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The changes in proviral chromatin that accompany morphological variation in avian sarcoma virus-infected rat cells

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ABSTRACT

The clone A11 of avian sarcoma virus B77-infected Rat-1 cells comprises both morphologically normal and morphologically transformed derivatives. Transformed subclones, in which virus-specific RNA is readily detectable, contain a provirus that is very sensitive to DNase 1 digestion of chromatin, and show DNase 1 hypersensitive sites at the 5' end of the provirus and in 5' flanking cell DNA. Normal subclones with no detectable virus-specific RNA, whether infected cells that have never been transformed or revertants derived from transformed cells, contain a provirus that is far more resistant to DNase 1 digestion. Moreover this provirus lacks hypersensitive sites at its 5' end, although DNase 1 hypersensitive sites are present in 5' flanking cell DNA. No DNase 1 hypersensitive sites were detected at the 3' end of the provirus in either normal or transformed clones. The pattern of cytosine methylation in the proviral restriction sites of the isoschizomers Msp I and Hpa II differed between transformed and revertant clones; the revertants show additional methylation at some CpG doublets.

INTRODUCTION

The expression of integrated retrovirus genomes varies in different host cells (reviewed in 1). In some cases such variation may be mediated by host cell control mechanisms and this, coupled with the ease with which retroviral genomes can be manipulated, makes modulation in provirus expression a versatile model for the study of eukaryote gene regulation. In pursuit of this latter aim we isolated a series of clones of the Rat-1 cell line non-permissively infected with avian sarcoma virus, B77 (2). One of these clones, A11, was initially of normal Rat-1 morphology but it segregated daughter clones that were either morphologically normal or transformed. The transformed clones in turn gave rise to a small proportion of revertant subclones that had regained normal morphology but could themselves generate re-transformed segregants (3). In all these A11 derivatives, whether normal or transformed, the single integrated provirus contains a competent transforming src gene and proviral structure and location is apparently unaltered. However,

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levels of virus-specific RNA in the morphologically normal clones are at least fifty fold lower than the levels in their transformed siblings (3). We now show that this clonal variation in morphology and viral RNA levels is accompanied by variations in chromatin structure as revealed by sensitivity of the provirus to DNase 1 digestion (4,5). The provirus in both morphological revertants and in infected clones that have never been transformed is far more resistant to DNase 1 than the provirus in transformed clones. Moreover, in a transformed clone there are DNase 1 hypersensitive sites that are located in or close to the 5' proviral long terminal repeat (LTR) only and are absent in a revertant clone. The altered DNase 1 sensitivity in the revertant is accompanied by cytosine methylation at a proportion of proviral CpG doublets.

MATERIALS AND METHODS

Cells

The origin of clone A11 and its various derivatives has been described in full previously (2,3). The ancestry and morphology of the subclones studied here are summarized in Fig. 1, the predominant cell morphology being indicated by the suffix N or T (3). Cells were cultured as described previously (2).

Isolation of nuclei

Cells were scraped into 25 mM Tris-HCl pH 7.4, 25 mM NaCl, 5 mM $MgCl_2$, 140 mM sucrose using a rubber policeman. After incubating on ice for 10 min the cells were lysed in a type B Dounce homogenizer (10 strokes). Nuclei were pelleted through a sucrose cushion (250 mM sucrose, 5 mM $MgCl_2$, 25 mM Tris-HCl pH 7.4) and washed once with the same solution.

DNase 1 digestions

Nuclei (2×10^7) were resuspended in 1 ml of DNase 1 buffer (60 mM KCl, 15 mM NaCl, 15 mM Tris-HCl pH 7.4, 0.5 mM dithiothreitol, 250 mM sucrose, 0.05 M $CaCl_2$, 3.0 mM $MgCl_2$). DNase 1 (Worthington) was added to the concentration described in the figure legends and the reaction incubated at room temperature for 3 min. The reaction was stopped by the addition of EDTA to 25 mM and DNA was extracted as described previously (3).

Electrophoresis and Hybridization

Restriction endonuclease digestion, agarose gel electrophoresis, transfer to nitrocellulose filters and hybridization were performed as previously (3). The probes used were derived from a cloned DNA provirus from Schmidt-Ruppin strain Rous sarcoma virus infected cells, pSRA2 (7; kindly provided by Dr J.M. Bishop). Subclone pgag is the Bam HI fragment containing part of the

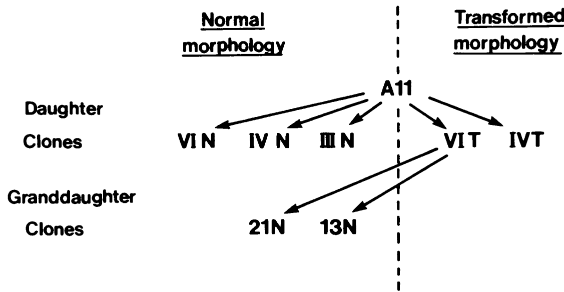

Fig. 1. Interrelationships of the derivatives of A11 studied here.

gag region (approximately from nucleotides 530 to 1920) and cloned in pAT153. Subclone pPst695 is a 695 base pair Pst I fragment largely upstream from the src gene coding region (approximately from nucleotides 6485-7180), also cloned in pAT153 (see Fig. 5). A probe was also prepared from a cDNA clone (A34) of an androgen-responsive rat gene (8), kindly provided by Dr M. Parker.

RESULTS

DNase 1 sensitivity of the B77 provirus in A11 derivatives

Nuclei were isolated from the transformed subclone of A11, IVT, and from its normal sibling VIN, digested with various amounts of DNase 1, and isolated DNA was then electrophoresed on agarose gels as described in Materials and Methods. DNA in the gels was stained with ethidium bromide (Fig. 2, lanes a-f) and transferred to nitrocellulose filters and hybridized with ^{32}P-labeled pSRA2, a probe that recognizes the whole provirus (Fig. 2, lanes g-l). By comparing equivalent lanes on the left and right hand sides of Fig. 2 it can be seen that the proviral sequences in IVT are markedly sensitive to DNase 1. Thus, in lanes a and g both total DNA and proviral sequences are at the top of the gel, but whereas most of the DNA migrates in the top half of the gel in lane b, virus-specific sequences have been lost completely (lane h). In contrast, in VIN, proviral sequences are perhaps more resistant than total DNA to DNase 1 digestion. Thus, in lane d, VIN is digested to about the same extent as is IVT DNA in lane b. However, the VIN provirus is clearly present in fragments of 10kb or larger (lane j). Even after more extensive digestion with DNase 1 a proviral fragment of about 10kb can be clearly seen (lane k) when most of the cell DNA is of smaller size (lane e). Results similar to those with VIN were also obtained with its morphologically normal

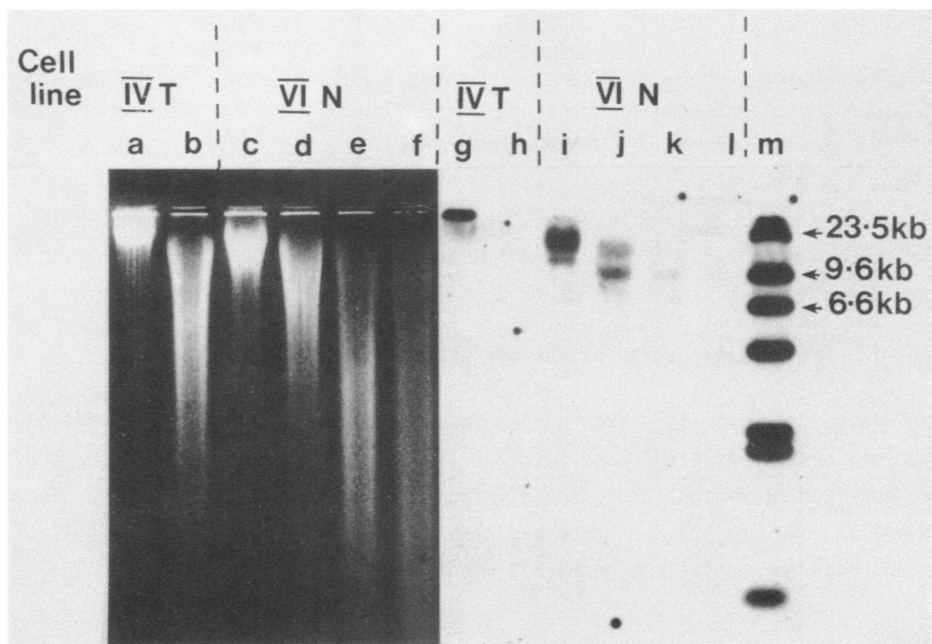

Fig. 2. DNase 1 sensitivity of chromatin from a morphologically transformed (IVT) and morphologically normal (VIN) subclone of A11. Lanes a-f show ethidium bromide staining of DNA extracted after digestion of chromatin with between 6 and 16 units of DNase 1. Lanes g-h show the same gel transferred to nitrocellulose and probed with pSRA2. 10ug of DNA were applied to each lane in this and other figures. Marker DNA in lane m is phage λ digested with Hind III.

sister clones IIIN and IVN and with a revertant clone, 13N, derived from the transformed sibling VIT (data not shown). When comparable digestions of IVT and VIN were hybridized with the cDNA of the androgen responsive rat gene, the DNase sensitivity of this gene was found to be in the same relatively resistant configuration in both cell clones (data not shown). This further supports the conclusion that the provirus is differentially DNase sensitive in the two clones.

Although the provirus in VIN appeared as or more resistant to DNase 1 than the bulk of the cell DNA, the discrete bands detected in Fig. 2 by pSRA2 suggest the presence of specific DNase 1 sensitive sites in the region of the integrated provirus. To locate these sites, DNA obtained after DNase 1 digestion of VIN nuclei was digested to completion with the restriction endonuclease Hind III, electrophoresed, transferred to nitrocellulose and hybridized with either pSRA2 or the gag gene specific probe, pgag (see Materials

and Methods). Hind III cleaves the B77 provirus twice, approximately at nucleotides 2740 and 2870 (9,10) and, as shown previously (2,3), generates in All and its derivatives 2 detectable "junction" fragments of 11.6kb and 7.4kb (Fig. 3A). Since only the 7.4kb fragment is detected by the pgag probe (Fig. 3A) this is the 5' junction fragment and thus the Hind III sites in and around the All provirus are as shown in Fig. 5.

Combined DNase 1 and Hind III digestion reveals two novel fragments of 5.6kb and 4.9kb that hybridize with the pgag probe (Fig. 3A). Since pgag represents a 1.4kb sequence between the 5' end of the provirus and the Hind III site at about base 2740 it follows that: 1) these fragments must be derived from the 3' end of the 7.4kb Hind III junction fragment; were they derived from the 5' end then the probe would detect smaller fragments (1.8kb and 2.5kb) that are clearly absent; 2) the two new fragments must overlap and be generated by two DNase 1 sensitive sites located approximately 1.9kb and 2.6kb 5' to the end of the LTR (Fig. 5). These conclusions are supported by double digestion of VIN with DNase 1 and Kpn 1, a restriction endonuclease that cleaves the provirus once, approximately at nucleotide 5000 (9,10). We find, as previously (2,3), that Kpn 1 digestion of All yields fragments of 12.4kb and 6.6kb and it is the former that hybridizes with pgag and is thus the 5' junction fragment (Figs. 3B and 5). In the double digestion with DNase 1 and Kpn 1 a diffuse new band appears of approximately 7.6kb. This fragment is recognized by pgag and would thus be generated by a DNase 1 sensitive site or sites about 2.3kb 5' to the end of LTR. This agrees with the location of the DNase 1 sites determined with Hind III, although with Kpn 1 the two discrete fragments generated by DNase 1 digestion are less well resolved. It is also noteworthy that with increasing DNase 1 digestion the 12.4kb fragment is replaced by a band of approximately 10.25kb. We do not know whether this represents another DNase 1 sensitive site about 5kb 5' to the provirus or whether it results from non-specific degradation of the larger fragment.

The location of DNase 1 hypersensitive sites in and around the provirus in transformed and revertant clones

Sites hypersensitive to DNase 1 have been reported in the chromatin at or near the 5' ends of several active genes (6,11-16). To test for such hypersensitive sites in the All lineage we compared the effects of mild DNase 1 digestion on the B77 provirus in the transformed clone VIT and its revertant daughter, 13N. In VIT levels of virus-specific RNA are at least 70 fold higher than in the revertant subclone (3).

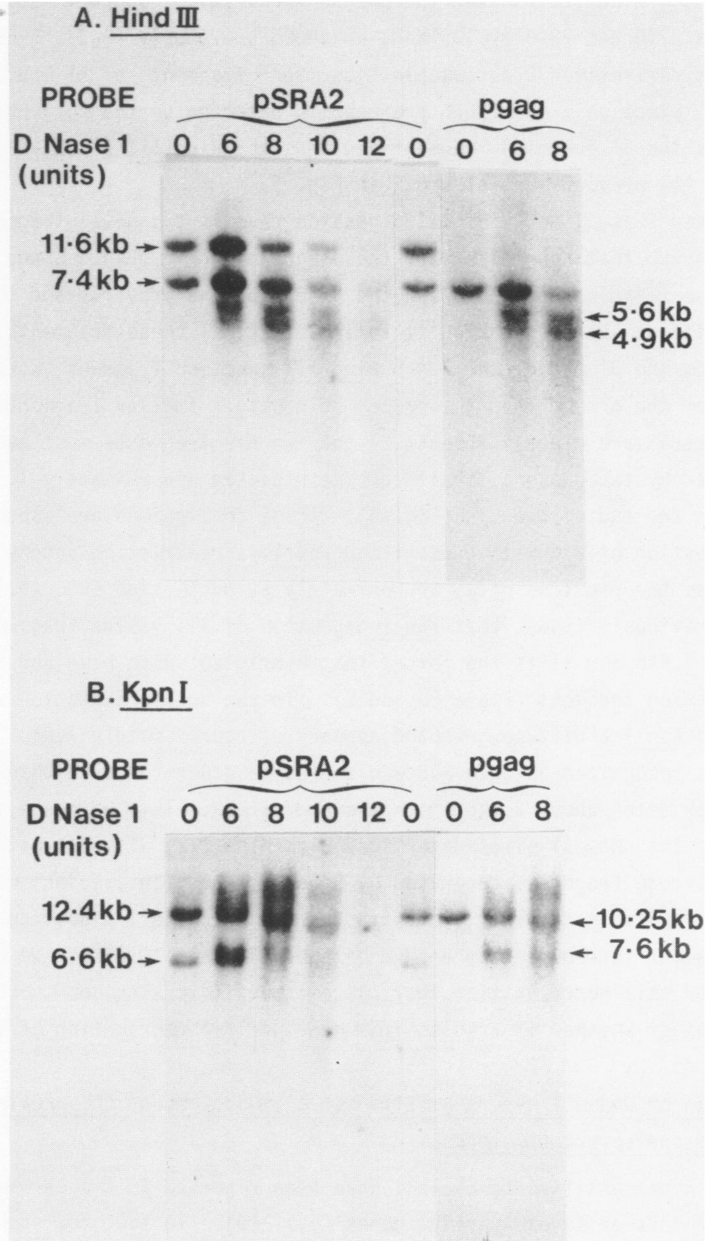

Fig. 3. The mapping of DNase 1 sensitive sites in the morphologically normal clone A11 VIN by DNase 1 digestion of chromatin followed by digestion of DNA with A. Hind III and B. Kpn 1.

Nuclei were digested with levels of DNase 1 that did not affect the size of total cell DNA, and the DNA was then digested with Hind III. Fig. 4, panel C, shows that very mild DNase 1 digestion of 13N chromatin generates 5.6kb and 4.9kb fragments that contain 5' proviral sequences and are presumably the same as those seen with clone VIN (Fig. 3). A 5.6kb 5' fragment is also seen after digesting VIT chromatin with low levels of DNase 1 (Fig. 4, panels A and B). Thus there are DNase 1 hypersensitive sites located about 2 kilobases to the 5' side of the provirus irrespective of the apparent level of proviral expression.

The 5.6kb fragment is only faint in digestions of VIT, but a far more obvious band appears at 2.9kb, followed by a fragment of 1.75kb (Fig. 4, panel A). These latter two fragments are detected by the pgag probe (Fig. 4, panel B) and are thus derived from the 5' end of the provirus. It is possible that they are contiguous sequences generated by a hypersensitive site within the region of provirus recognized by pgag and another site 2.9kb further left (in flanking cell DNA). However, because the 2.9kb fragment is far more intense and appears before the 1.75kb band we think it more likely that they are overlapping sequences, as depicted in Fig. 5. If so, then the DNase 1 hypersensitive site 2.9kb 5' to the Hind III site at nucleotide 2740 (approx.) is in the U3 portion of the left hand LTR, the location of putative regulators for viral RNA synthesis (17). It is thus significant that neither the 2.9kb nor the 1.75kb fragments are seen upon digestion of revertant 13N chromatin (Fig. 4C), nor are they observed after digesting chromatin from other revertants (data not shown).

To determine whether equivalent DNase 1 hypersensitive sites exist in or near the 3' proviral LTR the filters shown in Fig. 4, panels B and C, were hybridized with DNA from pPst695, a subclone of pSRA2 containing a 695 base Pst 1 fragment spanning the 3' end of the env gene and the non-coding region between env and the src gene (see Materials and Methods). As expected, this probe detected the 11.6kb Hind III fragment in both VIT and 13N (Fig. 4, panels D and E), and in both these clones this fragment generates no discrete sub-fragments after mild DNase 1 digestion and thus contains no apparent hypersensitive sites. We conclude that the chromatin containing the 3' LTR differs in structure from that containing the 5' LTR. However, pPst695 does appear to detect a smear of small molecular weight DNA that may represent non-specific digestion (compare for example, panels D and B in Fig. 4). This suggests that the 3' portion of the provirus is generally more sensitive to digestion than the 5' sequences. This conclusion also accords with the find-

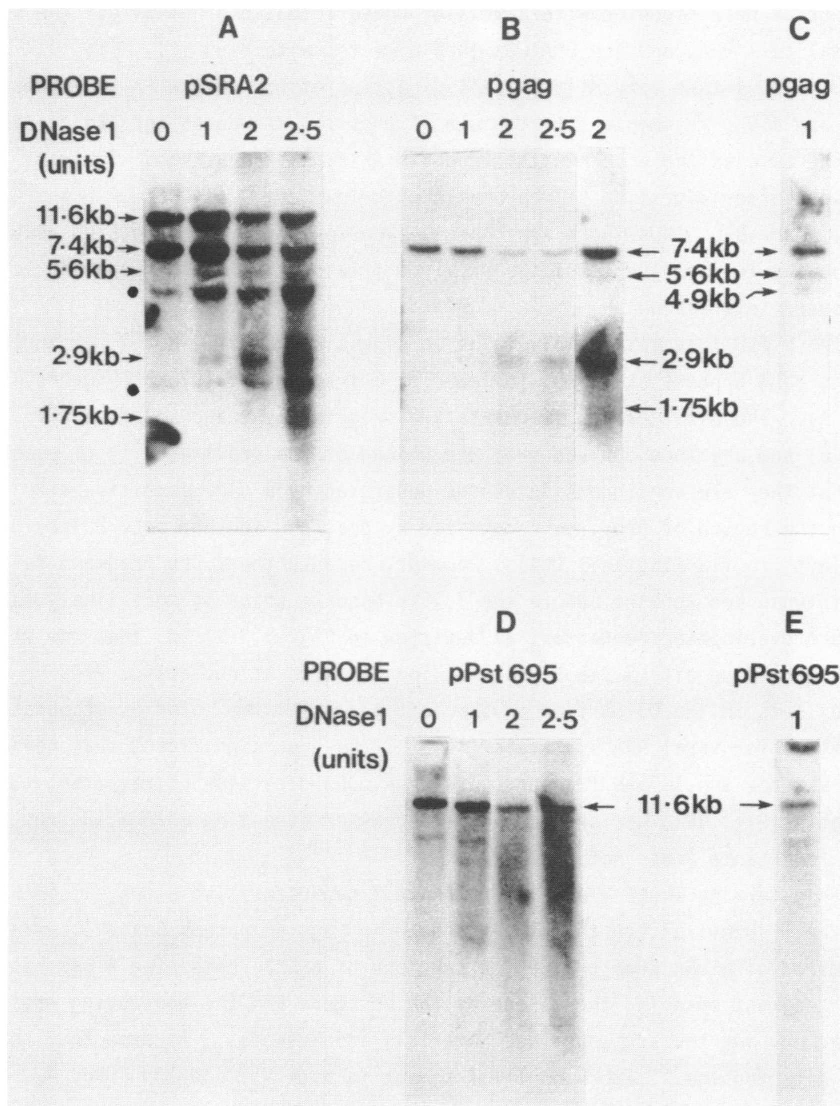

Fig. 4. The detection of DNase 1 hypersensitive sites by mild DNase 1 digestion, followed by Hind III digestion of All VIT (panels A,B,D) and its revertant subclone 13N (panels C,E). Panels B and D, and C and E show the same filters. Panel A is from a separate experiment. The fragments in panel A identified with ● are present with and without DNase treatment and are also frequently seen when hybridizing pSRA2 to normal Rat-1 DNA (data not shown). We presume they represent cellular sarc sequences and it is noteworthy that they are insensitive to DNase 1. The right hand lane in panel B is a longer exposure of the 2 units DNase 1 digestion, to show the 5.6kb fragment.

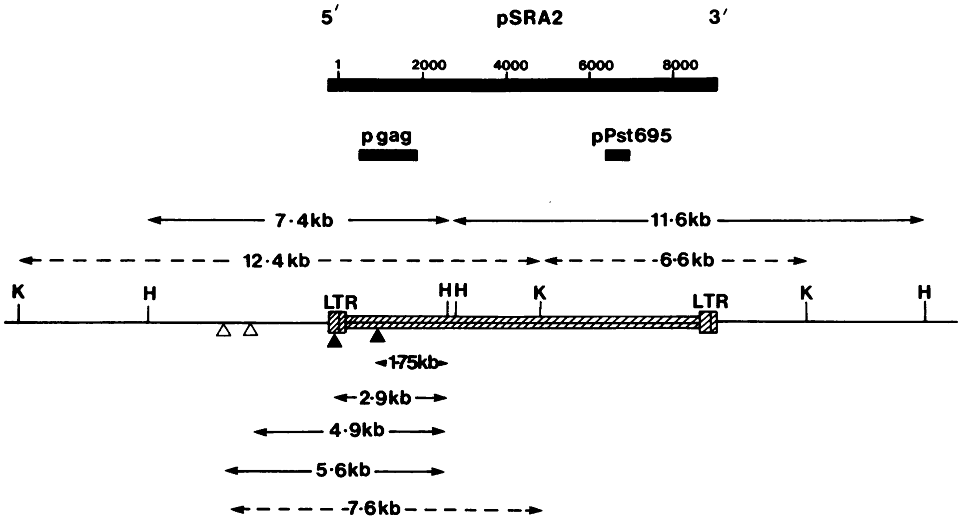

Fig. 5. Map of the A11 provirus and flanking cell DNA. H-cleavage sites for Hind III. K-cleavage sites for Kpn 1. △ DNase 1 sensitive sites detected in normal and transformed clones. ▲ DNase 1 sensitive sites seen in transformed clones only. The solid bars above the map show the regions detected by the cloned probes: top - pSRA2; lower left - pgag; lower right - pPst695. The numbers on the pSRA2 probe are nucleotides, beginning at the first nucleotide in viral RNA (D. Schwartz, personal communication).

ing that digestion of VIN chromatin with DNase 1 alone generates broad but discrete fragments of about 10kb and 15kb (Fig. 2). Since there are DNase 1 sensitive sites 5' to the provirus (Fig. 3), such fragments would be obtained by further cleavages in the 3' third of the provirus and in the 3' flanking host DNA.

The pPst695 probe also detects a faint band of about 7.4kb (Fig. 4, panels D and E). The nature of this fragment is unknown, but since it is present in DNA digested by Hind III alone and is apparently DNase 1 sensitive it may be the 5' proviral junction fragment detected by some homology in pPst695 to sequences at the 5' end of the provirus. We hope to clarify this point when cloned proviruses from these cell lines are available for investigation.

Methylation of proviral DNA in transformed and revertant clones

Hypomethylation of cytosine residues is a feature common to sequences in and around many active eukaryotic genes (18-22). Most methylated cytosines occur in the doublet CpG and one in sixteen of such doublets exists in the

sequence CCGG, the recognition site for the restriction isoschizomers Msp 1 and Hpa II. This pair of enzymes is very useful for studying overall levels of cytosine methylation, for Msp 1 cleaves the recognition sequence regardless of whether the internal cytosine is methylated, whereas Hpa II will cut only when this base is unmethylated. Nonetheless, it must be remembered on the one hand that these enzymes are detecting only a minority of CpG doublets and on the other hand, that they generate a very complex cleavage pattern in large DNA sequences.

Figure 6 shows the result of digesting A11 VIT and its revertant subclone 13N with either Msp 1 or Hpa II and hybridizing with pSRA2. Although the known sequence of the integrated provirus of Prague C strain Rous sarcoma virus contains 43 cleavage sites for these enzymes (D. Schwartz, personal communication) and B77 is likely to be very similar, there are enough large fragments to generate distinctive restriction patterns. With VIT (Fig. 6, lanes c,d) and other transformed clones (data not shown) digestion with either enzyme gives a very similar pattern, showing that none of the detected sites is methylated. One possible exception is the replacement of the 1.6kb fragment generated by Mst 1 (lane d) with a slightly larger fragment after Hpa II digestion (lane c). Although this apparent increase in size may be due to a methylated CpG doublet it could also be an artefact, migration being retarded by the generally larger cell DNA fragments generated after Hpa II cleavage. The Msp 1 cleavage patterns of 13N DNA (lane b) and of 21N DNA (lane f) are the same as those of VIT (lane d). However, Hpa II digestions of the revertants differ markedly (lanes a and e). In 13N, not only is the band around 1.6kb broader, but there is an additional major band of about 1.0kb and fainter fragments at about 1.3kb and of higher molecular weight. This suggests that several proviral CpG doublets are methylated only in the revertant, but the presence of discrete fragments in lane a, including some of only 0.4 to 0.6kb, shows that many Hpa II recognition sites in the 13N provirus remain unmethylated. In 21N a fragment of about 1.0kb is also seen after Hpa II digestion, but again many smaller fragments persist, strengthening the conclusion that cytosine hypermethylation in the revertants is not universal but occurs at limited, and perhaps specific, locations.

DISCUSSION

Our findings on the DNase 1 sensitivity of chromatin encompassing the B77 provirus in A11 derivatives are summarized in Fig. 5. In transformed clones that contain detectable levels of virus-specific RNA (3) the provirus

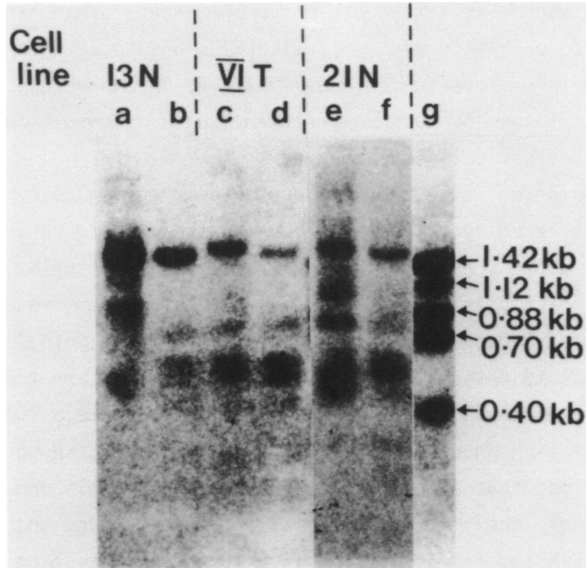

Fig. 6. Proviral DNA methylation in the revertant 13N (lanes a,b) and 21N (lanes e,f) and their transformed parent VIT (lanes c,d). Lanes a,c and e are of DNA digested with Hpa II and lanes b,d and f are DNA digested with Msp I. Markers in lane g are polyoma virus DNA cleaved with Hpa II.

is markedly DNase 1 sensitive (Fig. 2) but hypersensitive sites can be detected in and adjacent to its 5' end, although not in corresponding regions at the 3' end of proviral DNA (Fig. 4). The inactive conformation of the viral promoter at the 3' end of the provirus correlates with a failure to demonstrate in either transformed or normal cells non-viral RNAs that hybridize to a probe specific for the proviral LTR (unpublished observations). In morphologically normal derivatives, in which viral RNA levels are much reduced (3) the provirus is far less sensitive to DNase 1 (Fig. 2), the hypersensitive sites within the provirus are absent (Fig. 4) but hypersensitive sites in flanking DNA approximately 2kb 5' to the provirus are retained (Fig. 3).

The DNase 1 sensitivity of active proviruses, the presence of DNase 1 hypersensitive sites at the 5' end of active proviruses and their absence from inactive counterparts accords with studies on other genes (4-6,11-16) including other retroviruses (23-26). Our findings strengthen the association between chromatin structure and gene expression without proving a casual relationship. However, the reversibility of B77 expression and chromatin structure in Rat-1 cells makes these data particularly persuasive. Features of chromatin struc-

ture that appear when a cell that has never been transformed, such as VIN (3), gives rise to a transformed cell, like VIT, disappear when that transformed line segregates revertants (e.g. 13N). As far as we are aware, this is the first description of such reversible changes of chromatin structure within a single defined cell lineage.

We find, moreover, that the proviruses in morphologically normal clones contain more methylated cytosine than those in transformed derivatives of All (Fig. 6). Other workers have associated proviral hypermethylation with transcriptional inactivity (25-30) but in general the inactive proviruses have been unrelated to active counterparts and so highly methylated that it has been difficult to assess the significance of this observation. Since the clones studied here belong to a single lineage and show relatively minor changes in methylation when proviral activity is altered, we hope to identify specific alterations that affect gene activity, and to this end we are now attempting to locate the sites of methylation in and around the provirus, particularly in 21N and its derivatives. A further interesting feature of the All series is that the provirus in the transformed cell is closer to the "nuclear cage" (31,32) than it is in normal cells (33).

The versatility of the All system should help unravel the regulatory mechanisms that link the phenomena of proviral transcription, chromatin structure and proximity to the nuclear cage. On the basis of previous work (34-40) we anticipate that host cell elements in *cis* may be important in determining proviral expression. However, studies on the suppression of B77-induced transformation in cell hybrids (39,42) indicate that *trans*-acting factors may also be involved. To answer these questions information is required on the structure and activity of proviral regulatory elements and of host sequences flanking the provirus. To obtain such knowledge we are now cloning in bacteriophage λ integrated proviruses from both normal and transformed derivatives of All. Such clones will help to study the extent and nature of changes in chromatin structure that accompany phenotypic variation and whether or not changes in chromatin structure are accompanied by any alterations in primary DNA sequence. In concert with DNA transfer experiments, utilizing proviruses and variable amounts of flanking DNA from members of the All series, they should also help locate at least some of the cell elements that modulate proviral expression in these cells.

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