Volume 16 Number 1 1988 Nucleic Acids Research

High resolution psoralen mapping reveals an altered DNA helical structure in the SV40 regulatory region

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Received September 9, 1987; Revised and Accepted November 20, 1987

ABSTRACT

Preferential psoralen photobinding sites have been mapped in vitro on restriction fragments spanning the SV40 origin region and surrounding sequences by a new fine structure analysis technique. Purified DNA fragments were photoreacted with ^{3}H-5-methylisopsoralen (^{3}H-5-MIP), a psoralen derivative which forms only monoadducts. Fragments were then end-labeled and digested with λ exonuclease, a 5' processive enzyme which we have determined pauses at 5-MIP monoadducts. When photobinding sites were mapped on denaturing sequencing gels, it was observed that 5-MIP binds preferentially to 5'-TA sites, and to a lesser degree to 5'-AT sites. Utilizing this approach, we have identified a psoralen hypersensitive region in which the binding sites were much stronger than those in the surrounding sequences. This region extends from 150 base pairs (bp) to the late side of the enhancers to the early enhancer/promoter boundary. We suggest that this region contains a sequence directed structural alteration of the DNA helix which can be detected by the psoralen mapping approach described.

INTRODUCTION

Psoralens are three ring heterocyclic compounds that have become powerful tools for the in vivo analysis of DNA nucleoprotein structure and RNA secondary structure. These compounds have the ability to penetrate living cells and viruses without disruption of membranes and then intercalate between the stacked bases of the helix (1-4). Upon irradiation with long wavelength UV light, the intercalated molecule undergoes cyclophotoaddition to the 5,6 double bond of a pyrimidine to form a monoadduct. With additional UV irradiation, some psoralen derivatives may undergo a second photoreaction and form a crosslink between pyrimidines located in adjacent and opposite positions on the helix (see reference 5 for a review). In the case of 5-methylisopsoralen (5-MIP), the angular nature of the molecule prevents correct alignment for crosslink formation so that only monoadducts are formed.

Since nucleosomal core DNA is preferentially protected from psoralen photoaddition, psoralen derivatives are valuable probes for examining the

structure of cellular and viral chromatin (2,3,6,7). We have previously used psoralen photoaddition to examine the structure of SV40 minichromosomes during a lytic infection and in assembled virus (8,9). In these experiments, a restriction fragment of about 360 bp encompassing the SV40 regulatory region was found to be preferentially accessible to psoralen in minichromosomes purified from infected cells, as well as in intracellular and extracellular virus. The spacing of nucleosomes along the SV40 genome has been shown to be nonrandom in this region on a subpopulation of minichromosomes, in which a nucleosome free region of approximately 300 bp has been detected by both nuclease sensitivity (10-14) and electron microscopy (15,16). This region spans the viral origin of replication, early and late promoters, the 72 bp repeats, which function as enhancers of viral transcription (17,18), and the T antigen binding sites. To date, the ability to characterize the chromatin structure of SV40 by psoralen photoaddition has been limited by an inability to localize psoralen monoadducts and crosslinks precisely at the nucleotide level in both *in vivo* and *in vitro* samples.

Piette and Hearst have mapped psoralen adducts by sizing the DNA products resulting from nick translation of circular ϕX174 templates previously photoreacted with 4'-hydroxymethyl-4,5',8-trimethylpsoralen (HMT) (19). Their results showed that *E. coli* pol I was blocked by psoralen crosslinks with termination sites of equal intensity appearing before each 5'-AT and 5'-TA sequence. Unfortunately, it is impossible to map monoadducts with this approach since they do not block DNA pol I activity. In addition, these experiments require synthesis of a complementary strand followed by hybridization to a single-stranded template, so this approach cannot readily be applied to the analysis of samples generated *in vivo*. Becker and Wang have developed a chemical method for mapping protein-DNA interactions utilizing UV light and have suggested its application for mapping psoralen photoadducts (20). Finally, Zhen *et al.* have very recently described a method for detecting psoralen crosslinks based on the inhibition of Bal 31 exonuclease activity. Like DNA pol I, this enzyme is only inhibited by intrastrand crosslinks and apparently cannot detect psoralen monoadducts (21).

In this report we have taken an enzymatic approach to mapping psoralen monoadducts generated *in vitro* on SV40 DNA. This approach, which readily lends itself to *in vivo* applications, takes advantage of several features of λ exonuclease, an enzyme required for general recombination in the bacteriophage λ (see reference 22 for a review). This enzyme cleaves

5'-mononucleotides from the 5' phosphoryl termini of double-stranded DNA, and preferentially initiates at 5'-recessed ends but not at nicks or gaps. We have determined that λ exonuclease pauses at 5-MIP monoadducts on a series of restriction fragments encompassing the SV40 origin region and regulatory sequences. When the locations of these pauses were mapped, it was determined that 5-MIP monoadducts were present at each 5'-TA in the sequence. Within these sequences, a small subregion was found to be hypersensitive to psoralen photoaddition in vitro. This segment includes the repeated 72 bp enhancers, the late promoters, and the major late DNase I hypersensitive sites.

METHODS AND MATERIALS

Purification of DNA

Supercoiled SV40 DNA was purified from virus as described by Kondoleon et al. or from infected cells by a modification of the Hirt procedure (23). The maintenance of cell cultures, preparation of SV40 stocks and infection of cells has been described elsewhere (8).

Electrophoresis and Restriction Fragment Isolation

Restriction enzymes were purchased from BRL (Gaithersburg, MD) and were used according to the manufacturer's specifications. Restriction fragments of interest were separated by agarose gel electrophoresis using Bio-Rad ultra pure agarose (Richmond, CA) and isolated by electroelution into dialysis bags (24). Each sample was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1), once with ether, and then ethanol precipitated. The DNA was resuspended in 1 ml TE (10 mM Tris, pH 8.0 and 1 mM EDTA) and further purified by passage over Elutip-d^{TM} minicolumns (Schleicher and Schuell, Keene, NH).

^{3}H-5-MIP Photoaddition

^{3}H-5-MIP was obtained from HRI, Inc. (Emeryville, CA) in ethanol stock solutions of approximately 90μg/ml. The specific activity was determined as previously described, and was approximately 9.54 x 10^{6} cpm/ug (12). DNA samples were suspended in 15-50μl of H_2O in 1.5ml microcentrifuge tubes for irradiation. The irradiations were for 5-20 minutes and were performed by suspending the tubes between two GE F15T8 BlB fluorescent light bulbs (3.0-4.4 mW/cm^{2}/tube) spaced 7 cm apart. Controls were irradiated under identical conditions in the absence of psoralen.

End-labeling and Lambda Exonuclease Reaction

DNA was end-labeled with the Klenow Fragment (Boehringer Mannheim Biochemicals, Indianapolis, IN) as described by Maniatis et al. (24).

Restriction enzymes were chosen such that only one end of a given restriction fragment would be labeled.

Following the Klenow reaction, 1μg of sheared salmon sperm DNA and 0.5 volumes of 7.5M ammonium acetate were added, and the reaction mixture was heated for 15 minutes at 70-80°C. The sample was then extracted twice with phenol/chloroform/isoamyl alcohol, and ethanol precipitated. This procedure was repeated a second time to insure that all protein and unincorporated nucleotides were removed.

Prior to digestion with λ exonuclease, samples were normalized so that the same number of counts went into each reaction. Reactions were carried out at 37°C in 67 mM glycine-KOH (pH 9.4), 3 mM MgCl, and 3 mM β-mercaptoethanol for 3 to 40 minutes. Twenty to 65 units of λ exonuclease (New England Biolabs, Beverly, MA) were used for each digestion. Following the reaction, samples were phenol extracted and ethanol precipitated as described previously, and resuspended in electrophoresis dye containing 80% deionized formamide, 50 mM tris-borate (pH 8.3), 1 mM EDTA, and 0.1% (w/v) xylene cyanol and bromphenol blue.

Sequencing Gels and Autoradiography

DNA sequencing reactions and preparation of denaturing gels were carried out as described by Maxam and Gilbert (25). Following electrophoresis, gels were fixed in a 5% methanol/5% acetic acid solution, vacuum dried onto 3 mm Whatman chromatography paper for 1 hour at 80°C, and exposed to Kodak X-omat AR film for 18-36 hours.

RESULTS

The locations of 5-MIP monoadducts on restriction fragments spanning the SV40 regulatory region and surrounding sequences were mapped following *in vitro* photoaddition. Since the angular nature of this particular psoralen derivative prohibits formation of crosslinks, the exact position of the monoadducts is determined by analysis on single-stranded sequencing gels following λ exonuclease digestion. The experimental approach is summarized in Figure 1 for a 362 bp Hind III-Kpn I (C_2) restriction fragment which extends on the labeled strand from nucleotides 294 to 5176 (see reference 26 and Figure 3 for the sequence). The fragment was purified as described and then photoreacted with ^{3}H-5-MIP. All controls were irradiated under parallel conditions in the absence of ^{3}H-5-MIP. Since initial reports characterizing λ exonuclease had concluded that 5'-recessed ends were important for initiation of digestion (see reference 22 for a review), the asymmetry of the

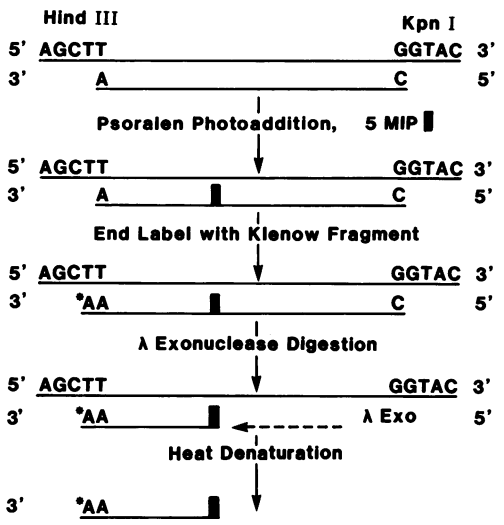

Figure 1. Experimental approach for mapping psoralen photoadducts using the enzyme lambda exonuclease. Purified DNA is photoreacted with the isopsoralen derivative 5-MIP. The DNA fragment is end-labeled at the 3'-recessed end with the Klenow fragment of DNA polymerase I and ^{32}P-dATP. The labeled fragment is then digested with lambda exonuclease, which initiates primarily at 5'-recessed ends, and fragments are analyzed on denaturing sequencing gels. Bands corresponding to λ exonuclease pauses, and hence the positions of 5-MIP adducts, are visualized by autoradiography.

fragment was maintained by end-labeling with [^{32}P]dATP alone. The enzyme should then preferentially initiate digestion on the ^{32}P labeled strand rather than the unlabeled strand, although the degree to which this occurs is unknown.

The experiment shown in Figure 2 demonstrates that λ exonuclease pauses at 5-MIP monoadducts. Lane 3 contains the undigested experimental sample photoreacted with ^{3}H-5-MIP to a level of about one adduct per 100 bp. Digestion of that sample with an excess of λ exonuclease shows a unique pattern with specific bands of varying intensities (lane 4). Digestion of an irradiated control under the same conditions shows that the fragment is completely degraded in the absence of psoralen (lane 2). When the photoadduct level was decreased to approximately one adduct per 300 bp, the same discrete pattern of bands is observed (lane 7).

The positions of the psoralen binding sites were mapped by comparison to

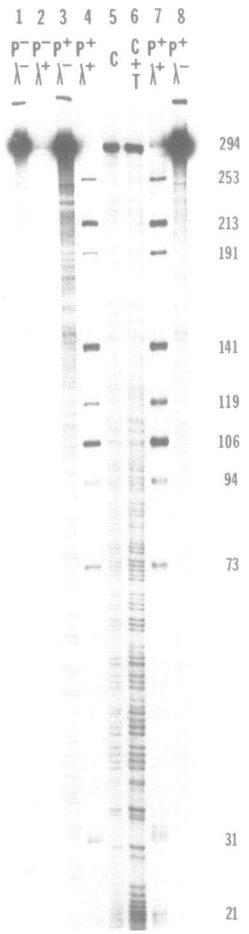

Figure 2. Fine structure mapping of 5-MIP monoadducts on a 362 bp Hind III-Kpn I restriction fragment (termed C_2) spanning the SV40 origin. Lanes 1,3 and 8 contain equal aliquots of the restriction fragment irradiated in the absence of 5-MIP (lane 1) or photoreacted to levels of 100 and 300 bp per 5-MIP adduct (lanes 3 and 8 respectively). Lanes 2, 4, and 7 are the corresponding samples treated with an excess of λ exonuclease. Lanes 5 and 6 are C and C+T Maxam-Gilbert sequencing reactions of the C_2 fragment. Vertical numbers indicate the nucleotide map positions of the psoralen binding sites. A nonrandom background nicking pattern is noted in a subpopulation of the DNA fragments in both the experimental and control samples (lanes 1,3,8). The extent and pattern of nicking was found to vary considerably from experiment to experiment; for example, see Figures 4 and 6 which show very low and moderately high levels of nicking in the undigested samples, respectively. The experiments have been repeated with restriction fragments purified both from agarose and polyacrylamide gels with various levels of nicking in the starting material, and the conclusions were identical in all cases regardless of the extent of nicking.

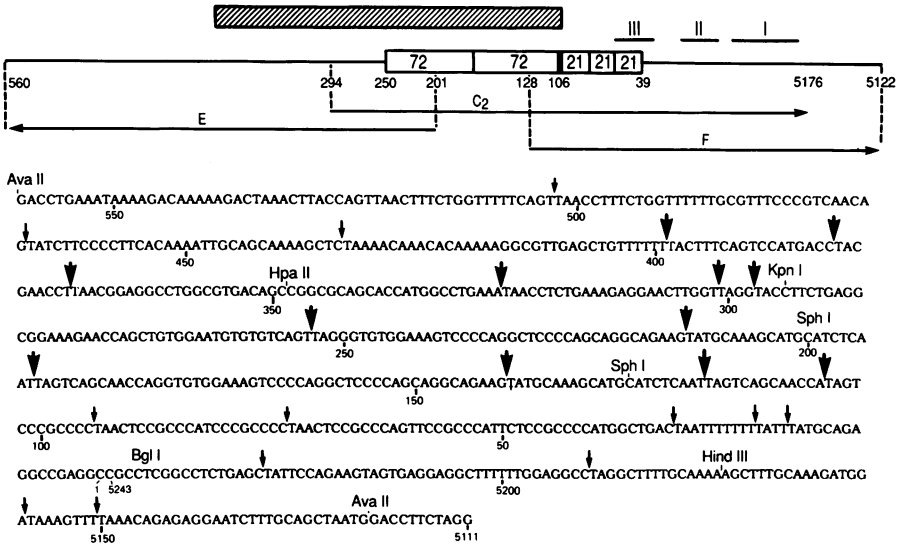

Figure 3. Localization of psoralen hypersensitive sites. The nucleotide sequence of the region mapped is shown. Early transcription is to the right, late to the left. Arrows in the line drawing above the sequence indicate pertinent restriction sites for fragments which were examined and the direction in which λ exonuclease traveled for each fragment. The 72 and 21 bp repeat regions, as well as T antigen binding sites I, II and III, are indicated. Arrows in the sequence indicate the position of each psoralen-associated pause. Pauses were found 5' to every 5'-TA in the regions examined, and the intensity of each arrow is indicative of the relative strength of that band on the gels. The striped bar delineates the psoralen hypersensitive region.

Maxam-Gilbert sequencing of the C_2 fragment. Additional experiments under different gel conditions allowed mapping of all psoralen binding sites within the fragment. These data are summarized in Figure 3. As the arrows indicate, a psoralen-associated band is found 5' to every 5'-TA dinucleotide. Very faint bands were mapped 5' to most 5'-AT sequences (data not shown). Because the psoralen-associated bands spanned a variable distance of 1-3 bp due to the retardation of electrophoretic mobility by psoralen intercalation, the results are accurate to within ±1 bp. The data clearly indicate that 5'-TA conformations are the primary sites of 5-MIP photoaddition.

The kinetics of the reaction were examined to determine whether 5-MIP adducts totally block λ exonuclease or merely slow the forward movement of the enzyme (Figure 4). The purified, end-labeled DNA fragments irradiated with and without ^{3}H-5-MIP are shown in lanes 1 and 7, respectively. The controls in lanes 8 through 11 were digested for 2, 20, 40, and 80 minutes

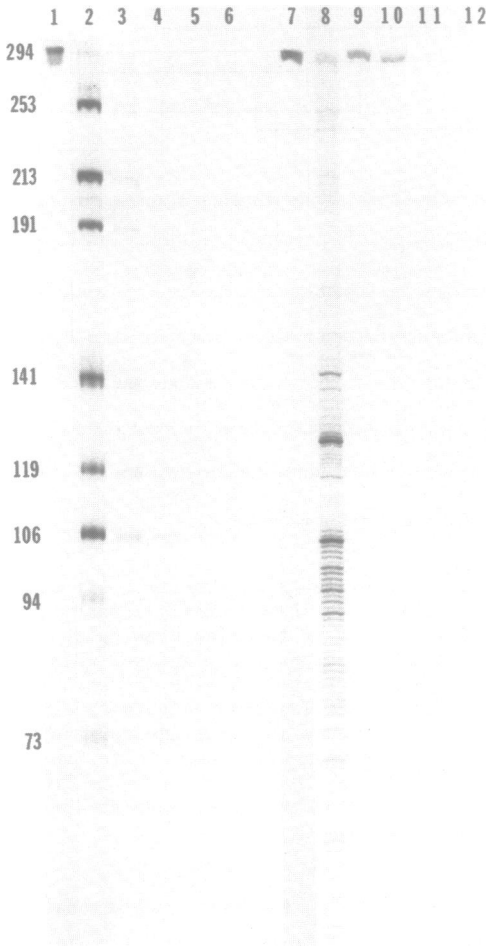

Figure 4. Kinetic analysis of λ exonuclease digestion on psoralen-labeled DNA. The SV40 C_2 fragment was end-labeled as described in the text, and equal amounts of radioactivity were used for the 10 experimental samples (lanes 2-6, 8-12). In lanes 1-5 the samples were irradiated with 5-MIP and digested with λ-exonuclease for 0,2,20,40, and 80 minutes respectively. The sample in lane 6 was digested for 80 minutes with a double aliquot of enzyme. Lanes 7 through 12 correspond to lanes 1 through 6 but were irradiated in the absence of 5-MIP. The photoaddition level was approximately 1 adduct/200 bp for this and subsequent experiments.

respectively. The control in lane 12 was digested for 80 minutes with a double aliquot of enzyme. The corresponding experimental samples in lanes 2 through 6 were photoreacted to a level of about one adduct per 200 bp. The

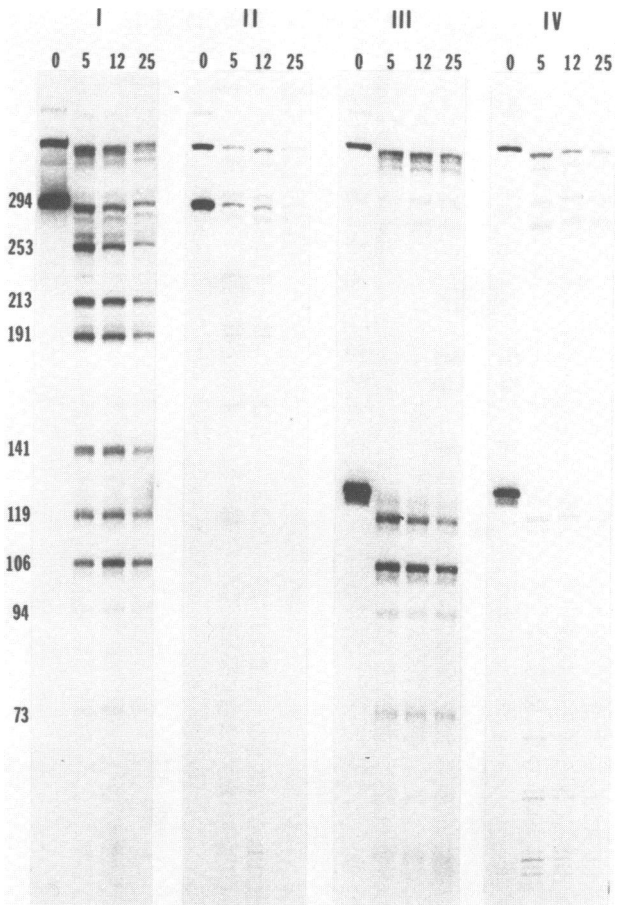

Figure 5. Effect of partial deletion of the enhancer region on psoralen photoaddition in the C_2 fragment. The purified fragment was digested with Sph I which cuts at nucleotides 200 and 128 on the labeled strand to remove four of the six 5'-TA sites in the enhancer region (see Figure 3). This leaves a 196 bp fragment which was treated with λ exonuclease following irradiation in the presence and absence of 5-MIP (Panels III and IV). Panels I and II show the full length C_2 Hind III-Kpn I fragment irradiated in the presence and absence of 5-MIP, respectively, and treated with λ exonuclease. Arabic numerals at the top of each lane refer to the length of time in minutes of λ exonuclease digestion. Vertical numbers indicate nucleotide map positions.

gradual disappearance of bands in the ^{3}H-5-MIP reacted samples with increasing time of digestion indicates that psoralen photoadducts inhibit the processive nature of the enzyme but do not completely block the reaction.

Interestingly, in both experiments described above the strongest bands

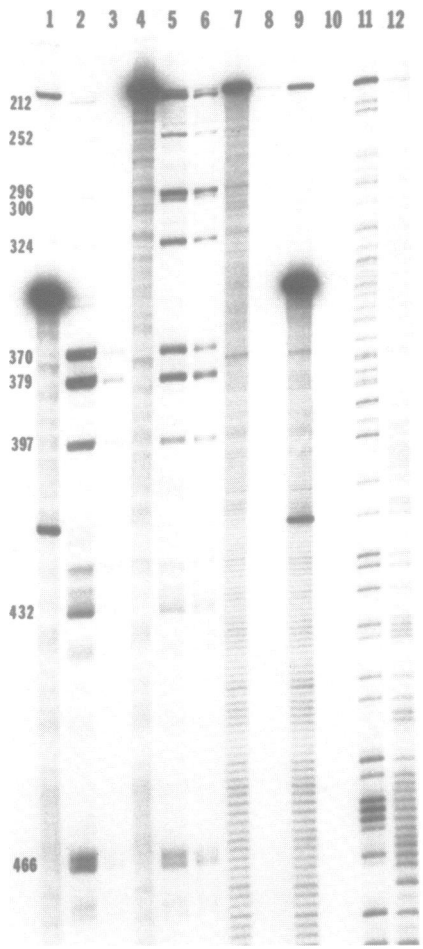

Figure 6. Psoralen accessibility pattern on the late side of the origin region. The Ava II-Sph I fragment in lanes 4-6 was psoralen photoreacted and then digested for 0, 5, and 20 minutes respectively with 65 U of λ exonuclease. In lanes 1-3 a portion of the sample shown in lane 4 was cleaved with Hpa II at bp 348 and then digested for 0, 5, and 20 minutes with 65 U of λ exonuclease. Lanes 7 and 8 show the Ava II-Sph I samples irradiated in the absence of 5-MIP and digested for 0 and 20 minutes with λ exonuclease. Lanes 9 and 10 show the corresponding Hpa II-Sph I samples treated for 0 and 20 minutes with λ exonuclease. Lanes 11-12 are the G and A+G Maxam-Gilbert sequencing lanes of the E fragment. A small region 5' to nucleotide 500 was too close to the end of the fragment to be sequenced.

were mapped to the two 72 bp direct repeats which function as bidirectional enhancers of transcription. The fainter bands correspond to the direct 21 bp repeats and T antigen binding sites. Since the enzyme digests from both 5'

ends, but may slow down when the fragment has been reduced to two half-length single strands, it was possible that the enzyme did not always reach the 3' end of the 362 bp molecule, thus accounting for the weak psoralen bands in the promoter region. In order to eliminate this possibility, the C_2 fragment was digested with Sph I, which cleaves at nucleotides 200 and 128. When the remaining end-labeled 196 bp fragment was digested with λ exonuclease, the pauses associated with the two remaining 5'-TA sites in the enhancer region were as strong as those seen in the samples which were not cleaved with Sph I, and the pauses associated with 5'-TA sites in the rest of the molecule were as faint as those seen in the full-length fragment (compare panels I and III, Figure 5). This suggests that the transition from strong to weak bands is probably sequence specific and unrelated to the distance of these sequences from the λ exonuclease start site at the 5' end of the fragment.

To determine whether sequences at the 3' end of the molecule were unusually psoralen inaccessible, or those associated with the enhancer were particularly psoralen accessible, Ava II-Sph I fragments which extended both 5' (nucleotides 201-560; termed E), and 3' (nucleotides 128-5122; termed F) from the origin region were analyzed. The additional psoralen stops found at the 3' end of the regulatory regions were as faint as those seen previously (data not shown). However, a second transition was noted 5' to the enhancers at approximately nucleotide 397 (see Figure 6, lanes 5 and 6). When this fragment was digested with Hpa II, which cleaves at nucleotide 348, and the remaining end-labeled fragment was digested, the conclusions were unchanged, indicating that these results were also independent of the relative position of sequences within the restriction fragment (Figure 6, lanes 2 and 3).

DISCUSSION

In these experiments 5-methylisopsoralen adducts on restriction fragments spanning the SV40 regulatory region have been mapped by a novel fine structure analysis. At multiple levels of photoaddition, 5-MIP monoadducts inhibit the forward movement of λ exonuclease and a series of specific bands of varying intensity are observed. The absence of bands in the irradiated control DNA indicates that the observed pattern is not related to an inherent difficulty of the enzyme to digest these sequences.

When the psoralen photobinding sites were mapped by comparing band positions to Maxam-Gilbert sequencing data, 5-MIP monoadducts were found 5' to each TA dinucleotide in the fragment. This work is in agreement with

previous reports showing that psoralens establish covalent bonds preferentially at 5'-TA sites. Initially, crystallographic and solution studies (summarized by Sobell et al., reference 27) indicated that simple intercalating agents like psoralen show a preference for binding 5'-CG and 5'-TA sequences in short chain oligomers. Gamper et al. subsequently showed that a double-stranded Kpn I linker is readily crosslinked by HMT while under identical conditions a corresponding Bam HI linker is resistant to modification (28). The linkers are identical except that a centrally located 5'-TA in the Kpn I linker is replaced by a 5'-AT in the Bam HI linker. They suggest that this preference is related to intercalation and reflects the ease of unstacking pyrimidine-purine sequences relative to purine-pyrimidine dimers. While this manuscript was in preparation, Zhen et al. reported that 5'-TA sites were preferred crosslinking sites for 4,5'8-trimethylpsoralen and 8-methoxypsoralen in restriction fragments isolated from pUC19 and pBR322 plasmids (21).

In these studies we show that 5-MIP photobinding sites within the enhancer region and immediately upstream were much more accessible than sequences elsewhere in the genome. A clear transition was noted immediately 3' to the enhancer region: by densitometer analysis the 5'-TA psoralen band at nucleotide 106 is at least 100 fold stronger than that at nucleotide 94 and those downstream (data not shown). Cleavage of the C_2 fragment with Sph I to remove most of the enhancer sequences and subsequent digestion with λ exonuclease gave the same result, verifying that this transition point was independent of the distance of the sequences from the 5' start site of exonuclease digestion. Approximately 150 bp upstream from the enhancers at bp 397, a second region of transition was observed. Digestion of the Ava II-Sph-I E fragment with Hpa II, which cleaves at bp 348, and subsequent treatment with λ exonuclease gave the same result, indicating that this transition is also independent of the location of the junction relative to the 5' end of the labeled strand.

The psoralen hypersensitive photobinding region spans sequences of unique biological significance. The 3' transition point (bp 106) occurs precisely at the 3' boundary of the enhancers. Jongstra et al. have shown that sequences contained mostly within the enhancer region are sufficient to generate a visible nucleosome gap in a subpopulation of minichromosomes (30). The psoralen hypersensitive sites overlap the major late promoter which initiates late transcription at bp 325. Sequences within this region are also critical for the formation of DNase I hypersensitive sites. Initially,

Cremisi reported the formation of three such hypersensitive sites on the late side of the Bgl I site (29). The first of these occurred around nucleotide 190 when the early genes are expressed. The other two are centered around nucleotides 270 and 370 and occur after or during viral replication when the late genes are expressed. More recently, Jongstra et al. have delineated two major regions of DNase I hypersensitivity in this region (30). One of these extends from approximately nucleotide 110, which coincides with the early border of the enhancers and the psoralen hypersensitive site, to nucleotide 323 ± 20. This is within the upstream transition point of the psoralen hypersensitive region.

The structural and molecular mechanisms underlying the generation of both the hypersensitive photobinding sites and the nucleosome free region in overlapping segments of the genome are unknown. Although regulatory sequences contained within the enhancer region have alternating purine pyrimidine tracts with the potential to form Z-DNA, and sequences within the T antigen binding sites have two sequence directed bends, there is no evidence that such perturbations can affect the specificity of psoralen photobinding (32-34). Since this technique detects only covalently bound adducts, it is not possible to determine the role that such deviation from regular B-form DNA might have on intercalation. It is also formally possible that this region is uniformly accessible to 5-MIP intercalation, but that either photoaddition is favored in the hypersensitive region or structural changes such as kinks are more readily induced by photobinding in and near the enhancer region, and these changes are more readily detected by λ exonuclease.

We suggest that sequences in this region contain a structural alteration that differs from those previously described. While Clarke et al. have shown by in vitro reconstitution experiments that nucleosomes can form over origin region DNA (35), both Wasylyk et al. and Hiwasa et al. have shown that this occurs with a much lower frequency than nucleosome formation over the rest of the genome, suggesting that sequences within the origin region cannot easily wrap around the histone core (36,37). Structural factors which affect the twist, tilt or roll of the helix might account for both this phenomenon as well as the hypersensitive photobinding properties of this region.

Previous reports have shown that neighboring sequences can play a role in the positioning of psoralen photoadducts. We have demonstrated that both HMT and 5-MIP display different binding preferences for the three unique Bgl I restriction sites in pBR322 (38). This preference cannot be explained

on the basis of the distribution of 5'-TA sequences within the restriction site. In addition, Zhen et al. found that only 70% of 20 5'-TA dinucleotides within the DNA fragments they analyzed appeared to constitute efficient crosslinking sites for HMT (21). Finally, in the Kpn I and Bam HI restriction site analyses mentioned previously, Gamper et al. showed that the 100 fold preference of HMT for Kpn I linkers over Bam HI linkers decreases to only 10 fold when the same restriction enzymes are examined on full length SV40 DNA (28). This suggests that neighboring sequences can markedly affect relative psoralen photobinding affinities.

The experiments described in this paper demonstrate the existence of a continuous region of altered psoralen photobinding affinity in the SV40 regulatory region. We suggest that these sequences contain a helical structure that differs significantly from that of the surrounding sequences. Such regions may be distinguished by their particular sensitivity to isopsoralen photoaddition, and may be detected by the psoralen mapping technique described.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Nancy Kurkinen for excellent technical assistance and graphic arts; Gary Wiesehahn, Alex Elbrecht, Gordon Robinson, Sophia Kondoleon and Stephen Isaacs for helpful discussions; and Cheryl Maslen for critically reading the manuscript. The authors would also like to thank James Shen and his group for their assistance with the initial Maxam-Gilbert sequencing. This work was supported by Public Health Service Grant 24799 to L.M.H. and a Tartar Trust Fellowship to E.A.O.

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