JOURNAL OF VIROLOGY, May 1980, p. 569-572
0022-538X/80/05-0569/04$02.00/0

Vol. 34, No. 2

NOTES

Modification of Avian Sarcoma Proviral DNA Sequences in Nonpermissive XC Cells but Not in Permissive Chicken Cells

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For the first time, we present evidence with restriction enzymes *Hpa*II and *Msp*I which indicates that the proviral DNA sequence of avian sarcoma virus is modified by methylation in a nonpermissive rat cell line but not in permissive chicken cells. Some of the endogenous viral sequences in the permissive cells were also methylated. No 5-methylcytosine could be detected in the unintegrated viral DNA.

Almost two decades ago, Svoboda (16) established a cell line (XC) from a Prague C strain of avian sarcoma virus-induced rat fibrosarcoma. Varmus et al. (20) have shown that these cells contain about 10 to 20 copies of viral genome per diploid cell genome. Direct experiments with XC DNA infection of permissive chicken cells indicate the presence of some complete genomes (6, 17). Despite the fact that many viral DNA (vDNA) copies are integrated in these cells, fewer than 200 molecules per cell (fewer than 10 to 20 viral 35S RNA equivalents per DNA copy) of viral RNA are synthesized (19), whereas in a permissive cell as many as 5,000 to 10,000 copies of viral RNA are transcribed per genome of proviral DNA (5). No satisfactory explanation for this differential level of transcription in these two host cells is available at this time.

In an attempt to study the pattern of integration of viral genome in these XC cells, we have made a surprising observation which indicates that restriction enzymes such as *Hha*I and *Hpa*II fail to cleave proviral DNA sequences. Since these enzymes do not cut DNA if the cytosine residue in the recognition sequence is methylated (2, 3, 9, 13, 21), we propose that proviral DNA is methylated. In this report, we present evidence for the occurrence of 5-methylcytosine in the proviral DNA of nonpermissive XC cells but not in permissive chicken cells. The implications of this finding are discussed.

XC cells and chicken embryo fibroblasts transformed by the Prague C strain of avian sarcoma virus (Pr.C-Ck), grown in minimal essential medium and medium 199, respectively, were harvested, and the DNA was isolated. Samples of the DNA were digested with restriction enzymes *Hha*I, *Hpa*II, and *Msp*I and analyzed on agarose gels. The gels were stained with ethidium bromide, the DNA in the gels was transferred to a nitrocellulose paper, and virus-specific sequences were detected by hybridization to ^{32}P-labeled virus-specific complementary DNA (7, 8, 12, 14). The enzyme *Hha*I recognizes the nucleotide sequence 5′-GCGC-3′, and the enzymes *Hpa*II and *Msp*I cleave DNA at 5′-CCGG-3′ (9). In contrast to *Hha*I and *Hpa*II, which do not digest at the recognition sequence if the cytosine is methylated, *Msp*I cleaves DNA regardless of methylation (3, 9). On the basis of four-nucleotide recognition, we anticipate that any given DNA should be digested extensively to yield very small fragments of about 250 base pairs.

In agreement with reports from other laboratories (3, 13, 15, 21), we found that both *Hha*I and *Hpa*II digested both rat and chicken DNAs to an average size of about 15 to 20 kilobases, whereas *Msp*I digested rat and chicken DNAs to an average size of less than 2 to 4 kilobases. Because the sites are not available to *Hha*I and *Hpa*II but are available to *Msp*I, we suggest that a majority of the sites in the cellular DNA are methylated in the cytosine residue.

When the DNA samples digested with *Hha*I and *Hpa*II were analyzed for viral sequences, we found that in XC cells the majority of vDNA was present in a form larger than 10 megadaltons (Md) (Fig. 1). In contrast, *Msp*I converted the viral sequences to small fragments ranging in size from 0.12 to 0.8 Md. Parallel experiments with unintegrated vDNA, purified from Prague-C-infected quail cells, indicated that the viral sequences were equally susceptible to all three

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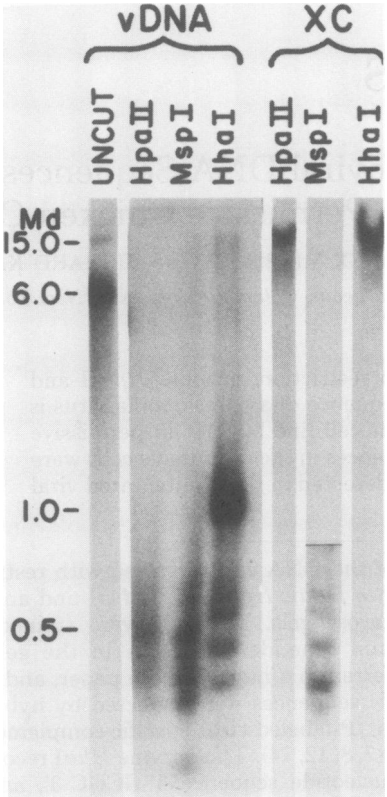

FIG. 1. *Large-molecular-weight DNA was isolated from XC and Pr.C-Ck cells as described previously (4). Approximately 10 μg of XC DNA and 20 to 30 μg of Pr.C-Ck DNA were digested with 40 to 80 U of HhaI, HpaII, or MspI under the conditions described by the suppliers of these enzymes (MspI and HhaI from New England Biolabs and HpaII from Bethesda Research Laboratories). To monitor digestion, ^{32}P-labeled adenovirus type 2 DNA was added to a sample of the reaction mixture, and the digestions were allowed to continue at 37°C for 2 to 3 h. The enzyme was inactivated either by heating at 68°C for 5 min or by phenol extraction followed by precipitation with ethanol at −20°C. The DNA digests, taken up in Tris-EDTA containing bromophenol blue dye marker, were applied in each slot of a 1% agarose slab gel and electrophoresed at 40 V for 16 to 18 h. DNA in the gel was denatured by alkali, transferred to nitrocellulose filter paper (Millipore Corp.), and hybridized with ^{32}P-labeled virus-specific complementary DNA by the method of Southern (14) with some modifications (8). ^{32}P-labeled viral complementary DNA (300 × 10^6 to 500 × 10^6 cpm/μg) was synthesized by the method of Taylor et al. (18), using calf thymus DNA primer and avian myeloblastosis virus reverse transcriptase (kindly supplied by J. Beard through the Office of Logistics and Resources of the National Institutes of Health). The hybridizations were carried out for 24 to 30 h at 68°C in sealed plastic bags containing 10 ng of ^{32}P-labeled complementary DNA per ml and 10 μg of HeLa cell rRNA per ml (14). We have observed that if we include rRNA during hybridization, no bands corresponding to rDNA are detected. The filters were washed by the method of Jeffreys and Flavell (8) with minor modifications, dried, and exposed against a Kodak X-Omat XR-1 film at −70°C with a Du Pont Cronex intensifying screen. A HindIII digest of λDNA was used as a marker.*

enzymes and that the fragments ranged from 0.12 to 1.2 Md (Fig. 1). In reconstruction experiments with ^{32}P-labeled DNA mixed with XC DNA, we ruled out incomplete digestion or the presence of nonspecific inhibitors in the XC DNA preparations. Similar results were obtained with two different clones of XC cells. From these results we conclude that the majority of the proviral DNA sequences in XC cells but not the unintegrated vDNA contain 5-methylcytosine.

Another set of experiments lends additional support to this conclusion. Instead of using whole DNA, XC, Pr.C-Ck, and chicken embryo fibroblast DNAs were first digested with the restriction enzyme *Eco*RI and then with *Hha*I, *Hpa*II, or *Msp*I; the resulting fragments were analyzed by the blotting method (7, 8, 12, 14) and then exposed against X-ray film for longer times to ensure against any incomplete digestion by the latter enzymes. Earlier, we generated cleavage maps of avian sarcoma vDNA for several restriction enzymes. On that basis, *Eco*RI should cleave vDNA at four sites to yield three defined fragments of 2.5, 2.0, and 1.5 Md. Figure 2 clearly shows the expected three fragments from both XC and Pr.C-Ck DNAs. In XC DNA another fragment of about 0.8 Md was also evident. Our evidence indicates that this fragment was generated by an additional *Eco*RI site which is present in many of the viral copies in XC DNA. In agreement with the data in Fig. 1, digestion by *Hha*I and *Hpa*II did not alter the pattern, whereas *Msp*I completely digested the DNA, as evidenced by the absence of all four major bands. By contrast, in the Pr.C-Ck DNA, both the 2.0- and the 1.5-Md fragments were completely digested by all three enzymes, and the intensity of the 2.5-Md fragment was greatly reduced. Unintegrated vDNA, either digested directly or after *Eco*RI digestion, showed complete susceptibility to *Hpa*II, *Msp*I, and *Hha*I (Fig. 2), providing strong evidence for the absence of any 5-methylcytosine in the recognition sequences for these enzymes. We have not yet derived a complete map for the cleavage sites of these enzymes, mainly because of a large number of small fragments which can be easily missed unless a very sensitive method is available. *Eco*RI enzyme results, as well as data with several enzymes such as *Hin*dIII and *Xba*I, which

are not affected by methylation, strongly argue against any possible artifacts in the restriction enzyme digestion. Further, these results indicate that the majority of the cytosine residues present in the recognition sequences for *Hha*I and *Hpa*II are methylated.

When XC DNA, Pr.C-Ck DNA, chicken embryo fibroblast DNA, and vDNA were digested with *Msp*I, a smear of small fragments was obtained (Fig. 1). To demonstrate the disappearance of *Eco*RI fragments and the appearance of several different small fragments as a result of *Msp*I digestion, we used a gradient agarose gel system. In this case, not only large fragments but also small fragments of up to 0.2 Md were detected. These were identical to those obtained with unintegrated vDNA (data not shown). *Hha*I generated slightly different-size fragments from vDNA and Pr.C-Ck DNA (Fig. 2).

Since the Pr.C-Ck DNA used in these experiments was isolated from the infected-cell nuclei and since the DNA was larger than 10 Md (Fig. 2), it is unlikely that the observed sensitivity of this DNA to *Hpa*II, *Msp*I, and *Hha*I was due to the presence of contaminating unintegrated vDNA, as there was no trace of 6.0-Md fragments in the uncleaved DNA (Fig. 2).

It should also be pointed out that *Eco*RI released not only the three expected 2.5-, 2.0-, and 1.5-Md fragments but also four other fragments of about 13.0, 10.0, 6.0, and 5.2 Md from the Pr.C-Ck DNA. In uninfected chicken embryo fibroblast DNA, only the 13.0-, 10.0-, 6.0-, 5.2-, and 2.5-Md bands were detected (Fig. 2). Two groups (1, 10) presented evidence for the presence of three *Eco*RI fragments derived from endogenous (Rous-associated virus type 0) viral sequences which correspond to 10.0-, 6.0-, 5.2-, and 2.5-Md fragments. In addition, McClements et al. (10) have shown that the 13-Md band contains sequences related to the endogenous *sarc* gene. Therefore, it is very likely that the 10.0-, 5.2-, and a portion of the 2.5-Md fragments are derived from endogenous Rous-associated virus type 0 sequences. Since the 5.2-, and 2.5-Md fragments were resistant to *Hha*I and *Hpa*II but completely susceptible to *Msp*I, we propose that, unlike the exogenous viral sequences, some of the endogenous sequences are also methylated. After *Hpa*II digestion, another prominent band of about 4.4 Md appeared. This might be due to the removal of adjacent cellular se-

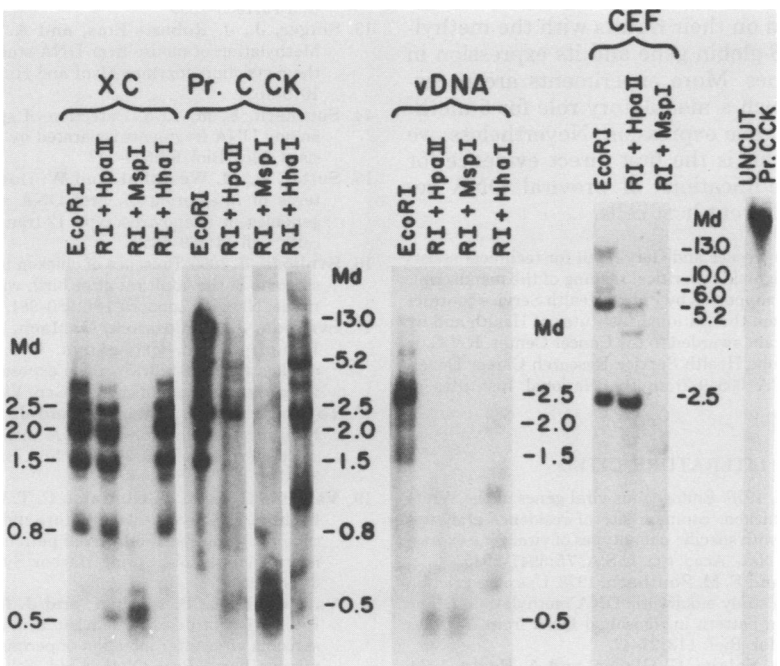

FIG. 2. *Purified cellular DNA was first digested with EcoRI (Bethesda Research Laboratories) at an enzyme-to-DNA ratio of about 8 to 10, phenol extracted, and precipitated with ethanol at −20°C. The precipitates were collected by centrifugation and suspended in the appropriate buffer, and a sample was subsequently treated with HhaI, HpaII, or MspI. The DNA digests were electrophoresed on 1% agarose gels, and virus-specific sequences were detected as described in the legend to Fig. 1. All chicken embryo fibroblast (CEF) cells were prepared from 12-day-old embryos which were obtained from SPAFAS.*

quences present in the 13.0-, 10.0-, and 6.0-Md fragments.

In summary, we have presented evidence for the methylation of proviral DNA sequences in a nonpermissive rat cell line as well as endogenous viral sequences that are normally present in chicken cells. In contrast, neither the unintegrated vDNA nor the integrated proviral DNA from chicken cells exogenously infected by avian sarcoma virus of different strains (Prague C, Prague B, and B77) is methylated. The fact that in both cases in which transcription was less efficient the proviral DNA was methylated whereas in chicken cells in which transcription was occurring no such methylation could be detected suggests, but does not prove, a correlation between the level of transcription and the degree of methylation. Also, the fact that the 2.0- and 1.5-Md *Eco*RI fragments, which lack 5-methylcytosine, represent the end fragments of the avian sarcoma virus genome and contain a possible promoter site (Guntaka et al., unpublished data) suggests a regulatory role for this odd base. The evidence presented here and by others (R. Sweet, personal communication) allows us to postulate a possible modulating role for 5-methylcytosine in gene regulation. McGhee and Ginder (11) recently arrived at similar conclusions based on their results with the methylation of the β-globin gene and its expression in different tissues. More experiments are necessary to test such a modulatory role for 5-methylcytosine in gene expression. Nevertheless, we believe that this is the first direct evidence for structural modifications of proviral DNA sequences in different host cells.

We thank Lou Yesner and Geryl Hall for technical assistance and D. H. Figurski for critical reading of the manuscript.

This work was supported by Public Health Service contract NO1 CP 71055 from the National Institutes of Health and by a core research grant awarded to the Cancer Center. R.V.G. is a recipient of Public Health Service Research Career Development award CA 00540 from the National Institutes of Health.

LITERATURE CITED

1. **Astrin, S. M.** 1978. Endogenous viral genes of the White Leghorn chicken: common site of residence and sites associated with specific phenotypes of viral gene expression. Proc. Natl. Acad. Sci. U.S.A. **75**:5941-5945.
2. **Bird, A. P., and E. M. Southern.** 1978. Use of restriction enzymes to study eukaryotic DNA methylation. I. The methylation pattern in ribosomal DNA from *Xenopus laevis*. J. Mol. Biol. **118**:27-47.
3. **Cedar, H., A. Solage, G. Glaser, and A. Razin.** 1979. Direct detection of methylated cytosine in DNA by use of the restriction enzyme MspI. Nucleic Acids Res. **6**: 2125-2132.
4. **Guntaka, R. V., O. C. Richards, P. R. Shank, H.-J. Kung, N. Davidson, E. Fritsch, J. M. Bishop, and H. E. Varmus.** 1976. Covalently closed circular DNA of avian sarcoma virus: purification from nuclei of infected quail tumor cells and measurement by electron microscopy and gel electrophoresis. J. Mol. Biol. **106**: 337-357.
5. **Hayward, W. S.** 1977. Size and genetic content of viral RNAs in avian oncovirus-infected cells. J. Virol. **24**:47-63.
6. **Hill, M., and J. Hillova.** 1974. RNA and DNA forms of the genetic material of C-type viruses and the integrated state of the DNA form in the cellular chromosome. B.B.A. Rev. Cancer **355**:7-31.
7. **Hsu, T. W., J. L. Sabran, G. E. Mark, R. V. Guntaka, and J. M. Taylor.** 1978. Analysis of unintegrated avian RNA tumor virus double-stranded DNA intermediates. J. Virol. **28**:810-818.
8. **Jeffreys, A. J., and R. A. Flavell.** 1977. A physical map of the DNA regions flanking the rabbit β-globin gene. Cell **12**:429-439.
9. **Mann, M. B., and H. O. Smith.** 1977. Specificity of HpaII and HaeII DNA methylases. Nucleic Acids Res. **4**:4211-4221.
10. **McClements, W., H. Hanafusa, S. Tilghman, and A. Skalka.** 1979. Structural studies on oncornavirus-related sequences in chicken genomic DNA. Proc. Natl. Acad. Sci. U.S.A. **76**:2165-2169.
11. **McGhee, J. D., and G. D. Ginder.** 1979. Specific DNA methylation sites in the vicinity of the chicken β-globin genes. Nature (London) **280**:419-420.
12. **Shank, P. R., S. H. Hughes, H. J. Kung, J. E. Majors, N. Quintrell, R. V. Guntaka, J. M. Bishop, and H. E. Varmus.** 1978. Mapping unintegrated avian sarcoma virus DNA: termini of linear DNA bear 300 nucleotides present once or twice in two species of circular DNA. Cell **15**:1383-1395.
13. **Singer, J., J. Roberts-Ems, and A. D. Riggs.** 1979. Methylation of mouse liver DNA studied by means of the restriction enzymes MspI and HpaII. Science **203**: 1019-1021.
14. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503-517.
15. **Sutter, D., M. Westphal, and W. Doerfler.** 1978. Patterns of integration of viral DNA sequences in the genomes of adenovirus type 12-transformed hamster cells. Cell **14**:569-585.
16. **Svoboda, J.** 1960. Presence of chicken tumor virus in the sarcoma of the adult rat after birth with Rous sarcoma tissue. Nature (London) **186**:980-981.
17. **Svoboda, J., I. Hlozanek, O. Mach, A. Michlova, J. Riman, and M. Urbankova.** 1973. Transfection of chicken fibroblasts with single exposure to DNA from virogenic mammalian cells. J. Gen. Virol. **21**:47-55.
18. **Taylor, J. M., R. Illmensee, and J. Summers.** 1976. Efficient transcription of RNA into DNA by avian sarcoma virus polymerase. Biochim. Biophys. Acta **442**: 324-330.
19. **Varmus, H. E., R. V. Guntaka, C. T. Deng, and J. M. Bishop.** 1974. Synthesis, structure and function of avian sarcoma virus-specific DNA in permissive and nonpermissive cells. Cold Spring Harbor Symp. Quant. Biol. **39**:987-996.
20. **Varmus, H. E., P. K. Vogt, and J. M. Bishop.** 1973. Integration of deoxyribonucleic acid specific for Rous sarcoma virus after infection of permissive and nonpermissive hosts. Proc. Natl. Acad. Sci. U.S.A. **70**:3067-3070.
21. **Wallwijk, C., and R. A. Flavell.** 1978. DNA methylation at a CCGG sequence in the large intron of the rabbit β-globin gene: tissue-specific variations. Nucleic Acids Res. **5**:3231-3236.