Modulation of Enediyne-Induced DNA Damage by Chromatin Structures in Transcriptionally Active Genes†

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ABSTRACT: To better understand how the cellular environment of DNA affects it as a target for genotoxins, we have used ligation-mediated PCR to map DNA damage produced by two DNA-cleaving enediyne antibiotics, esperamicins A1 and C, in the transcriptionally active human *p53* and phosphoglycerate kinase (*pgk1*) genes in vivo. Esperamicin A1, which is limited to damaging the linker region between nucleosome cores due to intercalation of an anthranilate moiety, did not detect the presence of a nucleosome proposed to reside between exons 5 and 6 of *p53*. This may be due to the absence of a nucleosome at this site in the *p53* gene or to the altered structure of nucleosomes in transcriptionally active genes. In studies of the upstream region of the active *pgk1* gene, we found that DNA damage produced by both enediynes was enhanced in sequences located between several transcription factor binding sites, while patterns of DNA damage within the binding sites were consistent with known drug binding modes and structures of the protein−DNA complexes. For both drugs, the DNA sequence appeared to be the major determinant of the location of DNA damage, with chromatin structures modulating the quantity of DNA damage.

The packaging of DNA as chromatin in cells (reviewed in refs *1* and *2*) alters the structure and dynamics of the DNA targets of genotoxic chemicals. Our previous studies have focused on the effect of nucleosome structure—the canonical nucleosomes of transcriptionally inactive genes—on the location and quantity of DNA damage produced by enediyne antibiotics (*3*−*6*). In the present work, we have turned our attention to the chromatin structures of transcriptionally active genes and their role in the selection of DNA targets by two related enediynes, esperamicins A1 and C. Specifically, we have used ligation-mediated PCR (LMPCR) to map DNA damage produced by the enediynes in the human *p53* and phosphoglycerate kinase (*pgk1*) genes.

Enediyne antibiotics present a unique opportunity to study the relationship between drug structure and selection of DNA targets in chromatin. Esperamicins A1 and C (Figure 1) share a common mechanism for producing DNA damage: the aglycone core forms a diradical intermediate that abstracts deoxyribose hydrogen atoms when situated in the minor groove of DNA (reviewed in refs *7* and *8*). The resulting damage produced by esperamicins A1 and C consists of single- and double-strand DNA lesions (*9*).

Despite this common mechanism, esperamicins A1 and C differ in the organization of functional groups attached to the enediyne core (Figure 1). Specifically, the deoxyfucose−anthranilate moiety of esperamicin A1, which intercalates in DNA, is missing from esperamicin C (Figure 1).

The structural differences between esperamicins A1 and C cause the two molecules to recognize different features of chromatin in cells. We have demonstrated that esperamicin A1 is limited to damaging the linker region between nucleosome cores due to intercalation of the anthranilate (*9*), while esperamicin C, a nonintercalating groove-binder, damaged both the core and linker DNA (*3*−*5*). Core DNA damage produced by esperamicin C was limited to sites where the minor groove faced away from the histone proteins.

We have now mapped the DNA damage produced by esperamicins A1 and C in the transcriptionally active *p53* gene and the active form of the *pgk1* gene. Because it is X-linked, the *pgk1* gene provides an opportunity to study the same DNA sequence in both transcriptionally active and inactive states. The inactive *pgk1* gene possess two nucleosomes spanning positions −330 to −200 and −150 to +1; while in the active state, the nucleosomes have been replaced by several transcription factors (Figure 2; *10*). We previously observed that damage produced by the intercalating esperamicin A1 was suppressed in the core region of the two *pgk1* nucleosomes, while the nonintercalating esperamicin C damaged both the core and linker regions of the nucleosomes (*6*). We have now examined the upstream region of the active form of *pgk1* at high resolution to define the effect of transcription factor binding on the damage produced by the two esperamicins.

We have also performed studies in the *p53* gene. The basis for these studies was the observation by Tornaletti et al. (*11*) and Drouin and Therrien (*12*) of a nucleosome-sized structure spanning the 3′-half of exon 5 and the adjacent intron in the transcriptionally active *p53* gene (Figure 2). Because nucleosomes vary in structure as a function of transcriptional activity (*1*, *13*, *14*), we were interested in assessing the ability

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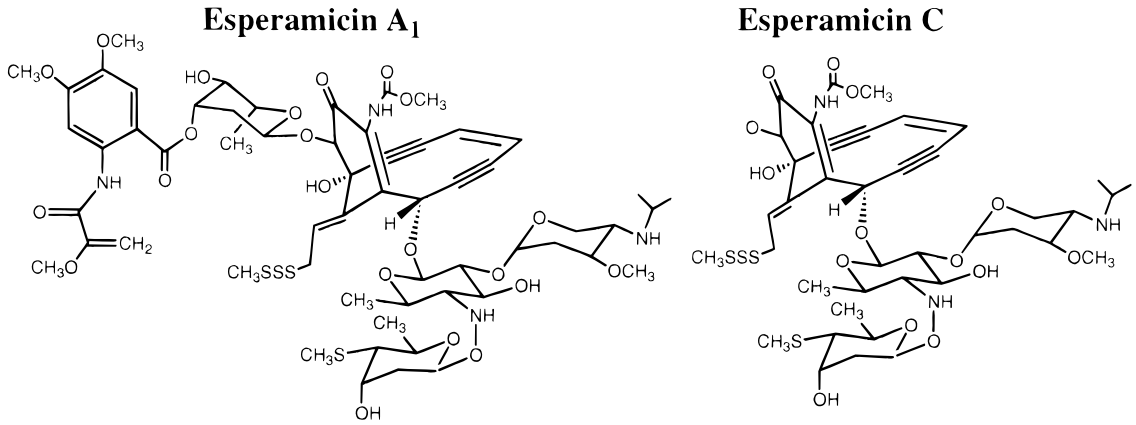

FIGURE 1: Structures of esperamicins A1 and C.

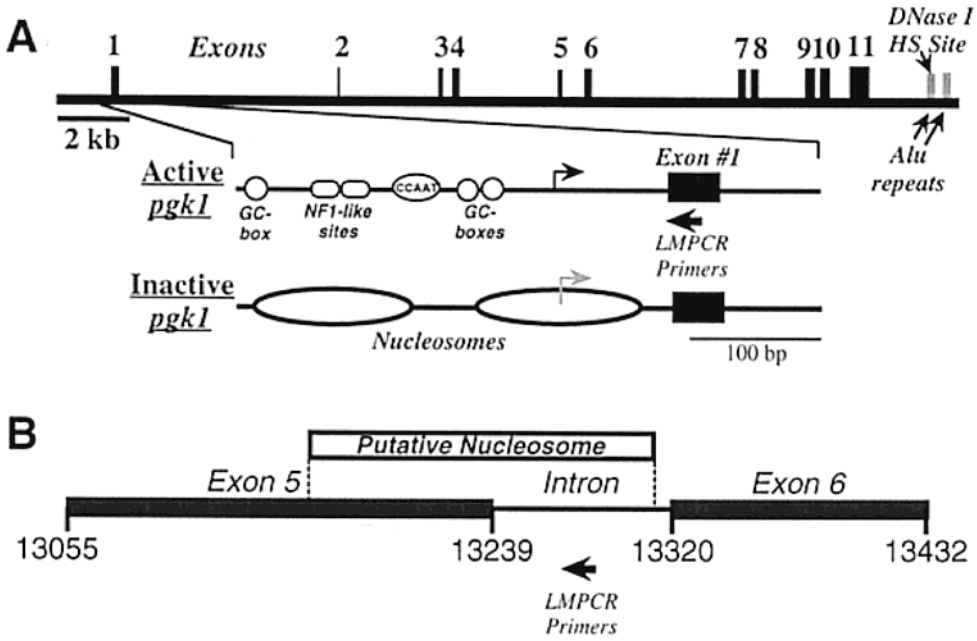

FIGURE 2: Structures of the *p53* and *pgk1* genes. The model for the *pgk1* gene was adapted from several sources (*10, 15*−*17, 33*). The position of the putative nucleosome in the *p53* gene is based on footprinting studies (*11, 12*).

of esperamicin A1 to recognize this putative nucleosome in an active gene.

The results of our studies suggest that the esperamicins are sensitive to the chromatin structures in transcriptionally active genes. In *p53*, we observed that DNA cleavage produced by esperamicin A1 was not affected by the presence of a nucleosome-like structure, while both esperamicins A1 and C produced cleavage patterns in *pgk1* consistent with the drug and transcription factor structures and binding modes.

MATERIALS AND METHODS

Cell Lines and Materials. HeLa S3 cells (for the *p53* gene) were grown in Joklik-modified Eagle's minimal essential medium with 10% newborn calf serum, 1% penicillin/streptomycin and 2 mM L-glutamine. Chinese hamster−human hybrids containing an active human X chromosome (cell line Y162-11C) were provided by Dr. Stanley Gartler (University of Washington, Seattle; *15, 16*), and cells were grown as a monolayer in RPMI 1640 with 10% fetal bovine serum and 40 μg/mL gentamicin (*15, 16*). A clone of the upstream region of the human *pgk1* gene, pBSHPGK1, was provided by Dr. Judith Singer-Sam (City of Hope Medical Center, Duarte, CA; *17*). Calicheamicin γ_1^I and esperamicins A1 and C were generous gifts from Dr. George Ellestad (Wyeth-Ayerst Research) and Dr. Jerzy Golik (Bristol-Myers Squibb), respectively. Glutathione and putrescine were purchased from Sigma Chemical Co. T4 polynucleotide kinase and restriction enzymes were purchased from New England Biolabs. T7 DNA polymerase (Sequenase version 2.0) was purchased from Amersham. T4 DNA ligase was purchased from Promega. *Thermus aquaticus* (*Taq*) DNA polymerase was purchased from Boehringer Mannheim. DNA primers were synthesized by Oligos Etc.

Treatment of DNA with Enediynes. After harvest, 10^7 cells were resuspended in 200 μL of phosphate-buffered saline (PBS), followed by purification of the DNA using a QiaAmp blood kit (Qiagen). The DNA damage reactions were performed by adding 2 μL of a methanolic solution of the drug (final methanol concentration was less than 1%) to a solution of DNA (0.1 mg/mL) in 10 mM glutathione, 50 mM HEPES, and 5 mM EDTA, pH 7.0. The reaction was allowed to proceed for 30 min at ambient temperature. To convert abasic sites to strand breaks, putrescine was added

to 100 mM, and the reaction mixture was incubated for 1 h at 37 °C (for example, see ref *15*). The damaged DNA was finally precipitated with ethanol and stored at 1 mg/mL in 10 mM HEPES and 0.1 mM EDTA, pH 7.7, at −80 °C. Maxam−Gilbert sequencing reactions were performed as described elsewhere (*19*).

Treatment of Cells with Esperamicins. An aliquot of the methanolic drug stock was added to 200 μL of PBS containing 10^7 cells harvested from culture medium (final methanol concentration was less than 1%). The reaction was allowed to proceed for 30 min at ambient temperature prior to DNA purification and putrescine treatment as described above.

Ligation-Mediated PCR. The primers used in the *p53* experiments were as described elsewhere (*11*). For the *pgk1* experiments, we used the following primers in the 5′ region of the human *pgk1* gene: CGTCCAGCTTGTCCAGC (+134 to +118; primer B1); TCCAGCGTCAGCTTGTTAGAAAGCG (+123 to +99; primer B2); TGGGGAGAGAGGTCGGTGATTCGGTCA (+80 to +54; primer B3). The sequences of the linker and its primer as well as the LMPCR reactions are described elsewhere (*20*). The amplified DNA samples were resolved on 6% or 8% polyacrylamide/8 M urea sequencing gels at 80 W constant power. The DNA was transferred to a charged nylon membrane (Qiagen) using an HEP3 electroblotting apparatus (Owl Scientific Inc.) per manufacturer's instructions, and the blotted DNA was UV-cross-linked (1200 J/m^2) to the membrane (*20*). Hybridization probes were prepared by repeated primer extension from PCR-amplified templates of the *p53* gene (*21*) or from a cloned *pgk1* template (pBSHPGK1; *19*) using primer B3 and *Taq* polymerase. Hybridized membranes were subjected to phosphorimager analysis (Molecular Dynamics).

Data Analysis. To compare damage frequency in isolated and cellular DNA, we performed the experiments with drug concentrations that produced roughly similar levels of DNA damage in the two situations. This necessitated a higher drug concentration for cells than for isolated DNA. Phosphorimager analysis was performed essentially as described elsewhere (*6*). Each pixel value was first divided by the sum of the total pixel values in the lane to account for lane-to-lane variations in sample loading. Normalized pixel values in each lane were then plotted as a line graph.

RESULTS

Enediyne-Induced DNA Damage in the p53 Gene. We first compared in vivo and in vitro damage patterns produced by esperamicin A1 in the *p53* sequence spanning exons 5 and 6, as shown in the sequencing gel in Figure 3. It is apparent that higher concentrations of esperamicin A1 were required to produce comparable amounts of DNA damage in cellular DNA as compared to isolated DNA. This effect was observed in previous studies (*6*), and it is likely due to factors such as sequestration of the drug in lipid membranes in the cells or deactivation of the drugs in the cytoplasm.

The line graph in Figure 4 represents signal intensity of the bands present in lanes 4 (isolated DNA, 80 nM esperamicin A1) and 8 (cellular DNA, 6.5 μM esperamicin A1) in Figure 3. It is apparent from Figure 4 that both the location and the quantity of DNA damage produced by esperamicin A1 in exon 5 of *p53* in isolated DNA are identical to the damage produced in whole cells, including the region proposed to be occupied by a nucleosome core (*11*, *12*).

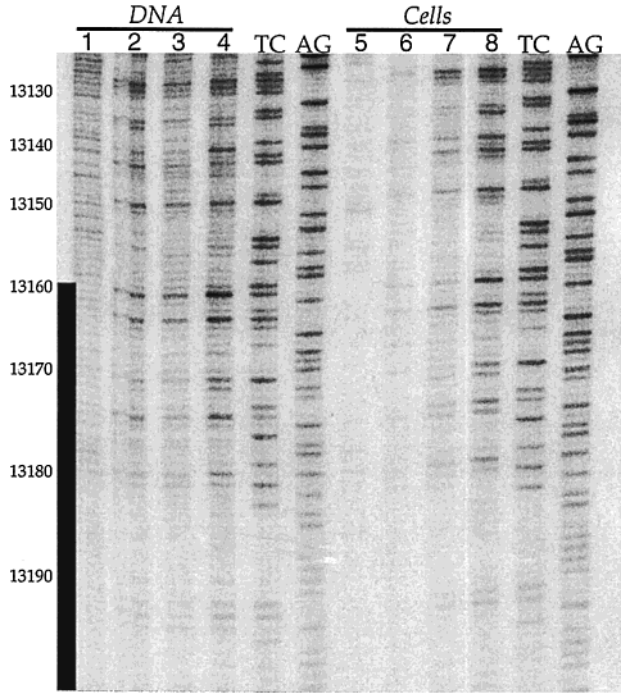

FIGURE 3: Sequencing gel analysis of the DNA damage produced by esperamicin A1 in the 3′-half of exon 5 of the *p53* gene in vitro and in vivo. Drug-induced damage was studied in isolated DNA (lanes 1−4) and intact cells (lanes 5−8). GA and TC lanes represent Maxam−Gilbert sequencing reactions (*19*). Lanes 1−4: 0, 20, 40, and 80 nM esperamicin A1; lanes 5−8: 0, 0.4, 1.6, and 6.5 μM esperamicin A1. The amplified DNA was resolved on 8% sequencing gel. The location of a putative nucleosome core region is indicated by the bar in the left margin along with sequence positions.

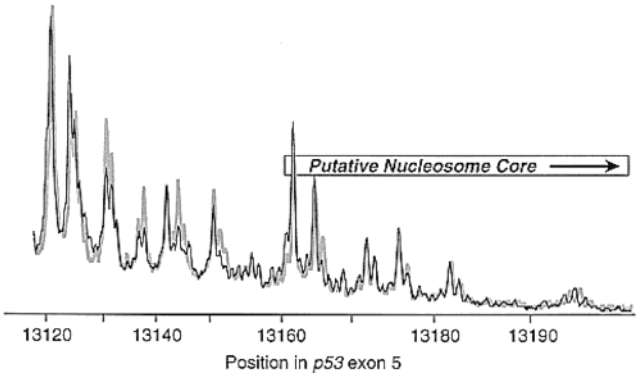

FIGURE 4: Comparison of esperamicin A1-induced DNA damage in 3′-half of exon 5 of the *p53* gene in isolated DNA (black line) and cellular DNA (gray line). Lanes 4 and 8 in the gel shown in Figure 2 were subjected to phosphorimager analysis, with data presented as overlaying line graphs of damage frequency along the gene sequence. The position of the putative nucleosome is indicated by the box.

*Enediyne-Induced DNA Damage in the Active Human pgk1 Gene***.** We next examined the location and quantity of DNA damage produced by esperamicins A1 and C in the 5′ promoter region of human *pgk1*. The DNA damage patterns produced by the esperamicins A1 and C in isolated DNA and in cells containing an active *pgk1* are shown in Figure 5. The data are summarized in the line graphs in Figure 6 along with the positions of the putative transcription factors

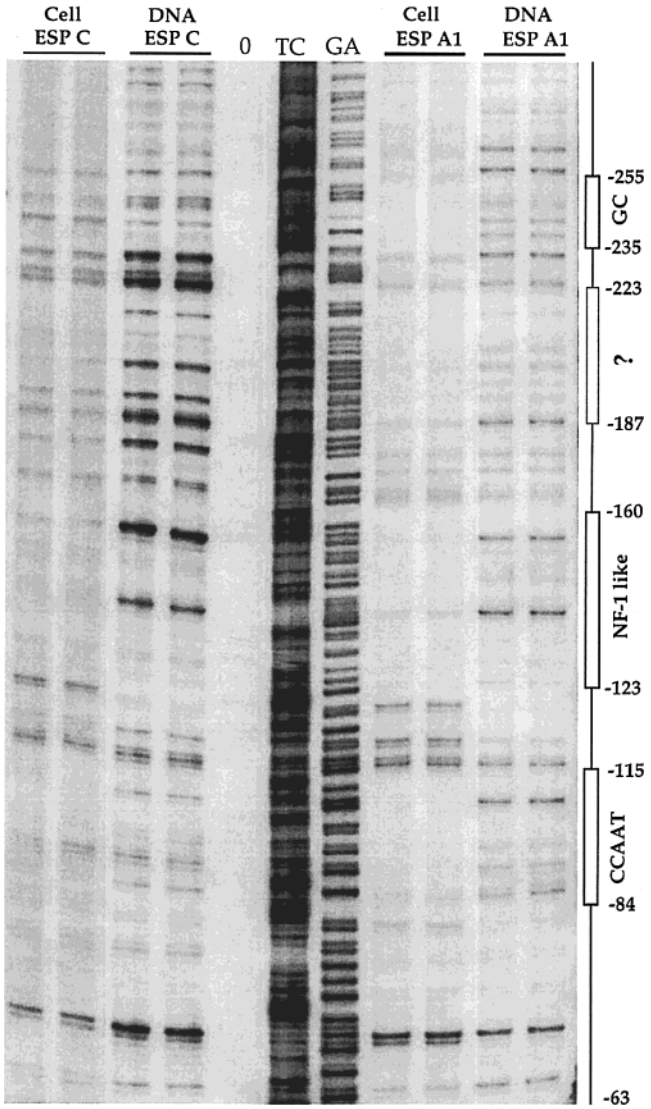

FIGURE 5: Sequencing gel analysis of the DNA damage produced by esperamicins A1 and C in the active human *pgk1* gene in vitro and in vivo. The left four lanes contain duplicate samples of cellular DNA or isolated DNA treated with 20 or 2 μM esperamicin C, respectively. The right four lanes contain duplicate samples of cellular DNA and isolated DNA damaged by 2 and 0.4 μM esperamicin A1, respectively. The lane marked 0 represents untreated control DNA subjected to LMPCR amplification. TC and GA represent Maxam−Gilbert sequencing markers (*19*). The LMPCR-amplified DNA was resolved on an 8% sequencing gel, electroblotted onto a nylon membrane and hybridized with a human *pgk1* probe. The positions of transcription factor binding sites in the *pgk1* gene are shown in the right margin (*12*, *24*, *25*). A single lane located between the esperamicin C-treated isolated DNA and cellular DNA samples was removed for clarity.

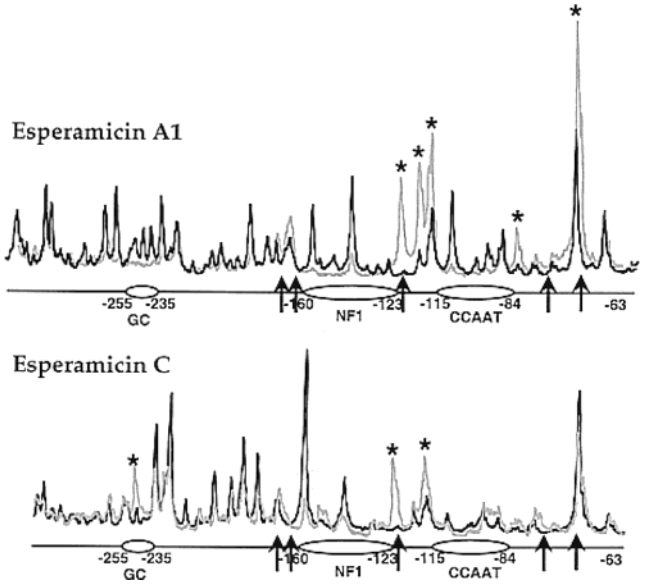

FIGURE 6: Comparison of enediyne-induced DNA damage in the active *pgk1* gene. The gel shown in Figure 5 was subjected to phosphorimager analysis, and the data are presented as line graphs of relative damage frequency along the human *pgk1* gene. Black lines represent damage in isolated DNA, and gray lines represent damage in cellular DNA. The locations of transcription factor binding sites in the *pgk1* gene are indicated below each graph. Damage enhancement sites in cellular DNA as compared to isolated DNA are indicated by asterisks. DNase I sensitive sites in cells (*12*) are indicated by upward arrows.

derived from DNase I and dimethyl sulfate footprinting studies (*10*, *22*).

As observed in previous studies (*6*, *9*), esperamicins A1 and C produce similar cleavage patterns in isolated DNA (Figure 6), despite the lowered binding affinity and altered damage chemistry produced by removal of the anthranilate (*9*). It is also apparent in Figure 6 that, with a few exceptions, the sequence selectivity for both drugs is similar in isolated DNA and in cells.

There are two notable features of the damage pattern produced by the esperamicins in the *pgk1* gene. First, it is apparent that there is suppression of DNA cleavage produced by esperamicin A1 at all transcription factor binding sites in the cells (Figures 5 and 6). Suppression of esperamicin C-mediated cleavage, however, occurs only at the NF1-like binding site between positions −123 and −160 (Figure 6). Esperamicin C-induced damage is enhanced in the GC box located between positions −235 and −255 (Figure 6).

The other unusual feature of the damage patterns is the strong enhancement of cleavage adjacent to transcription factor binding sites. There is an increase in damage for both esperamicins A1 and C in the region bounded by positions −115 and −123 in the cellular DNA and an increase in esperamicin A1-induced DNA damage between positions −63 and −84 (Figure 6). These two regions coincide with DNase I sensitive sites identified by Pfeifer and co-workers (*22*) (upward arrows in Figure 6).

DISCUSSION

Nucleosome Positioning in the p53 Gene. We have previously demonstrated that esperamicin A1 is sensitive to the structure of the nucleosomes found in transcriptionally silent chromatin in vitro and in vivo (*4*, *6*, *9*). The intercalating anthranilate of esperamicin A1 limits drug binding to the dynamically unconstrained DNA of the nucleosome linker (*4*, *6*, *9*). Removal of the anthranilate to form esperamicin C permits drug binding and thus DNA cleavage in both the core and linker regions of the nucleosome (*3*, *4*, *6*, *9*).

However, in the transcriptionally active *p53* gene, esperamicin A1 produced identical cleavage patterns in cellular and isolated DNA in a region proposed to contain a nucleosome. The presence of a nucleosome-sized footprint has been demonstrated independently by two groups using DNase I (*11*) and photofootprinting (*12*).

There are several explanations for this apparent discrepancy. First, it is possible that the HeLa cells used in the present studies do not possess a nucleosome at the same position in the *p53* gene as the fibroblasts used in the studies of Tornaletti et al. (*11*) and Drouin and Therrien (*12*). This seems unlikely given the conservation of chromatin structure in the same active gene in different cell types, such as the similar architecture of the *pgk1* gene in human lymphocytes and hamster−human hybrid cell lines (*22*).

A second possibility is that the DNA damage produced by esperamicin A1 is not affected by the altered structure of nucleosomes in transcriptionally active genes. The accessibility, structure, and dynamic properties of nucleosomal DNA are known to be affected by modifications of the histone core. For example, acetylation of lysine residues in the N-terminal tails of core histone proteins alters DNA structure and accessibility (*1*, *2*, *23*, *24*), with increased overall susceptibility to DNase I cleavage that is most marked ~60 bp from the ends of the core DNA (*25*). Allfrey and co-workers have also described a nucleosome variant isolated from active genes that appears to have a grossly altered structure as compared to canonical nucleosomes, with altered migration in sucrose gradients, a cracked appearance by electron microscopy, and increased sensitivity to nuclease digestion (*13*, *26*). It is possible that esperamicin A1 is able to bind to and cleave DNA in these nucleosome variants in active genes. Regardless of the basis for the observed effect, the possibility that esperamicin A1 differentially recognizes nucleosomes in active and inactive genes warrants further investigation.

Transcription Factors Bound to the Active Human pgk1 Gene. As in the *p53* gene, the sequence selectivity for esperamicins A1 and C was similar for both isolated DNA and cells. This is consistent with DNA sequence and, thus, DNA structure as a major determinant of target selection by the esperamicins. However, the chromatin structures present in the upstream region of the active *pgk1* gene affected DNA damage produced by esperamicins A1 and C differently. The results are consistent with the known structures and DNA binding modes of the esperamicins and transcription factors.

For example, binding of transcription factors to the GC boxes and CCAAT motifs involves major groove binding elements, such as the zinc finger of SP1 and the leucine zipper of C/EBP, respectively (*27*−*30*). In the case of esperamicin C, the presence of these two transcription factors does not inhibit binding of the drug in the minor groove and, in the case of the GC box, appears to enhance drug binding (Figure 6). However, cleavage by esperamicin A1 is inhibited at both the GC box and CCAAT site. This is understandable in terms of inhibition of intercalation of the anthranilate of esperamicin A1 due to the dynamic constraints imposed on DNA by binding of the transcription factors to these sites. Such is the case in the inhibition of esperamicin A1 binding to the nucleosome core in which the DNA is dynamically constrained by the contacts with histone proteins (*4*, *6*, *9*).

In the case of the NF1-like sequence, damage is inhibited for both esperamicin A1 and C (Figure 6). This may be due to the fact that transcription factor NF1 binds to DNA circumferentially, thus occupying both the major and minor grooves at the binding site (*31*). Occupation of the minor groove by NF1 would prevent binding by the minor groove-specific esperamicins A1 and C.

It is less clear why the damage produced by esperamicins A1 and C is enhanced in the region between the NF1 and CCAAT sequences (Figure 6). One possible scenario involves altered DNA structure or dynamics at this site caused by interactions between the occupied transcription factor binding sites. It is known that the trans-activating domains of many bound transcription factors interact with each other, which causes the intervening DNA to form a loop (*32*). This looping will alter DNA structure by bending and twisting the helix and widening of the minor groove at some sites. Although the esperamicins are not known to be affected by DNA bending, there is clearly an effect of local DNA structure between the NF1 and CCAAT sites that enhances cleavage by esperamicin A1 and C.

In summary, DNA sequence appeared to be the major determinant of the location of DNA damage produced by esperamicins A1 and C in the *p53* and *pgk1* genes. Though esperamicin A1 did not detect a nucleosome in the transcriptionally active *p53*, transcription factors bound to the upstream region of *pgk1* modulated the quantity of DNA damage of both esperamicins A1 and C.

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