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O^6-methylguanine inhibits the binding of transcription factors to DNA

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ABSTRACT

To study the effect of methylation of O6-guanine on the binding of cellular factors to different DNA sequences, modified oligonucleotides were constructed, in which O6-Methylguanine (O6-MeG) replaced some guanines. The DNA sequences utilized were: the region of the c-fos promoter containing the binding site for serum response factor (SRF); the region of the HIV LTR containing two binding sites for the transcription factor NFκB; the region of the HIV LTR containing three binding sites for the cellular factor sp1. After incubation of labeled oligonucleotides, either unmodified or containing O6-MeG, with nuclear extracts obtained from different cell lines, gel retardation assays indicated that the presence of O6-MeG resulted in inhibition of binding of cellular factors to DNA sequences located in the promoter regions of genes. This inhibition was not the same for all modified oligonucleotides but dependent on the position in which O6-MeG was located. The results obtained indicate the alkylation of O6-guanine affects the binding of transcription factors and thereby possibly the regulation of genes expression.

INTRODUCTION

Alkylation of O6 of guanine in DNA is considered to play an important role in the mutagenic effects of methylating agents (1, 2) since it may cause mispair of guanine with thymine, thus leading to a G:C/A:T transition (3). In addition, methylation of O6-guanine is an important step in the cytotoxic effects of different antineoplastic alkylating agents such as methyltriazenes and nitrosoureas (4, 5). This is supported by the fact that the sensitivity of different cell lines to O6-guanine alkylating agents correlates with the amount of O6-Alkylguanine-DNA Alkyltransferase (AGT) (5), an enzyme that repairs the lesions at O6 position of guanine by removing the alkyl group and leaving unaltered the guanine in DNA (4, 6). Furthermore, cell lines resistant to chloroethylnitrosoureas and methyltriazenes contain higher levels of AGT than the sensitive, parental lines (7).

Little is known about the precise mechanism by which the lesion at O6-guanine leads to cytotoxicity. The alkylation and repair of O6-guanine was reported to be dependent on the sequence flanking the alkylated guanine in synthetic oligonucleotides (8). Recently the presence of O6-MeG in the SV40 origin of replication was shown to block the binding of the large T antigen (9,10).

These findings prompted us to investigate whether the presence of a methyl group in guanines located in specific sequence altered the binding of transcription factors to DNA. We selected two sequences known to contain the binding site of two widely distributed transcription factors (NFκB and sp1) (11,12) and one sequence present in the promoter of the c-fos protooncogene which binds a protein, SRF, responsible for serum and growth factor activation of this gene (13).

MATERIALS AND METHODS

Oligonucleotides

The DNA sequences utilized were:

– the region of the c-fos promoter from −329 to −290 (F) 5′-CCCCTTACACAGGATGTCCATATTAGGACATCTGCG-TCAG containing the binding site for the SRF.

– the region of the HIV LTR from −115 to −81 (E), 5′-GCTTGCTACAAGGGACTTTCCGCTGGGGACTTTCC-TGCA containing two binding sites for the transcription factor NFκB.

– the region of the HIV LTR from −83 to −43 (S), containing three binding sites for the cellular factor sp1, 5′-TTCCAGGAGGCGTGGCCTGGGCGGGACTGGGG-AGTGGCGT.

All these oligonucleotides were synthetized using a Beckman Sys-200 oligosynthetizer (Beckman, Palo Alto, CA).

O6-Methyl Guanosine CED™ Phosphoramidite was purchased from ABN (USA). The O6 modified base was inserted in different positions in all three oligonucleotides selected on the basis of available data obtained with methylation interference or mutational analysis (14–16) as specified in the Results section. The oligonucleotides were purified on 10% polyacrylamyde/7M urea gels.

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The presence of O6-MeG in the oligonucleotide was verified using an immuno slot-blot procedure essentially as described by Nehls et al. (17). Briefly, 2 μg of oligonucleotides were immobilized on nitrocellulose filters using a Minifold II apparatus (Schleicher & Schuell, Keene, N.H.). The filters were presoaked in 1 M ammonium acetate, soaked in 5×SSC (1×SSC is 15mM NaCl-1.5mM sodium citrate) and baked for two hours at 80°C. The filters were then treated for 60 min at room temperature with an excess of blocking solution (10% instant non-fat dry milk in phosphate buffered saline (PBS)) and incubated at room temperature for 90 min with a specific monoclonal antibody (anti-O6-MeG, a generous gift from Dr. J.Boyle, Paterson Institute for Cancer Research, Manchester, UK) diluted 1:100 in incubation solution (10% instant non-fat dry milk, 0.3% Tween 20 in PBS).

After several washes in 0.3% Tween 20 in PBS, the filters were reacted with mouse Ig ^{125}I labeled whole antibody (Amersham, UK, 200000 cpm/ml) for 90 min at room temperature. After several washes in 0.3% Tween 20 in PBS, the filters were dried and autoradiographed.

Cell lines

We utilized the following cell lines: a human T cell leukemia (Jurkat), a human erythroleukemia (K562), a human promonocytic leukemia (U937) and a murine T cell lymphoma (EL4). Nuclear extracts were obtained from logarithmically growing cells according to Dignam et al. (18). T cell derived cell lines were stimulated with 50 ng/ml Phorbol 12-myristate 13-acetate (PMA) and 1 μg/ml Phytohemagglutinin (PHA) for 12 hours before preparation of the extracts.

Electromobility shift assay

The bottom strand of each oligonucleotide was labeled at the 5'end with γ^{32}P-ATP and T4 kinase, then annealed with the complementary strand to give a double-strand fragment.

The labeled oligonucleotides (0.1−0.5 ng), either unmodified or containing O6-MeG, were incubated with nuclear extracts (2.5 or 5 μg) obtained from different cell lines in the presence of 3 μg of poly (dI-dC) for 30 min. Free DNA was separated from protein-bound DNA on 4.5% polyacrylamide gels with a Tris-boric acid−EDTA buffer system and autoradiographed (19).

RESULTS

Fig. 1 shows the immunoblots obtained by incubating oligonucleotides immobilized on nitrocellulose filters with monoclonal antibodies raised against O6-MeG. All the modified oligonucleotides reacted with the antibody, the intensity being proportional to the number of modified guanines present in the sequence. The unmodified oligonucleotides (F, S and E) only slightly cross-reacted with the antibody.

Incubation of the c-fos promoter fragment containing the Serum response element (SRE) with nuclear extracts from Jurkat cells resulted in the formation of a retarded band on polyacrylamide gels (Fig. 2, panel A). This retarded band was specific for the SRE since it was eliminated by a 50-fold molar excess of unlabeled homologous competitor (lane 13). The use of an excess of non-homologous DNA did not alter the binding (lane 14). The presence of O6-MeG completely inhibited the binding of SRF to XF1 and XF2, both containing two modified guanines in the binding site(see Fig.2). XF3, which contains only one O6-MeG in the SRE, showed strongly reduced binding, but the retarded band was still visible (lanes 8 and 9). These results were confirmed using nuclear extracts from K562 (panel B) and U937 (panel C).

Fig. 3 shows the results of incubating nuclear extracts with wild type and O6-MeG containing oligonucleotides derived from HIV LTR (E). This region contains two binding sites for NFκB.

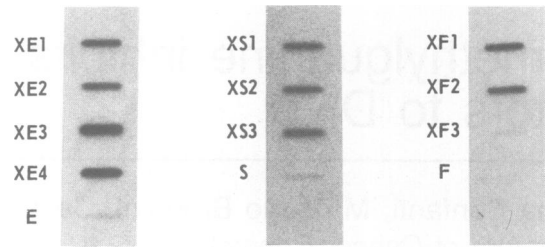

Fig. 1. Immuno-slot-blot of normal and O6-MeG modified oligonucleotides immobilized on nitrocellulose filters. The numbers XE1−XE4, XS1−XS3 and XF1−XF3, indicate oligonucleotides in which O6-MeG is located in different position. E,S and F indicate unmodified oligonucleotides. The sequence of the oligonucleotides utilized is shown in figures 2, 3 and 4.

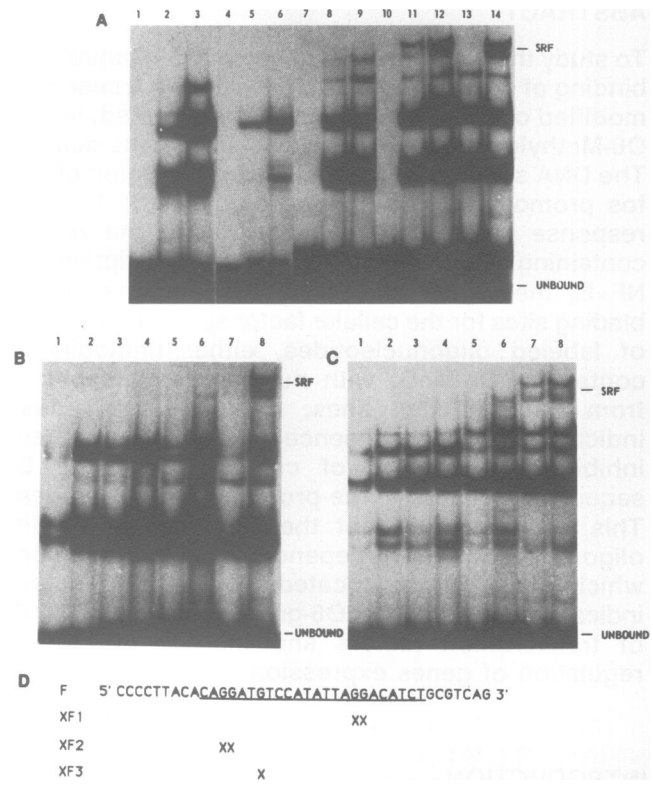

Fig. 2. Panel A. Binding of SRF from Jurkat nuclear extracts to a sequence of c-fos promoter containing the SRE element. 0, 2 or 4 μg of nuclear extracts were incubated respectively with XF1 (lanes 1,2,3), XF2 (lanes 4,5,6), XF3 (lanes 7,8,9) or F (lanes 10,11,12). The last two lanes show the reaction of 4 μg of nuclear extracts with F in the presence of a 50-fold molar excess of homologous (lane 13) or non-homologous (lane 14) competitor. Panel B. Binding of 2 or 4 μg of nuclear extracts from K562 to XF1 (lanes 1,2) XF2 (lanes 3,4) XF3 (lanes 5,6) and F (lanes 7,8). Panel C. Same as Panel B except that nuclear extracts were from U937 cells. Panel D. Sequence of oligonucleotides utilized. X indicates the position of the modified O6-MeG. The binding site for SRF is underlined.

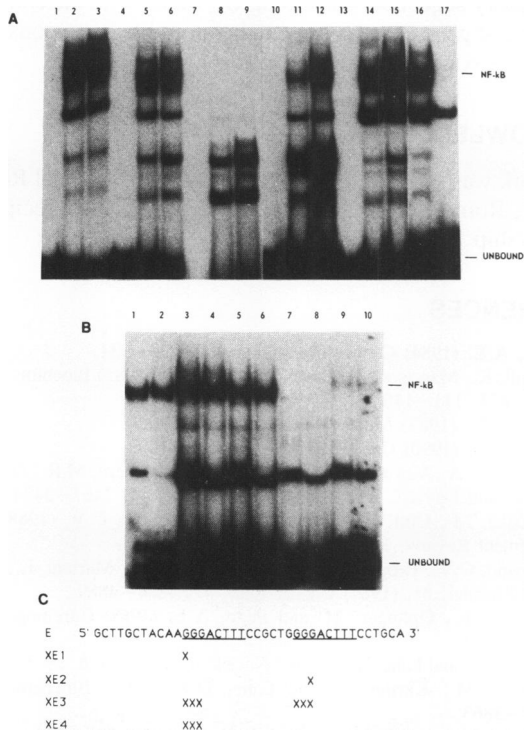

Fig. 3. Incubation of nuclear extracts with E oligonucleotides. Panel A. Binding of 0, 2 or 4 μg of Jurkat nuclear extracts respectively with XE1 (lanes 1,2,3), XE2 (lanes 4,5,6), XE3 (lanes 7,8,9), XE4 (lanes 10,11,12) or E (lanes 13,14,15). Lanes 16 and 17 show the incubation of 4 μg nuclear extracts with E in the presence respectively of an excess of non-homologous and homologous competitor. Panel B. Binding of 2 or 4 μg of nuclear extracts from EL4 cells with E (lanes 1,2) XE1 (lanes 3,4), XE2 (lanes 5,6), XE3 (lanes 7,8) or XE4 (lanes 9,10). Panel C. Sequence of the five oligonucleotides utilized. X indicates the position of the modified O6-MeG. The binding sites for NFκB are underlined.

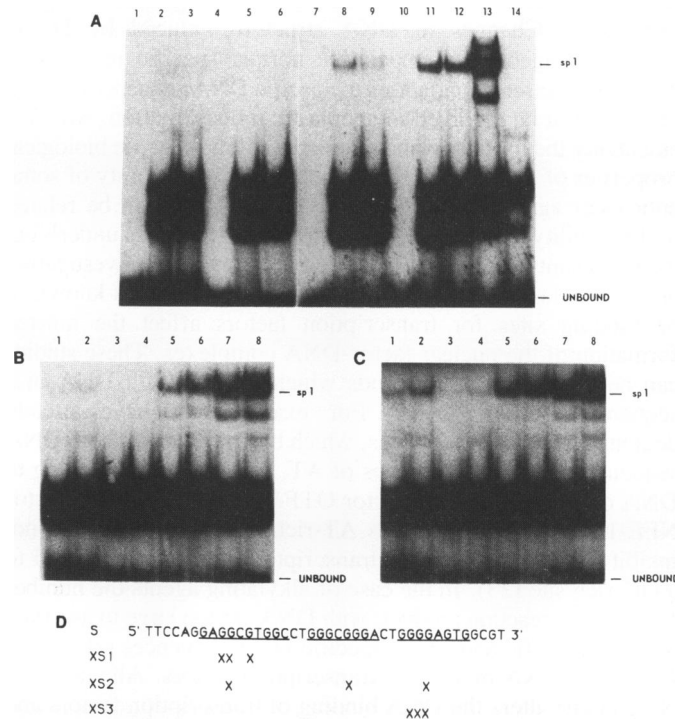

Fig. 4. Incubation of sp1 derived oligonucleotides with nuclear extracts. Panel A. Incubation of 0, 2 or 4 μg of Jurkat nuclear extracts respectively with XS1 (lanes 1,2 and 3), XS2 (lanes 4,5 and 6), XS3 (lanes 7,8 and 9) or S (lanes 10,11 and 12). The last two lanes show the incubation of 4 μg of nuclear extracts with S oligo in the presence of a 50-fold molar excess of non-homologous (lane 13) or homologous (lane 14) competitor. Panel B. Incubation of 2 or 4 μg of K562 nuclear extracts with XS1 (lane 1 and 2), XS2 (lanes 3 and 4), XS3 (lanes 5 and 6) or S (lanes 7 and 8). Panel C. Same as panel B but using nuclear extracts from U937 cells. Panel D. Sequence of oligonucleotides utilized. X indicates the position of O6-MeG. The binding sites for sp1 are underlined.

In this case we employed two different T cell derived cell lines which normally have NFκB in the cytoplasm; after stimulation with PMA and PHA, NFκB becomes available for the nucleus and in the gel retardation assay a typical retarded band was observable (20).

The effect of O6-MeG was studied by inserting one modified guanine in the first (XE1) or second (XE2) NFκB binding site. XE3 contains six O6-MeG, three in each binding site, while in the last (XE4) one NFκB binding site was completely modified, leaving the second unaltered.

Nuclear extracts from stimulated Jurkat cells bound to wild type E resulting in the formation of a complex (Fig. 3, panel A, lanes 14 and 15). The presence of an excess of unlabeled homologous competitor resulted in the loss of the specific retarded band (lane 17), while an excess of non-specific competitor had no such effect (lane 16).

O6-MeG inhibited the binding of NFκB only in XE3 (panel A, lanes 8 and 9) while XE4, which contains O6-MeG in only one binding site, showed a decrease in the intensity of the band (lanes 11 and 12). XE1 and XE2 had the same capacity as the wild type E to complex NFκB. Similar results were obtained using nuclear extracts from a murine T cell derived cell line (EL4) after stimulation with PMA and PHA (panel B).

The effects of O6-MeG on the binding of the cellular factor sp1 are shown in Fig. 4. We used part of the HIV LTR which contains three binding sites for sp1. The modified oligonucleotides contained one O6-MeG in each sp1 binding site (XS2), three O6-MeG in the first sp1 binding domain (XS1) and three O6-MeG in the third binding site (XS3).

The presence of one O6-MeG in all three sp1 binding sites completely inhibited the binding of sp1 contained in nuclear extracts from Jurkat cells (compare lanes 5 and 6 with lanes 11 and 12 in Fig. 4, panel A). Binding was markedly reduced with XS1 (lanes 2 and 3) but only slightly reduced with XS3 (lanes 8 and 9).

Panels B and C show the same results obtained with K562 and U937.

DISCUSSION

The regulation of gene transcription is mediated by the formation of stable complexes between DNA-binding proteins (trans-activating factors or transcription factors) and DNA sequences (cis-elements) in promoter and enhancer regions (12, 21). For some genes the binding of transcription factors governs the tissue specificity of gene expression, while for other genes it mediates the gene response to external stimuli (11, 13, 22). The regulatory sequences of genes contain the binding sites for transcription factors, some of which are general (i.e. they activate transcription of all or several genes), while others are specific for a given gene. Hypothetically, any alteration in the binding of transcription

factors to DNA could result in abnormal regulation of gene expression. Changes in DNA structure induced by DNA-interacting agents can impair the normal specific recognition pattern of transcription factors to specific DNA sequences. These effects could be involved in neoplastic transformation, which is essentially the loss of normal regulation of the specific biological properties of a given cell. On the other hand the ability of some anticancer agents to block tumor cell growth might be related to their ability to affect the transcription of the genes underlying the malignant behaviour . Therefore it seems worth investigating how DNA lesions caused by chemicals in sequences known to be binding sites for transcription factors affect the normal formation of the nuclear factor-DNA complexes. These studies can be done using compounds which interact with DNA in a sequence-specific manner. For example we have already demonstrated that distamycins, which bind preferentially to DNA sequences containing streches of AT, can inhibit the binding to DNA of octamer binding factor OTF-1 and the erythroid factor NFE-1 whose binding site is AT-rich; the same drugs did not inhibit the binding of other transcription factors which bind to a GC-rich site (23). In the case of alkylating agents the number and types of reaction products with DNA are too large to ascertain whether specific adducts in specific DNA sequences selectively impair the DNA binding of transcription factors. Alkylation of N7-guanine alters the DNA binding of transcription factors and is in fact the basis of methylation interference studies (19). The present study shows that the alkylation of O6-guanine too can block the DNA binding of cellular factors such as NF$\varkappa$B, sp 1 and SRF. These data are in keeping with the previous report that O6-MeG inhibits the binding of SV 40 large T antigen (9, 10).

To our knowledge this is the first evidence that the binding of cellular factors to DNA sequences located in the promoter regions of genes can be altered by the alkylation of O6-guanine. This phenomenom appears generalizable to all cell lines investigated such as T cell derived human and rodent leukemias, human erytroleukemia and promonocytic leukemia. It is interesting that not all the substitutions of guanine in the binding site with O6-MeG resulted in inhibition of binding but only specific modified guanines (for example in the sp1 oligonucleotide the presence of O6-MeG in the first binding site (XS1) has much more effect in inhibiting the binding of sp1 than when is present in the third binding site (XS3)).

Some studies have appeared on the sequence-specificity of N7 alkylation for a number of compounds (24). It appears that N7-guanine alkylation is not a random phenomenon, but depends on the physicochemical properties of the compound (i.e. charge, size and three-dimensional structure) and on the base composition of the sequence preceeding and following the alkylated guanine (24). Some initial results have been reported for O6-guanine alkylation using synthetic oligonucleotides containing O6-guanine in different positions (8). The information available is still very limited and new methods are needed to measure the pattern of sequence-specificity of O6-guanine alkylation in fragments of DNA treated with alkylating agents. This information is of particularly interest in light of the observation shown in the present paper, that alkylation of O6-guanine affects the binding of transcription factors and thus presumably the regulation of gene expression. Unless there is a prolonged exposure to chemicals which alkylate guanine-O6, or there is a repair deficiency, it is expected that this effect is present for a short time, i.e. before that O6-MeG is repaired, and is not inherited by daughter cells. Therefore the inhibition of the binding of transcription factors to regulatory sequences containing O6-MeG, with the consequent alteration of gene expression, can be more likely responsible for acute cytotoxic effects of alkylating agents.

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