RANDOM PROTECTION OF SINGLE CUT RESTRICTION ENDONUCLEASE SITES IN SV40 MINI-CHROMOSOMES ASSEMBLED IN VITRO

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1. Introduction

The question of phasing of nucleosomes in chromatin is particularly germane to mechanisms whereby regulatory proteins might interact with genomic DNA sequences. In cellular chromatin, the bulk of the evidence thus far available suggests random placement of histone octamers with respect to DNA sequence [1–5]. In contrast, for the SV40 mini-chromosome, there is good evidence suggesting nonrandom placement of histones for a region of the viral chromatin. While *Eco* RI and *Bam* HI cut ~1/3rd of native mini-chromosomes (consistent with random nucleosome placement around their sites), *Bgl* I cuts nearly 100% of native mini-chromosomes at the origin of replication [6]. Extension of this observation has shown that several other nucleases preferentially cut the mini-chromosome in this area: DNAase I [7], an endogenous nuclease [8] and staphylococcal nuclease [9]. Using multiple cut restriction endonucleases, the region of nuclease sensitivity has been defined as a 400 basepair segment which includes the origin and a part of the late region [10].

Making the tacit assumption that accessibility to nuclease reflects the absence of canonical nucleosomes, 3 possible mechanisms could explain these results:

(i) Specific features of the DNA sequence could preclude wrapping of the nucleic acid around a histone octamer;
(ii) Nonhistone proteins might bind to the DNA region, blocking formation of nucleosomes;
(iii) Nonfolding of DNA into nucleosomes might arise at the time of, and as a result of, chromatin replication in vivo.

Here, we have addressed the first of these possibilities by studying the susceptibility to 3 single-cut restriction endonucleases of mini-chromosomes assembled in vitro from SV40 DNA and histones; our results suggest that the first possibility above is not the basis for preferential exposure of the origin region to nucleases in native mini-chromosomes.

2. Experimental

Histones were isolated from chicken erythrocytes (Pel Freez Biol.) or HeLa cells, after treatment with 5 mM sodium *n*-butyrate for 24 h to induce hyperacetylation of H3 and H4 [11]. Chromatin preparations were made [12], washed with 0.35 M NaCl, adjusted to 2.5 M NaCl and sheared for 30 s at 90 V in a Waring blender. DNA was pelleted by centrifugation and the supernatant concentrated by ultrafiltration. Histones H1 and H5 were removed from total histone preparations, when desired, by gel filtration on Sephacryl S200 in 2.5 M NaCl, 10 mM Tris–HCl (pH 8.0), 1 mM EDTA.

SV40 DNA form I was purchased from Bethesda Res. Labs. Partially purified DNA topoisomerase I (nicking–closing enzyme) from LA9 cells was a gift from Dr M. Bina. DNA I^r was prepared using these two reagents as in [13].

Association of DNA and histones was carried out at protein:DNA ratios of 0.8 g/g; [histones] were measured using 0.43 as the A_{275} of a 1 mg/ml solution of histones. Salt step mixing began with histones and DNA in 2.5 M NaCl, 0.1 M Tris–HCl (pH 8.0), 10 mM EDTA. At intervals of 1 h NaCl was decreased to 2.0, 1.8, 1.6, 1.4 and 1.2 M. After 1.5 h at 1.2, 1.0 and 0.8 M NaCl, samples were incubated 16 h in 0.6 M NaCl. Association was completed by 2 h incubations in 0.3 and 0.1 M NaCl. Generally, this proce-

dure was done at 22°C. In direct mixing association reactions, histones and DNA were mixed in 0.2 M NaCl, 10 mM Tris–HCl (pH 8.0), 1 mM EDTA and incubated at 37°C for 8 h. When poly(L-glutamic acid) was used to facilitate chromatin assembly [14], incubation was for 1 h at 37°C and ionic strength 0.1 M.

At the conclusion of the association, samples were diluted with concentrated digestion buffer to give final conditions of 50 mM NaCl, 10 mM Tris–HCl (pH 7.5), 7.5 mM $MgCl_2$, 1 mM dithiothreitol for restriction enzyme digestions. Restriction enzymes (New England Biolabs) were used at 10-times the concentration necessary to degrade protein-free DNA I to linear molecules under identical digestion conditions. Digestions were for 20 min at 37°C and were terminated by addition of SDS and EDTA to 1% and 25 mM, respectively. DNA was precipitated by addition of 2.5 vol. EtOH and incubation at −20°C for >3 h.

DNA samples were dissolved in sample buffer [6], heated to 55°C for 15 min and electrophoresed on horizontal 1% agarose gels in 36 mM Tris–phosphate (pH 7.8), 1 mM EDTA for 6–8 h at 50 V. Histones were analyzed by electrophoresis on SDS–polyacrylamide gels or, acid–urea polyacrylamide gels [15].

3. Results

The experimental approach used is to form SV40 mini-chromosomes in vitro, using defined histone types and DNA of known topological properties, and then ask, using single-cut restriction endonucleases, whether the disposition of histones around the 3-sites (which differs in native mini-chromosomes [6]) is random or DNA sequence-dependent. Fig.1 shows the results of a typical experiment; the results are representative of all experiments we have done. The starting material, SV40 superhelical DNA (slot A) is digested to a linear form by the 3 endonucleases (slots B–D). After DNA is associated with chicken core histones, the electrophoretic properties of the DNA are identical to those of the starting DNA (slot E). Not shown for this experiment, control studies with DNA topoisomerase I demonstrated for all samples that association with histones had generated the appropriate torsional constraints on the DNA. Digestion of the in vitro assembled mini-chomosomes with the 3 restriction endonucleases leads, in each case, to

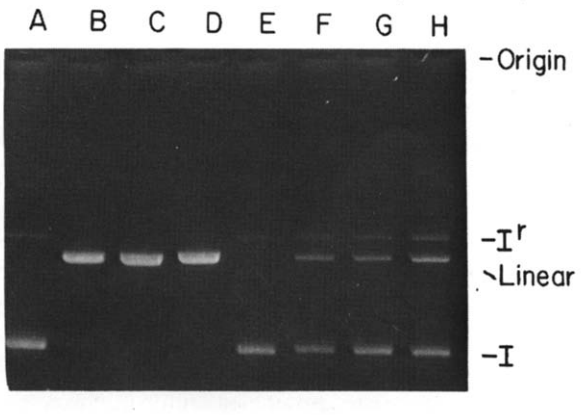

Fig.1. Digestion of SV40 DNA I (A–D) and in vitro assembled mini-chromosomes (E–H) by restriction endonucleases. Mini-chromosomes were assembled by the salt step procedure from DNA I and chicken erythrocyte core histones. Nuclease digestions were with no enzyme (A,E), *Bam* HI (B,F), *Bgl* I (C,G) and *Eco* RI (D,H). Electrophoretic mobilities of form I, form I^r and linear SV40 DNAs are indicated.

~35–50% of the DNA molecules being cut to linear forms while the remainder are resistant and electrophorese as superhelical closed circular DNA molecules (slots F–H). Presumably, the resistant molecules are those in which histone octamer bind the endonuclease susceptible site; in the susceptible molecules, the site is located between nucleosomes.

Several variables in the association method were investigated; study of all possibilities in the three-dimensional matrix including:

(1) Nature of histones (unmodified, hyperacetylated, ± H1 and/or H5);
(2) Topological state of DNA (superhelical or relaxed circular);
(3) Method of association (salt step or direct mixing ± acidic proteins) was not attempted.

With form I DNA, results similar to those depicted in fig.1 were obtained with erythrocyte or hyperacetylated HeLa core histones; in either case the presence or absence of lysine-rich histones was without effect. For all these samples, salt step association and direct mixing gave identical results. With erythrocyte core histones, variation of temperature of association from 4–37°C using the salt step method was without effect on exposure of the *Bgl* I site. Similarly, direct mixing with excesses of poly(L-glutamic acid) from 2:1 to 4:1 over histones did not affect the result, using erythrocyte core histones. Form I^r DNA gave results

like those above with either erythrocyte or butyrate-treated HeLa core histones, irrespective of whether association was performed by salt step or by direct mixing. The data thus demonstrate that, with several substrates and under a variety of association protocols, histones form nucleosomes on SV40 DNA near the 3 single-cut restriction endonuclease sites in a random fashion, unrelated to DNA sequence, when the assembly of the mini-chromosome is carried out in vitro. To emphasize, in no case was the *Bgl* I site, fully accessible in the native mini-chromosome [6], more available for cleavage than the *Bam* HI or *Eco* RI sites in the in vitro assembled mini-chromosomes.

4. Discussion

A variety of nuclease probes have demonstrated the availability for cleavage of the region near the origin of native SV40 mini-chromosomes [6–10]. Features of the sequence of this segment of the genome, particularly the fact that it contains a high portion of the G and C runs of the whole DNA [16], suggested that sequence-dependent phasing of nucleosomes on the viral DNA could lead to the observed results [17]. Here, we demonstrate that if this is true it does not arise simply from the inability of certain DNA sequences to be folded by the inner histones.

After these experiments were completed, a report appeared [18] which came to the opposite conclusion, namely that the site near the origin is unfavorable for formation of nucleosomes. The studies in [18] were done at low histone:DNA ratios (0.4 g/g) and the method used for localization of nucleosomes (cutting form I reconstitutes with *Eco* RI, *Bam* HI or *Msp* I followed by electron microscopy) would lead to low estimates of the formation of nucleosomes at the cutting sites, since uncut molecules are not scored. In our hands, using mini-chromosomes assembled in vitro, the *Bgl* I site at the origin is protected equally as well as the other two single cut restriction endonuclease sites investigated. Thus, there is nothing about the sequence near the origin which a priori precludes its being wrapped around a histone octamer.

Localized phasing of nucleosomes around the origin of native SV40 mini-chromosomes is the most plausible explanation for the selective nuclease sensitivity of this region. Ths inability to reproduce this phasing on assembly of mini-chromosomes from histones and DNA in vitro dictates that other features of viral composition or the assembly mechanism in vivo must underlie the observation. The possible presence of T antigen, known to bind near the origin of SV40 [19,20], must be considered, as must the fact that the in vivo assembly process might lead to localized phasing near the origin of replication which decays away from the origin.

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References

[1] Prunell, A. and Kornberg, R. D. (1978) Cold Spring Harbor Symp. Quant. Biol. 42, 103–108.
[2] Garel, A. and Axel, R. (1978) Cold Spring Harbor Symp. Quant. Biol. 42, 701–708.
[3] Musich, P. R., Maio, J. J. and Brown, F. L. (1977) J. Mol. Biol. 117, 637–655.
[4] Fittler, F. and Zachau, H. G. (1979) Nucleic Acids Res. 7, 1–13.
[5] Singer, D. (1979) J. Biol. Chem. 254, 5506–5514.
[6] Varshavsky, A. J., Sundin, O. H. and Bohn, M. J. (1978) Nucleic Acids Res. 5, 3469–3478.
[7] Scott, W. A. and Wigmore, D. J. (1978) Cell 15, 1511–1518.
[8] Waldeck, W., Fohring, G., Chowdhury, A., Gruss, P. and Sauer, G. (1978) Proc. Natl. Acad. Sci. USA 75, 5964–5968.
[9] Sundin, O. and Varshavsky, A. (1979) J. Mol. Biol. 132, 535–546.
[10] Varshavsky, A. J., Sundin, O. H. and Bohn, M. (1979) Cell 16, 453–466.
[11] Riggs, M. G., Whittaker, R. G., Neumann, J. R. and Ingram, V. (1977) Nature 268, 462–464.
[12] Simpson, R. T. and Sober, H. A. (1970) Biochemistry 9, 3103–3109.
[13] Bina-Stein, M. and Simpson, R. T. (1977) Cell 11, 609–618.
[14] Stein, A., Whitlock, J. P., jr and Bina, M. (1979) Proc. Natl. Acad. Sci. USA 76, 5000–5004.
[15] Simpson, R. T. (1978) Cell 13, 691–699.
[16] Reddy, V. B., Thimmappaya, B., Dhar, R., Subramanian, K. N., Zain, B. S., Pan, J., Ghosh, P. K., Celma, M. L. and Weissman, S. M. (1978) Science 200, 494–502.
[17] Simpson, R. T. and Kunzler, P. (1979) Nucleic Acids Res. 6, 1387–1415.
[18] Wasylyk, B., Oudet, P. and Chambon, P. (1979) Nucleic Acids Res. 7, 705–713.
[19] Jessel, D., Landau, T., Hudson, J., Lalor, T., Tenen, D. and Livingston, D. (1976) Cell 8, 535–545.
[20] Tjian, R. (1978) Cell 13, 165–179.