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Electron microscopic visualization of *N*-acetoxy-*N*-2-acetylaminofluorene binding sites in ColE1 DNA by means of specific antibodies

(chemical carcinogenesis/aromatic amides)

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ABSTRACT **ColE1 DNA has been allowed to react *in vitro* with *N*-acetoxy-*N*-2-[^{14}C]acetylaminofluorene in the range of 0–15 *N*-2-[^{14}C]acetylaminofluorene residues bound per molecule of DNA, at the C8 of guanine residues. Purified rabbit antibodies to both *N*-2-(guanosine-8-yl)-acetylaminofluorene and native DNA that had reacted with *N*-acetoxy-*N*-2-acetylaminofluorene were shown by electron microscopy to recognize specifically the acetylaminofluorene-modified ColE1 DNA. The antibodies bound to DNA were visualized either per se or after reaction with goat anti-rabbit immunoglobulins coupled with ferritin. There was a linear relationship between the average number of antibodies bound per DNA molecule and the number of *N*-2-(deoxyguanosine-8-yl)-acetylaminofluorene residues per DNA molecule. The slope of this straight line was equal to 0.4. Due to the bivalence of the immunoglobulins one would expect a value of 0.5; we actually observed an important fraction of the bound antibodies crosslinking two parts of the same (or of another) DNA molecule.**

An important step in the carcinogenic process is thought to be the initial attack of the DNA molecule by the so-called ultimate carcinogens. It has been shown in recent studies from our laboratories (1, 2) that native DNA modified *in vitro* with the model ultimate carcinogen *N*-acetoxy-*N*-2-acetylaminofluorene (*N*-AcO-AAF) can induce in rabbits the synthesis of antibodies that specifically recognize acetylaminofluorene (AAF)-substituted guanine residues in DNA. These antibodies have been purified by affinity chromatography and shown by radioimmunoassay to recognize almost equally well *N*-2-(guanosine-8-yl)-acetylaminofluorene (Guo-C8-AAF) and native and denatured DNA modified with AAF (DNA- AAF) (1, 2). On the other hand, guanosine 5′-monophosphate, *N*-hydroxy-*N*-2-acetylaminofluorene, denatured DNA, and native DNA are not recognized (2). Guo-C8-AAF coupled with bovine serum albumin has also been shown to trigger the synthesis of specific antibodies in rabbits (3, 4). Such antibodies are a specific tool enabling the titration of carcinogen binding sites in DNA by radioimmunoassay (2, 5). In this paper we report the direct electron microscopic visualization of both types of antibodies (against native DNA-AAF or against Guo-C8-AAF) bound to ColE1 DNA that has been modified *in vitro* by reaction with *N*-AcO-[^{14}C]AAF.

MATERIAL AND METHODS

ColE1 DNA was prepared as described by Clewell (6). *N*-AcO-[^{14}C]AAF [specific activity 50 mCi/mmol (1 Ci = 3.7 × 10^{10} becquerels)] was prepared as described (7). ColE1 DNA at a concentration of approximately 60 μM (in phosphate residues) in 2 mM sodium citrate buffer (pH 7) was allowed to react at 37°C for 3 hr with the carcinogen, under agitation and an argon atmosphere in the dark. Unreacted labeled carcinogen was removed by three extractions with ethyl ether, after which the DNA was extensively dialyzed against 2 mM sodium citrate buffer (pH 7) and precipitated with ethanol. Control DNA was obtained similarly by omission of the ultimate carcinogen in the incubation mixture. The total amount of binding was obtained by measuring the [^{14}C]AAF radioactivity and by measuring the nucleotide concentration from the 260-nm UV absorbance. The amount of binding to C8 of guanine residues was determined by using the radiochemical assay described by Fuchs (8). Supercoiled ColE1 (form I) DNA samples were converted to linear molecules (form III) with the restriction enzyme *Eco*RI (Boehringer, Mannheim). Antibodies to native DNA-AAF and to Guo-C8-AAF linked to bovine serum albumin were obtained and purified as described in refs. 1–3.

The antibodies were allowed to react with the carcinogen-modified ColE1 DNA (or control DNA) at 20°C for 30 min at pH 7.5 in 10 mM Tris·HCl buffer with various salt concentrations (0, 50, 100, or 150 mM NaCl) and with various ratios of antibody to antigen (ranging from 2 to 40). DNA concentration was in the order of 15 μg/ml. After incubation the excess antibodies were removed by gel filtration on a Sepharose 4B column (0.8 × 15 cm) in the corresponding ionic strength. Gel electrophoresis in agarose (9) was used to determine the optimal incubation conditions (see *Results and Discussion*). Goat anti-rabbit immunoglobulins coupled with ferritin (Miles Yeda, Rehovot, Israel) were incubated at 20°C, for 30 min, with the specific antibody–ColE1 DNA-AAF complex in 100 mM NaCl/10 mM Tris·HCl, pH 7.5. Unbound immunoglobulins were removed by filtration on a Sepharose 4B column.

Samples of ColE1 DNA (0.5 μg/ml) or ColE1 DNA complexed with IgG in the incubation buffer were spread on positively charged carbon-coated grids (400 mesh) according to the procedure of Dubochet *et al.* (10). A 50-μl drop of the sample was deposited on a Parafilm sheet and immediately covered with the grid. After 5-min adsorption, the grids were transfered into a 50-μl drop of uranyl acetate (1% in H_2O) for 30 sec, briefly rinsed in a 50-μl drop of distilled water, air dried, and rotary shadowed with platinium at an angle of 7° as described (11). The grids were examined in a Siemens 101 Elmiskop. For the determination of the average number of IgG molecules per

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Abbreviations: AAF, acetylaminofluorene; Guo-C8-AAF, *N*-2-(guanosine-8-yl)-acetylaminofluorene; *N*-AcO-AAF, *N*-acetoxy-*N*-2-acetylaminofluorene; DNA-AAF, DNA that has reacted with *N*-AcO-AAF.

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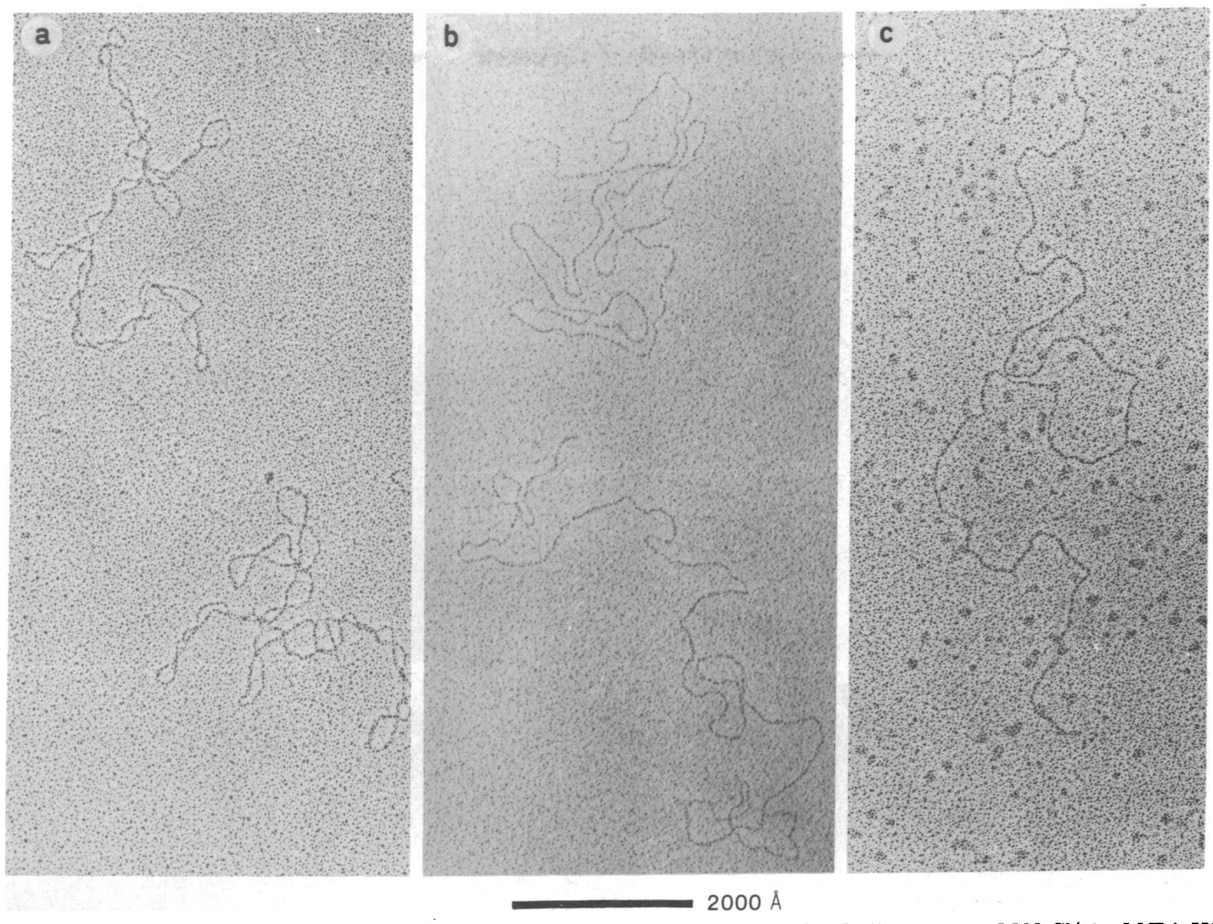

FIG. 1. Electron micrographs of ColE1 DNA-AAF having 0.07% modified bases. The spreading buffer was 100 mM NaCl/10 mM Tris·HCl/0.2 mM EDTA, pH 7.5, and the concentration of DNA was 0.5 μg/ml. (*a*) ColE1 DNA-AAF form I. (*b*) ColE1 DNA-AAF form III. (*c*) ColE1 DNA-AAF form III incubated with antibodies against native DNA-AAF at an antibody-to-antigen ratio of 10.

DNA molecule the grids were examined in a Philips EM 301 microscope equipped with a video system. The measurements were performed directly on the television screen. Calibration was routinely checked by examination of a carbon grating replica (Fullam, Schenectady, NY) photographed after each series of measurements.

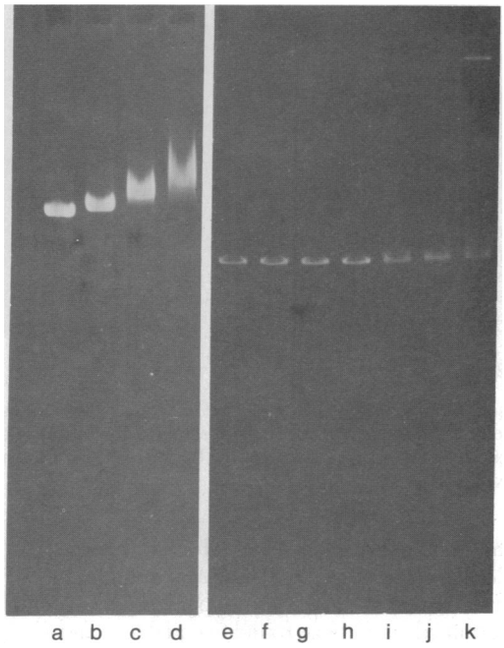

FIG. 2. Slab gel electrophoresis of linear ColE1 DNA incubated with anti Guo-C8-AAF antibodies as described in Table 1.

Table 1. Description of electrophoresis samples shown in Fig. 2.

Lane	DNA	Ionic strength*	IgG-to-antigen ratio†	IgG/nucleotide
a	Unmodified	Low	—	0
b	Unmodified	Low	—	0.0003
c	Unmodified	Low	—	0.0015
d	Unmodified	Low	—	0.0060
e	Unmodified	High	—	0
f	Unmodified	High	—	0.0003
g	Unmodified	High	—	0.0015
h	Unmodified	High	—	0.0060
i	0.15% AAF	High	2	0.0003
j	0.15% AAF	High	10	0.0015
k	0.15% AAF	High	40	0.0060

* Low and high ionic strength are 10 mM Tris·HCl, pH 7.5, and 100 mM NaCl/10 mM Tris·HCl, pH 7.5, respectively. For a given sample, the same ionic strength was used for incubation, gel, and chamber buffers. Both low ionic strength gel (lanes a–d) and high ionic strength gel (lanes e–k) were run at constant voltage (110 V) for 6 hr.

† For the unmodified DNA samples the IgG-to-antigen ratio is not defined because there were no AAF residues in these samples. Each lane was loaded with 0.2 μg of DNA (20 μl).

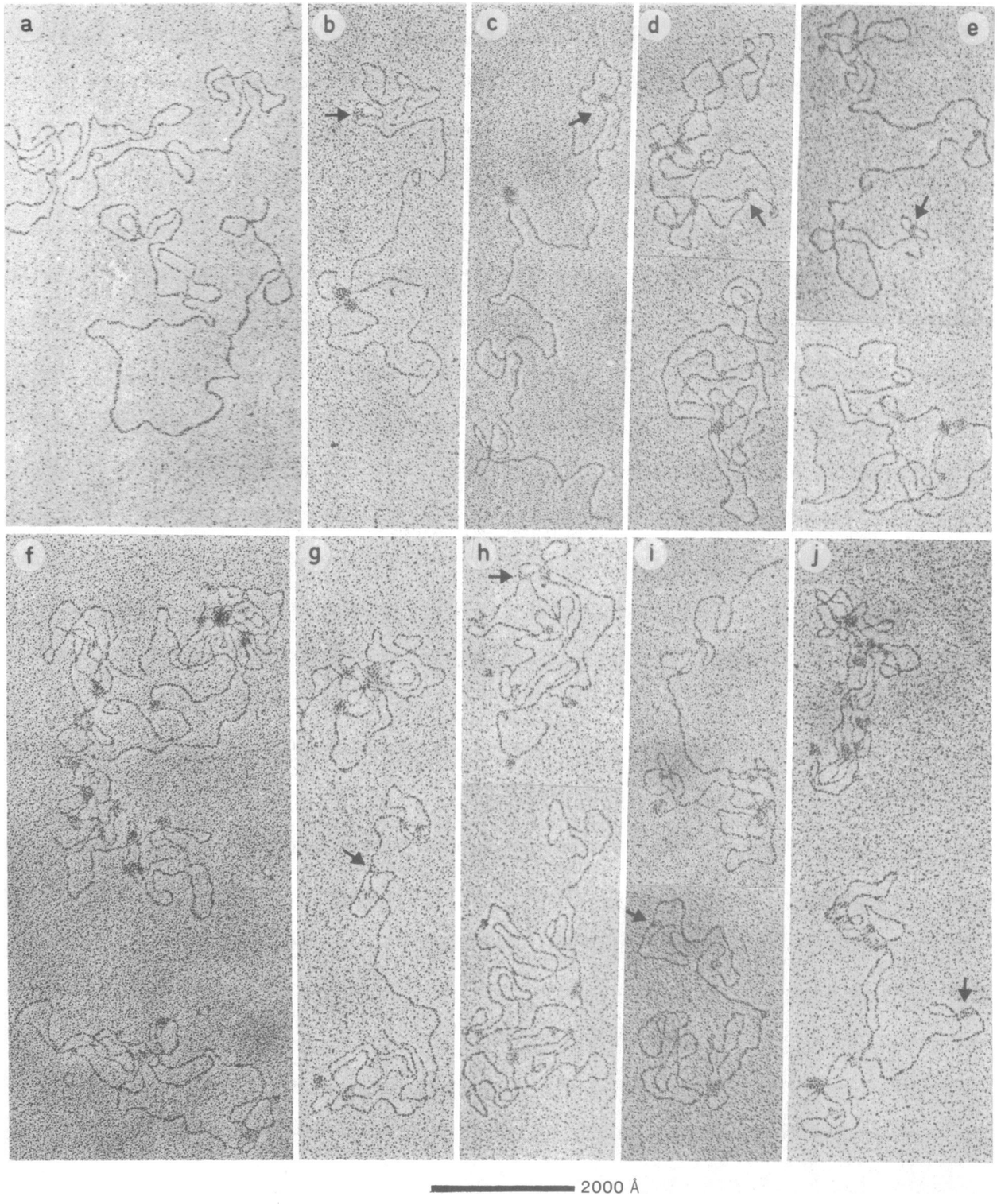

FIG. 3. Electron micrographs of ColE1 DNA form III having 0% modified bases (control) (*a*); 0.07% (*b–e*); 0.15% (*f–j*). All the samples were purified on Sepharose 4B after incubation with anti-Guo-C8-AAF IgG at an antibody-to-antigen ratio of 10. The arrows show intramolecular crosslinks resulting from the binding of an IgG to two antigenic determinants of a given DNA molecule. The spreading buffer was 100 mM NaCl/10 mM Tris·HCl, pH 7.5. Similar pictures were obtained with antibodies against native DNA-AAF.

RESULTS AND DISCUSSION

ColE1 DNA (form I) samples (Fig. 1*a*) with 0, 0.07, and 0.15% of the bases modified with the model ultimate carcinogen *N*-AcO-[^{14}C]AAF were prepared. The circular molecules were converted to linear duplexes (Fig. 1*b*) with the restriction enzyme *Eco*RI, which cuts ColE1 DNA at a single site (12). The number of dGuo-C8-AAF residues per ColE1 DNA molecule was determined (8) and was found to equal 0, 7, and 15 residues per DNA molecule, respectively (assuming a molecular weight of $4 \cdot 10^6$ for the ColE1 DNA molecule) (13).

Gel electrophoresis on agarose as a rapid screen for the determination of the optimal incubation conditions

At low ionic strength (10 mM) control DNA (i.e., without carcinogen) incubated with anti-Guo-AAF antibodies was found to have an altered migration on slab electrophoresis (Fig. 2, lanes a–d). Under those conditions electron micrographs showed unspecific (probably electrostatic) interaction between the DNA and the antibody molecules (data not shown). When ionic strength was increased (>50 mM NaCl) no change of the electrophoretic mobility of the control DNA could be detected even at high antibody concentration (Fig. 2, lanes f–h). Likewise, as seen under the electron microscope, after filtration on a Sepharose 4B column, control DNA did not show any bound antibody when the salt concentration was kept greater than 50 mM (Fig. 3*a*). Gel electrophoresis of ColE1 DNA-AAF samples incubated at ionic strength >50 mM showed (*i*) slower mobility and broadening of the DNA band and (*ii*) appearance of increasing amounts of aggregates (i.e., material that did not migrate) when the antibody-to-antigen ratio was increased (Fig. 2, lanes i–k). An antibody-to-antigen ratio equal to 10 was chosen for the electron microscopy studies because greater values of this ratio did not further alter the electrophoretic mobility of the DNA molecule but resulted only in increased amounts of aggregates. This aggregation phenomenon is most likely due to the bivalence of the immunoglobulins and the related formation of intermolecular crosslinks.

Electron microscopy

Fig. 1*c* is an electron micrograph of ColE1 DNA-AAF (0.07%) incubated with rabbit anti-DNA-AAF IgG at an antibody-to-antigen ratio equal to 10. No aggregates due to incubation with the IgG are visible on the carbon film. Under our experimental conditions of staining and shadowing the IgG molecules are circular with an average diameter of 90 Å. The number of bound antibodies per DNA molecule was scored for the different ColE1 DNA samples after purification of the complexes on a Sepharose 4B column. Figure 3*a* shows that, at a concentration of 100 mM NaCl, the unmodified ColE1 DNA bound no antibodies, in agreement with the electrophoresis data. Conversely the DNA-AAF samples present dark dots having a diameter close to that of the free IgG (Fig. 3 *b–j*). These DNA-bound dots were identified as IgG in a second labeling experiment, using ferritin labeled anti rabbit IgG (Fig. 4). We could detect highly contrasted ferritin-labeled IgG conjugates only when ColE1 DNA modified with AAF was first incubated with the carcinogen-specific antibodies (Fig. 4 *b* and *c*). There was no unspecific type of binding when ColE1 DNA modified with AAF was incubated with the ferritin-labeled IgG molecules (Fig. 4*a*). Unexpectedly, the sizes of the dots in Figs. 3 and 4 look similar with, however, much higher contrast in the ferritin experiment. Presumably we only see the iron oxide core of the ferritin-labeled protein in Fig. 4 and the whole protein in Fig. 3.

The histograms (Fig. 5) were obtained from the examination of about 120 molecules of both DNA-AAF samples and the control sample. The average values of the number of antibodies per DNA molecule are plotted against the number of dGuo-C8-AAF residues per DNA molecule (Fig. 6) as determined directly (8).

We observe a straight line with slope equal to 0.4. Due to the bivalence of the antibody molecule one would expect a 0.5 value for this slope, provided that all bound antibodies recognize two antigens. This is obviously not the case, but a large proportion of the antibodies do actually crosslink two points in a given DNA molecule (see arrows, Fig. 3 *b–j*). As shown earlier in this paper, increasing amounts of antibodies in the incubation mixture did not increase the average number of bound anti-

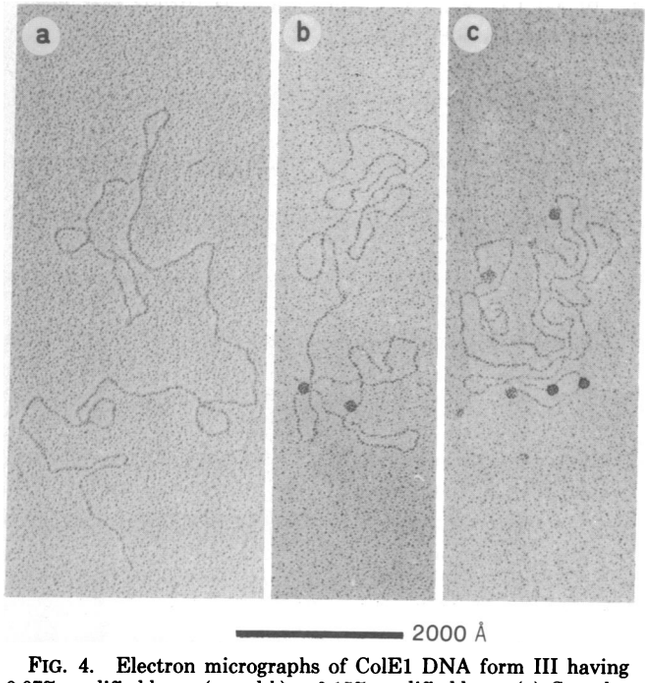

FIG. 4. Electron micrographs of ColE1 DNA form III having 0.07% modified bases (*a* and *b*) or 0.15% modified bases (*c*). Samples *b* and *c* were incubated with anti-Guo-C8-AAF antibodies, purified as in Fig. 3, incubated with ferritin-labeled goat anti-rabbit IgG, and then purified by filtration on a Sepharose 4B column. Sample *a* was treated similarly except that the incubation with the anti-Guo-C8-AAF antibodies was omitted. Spreading conditions as in Fig. 3.

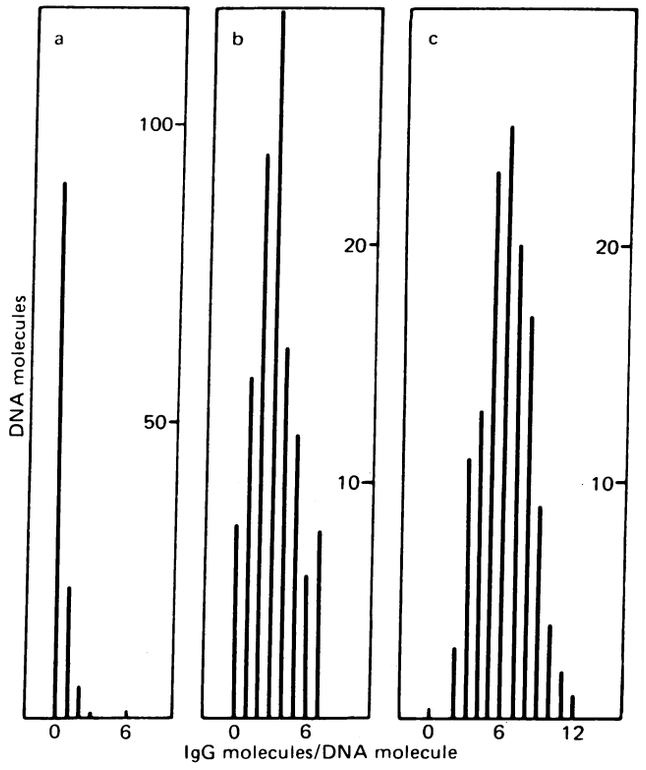

FIG. 5. Distribution of the number of anti-Guo-C8-AAF antibodies per ColE1 DNA molecule having: 0% modified bases (*a*), 0.07% (*b*), 0.15% (*c*). Respectively, 118, 120, and 128 DNA molecules were scored.

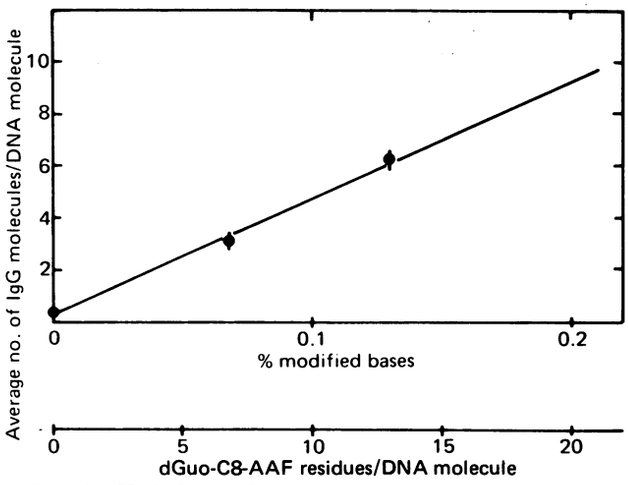

FIG. 6. Variation of the average number of anti-Guo-C8-AAF antibodies bound per ColE1 DNA molecule as a function of the number of dGuo-C8-AAF residues per DNA molecule. The error bars correspond to the confidence interval $2\sigma/\sqrt{n}$, σ being the standard deviation and n the number of measurements.

bodies per DNA molecule but resulted in a huge network formed of DNA molecules linked together by the divalent antibodies. This problem might be circumvented by using monovalent Fab fragments instead of IgG. It is known that the *in vitro* reaction of N-AcO-AAF with native DNA yields beside the C8 adduct a so-called minor adduct to N^2 of guanine, 3-(deoxyguanosine-N^2-yl)-acetylaminofluorene (14, 15). The antibodies raised against Guo-C8-AAF linked to bovine serum albumin have been shown not to crossreact with the N^2 adduct (5). On the other hand, the anti-native DNA-AAF antibodies were purified on a denatured DNA-AAF affinity column; because denatured DNA-AAF contains almost exclusively C8 adducts (8), it is most likely that the purified antibodies do not contain any N^2 adduct-specific antibodies, even though such antibodies may be present in the crude serum.

This paper shows clearly that specific antibodies can be used to map the carcinogen binding sites along a DNA molecule. For example, by using well-defined oligonucleosomes, this technique might be applied to determine the distribution of the carcinogen along the DNA in the chromatin.

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1. Leng, M., Sage, E., Fuchs, R. P. P. & Daune, M. P. (1978) *FEBS Lett.* **92**, 207–210.
2. Sage, E., Fuchs, R. P. P. & Leng, M. (1979) *Biochemistry* **18**, 1328–1332.
3. Guigues, M. & Leng, M. (1979) *Nucleic Acids Res.* **6**, 733–744.
4. Poirier, M. C., Yuspa, S. H., Weinstein, I. B. & Blobstein, S. (1977) *Nature (London)* **270**, 186–188.
5. Poirier, M. C., Dubin, M. A. & Yuspa, S. H. (1979) *Cancer Res.* **39**, 1377–1381.
6. Clewell, D. B. (1972) *J. Bacteriol.* **110**, 667–676.
7. Lefèvre, J. F., Fuchs, R. P. P. & Daune, M. P. (1978) *Biochemistry* **17**, 2561–2567.
8. Fuchs, R. P. P. (1978) *Anal. Biochem.* **91**, 663–673.
9. Lang, M. C. E., Freund, A. M., de Murcia, G., Fuchs, R. P. P. & Daune, M. P. (1979) *Chem.-Biol. Interact.*, in press.
10. Dubochet, J., Ducommun, M., Zollinger, M. & Kellenberger, E. (1971) *J. Ultrastruct. Res.* **35**, 147–167.
11. de Murcia, G., Das, G. C., Erard, M. & Daune, M. P. (1978) *Nucleic Acids Res.* **5**, 523–535.
12. Tomizawa, J.-J., Sakakibara, Y. & Kakefuda, T. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 2260–2264.
13. Voordouw, G., Kam, Z., Borochov, N. & Eisenberg, H. (1978) *Biophys. Chem.* **8**, 171–189.
14. Kriek, E. (1972) *Cancer Res.* **32**, 2042–2048.
15. Westra, J. G., Kriek, E. & Hittenhausen, H. (1976) *Chem.-Biol. Interact.* **15**, 149–164.