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Effects of 8-oxo-7,8-dihydro-2′-deoxyguanosine on the Binding of the Transcription Factor Sp1 to its Cognate Target DNA Sequence (GC box)

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Emphasis was placed in this work on the assessment of the role of guanine bases in the interaction of transcription factor Sp1 with its cognate DNA sequence. For this purpose, each guanine residue of the 5′-GGGGCG-GGG-3′ (GC box) target DNA sequence was substituted in turn by 8-oxo-7,8-dihydro-2′-deoxyguanosine. The latter oxidized nucleotide which is likely to be present in mammalian DNA and exhibit mutogenic features is expected to be involved in age-related diseases and cancer. The effect of the incorporation of 8-oxodGuo into DNA on the binding of transcription factor Sp1 was studied using electrophoretic mobility shift assays with nuclear extracts from HeLa cells. When guanines at position G′2, G′3, G′4, G′5 and G′6 were replaced with 8-oxodGuo, binding of Sp1 was only 28%, 30%, 7%, 5% and 21%, respectively, to that of the non-substituted oligonucleotide. The binding is less affected when guanines at position G′1, G′7, G′8 and G′9 were substituted by 8-oxodGuo. Results show up the importance of the core of the GC box and the stronger contribution of the second and the third zinc finger to the binding with DNA. All together, this suggests that incorporation of 8-oxodGuo may alter the expression of the gene regulated by Sp1 and affect the response of the cell.

Keywords: Electromobility shift assay, 8-oxodGuo, transcription factor Sp1, GC box

Abbreviations: 8-OxodGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine; DTT, dithiothreitol; EMSA, electromobility shift assay; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethylsulfonylfluoride; Temed, *N,N,N′,N′*-tetramethyl-ethylenediamide; Tris-HCl, Tris(hydroxymethyl)-aminomethane hydrochloride

INTRODUCTION

Reactive oxygen species which are formed *in vivo* upon exposure to oxidizing agents,[1] ionizing radiation[2] or during cellular oxidative metabolism[3] have various biological targets. These include proteins,[4] lipids[5] and DNA.[6] In particular, single or double DNA strand breaks, protein–DNA cross-links, apurinic/apyrimidic sites, modified bases and sugar lesions represent

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the main classes of oxidative damage to DNA.[7,8] The latter modifications are likely to be involved in aging,[9] mutagenesis,[10,11] carcinogenesis[12] or to alter replication and gene expression.[13,14]

The regulation of gene expression is dependent upon the recognition of specific DNA sequences by their cognate transcriptional regulatory proteins. Recognition of target DNA sequences is based on the ability for the protein to 'read' local DNA topography.[15] The transcription factor Sp1[16,17] is a sequence specific DNA binding protein that binds to proximal promoter sequences of various cellular and viral genes. In addition, it activates the transcription of the latter genes by RNA polymerase II.[18] The DNA binding domain is located at the C-terminus of the protein. It contains three contiguous repeats of classical Cys_2–His_2 type of zinc finger motif and it was found to interact with the consensus sequence 5′-GGGGCGGGG-3′ (GC box). It was recently reported that the ability for Sp1 to bind its cognate DNA sequence may be modified by changes in the redox status of the cell.[19,20] In this respect, the zinc finger in an oxidized form can abolish the fixation of the transition metal.[21,22] Moreover, several studies revealed the importance of each of the amino acids present in the zinc finger domain and provided information on the interactions between amino acid residues of Sp1 protein and DNA base pairs.[23] A change of one parameter induces a decrease in the DNA-binding efficiency of Sp1.[24–27] It could lead to a decrease in the expression of the genes down-regulated by Sp1.

8-Oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) that results from the addition of $OH^{\bullet}$ at the C8 position of 2′-deoxyguanosine (dGuo) is one of the 20 main oxidative base lesions to DNA. 8-OxodGuo which is present in mammalian DNA is considered as a biomarker of *in vivo* oxidative DNA damage.[28,29] 8-OxodGuo is mutagenic[30,31] and attempts were made to correlate its level in either DNA or urine and the incidence of cancer and other age-related degenerative diseases.[32] Furthermore, the presence of a lesion such as 8-oxodGuo into the GC box may alter the global DNA conformation. As a result, this may affect the affinity of the GC box for the transcription factor Sp1.[33]

In order to investigate the ability for 8-oxodGuo to affect the interactions between the protein Sp1 and DNA, we have synthesized oligonucleotides that exhibit 8-oxodGuo modifications in the Sp1 consensus sequence. The substitution of dGuo by 8-oxodGuo was made at various nucleotide positions. A G-substituted variant (5′-GGGGGGG-GG-3′) of the GC box which binds 30-fold more weakly than the consensus site was also used.[34] The ability for the transcription factor Sp1 to bind to the 8-oxodGuo containing oligonucleotides was assessed by electromobility shift assay (EMSA)[35] using either purified Sp1 or crude extracts of HeLa cells. The composition of the binding buffer, especially the concentration of poly(dI-dC)·poly(dI-dC) and dithiothreitol (DTT), was optimized. The aim was to study the effect of the 8-oxodGuo substitution at each of the dGuo sites of the GC box on the binding of Sp1 to its cognate DNA sequence.

MATERIALS AND METHODS

Chemicals

All chemicals used for the oligonucleotide synthesis with the phosphoramidite method were obtained from Applied Biosystems (Foster City, CA). Acrylamide, bis-acrylamide, ammonium persulfate, urea and *N,N,N′,N′*-tetramethylethylenediamide (Temed) were purchased from SERVA (Heidelberg, Germany). Boric acid and tris(hydroxymethyl)aminomethane (Tris) were obtained from Merck (Darmstadt, Germany), whereas EDTA was from Sigma (St. Louis, MO). Triethylamine and acetonitrile were obtained from Prolabo (Fontenay-Qux-Roses, France). Nuclease P1 (*Penicillium citrium*) and alkaline phosphatase were purchased from Boehringer-Mannheim (Mannheim, Germany). Poly(dI-dC)·poly(dI-dC) came from Pharmacia (Uppsala, Sweden). Buffers for high performance liquid chromatography (HPLC) were prepared using

water obtained from a Milli-Q system (Millipore, Milford, MA). Optimem buffer, phosphate buffer saline (PBS), foetal calf serum (FCS) and trypsin used for cell culture were obtained from GibcoBRL (Life Technology, Paris, France). Other chemicals were of the best grade commercially available.

Preparation and Extraction of Crude Nuclear Extracts

Crude nuclear extracts from HeLa cells were prepared essentially according to the protocol of Dignam *et al.*[36] Briefly, subconfluent HeLa cells (2×10^7 in 75 ml cell culture flasks) growing in Optimem buffer complemented with 4% FCS were washed three times with 7 ml of PBS without calcium and magnesium. Then, the cells were harvested with 3 ml of trypsin (1/10 in PBS) prior to be pelleted by centrifugation at 2000*g* for 2 min at 4°C. The pellet was washed with 10 ml of cold PBS and the cells were suspended in 500 µl of cold hypotonic swelling buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF). The solution was centrifuged at 2000*g* for 2 min at 4°C. Then, the pellet was suspended in 1 ml of cold (4°C) hypotonic swelling buffer and put on ice for 15 min after homogenization. Then, 100 µl of Nonidet P40 was added and the resulting mixture was homogenized for 10 s and immediately centrifuged for 30 s at 14000*g* at 4°C. The cytoplasm was removed and the cellular pellet was suspended in 40 µl of cold hypertonic buffer (10 mM HEPES, pH 7.9, 0.1 mM EDTA, 50 mM KCl, 300 mM NaCl, 10% glycerol, 0.5 mM DTT, 0.2 mM PMSF). The nuclei were rocked on ice for 30 min and then centrifuged at 14000*g* for 10 min at 4°C. The supernatant was divided into aliquots and stored at −80°C until use. The protein content of the nuclear extract was determined using a protein assay kit (Pierce, Rockford, IL).

Synthesis of Oligonucleotides

In order to evaluate the individual contribution of the nine dGuo residues of the GC box to the Sp1 binding, a series of 13 oligonucleotide probes were synthesized (Table I): These include the

TABLE I Sequences and name of duplex oligonucleotides used for Sp1 electromobility shift assay. X indicates the substitution of 2′-deoxyguanosine by 8-oxodGuo

Oligomer duplex	Sequence
G	5′-ACGTATTCGATC**GGGGCGGGG**CGAGC-3′
	3′-TAAGCTAG**CCCCGCCCCG**CTCGTGCA-5′
G′1	5′-ACGTATTCGATC**X**GGGCGGGGCGAGC-3′
	3′-TAAGCTAGCCCCGCCCCGCTCGTGCA-5′
G′2	5′-ACGTATTCGATC**GX**GGCGGGGCGAGC-3′
	3′-TAAGCTAGCCCCGCCCCGCTCGTGCA-5′
G′3	5′-ACGTATTCGATC**GGX**GCGGGGCGAGC-3′
	3′-TAAGCTAGCCCCGCCCCGCTCGTGCA-5′
G′4	5′-ACGTATTATC**GGGX**CGGGGCGAGC-3′
	3′-TAAGCTAGCCCCGCCCCGCTCGTGCA-5′
G′5	5′-ACGTATTCGATC**GGGGCGGGG**CGAGC-3′
	3′-TAAGCTAGCCCC**X**CCCCGCTCGTGCA-5′
G′6	5′-ACGTATTCGATC**GGGGCXGGG**CGAGC-3′
	3′-TAAGCTAGCCCCGCCCCGCTCGTGCA-5′
G′7	5′-ACGTATTCGATC**GGGGCGXGG**CGAGC-3′
	3′-TAAGCTAGCCCCGCCCCGCTCGTGCA-5′
G′8	5′-ACGTATTCGATC**GGGGCGGXG**CGAGC-3′
	3′-TAAGCTAGCCCCGCCCCGCTCGTGCA-5′
G′9	5′-ACGTATTCGATC**GGGGCGGGX**CGAGC-3′
	3′-TAAGCTAGCCCCGCCCCGCTCGTGCA-5′
T	5′-ACGTATTCGATC**GGGGGGGGG**CGAGC-3′
	3′-TAAGCTAGCCCCCCCCCGCTCGTGCA-5′

9 different probes (G'1 → G'9) that contained a unique 8-oxodGuo at each of the nine dGuo positions (8 in the G rich strand and 1 in the C rich strand) in the consensus site, the normal non-substituted probe (G), the G-substituted variant (T), and the corresponding complementary sequences. The G-substituted probe, namely T, was dedicated to the optimization of the experimental conditions for EMSA.

The oligonucleotides were synthesized on an Applied Biosystems 392 RNA/DNA synthesizer using 1 μmol of solid support controlled pore glass (CPG). Oligonucleotides were synthesized "trityl on" for HPLC purification and "trityl off" for PAGE purification. The oligonucleotides were removed from the CPG by incubation in 2 ml of a 33% ammonia solution for 24 h at 55°C. To avoid oxidation of 8-oxodGuo, a 0.25 M β-mercaptoethanol solution (40 μl) was added.[37] The support was removed by centrifugation and the supernatant was evaporated under reduced pressure using a Speed-vac apparatus. Then, the solid residue was dissolved in 300 μl of distilled water for subsequent purification on polyacrylamide gel electrophoresis (PAGE). An alternative method of purification involved reverse phase HPLC after the oligonucleotides were dissolved into 2 ml of 25 mM triethylammonium acetate (TEAA).

Oligonucleotides Purification

Purification on PAGE

Synthesized oligonucleotides were purified on a 20% polyacrylamide gel (acrylamide/bisacrylamide: 19/1) under denaturing conditions with 8 M urea. The oligonucleotides were detected by UV shadowing. The related zones were cut out and subsequently eluted in 5 ml of 0.05 M ammonium bicarbonate overnight at 37°C. Then, purified oligonucleotides were desalted on NAP-25 column (Pharmacia Biotech, Uppsala, Sweden). The concentration of the oligonucleotides was determined by measuring their absorbance at 260 nm.

HPLC Purification

A fraction of the oligonucleotide solution was injected onto a HPLC column (Hamilton, 7 μm, 7.0 × 300 mm) and eluted with a linear gradient of acetonitrile (0–13% in 25 mM TEAA buffer, pH = 7). The resulting solution was lyophilized and desalted on a NAP-25 column.

Characterization of the Oligonucleotides

Determination of the Purity

The analysis was achieved on an analytical acrylamide gel, after 5'-end ^{32}P labeling of the oligonucleotides. Labeling was performed by adding 1 μl of the oligonucleotide solution (30 μg/μl) and 0.1 μl of [γ-^{32}P] ATP (3000 Ci/mmol, Amersham, Buckinghamshire, UK) to 10 μl of an aqueous solution of 10 mM Tris-acetate, 10 mM magnesium acetate and 50 mM potassium acetate that contained 2 U of T4 polynucleotide kinase (Pharmacia Biotech). The resulting mixture was incubated for 30 min at 37°C and the reaction was stopped by the addition of 2 μl of 0.5 M EDTA, pH = 8.0. Then, 3 μl of a blue dye solution was added and 6 μl of the resulting mixture was loaded onto 20% acrylamide gel (acrylamide–bisacrylamide: 19/1). The yield of synthesis varied between 1.9% and 7.8% for the purification on PAGE and 12.8% and 20% for the purification by HPLC. Purification of the probes on PAGE was more efficient than by HPLC. In the latter case, shorter sequence contamination was still in the target oligonucleotides. Purification on PAGE was the best way to obtain high purity oligonucleotides required for the molecular biology experiments reported in the present study.

Analysis of the Nucleotide Composition of Unmodified and Modified Oligonucleotides

To 15 μg of the 26-mer oligonucleotides suspended in 0.5 ml of distilled water was added 50 μl of 10 × 0.3 M sodium acetate buffer + 1 M

$ZnCl_2$ (pH = 5.3), and 10 µl of nuclease P1 (3 U.I.). The digestion reaction was carried out for 30 min at 37°C, leading to a solution of nucleoside 5′-monophosphates. The pH was adjusted at 8.2 with 55 µl of 1 M Tris-HCl, 1 M $MgCl_2$ and 1 M $ZnC1_2$ aqueous buffer. Then, 2 µl of alkaline phosphatase (*8 U.I.*) was added and the reaction was run for 2 h at 37°C. The nucleoside solution was analyzed by HPLC (Hypersil C18 column 5 µm, 4–6 × 250 mm, Interchim, Montlugon, France) with a linear gradient of acetonitrile (0–7% acetonitrile in 25 mM, TEAA buffer, pH = 7) over 40 min. The relative content of constituent nucleosides was calculated by dividing the integrated HPLC peak area corresponding to each nucleoside by the related extinction coefficient at 260 nm (data not shown).

Further strucural information on the oligonucleotides was inferred from electrospray ionization mass spectrometric analysis in the negative mode (Micromass Platform 3000, Manchester, UK). Typically, the analysis of the mixture was performed with 3 µg of oligonucleotide dissolved in water/acetonitrile (50/50, v/v) that contained 1% triethylamine. The measured molecular mass of the 8-oxodGuo containing oligonucleotides (MW = 8109) was in agreement with the results obtained by exonuclease digestion (*vide supra*) (data not shown).

Preparation of Radiolabeled Probes

Complementary oligonucleotides were annealed as follows: 500 pmol of each oligonucleotide was mixed in 10 µl of TNE buffer (10 mM Tris-HCl, 10 mM NaCl, 0.5 M EDTA, pH = 8). The annealing reaction mixture was incubated at 75°C for 15 min and then gradually cooled to 25°C. Double-stranded oligonucleotides were ^{32}P end-labeled in a reaction mixture consisting of 100 pg of double-stranded oligonucleotides, 50 mM Tris-HCl, 10 mM $MgCl_2$, 0.25 mM dGTP, 0.25 mM dTTP, [α-^{32}P]dATP, [α-^{32}P]dCTP (3000 Ci/mmol) and 2 U of Klenow enzyme in a total volume of 10 µl. Non-incorporated radiolabeled nucleotides were removed using Sephadex G-25 spin columns (Boehringer-Mannheim, Germany). The specific activity of the radiolabeled probes ranged from 2.5×10^7 to 3.1×10^7 cpm/ng. The radiolabeled oligonucleotides were diluted 15-fold with TNE buffer just before use.

Sp1 Electrophoretic Mobility Shift Assays

It has been shown that the DNA-binding efficiency of transcription factor Sp1 is affected by redox changes that are conferred by the redox-susceptibility of the thiol groups of the zinc finger. Then, it was necessary to determine the concentration in a reducing agent such as DTT which allowed the maximal DNA-binding efficiency.

The influence of the addition of poly(dI-dC) · poly(dI-dC) on the binding of either purified Sp1 or Sp1 in the crude extracts, to its cognate sequence was studied. Typically, the experiments with purified Sp1 were performed using 1 µg of BSA (Bovine Serum Albumin) in the reaction mixture that contained 1 mM DTT but no poly(dI- dC) · poly(dI-dC).

The binding reaction consisted of either 6–8 µg of HeLa cell nuclear extracts or 10 ng of purified Sp1 dissolved in 1 µl of 10× binding buffer (200 mM HEPES, pH = 7.9, 75 mM NaCl, 1 mM EDTA, 0.5 mM $MgCl_2$, 1 mM DTT and 50% glycerol). The final volume of reaction mixture was 10 µl and contained also 1.3 µg of poly(dI-dC) · poly(dI-dC), 1.0 µg of BSA and 0.2 pg of the ^{32}P labeled probe. After 15 min of reaction at room temperature, 2.5 µl of 50% glycerol–0.5% bromophenol blue was added to facilitate gel loading. The binding complex was separated on a 4% non-denaturing polyacrylamide gel (acrylamide/bisacrylamide: 29/1) and electrophoresed in 0.25× TBE buffer (0.25 M Tris, 0.25 M $Na_2B_4O_7$, 0.5 mM EDTA, pH = 8.3.) for 3 h at 180 V. The specificity of the complex was checked using 10 ng of anti-Sp1 antibody (PEP, Santa Cruz Biotechnology, CA) which forms a new larger DNA–Sp1–antibody complex. The antibody was incubated with the protein 15 min prior to the addition of the probe.

Following electrophoresis, the gel was dried under vacuum and exposed either to a X-ray film or a phosphorimaging plate. The complexes were quantified by scanning within a Molecular Dynamics Phosphorimager (Molecular Dynamics, Sunnyvale, CA). For competition studies, cold competitor was added to the binding reaction 10 min prior to the addition of the ^{32}P-labeled probe. The concentration of the cold competitor was comprised between 1.5×10^{-11} and 6.2×10^{-10} M.

RESULTS

Characterization of Sp1–DNA Duplex

The reaction conditions were optimized using the G and T probes (Figure 1). The specificity of the Sp1–DNA duplexes was checked using two methods. First, the addition of anti-Sp1 antibody induced the disappearance of Sp1–DNA specific duplex (lines e and e′) and the formation of antibody–Sp1–DNA triplex which reduces the mobility of the complex. On the other hand, the unlabeled probe was used as a cold competitor. An increase in the concentration of the latter probe in the binding mixture led to a decrease in the Sp1–DNA specific duplex (lines b, c, d and b′, c′, d′).

The present results are in agreement with those obtained by Letovsky and Dynan.[34] In the latter work, a drop in the affinity of Sp1 for the G-substituted variant compared to the normal GC box was observed using a footprinting assay. The calculated affinity of the T probe duplex for Sp1, measured by integration of the band intensity, was 20-fold weaker than for the G probe duplex (lines b and b′).

Influence of DTT Concentration

In the concentration range tested [0–50 mM], it was observed an increase in the DNA-binding efficiency of Sp1 up to 1 mM DTT and then a

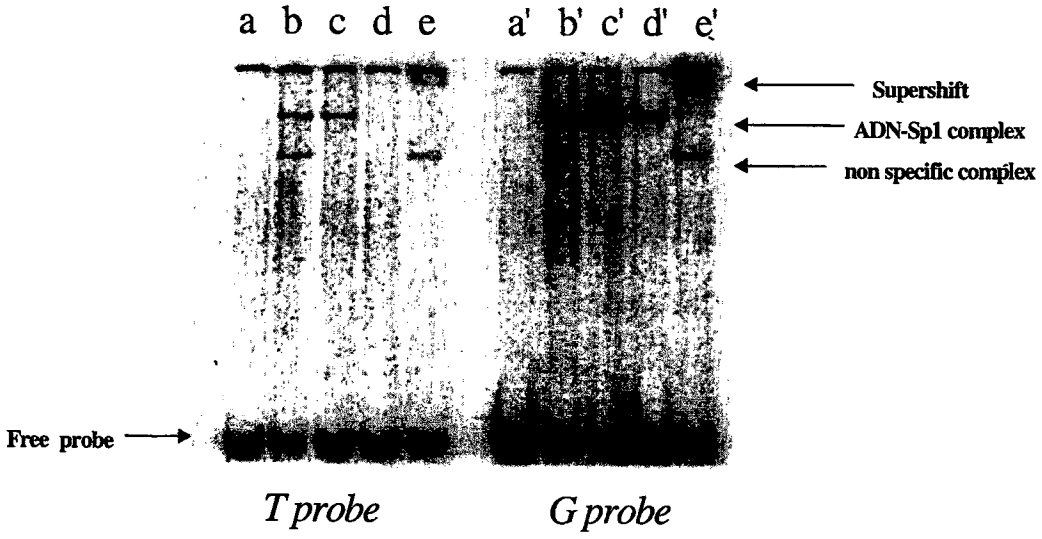

FIGURE 1 Electrophoretic Mobility Shift Assay (EMSA). Characterization of Sp1–DNA duplex using the T (G substituted variant) and the G normal probes. The experiments were performed with 2.5 pg of labeled probe and 10 ng of purified Sp1: a,a′: free labeled probe. b,b′: labeled probe + Sp1. c,c′: labeled probe + purified Sp1 + 0.1 ng of non-labeled probe. d,d′: labeled probe + Sp1 + 20 ng of non-labeled probe. e,e′: labeled probe + Sp1 + 10 ng of anti-Sp1 antibody.

decrease for higher concentrations. Similar results were obtained using nuclear extracts (data not shown). For concentrations lower than 1 mM DTT, the cysteine residues are oxidized and are not able to participate in the binding of zinc. The zinc finger domain is unfold and, thus, this leads to a decrease in the affinity binding. Higher DTT concentrations may affect other cysteines involved in disulfide bridges, which are responsible for the refolding of the protein.

Influence of Poly(dI-dC) · poly(dI-dC) Concentration

The procedures used for investigating DNA–protein interactions, such as EMSA, require the addition of exogenous or synthetic nucleic acids to the reaction mixture in order to either prevent or reduce the non-specific interactions of nuclear proteins with the specific probe. The most commonly selected competitor remains the synthetic polymer poly(dI-dC) · poly(dI-dC). Its usefulness is unquestionable when crude or nonenriched extracts of nuclear proteins are used in EMSA.[38] However, evidence was provided that poly(dI-dC) · poly(dI-dC) impairs or totally prevents the formation of specific DNA–Sp1 complex when purified Sp1 was used (data not shown). The decrease in the specific interactions was marked for variants of low affinity for Sp1 such as the T probe. In the presence of poly(dI-dC) · poly(dI-dC), Sp1 was found to bind 80-fold less tightly to the T probe than to the consensus probe and only 20-fold less if no poly(dI-dC) · poly(dI-dC) was present. Footprinting experiments[34] had also revealed an affinity of the T probe for Sp1, 30-fold weaker than shown by the control probe. This indicates that poly(dI-dC) · poly(dI-dC) may interfere with the specific formation of the DNA–protein complex leading to a false estimation of the binding affinity of the variant sequences. The result is in agreement with a previous study involving purified Sp1.[39] Thus, a 33% drop in the formation of Sp1–DNA complex was found when 0.1 μg of poly(dI-dC) · poly(dI-dC) was present by comparison to in the absence of competitor. The finding is not very surprising since the competitor is in large excess with respect to the amount of the labeled probe (1.3 μg and 2.5 pg respectively).

Gel Shift Analysis of the Binding of Purified Sp1 to Non-modified and 8-oxodGuo-substituted Duplex DNA

The oligonucleotides corresponding to the G rich and the C rich strands of the Sp1 consensus sequence that were substituted by 8-oxodGuo at the indicated dGuo positions are shown in Figure 2A. The binding of Sp1 to the non-substituted oligonucleotide duplex DNA is shown in Figure 2C in the lane marked G. This value was assumed to represent 100% binding efficiency. Substitution of dGuo by 8-oxodGuo at positions G′1, G′7, G′8 and G′9 respectively, resulted in a slight decrease in the binding of the related oligonucleotides to Sp1. This was estimated to be 72%, 53%, 94% and 58% respectively compared to the control oligonucleotide duplex. On the other hand, when 8-oxodGuo replaced dGuo at positions G′2, G′3, G′4, G′5 and G′6, binding to Sp1 was only 28%, 30%, 18%, 7%, and 21% respectively. Thus, the substitution of dGuo by 8-oxodGuo at positions which are critical for the binding Sp1, led to profound decreases in the affinity. In contrast, the binding of Sp1 is slightly affected when 8-oxodGuo is present at positions G′1, G′7, G′8 and G′9. In this respect, it was suggested that some guanines of the GC box participate more strongly than others to the DNA–protein interactions.[40]

Gel Shift Analysis of Cellular Nuclear Extracts Binding to Non-modified and 8-oxodGuo-substituted Duplex DNA

In a similar way, the affinity of the different Sp1 variants was tested upon incubation of the targeted oligonucleotides with HeLa cell nuclear extracts. It was also found that the substituted bases corresponding to the positions in contact

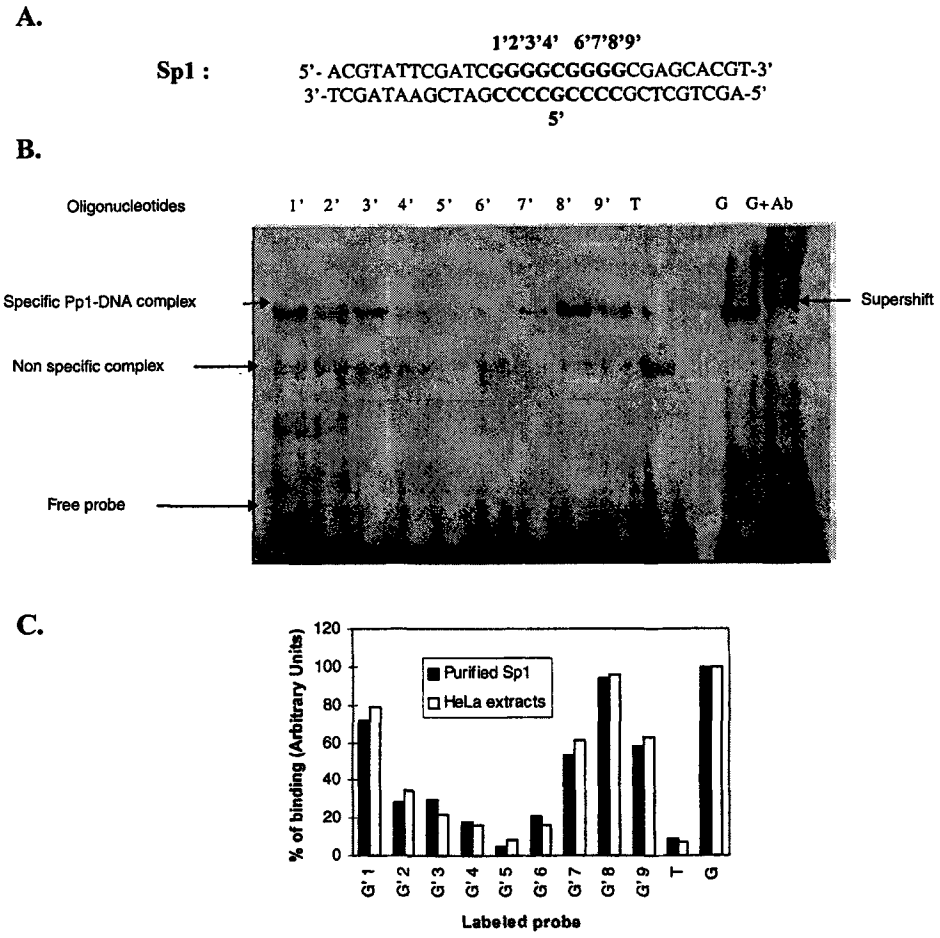

FIGURE 2 The effect of incorporation of 8-oxodGuo on binding of the either purified transcription factor Sp1 or extracts from HeLa cell, to their target DNA sequence. (A) Sequence of the DNA probe containing the consensus Sp1 binding site used in this study. The guanine nucleosides substituted by 8-oxodGuo are indicated (G'1→G'9). (B) Binding of Sp1 extracted from HeLa cells was assayed using a non-substituted consensus Sp1 (G), 8-oxodGuo-substituted probes (G'1→G'9) and the G-substituted variant (T), analyzed by gel elecrophoresis and exposed to phosphorimaging plates as described in Materials and Methods. (C) Percentage of binding of the different probes was calculated with the Phosphorimager: The extent of Sp1 binding to the consensus Sp1 probe was set to 100%. The extent of Sp1 binding to each probe was normalized to the specific activity of that particular probe. Each value represents the average of three independent experiments. Standard deviations between each value are not significant.

with the second and the third zinc fingers of Sp1, appeared to be critical for the interaction between the transcription factor Sp1 and DNA (Figure 2B and C). The binding affinity of Sp1 decreased strongly when the positions G'2, G'3, G'4, G'5 and G'6 of the oligonucleotides were substituted by 8-oxodGuo. Thus, the relative affinity was 35%, 22%, 14%, 8% and 16% respectively. The substitutions at the other positions did not significantly affect the binding rate. Interestingly, it was found that the position G'7 of the GC box is likely to play an important role in the interaction between the DNA and the zinc finger domain of the protein. This was inferred from the 50% decrease in the affinity when the 7'-guanine was oxidized. As observed with the purified Sp1, only a slight decrease in the binding was observed upon substitution of positions G'1, G'8 and G'9.

Binding of Sp1 to Non-modified and 8-oxodGuo-substituted Sequences in the Presence of Cold Competitor DNA

To confirm the difference of affinity between the non-substituted and substituted probes, competition reactions were carried out using non-substituted sequences as cold competitor DNAs (G probe). Sp1 binding to the different 8-oxodGuo-substituted sequences was assayed in the presence of increasing amounts (0–40-fold molar excess) of cold competitor DNAs (Figure 3A). The extent of binding, as indicated by the specific complex formation, was quantified using the

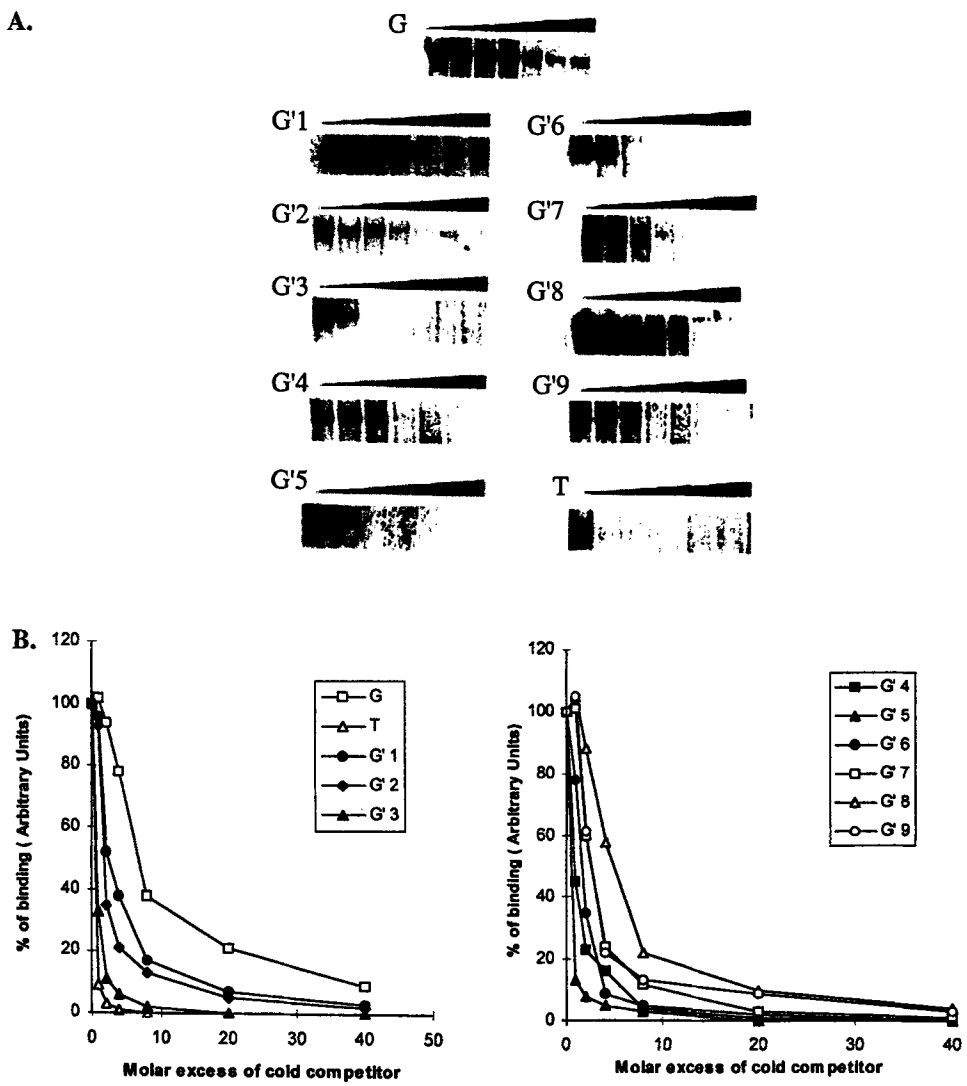

FIGURE 3 Competition reactions. The binding of Sp1 to the different 8-oxodGuo-substituted sequences was assayed in the presence of increasing amounts of unsubstituted target sequence as cold competitors (0, 1, 2, 4, 8, 20 and 40-fold molar excess), as described in Materials and Methods. The autoradiograms (A) were scanned and the extent of Sp1 binding was calculated and plotted (B). Each point represents the average of three independent experiments. Standard deviation between each value are not significant. The extent of Sp1 binding to modified and non-modified probe was set to 100% in the absence of cold competitors.

TABLE II Competition studies with non-substituted probes as cold competitors

Labeled probe[a]	IC90[b]	Relative affinity[c]
G	36.8	1
G′1	17.1	0.46
G′2	12.5	0.34
G′3	2.3	0.06
G′4	3.8	0.1
G′5	1.9	0.05
G′6	3.6	0.1
G′7	8.6	0.23
G′8	34.2	0.92
G′9	14.3	0.38
T	0.9	0.025

[a] For location of each 8-oxodGuo, see Figure 2A.
[b] Amount of cold competitor (in fold molar excess) required to reduce Sp1 binding by 90%.
[c] Relative affinity calculated by dividing the IC90 of the Sp1 probe (36.8) into the IC90 of each probe containing an 8-oxodGuo substitution.

phosphorimager (Figure 3B). In addition, the relative affinity of each sequence was calculated by applying the method of Reed and Muench.[41] The amount of cold competitor required to reduce the binding of Sp1 by 90% was determined. Furthermore, the relative affinity was calculated by dividing the IC90 of the Sp1 probe into the IC90 of each probe that contained a 8-oxodGuo residue (Table II). As shown in Figure 3A, with the exception of G′8, the different 8-oxodGuo-substituted sequences were less able to compete with the non-substituted sequence as cold competitor for binding to Sp1, G′3, G′4, G′5 and G′6 positions displayed the least relative affinity for binding Sp1, 0.06, 0.1, 0.05 and 0.1 respectively. Sequences substituted in positions, G′1, G′2, G′7 and G′9 displayed intermediate affinities for binding Sp1 and substitution of guanine with 8-oxodGuo in position G′8 did not affect the affinity of the substituted oligonucleotide for Sp1.

DISCUSSION

DNA represents a major cellular target to reactive oxygen species. In particular, oxidation of critical gene sequences may affect transcription. The transcription factor Sp1 and its DNA target sequence (GC box) in which each of the dGuo sites was substituted in turn by a 8-oxodGuo residue, were used as model systems. The aim of this approach was to evaluate the contribution of each dGuo position to the binding affinity of Sp1 transcription factor.

Binding of Sp1 transcription factor to the non-modified and 8-oxodGuo-substituted DNA probes respectively was evaluated by gel shift analysis. The results indicated a decrease in the binding when each of the guanine bases of the GC box was oxidized into 8-oxodGuo. The drop was found to be position dependent, confirming[40,42] that the guanine bases are not equivalent in the DNA–protein interactions. In fact, positions G′2, G′3, G′4, G′5 and G′6 appeared to be more critical compared with positions G′1, G′7, G′8 and G′9. Two hypotheses may be considered: either the incorporation of 8-oxodGuo led to conformational changes in the DNA duplex, or oxidation of guanine bases modified the interactions between the DNA sequence and the zinc finger domains of Sp1. Previous studies have shown that conversion of dGuo into 8-oxodGuo does not alter the global DNA duplex conformation[43] since the C8 position does not participate in G–C interactions. The decrease in Sp1 binding could thus be caused by a direct perturbation of DNA–Sp1 interactions. Oxidation of guanine base induces important changes in hydrogen bonding ability of the imidazole ring of guanine. This leads to the formation of an hydrogen donor site at *N7* position of 8-oxodGuo instead of an hydrogen acceptor site at the N7 position of guanine.

The Sp1 transcription factor contains three tandem arrays of zinc finger domain (Cys_2–His_2 zinc finger). Each of them is close to the sequence (Tyr, Phe)-Xaa-Cys-Xaa-Xaa-Cys-Xaa-Xaa-Xaa-Phe-Xaa-**Xaa13**-Xaa-Xaa-**Xaa16**-Leu-Xaa-**Xaa19**-His-Xaa-Xaa-Xaa-His-Thr-Gly-Glu-Lys.[44,45] The interaction of Sp1 with the GC box involves primarily zinc finger contacts in the major groove of DNA. The predominant sequence specific contacts are made by the residues Xaa13, Xaa16 and Xaa19 in a region of helical structure. Each

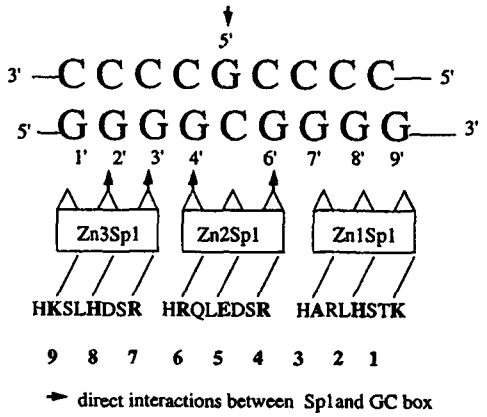

FIGURE 4 Plausible model of DNA–protein interactions of Sp1 with its target site. Arrows indicate strong guanine contacts between Sp1 and the amino acid residues (greasy letters) predicted to be involved in specific base recognition.

domain is associated with 3 base pairs of DNA (either 5′-GGG, GCG, or GGG-3′; Figure 4). Xaa13, Xaa16 and Xaa19 are highly conserved in zinc finger proteins but are different for each domains.[46] Attempts to rationalize the EMSA results of our study were made on the basis of this knowledge. It appears that 5 guanine bases, from positions G′2 to position G′6, are strongly involved in the interactions between the DNA duplex and the Sp1 protein, mainly at the zinc finger domains 2 and 3.

It may be inferred that the guanine bases at position G′2 and G′3 participate strongly to the interactions between the DNA duplex and the third zinc finger of Sp1. This is illustrated by the decrease in the binding from 100% to 28% and 30% respectively, when the related positions were oxidized into 8-oxodGuo. In fact, the G′2 and G′3 guanines interact respectively with histidine H8 and arginine R7 of the third zinc finger.[45] Another interaction may occur between the positions G′4 and G′6 of the DNA sequence and the second zinc finger of Sp1 through the intermediary of arginines R4 and R6. Interestingly, oxidation of the latter guanine residues into 8-oxodGuo leads to a pronounced decrease in the binding of Sp1 down to 18% and 21%. The results of EMSA provide further confirmation to the fact that the transcription factor Sp1 interacts with the consensus sequence only at the levels of arginine and histidine residues.[40]

The G′1 position appears to be less important in the duplex formation since its oxidation leads only to a weak decrease of 30% in Sp1 binding. Nevertheless, lysine K9 which is associated with G′1 is able to bind to the base. The same feature applies to the lysine K1 associated with G′9, whose oxidation does not affect the binding of Sp1. In fact, the length of the lysine side chain allows a great variety of base targets (dAdo, dThd, dGuo, dCyd), including 8-oxodGuo. Indeed, the G′1 and G′9 positions are not well conserved among the Sp1 consensus sequences as they can be replaced by the thymine and adenine bases respectively.[17] The oxidation of G′7 leads to a significant but not drastic decrease in the binding. The interaction mode of alanine A3 with DNA is not characterized but this amino acid seems to be important since it is highly conserved. This indicated the complexity of DNA–Sp1 interactions. Another example of this complexity is the role of histidine H2 and glutamine E5. The histidine H2 which is associated with G′8 might have the same features than histidine H8, but oxidation of the guanine residue does not affect too much for the binding. This shows that the contribution of each individual zinc finger domain to the specific binding of Sp1 to the GC box is nonequivalent. In addition, EMSA confirmed[47] that the 5′ portion of the GC box DNA sequence (GGG GCG) contributes more strongly to the total energy than the 3′ portion (GGG).

The C(5)–G′(5) base pair is well conserved in the center of the GC box. However, it is established that only weak contacts between the zinc finger domains and the C-rich strand of DNA occur. Nevertheless, this base pair is the core of the GC box and modifications such as the oxidation of dGuo into 8-oxodGuo (probe G′5) or mutation such as G to C (probe T) led to noticeable decrease in the binding which is closed to 8% and 7% respectively.

Figure 4 summarizes a plausible direct interaction mode of Sp1 with the GC box. Interestingly, in recent studies, the occurrence of two other direct contacts between zinc finger domains and C(4) and C(7) cytosines on the C-rich strand of DNA was found.[48]

In conclusion, we demonstrate that five guanines on both strands are important for the GC box recognition. In addition, these bases are in close contact with amino acids which belong to the third and the second zinc finger of Sp1. This means that the 5′ portion of the GC box is more important for the recognition of the transcription factor Sp1 than the 3′ portion. Nevertheless, previous works have demonstrated that finger 1 is required for high-affinity binding.

Our study can be considered as a new approach for investigating the key role played by the guanine and cytosine bases in the GC box. The contribution of each individual base to the binding of Sp1 to its target sequence was delineated. This pointed out the heterogeneous role of guanine positions into protein–DNA interactions, in agreement with previous works. It also provides a means to understand the potential effect of oxidation on the cellular regulation by targeting G arrays with emphasis on the role of the mutagenic 8-oxodGuo lesion.

It would be of interest to extend similar type of studies to other important transcription factor binding sites including AP-1 or NF-κB. In this respect, we note a paucity of information dealing with the effects of DNA modifications on the binding of the transcription factors, at the exception of two studies. One concerns the modulation of the binding of AP-1 and TFIID to DNA in which 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil (FIAU) was incorporated.[41] It was found that the affinity of AP-1 to modified DNA is significantly decreased whereas the binding of TFIID is increased. In the second investigation,[14] it was observed that the presence of benzo[a]pyrene diol epoxide bound to the GC box have no effect on the binding of Sp1 to its DNA target site.

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