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An electron microscopic method for the mapping of proteins attached to nucleic acids

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ABSTRACT

An electron microscopic method for demonstrating the presence of and mapping the positions of proteins specifically bound to nucleic acids is described. The nucleic acid-protein complex is treated with dinitrofluorobenzene under conditions such that dinitrophenyl (DNP) groups are attached to nucleophilic groups on the protein, with only a low level of random attachment to the nucleic acid. This product is treated with rabbit anti-DNP IgG. The position of the protein-$(DNP)_n(IgG)_m$ complex on the nucleic acid strand can be observed by electron microscopy by protein free spreading methods and, in many cases, by cytochrome-c spreading. If necessary for visualization by the latter method, the size of the labeled region can be increased by treatment with goat anti-rabbit IgG. High efficiency of electron microscopic labeling is achieved. Examples studied are: the adenovirus-2 DNA terminal protein, a protein covalently bound to SV40 DNA, DNA polymerase I bound to DNA, E. coli RNA polymerase bound to T7 DNA, and proteins UV crosslinked to avian sarcoma virus RNA.

INTRODUCTION

There have been a number of studies in which proteins attached to nucleic acids have been recognized and mapped by electron microscopy. In most of these, high resolution protein free spreading methods have been used; for example, RNA polymerase and lac repressor bound to specific sites on appropriate DNAs (1, 2, 3, 4), Qβ replicase bound to its RNA (5), restriction endonucleases bound to their recognition sites (6), DNA polymerase bound to nicks in DNA (7), protein aggregates or a protein bound close to the origin of replication of SV40 DNA (8, 28), and a membrane protein aggregate bound to mitochrondrial DNA (9). These direct

ABBREVIATIONS

DNP:dinitrophenyl. DNFB: dinitrofluorobenzene. DMSO: dimethylsulfoxide. NaP: sodium phosphate. BED buffer: 0.125 M sodium borate (pH 9.0), 6.25 mM Na_3EDTA and 10% v/v DMSO. ENTc buffer: 5 mM Na_3EDTA, 0.2 M NaCl, 0.05 M Tricine buffer (pH 8.0). GuHCL: guanidinium hydrochloride.

observation high resolution methods are limited to some extent in that proteins of intermediate molecular weight, such as the 55,000 dalton protein bound to the ends of adenovirus-2 DNA (10), are too small to be seen. Furthermore protein free techniques for mounting DNA and RNA for electron microscopy are technically more difficult and less reproducible than the basic protein film method developed by Kleinschmidt and co-workers (11) and modified for application to single stranded nucleic acids by later workers (12, 13). In a cytochrome-c film the nucleic acid thread has a thick layer of denatured cytochrome-c collapsed around it so that proteins with molecular weights as large as 2×10^5 daltons cannot be seen and proteins as large as RNA polymerase (4.8×10^5 daltons) are visible only dimly. SV40 T-antigen bound to SV40 DNA has been identified and mapped by a cytochrome-c spreading method, using a ferritin labeled second antibody to hamster anti-T serum (14). Broker and Chow have described an effective method for identifying proteins attached to nucleic acids by acylation of lysine amino groups of the protein with the N-hydroxy-succinimidyl ester of biotin, affinity labeling of the biotin with a ferritin-avidin conjugate and observation of the ferritin labels attached to DNA in cytochrome-c spreads. This method was tested by identifying at a high efficiency the protein attached to the ends of adenovirus-2 DNA (15).

We report here our initial studies of an alternative method of mapping proteins using cytochrome-c spreads. The method is based on the attachment of the dinitrophenyl (DNP) hapten to lysine (and perhaps other) residues of the protein by reaction with dinitrofluorobenzene (DNFB) followed by binding of rabbit anti-DNP IgG molecules to the DNP haptens. If there are a number of DNP residues bound to a single protein, the complex with several IgG molecules can be directly visualized in cytochrome-c spreads. Where necessary a second antibody stain can be used to increase the size of the complex. We believe this method has advantages of convenience, simplicity, and high labeling efficiency.

MATERIALS AND METHODS

Materials. Materials were purchased from commercial sources as follows: 1-fluoro-2, 4-dinitro (3,5-^{3}H) benzene with a specific activity of 14.5 ci/mmole, Amersham-Searle; unlabeled fluorodinitrobenzene (DNFB), polyvinyl pyrrolidone (PVP), and Tricine, Calbiochem; RNA polymerase from E. coli MRE 600 and DNA polymerase (Grade II), Boehringer-Mannheim; glutaraldehyde EM grade, Polyscience; Proteinase K, EM Laboratories, Inc.;

dimethylsulfoxide (DMSO) reagent grade, Baker; Sepharose, Sephadex, and Ficoll products, Pharmacia; rabbit anti-DNP IgG, goat anti-serum to rabbit IgG, Gateway Immunosera Co.; E. coli 23S ribosomal RNA and EcoRl restriction endonculease, New England Biolabs; ovalbumin, Nutritional Biochemicals, Inc.; bovine serum albumin (BSA), Miles-Pentex.

T7 DNA was a gift from Dr. P. Server. Adenovirus-2 was a gift from Dr. M.T. Hsu. In vivo labeled ^{3}H E. coli DNA with a specific activity of 1.2×10^{5} cpm/μg was a gift from Dr. Mark Guyer. UV irradiated avian sarcoma virus (Prague B) was kindly prepared by Dr. Klaus Bister. Circular trypanosome mitochondrial DNA with poly(dBrU) tails was a gift from Dr. Welcome Bender.

Ovalbumin was purified by passage through a 0.2 x 20 cm Sephadex G100 column equilibrated with 0.05 M sodium phosphate (NaP) buffer, pH 7.2, 0.1 M NaCl. Supercoiled SV40 DNA, ϕX174 single stranded DNA and E. coli 23S ribosomal RNA were purified by treatment with proteinase K, phenol extraction and CsCl banding.

Dinitrophenylation reactions. Reaction conditions were modified from those of Sanger (16) and Bunnett and Hermann (17). Fresh 0.21 M DNFB stock solution was prepared in absolute ethanol and stored in the dark at 4°C for no more than two weeks. 40 μl solution containing DNA, RNA or protein with concentrations ranging from 5 μg/ml to 10 mg/ml were dialyzed against 0.125 M sodium borate (moles of $Na_2B_4O_7$) buffer (pH 9.0), 6.25 mM Na_3EDTA and 10% v/v dimethyl sulfoxide (BED buffer) at room temperature for two hours. 10μl of 0.21 M DNFB stock solution was added with good mixing. When there is a considerable volume change during dialysis, the volume of DNFB solution should be modified to be 1/4 of the final volume of the macromolecule solution. The reaction mixture which appears turbid because of the low aqueous solubility of DNFB was incubated at 37° in the dark for the required period of time. (The solubility of DNFB in water at 15°C has been measured as 0.86×10^{-2} M (18)). Excess DNFB was removed by three extractions with equal volumes of ether. Hydrolyzed DNFB was removed by dialysis against 5 mM Na_3EDTA, 0.2 M NaCl, 0.05 M Tricine buffer (pH 8.0) (ENTc buffer). The extent of modification was estimated by absorbance measurements at 365 nm or radioactivity determination. To interpret the absorbance measurements, we use $\varepsilon = 1.6 \times 10^{4}$ M^{-1} cm^{-1} at 365 nm for DNP groups bound to proteins (19). The absorbance of dinitrophenol at pH 8 is about the same. Degradation of DNA or RNA during the DNFB reaction was checked by measurements of A_{260} before and

after modification and by electron microscopic observations of the modified molecules.

In some cases protein or protein-DNA complexes were first treated at 37° for 15 min. with 0.2% glutaraldehyde in an incubation mixture without any primary amine as described under the particular experiments. The solution was dialyzed against BED buffer containing 0.02% glutaraldhyde at room temperature for two hours. One quarter volume of DNFB stock solution was added and the reaction carried out as described above, except that 0.02% glutaraldhyde was added to all dialysis solutions.

In all experiments with DNFB, glassware and dialysis tubing were pretreated with a solution containing 0.1% Ficoll, 0.01% polyvinyl pyrrolidone and 0.01% BSA to reduce surface absorption of dinitrophenylated proteins.

For optimum results on these protein-nucleic acid complexes for which the protein is not covalently bound to the DNA, the entire process up through the spreading for the electron microscope should be completed within twelve hours.

Purification of antibodies. Ten ml of a 10 mg/ml rabbit anti-DNP IgG solution in 0.01 M Na borate buffer (pH 8.0) was clarified by centrifugation at 16,000 rpm in a Sorvall SS34 rotor at 4°C for 30 min. The precipitate was discarded. The clear supernatant was mixed with 6.6 ml of saturated ammonium sulfate solution and allowed to stand at 0°C for 30 min. The precipitate was collected by centrifugation at 12,000 rpm (SS34 rotor) for 10 min, resuspended in 5 ml of 0.01 M borate buffer and dialyzed against 0.01 M Na phosphate (pH 7.5), 0.01 M NaCl. One ml of this solution was applied to a small column containing 2.5 ml of DEAE cellulose overlayed with 2.5 ml of CM cellulose equilibrated with 0.01 M phosphate buffer (pH 7.5), 0.015 M NaCl (20). Elution of IgG from the column by washing with the above buffer was monitored by A_{280} measurements. Peak fractions were pooled and used as described.

Nuclease contamination of the purified IgG was estimated by incubating a mixture of 0.25 mg of IgG and 0.75 µg of DNA or RNA in 50 µl of ENTc buffer for two hours at 37°C. IgG was removed by passage through a Sepharose 2B column and the DNA or RNA in the void column was mounted for observation by electron microscopy.

One batch of rabbit anti-DNP IgG was found to be satisfactorily free of nuclease after the above purification procedure; other batches were further purified by a 40% ammonium sulfate precipitation followed by size

fractionation on a 0.9 by 20 cm Sephadex G-100 column equilibrated with 0.1 M Na borate (pH 8.0), 0.1 M NaCl solution.

Goat anti-rabbit IgG was purified from the anti-serum by the same procedure.

Univalent anti-DNP Fab was prepared from purified anti-DNP IgG by pepsin digestion and reduction with dithiothreitol as described (21).

Preparation and purification of protein-nucleic acid complexes.

Adenovirus-2 DNA with its covalently attached protein was purified according to Robinson and Bellet (22). Virions were disrupted with 4 M guanidinium hydrochloride (GuHCl) at 0°C, banded to equilibrium in a ρ = 1.48 g ml^{-1} CsCl gradient containing 4 M GuHCl, and sedimented through a 5-20% sucrose gradient in 4 M GuHCl. The fast sedimenting fractions were pooled.

UV-irradiated avian sarcoma virus (Pr-B) was disrupted in 1% SDS at 60°C for 1 min, sedimented through a 5-20% sucrose gradient in 0.01 M Tris (pH 7.5), 0.15 M NaCl, 1 mM EDTA, 0.01% SDS. The rapidly sedminenting RNA fractions were pooled, mixed with 2.5 volumes of absolute ethanol and stored at -20°C. Before modification with DNFB the ethanol precipitate was sedimented, dissolved, and further purified from free proteins by the airfuge procedure described below.

A complex of RNA polymerase with T7 DNA was prepared by minor modifications of the procedure of Hinkle and Chamberlin (23). The incubation mixture contained 50 µg/ml of T7 DNA and 10 µg/ml of RNA polymerase in 10 mM Tricine buffer (pH 7.5), 10 mM Mg Cl_2, 50 mM NaCl, 5 mM dithiothreitol, 0.1 mM EDTA, 100 µg/ml BSA. After incubation at 37° for 10 min, glutaraldehyde was added to a final concentration of 0.2%, and the solution incubated at 37° for 15 min.

For the experiments on DNA polymerase binding, the polymerase (26 µg/ml) and EcoR1 cut SV40 linear DNA (50 µg/ml) were incubated in 25 mM K phosphate (pH 7.4), 5 mM $MgCl_2$, 1 mM DTT and 50 µg/ml BSA at 20°C for 10 min. 0.2% glutaraldhyde was added, followed by further incubation for 30 min at 20°C. This procedure is a modification of that given by Tabak et al., (24).

Reaction of anti DNP IgG with DNP modified protein-nucleic acid complexes. In a typical reaction, 200 µg of rabbit anti-DNP IgG was added to 0.5 to 2 µg of the DNP modified protein-nucleic acid complex in 40 µl of ENTc buffer and incubated for 1 hr at 37°C. Unbound protein was removed either by gel filtration or by centrifugation. In the former

method, a column with 0.5 ml of Sepharose 2B in a Pasteur pipette was washed with ENTc buffer. 40 µl of protein-DNA mixture was loaded on top and eluted with the same buffer. The DNA-protein complex was recovered in the void volume and used immediately for electron microscope spreading. In an alternative procedure 50 µl of the incubation mixture was mixed with 50 µl of ρ = 1.878 gm/ml CsCl solution (final density approximately 1.42) and centrifuged at 25 psi (approx. 125,000 g) in a Beckman Airfuge for 2.5 hr at room temperature. This procedure results in pelleting of nucleic acids. The upper part of the tube with approximately 50 µl supernatant solution was severed with a razor blade and removed; the rest of the supernatant solution was carefully removed by microaspiration. The pellet was dissolved in 10-20 µl of 0.01 M Tris (pH 7.5), 1 mM EDTA and used for electron microscopy. A similar procedure was used for further purification of UV irradiated avian sarcoma virus RNA as recovered from a sucrose gradient. In this case the pellet was dissolved in 0.01 M Na borate ($Na_2B_4O_7$, acidified with HCl) buffer (pH 7.5) for treatment with DNFB.

Reaction with second antibody. The adduct of rabbit anti-DNP IgG and DNA-protein-DNP complex prepared as described above and eluted from the Sepharose 2B column was treated with 0.02% glutaraldehyde at 30°C for 30 min. It was then mixed with an equal volume of 10 mg/ml of goat-anti rabbit IgG, incubated at 30°C for one hr, passed through a Sepharose 2B column that had been equilibrated with ENTc buffer to remove IgG, and spread for electron microscopy. In this procedure it is important to keep the glutaraldehyde concentration equal to or less than 0.1%, otherwise some IgG will form a precipitate during the incubation.

Electron microscopy. The standard formamide cytochrome-c spreading method was used (13), normally with 50% formamide, 0.06 M Tris (pH 8.2), 6 mM EDTA and 0.05 NaCl in the spreading solution, and 15% formamide, 0.01 M Tris (pH 8.2) 1 mM EDTA in the hypophase. Ethidium bromide (EtdBr) mounting was carried out as previously described (8). Benzyldimethylalkyl ammonium chloride (BAC) spreading was modified from the procedure of Vallenweider et al. (25). The DNA sample ($\sim$0.4 µg/ml) in 40% urea-formamide solvent (26), 10 mM triethanolamine buffer (pH 7.5), 1% CH_2O, was spread over a hypophase of distilled water, picked up with ionized carbon grids, stained with uranyl acetate, and shadowed with platinum.

RESULTS

Dinitrophenylation of proteins and nucleic acids. The reaction of DNFB with proteins has been extensively studied because of its applications for protein modification, end group analysis, and identification of peptide fragments. The rate of reaction with amino groups increases with increasing pH and with the addition of dimethyl sulfoxide to the aqueous reaction medium (16, 17). We have studied the reaction of DNFB with proteins, the hydrolysis rate of DNFB and the degree of reaction with nucleic acids in phosphate, borate, and bicarbonate buffer systems from pH 7 to 10 and at DMSO concentrations between 5 and 25%. In our hands, the incubation conditions presented in Materials and Methods give satisfactory results. The data in table 1 show that by varying the reaction time, one can control the extent of reaction per ovalbumin molecule over a practically useful range of from 6 to 30 DNP's per protein. Incubations

Table 1

Reaction of DNFB with Ovalbumin

Conc. of ovalbumin (mg/ml)	Reaction time min	Radioactivity assay cpm bound/ 500 µl	Radioactivity assay No of DNP per molecule	Absorbance assay A_{365}	Absorbance assay No of DNP per molecule
0.1	10	80	6.12	0.02	5
	60	230	17.60	0.06	18
	120	370	28.31	0.08	22
1	10	1,070	8.18	0.22	6.1
	60	2,430	18.59	0.63	17
	120	3,710	28.44	0.91	24.7
10	10	8,580	6.56	2.19	6
	60	21,930	16.77	2.67x2	14.52
	120	30,150	23.05	2.13x5	28.94

Experiments were performed in duplicate using DNFB of specific activity 11,360 cpm/µmole under the reaction conditions described in Materials and Methods. The molecular weight of ovalbumin was taken as 43,500.

for periods of time longer than 2 hours with consequent further modification of the protein resulted in reduced solubility and eventual precipitation of the protein.

The rate of hydrolysis of DNFB in the buffer solution used was observed by A_{365} measurements to be approximately 6 x 10^{-6} M hr which is negligible during the reaction times studied.

The reaction of DNFB with nucleic acids that do not have any bound proteins was assayed using ^{3}H labeled DNFB (Table 2).

Table 2

Reaction of DNFB with Nucleic Acids

Sample	Reaction time (min)	cpm/25 µg nucleic acid	No. of DNP per molecule	Electron microscopic assays (N=40) (dots/molecule) Single antibody label	Double antibody label
ϕX174	0	63	0.18	0	0.05
(SS DNA)	30	150	0.42	0.05	0.075
	60	326	0.90	0	0.4
	120	410	1.44	0.15	0.75
SV40	0	27	0.22	0	0.025
(DS DNA)	30	68	0.38	0	0.075
	60	193	1.08	0.125	0.4
	120	229	1.27	0.1	0.7
rRNA	0	42	0.06	0	0.05
	30	170	0.27	0.02	0.5
	60	269	0.42	0.15	0.75
	120	680	1.08	0.2	1.25

Experiments were performed as described in Materials and Methods (radioactivity assay experiments in duplicate). The specific activity of the DNFB was 2.29 x 10^{7} cpm/µmole. We take 360, 180, and 630 cpm/25 µg as one DNP per single-stranded ϕX DNA, double stranded SV40 DNA, and 3.0 kb 23S rRNA molecule respectively. Excess IgG was removed by the Sepharose 2B procedure (see Methods).

As a control for the results of electron microscopic observation of specific labeling of proteins bound to nucleic acids as reported below, we also estimated the number of dots seen per nucleic acid molecule by electron microscopy of cytochrome-c spreads after incubating the DNFB treated nucleic acids with rabbit anti-DNP IgG, and after additional labeling with goat anti-rabbit IgG. These results (Table 2) indicate that from 0.5 to 1 of the DNP molecules measured as bound by the radioactivity assay are recognizably labeled by the double antibody method, but that only 0.15 $\pm$ 0.15 as many dots are seen by single antibody staining. The data in Table 2 show that the number of DNP's recognized by the double antibody method is approximately 0.7 per 5 kb of single or double stranded DNA and 1.3 per 3 kb of RNA, after two hour incubations with DNFB. This is a tolerable background of nonspecific labeling in most studies for identifying a protein at a specific site on a nucleic acid. The non-specific background will be reduced by a factor of two if only an one hour incubation is used.

For proteins non-covalently attached to a nucleic acid, it is usually necessary to stabilize the complex against dissociation by crosslinking with glutaraldehyde before DNFB labeling. Table 3 presents the results of experiments in which ovalbumin was pretreated with glutaraldehyde for

Table 3

Reaction of DNFB with Glutaraldehyde Treated Ovalbumin

conc. ovalbumin (mg/ml)	Time of reaction with glutaraldehyde (min.)	cpm/25µl	No. of DNP/ molecule
1	0	3,781	18.39
	30	2,196	10.68
	60	1,428	7.14
10	0	24,096	16.17
	30	15,840	10.63
	60	10,304	6.91

The reaction was carried out as described in Methods. The glutaraldehyde concentration was 0.2% during incubation (0.05 M NaP buffer, 5 mM EDTA, pH 7.5, 37°) and 0.02% during the DNFB reaction (1 hr, 37°, specific activity 259,000 cpm/µmole).

various periods of time under the specified conditions. The glutaraldehyde concentration was then reduced from 0.2% to 0.02% and the protein treated with DNFB for 1 hr. Table 3 shows that the extent of reaction with DNFB decreases with the time of preincubation with glutaraldehyde; however, with a 30 min. incubation with 0.2% of glutaraldehyde a satisfactory degree of later modification by DNFB was achieved. In our tests with the T7 DNA-RNA polymerase complex as reported below, we observed that the glutaraldehyde crosslinked complex tended to lose RNA polymerase during incubation with DNFB unless a low concentration of glutaraldehyde (0.02%) was maintained in the reaction mixture. Therefore the reaction of DNFB with glutaraldehyde crosslinked protein-nucleic acid complexes was carried out under these conditions.

Purification of IgG's. Approximately 60-80% of the initial protein in the commercial rabbit anti-DNP IgG preparation was recovered after the centrifugation, ammonium sulfate precipitation, CM-DEAE cellulose column chromatography purification procedure (A_{280} measurements). Starting with goat anti-rabbit serum, the IgG yield was 6-15% of input protein; the yield was 70-80% in a second step of purification involving ammonium sulfate precipitation and Sephadex chromatography.

Table 4 shows the results of experiments for estimating the level of nuclease contamination in the purified IgG's by incubation with several nucleic acids. Under conditions of incubation similar to those used in labeling experiments, the nucleolytic activity on duplex SV40 closed

Table 4

Nuclease Tests of Purified IgG's

IgG		SV40 form I	SV40 form II + linear	ϕX 174 Circles	ϕX 174 linear	23S rRNA Intact	23S rRNA Partial
Anti-DNP	before	46	4	38	12	26	24
	after	41	9	32	18	17	33
	% not changed	90 %		85%		66%	
Goat anti rabbit	before	47	3	36	14	24	26
	after	40	10	28	22	14	36
	% not changed	85%		78%		60%	

The incubation was carried out in 0.05 M Tricine (pH 8.0), 0.5 mM EDTA, 0.15 M NaCl at 37° for 2 hours.

circular DNA was about 1 hit per 10 SV40 duplexes, 1 per 6 ϕX174 single strand molecules and 1 per 3 23S rRNA molecules respectively.

Several qualitative experiments using electron microscopic observation of unmodified nucleic acids indicated that the amount of nonspecific binding of IgG to the nucleic acids was adequately small at 0.15 M NaCl for duplex DNA and at 0.2 M NaCl concentrations for single stranded DNA, but was significant at lower salt concentrations.

The extent of reaction of DNPylated ovalbumin with the rabbit antibody was studied as a function of NaCl concentration by ammonium sulfate precipitation experiments. It was observed that under practical circumstances over 95% of the ^{3}H-DNP-ovalbumin was precipitated from 0.1 M NaCl, 92% from 0.2 M NaCl and 60% from 0.5 M NaCl. Thus there is a slight electrolyte effect on the antigen-antibody reaction but NaCl concentrations of 0.2 M or less give a satisfactory extent of reaction. It was also observed that high concentrations (greater than 10 mM) of EDTA interfered with the antigen antibody reaction. However, a low concentration of EDTA was included in the incubation mixture to inhibit nuclease activities. As a result of these tests, the electrolyte concentrations chosen for antibody labeling of DNP groups attached to protein-nucleic acid complexes were: 0.15 M NaCl, 0.05 M Tricine (pH 8.0), and 0.5 mM EDTA for double stranded DNA. For single stranded DNA or RNA, the NaCl concentration was raised to 0.2 M.

Mapping of proteins covalently linked to DNA.

a) The terminal protein of adenovirus-2 DNA. Adenovirus-2 (Ad-2) DNA molecules have a protein of molecular weight of ~55,000 daltons covalently attached at each end of the 35 kb molecule. These molecules appear, at a high frequency, as circles and oligomers in cytochrome-c spreads, presumably because of a hydrophobic interaction between the terminal proteins. Pronase digested DNA molecules do not circularize (10,27).

When our preparation of Ad-2 DNA, purified from virions by disruption with GuHCl and banding in CsCl-GuHCl, was spread by the aqueous cytochrome-c method, 65% of the intact monomers (N=50) were circles. No terminal "dot" attributable to the protein was visible on either circular or linear molecules when spread by either the BAC or the EtdBr protein free method, nor of course, in cytochrome-c spreads.

When the Ad-2 DNA-protein complex is incubated with DNFB for 1 hour, then with rabbit anti-DNP IgG, and spread with cytochrome-c from 50% formamide onto 15% formamide, many of the molecules had clearly visible

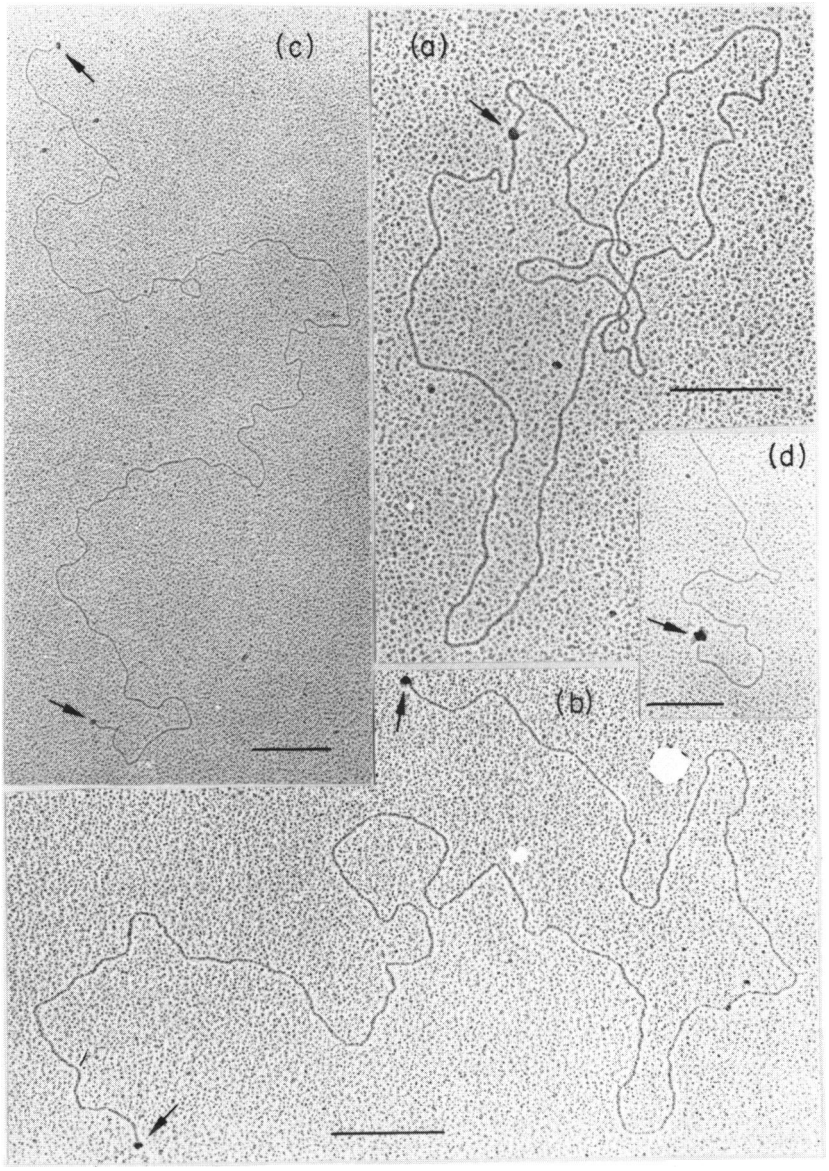

Figure 1. Electron micrographs of Ad-2 DNA-protein complexes labeled by treatment with DNFB and rabbit anti-DNP IgG and spread by the cytochrome-c method. Arrows point to the several amplified protein dots. The bar represents 0.5 μm. a) a circular DNA molecule with one cluster of anti-DNP IgG b)-c) Linear molecules with both ends clearly labeled with anti-DNP IgG clusters d) one end of a linear molecule labeled with anti-DNP IgG and goat anti-rabbit IgG.

dots at one or both ends. Dots were not seen on DNA molecules treated with DNFB unless antibody was added. Examples of labeled molecules are shown in Fig. 1. The size of the dots ranged from 18 to 30 nm, whereas the average width of the DNA was about 12 nm. Table 5 gives statistical data on the different structures observed. In a sample of 50 unbroken monomer molecules, 16 were circles, and 34 were linears. Of the 16 circles,

Table 5

Labeling of the Terminal Protein of Ad-2 DNA

	Linear molecules ends labeled			Circular molecules with		
	2	1	0	1 dot	2 dots	no dots
			(1 hr reaction with DNFB)			
No. of molecules	23	7	4	14	1	1
No. of internal dots	2	0	0			
			(0.5 hr reaction with DNFB)			
No. of molecules	18	8	5	17	0	2
No. of internal dots	1	1	0			

For each series of experiments, 50 molecules were scored.

14 had a clearly recognized dot at one point (Fig. 1a), one circle had two dots, and one had none. 23 of the 34 linear molecules had both ends clearly labeled with IgG clusters (Fig. 1b, c), seven had one end labeled, and four had none. That is, we observed for linear molecules that 53 out of 68 ends (78%) had clearly visible IgG aggregates. Therefore the labeling efficiency is at least this high. Table 5 also shows that reduction of the time of incubation with DNFB from one to 0.5 hours decreases the efficiency of observable labels slightly. The data in the table also show that the ratio of nonspecific (internal) dots to specific (terminal) dots is 2:53.

These results show that it is possible to modify the terminal protein of Ad-2 DNA with sufficient DNP groups so that several IgG molecules bind at each end to produce an aggregate that is sufficiently large to be

seen directly in cytochrome-c spreads without further amplification with a second antibody.

b). The SV40 DNA-protein complex; amplification with a second antibody. Kasamatsu and Wu discovered that if SV40 DNA is extracted from virions by a particular procedure, a protein is covalently attached to the duplex DNA at a specific position relative to the EcoR1 endonuclease site (8). This protein has a molecular weight of approximately 90,000 daltons (28, and H. Kasamatsu, private communication) and is visible by electron microscopy when the DNA is spread by anyone of several protein free spreading methods, but is not visible in cytochrome-c spreads. Since the presence of the protein can be independently observed, this system was chosen to measure the labeling efficiency in our present procedure. One disadvantage of the system is that the fraction of DNA molecules with the protein specifically attached varies in different preparations.

A preparation of the duplex SV40 DNA-protein complex was digested with EcoR1 restriction endonuclease and spread for electron microscopy by the EtdBr technique (Fig. 2a). The specific dot at 0.67 map units and

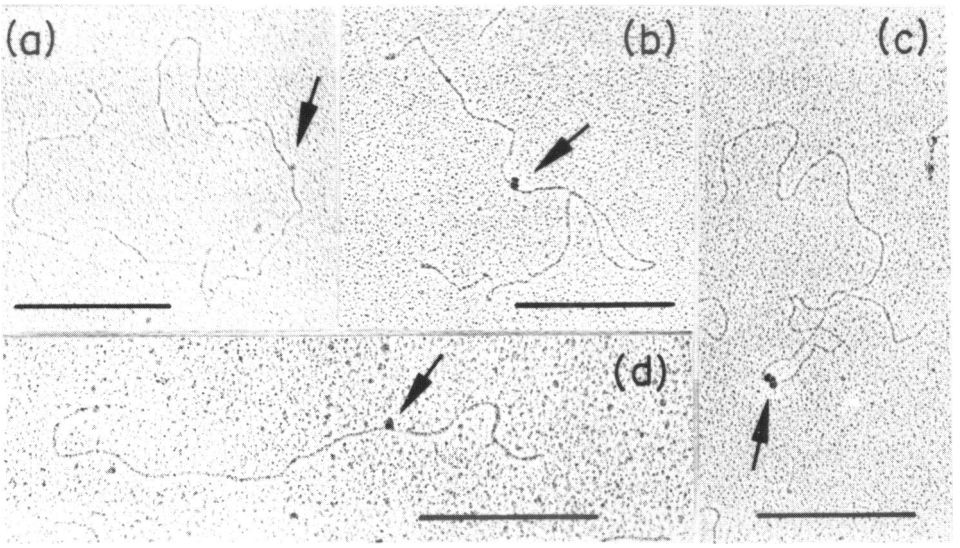

Figure 2. a) Electron micrograph of DNA-protein complex prepared from EcoR1 restriction endonuclease digested SV40. b). same DNA-protein complex after amplification with DNFB and anti-DNP IgG as described in text. c)-d). the DNA-protein complex after amplification with both anti-DNP IgG and goat anti-rabbit IgG. The molecules in Fig. 2a, b, c, were mounted by the EtdBr method. Fig. 2d was prepared by the cytochrome-c spreading method. Arrows point to the protein dot or the IgG clusters. Bars represent 0.5 μm. Intact SV40 DNA molecules measured 2.80 μm by the EtdBr method and 1.75μm in cytochrome-c spreads.

dots located at other positions were scored (Table 6). The complex was then reacted with DNFB for 1 hr and incubated with anti-DNP IgG for 1 hr. It may be seen that after labeling with IgG, the protein-DNP-IgG complex is larger in EtdBr spreads (Fig. 2b) than is the protein alone (Fig. 2a). The number of dots was again scored. However, the size of this amplified protein region is less than that seen for the Ad-2 terminal protein case. This preparation was therefore treated with goat anti-rabbit IgG. The doubly labeled protein aggregates were then very clearly visualized in cytochrome-c spreads (Fig. 2d). As shown in Fig. 2c, when molecules labeled with antibodies are spread by the high resolution EtdBr method the big dot seen in the cytochrome-c spreads appear as an aggregate of many protein molecules. In general reaction with the second antibody always increased the size of the protein label, however, there was also a greater number of background dots and more aggregates.

Figure 1d shows one end of a typical Ad-2 DNA molecule after double antibody labeling. In this case, a large protein aggregate is seen at the terminal position.

The statistics for the various numbers of structures seen in the SV40 experiments are given in Table 6. These data show that the labeling efficiency by the single and by the double antibody method is around 85-90%.

Table 6

Labeling of the Covalently Bound Protein of SV40

Treatment	Spreading Method	No. of molecules with dot at correct position	No. of molecules with dot at incorrect position	Total no. of molecules observed
Control	EtdBr	20	6	50
After incubation with anti-DNP IgG	EtdBr	17	3	50
After incubation with anti-DNP and goat anti-rabbit IgG	Cyt c	18	7	50

Mapping of proteins which are noncovalently bound to DNA.

a) DNA polymerase. *E. coli* DNA polymerase I, a protein of molecular weight 109,000 daltons, binds noncovalently to nicks, to ends, and to

junctions of duplex with single stranded DNA that have free 3'OH ends (24). Cleavage of duplex DNA by EcoR1 endonuclease generates such junctions. Supercoiled SV40 DNA was treated with EcoR1 restriction endonuclease, incubated with DNA polymerase, fixed with glutaraldehyde and prepared for electron microscopic observation by the EtdBr technique. The terminal DNA polymerase can be seen by the EtdBr method (Fig. 3a). As shown in Table 7, 34 out of 50 ends had dots. In the control experiments with no

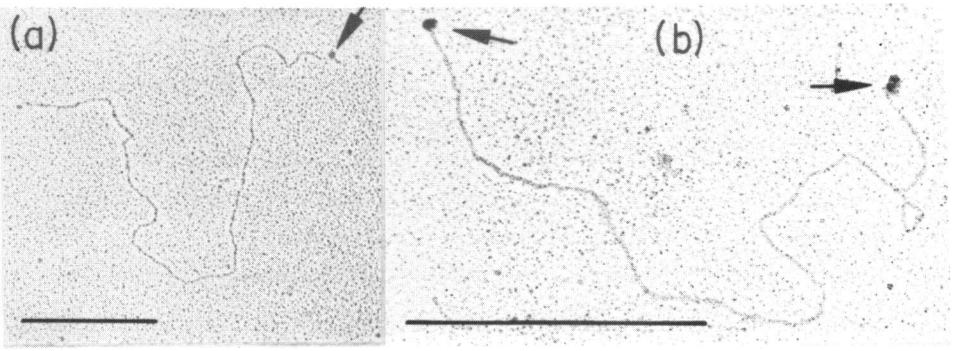

Figure 3. a) Electron micrograph of DNA polymerase attached to the end of an EcoR1 endonuclease treated SV40 DNA. The arrow points to a dot scored as DNA polymerase. The small dot on the other end was not scored. The sample was prepared for microscopy by the EtdBr method. b) Micrograph of a DNA polymerase-SV40 DNA complex after amplification with anti-DNP IgG. The larger cluster at the right end may represent a DNA polymerase dimer. The sample was prepared by cytochrome-c spreading. The bar represents 0.5 μm.

Table 7

Electron Microscopic Labeling of DNA Polymerase-EcoR1 Cut SV40 Complexes

Spreading method and treatment	-DNA polymerase No. of dots at end	-DNA polymerase No. of internal dots	+ DNA polymerase No. of dots at end	+ DNA polymerase No. of internal dots
EtdBr (before DNPlation)	3	2	34	5
EtdBr (after DNPlation)	2	2	22	5
Cyt c (after incubation with anti-DNP)	0	0	19	2

* For each treatment, 25 molecules, or 50 ends were studied.

added DNA polymerase, only 3 ends had dots. The protein-DNA complexes were treated with DNFB and with rabbit anti DNP IgG. Fig. 3b shows that the DNP labeled DNA polymerase, after incubation with anti DNP IgG, is readily identified in cytochrome-c spreads, although DNA-polymerase by itself is not. Further treatment with goat anti-rabbit IgG is not necessary. Data on the efficiency of labeling (Table 7) indicate that DNA polymerase binds to EcoR1 digested ends with an efficiency of about 68% with a fairly low background of internally labeled sites, and that there are very few dots in the absence of DNA polymerase. The efficiency with which terminal DNA polymerase molecules are labeled by DNP and the first antibody is approximately 55%.

b) RNA polymerase-T7 DNA. RNA polymerase is a large protein (480,000 daltons) and is readily observed by protein free spreading methods. Its binding to T7 DNA has been carefully studied (1, 2, 3). There are four specific binding sites at 0.2, 0.45, 0.58, 0.7 kb from one end. The DNP labeling procedure was tested in this system. The T7 DNA-RNA polymerase complex was prepared by standard methods, fixed with glutaraldehyde, and treated with DNFB. Figure 4a shows a cytochrome-c spread of such a molecule. The bound RNA polymerase molecules are observable but are not very prominent. After treatment with rabbit anti-DNP IgG, the labeled RNA polymerase molecules are much more readily seen in cytochrome-c spreads (Figs. 4b, c). After treatment with DNFB for one hour there was a tendency for the separate RNA polymerases bound to neighboring sites on T7 DNA to be clustered together possibly due to hydrophobic interactions between neighboring IgG molecules or to binding of a single divalent IgG to DNP haptens on adjacent polymerases (Fig. 4b). Thus, it was difficult to observe the intermolecular distances between binding sites by this method. However when time of treatment with DNFB was reduced to 30 minutes and the modified protein labeled with the monovalent Fab antibody, distinct RNA polymerase molecules are readily observed and the spacing between them can be estimated (Fig. 4d, 4e). The data in Table 8 show that the overall labeling efficiency with a one hour treatment with DNFB and labeling with rabbit anti-DNP IgG was approximately 50%. We suspect that the cause of this low efficiency is the dissociation of some RNA polymerase molecules during the reaction with DNFB.

Preliminary Study of Proteins Crosslinked to Avian Sarcoma Virus by UV Irradiation.

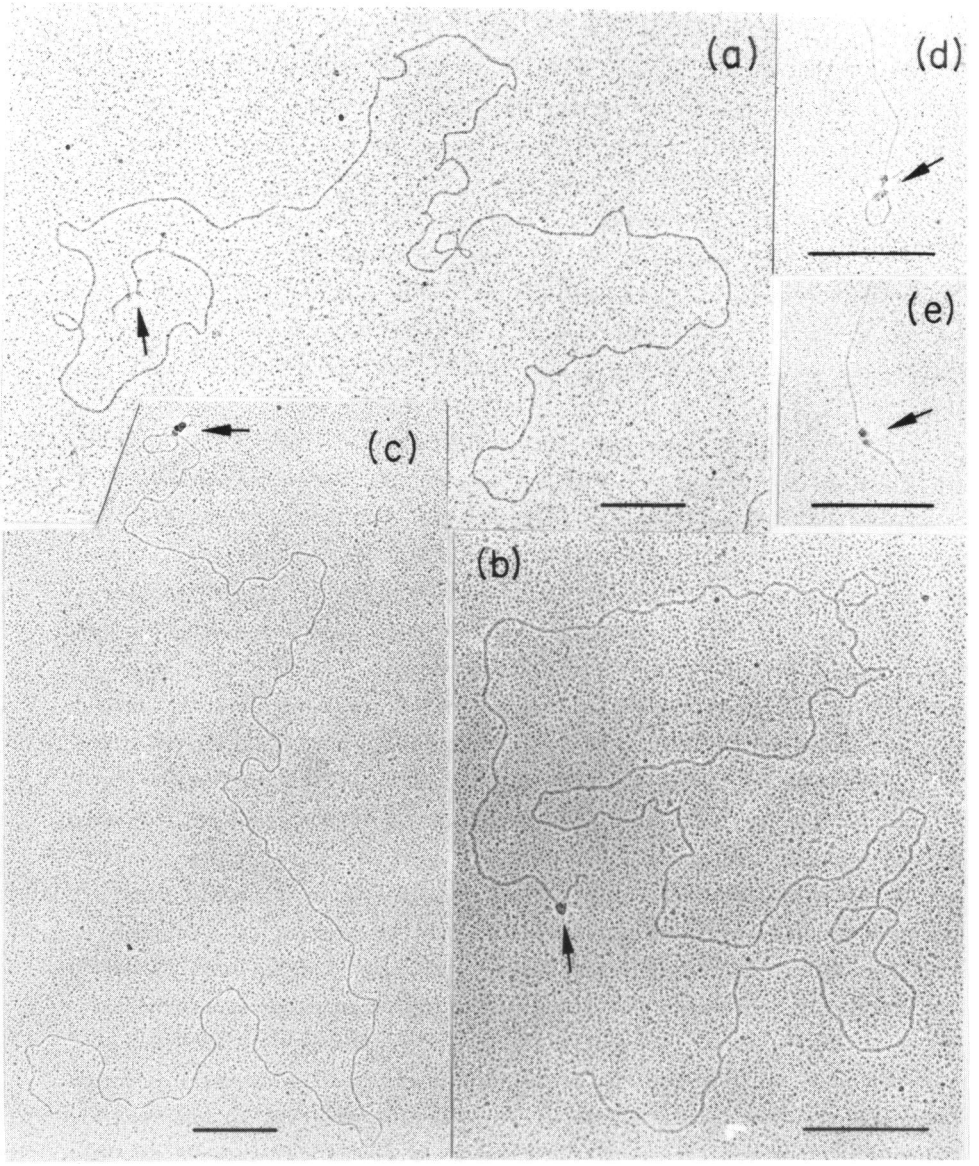

Figure 4. Electron micrographs of RNA polymerase-T7 DNA complexes prepared by the cytochrome-c spreading method. The arrow points to RNA polymerase and the bar represents 0.5 μm in each picture. a) The complex was treated with DNFB only. b)-c) The complex was treated with DNFB and then with anti-DNP IgG. d)-e) a part of a DNA molecule showing a complex after treatment with DNFB and the monovalent Fab fragment of anti-DNP IgG.

Several lines of evidence indicate that ultraviolet irradiation of avian sarcoma virions induces covalent binding of one or several proteins

Table 8

Electron Microscopic Labeling of
RNA Polymerase-T7 DNA Complexes

Spreading method and treatment	No. of molecules with 0 dot	1 dot	2 dots	3 dots	4 dots	Total No. of dots
BAC (before DNPlation)	0	5	10	20	15	145
BAC (after DNPlation)	2	30	11	6	1	74
Cyt c (after incubation with anti DNP)	1	28	14	7	1	81

For each treatment 50 molecules were scored.

to the viral RNA (29, 30). We are engaged in a study of this problem in collaboration with Drs. K. Bister and P. Vogt of the University of Southern California. One preliminary observation is presented here to illustrate the application of the present technique to the mapping of proteins attached to RNA molecules.

RNA was isolated from UV irradiated avian sarcoma virions (Prague-B) by methods which remove noncovalently attached proteins, treated with DNFB, rabbit anti-DNP and spread with cytochrome-c from 70% formamide onto 30% formamide. Many partially extended RNA molecules with dots at one end were seen. In order to extend the RNA more effectively, it was treated with DNFB, then with glyoxal (31) then with rabbit anti-DNP IgG, and spread in the presence of a small circular DNA (trypanosome mitochondrial DNA) tailed with poly(dBrU) in order to label the poly(A) ends of the RNA (32). A typical observation, as shown in Fig.5, is of a molecule with a protein dot or dots very close to the 3' end which is labeled with a circle-poly(dBrU). Similar dots were not seen at a comparable frequency in unirradiated controls. In the molecule in Fig. 5, there seem to be two closely spaced clusters, indicating that there may be two or more proteins at slightly different sites. The UV bound protein(s) does not completely block the terminal poly(A) segment, because the poly(dBrU) label is able to hybridize to it.

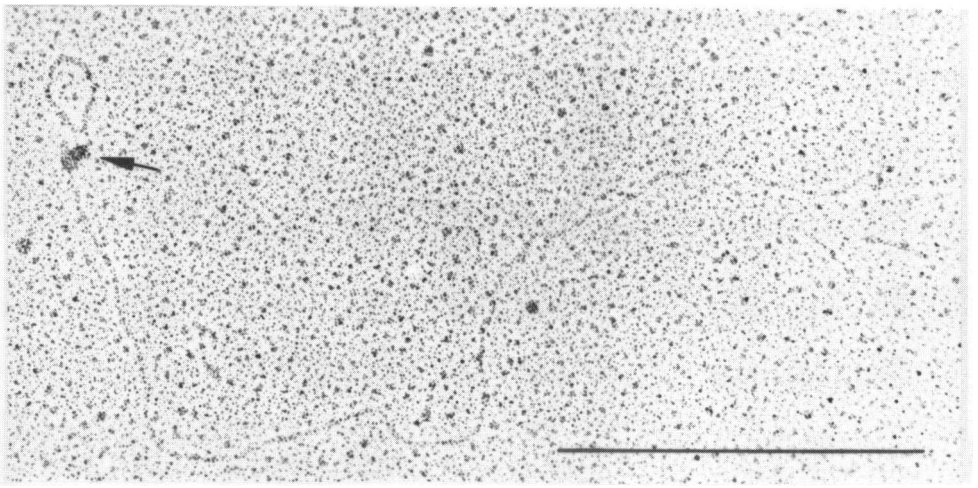

Figure 5. An RNA molecule isolated form UV irradiated avian sarcoma virions. The RNA was treated with DNFB and anti-DNP IgG and spread in a cytochrome-c film as described. The poly(A) end was labeled with the small circle of trypanosome mitochrondrial DNA. The arrow points to the IgG cluster(s). The bar represents 0.5 μm.

DISCUSSION

We believe that the technique described here will be useful for the identification of proteins attached to nucleic acid single or double strands. It requires only small amounts of material and involves comparatively simple and brief manipulations. The most widely used and generally effective method of studying nucleic acids by electron microscopy is by cytochrome-c spreading; proteins labeled by the present technique can be visualized in cytochrome-c spreads.

The full scope and limitations of the present labeling method have not yet been fully explored. We know, for example, that the terminal protein of adenovirus-2 even though it is not itself directly visible by protein free spreading methods, is labeled with a sufficient number of DNP residues so that the aggregate formed by reaction with anti-DNP can be seen directly in cytochrome-c spreads. We have no method of measuring how many DNP's have reacted per terminal protein, nor how many IgG's are attached. In some cases, especially for smaller proteins it is advantageous to amplify the label with a second antibody.

For noncovalently bound proteins, it is necessary to crosslink the protein to the nucleic acid with glutaraldehyde, otherwise there is a tendency to displace the protein during the DNFB treatment. DNFB and glutaraldehyde react with the same nucleophilic groups. Nevertheless, by

pretreatment of the protein-nucleic acid complex with 0.2% glutaraldehyde and incubation with DNFB in the presence of 0.02% glutaraldehyde, the protein-nucleic acid corsslinks are not displaced completely by DNFB but the glutaraldehyde binding to uncrosslinked nucleophilic groups of the protein (presumably mainly lysines) is sufficiently weak so that a sufficient degree of DNPylation is achieved. Similarly, treatment with 0.02% glutaraldehyde does not prevent reaction of anti-DNP IgG with its antibody, although more extensive treatment with glutaraldehyde does (33).

The present technique in its simpler form, with fairly extensive reaction with DNFB, and labeling with intact anti-DNP IgG may not be satisfactory for resolving closely spaced proteins on a nucleic acid strand, because the labeled proteins seem to aggregate with each other due to the divalent character of the IgG. Our brief tests suggest that less extensive DNPylation and use of a monovalent Fab antibody fragment will overcome this difficulty.

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