of BaP diol epoxide to minichromosonal DNA suggested the existence of a nonrandom binding pattern; this was investigated with staphylococcal nuclease.

Viral chromatin was reacted with BaP diol epoxide and digested with staphylococcal nuclease immediately after isolation. The digestion of bulk DNA and BaP diol epoxide modified nucleotides was followed by counting ethanol precipitable ^{32}P and ^{3}H radioactivity, respectively, after removal of RNA, protein, and unreacted hydrocarbon. By labeling the DNA with ^{32}P the release of all four nucleotides could be monitored thereby avoiding problems associated with the preferential release of radioactive thymidine by staphylococcal nuclease (42). Control experiments indicated that low modification levels (< 5 DNA adducts/genome) did not affect the fragmentation pattern or the digestion kinetics of minichromosomal DNA (Figure 2). Approximately 50% of the DNA was rendered ethanol soluble, a value characteristic of the nucleoprotein complex. Modified protein-free viral DNA was almost completely digested by staphylococcal nuclease (Figure 6). However, the covalently bound BaP diol epoxide was less completely solubilized and suggested that certain alkylation sites were resistant to digestion.

When BaP diol epoxide modified minichromosomes were incubated with staphylococcal nuclease, bulk DNA and covalently bound hydrocarbon were digested with the kinetics shown in Figure 7. After 20 min of incubation 60% of the DNA and 56% of the adducts were released, clearly indicative of a uniform distribution of adducts at the time of digestion. Following deproteinization the residual nuclease-resistant ^{32}P and ^{3}H radioactivity

was almost totally solubilized by the enzyme, discounting the possibility that hydrocarbon modified macromolecules other than DNA were present in the ethanol pellet. The ratio of ^{32}P to ^{3}H was also determined for total DNA prior to digestion and for nucleosomal core DNA generated by the nuclease and isolated by gel electrophoresis. Digestion of the minichromosomes increased the ratio from 0.15 $\pm$ 0.02 to 0.18 $\pm$ 0.02, again indicative of random alkylation. The slight rise in the ratio is attributed to resistance of some modified sites to nuclease digestion.

The staphylococcal nuclease digestion described above was carried out in 0.13 M NaCl. Had the minichromosomes contained a normal complement of histone H1 they would have been refractile to the enzyme (43). When the same incubation was carried out in the absence of salt, the kinetics in Figure 8 were obtained. The two digestions are not strictly comparable since modification at low ionic strength generated 35-fold more DNA adducts. The extensive DNA digestion may reflect a perturbation of basic nucleosomal structure by the high modification. In any case, the release of covalently bound BaP diol epoxide paralleled the digestion of DNA albeit at a lower level.

DISCUSSION

The dramatic inhibition by NaCl of SV40 DNA alkylation and strand scission is attributed to charge neutralization. By acting as a counterion, Na^+ reduces the repulsion of negatively charged DNA phosphates and stabilizes the helix (44). In so doing it reduces the physical, possibly intercalative, association which precedes covalent reaction of BaP diol epoxide with DNA. Nicking also requires an initial alkylation event and is inhibited by the same mechanism or by direct masking of the DNA reaction sites by bound Na^+ (45,46). An additional factor which may reduce

BaP diol epoxide alkylation and nicking of minichromosomal DNA in 0.13 M NaCl is physical compaction of the minichromosomes (13, 24, 43). However, the viral chromatin employed here is depleted in histone H1 and only undergoes minor compaction in 0.13 M NaCl. Even so, a comparison of the percent inhibition values for naked and minichromosomal DNA in Table 1 indicates that this compaction slightly reduces the accessibility of DNA to nicking and binding.

The enhancement of strand scission in minichromosomal relative to naked DNA was unexpected but had several possible explanations. First, the chromatin preparation may contain an endogenous endonuclease which nicks adjacent to BaP diol epoxide adducts. Second, the minichromosomal histones may slightly alter the distribution of DNA adducts favoring those which rearrange with strand scission. Third, the histones may catalyze the rearrangement leading to strand scission. This explanation is considered the most likely since the primary mechanism of nicking is depurination strand scission (8, 9; Gamper et al., unpublished experiments). Rearrangement of deoxyribose at apurinic sites is known to be catalyzed by lysine, arginine, and histidine and by basic proteins which contain these amino acids (47).

The two-fold reduction in alkylation of viral DNA when it was reacted as a nucleoprotein complex can be attributed to minichromosomal protein which competes with the viral DNA for BaP diol epoxide. Furthermore, studies with cellular chromatin indicate that the histone cores can reduce the availability of nucleosomal DNA to the epoxide (31, 48, 49). This may reflect a local tightening of the DNA helix in the nucleosomes due to charge stabilization.

The random distribution of BaP diol epoxide DNA adducts which we have found in the SV40 minichromosome implies a rather uniform decrease in the accessibility of all regions of minichromosomal DNA and was unexpected in light of several recently published studies with cellular chromatin which indicate that both BaP diol epoxide and metabolically activated BaP preferentially alkylate internucleosomal DNA (31, 39, 48). SV40 minichromosomes resemble transcriptionally active chromatin (50). The nucleosomal core histones H3 and H4 are highly acetylated and phosphorylated (26, 51, 52). Weintraub and Groudine (53) have shown that transcriptionally active genes are readily digested by DNase I and have postulated that such genes have a conformation different from inactive chromatin. It is tempting to speculate that the acetylation and phosphorylation of core histones which renders DNA more susceptible to DNase I also makes it more accessible to BaP diol epoxide.

The viral minichromosomes also differ from cellular chromatin in their low content of histone H1. Loss of this histone from chromatin eliminates a very high affinity binding site for ethidium bromide (54). If BaP diol epoxide intercalates prior to reaction, preferential alkylation of linker DNA may be reduced in the histone H1 depleted minichromosomes. It should be noted, however, that when these same minichromosomes were photoreacted with psoralen, the reaction was highly specific for linker DNA (15).

A third possibility which may explain the equal accessibility of nucleosomal and linker DNA involves migration of the nucleosomal histone cores along the DNA effectively randomizing the sites of alkylation. Beard (5) has found that histone cores can slowly migrate in H1 depleted minichromosomes but was unable to measure the rate. It is conceivable that sufficient migration may occur, possibly facilitated by BaP diol epoxide

adducts or physically bound hydrocarbon, to randomize the DNA alkylation sites. The effect of nonhistone proteins present in the minichromosome preparation on DNA accessibility or histone migration is unknown.

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Table 1. Inhibition of BaP diol Epoxide Modification of SV40 DNA by NaCl*

[NaCl]	Adducts/Nick	Relative Percent Binding	Relative Percent Nicking
Naked DNA			
0 mM	117 ± 9	100	100
130 mM	197 ± 23	82.7	89.8
Minichrosomal DNA			
0 mM	40 ± 5	100	100
130 mM	60 ± 12	89.1	92.6

* The data are average values obtained from the curves in Figure 5.

FIGURE LEGENDS

Figure 1. (A) Preparative sucrose density gradient centrifugation of SV40 minichromosomes. SV40 [^{3}H]-chromatin was layered onto a 5-40% sucrose gradient and centrifuged for 5 hr at 25,000 rpm. (B) Agarose slab gel of sucrose density gradient fractions containing SV40 minichromosomes. Aliquots from fractions 11-19 of the above gradient were deproteinized with SDS and electrophoresed as DNA. The direction of electrophoresis was downward. Forms I and II DNA refer to superhelical and nicked circular SV40 DNA, respectively.

Figure 2. Time course of staphylococcal nuclease digestion of (A) control and (B) BaP diol epoxide modified SV40 minichromosomes. Autoradiograph of a 6% polyacrylamide gel. SV40 [^{32}P]-chromatin was incubated with [^{3}H]-BaP diol epoxide (molar reaction ratio = 0 or 0.32) in the gradient isolation buffer for 20 min at 37°. The minichromosomal DNA of both samples was immediately digested with staphylococcal nuclease (1.8 E.U./μg viral DNA). Aliquots containing 5-10 μg of viral DNA were loaded onto each gel slot after removal of DNA, protein, and excess hydrocarbon. The alkylated chromatin contained 3.0 DNA adducts/minichromosome.

Figure 3. Sedimentation pattern of SV40 minichromosomes reacted with BaP diol epoxide. SV40 [^{3}H]-chromatin was reacted at 37° for 24 hr with a concentration series of BaP diol epoxide in 20 mM Tris-HCl, pH 8.0-0.5 mM EDTA-5% dimethyl sulfoxide. The modified chromatin was layered onto 5.0 ml 5-30% sucrose gradients and centrifuged for 90 min at 50,000 rpm. Molar reaction ratios of BaP diol epoxide to DNA mononucleotide were 0 (●), 0.2 (o), 0.8 (△), 1.5 (▲), and 3.0 (◇).

Figure 4. Electrophoretic pattern of SV40 minichromosomes reacted with BaP diol epoxide and analyzed as (A) DNA and (B) chromatin. SV40 chromatin was reacted with a concentration series of BaP diol epoxide in 20 mM Tris-HCl, pH 8.0-0.5 mM EDTA-5% dimethyl sulfoxide. After 24 hr at 37°, the reaction mixtures were divided into two sets. One set was deproteinized and electrophoresed as DNA. The other set was electrophoresed as chromatin. In both gels the direction of electrophoresis was downward. The molar reaction ratios of BaP diol epoxide to DNA mononucleotide were (a) 0, (b) 0.05, (c) 0.10, (d) 0.15, (e) 0.20, (f) 0.30, (g) 0.40, (h) 0.60, (i) 0.80, (j) 1.0, (k) 1.5, (l) 3.0.

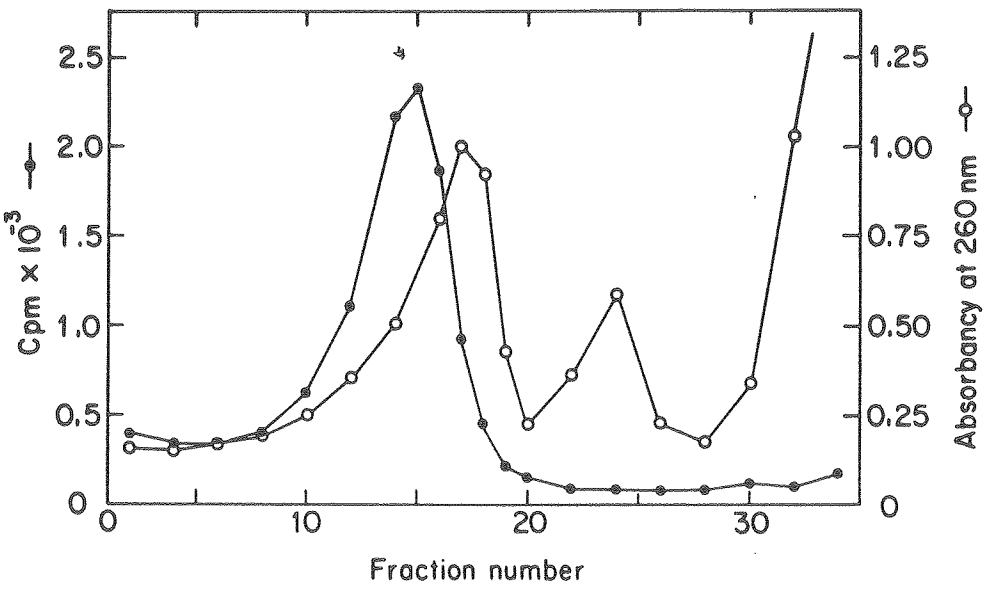
Figure 5. Comparison of BaP diol epoxide dependent DNA alkylation and strand scission in SV40 DNA and SV40 chromatin in the presence and absence of 0.13 M NaCl. (●—●), protein-free DNA in 0 M NaCl; (o—o), minichromosomal DNA in 0 M NaCl; (●---●), protein-free DNA in 0.13 M NaCl; (o---o), minichromosomal DNA in 0.13 M NaCl.

Figure 6. Staphylococcal nuclease digestion of SV40 DNA after prior reaction with BaP diol epoxide. SV40 form I [^{32}P]-DNA containing 2.2 [^{3}H]-BaP diol epoxide adducts/genome was digested with 0.9 E.U. staphylococcal nuclease /µg DNA in the presence of 0.20 M NaCl. Aliquots were counted after ethyl acetate extraction and ethanol precipitation. (●), DNA; (o), BaP diol epoxide adducts. 100% = 3,200 cpm ^{32}P and 350 cpm ^{3}H.

Figure 7. Staphylococcal nuclease digestion of "compact" SV40 minichromosomes after prior reaction with BaP diol epoxide. SV40 [^{32}P]-chromatin was incubated 20 min at 37° with [^{3}H]-BaP diol epoxide (molar reaction ratio = 0.55) in the gradient isolation buffer. The alkylated chromatin (0.74 DNA adducts/minichromosome) was immediately digested with 4.3 E.U. staphylococcal nuclease/µg viral DNA. Aliquots were taken over a 20 min period into EDTA. Ethanol precipitable radioactivity was determined after deproteinization and ethyl acetate extraction. (●), minichromosomal DNA; (o), BaP diol epoxide DNA adducts. 100% = 12,800 cpm ^{32}P and 650 cpm ^{3}H.

Figure 8. Staphylococcal nuclease digestion of "extended" SV40 minichromosomes after prior reaction with BaP diol epoxide. SV40 [^{32}P]-chromatin was incubated 20 min at 37° with [^{3}H]-BaP diol epoxide (molar reaction ratio = 0.44) in 20 mM Tris-HCl, pH 8.0-0.5 mM EDTA-3% dimethyl sulfoxide. The alkylated chromatin (27.5 adducts/minichromosome) was immediately digested with 9.5 E.U. staphylococcal nuclease/µg viral DNA in the absence of NaCl. Aliquots were analyzed as outlined in Figure 7. (●), minichromosomal DNA; (o), BaP diol epoxide DNA adducts. 100% = 6,000 cpm ^{32}P and 5,100 cpm ^{3}H.

a.


Cpm x 10^-3
2.5
2.0
1.5
1.0
0.5
0
0
10
20
30
Fraction number
1.25
1.00
0.75
0.50
0.25
0
Absorbancy at 260 nm

b.

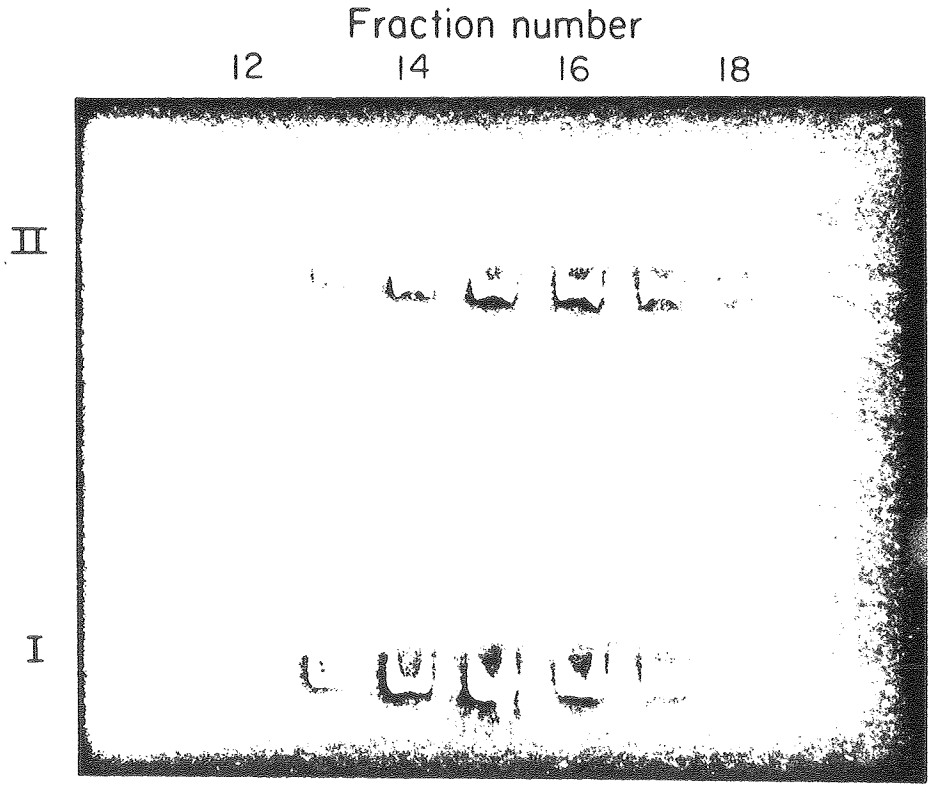

Fraction number
12
14
16
18
II
I

Fig. 1.

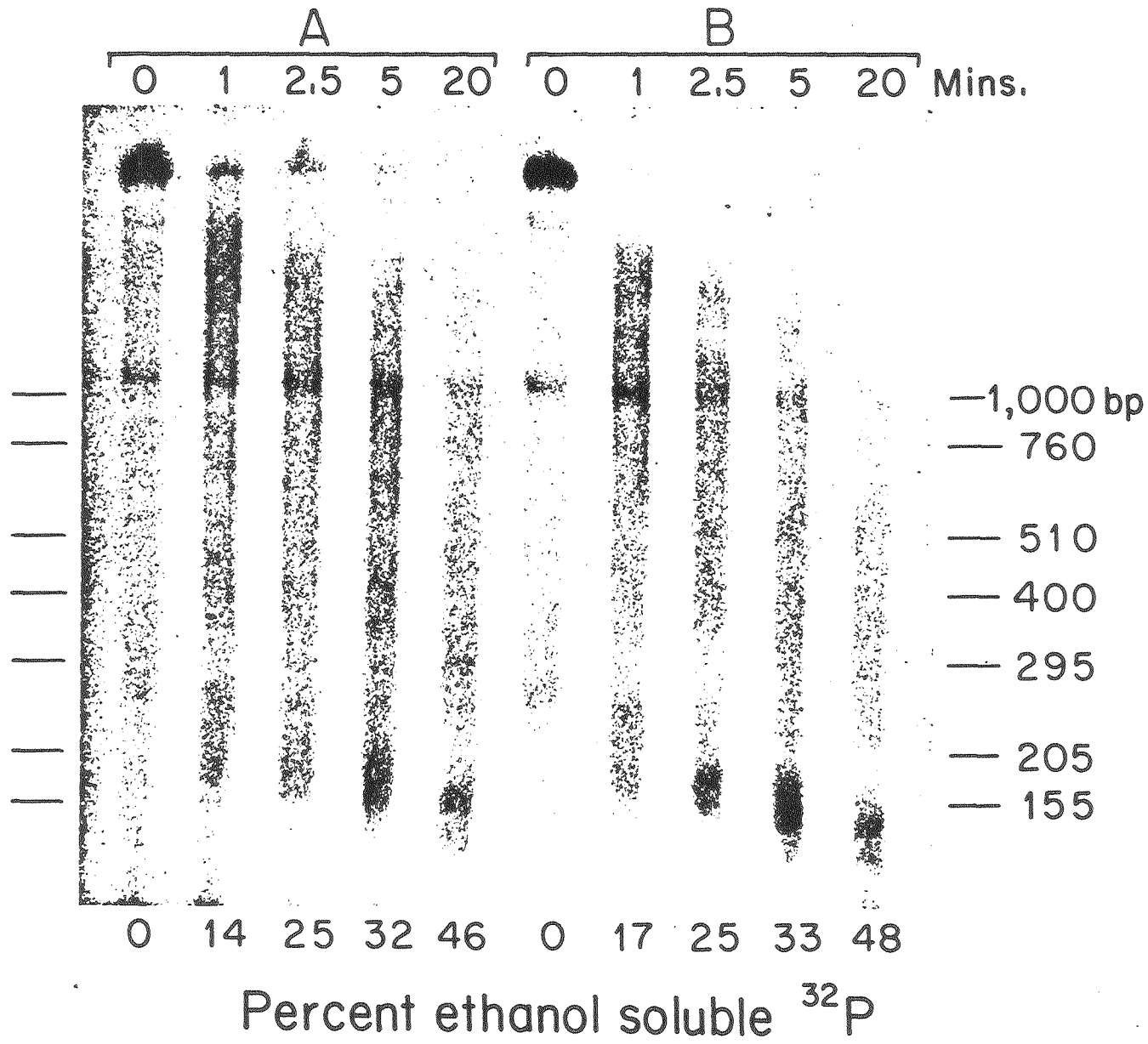

A
B
0 1 2.5 5 20 0 1 2.5 5 20 Mins.
1,000 bp
760
510
400
295
205
155
0 14 25 32 46 0 17 25 33 48
Percent ethanol soluble ^{32}P

Fig. 2

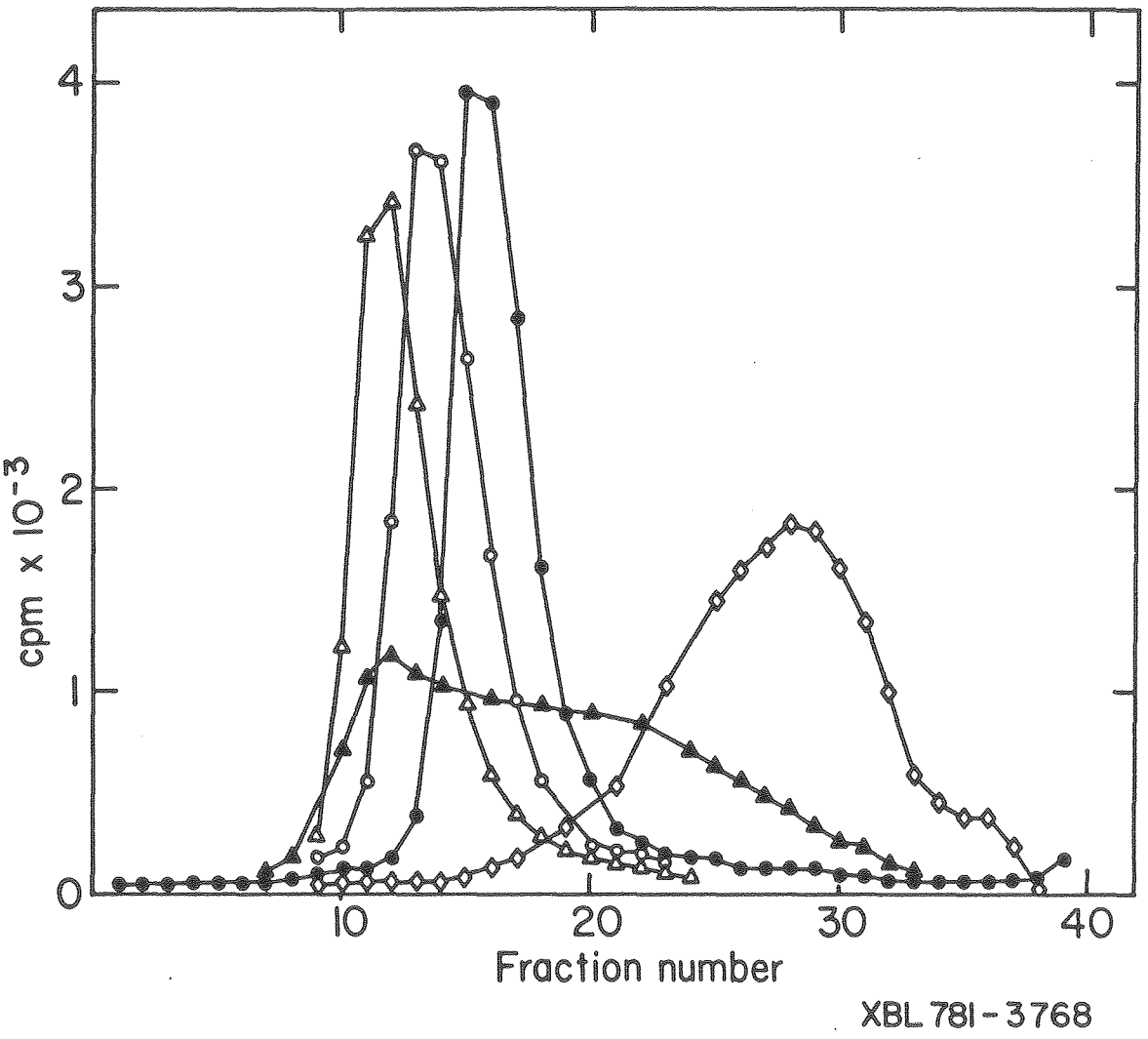


Fig. 3

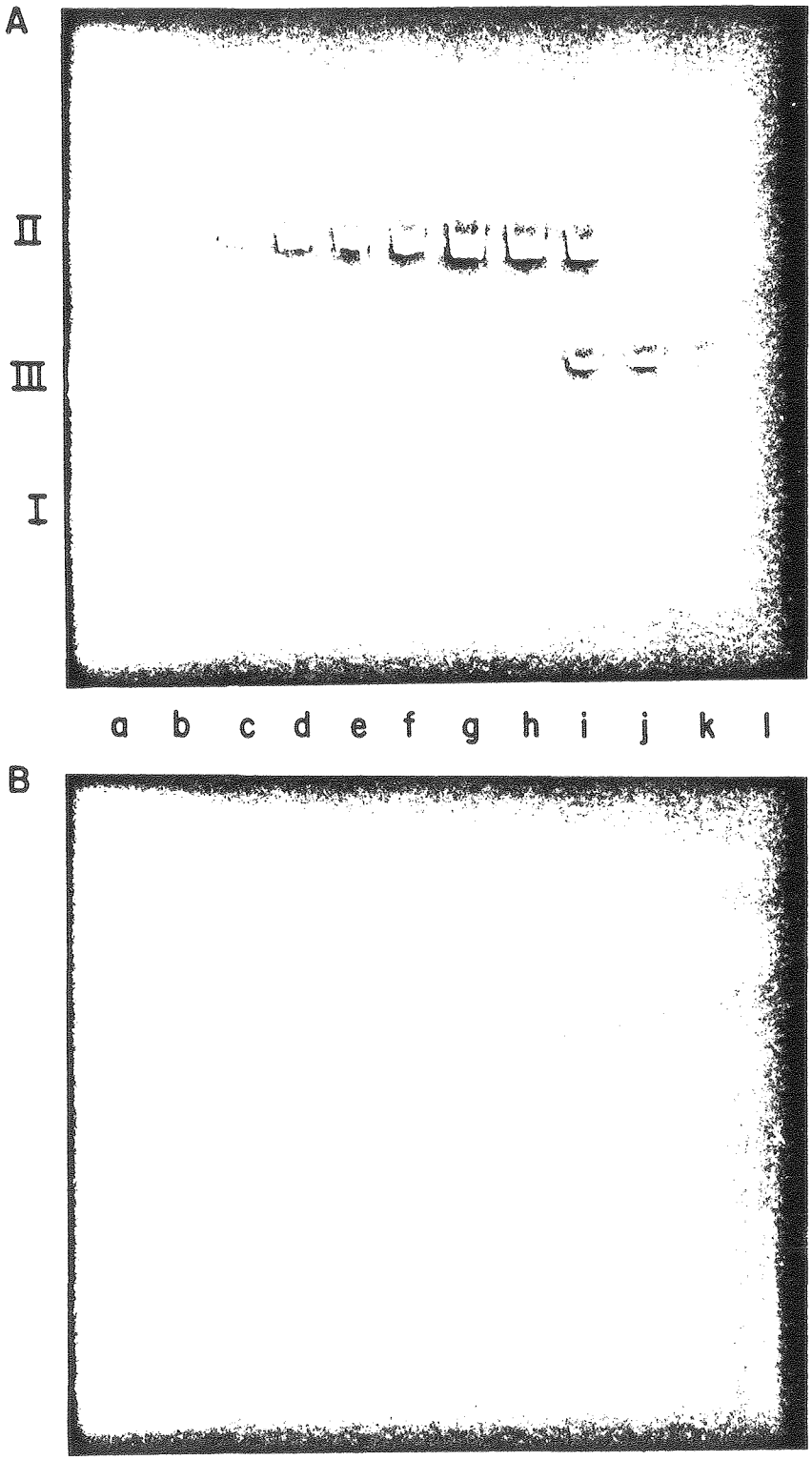


Fig. 4

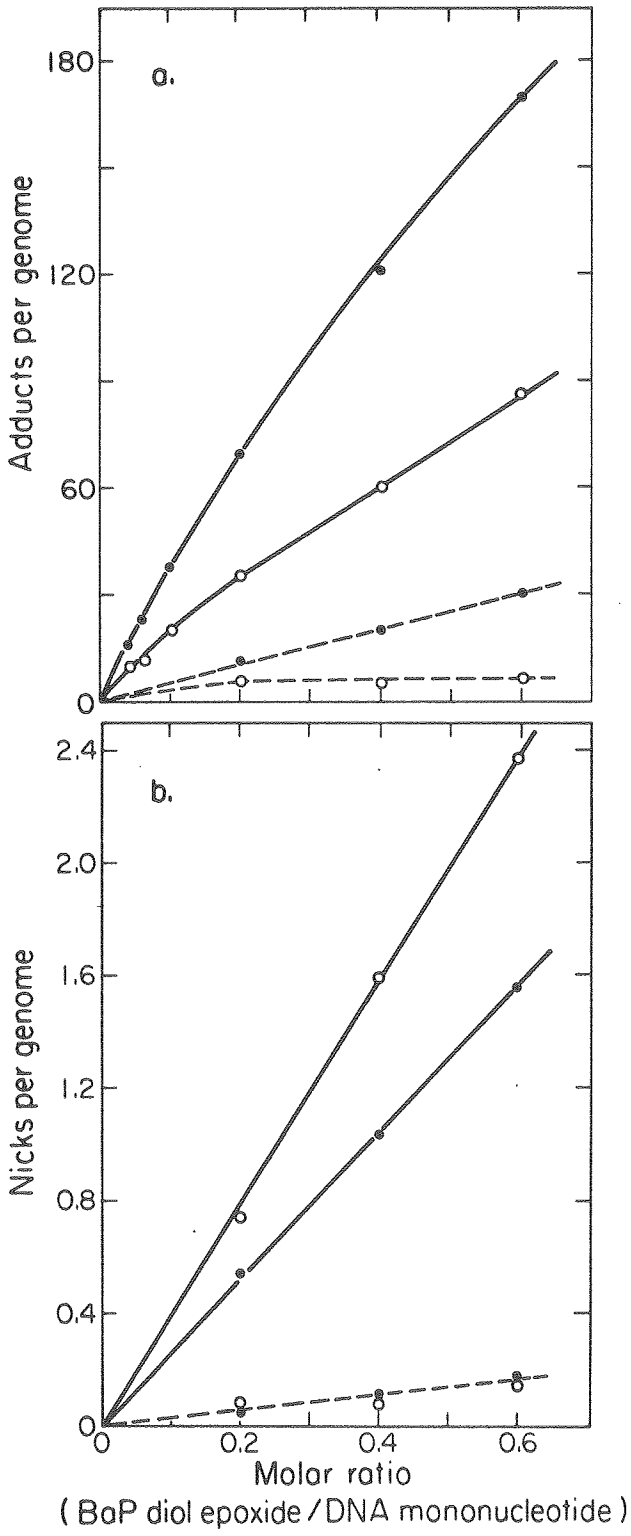
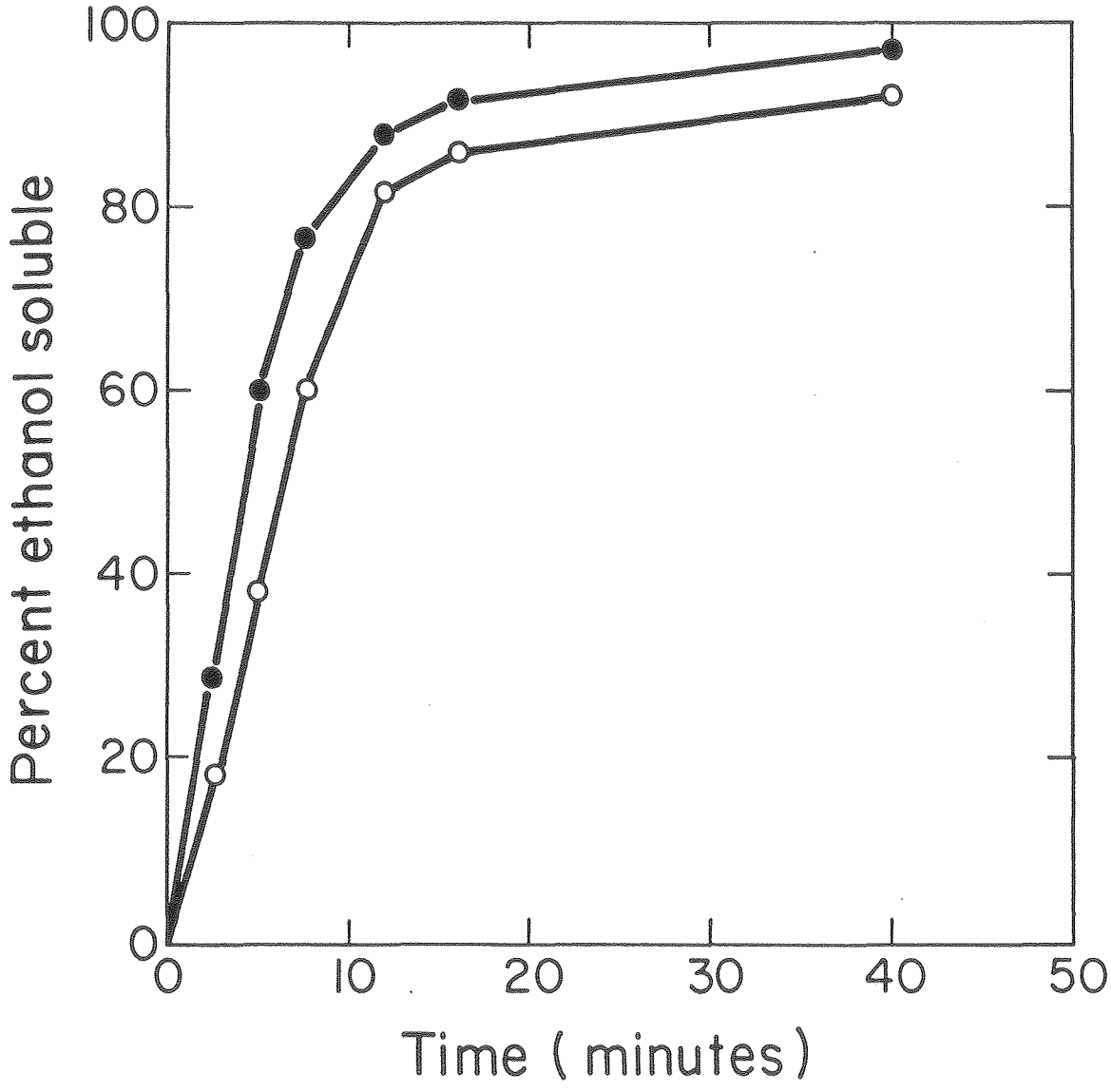
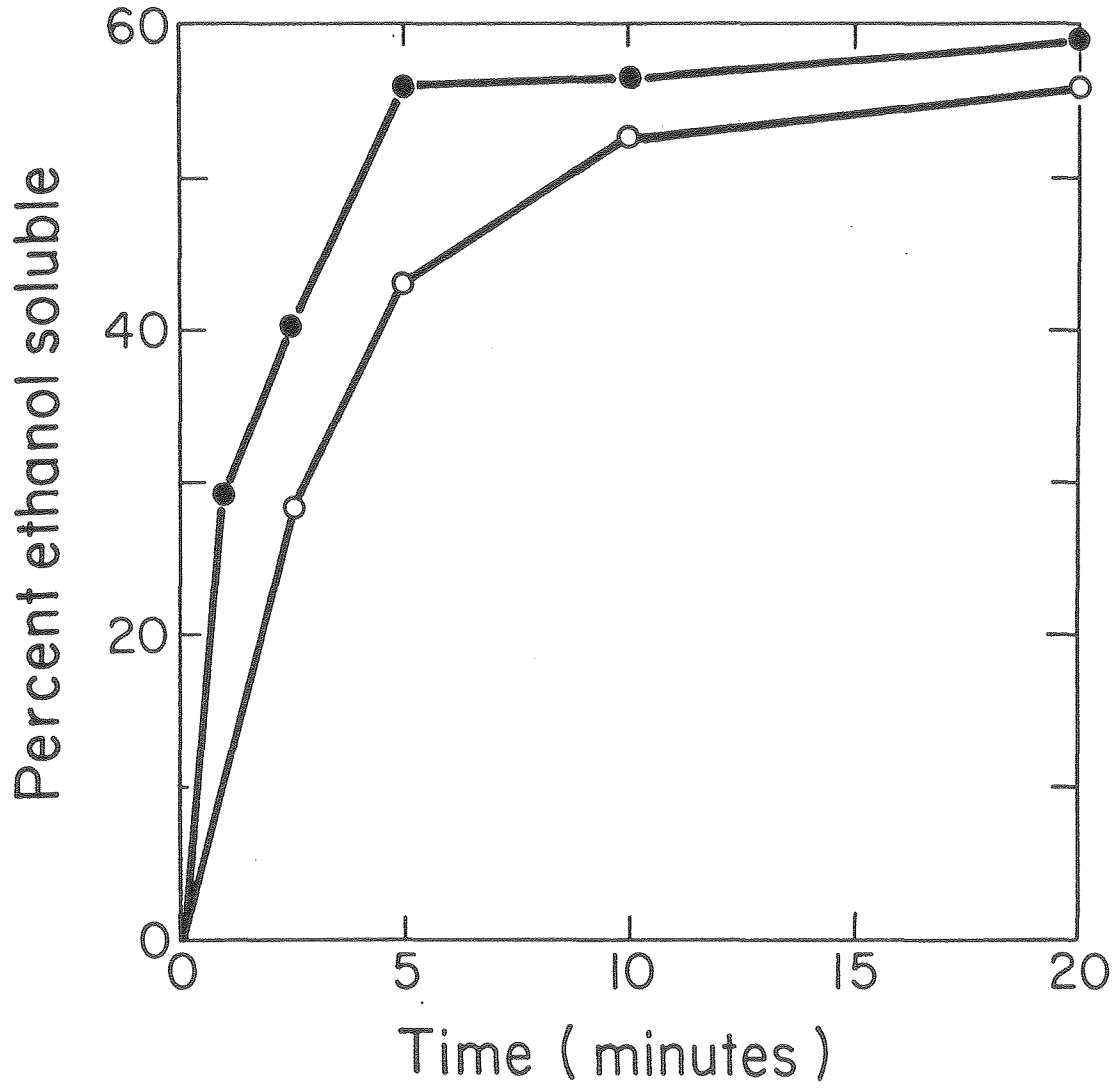

a.
b.
Adducts per genome
Nicks per genome
180
120
60
0
2.4
2.0
1.6
1.2
0.8
0.4
0
0.2
0.4
0.6
Molar ratio
(BaP diol epoxide / DNA mononucleotide)
XBL796-4813

Fig. 5



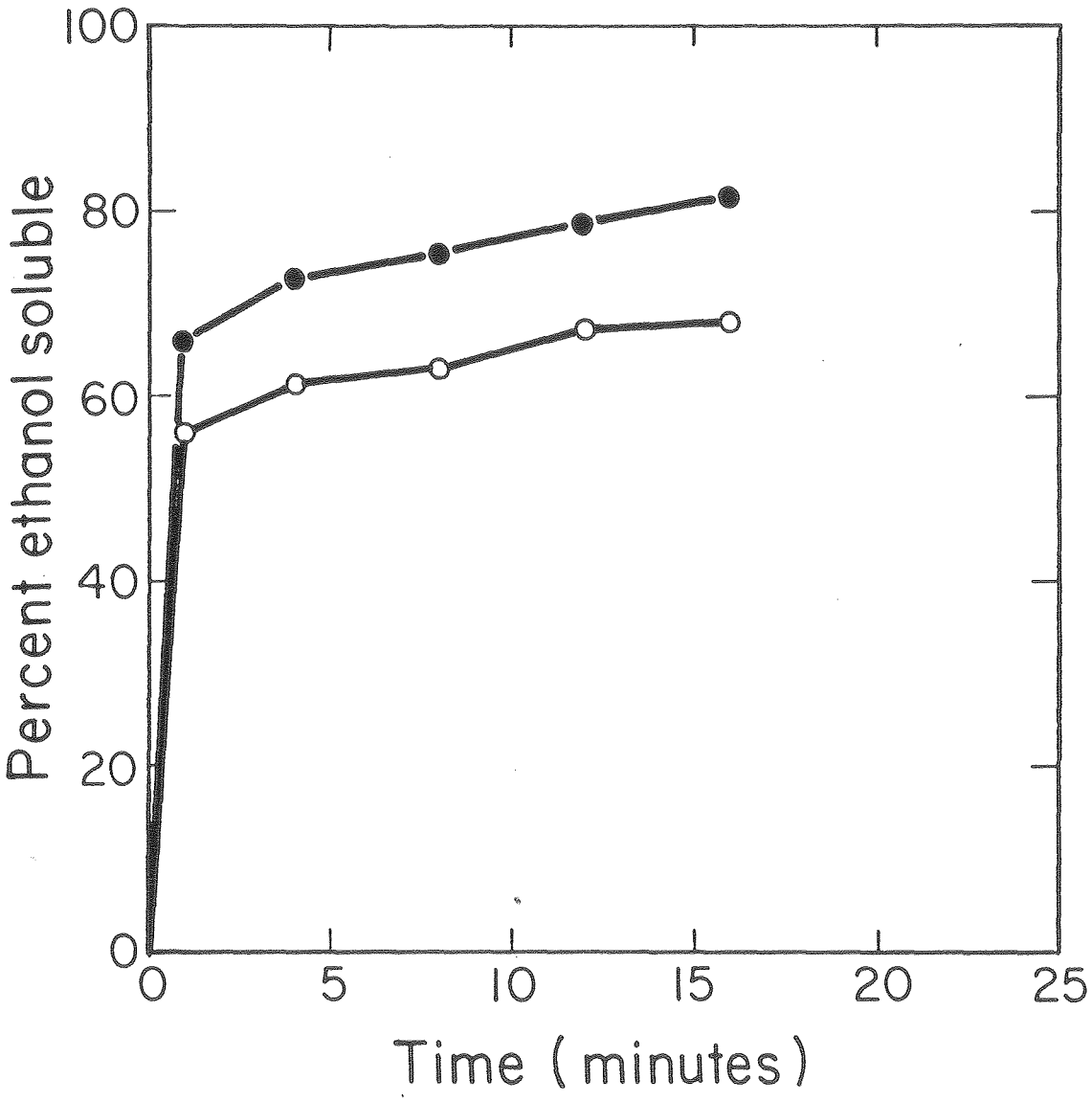
XBL7810-4314

Fig. 6



XBL7810-4315

Fig. 7



XBL 7810-4316

Fig. 8