NOTES

Enhancer Sequences Responsible for DNase I Hypersensitivity in Polyomavirus Chromatin

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DNase I preferentially cleaves polyomavirus minichromosomes at two sites in the enhancer, each of which comprises the sequence AAGCAPuPuAAG flanked by short inverted repeats. A tandem duplication of this sequence generates an additional hypersensitive locus. Mutations which alter either the AAGCAPuPuAAG or flanking repeats diminish hypersensitivity. This region must determine the chromatin conformation recognized by DNase I.

The chromatin of the 5' flanking regions of many active or potentially active genes is unusually susceptible to nuclease attack (7). This 5' hypersensitivity probably reflects the accessibility of specific sequences in the DNA, but its physical nature and functional significance remain speculative (2, 7, 17). Polyomavirus is a useful system with which to study sequences conferring DNase I hypersensitivity to chromatin since its DNA can be manipulated and then reintroduced into mouse cells, in which it is packaged into minichromosomes. That region of the viral genome from the origin of replication to the sites of initiation of transcription of late mRNAs is hypersensitive to various nucleases and frequently lacks nucleosome morphology (3, 14). These sequences are required both for DNA replication and early mRNA transcription and can serve as a transcriptional enhancer for *cis*-linked cellular genes (5, 6, 13, 15, 16, 25, 26).

We examined the effect of mutations in this region on DNase I hypersensitivity. Our data indicated that altered chromatin structure is determined at the site of DNase I cleavage by the sequence AAGCAPuPuAAG flanked by small inverted repeats.

Herbomel et al. (14) previously demonstrated that the enhancer region of the polyomavirus strain A2 contains two regions exhibiting DNase I hypersensitivity, but their analysis did not establish whether cleavage by DNase I was determined by sequences within or outside of the enhancer. To precisely map these DNase I-hypersensitivity sites, we used the technique of indirect end labeling (28) with the *Bam*HI site as reference point (Fig. 1A).

Mouse 3T6 cells were infected with polyomavirus strain A3 at 0.5 PFU per cell. After 24 h, the nuclei were isolated and digested with DNase I to produce approximately 1 cut per 10 kilobases. The DNA was purified, digested with *Bam*HI, and fractionated by agarose gel electrophoresis, and then probed with a radiolabeled fragment extending from nucleotide 4659 to 5048. Of the polyomavirus minichromosomes cut with DNase I, most are cleaved in a region 470 to 570 base pairs (bp) from the *Bam*HI site (Fig. 1C). DNase I digestion of purified DNA produces an entirely different

pattern (Fig. 2) and shows that specific cutting between the *Bam*HI site and the site of origin of DNA replication is dependent on chromatin structure. Using densitometric scans, we established the two main locations of cutting to within 5 bp. The two hypersensitive sequences resemble each other in that cleavage occurs within a region containing the sequence AAGCAPuPuAAG flanked by short inverted repeats (Fig. 1B).

We wished to determine whether DNase I cleavage is specified by the cutting site itself or by distal sequences. Crawford small-plaque (CSP) polyomavirus contains a tandemly duplicated 44-bp segment of DNA (22) that includes one of the hypersensitive sequences (Fig. 1A). The DNase I digestion pattern of the chromatin of this virus (Fig. 2) reveals three distinct hypersensitive sites. The site closest to the *Bam*HI reference point occurs in the identical position on the chromatin of strains A2 and A3. Site 2 is 44 bp upstream and corresponds to cleavages within the CSP duplicated sequence, and site 3 is located at the same origin-proximal sequence cut in strains A2 and A3 of the virus, however, in CSP virus, it is shifted away from the *Bam*HI site by approximately 44 bp. This result demonstrates that the duplicated DNA is sufficient to encode a cutting site within the context of this local sequence environment. This is the smallest segment of DNA that has been demonstrated to be capable of inducing such a chromatin feature. Furthermore, that such a segment of DNA can induce hypersensitivity within itself is strikingly different from what has been reported for DNase I-hypersensitive sequences upstream of the *Drosophila melanogaster hsp70* gene, where external sequences appear to dictate cutting when the DNA is replicated in yeast cells (4).

To determine whether the 44-bp sequence is sufficient to specify DNase I hypersensitivity in other regions of the polyomavirus genome, we inserted it next to the origin of DNA replication (at nucleotide 5295) of polyomavirus strain A2. This virus was viable, and its chromatin displayed only very slight DNase I cutting at the sequences next to the origin of DNA replication (data not shown). We concluded that the 44-bp duplication determines DNase I hypersensitivity only in the proper context. The basis of such a context is as yet undetermined; however, it may be related to the lack of nucleosome organization of the enhancer sequences.

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FIG. 1. DNA and chromatin structure within the polyomavirus enhancer region. (A) Restriction sites within the polyomavirus enhancer region and the direction of early and late transcription are indicated. Heavy solid line denote 44 bp tandemly duplicated in polyomavirus CSP DNA (22). (B) Nucleotide sequence surrounding DNase I-hypersensitive site. Underlining indicates AAGCAPuPuAAG motif; arrows indicate inverted repeats. (C) Indirect end labeling of DNase I-digested *Bam*HI-cleaved polyomavirus strain A3 chromatin (lane 1) with *Hae*III partial digest (lane 2) of polyomavirus A3 DNA used as a reference marker.

To examine the precise nucleotide sequences responsible for DNase I hypersensitivity, we introduced point mutations and deletions into the tandemly repeated sequences of CSP DNA. Point mutants were isolated after a bisulfite treatment of heteroduplexed CSP and strain A3 DNAs by using conditions described by Folk and Hofstetter (10). Mutants with double and triple nucleotide changes in the AAGCAPuPuAAG sequence (Fig. 3, lanes 1 and 3) resulted in diminished cutting at the corresponding sites compared with that of CSP chromatin. A mutant with three changes in the AAGCAPuPuAAG sequence also showed altered cutting with a loss of resolution between individual cutting sites (Fig. 3, lane 4). Mutations not involving the AAGCAPuPuAAG sequences had no apparent effect on the DNase I hypersensitivity pattern (Fig. 3, lane 2).

Because we were unable to mutate the repeats flanking the AAGCAPuPuAAG sequence, we resorted to generating unidirectional deletions extending from the *Pvu*II site at nucleotide 5155. First, we inserted into this site an *Xho*I linker (Fig. 4). This insertion had no effect on the DNase I digestion pattern except to separate hypersensitive sites by the predicted amount (Fig. 4, lane 1). We linearized this DNA with *Xho*I and used T4 DNA polymerase to resect its ends. After adding *Xho*I linkers, and then recloning and sequencing the deleted molecules, we substituted several shortened *Bam*HI-*Xho*I fragments back into CSP in place of the original DNA. Analysis of the DNase I digestion of these mutants indicated that the repeats flanking the AAGCAPuPuAAG sequence are essential for DNase I cutting. Removing half the flanking sequence without affecting the conserved sequence essentially abolishes cutting at the site. (This mutant deletes 14 bp from the *Xho*I site, but because of the insertion of the 8-bp linker, the net change is only 6 bp [Fig. 4, lane 2].) A mutant lacking all of one repeat and part of the conserved sequence was resistant to cutting, as expected (Fig. 4, lane 3). We concluded that the informa-

tion or structure or both contributed by the inverted repeats is also required for DNase I hypersensitivity.

The inverted repeats may serve as part of a recognition site for a regulatory molecule or they may assist the DNA in

FIG. 2. DNase I digestion pattern of polyomavirus CSP and strain A2 chromatin. Lanes: A and E, *Hae*III partial digest of polyomavirus strain A3 DNA; B, DNase I digest of CSP chromatin; C, DNase I digest of strain A2 DNA (purified); D, DNase I digest of strain A2 chromatin.

FIG. 3. DNase I digestion patterns of point mutants of CSP virus. Autoradiograph and densitometric tracings show changes in chromatin structure as a result of various combinations of point mutations within the duplicated region of CSP. Lanes 1 through 4 show four mutants. Specific G→A transitions in each lane are identified on the DNA sequence by corresponding number.

forming nonhelical secondary structures. DNase I hypersensitivity is correlated with conformations of DNA varying from the regular B structure (19). The existence of other sequences in the polyomavirus genome containing inverted repeats (e.g., at the origin of DNA replication) which are not sites of enhanced DNase I cleavage indicates that such repeats are not in themselves sufficient for DNase I hypersensitivity. It was apparent from the pattern of digestion of

naked DNA to DNase I that chromatin components are of major importance in determining DNase I hypersensitivity.

The polyomavirus DNase I hypersensitive sites overlap sequence elements which exert a major effect on RNA transcription and DNA replication (5, 6, 13, 15, 16, 18, 25, 26), and which are homologous to adenovirus, simian virus 40, and immunoglobulin G transcriptional enhancers (1, 11, 12, 21, 27). Such enhancement is dependent on *trans*-acting

FIG. 4. DNase I digestion patterns of deletion mutants of CSP virus. Autoradiograph and densitometric tracings show the results of deletions from the *Xho*I site (shown schematically) into the hypersensitive region. Deletion -14 removes one inverted repeat but not the sequence AAGCAAPuPuG. Deletion -21 removes inverted repeat plus one-half of the conserved sequence.

factors (23, 24). It is likely that the sequences we have identified may associate with such factors. Recently, Piette et al. have detected mouse 3T6 proteins binding to the polyomavirus enhancer region which induce altered cutting by DNase I within the origin-proximal enhancer element (20). Either these or related proteins might induce DNase hypersensitivity by binding to DNA in a fashion analogous to that observed upstream of the chicken adult β -globin gene (8, 9).

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