Studies on the Methylation of Avian Sarcoma Proviruses in Permissive and Non-permissive Cells

(Accepted 21 September 1982)

SUMMARY

The extent of methylation of several avian oncogenic proviruses was determined by using the restriction endonucleases HpaII and MspI. The results indicated that the transformation-defective proviruses (RAV-O or B77-td), which are exogenously introduced into avian host cells, were not methylated. However, endogenous proviruses (RAV-O) or ASV proviruses present in non-permissive host cells were found to be partly or completely methylated. The methyl-sensitive restriction endonuclease PvuI, which recognizes a unique site within the long terminal repeat in the ASV genome, failed to cleave proviruses present in several non-permissive host cells. From these results we suggest that modification of the sequence around the PvuI site results in reduced levels of transcription.

The DNA of higher eukaryotes contains 5-methylcytosine (MeC) at a frequency of about 2 to 7\% of the total cytosine residues (Vanyushin et al., 1970). The majority of these residues are found in the dinucleotide CpG (Doscocil & Sorm, 1962). The function of MeC in eukaryotic DNA, however, remains obscure. Using restriction enzymes HpaII and Hhal, which fail to cleave DNA if MeC is present in the recognition sequence, Bird & Southern (1978) have demonstrated that a defined methylation pattern is inherited during eukaryotic DNA replication. Using this method, the extent of methylation of many eukaryotic as well as virus genes has been determined (Razin & Riggs, 1980; Doerfler, 1981). We have previously reported hypermethylation of integrated avian retrovirus genomes in the non-permissive rat cell line, XC, while exogenously introduced genomes present in permissive chicken cells are not detectably methylated (Guntaka et al., 1980). In addition, as also shown by Humphries et al. (1979), we have shown that endogenous virus sequences (RAV-O) present in normal chicken cells are also methylated (Guntaka et al., 1980). Likewise, in the mov-3 substrain of mice, the integrated M-MuLV provirus has been shown to be highly methylated whereas the same provirus cloned in bacteria is not methylated (Harbers et al., 1982). Further experiments with several other non-permissive and permissive cells lend additional support for the modification of a portion or the entire provirus genome in the non-permissive systems.

In this work, we have analysed avian sarcoma virus (ASV) proviruses found in several different cells or cell lines. These included RR1022, which was established from a tumour induced in rat by the Schmidt–Ruppin strain of avian sarcoma virus (Nichols, 1963), and several virus-infected chicken embryo fibroblasts and quail tumour cell line, QT6. The methylation pattern of integrated ASV proviruses in RR1022 cells was examined using HpaII and MspI. HpaII does not cleave if the internal C in the recognition sequence (5'-CCGG-3') is methylated whereas MspI, an isoschizomer of HpaII, cleaves regardless of methylation (Cedar et al., 1979). High molecular weight cellular DNA was digested with BamHI and then with HpaII or MspI. The DNA was electrophoresed, transferred to a nitrocellulose paper by the method of Southern (1975) and hybridized with 32P-labelled cDNA prepared from ASV RNA (Guntaka et al., 1980). Digestion with BamHI produced two virus-specific fragments of 1.1 and 0.8 x 10^6 daltons (Md) corresponding to the left end internal fragments (De Lorbe et al., 1980) (Fig. 1, lane 1). A series of virus–cellular junction fragments, ranging in size from 2.6 to 15 Md were also observed, indicating the presence of multiple proviruses. The internal fragments of 1.1 and 0.8 Md, derived from the left half (corresponding to the gag and pol regions) of the genome, were resistant to cleavage by HpaII (lane 2) but sensitive to MspI digestion (lane 3), indicating
Fig. 1. Southern blot analysis of RR1022 DNA. High molecular weight DNA was prepared from RR1022 cells (obtained from the ATCC) as described (Guntaka et al., 1980). Approximately 30 μg was digested with BamHI and two 10 μg lots were further digested with HpaII or MspI under the conditions described by the supplier. The DNA was electrophoresed (10 μg/lane) in a 1% agarose gel, transferred to nitrocellulose and virus-specific fragments were detected using 32p-labelled cDNA as described previously (Guntaka et al., 1980). Lane 1, BamHI; lane 2, BamHI plus HpaII; lane 3, BamHI plus MspI. The other DNAs and the restriction enzymes used are indicated above the figure.

hypermethylation at the HpaII/MspI sites. The size of the 15 to 2.6 Md fragments was reduced to 2.2 to 1.2 Md upon HpaII digestion, indicating that at least some of the HpaII sites in the right half of the genome were not methylated (Fig. 1, lane 2). Analysis by HpaII/MspI enzymes was carried out with cellular DNA prepared from three different cells: (i) RAV-O-infected QT6 cells, (ii) chicken embryo fibroblasts (CEF) infected with B77 transformation-defective (td) virus and (iii) CEF cells infected with Pr.C virus, rescued from XC cells (kindly given by Dr H. Hanafusa of the Rockefeller University, New York, U.S.A.). In each case the cellular DNA was first digested with EcoRI and then with HpaII or MspI. As shown in Fig. 1, RAV-O-infected QT6 cells contained a virus-specific EcoRI fragment of 2.5 Md as previously described (Guntaka et al., 1980). No virus–cellular junction fragments were observed, probably due to random integration. The 2.5 Md fragment was completely sensitive to both HpaII and MspI digestion, indicating that this fragment was unmethylated (Fig. 1). In agreement with the results described previously (Guntaka et al., 1980), the 2.5 Md EcoRI fragment in normal CEF DNA which is specific for the endogenous RAV-O, was resistant to cleavage by HpaII (Fig. 1). Cleavage of DNA from B77 td-infected CEF cells with EcoRI produced the expected fragments of 1.5, 2.5 and 0.8 Md (Shank et al., 1978). These fragments, with the exception of the endogenously derived 2.5 Md fragment was also sensitive to HpaII digestion (Fig. 1). Digestion of DNA from CEF cells infected with virus rescued from XC gave similar results (data not shown). In contrast to XC, RR1022 and ev-1 locus of CEF, we have found that in Pr.C-3T3,
Fig. 2. HpaII and MspI digestion of Pr. C-3T3 DNA. High molecular weight DNA from Pr. C-3T3 cells was isolated and digested first with EcoRI. Then it was digested with HpaII or MspI and run on an agarose gel. The DNA was blotted on to cellulose nitrate paper and virus-specific sequences were detected by hybridization with radiolabelled virus cDNA.

which contains a single ASV provirus, the genome is apparently intact, as indicated by the presence of terminal EcoRI sites (Copeland et al., 1981) but digestion of the Pr.C-3T3 DNA with EcoRI followed by HpaII/MspI provided no evidence of methylation because all three EcoRI fragments were totally susceptible to HpaII (Fig. 2).

It has become evident that the LTR (long terminal repeated sequence) region of the ASV genome contains transcriptional regulatory sequences (Temin, 1982). Since the restriction enzyme PvuI cleaves at a unique site in the ASV genome within the LTR region (Hsu et al., 1978; Shank et al., 1978), it can be used to test for the presence of intact LTR sequences in the proviruses. As shown in Fig. 3, cleavage of cellular DNA from Pr.C-ASV-infected CEF cells produced genome-size virus-specific fragments of 4.6 and 5.8 Md. These fragments correspond to the transformation-defective and non-defective genomes, respectively. Virus-specific fragments migrating in the high mol. wt region of the gel (> 15 Md) represent endogenous RAV-O sequences (McClements et al., 1979), which do not contain the PvuI cleavage sites (Fig. 3a). Digestion of the cellular DNA from RAV-O-infected QT6 cells or uninfected CEF with PvuI also did not produce genome-size fragments (Fig. 3a). In contrast to permissive cells, cleavage of DNA from non-permissive mammalian host cells (XC, RR1022 and Pr.C-3T3) with PvuI failed to produce the expected genome-length (5.8 Md) fragment (Fig. 3a). This might be due to
deletion of a sequence containing the PvuI site or PvuI may be a methyl-sensitive restriction endonuclease. That PvuI is indeed a methyl-sensitive enzyme can be demonstrated by comparing methylated genomic DNA to that of cloned DNA fragments. We have observed that ribosomal RNA genes in XC cells, like in H4 cells (Tantravahi et al., 1981), are highly methylated. Therefore, we compared the digestion pattern of a 3.6 Md 18S HindIII rDNA fragment from XC cells with that of the same fragment cloned and amplified in *Escherichia coli.* PvuI did not cleave the cellular gene but cleaved the unmethylated cloned fragment (Fig. 3b). Thus, we conclude that PvuI does not cleave if the site is modified. On the basis of this observation, we suggest that the proviruses present in these non-permissive cells are either methylated or deleted within at least one LTR.

To test for the presence of sequences surrounding the PvuI site, HindIII-digested XC DNA was separated by gel electrophoresis, transferred to cellulose nitrate paper and hybridized with a radiolabelled probe derived from the PvuI to EcoRI sites (55 bp) in the LTR, which was cloned in pBR322. Since this probe detects sequences to the left of EcoRI which is located in the LTR and since this probe hybridized to multiple junction fragments (data not shown), we conclude that at least a part or the whole sequence between the EcoRI and the PvuI sites is present in the majority of proviruses.
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9.8- ~ --9.8
3.6--
3.1-
2.7--

Fig. 4. Evidence for the presence of LTR sequences. Prague-C ASV infected chicken cell and Pr. C-3T3 DNAs were digested with PvuI and KpnI (lanes 1 and 2) or RsaI (lanes 3 and 4), and electrophoresed on agarose gels. The DNA was blotted on to cellulose nitrate paper and hybridized with representative cDNA (lanes 1 and 2) or gag-specific probe which was obtained by isolating the 1.4 kb BamHI fragment between the nucleotides +532 and +1916 (numbers were from the nucleotide sequence of ASV DNA; D. Schwartz, R. Tizard & W. Gilbert, personal communication) followed by labelling with [32P]dCTP.

In Pr.C-3T3 only a single provirus is present. Since the restriction enzyme KpnI has a single site in the virus DNA, near the 5' end of the env gene, double digestion with KpnI and PvuI should yield two fragments of 3.1 and 2.7 Md. When Pr.C ASV-infected chicken cells were cleaved with these enzymes, both these fragments appeared, in addition to the fragment derived from endogenous provirus units (Fig. 4, lane 1). However, similar digestion of Pr.C-3T3 DNA yielded two fragments of 3.6 and 9.8 Md which are clearly larger than the expected sizes (Fig. 4, lane 2). Thus we conclude that PvuI, instead of cleaving in the LTR, is digesting at a site in the flanking cellular sequences and therefore we infer that both PvuI sites are modified or deleted.

To establish unequivocally the presence of the sequence upstream from the PvuI site in the provirus of Pr.C-3T3 cells, the DNA was digested with RsaI. This enzyme, which recognizes the sequence 5’-GTAC-3'/3'-CATG-5' cleaves the virus DNA at multiple sites. One site is located within the LTR, only three nucleotides (-116) to the left of the PvuI (-113) site and the 3' end cytosine residue of the RsaI recognition sequence overlaps with the PvuI recognition sequence 5’-CGATCG-3'/3'-GCTAGC-5’. The next two RsaI sites are located in the gag and pol genes at nucleotides +1071 and +2989 with respect to the cap site of virion RNA. Therefore, if we use a probe specific for these sequences, and if the RsaI site in the LTR is present in the provirus of Pr.C-3T3, we expect hybridization to two fragments of 0.7 and 1.2 Md. For this purpose, we isolated the 1.4 kb (0.88 Md) BamHI fragment (between nucleotides +532 and +1916) from recombinant plasmid pATV-8 (Katz et al., 1982), labelled with [32P]dCTP by nick-translation and used it as a probe. The results presented in Fig. 4 (lanes 3 and 4) clearly show hybridization to the expected two left end RsaI fragments derived from the provirus of 3T3 cells. In Pr.C-ASV-infected chicken cells the same 1.19 kilobase (0.7 Md) and 1.9 kb (1.2 Md) fragments appeared (lane 1). In addition, two more bands of 2.3 (1.5 Md) and 3.0 kb (1.9 Md) fragments were also revealed. These, we believe, are probably derived from endogenous proviruses. Since the RsaI site in LTR is present in Pr.C-3T3 provirus, we conclude that the PvuI site is present in the single provirus of Pr.C-3T3 cell line but methylated. This would account for its failure to cleave the proviral DNA. It should, however, be emphasized that the possibility that PvuI failed to cleave proviruses due to single base changes has not been eliminated by these experiments.
Retroviruses provide an excellent model system for studying eukaryotic gene expression. The integrated virus genome can exist in multiple forms which are structurally similar but differ in their apparent transcriptional capacities. We have investigated the methylation pattern of avian retrovirus genomes present in active (permissive) and inactive (non-permissive) states to determine whether there is a relationship between methylation and virus gene expression. Our results clearly indicate that methylation and/or deletion of at least one region in the LTR is important in the regulation of transcription.

We have extended our initial observation that non-permissive mammalian cell lines, derived from a tumour induced by the Schmidt–Ruppin strain of ASV contain methylated proviruses. The extent of methylation of the ASV genome observed in RR1022 cells was identical to what we previously observed in the cell line XC (Guntaka et al., 1980). In addition, we have shown that methylated proviruses removed from a non-permissive environment are usually unmethylated in a permissive environment. For example, the virus genome rescued from XC cells is unmethylated in a chicken cell host. This suggests that the virus genome does not contain information which encodes its own methylation but rather that the extent of methylation is determined by the cellular environment. A similar observation is made with RAV-O, which is unmethylated when introduced into permissive cells.

We have also observed that proviruses present in several non-permissive host cells are methylated within at least one LTR. An alternative possibility for the resistance of proviruses in cells could be that a portion of the LTR, including some critical regulatory elements such as the enhancer sequences, was deleted in the integrated genome. In this connection it should be pointed out that recently we have obtained evidence for the requirement of sequences between −299 and −110 (relative to the transcription initiation site) for virus DNA transcription (S. A. Mitsialis & R. V. Guntaka, unpublished results). Therefore, deletion or modification of this sequence may have a profound effect in gene regulation.

We have also obtained evidence which suggests that partial demethylation of a large number of internal HpaII sites by treating XC cells with 5-azacytidine (Jones & Taylor, 1980) does not enhance virus-specific transcription (data not shown). This suggests that under-methylation at the internal sites is not sufficient to enhance transcription levels. However, Groudine et al. (1981) have reported that endogenous avian viruses (RAV-O) could be induced from normal chicken cells by 5-azacytidine, but proviruses cannot be induced from non-permissive cells. Unless provirus genomes including the LTRs are completely demethylated, it is difficult to conclude that mammalian cells may contain additional transcription blocks. Studies with demethylated DNA are in progress.

We thank Drs H. Hanafusa, G. Cooper and J. Coffin for the viruses and cells and S. Malik for help with experiments. This work was supported by a contract (CP71055) from the National Cancer Institute. R.V.G. is a recipient of a Research Career Development Award from the NIH.

Note added in proof. Recently, we have observed modification of the PvuI sites in the LTRs of proviruses in two different hamster tumour cell lines (S. Malik, J. Svoboda & R. V. Guntaka, unpublished results).

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(Received 18 March 1982)