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Mapping the In Vivo Arrangement of Nucleosomes on Simian Virus 40 Chromatin by the Photoaddition of Radioactive Hydroxymethyltrimethylpsoralen

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Intracellular simian virus 40 (SV40) chromatin was photoreacted with a ^{3}H-labeled psoralen derivative, hydroxymethyltrimethylpsoralen (HMT), at 48 h postinfection. Psoralen compounds have been shown to readily penetrate intact cells and, in the presence of long-wavelength UV light, form covalent adducts to DNA, preferentially at regions unprotected by nucleosomes. The average distribution pattern of [^{3}H]HMT on the SV40 genome was determined by specific activity measurements of the DNA fragments generated by *Hin*dIII plus *Hpa*II or by *Atu*I restriction enzyme digestion. At levels of 1 to 10 [^{3}H]HMT photoadducts per SV40 molecule, the radiolabel was found to be distributed nonrandomly. Comparison of the labeling pattern in vivo with that of purified SV40 DNA labeled in vitro revealed one major difference. A region of approximately 400 base pairs, located between 0.65 and 0.73 on the physical map, was preferentially labeled under in vivo conditions. This finding strongly suggests that the highly accessible region near the origin of replication, previously observed on isolated SV40 "minichromosomes," exists on SV40 chromatin in vivo during a lytic infection.

The intracellular and viral nucleoprotein complexes of simian virus 40 (SV40) have a repeating structure of nucleosome beads very similar to that of cellular chromatin (for reviews, see 6, 24, 32). Both cellular and viral nucleosomes contain a core particle that consists of 146 base pairs (bp) of DNA (29, 50) wound around an octamer of proteins, two molecules each of the histones H_2A, H_2B, H_3, and H_4 (see reviews cited above). Histone H_1 is thought to be associated with the internucleosomal or linker regions both of cellular chromatin (55, 58, 60, 64) and of the intracellular viral complex (50, 59). The small size of SV40 and the availability of molecular information on the sequence and expression of the viral genome make it an attractive model for cellular chromatin structure and replication. A number of early studies utilizing SV40 chromatin supported the idea that nucleosomes are positioned randomly with respect to the DNA sequence (7, 9, 28, 37, 38). However, several recent investigations have indicated that a nonrandom arrangement exists on at least a subpopulation of SV40 chromatin molecules. A nucleosome-free region of approximately 300 to 400 bp has been detected at map positions 0.66 to 0.75 on isolated SV40 nucleoprotein complexes by electron microscopy (22, 45) and by endonuclease digestion (47, 53, 61–63). The function of this "open region" is unknown; however, these sequences contain the origin of replication (15, 35, 52), the region encoding the 5′ ends of early and late SV40 mRNA (13, 40, 56), as well as the preferred binding sites for T antigen (23, 42, 51, 57).

We have developed a system to analyze the nucleosome phasing on SV40 DNA in vivo, using the photoreaction of furocoumarin (psoralen) derivatives with intracellular DNA. These compounds have the ability to penetrate intact cells, intercalate into DNA, and, under the influence of long-wavelength UV light, covalently bind to pyrimidines of the DNA (4, 25, 33, 36). Because nucleosomal core DNA is preferentially protected from psoralen photoaddition (2, 65, 66), these derivatives are useful probes for studying the structure of both cellular (2, 3, 17, 66) and viral (16) chromatin.

In the experiments described below, a radioactive psoralen derivative was used as a reagent for labeling accessible regions of DNA in intracellular SV40 chromatin. SV40 DNA was isolated and restriction fragments were examined to determine the distribution of radioactive drug on the SV40 genome. This approach eliminates the extraction or fixation of SV40 chromatin required by previous in vitro nucleosome analyses. A region around the SV40 replication origin preferentially accessible to photoaddition was identified, demonstrating that there is a nonran-

dom distribution of nucleosomes on SV40 chromatin in vivo.

MATERIALS AND METHODS

Cells and virus stocks. All infections and virus preparations were carried out in CV-1 cells, an African green monkey kidney line obtained from P. Berg. SV40 strain 776 was obtained from K. Danna. Cells were grown in Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 5% fetal calf serum (Sterile Systems, Ogden, Utah). Virus stocks were prepared by low-multiplicity infection (0.01 PFU/cell) from plaque-purified stocks and were titered on CV-IP cells, a derivative of CV-1 cells obtained from P. Berg. Infections used for in vivo labeling experiments were carried out at a multiplicity of 1 to 10 by adsorbing virus at 37°C for 1 h in Tris-buffered salt solution (0.14 M NaCl, 5 mM KC1, 0.7 mM Na_2HPO_4, 5.5 mM glucose, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 25 mM Trizma base [Sigma Chemical Co., St. Louis, Mo.], pH 7.4), with occasional rocking. Medium was then added, and the infection was allowed to proceed at 37°C for 46 to 48 h, a time when SV40 DNA replication is maximal under these conditions (16; S. Kondoleon, personal communication).

HMT. Tritiated 4'-hydroxymethyl-4,5',8-trimethylpsoralen (HMT) was generously provided by S. Isaacs and J. Hearst. Stock solutions of approximately 80 μg/ml were made in ethanol. Radioactivity measurements were determined by counting equal portions in a mixture of 1 ml of water and 10 ml of Omnifluor-Triton scintillation fluid (2 liters of toluene, 1 liter of Triton X-100 [Research Products International Corp., Elk Grove Village, Ill.], and 12 g of Omnifluor [New England Nuclear Corp., Boston, Mass.]) in a Beckman LS-345 scintillation counter. The absorbance at 249 nm of an aqueous dilution of HMT was measured in a Beckman Acta III spectrophotometer, and specific activities were calculated by using an extinction coefficient in 100% water at this wavelength of 25,000 M^{-1} cm^{-1} (21). The HMT stock solutions in the reported experiments had a specific activity of 4×10^7 cpm/μg of HMT.

[^{3}H]HMT labeling of infected cells. The medium was removed from cells at 46 h postinfection and replaced with "albino" medium lacking UV absorbents as described by Hallick et al. (16). Cells were then labeled with [^{3}H]HMT by one of two methods. In the first method, a large tissue culture plate (500 cm^2; Vanguard International, Inc., Neptune, N.J.) of infected cells was preincubated for 30 min with the fresh medium, after which time all but 10 ml of it was aspirated. Tritiated HMT was added at a concentration of 1.2 to 12.0 μg/10^8 cells, and the plate was irradiated directly on a bank of six General Electric F15T8 BLB fluorescent tubes at an incident light intensity of 2.2 mW/cm^2 for 30 min. The tissue culture dish was rocked and rotated frequently to ensure uniform drug distribution and irradiation. Irradiations were performed in a cold room to counteract the heat from the light source; the temperature of the medium equilibrated to 22°C after 5 min and remained constant throughout the course of the irradiation.

In the second method, cells from five tissue culture dishes were scraped with a rubber policeman and pelleted by centrifugation for 5 min at 600 × g. Cells were suspended in 2.0 ml of albino medium and transferred to a 100-mm bacterial culture dish (Falcon Plastics, Oxnard, Calif.) to prevent reattachment of the cells. Drug addition and cell irradiation took place exactly as described in the first method.

Isolation of SV40 DNA. SV40 DNA was isolated from infected cells by a modification of the Hirt procedure (19) as described by Hallick et al. (16). [^{3}H]HMT-labeled cell suspensions were washed with Tris-saline and pelleted by centrifugation for 5 min at 600 × g. After the Hirt extraction, SV40 DNA in the supernatant was deproteinized with self-digested pronase (Sigma) and banded by density equilibrium centrifugation in ethidium bromide-CsCl (4.10 gm of CsCl [Merck & Co., Inc., Rahway, N.J.], 4.15 ml of DNA solution, and 50 μl of 20-mg/ml ethidium bromide for 16 to 19 h at 53,000 rpm and 20°C in the Beckman VTi 65 rotor) to separate supercoiled SV40 DNA from nicked circular and linear DNA. The supercoiled DNA was extracted five times with butanol and dialyzed against TE buffer (10 mM Tris-hydrochloride–1 mM EDTA, pH 8.1).

[^{3}H]HMT labeling of purified SV40 DNA. Unlabeled SV40 DNA purified as described above and twice banded on ethidium bromide-CsCl gradients (>90% supercoiled molecules) was used for preparing in vitro psoralen-labeled DNA samples. SV40 DNA was diluted into 1.0 ml of TE buffer or, where indicated, TE buffer plus 0.15 M NaCl. Tritiated HMT was added at a concentration of 140 to 4,300 pg of drug per μg of DNA, and the mixture was irradiated for 30 min in a 35-mm tissue culture dish (Falcon) on the bank of fluorescent lights previously described. After irradiation, the [^{3}H]HMT-labeled DNA solution was made 1.0 M in NaCl and extracted three times with chloroform-isoamyl alcohol (24:1) to remove noncovalently bound [^{3}H]HMT and dialyzed against TE buffer.

Restriction enzyme digestions. Supercoiled SV40 DNA, [^{3}H]HMT labeled either in vitro or in vivo, was concentrated by ethanol precipitation at −20°C. Precipitates were collected after centrifugation at 12,000 rpm for 30 min in a Sorvall HB-4 rotor and suspended in TE buffer at a concentration of 1 μg of DNA/μl.

Restriction endonucleases *Hin*dIII, *Hpa*II, and *Atu*I were obtained from Bethesda Research Laboratories (Bethesda, Md.). Figure 1 shows the restriction map of SV40 for these three enzymes. SV40 DNA digestions with *Atu*I were performed at 37°C for 4 h in 20 mM Tris-hydrochloride, pH 7.5, 20 mM $MgSO_4$, 10 mM dithiothreitol, and 500 μg of gelatin per ml. *Atu*I concentration was 2 U/μg of DNA. The enzymes *Hin*dIII and *Hpa*II were used together at 37°C for 2 h in 20 mM Tris-hydrochloride, pH 7.4, 7 mM $MgSO_4$, 60 mM NaCl, 10 mM dithiothreitol, and 500 μg of gelatin per ml. The *Hin*dIII concentration was 5 U/μg of DNA, and the *Hpa*II concentration was 3 U/μg of DNA.

Gel electrophoresis. The DNA fragments, containing 150 μg of bromphenol blue per ml, 5% (wt/vol) Ficoll, 0.3% sodium dodecyl sulfate, and 10 mM Tris-hydrochloride, pH 8.0, were separated in vertical 4.5% polyacrylamide slab gels (20:1, acrylamide/bisacrylamide; 15 by 14 by 0.15 cm) in E buffer (40 mM Tris, pH 7.1, 20 mM sodium acetate, and 2 mM EDTA). The restriction digests were electrophoresed at 60 V for 17

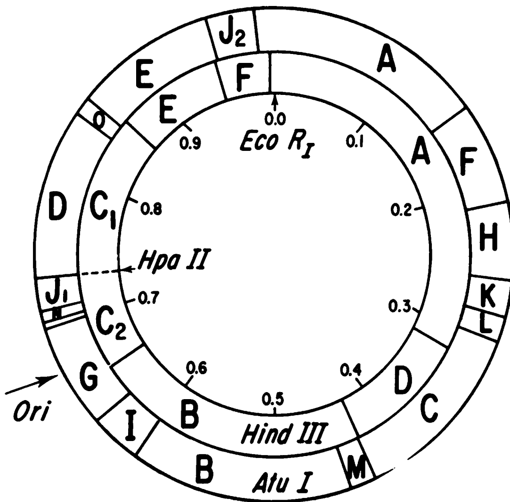

FIG. 1. Physical map of SV40 DNA. The sites of cleavage by the various restriction enzymes are shown relative to the single cleavage site for *Eco*RI. There are six cleavage sites for *Hin*dIII and one site for *Hpa*II (8), and 17 sites for *Atu*I, an isoschizomer of *Eco*RII (27). The location of the replication origin is also indicated.

h (*Hin*dIII-*Hpa*II digests) or at 42 V for 18 h (*Atu*I digests). Buffer was continuously recirculated between the top and bottom chambers during electrophoresis.

Assessment of DNA distribution in gels. Polyacrylamide gels were stained for 30 min in E buffer which contained ethidium bromide (2.5 μg/ml), and DNA fragments were visualized on a Chromatovue transilluminator (Ultra-Violet Products, Inc., San Gabriel, Calif.). DNA distributions in individual gel lanes were estimated from ethidium bromide fluorescence by a modification of the method of Pulleyblank et al. (39). Photographic negatives of the stained gel were produced by using Kodak Tri-X film (4 by 5 inch [ca. 10 by 12.5 cm]) and a Linhoff-Technikoff camera equipped with a Wratten 23A filter. Negatives were scanned with a densitometer (Transidyne, Ann Arbor, Mich.).

Determination of radioactivity in restriction enzyme digest fragments. Individual gel lanes were sliced into 1.3-mm fractions and solubilized in scintillation vials by the method of Mahin and Lofberg (30) (0.2 ml of 60% perchloric acid and 0.4 ml of 30% hydrogen peroxide, incubated with shaking for 4.5 h at 60°C). Fractions were counted after adding 0.5 ml of water and 10 ml of Omnifluor-Triton scintillation fluid and allowing 24 h for dark adaptation. The amount of tritium radioactivity present in DNA bands was determined by pooling appropriate fractions after aligning the peaks from parallel gel lanes of in vivo- and in vitro-labeled samples. Thus, the same number of fractions was included for a given fragment in both samples.

RESULTS

Photoaddition of [^{3}H]HMT to SV40 DNA. To determine the in vivo distribution of nucleosomes on the SV40 genome, SV40 DNA was labeled intracellularly with the psoralen derivative [^{3}H]HMT by irradiation with UV light 48 h after infection (Table 1). A low level of HMT photoaddition (1 to 10 drug molecules/SV40 genome) was chosen to minimize alterations in chromatin structure and interference with subsequent restriction enzyme digestion which might occur with the intercalation of high levels of psoralen derivatives. The first two samples were prepared by irradiating a suspension of cells, whereas the third sample was irradiated as a monolayer of infected cells attached to a single large tissue culture dish. The higher specific activity and labeling density achieved with a cell monolayer probably reflects the greater efficiency of irradiation under these conditions.

Control samples of purified DNA were labeled over approximately the same 10-fold range as the in vivo-labeled samples (Table 1). This in vitro-labeled DNA serves as a model for labeled chromatin in the case where nucleosomes are positioned randomly with respect to the SV40 map. In both situations, all sites on the genome should be equally accessible to photoaddition.

TABLE 1. [^{3}H]HMT-labeled SV40 DNA preparations[a]

Expt	Irradiation condition	μg of HMT/ 10^8 cells	pg of HMT/ μg of SV40 DNA	HMT adducts/ SV40 genome	Spc act (^{3}H cpm/μg of SV40 DNA)
Prepn					
In vivo labeled					
1	Suspension	1.2		1.3	3,800
2	Suspension	12.0		6.3	18,600
3	Monolayer	8.7		11.0	31,300
In vitro labeled					
1	TE buffer		135	1.6	4,700
2	TE buffer		935	8.2	24,500
3	TE buffer		1,190	10.1	30,000
4	TE buffer		1,350	13.6	38,000
5	TE buffer + 0.15 M NaCl		4,270	19.1	56,800

[a] Samples were irradiated, purified, and analyzed for specific activity as described in the text.

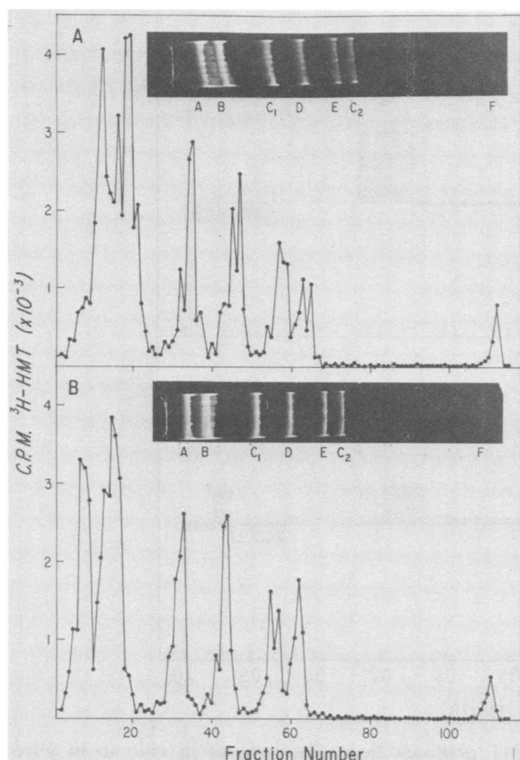

FIG. 2. *Hin*dIII-*Hpa*II cleavage pattern of SV40 DNA. The DNA was labeled with [^{3}H]HMT under in vitro (A) or in vivo (B) conditions as described in the text. SV40 form I DNA was purified and digested with *Hin*dIII and *Hpa*II restriction endonucleases. The resulting fragments were separated by gel electrophoresis and analyzed for radioactivity as described in the text. Inserts show photographs of ethidium bromide-stained gels.

In vitro DNA also provides a control for any sequence-specific binding of psoralens to particular regions of the SV40 genome which might occur over that range of drug additions.

In all of the in vitro-labeled samples but the last one (no. 5), the photoaddition of HMT to purified SV40 DNA was carried out in TE buffer. In those samples, the labeling density achieved was roughly proportional to the amount of drug added. In the last sample, the DNA was irradiated in TE buffer plus physiological saline. As expected from the results of Hyde and Hearst (20), the efficiency of photoaddition was twofold lower at this elevated ionic strength.

Quantitation of [^{3}H]HMT photoaddition. SV40 DNA labeled with [^{3}H]HMT intracellularly or under in vitro conditions was examined for the distribution of drug on the viral genome. Samples were digested simultaneously with the restriction enzymes *Hin*dIII and *Hpa*II (see Fig. 1), and the resulting DNA fragments were separated by electrophoresis on 4.5% polyacrylamide slab gels (Fig. 2). In all cases, digests of in vivo- and in vitro-labeled samples were electrophoresed in parallel on the same gel to control for any losses of smaller fragments during gel staining. Data obtained by the use of a photographic method of DNA quantitation indicated that such losses did occur to a limited extent, but that the overall distributions of DNA in parallel gel lanes were equivalent (G. W. Robinson and L. M. Hallick, unpublished data).

The gels containing the digested fragments were sliced, and the DNA was analyzed for radioactivity (Fig. 2 and Table 2). The amount of radioactive HMT bound to each restriction fragment is expressed in two ways. In the first method, the amount of radioactivity in each fragment is given as a percentage of the total radioactivity for all restriction fragments recovered (Table 2). The only significant difference in labeling density between the in vivo and in vitro samples was found with the C_2 fragment. As can be seen from the ratio of the in vivo to the in vitro samples (Fig. 3), the fragment labeled

TABLE 2. *Hin*dIII-*Hpa*II restriction enzyme analysis

Fragment	% Total [^{3}H]HMT[a]				Normalized sp act[b]			
	In vivo (n = 4)	In vitro (n = 4)	Ratio	Significance	In vivo (n = 4)	In vitro (n = 4)	Ratio	Significance
A[c] B	53.36 ± 0.82	54.07 ± 1.39	0.99	NS[d]	0.85 ± 0.02	0.88 ± 0.08	0.97	NS
C_1	14.93 ± 0.43	14.93 ± 0.95	1.00	NS	1.00 ± 0.00	1.00 ± 0.00	1.00	
D	12.60 ± 0.50	13.53 ± 0.36	0.88	NS	1.13 ± 0.08	1.22 ± 0.08	0.93	NS
E	8.65 ± 0.34	9.63 ± 0.31	0.90	NS	0.91 ± 0.05	1.02 ± 0.07	0.89	NS
C_2	8.45 ± 0.27	5.51 ± 0.18	1.53	$P < 0.001$	1.00 ± 0.05	0.65 ± 0.04	1.54	$P < 0.005$
F	2.03 ± 0.20	2.34 ± 0.18	0.87	NS	0.45 ± 0.05	0.52 ± 0.07	0.87	NS

[a] The percentage of total radioactivity recovered was calculated for each of four experiments, and the values were averaged. The data are presented as this average ± the standard error of the mean.

[b] Specific activities were calculated by dividing the counts per minute by the molecular weight of the fragment, normalizing to fragment C_1 as 1.0, and averaging four experiments as described in footnote *a*.

[c] Fragments A and B were combined for analysis due to incomplete separation of these bands on the gels.

[d] NS, Not significant at $P < 0.05$ by Student's *t*-test.

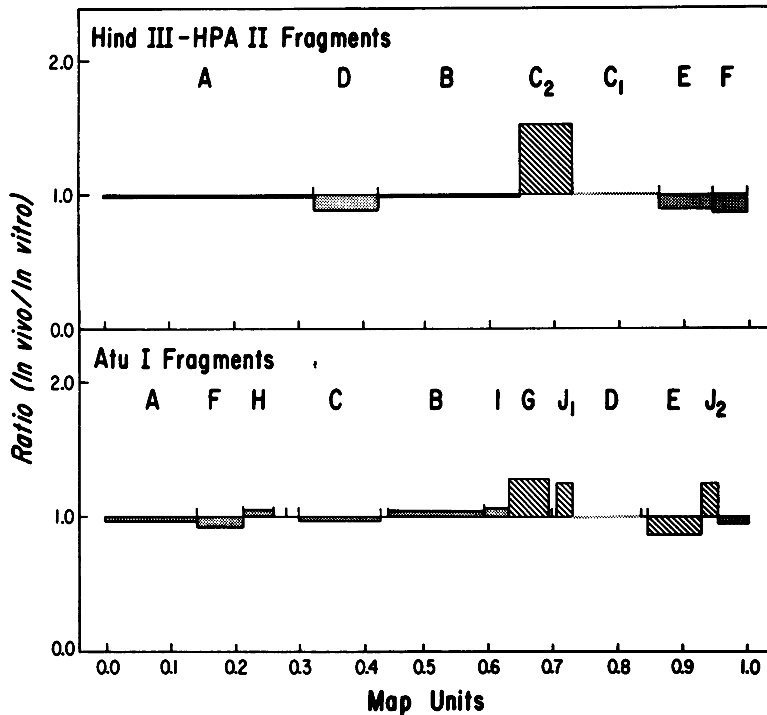

FIG. 3. Accessibility of the SV40 genome to [^{3}H]HMT photoaddition. The ratio of in vivo to in vitro percentage of total ^{3}H counts is indicated. Cross-hatched bars indicate fragments for which the ratios of in vivo/ in vitro values were statistically different than 1.0.

intracellularly contained 53% more radioactive label than the control.

Fragment radioactivities expressed as percentage of total [^{3}H]HMT can bias the data slightly because all fragment values are pooled for the calculation and thus become dependent on one another. For this reason, a second method of analysis was used in which specific activities were calculated for each fragment by dividing ^{3}H counts per minute recovered by the molecular weight for that fragment predicted by the sequence (41). Specific activities of all fragments were then normalized to the specific activity of fragment C_1, which was arbitrarily assigned a value of 1.00. The ratio of in vivo to in vitro values was then calculated as before. The results obtained by this method of analysis indicated that the in vivo-prepared sample contained 54% more HMT on the C_2 fragment than the in vitro-prepared sample (Table 2), in complete agreement with the calculation of percentage of total radioactivity described previously.

To further map the [^{3}H]HMT-labeled regions on the SV40 genome, a second restriction enzyme was used. *Atu*I was used because it allowed regions within the C_2 fragment and the unseparated A + B fragment in the *Hin*dIII-*Hpa*II digest to be examined (see Fig. 1). When the [^{3}H]HMT-labeled DNA samples were digested with *Atu*I and the distribution of radioactivity on polyacrylamide gels was determined (Fig. 4), significant differences between the in vivo and in vitro samples were found on three restriction fragments (Table 3 and Fig. 3). By using the methods of analysis previously described (percentage of total [^{3}H]HMT and normalized specific activity), *Atu*I fragments G and J of the in vivo-labeled samples were found to contain 28% more [^{3}H]HMT than the in vitro-labeled samples. In contrast, fragment E contained 13% less HMT in the in vivo samples than in the in vitro samples.

In all of the experiments described above, in vivo- and in vitro-prepared samples were labeled with HMT to the same molar ratio and electrophoresed in the same gel to reduce the effect of systematic experimental errors. No significant differences by the chi-square test of homogeneity were ever noted between different in vitro samples labeled over the indicated range of HMT densities or labeled in the presence or absence of 0.15 M NaCl. Similarly, no differences were noted among in vivo samples labeled over the range of HMT densities or among samples irradiated as cell suspensions or monolayers. The data from all appropriate experiments were therefore pooled, and averages are presented in Tables 2 and 3.

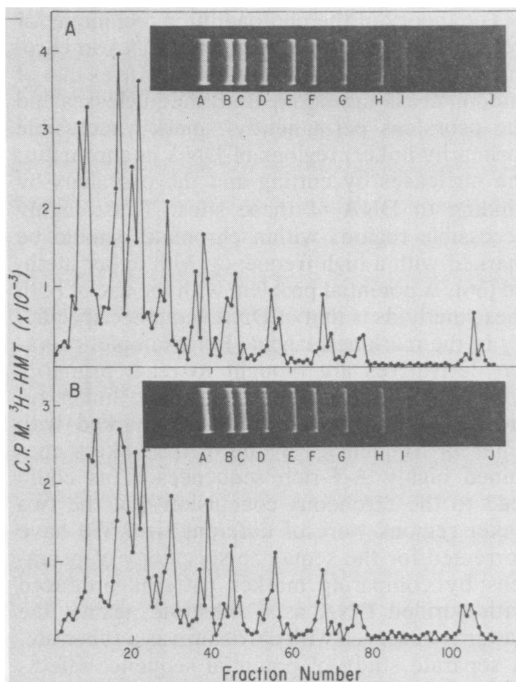

FIG. 4. *AtuI* cleavage pattern of SV40 DNA. The DNA was labeled with [^{3}H]HMT under in vitro (A) or in vivo (B) conditions as described in the text. Labeled SV40 form I DNA was purified and digested with *AtuI*, and the resulting fragments were separated by gel electrophoresis and analyzed for radioactivity as described in the text. Inserts show photographs of ethidium bromide-stained gels.

From the restriction map of the SV40 genome (Fig. 1), it can be seen that the *HindIII-HpaII* fragment C_2 and the *AtuI* fragments G and J all mapped between 0.646 and 0.726 map units. The results from Tables 2 and 3 indicate that this region was preferentially photolabeled with HMT under in vivo labeling conditions relative to in vitro labeling conditions.

DISCUSSION

The sites of intracellular SV40 chromatin most accessible to psoralens are not randomly distributed over the genome. The results from the analysis of psoralen distribution patterns using two sets of restriction enzymes show that the region encompassing the origin of SV40 DNA replication, the T-antigen-binding sites, and the promoters of early and late transcription is preferentially labeled. The coordinates of this region agree remarkably well with those previously determined for the location of a nucleosome-free region on extracted minichromatin. The open region detected by either endonuclease digestion or electron microscopy generally extends from 0.66 to 0.75 map units.

In the *AtuI* digestion (Table 3), two additional fragments have specific activities that are significantly different ($P < 0.05$) for in vivo DNA and in vitro control DNA. Fragment E (0.84 to 0.93 map units) was protected from photoaddition in vivo, whereas fragment J_2 (0.93 to 0.95 map units) exhibited an enhanced specific activity. A comparison with the *HindIII-HpaII* results (Table 2) suggests that the protected region may in fact be real, although of uncertain significance. The enhanced J_2 region is probably an artifact that results from unseparated J_1 and J_2 fragments. Thus, the actual increase in specific activity of the J_1 fragment may be as high as 50%, equivalent to the increase observed in the *HindIII-HpaII* digests.

The size of the accessible region of in vivo chromatin can be estimated if two assumptions are made. The first assumption is that the ease of psoralen photoaddition to this open region is comparable to that seen for other non-nucleoso-

TABLE 3. *AtuI* restriction enzyme analysis[a]

Fragment	% Total [^{3}H]HMT				Normalized sp act			
	In vivo (n = 8)	In vitro (n = 6)	Ratio	Significance	In vivo (n = 8)	In vitro (n = 6)	Ratio	Significance
A	20.79 ± 0.81	21.66 ± 0.56	0.96	NS[b]	1.10 ± 0.05	1.15 ± 0.06	0.96	NS
B	20.87 ± 1.21	20.13 ± 0.98	1.04	NS	1.33 ± 0.08	1.28 ± 0.06	1.04	NS
C	16.24 ± 0.79	16.69 ± 0.98	0.97	NS	1.27 ± 0.07	1.31 ± 0.11	0.97	NS
D	10.56 ± 0.25	10.59 ± 0.37	1.00	NS	1.00 ± 0.00	1.00 ± 0.00	1.00	
E	8.29 ± 0.07	9.49 ± 0.20	0.87	$P < 0.001$	0.98 ± 0.03	1.12 ± 0.04	0.88	$P < 0.001$
F	6.73 ± 0.31	7.35 ± 0.09	0.92	NS	0.96 ± 0.05	1.05 ± 0.04	0.91	NS
G	6.66 ± 0.24	5.22 ± 0.08	1.28	$P < 0.001$	1.13 ± 0.06	0.88 ± 0.02	1.28	$P < 0.005$
H	4.22 ± 0.21	4.06 ± 0.14	1.04	NS	0.89 ± 0.06	0.86 ± 0.04	1.03	NS
I	2.32 ± 0.08	2.19 ± 0.04	1.06	NS	0.61 ± 0.02	0.58 ± 0.03	1.05	NS
J[c]	3.31 ± 0.17	2.64 ± 0.08	1.25	$P < 0.01$	0.69 ± 0.05	0.54 ± 0.02	1.28	$P < 0.025$

[a] Percentage of total radioactivity recovered and specific activities were calculated as described in Table 2. Normalization was to fragment D.

[b] NS, Not significant at $P < 0.05$ by Student's t-test.

[c] Fragments K through P represent 5% of the total genome and were run off the gel during electrophoresis; fragment J is composed of J_1 (127 bp) and J_2 (126 bp).

mal regions of chromatin. The second assumption, frequently made in nuclease digestion studies, is that DNA treated in vitro serves as a model for chromatin with randomly positioned nucleosomes. SV40 minichromosomes contain approximately 24 nucleosomes (45). Thus, each nucleosome is separated by an average of 72 bp of internucleosomal (accessible) DNA and protects 146 bp of core DNA. Table 4 shows the portion of each restriction fragment in a *Hin*dIII-*Hpa*II digest that would be accessible to [^{3}H]HMT photoaddition if the nucleosomes were positioned randomly. It can be seen that 138 of 417 bp would be accessible to drug on the C_2 fragment. When the comparison was made between in vivo and in vitro (random chromatin) HMT-labeling patterns, approximately 50% more label was found on this fragment in vivo (Fig. 2 and Table 2). Therefore, an average of 213 of 417 bp on the C_2 fragment from intracellular SV40 chromatin should be accessible to drug. This value is based on labeling analyses of total SV40 chromatin; thus, it is a valid estimate only if all chromatin molecules have this nucleosome configuration. Our results do not distinguish between this possibility and an alternative situation, in which a fraction of the molecules contain a larger open region. Recent electron microscope studies have reported that approximately 20 to 25% of total SV40 minichromosomes possess a nucleosome-free region (22, 45). If we take 25% of the molecules as an average figure, we can then estimate that 414 of 417 bp should be exposed to psoralen derivatives on this fraction of total chromatin molecules. This size estimate is consistent with the reported electron micrograph measurements (250 to 320 bp) of the accessible region (22, 45) and also with the estimates reported in nuclease digestion experiments (315 to 400 bp) (47, 62).

TABLE 4. Accessibility of *Hin*dIII-*Hpa*II restriction fragments with randomly positioned nucleosomes[a]

Fragment	Molecular length (bp)	Protected DNA (bp)	Accessible DNA (bp)
A	2,937	1,968	969
B			
C_1	701	470	231
D	526	352	174
E	447	299	148
C_2	417	279	138
F	215	144	71
Total	5,243	3,512	1,731

[a] This model is based on SV40 minichromosomes that possess 5,243 bp of DNA and 24 nucleosomes randomly distributed around the genome. Each nucleosome protects 146 bp of DNA and is separated from the adjacent nucleosomes by an average of 72 bp. It is assumed that 72/218 = 0.33 of each fragment is available for psoralen photoaddition.

The theory of the photoaddition technique for determining accessible regions of DNA in chromatin is based upon the same rationale as that of endonuclease digestion. Both the nucleases and the psoralens permanently "mark" accessible (primarily linker) regions of DNA in chromatin, the nucleases by cutting and the psoralens by binding to DNA at these sites. Thus, highly accessible regions within chromatin should be marked with a high frequency with either method (66). A potential problem with the use of both these methods is that of DNA sequence specificity in the marking reagent. For example, psoralen derivatives are thought to react primarily with thymidine residues. Thus, two linker regions of identical size could be marked with different frequencies if one of the linkers contained highly A-T-rich sequences. This could lead to the erroneous conclusion that the two linker regions were of different size. We have corrected for the sequence specificity of psoralens by comparing marker patterns produced with purified DNA as a substrate against the patterns observed with chromatin as a substrate. A separate study of potential sequence effects on psoralen photoaddition will be reported elsewhere.

Psoralen photoaddition appears to be a powerful technique for examining the accessibility of DNA in chromatin structures. The major advantage of this approach over conventional approaches which use isolated minichromatin for analysis is that chromatin structure can be probed within intact cells with little if any disruption of nuclear organization. The covalent nature of psoralen photoadducts ensures that a stable record of nucleosome position remains on the DNA, and thereby makes it unnecessary to maintain chromatin structure once the actual labeling is completed. Therefore, the labeled DNA can be quantitatively extracted and rigorously purified before the determination of drug distribution. Thus, DNA samples used in the experiments reported here accurately represent the total population of intracellular SV40 supercoiled molecules.

When type II restriction enzymes are used for analyses of SV40 chromatin structure, they yield information that is relevant to DNA accessibility at enzyme recognition sites, or at best small regions around the sites. A poor or limited choice of enzymes would lead to conclusions that are not based on accessibility over the entire genome. Like the enzyme micrococcal nuclease, the psoralen derivatives lack strong DNA site specificity. Thus, determinations with the latter two reagents should give information that pertains to the most accessible region on the whole minichromatin molecule.

At present, there is uncertainty over the role

played by the psoralen and nuclease-accessible region of SV40 chromatin. Late during infection (40 to 60 h), various subpopulations of chromatin can be isolated that are undergoing replication (31, 43, 48), transcription (12, 14), or encapsidation into virions (11, 49). Any or all of these processes may require a chromatin substrate that has a nucleosome-free region. The finding of an SV40 chromatin subfraction enriched in molecules with an open region would suggest a functional role of this structure. Using electron microscopy and site-specific endonucleases, L. Tack, P. Wassarman, and M. DePamphilis (J. Biol. Chem., in press) have analyzed pulse-labeled replicating chromatin and report that this population is not enriched for open-origin molecules. Virion and intracellular previrion chromatin complexes have been examined by DNase I digestion, and it has been found that preferential cleavage in the open region is lost or altered during encapsidation (18). A correlation between transcription and this open region has not, as yet, been reported.

The intriguing question as to whether T antigen interacts with this nucleosome-free region has also not been addressed. T antigen is required for initiation of DNA replication and for regulation of transcription (5, 26, 44, 46, 54). On the basis of in vitro studies, this protein presumably functions in vivo only after it has become bound to SV40 origin sequences (31, 34, 43, 48). We are currently using temperature-sensitive T-antigen mutants and deletions of small t antigen in psoralen-labeling experiments to define the relationship between T-antigen binding and chromatin molecules that possess an open region.

While this manuscript was in preparation, we learned of results obtained by P. Beard and his colleagues (1) on the binding of acetylaminofluorene to intracellular SV40 nucleoprotein complexes. Unlike psoralen derivatives, this compound reacts primarily with guanine residues, and yet the levels bound to a DNA fragment near the origin of replication (0.663 to 0.715 map units) were 1.5- to 2.0-fold higher than the levels bound at other genomic locations. The results of acetylaminofluorene binding are thus in fundamental agreement with our analysis of psoralen binding.

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