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Incorporation of 5-bromodeoxycytidine in the adenovirus 2 replication origin interferes with nuclear factor 1 binding

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ABSTRACT

We have studied the binding of nuclear factor 1 (NFI), a human sequence-specific DNA-binding protein, to a DNA fragment substituted in vitro with 5-bromodeoxycytidine (5-BrdC). Even at low substitution grades binding of NFI to its recognition sequence was considerably lower than with the unsubstituted control fragment. We developed a procedure to cleave substituted DNA specifically at a BrdC residue and searched for contacts between NFI and 5-BrdC residues by an interference assay. Surprisingly, no specific contacts were found in or near the recognition sequence. It appeared instead that interference was inversely related to the distance of a 5-BrdC residue from the NFI binding site. Models to explain these results, including a possible sliding mechanism, are discussed.

INTRODUCTION

Nuclear factor I (NFI) is a sequence-specific DNA-binding protein from HeLa cell nuclei first isolated by virtue of its ability to stimulate adenovirus 2 (Ad2) DNA replication in vitro (1). Stimulation of replication requires the binding of NFI to its recognition site on the Ad2 origin (2,3,4,5,6,7). A large number of cellular and viral NFI binding sites have since been identified (8,9,10,11,12) with a consensus sequence $TGGN_6GCCAA$. In addition to the role of NFI in the initiation of Ad DNA replication evidence has been obtained for involvement of NFI in transcription control (13,14). Recently it has been reported that NFI is identical to the CAAT-box binding transcription factor CTF (15). This protein has been purified to near homogeneity and SDS polyacrylamide gel-electrophoresis reveals a large number of species migrating between 52 KDa and 66 kDa. However, others have purified NFI with a molecular weight of 160 kDa (16) or make use of a NFI preparation showing slightly different binding characteristics as CTF (13), suggesting that there might be a group of possibly related proteins binding to the same sequence.

As a first step to elucidate the mechanism of NFI action we performed a detailed contactpoint analysis of NFI with its binding site (17). The results demonstrated that NFI binds in a symmetrical fashion, probably as a homo-dimer, and is located at one side of the DNA-helix.

One of the methods used in this study employed DNA in which T-residues were substituted in vitro by 5-Bromodeoxyuridine (5-BrdU). UV-irradiation of this DNA in complex with NFI identified close contacts between T-residues and NFI. In a similar way we tried to detect contacts between C-residues and NFI by substituting DNA with 5-Bromodeoxycytidine (5-BrdC) in vitro. This experiment was unsuccessful, probably due to the weak binding of NFI to 5-BrdC substituted DNA. We have now investigated the nature of this weak binding in more detail and under various reaction conditions. We show that the 5-BrdC substituted DNA can be specifically broken at the 5-BrdC residues by chemical cleavage with piperidine. This observation allowed the use of an interference assay in an attempt to contribute the decreased binding to specific 5-BrdC residues.

MATERIALS AND METHODS

In vitro substitution with 5-BrdC

5'-end labeling with T_4 polynucleotide kinase of primer B containing the sequence 5'-ACGCCCCGCGCCACGTC complementary to nucleotides 65-81 in the Ad 2 origin was performed as described (17). 5ng of endlabeled primer B and 5ng M13 sequencing primer were hybridized to 3 µg of ssMXE-2 containing the 1338 left terminal Ad2 sequence (r-strand) in M13 mp8 (17) in a buffer containing 50mM NaCl, 66mM Tris-HCl (pH7.5) and 6.6mM $MgCl_2$ by heating for 3 min at 80°C followed by 2 hrs at 20°C. Primer extension was carried out in the same buffer with addition of 5mM dithiothreitol, 5U E.coli DNA polymerase I (Klenow fragment, Boehringer), 50 µM ATP and 250 µM of dNTP's. The total concentration of dCTP and 5-BrdCTP (Pharmacia) together was always 250 µM whereas the ratio of 5-BrdCTP to dCTP is varied as indicated in the text. The reaction was performed for 2 hrs at 30°C in a total volume of 30 µl. DNA was purified and separated from (γ-^{32}P)-ATP by filtration over Sepharose Cl-4B. The DNA was digested with PvuII, phenol extracted, precipitated with ethanol and separated on a 1.0% agarose gel (Pharmacia, NA grade). Upon staining with ethidiumbromide all bands expected are visible at corresponding intensities indicating that both primers are efficiently elongated. The 666bp fragments were recovered by electroelution (18), phenol extracted, purified by filtration over Sepharose Cl-4B and ethanol precipitated. The yield was identical at all 5BrdCTP to dCTP input ratios.

Nitrocellulose filterbinding assays

DNA fragments (666bp, 0.2 ng, 3000 cpm) were incubated in 20 µl total volume with NFI in a buffer containing 15 mM Tris-HCl (pH 7.5), 5mM $MgCl_2$, 2mM $NaPO_4$ (pH 7.0), 0.02% NP40, 5ng/µl poly (dI-dC). poly(dI-dC) at 0°C. NaCl concentration, NFI concentration, incubation time and substitution grade of the DNA fragments used are indicated in the legends. The mixture was passed over a nitrocellulose filter (Millipore, HAWP) at a speed of 1µl/s. Filters were washed twice with 20µl of incubation buffer.

5-BrdC interference assay

0.75ng (10.000 cpm) of a DNA fragment synthesized at a 5-BrdCTP to dCTP ratio of 0.5 was incubated for 45 min. at 0°C with 7.5 µl NFI in a buffer containing 15 mM Tris-HCl (pH 7.5), 5mM $MgCl_2$, 2mM $NaPO_4$ (pH 7.0), 0.02% NP40, 5 ng/µl poly (dI-dC).poly (dI-dC), 100 mM NaCl (total volume 50 µl). Filtration over nitrocellulose was followed by two washes of 50 µl with buffer. Filterbound material was eluted in 0.5 ml 0.3 M NaAc (pH 5.4), 0.1% SDS for 1 hr at 37°C. Filterbound and filtrate fractions were extracted with phenol and chloroform and precipitated with 0.5ml isopropanol in the presence of 1µg tRNA. The pellet was dissolved in 100 µl 1M piperidine and after heating for 45 min at 95°C the mixture was freeze dried. Subsequently the pellet was dissolved in 50 µl H_2O followed by freeze drying (repeated three times). The pellet was dissolved in 2µl sample buffer (80% formamide, 20mM NaOH) and products were analyzed on a 10% denaturing polyacrylamide gel.

Purification of NFI

Preparation of HeLa cell nuclear extract and the first purification steps (DEAE-cellulose and phosphocellulose chromatography) were performed as described (4). NFI containing fractions were diluted to 0.2M NaCl in buffer B (55 ml) and loaded on a 10 ml double-stranded calf thymus DNA-cellulose column. The column was washed with 30 ml buffer and proteins were eluted with a linear salt gradient (250 mM-750 mM; 50 ml). NFI eluted around 400mM NaCl. After dilution to 250 mM NaCl in buffer B, NFI was further purified by recognition-site affinity chromatography essentially as described by Rosenfeld et al. and Diffley and Stillman (19,16). 12.5 ml was loaded on a 1 ml pKB 67-88 DNA-cellulose column followed by a wash (5 ml) and elution with a linear gradient (250 mM- 1 M NaCl; 20 ml). NFI eluted between 500 mM and 550 mM NaCl. NFI containing fractions were pooled and 10 µl was analyzed on a SDS-polyacrylamide gel. Silver staining of the gel revealed only three protein bands indicating that NFI was highly purified (see Fig. 1). The band most prominently retained on the specific DNA-cellulose column migrated at

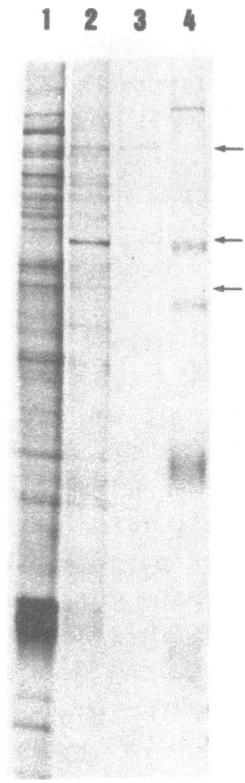

Figure 1 Purification of NFI
NFI containing fractions of the phosphocellulose column (lane 1) the double-stranded calf thymus DNA-cellulose column (lane 2) and the specific DNA-cellulose column (lane 3) were pooled and 10 µl of each pool was analyzed on a 12% SDS-polyacrylamide gel (27). Proteins were made visible by silver-staining (28). Arrows indicate the three bands visible in the material from the last column which was used for all further experiments. Lane 4 shows 50ng each of the following molecular weight markers: Ovalbumin (45kD), Bovine albumin (66kD), Phosphorylase b (97,4kD), β-Galactosidase (116kD), Myosin (205kD).

about 160 kD, corresponding to a previously reported molecular weight for NFI (16). However, we have not yet further identified which band corresponds to NFI. The NFI containing fractions of both DNA-cellulose columns were tested for DNA binding by DNase I and ExoIII footprinting and for stimulation of Ad2 DNA replication in vitro. Binding and stimulation were comparable to previously used NFI preparations (4,5,17) and co-purified on both columns. The protein concentration as estimated from the gel was less then 10 ng/µl.

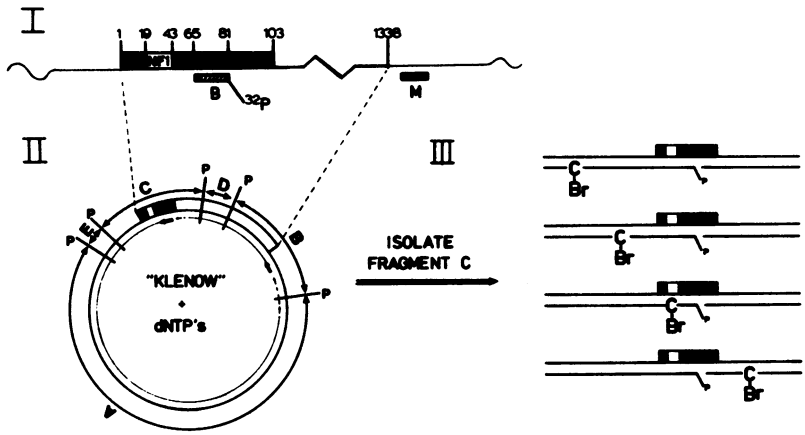

Figure 2 Synthesis of 5-BrdC substituted DNA in vitro.
(I) 5'-endlabeled primer B and M13 sequencing primer (M) are hybridized with single-stranded MXE-2. Numbering starts a the first nucleotide of Ad2. The 103 bp inverted terminal repeat is indicated at an enlarged scale. Primer B (65-81) hybridizes just in front of the NFI site (19-43). (II) Primers are elongated by DNA polymerase I large fragment at variable 5-BrdCTP to dCTP ratios. P indicates PvuII restriction sites. (III) A PvuII digest of the elongation products is separated on a 1% agarose gel. Fragment C is isolated and consists of a collection of 666 bp fragments that are brominated at different positions as indicated schematically.

RESULTS

Preliminary experiments (17) indicated that in a DNA fragment, containing an NFI binding site and highly substituted with BrdC, no significant binding of NFI could be demonstrated by DNase I footprinting. This raised the possibility that certain 5-BrdC residues in the recognition sequence interfere with NFI binding. For further characterization of this interference we made use of a nitrocellulose filter binding assay. In Figure 2 the procedure to obtain a 5-BrdC substituted, double-stranded DNA fragment labeled at a unique position is outlined. An oligonucleotide hybridizing close to the NFI recognition sequence is 5'-end labeled with ^{32}P and hybridized to ssMXE-2 containing the Ad2 origin in M13mp8 together with the 15-mer M13 sequencing primer. Subsequent strand elongation by DNA polymerase I large fragment in the presence of all four dNTP's (fragment C) or four dNTP's and 5-BrdCTP (fragment BrC) is followed by digestion with PvuII. The PvuII fragments were separated by agarose gel electrophoresis and the 666bp fragments containing the NFI site were recovered by electroelution. The fragments

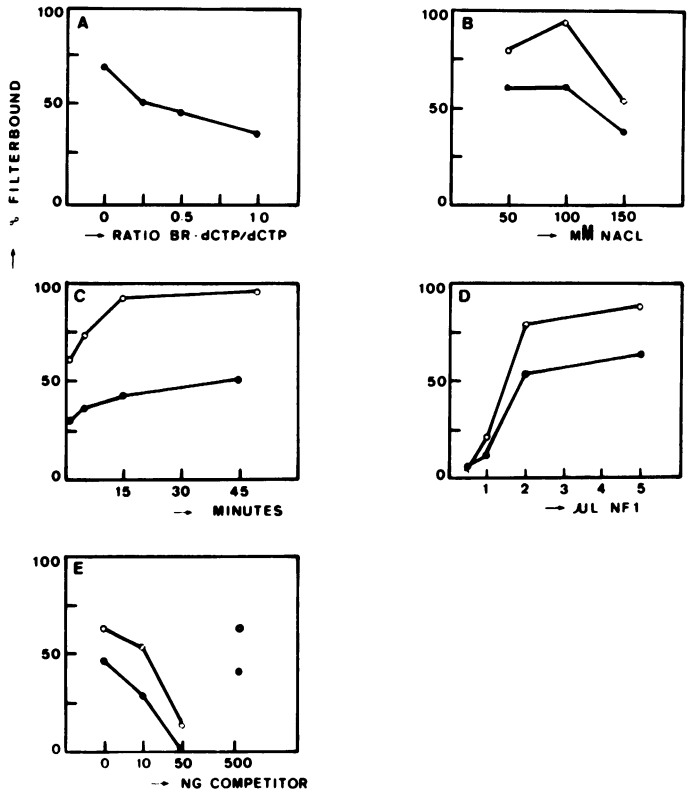

Figure 3 Inhibition of NFI binding to BrdC substituted DNA
The vertical axes indicate the percentage of input DNA that is bound by NFI. (A) shows the binding at increasing 5-BrdC substitution grades. The horizontal axis indicates the 5-BrdCTP to dCTP ratio at which fragments are synthesized. Binding is done at 100 mM NaCl, 2.0 µl NFI for 45 min. (B) The effect of NaCl concentration, 2.0 µl NFI, 45 min. (C) A time curve at 100 mM NaCl, 2.0 µl NFI. (D) NFI titration curve at 100 mM NaCl, 45 min incubation. (E) Specificity of binding is shown by competition with 10 or 50 ng XD7 or 500 ng pBr322. Competitor DNA is added together with the labeled fragment. Binding is performed at 100 mM NaCl, 2.0 µl NFI for 45 min. (o o) Fragment C, (o o) Fragment BrC.

were incubated under various conditions with NFI and binding affinities were compared by trapping complexed DNA on nitrocellulose filters and counting the radioactivity. In figure 3A the effect of the 5-BrdC/dC ratio is demonstrated. When only dC is incorporated over 70% of the DNA is complexed to NFI whereas a fragment that is elongated at a BrdCTP/dCTP ratio of 1.0 is complexed for only 36% under the same conditions. All subsequent experiments were performed with fragments

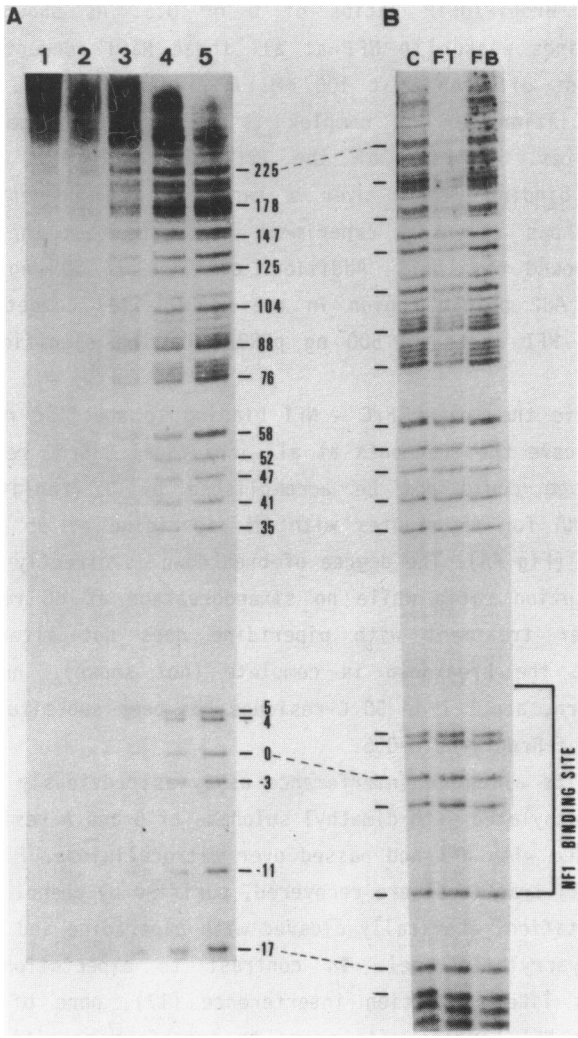

Figure 4 Analysis of NFI-BrdC contacts by specific cleavage with piperidine
(A) shows the breakdown products obtained by treatment with piperidine of fragments synthesized at BrdCTP/dCTP ratios of 1/256, 1/64, 1/16, 1/4, 1/2 (lanes 1 to 5). The pattern can be read as a C-lane from a sequencing gel. Numbers indicate the distance of the BrdC-residues to the center of the NFI bindingsite (position 0, which is GC-basepair 31 in the conventional numbering of the Ad2 genome as used in figure 2). (B) shows the interference experiment. Fragment BrC was incubated with NFI and after filtration over nitrocellulose proteinbound (FB) and unbound (FT) fractions were treated with piperidine and analyzed on gel. In lane C unseparated DNA is shown for comparison. The region protected by NFI against DNase I digestion is indicated by a bracket.

synthesized at BrdCTP/dCTP ratios of 0 or 0.5. As shown in Fig. 3B, fragment BrC binds weaker to NFI at all three NaCl concentrations tested with the largest difference at 100 mM. A time-curve (Fig. 3C) indicates that the association of the complex is slower for fragment BrC. Even after 45 minutes formation of the BrC-NFI complex is not complete. Inhibition of binding occurs over a range of NFI concentrations (Fig. 3D). Fig. 3E shows a control experiment to assure that the fragments are specifically bound to NFI. Addition of 10 or 50 ng XD7 (pBR322 containing the Ad2 origin region in the EcoRI site) competes efficiently for binding to NFI where as 500 ng pBR322 has no significant effect on binding.

To attribute the weaker BrC - NFI binding to specific residues it was necessary to cleave the fragments at all individual 5-BrdC residues. Such a complete cleavage could not be accomplished by UV radiation. However, treating the DNA for 45 minutes with 1M piperidine at 95° C led to the desired results (Fig 4A). The degree of breakdown is directly related to the BrdC/dC substitution ratio while no strandbreakage at dC residues can be detected. Longer treatment with piperidine does not alter the pattern indicating that the breakdown is complete (not shown). Judged from the breakdown pattern, about 1 in 50 C-residues has been substituted by BrdC at an input ratio of BrdCTP/dCTP=0.5.

These results allow an interference assay as previously performed with DNA fragments methylated with dimethyl sulphate at G and A residues (17). BrC DNA was complexed with NFI and passed over nitrocellulose. Filterbound (FB) and filtrate (FT) fractions were recovered, purified by phenol extraction and ethanol precipitation, chemically cleaved with piperidine and separated on a denaturing polyacrylamide gel. In contrast to expectations based upon previous assays like alkylation interference (17), none of the four BrC residues in the NFI binding site seems to interfere specifically with NFI binding to any greater extent than all other BrC-residues. Nevertheless there is a remarkable difference between filterbound and filtrate fractions. The bands in or just beside the NFI binding site show up in the filtrate fraction for more than 50%. This effect is slowly fading out when the distance to the NFI site increases and at large distances all bands are completely in the filterbound fraction. Thus molecules containing a BrC residue at a large distance from the NFI site (upper part of the gel) bind NFI well while BrC-residues close to the NFI site interfere considerably with binding. This finding is substantiated when the gel is scanned and the percentage

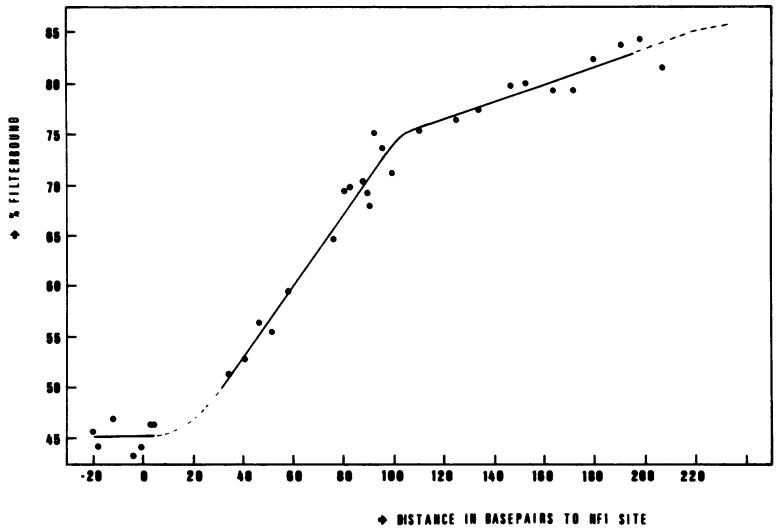

Figure 5 Interference is inversely related to the distance to the NFI site
The autoradiogram of Figure 4B was quantified by densitometric scanning. On the X-axis the distance of a BrdC-residue to the center of the NFI binding site is plotted. On the Y-axis the percentage complexed with NFI is indicated as calculated by the formula FB/(FT+FB) x 100%. As a control we calculated for every band the ratio C/(FT+FB) which theoretically should be 0.500 and was in this case 0.488 ± 0.042 (standard deviation). No C-residues are present between 5 and 35bp. Therefore an interrupted line is drawn as its real shape is unknown in this region.

filterbound for each BrdC peak is plotted against the distance from the NFI site (Fig. 5). Two straight lines can be drawn with correlations of 0.972 between 35 and 100 bp and 0.914 between 112 and 200 bp, respectively. Although the significance of the two slopes is unclear at present, we conclude that the degree of interference by a 5-BrdC residue is inversely related to its distance to the NFI binding site.

DISCUSSION

We have shown that the substitution of a DNA fragment containing the NFI recognition sequence with 5-BrdC residues interferes with its binding to NFI. A priori this effect can be explained in at least two ways. Firstly, bromination of C-residues might induce a three-dimensional structural change in the NFI binding site which decreases its binding affinity for NFI. A local structural change is unavoidable when a dC residue in the binding site itself is substituted. It is also possible that substitution of a dC-residue at a

distance of the NFI binding site introduces a structural change in the DNA helix that is propagated into the NFI binding site. Secondly, the association rate of the complex could be seriously reduced by substitution of certain dC-residues. Depending upon the dissociation rate this could lead to an overall reduction in binding. Our results do not allow to distinguish between these two possibilities. In these experiments we used a double-stranded fragment uniquely labeled at about 40bp downstream from the NFI recognition site. The labeling procedure (figure 1) leads to a nick or possibly a small gap at the position of the label. It is very unlikely that this causes the observed effects because the control fragment C is synthesized in the same way and this fragment binds to NFI as efficient as fragments end-labeled by standard procedures previously used (17).

The finding that piperidine specifically cleaves the DNA at 5-BrdC residues enables the use of 5-BrdC substituted DNA for contactpoint studies. We recently identified contacts between NFI and guanosines and thymidines in the major groove of the DNA helix (17). The bromine atom of 5-BrdC is also located in the major groove. Therefore it seemed feasible that 5-BrdC substitution of the dC residues in the NFI binding site might interfere with NFI binding. As can be seen in figure 3, interference is not only restricted to these dC residues but is inversely related to the distance of a 5-BrdC residue to the NFI binding site. This observation excludes a simple contact inhibition by a bromine as the only explanation for interference. An already mentioned possibility is a structural change in the DNA-helix induced by introduction of a bromine group. Several reports have mentioned the stabilizing influence of 5-BrdC or 5-methyldC residues on "Z-DNA like" structures (20,21,22). Such a stabilization might require the presence of several consecutive substituted C-residues, a situation which differs from the one in our experiments, where only one out of fifty or more C-residues is substituted. An attractive model is based on the assumption that NFI selects its specific binding sites by one-dimensional sliding along the DNA. The obtained 5-BrdC interference results of figure 4 could then be explained by assuming that a bromine residue might obstruct sliding of NFI. If NFI starts sliding at any random position on a DNA fragment a bromine group just upstream of a NFI binding site would have a larger effect than a bromine group near the end of a fragment, just by matter of chance distribution. For the time being such a model must be regarded as speculative since the way in which NFI locates its binding site is unknown. Even if sliding is involved e.g., as proposed for the lac repressor (23,24) or cro protein (25), this is

possibly purely based on electrostatic interactions with the DNA surface without any sequence-dependent components (26). The effect would then be caused by an electrostatic change in the DNA surface by the bromine residue, which is theoretically possible.

Finally we want to point out that the bromine group is attached to the C5 atom of cytosine, the same position at which eukaryotic DNA methylation takes place. Although a bromine group has a somewhat larger Van der Waals radius and negative charge than a methylgroup, 5-MeC residues might have a comparable effect as 5-BrdC residues. In view of the important role of hyper- and hypomethylation of DNA on gene expression our finding of reduced binding of a sequence-specific protein involved in transcription might bear relevance to the mechanism of transcription control by methylation. Unfortunately, a similar study of NFI binding to 5 MeC-substituted DNA is hampered by the lack of a specific cleavage reaction for methyl-C substituted DNA.

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