New procedure using a psoralen derivative for analysis of nucleosome associated DNA sequences in chromatin of living cells

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ABSTRACT

Sites for restriction endonuclease cleavage in double helical DNA are blocked from cleavage when the photoaffinity drug trimethylpsoralen is photo-bound at or near the site. In general, Hind III sites are about 15 fold more sensitive to inactivation than the other restriction sites which were tested, although sensitivity of different Hind III sites seems to vary somewhat depending on base sequences adjacent to the site. Hind III sites can be inactivated in two ways; one which completely blocks action of the specific restriction endonuclease and one permitting the introduction of a swivel which relaxes DNA supercoiling without producing a double strand break. Nucleosomes and perhaps other protein-DNA complexes can protect the underlying DNA sequence from trimethylpsoralen photobinding and thus protect restriction sites from inactivation. This property can be exploited to determine if specific sites are accessible to the psoralen probe *in vivo* and thus to establish if specific nucleotide sequences are nucleosome associated. Using this procedure evidence is obtained that nucleosomes on SV40 DNA in living infected cells are either distributed randomly or at many discrete alternate sites that approach a random distribution.

INTRODUCTION

Recent studies of chromatin structure have sought to establish the location of nucleosomes on specific DNA sequences. One approach used digestion of isolated nuclei or chromatin with micrococcal nuclease followed by mapping the position of the micrococcal nuclease cuts with respect to various restriction endonuclease cleavage sites. This approach has indicated that there may be some DNA sequences on which each nucleosome is precisely positioned (example, the non-transcribed spacer of *Drosophila* histone genes (1); however, more commonly, nucleosomes on sequences of viral DNA (2), structural genes of chromosomal DNAs (1,3-6) and on reiterated chromosomal DNA (7) may occupy a discrete set of alternate positions. The experimental approach of this research has been questioned because micrococcal nuclease was used to cleave internucleosomal DNA and the influence of the strong sequence selectivity of this enzyme has not been clearly resolved (8,9). However, cuts oc-

cur at similar sites, at least in some systems, when the same sequences are treated with DNase I (1,10), so it seems that the general conclusions concerning the distributions of nucleosomes on the nucleoprotein complexes could be correct. Another approach applied to isolated SV40 minichromosomes has been to examine the availability of various restriction enzyme sites for cleavage. These studies have indicated that nucleosomes are positioned randomly or near randomly with respect to specific restriction sites (2,11-13), although a specific nucleosome free region was located near the origin of replication in a subset of the minichromosomes (14,15).

Concern has also often been expressed about another feature of the experimental approach of this research. The nucleosome mapping studies have examined distributions existing in isolated nuclei or in nucleoprotein complexes removed from their normal intracellular environment after having been subjected to nucleolytic cleavage and exposure to non-physiologic solutions. It is uncertain whether or not non-histone chromosomal proteins involved in aligning nucleosomes may be released or that manipulations in vitro may otherwise induce nucleosomal translocations. Thus there is a possibility that the alternate or even random nucleosomal sites observed on viral DNAs and on gene sequences of nuclear DNAs could be induced by the experimental conditions. While different control experiments have explored these possibilities, uncertainties such as these are difficult to resolve by purely in vitro studies. What is required is an approach for examining the DNA sequences bound to nucleosomes in the living cells. The present report focuses on the development of an approach which may assist in attaining this goal.

Psoralen derivatives used as photoaffinity probes have been applied in the past to investigate similar problems. Certain derivatives such as trimethylpsoralen (me₃psoralen) enter cells readily and upon absorption of 340 to 380 nm light photobind at accessible sites of attachment on nucleic acids. The advantage of this approach is that the sites of photobinding are determined in vivo. The cells can subsequently be disrupted and chromatin fractionated in the absence of the photoactivating light without changing the distribution of the covalently bound adducts. Previously two different methods were developed to probe the structure of nucleoprotein complexes. The first application utilized large doses of psoralen and light to approach saturation of accessible DNA in the chromatin. It was shown that internucleosomal DNA was preferentially accessible to the probe and could thereby become interstrand crosslinked in regular repeating arrays (16-18). Studies of this kind provided some of the early supportive evidence for the nucleosomal structure of

chromatin. A second distinct procedure has studied the rate of intercalative photobinding to DNA at low doses of psoralen and light. Since binding of intercalative agents generally involves unwinding the double-helix, the rate of binding is sensitive to the level of torsional tension in the double-helix. Analysis of the photobinding rate to DNA in eukaryotic and prokaryotic cells has enabled quantitative assay of the unrestrained superhelical tension in these chromosomal DNAs (19) and has also enabled the determination of the number of domains of supercoiling in chromosomes of living cells (20).

A tacit, though not critical, assumption in this earlier research was that psoralen derivatives bind to DNA with little sequence specificity. Studies using bacterial DNAs of various base compositions (21), different satellite and main band DNAs from *Drosophila melanogaster* (22) and synthetic DNAs (21) suggested that there was no marked dependence of rate and extent of binding of psoralen derivatives on base composition; however, binding to the homopolymers dG:dC and dA:dT was much reduced compared to the respective alternating copolymers dGC:dGC and dAT:dAT (21).

Here we show that the cleavage of DNA by restriction endonucleases at their specific recognition sites is blocked when the DNA has photobound me_3 -psoralen. All of the restriction sites that were tested were approximately equally sensitive to inactivation by me_3 -psoralen with the exception of the Hind III site, which was unusually sensitive. The results support the conclusion that psoralen adducts which block restriction endonucleases are formed preferentially at or near the recognition sequence for Hind III. We also develop an approach exploiting this phenomenon, to probe the accessibility to me_3 -psoralen of specific Hind III sites on the SV40 genome *in vivo*. We then describe how this method can be applied to investigate the sequence association of nucleosomes in living cells.

MATERIALS AND METHODS

Enzymes and chemicals: 4,5',8'-trimethylpsoralen (me_3 -psoralen) was obtained from the Paul B. Elder Company (Bryan, Ohio). The ^3H labeled me_3 -psoralen was prepared commercially by New England Nuclear Corp. It was repurified at about 2 month intervals by thin layer chromatography as described (19) and used at a specific activity of 1.1×10^6 cpm/ μg (counting efficiency about 40%). The restriction endonucleases (Hind III, EcoRI, PstI, SalI, BamHI and SmaI) were purchased from Boehringer Mannheim Biochemicals.

Growth and radioactive labeling of SV40 infected cells: SV40 virus strain Rh 911 was propagated at low multiplicity from single plaques and grown in CV-1

cells in 90 mm plastic petri dishes in MEM medium containing 5% to 10% fetal calf serum. Subconfluent cultures were infected with 5 pfu per cell of purified virus. Labeling with ^{14}C -thymidine (0.1 to 0.6 uCi/ml, 51 mCi/mmmole) was begun 20-28 hr after infection. In studies of nucleosome distribution in vivo labeling was for 4 hrs beginning 44 hrs post infection.

Purification of pMB9, SV40 DNA and minichromosomes: The ^{14}C -thymidine labeled pMB9 DNA was purified from *E. coli* HB101 using described procedures (19). For studies of PstI and SmaI sites which are absent from pMB9 DNA, PBR322 and cDm703 DNAs were prepared by similar procedures. Covalently continuous molecules were purified by equilibrium centrifugation on ethidium bromide - CsCl density gradients (19). The ^{14}C -thymidine labeled SV40 DNA was purified from SV40 infected cells prepared as described above from cells two days post-infection. Viral DNA was purified from the SDS lysed cells by the method of Hirt (23) and covalently continuous circular SV40 DNA molecules obtained by equilibrium density gradient centrifugation as above.

Minichromosomes were prepared from SV40 virus which had been purified using a described procedure (24) from CV-1 cells harvested 5 to 8 days post infection. The purified virus were dialyzed from CsCl into a solution containing 0.01 M Tris (pH 7.5) and 0.10 M NaCl. They were disrupted by adding an equal volume of a solution containing 0.2 M glycine (pH 10.0), 1 mM dithiothreitol, and 1 mM EDTA. Minichromosomes were purified by sedimentation (1 hr, 50,000 rpm) in a 5-20% sucrose gradient containing 0.01 M Tris (pH 7.5 at 4°C), 0.10 M NaCl, 0.1% Triton X-100, 1 mM EDTA using a Beckman SW-55Ti rotor. This procedure follows one previously described (24). Fractions containing the ^{14}C -labeled SV40 DNA were pooled, diluted appropriately and used immediately in the experiments described below. Analysis of the minichromosome associated proteins by SDS polyacrylamide gel electrophoresis indicated a composition similar to that described before (24).

Photobinding of $\text{me}_3\text{psoralen}$: Methods were as described previously (19). For reactions with purified DNA or minichromosomes ^3H - $\text{me}_3\text{psoralen}$ was added to a concentration 1.2 ug/ml from a stock ethanolic solution to solutions containing 0.01 M Tris (pH=7.5), 0.10 M NaCl and 1 mM EDTA (and in the case of minichromosomes approximately 0.1% Triton X-100). The mixture was allowed to equilibrate with the DNA for 5 min and irradiated with either a Burton model 9110 lamp (Cavitron Corp., Van Nuys, California) or for higher doses, a Model B-100A Blak-ray ultraviolet lamp (Ultra-Violet Products, Inc., San Gabriel, California). At the sample positions the incident light intensities were roughly 9 KJ/M^2 per min and 120 KJ/M^2 per min respectively. A short wave-

length UV filter was used in all experiments and in some experiments for exposures exceeding 10 min a 1 cm water filter was also employed. To obtain high psoralen: DNA ratios (in excess of 10-20 molecules photobound per molecule) repeated additions of $\text{me}_3\text{psoralen}$ were done during irradiation. Mini-chromosomes were then deproteinized in the dark by incubating 10 min at 60°C with 0.1 to 0.2% SDS. The DNA was separated from unbound $\text{me}_3\text{psoralen}$ by repeated chloroform extraction. DNA was precipitated with ethanol and resuspended in buffer solutions appropriate for reaction with a specific restriction endonuclease which for Hind III was 0.05 M NaCl, 0.01 Tris (pH=7.6), 0.01 M MgCl and 14 mM dithiotriethiol.

Photobinding to DNA in SV40 infected CV-1 cells (2 days post infection) was done at 25°C on cells suspended at a concentration of 5×10^6 cells/ml in balanced salt solution. Other conditions were similar to those used with mini-chromosomes. Following photobinding, SV40 DNA was purified as described above including steps to eliminate unbound $\text{me}_3\text{psoralen}$.

Cleavage with restriction endonucleases: Purified DNA was dissolved at a concentration less than 100 $\mu\text{g/ml}$ in a solution suitable for the specific endonuclease. The concentrations of endonuclease added were 2-3 times the amount required to completely cleave the $\text{me}_3\text{psoralen}$ free DNA during a parallel reaction.

Electrophoretic analysis: DNA molecules and their restriction fragments were separated by electrophoresis for 16 to 18 hrs at 30-40 ma on horizontal 1.4% agarose gels at 6.3 volts/cm and 25°C . The gels were formed in a solution containing 0.05 M sodium phosphate pH=7.1, plus 1 mM EDTA. DNA samples (0.2 to 2 μg per lane) were dissolved in the same buffer plus 1% SDS, 5% glycerol, 50 $\mu\text{g/ml}$ bromophenyl blue and layered into slots in the gel. After electrophoresis, the DNA bands were stained by incubating 0.5 hr with 0.5 $\mu\text{g/ml}$ ethidium bromide and photographed on a UV transilluminator. Bands were excised, the gel solubilized by heating and suspended in a xylene-Triton based scintillation fluid.

RESULTS

Effect of trimethylpsoralen photoadducts in DNA on the action of restriction endonuclease:

Purified plasmid pMB9 DNA containing known amounts of photobound $\text{me}_3\text{psoralen}$ was reacted with restriction endonuclease Hind III and analysed to determine the number of DNA molecules which could be cut. Figure 1 shows the results of electrophoresis of this DNA on an agarose gel capable of separat-

Figure 1. Electrophoresis on an agarose gel of pMB9 DNA containing varied amounts of covalently bound me_3 psoralen after exhaustive reaction with restriction endonucleases.

Purified labeled pMB9 DNA (650 ^{14}C cts/min/ug) was mixed with ^3H - me_3 -psoralen and exposed to near UV light to photobind the me_3 psoralen. DNA samples removed after different doses were repurified free of non-covalently bound me_3 psoralen and the average number of psoralen residues per pMB9 DNA molecule was determined from the $^3\text{H}/^{14}\text{C}$ radioactivity ratios. Mixtures containing 0.5 ug of the photoreacted DNA, 2.0 units Hind III or 2.0 units EcoRI restriction endonuclease in a suitable reaction buffer were incubated at 37°C for 60 min using conditions sufficient to cleave all restriction sites in control DNA free of me_3 psoralen. Electrophoresis on agarose gels as described in Methods, the positions of covalently closed circular, ccc; open circular, oc; and linear, l, DNA are noted. Lanes a thru f show results of reactions with Hind III and Lanes h thru m with EcoRI. Lane g shows the unreacted control DNA containing predominant ccc supercoiled DNA. pMB9 DNA contained the following average numbers of me_3 psoralen adducts per DNA molecule. Lanes a and h, zero; lanes b and i, 19; lanes c and j, 32; lanes d and k, 44; lanes e and l, 53; lanes f and m, 56.

ing the covalently closed circular (ccc), linear and nicked circular isomers of the pMB9 DNA. The distribution in lane g shows that the unreacted DNA was composed predominantly of ccc molecules but that approximately 10% of the DNA was in the nicked circular form and that there were no detectable linear forms. All of the DNA free of me_3 psoralen was cut to the linear form by the Hind III endonuclease. The number of pMB9 DNA molecules which could be cleaved at their single Hind III site was decreased by an amount dependent on the average number of me_3 psoralen adducts per DNA molecule. The ccc DNA- me_3 psoralen complexes which were resistant to the Hind III endonuclease had electrophoretic mobilities reduced relative to the me_3 psoralen free ccc DNA. This occurred because the superhelical density of the DNA was reduced in proportion to the amount of photobound me_3 psoralen (25,26). There was also a slight decrease in the electrophoretic mobilities of the linear and nicked isomers

because of the increased molecular weight and stiffness of DNA containing me_3 -psoralen.

The endonuclease resistant pMB9 DNA fraction cannot be attributed to slower kinetics of hydrolysis or to irreversible inactivation of the Hind III endonuclease mediated by the psoralen-DNA complex. Fresh, untreated pMB9 DNA added to the mixture of Hind III endonuclease and me_3 psoralen-DNA after most of the sensitive DNA in the mixture was cleaved, was rapidly and completely hydrolysed to the intact linear DNA form (data not shown). Also the components obtained in experiments like that of Fig. 1 seem to be end-products; further incubation causes no further cleavage (data not shown).

The experiments described here used [^{14}C]-pMB9 DNA and [^3H]- me_3 psoralen. The radioactivity ratio provides a direct measurement of the number of psoralen adducts per pMB9 molecule. The bands on gels such as that shown in Figure 1 were cut out and the relative amounts of labeled DNA in the different DNA fractions determined by measuring the radioactivity. The fraction of the pMB9 DNA which could be converted to linear form by the Hind III endonuclease decreased with increasing numbers of psoralen residues per DNA molecule, while the fractions of both the open circular and ccc forms increased (Fig. 2A). This shows that the bound me_3 psoralen can have two effects on the Hind III reaction; one which completely blocks the endonuclease reaction and the other which permits only a loss of DNA supercoiling while maintaining the DNA in a circular form. These results suggest that the me_3 psoralen can bind at or near the Hind III site in at least two functionally different ways. A possible explanation for this difference is proposed in the Discussion section.

In comparison with the results obtained with Hind III endonuclease, cleavage of the same pMB9 DNA preparations with EcoRI endonuclease was less sensitive to the presence of bound me_3 psoralen (Fig. 1). When plotted on a logarithmic scale there was a linear decrease in the number of cleavable restriction sites with increasing me_3 psoralen content, but the slope of the least squares line for the Hind III inactivation was about 15-fold steeper than that for the EcoRI inactivation (Fig. 2B). When there were about 55 me_3 -psoralen adducts per pMB9 DNA or about one per 100 base pairs about 7% of the EcoRI sites could not be cleaved to form a double strand break. The EcoRI restriction enzyme reaction was, therefore, inactivated at a rate expected for a target of about 8 base pairs, which is about the size of the recognition sequence for the enzyme (six base pairs). Thus the EcoRI restriction site was no more likely to be affected by me_3 psoralen than any random sequence of that size. Preliminary experiments using BamHI, PstI, SalI and SmaI restriction en-

Figure 2. Effects of trimethylpsoralen on the cleavage of pMB9 DNA by Hind III and EcoRI endonuclease.

A) Results are compiled from repeated analyses of Hind III cut pMB9 DNA such as those shown in Figure 1. The bands from the agarose gel electrophoresis were cut out and radioactivity in the ¹⁴C-labeled DNA measured to determine the fractional amounts of DNA in the: ccc, ●—●; open circular, ▲—▲; and linear, ■—■ isomers.

B) Percent of cleavable pMB9 sites plotted on a logarithmic scale. A cleavable site is defined as one on which the restriction endonuclease can make a double-strand break. Hind III sites, ●—●; EcoRI sites, ■—■.

donucleases have shown sensitivities to me₃psoralen similar to EcoRI. It seems, therefore, that cleavage at the Hind III site is unusually sensitive to the me₃psoralen compared to these other restriction sites.

It is known that negatively supercoiled DNA's have enhanced photobinding reactions with me₃psoralen and the photobinding rate is stimulated in proportion to the negative torsional tension (19). When the experiment of Figure 1 was repeated using pMB9 DNA relaxed by reaction with *E. coli* topoisomerase I, the Hind III site was also preferentially inactivated by the me₃psoralen (data not shown). Thus the preferential inactivation of the Hind III site is

not determined by the torsional tension in the DNA.

Effect of $\text{me}_3\text{psoralen}$ on Hind III sites in SV40 DNA: The SV40 genome contains 5243 nucleotides and six Hind III sites (27). Exhaustive cleavage of the DNA with Hind III endonuclease resulted in six characteristic fragments (referred to as first order fragments), two of which are so close in size that they were not separated by gel electrophoresis (Fig. 3). When the SV40 DNA was reacted with $\text{me}_3\text{psoralen}$ before digestion with the restriction endonuclease, some of the Hind III sites could not be cleaved and as a result new, higher molecular weight fragments appeared after gel electrophoresis. Some of these new bands could be unambiguously identified from their electrophoretic mobilities, as being the products of a specific blocked Hind III site (Fig. 3). From this analysis it can be concluded that each of the Hind III sites that was tested was inactivated by the $\text{me}_3\text{psoralen}$ in a dose dependent manner over a similar range as the Hind III site in pMB9 DNA. The only site which was not tested quantitatively was the site between Fragments 2 and 4, which, when blocked, produced a larger fragment of only slightly smaller size than Fragment 7. However, in other experiments, it can be seen qualitatively (example, in Fig. 5) that this site was also inactivated over at least roughly a similar dose range. Assuming that the psoralen adduct can block the restriction endonuclease by either of the mechanisms observed with pMB9 DNA, the observed inactivation rate would include those events which entirely block the endonuclease as well as those that allow the nuclease to introduce only a swivel in the SV40 DNA.

When larger amounts of $\text{me}_3\text{psoralen}$ were bound to the SV40 DNA, a significant fraction of the DNA was completely refractory to the Hind III endonuclease and retained the properties of ccc DNA (Fig. 3). If the number of photoadducts per DNA molecule exceeded 200, most of the DNA remained circular. In this state a significant fraction of the DNA lost its supercoiling while retaining its circularity, suggesting that this DNA could still be nicked without cleavage. Thus, it seems that Hind III sites in SV40 DNA can be affected by $\text{me}_3\text{psoralen}$ in the same two ways observed with the single pMB9 site. Detailed analysis to be described below (Figs. 4-6) will show that even though all the Hind III sites are sensitive to psoralen adducts, the sites differ somewhat in their sensitivity. It appears, therefore, that the inactivation of Hind III sites is influenced by base sequences surrounding the actual recognition sequence.

$\text{Me}_3\text{psoralen}$ bound to SV40 minichromosomes: $\text{Me}_3\text{psoralen}$ was photobound to DNA in isolated minichromosomes, the DNA purified free of protein and treated ex-

Figure 3. The SV40 genome before and after cleavage with Hind III endonuclease. On the top is shown a map of the six Hind III sites in SV40 DNA. The numbers designate the fragments that would be expected (arranged in order of increasing size) after cleavage with Hind III endonuclease. Larger fragments shown in the outer rings are those expected if a single Hind III site is blocked. Sizes of the fragments are: #0, 0.215 kb; #1, 0.447 kb; #2, 0.526 kb; #3 0.662 kb; #4, 1.118 and 1.169 kb (not resolved); #5, 1.565 kb; #6, 1.768 kb; #7, 1.983 kb; #8, 2.294 and 2.387 kb (not well resolved). The fragment obtained by fusing #2 and #4 is not designated because it cannot be completely resolved from #6, although it can be seen in the electrophoretic

analyses (Fig. 5).

On the lower left is the electrophoretic analyses of SV40 DNA containing small amounts of $\text{me}_3\text{psoralen}$ after exhaustive reaction with Hind III endonuclease. On the lower right is a similar analysis of SV40 DNA containing larger amounts of $\text{me}_3\text{psoralen}$. The bands are labeled according to the system described above. Fragment #0 cannot be seen in the analysis on the left. (However, see Fig. 5). The average numbers of $\text{me}_3\text{psoralen}$ adducts per SV40 DNA molecule are: a) 0; b) 7; c) 15; d) 18; e) 23; f) 24; a') 0; b') 98; c') 200; d') 374; e') 549.

haustively with Hind III endonuclease. Results compiled in Fig. 4 describe the relative amounts of specific Hind III fragments or bands which were obtained at different $\text{me}_3\text{psoralen}$:DNA ratios. The effect of the psoralen adducts was changed markedly from that observed when the photobinding was directly on naked DNA. With naked DNA the amount of DNA in first order fragments (#0,1,2,4 and 6) declined progressively as the psoralen:DNA ratios increased, until at the highest ratios there were no observable first order bands. In contrast, when the $\text{me}_3\text{psoralen}$ was photobound to minichromosomes, the DNA purified from the chromosome was only partially resistant to the restriction endonuclease and even at the highest psoralen:DNA ratios first order bands were formed. For example, at the highest psoralen:DNA ratios examined, about 25% by mass of the total SV40 DNA could be cleaved to Fragment 6 and this fraction seemed nearly independent of psoralen:DNA ratio at ratios exceeding 40. In controls lacking $\text{me}_3\text{psoralen}$, Fragment 6 comprised 35±5% of the total DNA mass after similar exhaustive hydrolysis (the theoretical fraction is 33.8%). Thus, it would seem that roughly 25/35±70% of the SV40 molecules in minichromosomes have both Hind III sites defining the termini of Fragment 6 protected from inactivation.

Likewise the second order bands (#3,5,7 and 8) produced by blockage of a single Hind III site, were formed differently when $\text{me}_3\text{psoralen}$ was photobound to minichromosomes and free DNA. For example, the amount of band 8 obtained from naked photobound DNA reached a maximum at roughly 100 $\text{me}_3\text{psoralen}$ residues per SV40 DNA molecule and then declined to undetectable levels at higher $\text{me}_3\text{psoralen}$:DNA ratios. In contrast, the second order bands from the DNA of photobound minichromosomes reached a maximum at about 40 psoralens per DNA and then remained essentially constant at higher psoralen:DNA ratios. This is as expected if the Hind III sites at each end of the DNA sequence of a second order band were protected from inactivation in certain of the minichromosomes. In this kind of analysis binding of psoralen at a specific Hind III site is inferred from the appearance of a second order band which includes that site. Decrease in amounts of first order bands is quantitated to infer

Figure 4. Inactivation of Hind III sites in purified SV40 DNA and in SV40 minichromosomes treated with $\text{me}_3\text{psoralen}$. Isolated minichromosomes or purified SV40 DNA were treated with $\text{me}_3\text{psoralen}$ and light as in Fig. 1 and DNA was isolated free of proteins and unbound $\text{me}_3\text{psoralen}$. The DNAs were reacted exhaustively with Hind III endonuclease and fractionated on an agarose gel as in Fig. 3. Amounts of DNA in each band were determined and results from selected bands are shown above as fractions of total recovered DNA. Minichromosome DNA (upper figure): Fragment 6, $\circ$ --- $\circ$; Fragment 7, $\blacktriangle$ — $\blacktriangle$; Band 8 $\triangle$ — $\triangle$. Naked DNA (lower figure): Fragment 6, $\circ$ --- $\circ$; Band 4, $\square$ — $\square$; Band 8, $\triangle$ — $\triangle$.

In the lower figure the solid symbols $\bullet$ and $\blacksquare$ indicate the theoretical values for Fragments 6 and Band 4 respectively when all Hind III sites are cleaved.

accessibility of the two adjacent Hind III sites which define the termini of a specific fragment. Reduction in amount of a second order band infers accessibility of the Hind III sites adjacent to the site included in the fragment. With these assumptions we conclude from data of the kind shown in Fig. 4 that there are no Hind III sites in the isolated SV40 minichromosomes that are always accessible or never accessible to inactivation by $\text{me}_3\text{psoralen}$. The results agree with earlier studies where the cleavage of DNA in SV40 minichromosomes by restriction endonucleases was examined directly without the use of

psoralen probes (2,11-13,28,29). It was shown that specific sites are accessible in some minichromosomes but not in others and it was concluded that the distribution of nucleosomes on the underlying DNA sequence is either random or includes several discrete sets of alternate positions. While our findings support this conclusion, the result which is of most importance to present goals is that each Hind III site is protected from inactivation in certain minichromosomes. Thus it seems that Hind III sites, in spite of their sensitivity to inactivation by $\text{me}_3\text{psoralen}$, can be protected from photobinding and that the Hind III sites that are photobound are probably restricted to internucleosomal sequences just as occurs with the bulk of the DNA in nucleoprotein complexes (16-17,30). While it is possible that some non-histone proteins may also limit $\text{me}_3\text{psoralen}$ photobinding, quantitative analysis to be considered in the Discussion suggests that it is primarily the nucleosomes that limit photobinding.

It was noted that the initial rate of $\text{me}_3\text{psoralen}$ photobinding to minichromosomes was about 10 fold slower than to naked DNA at constant light flux, constant $\text{me}_3\text{psoralen}$ and DNA concentrations. Only a small part of this difference (2-fold) can be accounted for by the torsional tension present in the free supercoiled DNA (19). The primary reason for this difference is probably the limited accessibility of the DNA in minichromosomes. For example, in the data of Fig. 4, the highest light flux used with minichromosomes and purified DNA was similar, yet the maximal amount of photobound $\text{me}_3\text{psoralen}$ was about 5 fold less to the minichromosomal DNA. Since the psoralen adducts are restricted to internucleosomal sequences (see Ref. 30), the average linear density of the adducts should be about 5 fold greater in the internucleosomal DNA than in naked DNA at comparable total $\text{me}_3\text{psoralen}$:DNA ratios. Thus, one might expect saturation of accessible Hind III sites at lower total $\text{me}_3\text{psoralen}$:DNA ratios in minichromosomes than in naked DNA. This effect is shown in Fig. 4, where saturation of accessible Hind III sites in the minichromosome seems to occur in the range 40-80 $\text{me}_3\text{psoralen}$ molecules per SV40 DNA while with naked DNA this ratio is about 400.

Quantitative analysis of the accessibility of restriction sites in nucleosome DNA complexes: Since a DNA sequence associated in a nucleosome seems to be inaccessible to $\text{me}_3\text{psoralen}$, it would seem possible to exploit the above results to define the nucleosome association *in vivo* of specific restriction endonuclease sites. To carry out such analysis, it was necessary to develop a more precise analytical approach than that used for the *in vitro* studies. The absolute rates of $\text{me}_3\text{psoralen}$ photobinding to DNA and the inactivation of

Hind III sites differ substantially in vitro and in vivo because of a number of factors that are difficult to control. These may include the permeability of cell and nuclear membranes to $\text{me}_3\text{psoralen}$, competitive binding of the $\text{me}_3\text{-psoralen}$ to cellular components other than DNA, and light scattering by cells in suspension. It is therefore desirable to develop a parameter which can provide an internal control permitting direct comparison of data from in vivo experiments. In the analysis below, it is assumed that the total number of photobound $\text{me}_3\text{psoralen}$ adducts averaged over the entire SV40 DNA molecule provides such a normalization factor.

As was shown above (Fig. 2), the functional dependence of the number of Hind III sites remaining cleavable after binding a determined number of psoralen adducts is exponential. The equation for inactivation of a restriction site can be written

$$\frac{dN}{dR} = -SN \quad 1)$$

where N is the number of cleavable sites; $R=r/f$ where $\underline{r}$ is the number of $\text{me}_3\text{-psoralen}$ adducts per DNA molecule and $\underline{f}$ is the fraction of the total DNA sequence available for photobinding; $S=pk$, where p is the fraction of a specific restriction site available for photobinding inactivation and k is the intrinsic reactivity of that site for $\text{me}_3\text{psoralen}$. Then

$$\frac{dN}{N} = -(p/f)kdr \quad 2)$$

which can be integrated to obtain

$$\log N/N_0 = (-p/f)kr \quad 3)$$

where N_0 is the total number of a specific restriction site in the DNA population.

Equation 3 indicates that a plot of r (the number of photobound $\text{me}_3\text{-psoralen}$ adducts per DNA molecule) versus the logarithm of the fraction of cleavable restriction sites should yield a straight line having a slope of $-pk/f$. In purified DNA all sites are accessible to the psoralen probe and all DNA is also available. Therefore, both p and $f=1$ and the slope equals the reactivity, $-k$. If a particular Hind III site was always nucleosome associated, $p=0$ for that site and the slope would be zero. If nucleosomes were randomly distributed, the probability of a specific Hind III site being nucleosome associated would equal the fraction of DNA occupied by nucleosomes at any time or $p=f$ and therefore the slope $= -k$, just as in the case of naked DNA. The nucleosome repeat length is about 200 base pairs of which about 60 base pairs are in the internucleosomal region. It would be expected that $f =$

60/200 or about 0.3. If a particular Hind III site is always accessible, $p=1$ and the slope would be -3.3, or about three times as steep as the inactivation curve for naked DNA. Thus, studies of the inactivation of specific restriction sites by $\text{me}_3\text{psoralen}$ photobound in vivo should provide information about the association of nucleosomes to those sequences in vivo.

Inactivation of Hind III sites in SV40 DNA by $\text{me}_3\text{psoralen}$ in vivo: $\text{Me}_3\text{-psoralen}$ was photobound to SV40 DNA in living infected cells and the SV40 DNA purified and analyzed as shown in Fig. 5. Using reaction conditions sufficient to cleave all Hind III sites with the Hind III endonuclease, the same higher order bands were observed as seen with DNA photobound in vitro. In all the in vivo experiments reported here, the $\text{me}_3\text{psoralen}$:DNA ratio was kept at low levels (less than 1 $\text{me}_3\text{psoralen}$ per 400 base pairs DNA) to minimize the possibility that the photobound adducts may themselves disrupt the chromosome structure. At these levels the unwinding of the double-helix averaged over all photobound DNA is less than 0.06 degrees per base pair. It should also be

Figure 5. Inactivation of Hind III restriction endonuclease sites in SV40 DNA in vivo. (^3H)- $\text{me}_3\text{psoralen}$ was added to SV40 infected CV-1 cells and photobinding carried out as described in Methods. The SV40 DNA was purified, reacted with excess amounts of Hind III endonuclease, and the digests analyzed by agarose gel electrophoresis. The amounts of photobound $\text{me}_3\text{psoralen}$ per SV40 DNA molecule were: a) 0; b) 5; c) 6; d) 8; e) 10; f) 11; g) 12 molecules per molecule.

emphasized that the intercalation binding constant for $\text{me}_3\text{psoralen}$ is quite small, so that the number of intercalated (but not photobound) adducts is not expected to contribute significantly (21).

The second order fragments obtained after hydrolysis with the restriction endonuclease were analyzed quantitatively to test the accessibility of specific Hind III sites to the $\text{me}_3\text{psoralen}$ probe. Results for two such sites are shown in Fig. 6. It is apparent that the initial inactivation rates at low psoralen:DNA ratios for these two sites are indistinguishable when photobinding is carried out in infected cells or in vitro on purified naked DNA. Similar results within the margin of error were also obtained for the other two sites which could be accurately tested. The data clearly rule against the possibilities that these sites are never occupied by a nucleosome or always occupied by a nucleosome. Since the slopes of the in vivo inactivation curves are essentially equal to $-k$, as determined from the in vitro analysis, the results are suggestive of random or near random distribution of nucleosomes on the underlying SV40 DNA sequence. The Hind III site between fragments 4 which is near the origin of replication and sometimes free of nucleosomes (14,15), has not been specifically studied to test the possibility of non-random associations in this region.

It is apparent that the inactivation rates for sites 5 and 7 differ substantially. The difference is maintained whether photobinding is done in vivo or on purified DNA, indicating that the intrinsic rate constants k for the two sites differ. Other Hind III sites in SV40 DNA which have been examined show sensitivities in the range at or between that of sites 5 and 7. For example, site 3 seems to be similar to that of site 7. Thus, it seems likely that the base sequence around a Hind III site influences the $\text{me}_3\text{psoralen}$ photobinding events which can inactivate this site.

DISCUSSION

The above results demonstrated the use of psoralen photoaffinity probes for identifying nucleosomal and internucleosomal DNA sequences. In favorable situations application of this approach could yield quite clear cut conclusions. For example, if precise positioning occurs of nucleosomes on the non-transcribed DNA sequence of Drosophila histone gene spacer (1), specific restriction sites in this sequence should be either completely resistant or completely sensitive to inactivation by the psoralen probe depending on whether the site is always in either internucleosomal or nucleosome associated DNA. As was stressed above, the great advantage of

Figure 6. The rates of inactivation of specific Hind III restriction sites in SV40 DNA by $\text{me}_3\text{psoralen}$. DNA bands from analyses such as that shown in Figure 5 were cut out and the amounts of DNA in each band were determined. The above figures show plots of $\text{me}_3\text{psoralen}:\text{DNA}$ ratio versus the logarithm of the percentage of a specific Hind III site which was cleavable ($\log 100N/N_x$). N , the total number of a specific Hind III site, was calculated from the total recovered DNA in the gel, assuming one site per SV40 DNA molecule. N_x , the number of cleavable sites, was calculated from $N_x(F_T - F_x)/F_T$ where F_x is the fraction of the total recovered DNA found in second order band X and F_T equals S_x/S_T where S_x is the size in base pairs of the DNA fragment found in band X and S_T is the size of the SV40 genome. Inactivation curves for two of the six sites are shown above. Sites 5 and 7 refer to the sites which, when inactivated, give rise respectively to Fragments 5 and 7. The analyses include experiments where photobinding was done *in vitro* to purified SV40 DNA $\bullet$ ----- $\bullet$ and where photobinding was done *in vivo* in infected cells $\circ$ ----- $\circ$. The above curves are traces of computed print-outs of least square fits of the raw data.

the procedure is that it can be applied to study chromatin structure in living cells using conditions which should minimally perturb the complexes under investigation. Thus, the approach may augment existing in vitro methods for mapping nucleosome sites, especially in cases where there are possibilities of experimentally induced translocations or disruptions of nucleosomes.

The initial application of the procedure described in this report was to measure the accessibility of Hind III sites in SV40 minichromosomes. It was demonstrated that sites 3,5 and 7 (as defined above) are protected from photoinactivation in some minichromosomes in vivo and not in others. Quantitative analysis of the inactivation indicated a near random distribution among these sites of the components that protect them from inactivation, implying that nucleosomes are randomly or near randomly distributed about the sites. Although there is a possibility that certain non-histone proteins could also protect Hind III sites from psoralen inactivation, such proteins could not by themselves generate a random pattern of protection even if they bound randomly to internucleosomal Hind III sites (provided there is at least one Hind III site that is nucleosome bound). If nucleosomes were precisely positioned on the SV40 DNA sequence, those Hind III sites in nucleosomes would be completely protected from me_3 -psoralen inactivation in spite of any randomizing effects of internucleosomal non-histone proteins. Thus the conclusions of this report do not depend in general on the assumption that only nucleosomes can protect restriction sites. Actually, quantitative analysis of the number of protected Hind III sites in minichromosomes is consistent with the prediction that randomly distributed nucleosomes are the major source of protection. Since the internucleosomal DNA of SV40 amounts to about $60/200 \approx 0.3$ of the 200 base pair repeat (31), one would expect randomly distributed nucleosomes to protect about 70% of a specific Hind III site. When all non-protected Hind III sites are inactivated at conditions of saturating me_3 psoralen, a first order DNA fragment requiring two adjacent protected Hind III sites for its production would comprise $0.7 \times 0.7 \approx 0.5$ of the amount produced when no Hind III sites were inactivated. A second order fragment requiring an inactivated Hind III site flanked by two protected ones would comprise $0.7 \times 0.3 \times 0.7 = 0.15$ of the amount when no sites were inactivated. These fractions are not far removed from those that are actually observed. For example, the data in Fig. 4 show that at saturation the second order fragment #7 and the band #8 amount to 0.19 and 0.20 of the theoretical

amount when no sites are inactivated. Likewise, the first order fragment #6 saturates at roughly 0.70 the amount when no sites are inactivated.

It is not known why Hind III sites are more sensitive to inactivation than other restriction sites. There are two general possibilities: a) The reaction of $\text{me}_3\text{psoralen}$ with the Hind III site could be unusual so that inactivating psoralen adducts are preferentially formed at or near this nucleotide sequence; b) The Hind III endonuclease could be unusual so that it is sensitive to psoralen adducts far removed from the Hind III recognition sequence. In the latter case the preferential inactivation of the sensitive Hind III sites would be explained by the target for inactivation being 15 times larger ($15 \times 6 = 90$ base pairs instead of the usual 6 base pairs). It should be emphasized that none of the conclusions of this report depend on whether the target is 6 or 90 base pairs. In any case the target is being randomly protected in SV40 minichromosomes *in vivo*. However it should be noted that there are two lines of evidence favoring the idea that the Hind III target is not unusually large. 1) Fragment 7 of SV40 DNA is generated when Hind III site 7 is inactivated and site 3 only 215 base pairs away is not. If the Hind III target approached 200 base pairs in size, one would expect overlap of these two close targets and thus fragment 7 would be less frequently produced. Results described above showed that no second order fragment is more abundant than #7 at any psoralen:DNA ratio. 2) If the target for Hind III site inactivation was 90 base pairs, many of the randomly distributed nucleosomes which protect the target would protect only part of it, so that at saturating doses of $\text{me}_3\text{psoralen}$ a much higher fraction of the targets would be hit. One can show that one randomly distributed nucleosome per 200 base pairs would totally protect only 25% of a specific 90 base pair sequence in different minichromosomes while about 70% of a 6 base pair sequence would be protected. Thus the formation of a specific first order fragment which requires two adjacent protected targets could occur in only $25\% \times 25\% = 6\%$ of the minichromosomes if the targets were 90 base pairs and randomly protected, while this fraction would be 50% for a 6 base pair target. The experimental results reviewed above are much closer to 50%, favoring the smaller target. Thus it seems that the Hind III recognition site or nucleotides very near it are unusual in their reaction with $\text{me}_3\text{psoralen}$, however, the actual mechanism of inactivation will have to be worked out before this is certain.

Whatever the mechanism, it is clear that the psoralen adducts can

have at least two different effects on the Hind III endonuclease, one blocking completely the endonuclease activity and the other permitting the introduction of only a swivel. It is worth noting that the swivel could be the result of an asymmetric inhibition of the enzyme permitting the cleavage of only one DNA strand. On the other hand, it is also apparent that a diadduct crosslink between the two normal sites of endonucleolytic cleavage could prevent a double-strand break, yet allow relaxation of DNA supercoiling. Future research in progress will be required to confirm either of these speculations and to work out other mechanistic details of the inactivation.

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