Immunoassays for carbodiimide modified DNA-detection of unpairing transitions in supercoiled ColE1 DNA

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ABSTRACT

The water soluble reagent N-cyclohexyl-N'- β -(4-methylmorpholinium) ethyl carbodiimide-p-toluene sulphonate (CMC) can be used to probe for unpaired and mismatched sites in DNA. Polyclonal antibodies for CMC modified DNA were produced in order to develop immunological assays for the localization and quantitation of CMC adducts. Immunoslot blot analysis of modified DNA exhibited antibody binding proportional to the extent of CMC modification with adduct detection in the femtomole range. Unmodified DNA did not cross react under the conditions of the assay. The distribution of CMC reactivity for supercoiled ColE1 DNA modified at 100, 200 and 300mM NaCl was determined by immunoanalysis of EcoRI-Hae2-NruI restriction fragments Southern transferred to nylon membranes. Reactivity above random expectation occurred in the A2-II fragment which can be accounted for by its high A-T content of 71.3%. Reactivity below random expectation occurred in the C fragment which can be accounted for by its low AT content of 43%. CMC modification for the other restriction fragments appeared random.

INTRODUCTION

Chemical modification of unpaired bases has played a very important role in providing assays for regions of the DNA that have been converted to the single strand state (1-4). Such unpairing transitions have been involved in supercoiled induced cruciform extrusions, B to Z transitions and triple stranded structures (1-4). In addition to stable transconformations, supercoiling increases transient metastable unpairing events which can be detected by chemical modification or single stranded endonucleases (5-12). Another extremely important application of chemical modification of unpaired bases lies in its capacity to detect point mutations in genomic DNA, providing rapid and efficient identification of genetic defects (13,14).

This laboratory has focused on the use of the single strand specific reagent, N-cyclohexyl-N'- β -(4-methylmorpholinium) ethyl carbodiimide-p-toluene sulphonate (CMC) to probe for unpaired sites in supercoiled DNA coupled with an analysis of the effects of CMC modification on transcription (7-11). CMC is a bulky reagent that reacts exclusively with the imino sites of thymine (T) and guanine (G) residues (Fig.1), allowing for the detection of all unpaired

Fig. 1: Structure of CMC and its reaction with thymidine.

sites in DNA. For point mutation studies CMC has been utilized to modify mismatched T and G residues (13). Adduct formation was detected by the reduced mobility of modified DNA in gels. However, this assay is limited to small DNA fragments in order to resolve the mobility shift (13). CMC adducts are recognized by the *E. coli* Uvr ABC protein complex (15), however, this complex does not turn over without the action of the Uvr D protein and DNA polymerase I (16). The complexity of the repair system for removal of bulky adducts does not readily allow its adoption as an analytical tool. CMC modified residues and many other adducts stop DNA synthesis and can be precisely located using primer extension analysis (17). This powerful methodology has provided the experimental basis for studying the secondary and tertiary structure of RNA molecules either free or engaged in complexes with proteins. We have adopted the primer extension strategy to detect CMC adducts in modified supercoiled DNA (18). From the above considerations it is apparent that new methodology to facilitate the location and number of CMC modified sites for both DNA structural and genetic studies would be of considerable value. In recent years, immunological approaches capable of very sensitive specific detection of DNA base adducts have been developed (19-22). Hence it was of considerable interest for us to examine whether we could develop CMC adduct specific antibodies and immunoassays that can detect the level of CMC adducts with high sensitivity. The ability to immunologically identify and quantitate CMC adducts in restriction fragments would allow for a detailed analysis of CMC reactivity for supercoiled DNA molecules modified under a variety of conditions. A knowledge of the distribution of CMC adducts in different restriction fragments would rapidly allow for the selection of the oligonucleotides that would be needed for primer extension mapping to precisely locate all adduct sites.

Polyclonal antibodies to CMC modified DNA were prepared to meet the objectives described above and immunoassays were developed that can detect the level of CMC in DNA with femtomole sensitivity. Under the conditions of the assays no cross reactivity was seen with unmodified single stranded DNA. Since CMC adducts are removed by alkali it was necessary to examine Southern transfer methodologies that avoided alkaline conditions. Modified restriction fragments can be Southern transferred to a nitrocellulose filter membrane at neutral pH but the transfer efficiency appears to be a function of the number of CMC adducts in respective restriction fragments and therefore was not quantitative. However, quantitative transfer of modified restriction fragments to nylon membranes were possible under nondenaturing conditions. The membrane was immunoprobed using antisera specific for CMC adducts and a streptavidin-biotin complex system. The amount of total CMC modification in ColE1 plasmid molecules was obtained from buoyant density measurements. Densitometric analysis of the immunoprobed southern blot allowed for a quantitative determination of the colorimetric signal for each fragment and calculation of the number of CMC adducts per fragment. The extent of CMC modification for different regions of supercoiled ColE1 DNA was determined as a function of the ionic strength during CMC reaction. These results represent the first phase of a comprehensive immunoanalysis of unpairing events in ColE1 plasmids vs. environmental parameters.

MATERIALS AND METHODS

Preparation of antibodies

The antibodies against CMC modified DNA (see DNA modification below) were produced in one month old New Zealand white rabbits essentially as previously described (20,21). Single-stranded calf thymus DNA, extensively modified with CMC (CMC-CT DNA), was complexed with an equal amount of methylated BSA according to Plescia (23). One mg of the complex was emulsified with two volumes of complete Freund's adjuvant and injected in four rabbits (2 male, 2 female) bilaterally i.m. and s.c. Three weeks later, rabbits 1 and 2 received a booster immunization with 0.5 mg CMC-CT DNA/mBSA conjugate emulsified in incomplete Freund's adjuvant, by injection into 25-40 i.c. sites on the neck and back. The other two rabbits were similarly injected with 0.1 mg CMC-poly(dT) (rabbit 3) and 0.1 mg CMC-poly(dG) (rabbit 4) emulsified with incomplete Freund's adjuvant. At week 8 the rabbits were given an i.v. (ear vein) injection of 0.5 ml PBS containing 0.5 mg CMC-CT DNA (rabbit 1 and 2) 0.1 mg CMC-poly(dT) (rabbit 3) and 0.1 mg CMC-poly(dG) (rabbit 4). The sera

were weekly monitored for titers against CMC-CT DNA as antigen in ELISA, until the animals were sacrificed 10 days after last injection by cardiac puncture. The antisera were stored at 4°C as ammonium sulfate (40%) precipitates (20,21). Appropriate aliquots of the precipitate were recovered by centrifugation, the pellets redissolved in PBS containing 0.05% merthiolate and used in the reported experiments.

Polyclonal antibodies developed against CMC modified single stranded calf thymus DNA were initially titrated against unmodified and modified single stranded DNA by ELISA established in one of our laboratories (20,21). Upon screening all sera showed a high affinity for CMC modified DNA (data not shown). No cross reactivity was observed with unmodified single stranded calf thymus DNA run in parallel (see Results). Also, preimmune sera titrated against modified DNA did not exhibit any binding (data not shown). Antibody dilution that gives 1 O.D. (at 450 nm) during 60 min. incubation with 10ng of CMC-DNA substrate was found to be in excess of 2×10^{-5} . Although antibody dilution titers were comparable for the sera of the four immunized rabbits, antiserum CMC-3, obtained by an immunization schedule involving secondary booster injections with CMC-poly(dT), showed the highest titer. Consequently, antiserum CMC-3 was chosen as the primary antibody for the immunoassays of this report. Although secondary booster injections for rabbits 2 and 3 were made with different antigens, CMC-CT DNA and CMC-poly(dT) respectively, we did not find any difference in antibody specificity between the antisera (data not shown). CMC polyclonal antibody is available to investigators upon request.

Nucleic acid, protein, and reagent sources and buffers

Plasmid ColE1 was obtained from Calbiochem or was prepared as previously described (11). Ethidium bromide was obtained from Calbiochem. N-cyclohexyl-N'-β'-(4-methylmorpholinium) ethyl carbodiimide p-toluene sulfonate (CMC) was from Fluka or Aldrich and was used without further purification. Agarose was obtained from Seakem. Affinity purified goat anti-rabbit IgG-Biotin (GARI-B) was from Cappel or BMB and Streptavidin - alkaline phosphatase (StpAv-AP) conjugate from BRL. Restriction endonucleases were from BRL and New England Biolabs. Nonfat dry milk was purchased from a local supermarket. Nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate (p-toluidine salt), p-nitrophenyl phosphate (Sigma 104 phosphatase substrate tablets), and Tween-20 were from Sigma. All other reagents were the highest grade available.

Buffer, reagent and immunological assay solutions- TE: 10 mM Tris-HCl/pH7.5, 1 mM EDTA; PBS: 10 mM Na₂HPO₄/pH7.4, 140 mM NaCl; PBS-Blotto: PBS containing 5% (w/v) nonfat dry milk, 0.01% (v/v) Antifoam A concentrate (Sigma); AP7.5:100 mM Tris-HCl/pH7.5, 100 mM NaCl, 2 mM MgCl₂; SSC: 15 mM sodium citrate/pH7.0, 150 mM NaCl; Blotto-Tween Blocker: 2.5% nonfat dry milk, 50 mM Tris-HCl/pH7.5, 25 mM NaCl, 1 mM EDTA, 0.09% Tween-20; TBS-Tween: 20 mM Tris-HCl/pH7.5, 500 mM NaCl, 0.09% Tween-20; TAP7.5-TX100: 25 mM Tris-HCl/pH7.5, 100 mM NaCl, 0.07% (v/v) Triton X-100; TAP9.5: 100 mM Tris-HCl/pH9.5, 100 mM NaCl, 5 mM MgCl₂; NBT: 75 mg/ml nitroblue tetrazolium in 70% (v/v) dimethylformamide (DMF); BCIP: 50 mg/ml bromochloroindolyl phosphate in absolute DMF. NBT and BCIP solutions were stored in dark bottles at -20°C, and were used within 2-3 weeks.

Preparation of CMC modified DNA

CMC modification of Cole1 DNA in supercoiled conformation was performed in either a 1.9 mM or 10 mM sodium phosphate buffer/pH7 containing appropriate concentrations of salt. Reactions were at 37°C for 16 hours and contained approximately a 500:1 CMC to DNA phosphate molar ratio. Phenol deproteinized calf thymus DNA (Sigma) was heat denatured by boiling the DNA solution for 10 minutes followed by rapid cooling in an ice bath. Single stranded calf thymus DNA in 1.9 mM sodium phosphate was CMC modified as described above. The CMC reactions were terminated and the DNA purified by precipitation with ethanol/ammonium acetate or ethanol/sodium acetate and extensively washed with 70% ethanol. The pellets were dissolved in TE and stored frozen at -20°C. The concentration of DNA was monitored by UV absorbance at 260 and 280 nm (24) and also in some cases by a microfluorometric assay using a Hoechst 33258 (25).

Buoyant density measurement of CMC modification

The number of CMC adducts in Cole1 DNA were determined by CsCl buoyant density centrifugation in a Beckman model E analytical ultracentrifuge as previously described (11).

Immunoslot blot (ISB) assay

DNA samples (0.1 ml) containing various amounts (1 pg to 1 ng) of modified or unmodified heat denatured DNA were mixed with an equal volume of 2 M ammonium acetate and applied onto nitrocellulose (NC) filters using a BRL hybrislot Manifold filter device. Prior to use, the NC filters were pre-soaked uniformly in 1 M ammonium acetate by floating on the liquid for 5-10 min. After application of DNA under low vacuum, the slots were rinsed with 0.2 ml of 1 M ammonium acetate. The filters were removed from the manifold, soaked in 5X SSC

for 5min, dried and baked in a vacuum oven for 2 h at 80°C. The filters were incubated for 1 h at 37°C with PBS-Blotto in a heat-sealed plastic bag (Sears, seal-a-meal) to block the nonspecific binding of the reagents to the filter. All subsequent incubations at 37°C were carried out in the plastic bags. The filters were incubated with specific antibody (suitable dilution predetermined for antibodies by checker board titration) in 10-15 ml of PBS-Blotto at 37°C for 1 h. The filters were washed extensively in PBS/0.1% Triton X-100, with several buffer changes. The filters were then treated with 1:3000 dilution of GARI-B in PBS-Blotto. After 1 h incubation the filters were washed as above and incubated with StpAv-AP (1:1000 dilution in AP7.5 buffer). After washing as above, two additional washes were performed with AP7.5 buffer for 10 min each. Following the final washing step, the filter bound enzymatic activity was visualized by incubation of the NC filters in a solution of NBT/BCIP (400 ul NBT and 340 ul BCIP per 100 ml TAP9.5), as described by Leary et al. (26). Color development was stopped in PBS containing 10 mM EDTA. NC filters were stored wet in heat-sealed plastic bags prior to densitometric evaluation. The filters were scanned for the relative band intensities by using a LKB laser densitometric scanning procedure as described earlier (27,28).

Restriction enzyme digestion and gel electrophoresis

Double digestion of ColE1 DNA with restriction endonucleases EcoRI and HaeII or triple digests with EcoRI, HaeII and NruI were performed at 37°C in the reaction buffer (100 mM Tris-HCl/pH7.2, 5 mM MgCl₂, 50 mM NaCl) containing DNA (unmodified or CMC modified) and excess enzyme. The reaction was stopped after 2 h incubation by adding loading buffer consisting of EDTA/ficol/bromophenol blue or EDTA/glycerol/bromophenol blue/xylene cyanol. Suitable amounts of the reaction mixture were applied to a 1.8% agarose gel and separated according to size by electrophoresis in Tris/acetate/EDTA buffer at a constant voltage as described earlier (24,29). In some cases, the gels were stained with 0.5 ug/ml ethidium bromide, washed extensively under running tap water for 30 min and photographed.

Immobilization of separated fragments to membranes and immunoprobings

a.) Nitrocellulose Immunoprobings: The stained gel was soaked in 5X SSC for 30 min with gentle agitation. The DNA in the gel was then capillary blotted to nitrocellulose filter sheets by Southern transfer in 10X SSC (30). The filters were washed with 5X SSC and baked in a vacuum oven for 2 h at 80°C. The filters were processed for immunodetection of CMC adducts and quantitative densitometry as described in the ISB section above.

b.) Nylon Immunoprobng: Following electrophoresis the gel was soaked briefly in dH₂O and then capillary blotted to a nylon (FMC Nylon-GTG) membrane by Southern transfer in dH₂O. Capillary transfer was for about 12 hours at room temperature. The amount of DNA remaining in gel following capillary transfer to nitrocellulose or nylon was less than 5%. After transfer, the membrane was rinsed briefly in dH₂O and baked at 75°C for 2 hours. The membrane was blocked from nonspecific antibody binding by incubating in a seal-a-meal bag with Blotto-Tween Blocker for about 12 hours at 37°C with shaking. All three incubations with immuno reagents were for 1 h each at 37°C with shaking. CMC-3 antiserum was diluted 1:5,000 in the Blotto-Tween Blocker solution and GARI-B was diluted 1:3,000 in Blotto-Tween Blocker. In between each incubation the membrane was washed 3X for 10 min each in a large excess of TBS-Tween at room temperature with shaking. StpAv-AP was diluted 1:1,000 in AP7.5 buffer. Following the StpAv-AP incubation, the membrane was washed 3X for 10 min each with TAP7.5-TX100 and 2X further with AP7.5 buffer. Color development was as described in the ISB section above and maximal color development occurred in less than 20 minutes. Unlike the nitrocellulose membranes, the FMC-nylon membrane was not scanned directly. The texture of the nylon membrane was sufficiently rough that direct scanning with the laser densitometer created a high spike background. Instead, the wet nylon membrane was photographed via reflectance with Kodak Technical Pan Film with a Kodak gray scale (0.15 O.D. change per scale increment) placed adjacent to the blot to monitor the efficiency of linear transfer of intensity during photography. Both the sample lanes on the negative and the gray scale were scanned with an LKB laser densitometer. Using the FUNCTION option of GSXL software, all curves (including gray scale) were converted to an inverse and stored. The intensity transfer of gray scale to Tech Pan film was found not to be linear but to be exponential (correlation coefficient to exponential = 1.00). It was therefore necessary to correct for the deviation from linearity. To correct to linearity the log of the exponential gray scale curve was taken to give a linear relationship. The slope of this resulting line (m) was corrected by a factor to the true gray scale slope of 0.15 (i.e. $0.15/m$ = factor). Using the FUNCTION option of GSXL software the log of each curve (curves inversed earlier) was taken and multiplied by the correction factor and stored. The resulting corrected curves were integrated by GSXL software using the Gaussian integration method and the Valley Base Line Option. The background of negative of blot was much less than that observed when the membrane was scanned

directly and resulted in a greater degree of detection. However, direct scanning of the blot gave similar results to the negative albeit with poorer limits of detectability.

RESULTS

Specificity of antibodies toward CMC modified DNA and sensitivity of antibody detection of CMC adducts

The immunoslot blot (ISB), a non-competitive solid phase immunoassay has been established in one of our laboratories (27,28) for the quantitation of low levels of modified bases in very small DNA samples. In this assay, heat denatured, modified or unmodified DNA samples are blotted onto nitrocellulose filters. Subsequent treatment with antibodies and detection of specific binding is performed on the paper matrix. The application of a DNA sample with a relatively smaller area on the solid phase increases the effective concentration of the test sample. The application of this assay to detect CMC modified single-stranded calf thymus DNA using the CMC-3 antiserum is shown in Figure 2. The filters show a gradient of color intensity with increased amounts (1 to 100 pg) of CMC modified calf thymus DNA (Fig. 2A). Unmodified DNA is not detected with any antibody dilution employed when the same range of DNA is applied to the filter. When a 1000 fold higher concentration of unmodified DNA is used, the background binding is observed with only higher concentrations of antibody. The relative peak intensities in the ISB assay for varying amounts of modified DNA, are unaffected by the antibody dilution from 1:5000 to 1:50,000 (Fig.2B). Since nonspecific binding to excess unmodified DNA is overcome by using a lesser antibody concentration, the specific antigen detection by the ISB assay is performed with as low as 1:50,000 dilution of antibody without compromising the sensitivity of adduct identification. The signal obtained with 1 pg modified single-stranded calf thymus DNA is visually detected (Fig. 2A) and is quantitated by determining the intensity of the blot upon scanning of filters.

Localization of CMC in supercoiled DNA

A major objective of this study is the development of immunoassays for mapping the location of CMC adducts in modified supercoiled DNA. While it would be possible to isolate CMC modified restriction fragments and perform a slot blot as described earlier, this approach would be very laborious. Consequently, we examined the possibility of direct Southern blotting of restriction fragments from agarose gels. This blotting methodology usually employs a denaturation step in which the gel is treated with an alkaline solution and rapidly

Fig. 2: Immuno-slot blot analysis of specific antibody binding to CMC DNA. (A) Unmodified single stranded calf thymus DNA (SS-DNA; row a, c, e and g) at a concentration of 1, 2, 5, 10, 50 and 100 ng (slot 1 - 6) and CMC modified single stranded calf thymus DNA (CMC-SS DNA; row b, d, f and h) at a concentration of 1, 2, 5, 10, 50 and 100 pg (slot 1 - 6) were immobilized to NC filters. Incubation with antiserum CMC-3 at a dilution of 1:5000 (a, b), 1:10000 (c, d), 1:20000 (e, f) and 1:50000 (g, h) was followed with GARI-B and StpAv-AP and the bound enzymatic activity was determined by color development with NBT/BCIP substrate as described in materials and methods.

(B) Plot of slot intensity as a function of DNA concentration at different antibody dilutions. Absorbance values obtained after valley to valley subtraction of filter background are plotted for CMC calf thymus DNA (top) and compared with 1000- fold excess of control unmodified DNA (bottom).

Fig. 3. Linear map of ColE1 showing restriction sites and the percentage A-T content for regions in the DNA molecule. The location of the restriction sites for EcoRI (Ec), HaeII (H), and NruI (N) and the restriction fragments generated by these enzymes are shown. The location of the major cruciform in ColE1 is also designated. A moving average (window=25 bp; shift=1 bp) analysis of % A-T content is shown.

neutralized. The single-stranded DNA generated by the above treatment can then be transferred to the nitrocellulose filter because of its affinity for this matrix. However, alkaline denaturation can not be used to denature CMC modified DNA since this treatment would remove the CMC adducts (31). Furthermore, we need to establish that the CMC antibodies can recognize adducts in a double stranded sequence context. We therefore attempted to transfer native modified double stranded DNA directly to nitrocellulose filters without a prior denaturation step.

Supercoiled ColE1 DNA (ColE1-FI) was modified with CMC in the presence of either 0, 10, 100, 200, or 300 mM NaCl for 16 hrs at 37°C. Following purification, an aliquot of the modified or unmodified DNA samples were double-digested with EcoRI and HaeII. A map of ColE1 showing the location of these restriction sites is shown in Fig.3. A moving average analysis of percent A-T (32) for various regions of ColE1 is also shown in Fig. 3 as well as the position of an extruded cruciform. The digested samples were loaded onto an agarose gel and the restriction fragments resolved. Following electrophoresis, the gel was stained with ethidium bromide (Fig. 4, lanes a-f). Supercoiled DNA modified with CMC in the absence of salt or in the presence of 10 mM NaCl, showed incomplete restriction endonuclease digestion, generating a set of partial fragments (Fig. 4, lanes b & c). Due to extensive CMC reactivity at low ionic strength, it is conceivable that CMC modification within enzyme recognition sites in DNA interferes with their cleavage. Restriction fragments in digests of the 0 and 10 mM reactions show a retarded mobility in the gel (lanes b & c) compared to the unmodified DNA (lane a) or DNA modified in the presence of 100, 200, or 300 mM NaCl (lanes d-f,

Fig. 4. The localization of CMC reactivity in ColE1 form I DNA as a function of salt concentration (nitrocellulose immunoprobings). ColE1-FI DNA was reacted with CMC in a sodium phosphate buffer (pH 7) for 16 h at 37°C in a reaction mixture containing various concentrations of NaCl. The DNA was cleaved with a combination of restriction enzymes (EcoRI and HaeII) and the fragments separated on a 1.8% agarose gel. Fragments were Southern transferred and the level of CMC modification determined by immunoanalysis of the filter using the antibody CMC-3 at a dilution of 1:40000. Restriction digests of DNA (20 ng/lane) are shown after ethidium bromide staining (lanes a-f) and same gel after immunodetection of Southern blots (lanes g-l). DNA digested and electrophoresed were untreated (lanes a & g), or treated with CMC in 0 mM NaCl (lanes b & h), 10 mM NaCl (lanes c & i), 100 mM NaCl (lanes d & j), 200 mM NaCl (lanes e & k) and 300 mM NaCl (lanes f & l). Size in base pairs for various DNA fragments are indicated.

respectively). The altered electrophoretic mobility is consistent with the behavior of CMC modified DNA reported earlier (9,11,13). The separated fragments were Southern transferred to a nitrocellulose membrane under nondenaturing conditions and immunoprobed as described in Materials and Methods (Fig. 4, lanes g-l). The results of Fig. 4 were obtained using 20 ng of the restriction digest per lane. The immunogram showed the following: 1. No bands are visible in the digest of unreacted ColE1 (lane g); 2. the partial fragments that are faintly visible upon ethidium bromide staining are clearly distinguishable upon immunodetection, consequently CMC antibodies can

recognize adducts in duplex DNA; 3. decreased electrophoretic mobility of restriction fragments correlates with the extent of CMC modification in that fragment; and 4. Quantitative densitometry showed a correlation exists between the degree of modification and the optical signals observed (results not shown).

Of concern to us was the binding efficiency of restriction fragments for nitrocellulose in the absence of alkali pretreatment of the gel containing the DNA fragments. Was binding to the nitrocellulose membrane dependent on the amount of CMC modification in fragments? In order to answer this question, a portion of each EcoRI-HaeII digest of the unmodified and 100, 200, and 300 mM NaCl reactions was dephosphorylated and then 5' end-labeled with [γ - 32 P]ATP. Duplicate amounts of unmodified and modified labeled samples were resolved in an agarose gel. Following electrophoresis, the gel was cut into two halves, each half with identical amounts of modified and unmodified samples. One-half was treated with alkali following the standard Southern procedure (30), and neutralized. The other half was treated identically except no alkaline step was employed. The DNA was then allowed to capillary transfer to a nitrocellulose membrane. Following transfer, the membrane was removed, allowed to air dry, and exposed against an x-ray film. In the absence of alkaline treatment binding to nitrocellulose was dependent on the amount of CMC modification, with the 100 mM NaCl sample having the greatest binding (data not shown). The binding of fragments from the gel pre-treated with alkali was independent of the amount of CMC modification (data not shown). Consequently, quantitative transfer can only occur for single stranded DNA. The affinity of modified duplex DNA fragments for the nitrocellulose matrix probably occurred as a result of the positive charge of CMC and/or unpaired bases at the adduct sites.

To eliminate the problems encountered with nitrocellulose we sought an alternative DNA affinity matrix that would bind duplex fragments quantitatively. The capacity for nylon membranes to bind duplex DNA has been shown by others (33). Therefore we investigated the ability of CMC modified duplex DNA fragments to be quantitatively transferred to nylon membranes using end labeled DNA. It was found that binding of fragments to nylon, in the absence of an alkaline pre-treatment step, was independent of the amount of CMC modification with unmodified and modified DNA showing equivalent levels of binding (data not shown).

The direct transfer of CMC modified duplex DNA to nylon membranes enabled the examination of the CMC reactivity for different target sites in

the ColE1 molecule. The ColE1-FI DNA samples modified with CMC at 100, 200, and 300 mM NaCl or unmodified, were either double-digested with EcoRI and HaeII or triple-digested with EcoRI, HaeII, and NruI (Fig.3). In the double enzymatic digests, EcoRI cleaves the HaeII A fragment into two unequal fragments designated A1 and A2. Further digestion with NruI separates the A2 fragment into two components designated A2-I and A2-II. The A2-II fragment is 71.3% AT, whereas the A2-I fragment is 48.6% AT. Double or triple digested modified or unmodified ColE1 samples were resolved in a 1.8% agarose gel and directly capillary blotted to a nylon membrane. The membrane was then processed for immunoprobings using CMC-3 Ab, GARI-B, and StpAv-AP as described in procedures. Incubation of the membrane with a mixture of bromochloroindolyl phosphate and nitroblue tetrazolium results in the deposition of a purple precipitate at the CMC modified DNA bands. The intensity of each band is dependent on the amount of primary antibody (CMC-3) bound and therefore the amount of CMC in the fragment. As can be seen in the immunogram (Fig. 5) the unmodified ColE1 DNA digested with EcoRI-HaeII-NruI (lane c) gave no response. The total amount of digested DNA in each lane was 50 ng. Decreasing the NaCl concentration during CMC reaction results in an increase in the intensity of fragment bands. In the 100 (lanes b and f) and 200 (lane e) mM NaCl reaction samples there are some incompletely digested fragments. The amount of incompletely digested fragments increases as the amount of CMC modification increases. However, the partial fragments constitute a small percentage of the total amount of DNA as determined by separate experiments using ³²P end-labeled DNA (results not shown). Partial fragments are probably a result of CMC adducts at or near the recognition sequence of the restriction enzyme.

If the nucleotide sequence throughout ColE1 DNA was essentially uniform we would anticipate that CMC modification would occur randomly and the number of CMC adducts would be based exclusively on fragment size: B>C>A1>A2>D (Fig.3). However, the order of increasing reactivity appears to be B>A2>A1>C>D (Fig. 5). As expected, ionic shielding decreased the total reactivity as the ionic strength increased from 100-300 mM, however the distribution of CMC reactivity remained similar. While the B and D fragments showed the greatest and least number of CMC adducts respectively according to size expectations, the following deviations from random behavior were observed: 1. the A2 fragment (1011 bp) had the second greatest reactivity although it is 306 bp smaller than fragment C (1317 bp) and; 2. the A1 fragment (1228 bp), which is also smaller than the C fragment, appeared to have slightly more CMC adducts than the C fragment based on the greater

Fig. 5 Analysis of the distribution of CMC in supercoiled ColE1 DNA (immunoprobings of a nylon Southern blot). Supercoiled ColE1 DNA was reacted with CMC as described in Figure 4. The DNA was modified in the presence of either 100 (lanes b & f), 200 (lane e), or 300 (lanes a & d) mM NaCl. The modified DNA was either double digested with EcoRI and HaeII (lanes a & b) or triple digested with EcoRI, HaeII, and NruI (lanes d-f). Unmodified ColE1 DNA was also cleaved with EcoRI, HaeII, and NruI (lane c). The DNA was separated on a 1.8% agarose gel, Southern transferred to a nylon membrane, and immunoprobed with antisera specific for CMC adducts. For a description of the fragments generated by these restriction enzymes see Fig. 3.

optical signal. An examination of Fig. 3 shows that the ColE1 nucleotide sequence undergoes significant % AT content variations as reflected by the moving average. The probability of unpairing transitions would be much greater in AT vs. GC rich sequences and we would expect deviations from a random distribution. This expectation will be explored in greater detail below.

Quantitative determination of CMC adducts in ColE1 DNA restriction fragments

Buoyant density analysis, using analytical ultracentrifugation, has been used extensively by us to provide a quantitative measure of CMC reactivity for

intact plasmid and viral DNAs (5,10,11). This technique takes advantage of the shift toward a lower buoyant density which occurs in DNA modified with CMC. Comparison of the number of CMC adducts determined by buoyant density measurements vs. radioactive detection using ^3H and ^{14}C CMC of known specific activity has confirmed the validity of the buoyant density analysis (8,9,11). Accurate measurement of the buoyant density shift is dependent on the molecular weight of the DNA molecule. Progressively broader buoyant bands are generated as the molecular size of the DNA decreases, making accurate determination of amounts of CMC adducts difficult in molecules less than 2 kb. However, by combining buoyant density measurements for the amount of total CMC modification with immunoanalysis of CMC in restriction fragments it should be possible to measure the number of CMC adducts in each fragment from the relative intensity of each immunoprobed restriction fragment band.

To obtain the number of CMC adducts in each restriction fragment a negative of the immunoprobed blot of Fig. 5 was scanned using an LKB laser densitometer and an area analysis performed using GSXL software as described in Methods and Materials. For most of the restriction fragments it was possible to convert the area value into the number of CMC adducts per fragment using the total number of CMC adducts per ColE1 molecule as determined by buoyant density analysis. For the 100, 200 and 300 mM NaCl reaction conditions we obtained 108, 60 and 41 CMC adducts per ColE1 DNA molecule respectively. Area values for the HaeII B,C,A1,A2, and D fragments could be readily obtained (results not shown). However, modification for the HaeII E and F restriction fragments was too low to obtain reliable area values. Band spreading due to diffusion contributed to the decrease of the optical signals for the HaeII E and F fragments. To take into account the missing HaeII E and F restriction fragments, which are respectively 359 and 80 basepairs in size, the total the number of CMC adducts were corrected by 0.933 to 100.8 and 56 CMC adducts for the 100 and 200 mM NaCl reaction conditions respectively. Due to low total modification at the 300 mM NaCl reaction condition, densitometric scanning could no longer provide reliable area values for the A2-I, A2-II and D fragments and consequently CMC values could only be obtained for the B,C,A1 and A2 fragments. The buoyant density CMC value was corrected by a factor of 0.826 to 33.9 to account for the missing restriction fragments. These CMC corrections were made with the assumption that CMC modification occurred randomly; an hypothesis that we will examine below.

The respective corrected CMC values were used to determine the % area for each fragment from the total densitometric area value. The % area for each

Table 1. Analysis of the distribution of CMC adducts in supercoiled ColE1 by quantitative densitometry of the immunogram shown in Figure 5. The null hypothesis that CMC is distributed randomly throughout the DNA molecule was tested for each fragment by Chi-Squared statistical analysis.

Fragment (bp size)	100mM NaCl Reaction			200mM NaCl Reaction			300mM NaCl Reaction			χ^2	Null Hypothesis Accept/Reject (significance level)
	%Area	CMC/fragment	CMC/bp (x1000)	%Area	CMC/fragment	CMC/bp (x1000)	%Area	CMC/fragment	CMC/bp (x1000)		
B (1940)	36.1	36.4	18.8	34.8	19.5	10.1	46.2	15.7	8.1	1.10	Accept (50 < p < 70)
C (1317)	13.9	14.0	10.6	12.4	6.9	5.2	13.7	4.6	3.5	4.77	Reject (05 < p < 10)
A1 (1228)	14.6	14.7	12.0	18.1	10.1	8.2	17.1	5.8	4.7	1.57	Accept (30 < p < 50)
A2 (1011)	25.2	25.4	25.1	24.2	13.6	13.5	23.0	7.8	7.7	7.36	Reject (02 < p < 05)
D (710)	10.1	10.2	14.4	10.4	5.8	8.2	—	—	—	0.13	Accept (50 < p < 75)
A2-I (533)	7.0	7.1	13.3	5.4	3.0	5.6	—	—	—	1.32	Accept (25 < p < 50)
A2-II (478)	18.2	18.3	38.3	18.8	10.5	22.0	—	—	—	45.7	Reject (p < .001)

fragment, the number of CMC per fragment, and the number of CMC per bp are shown in Table 1. Unpairing transitions will occur in supercoiled DNA in order to reduce superhelical torsional stress and it would be anticipated from energetic considerations that unpairing events would occur with greater probability in less stable AT rich sequences of the supercoiled plasmid (34). However, sequences that allow the formation of stable transconformations can compete with transient unpairing events (34). The unpaired sites of an extruded structure or structures could serve as "hot" spots for CMC modification which could lead to further modification propagating from the site of the extruded structure. Based on the above considerations it would be anticipated that CMC modification should not be random in restriction fragments with high AT content and possibly in fragments containing sequences that can form stable transconformations containing unpaired sites. As a preliminary screen for deviation from random modification we used Chi Square Goodness of Fit analysis to test the null hypothesis that CMC reactivity is random over the ionic conditions investigated. The expected random number of CMC/bp was calculated by dividing the total number of CMC adducts per plasmid molecule (from the buoyant density analysis) by 6646, the total number of basepairs in ColE1 DNA (35). The values for the expected random number of CMC/bp x 1000 are 16.3, 9.0 and 6.2 for the 100, 200 and 300 mM NaCl reaction conditions respectively. The

Yates correction for continuity (36) was used for the calculation of Chi squared values of the D, A2-I, and A2-II fragments since the degrees of freedom were equal to one in each case. The Chi squared values are also shown in Table 1, as well as the acceptance or rejection of the null hypothesis (confidence limits are shown). The only fragments that result in the rejection of the null hypothesis are the C, A2 and A2-II fragments. The rejection of random modification for the A2 fragment is clearly a result of the A2-II fragment which is 71.3% AT as indicated earlier. The A2-I fragment of 48.6% AT was accepted to be randomly modified. The A1 fragment of 58.3% AT was also accepted to be randomly modified. The C fragment of 43% AT deviates from random modification, but this deviation is toward less CMC reactivity than random.

DISCUSSION

A polyclonal antibody was developed to CMC adducts using modified single stranded calf thymus DNA as an antigen. To characterize the specificity and sensitivity of adduct detection we first employed immunoslot blot analysis of modified and unmodified single stranded calf thymus DNA. This assay demonstrated that the polyclonal antibody is highly specific for adducts with essentially no cross reactivity with unmodified single strand DNA. Antibody binding is proportional to the concentration of modified DNA. One picogram of heavily modified single stranded calf thymus DNA could be detected which is equivalent to 3 femtomoles of nucleotides. Consequently the detection sensitivity using immunoslot blot analysis is in the range of 1.0 femtomole of adducts since only T and G residues can be modified and we would not anticipate that complete modification had occurred.

The immunoslot blot results strongly suggested that a quantitative analysis of the distribution of CMC adducts could be obtained through immunoanalysis of DNA bound to a solid matrix. As a test system supercoiled ColE1 DNA was CMC modified as a function salt concentration to provide modified DNA samples for the determination of the number of CMC adducts in different regions of the plasmid. To obtain an accurate assessment of the number of adducts in different regions of the plasmid, modified restriction fragments must be transferred quantitatively from the gel medium to an appropriate binding matrix. Since CMC adducts are removed from DNA by high pH we needed to employ transfer methodology that avoided alkaline treatment of the DNA. This goal was achieved by the Southern transfer of ColE1 DNA restriction fragments to a nylon membrane at neutral pH followed by

immunoanalysis to generate colorimetric signals proportional to the number of CMC adducts in each restriction fragment. Densitometry of the immunoblot provided area values for the respective restriction fragments. To convert the area data into CMC adducts we used the total CMC modification obtained from the buoyant density analysis.

The quantitative evaluation of the distribution of CMC adducts was presented in Table I. The A2-II restriction fragment, with an AT content of 71.3% , was the most highly modified region of ColE1 DNA on a CMC per basepair basis. In addition to the high AT content, the A2-II fragment also contains a region known to extrude a major cruciform under DNA supercoiling (Fig.3). The unpaired bases in the cruciform loop would be highly reactive toward CMC. In addition Lilley's laboratory (37) has found sequences in close proximity to the cruciform site which have enhanced unpairing dynamics and act to induce cruciform extrusion. All the above considerations would lead to the expectation of unusual high reactivity for the A2-II fragment. The quantitative analysis of the distribution of CMC adducts in different restriction fragments allowed us to examine whether the number of CMC adducts in restriction fragments could be accounted for by random reactivity. The Chi-Squared Goodness of Fit statistical test was performed to examine the random hypothesis. CMC modification of the B, A1 and D restriction fragments were accepted as random with A2 and C restriction fragments rejected as nonrandom. As can be seen in Table 1, the A2 fragment was rejected as nonrandom with a probability between 0.95 and 0.98. Subcleavage of this fragment into A2-I and A2-II fragments with NruI revealed that only the A2-II was nonrandom with a probability in excess of 0.999. As indicated above we can account for the non-random behavior of the A2-II fragment based on its high AT content. Nonrandom CMC reactivity for the C fragment can be accounted for by its low AT content. The appearance of random CMC reactivity could result if a restriction fragment contained both a region of higher than random reactivity and a region of lower than random reactivity. This possibility would be greatest in large size fragments. For the A1 fragment there is a gradient of increasing AT content from left to right with the right half of the fragment very rich in AT sequences (Fig.3). Would subcleavage of the A1 fragment generate fragments with lower and higher than random reactivities? This question points out that a more refined analysis of DNA structural fluctuations in ColE1 DNA is needed and that CMC analysis should be performed on the smallest size fragments possible. Only 50 ng of digested DNA was used in the immunoanalysis described in this study and the smallest fragment

detectable was 478bp. Theoretically if we used a 0.5 ug DNA sample we should be capable of immunodetection of CMC in fragments as small as 50 bp in plasmid molecules modified to the same extent. A 10 to 100 fold increase in DNA for a far more refined analysis would not present any difficulties. We have not attempted to provide an exhaustive analysis of CMC modification by extensive subcleavage, this will be forthcoming in future work.

As shown, unpairing transitions are diminished by the ionic shielding. It would also be anticipated that differences in cation interactions, e.g. Mg vs. Na ions, polyamine binding and temperature would influence unpairing events. We have initiated a study to examine the effects of solvent components and temperature on unpairing transitions in ColEI plasmids using CMC modification. For rapid screening of the total modification we have employed ELISA analysis. Empirical relationships between the slope of the absorbance change and the number of CMC adducts are being developed. By using modified ColEI DNA as a standard in ELISA assays investigators should be able to calibrate CMC antibodies developed in their own laboratories without the need to perform buoyant density measurements.

The immunoassays developed in this study for the sensitive detection of CMC adducts will greatly extend the utility of CMC as a reagent to probe unpairing transitions in DNA. The first phase of the analysis of CMC modification of ColEI illustrated the potential of the immunodetection of CMC by obtaining the distribution of adducts in different regions of the DNA. To our knowledge, this type of analysis has not been performed with any other reagent used to detect unpairing transitions in DNA. The quantitative determinations of adducts coupled with high resolution mapping by primer extension sequence analysis (17) will provide frequency of modification data. Knowledge of the precise location and frequency of CMC adducts will be utilized to develop relationships between supercoiled enhanced or induced unpairing transitions and promoter activity. We can also envision the development of assays for the detection of CMC modified mismatch sites for mutational analysis. In conjunction with this study, we point out the recent development of polyclonal antibodies to DNAs chemically modified with osmium structural probes (38) which open additional possibilities for studying conformational transitions in DNA using immunoassays.

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