Vol. 100, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1981 Pages 559-565

SYNTHESIS OF HYPOMETHYLATED EPSTEIN-BARR VIRAL DNA IS STIMULATED BY DIMETHYLSULFOXIDE TREATMENT OF LYMPHOBLASTOID CELLS

David Larocca, Delores Homisak and Wendy Clough

Molecular Biology Division, University of Southern California
Los Angeles, California, 90007

Received March 31, 1981

SUMMARY. Dimethylsulfoxide, previously described as an inducer of cell differentiation, induced Epstein-Barr viral DNA synthesis but inhibited host cell DNA synthesis in an Epstein-Barr virus producer lymphoblastoid cell line. The viral DNA, whose synthesis was stimulated, was hypomethylated, in contrast to the cellular DNA, which was highly methylated and whose synthesis was inhibited by dimethylsulfoxide treatment of the cells. No alteration of DNA methylation patterns was observed in treated cells. Dimethylsulfoxide therefore appeared to selectively stimulate synthesis of previously hypomethylated DNA while inhibiting synthesis of methylated DNA within this virus-producing cell line.

INTRODUCTION. We report here the effects of dimethylsulfoxide (DMSO) on the replication of the human herpes virus, Epstein-Barr (EBV), in a virus-producing cell line. DMSO has been studied extensively for its medicinal properties (1). It is also among several compounds that induce Friend erythroleukemic cells to differentiate into globin-producing cells (2). n-Butyrate, also an inducer of differentiation of Friend cells, has recently been shown to induce EBV antigen production in EBV producer cells (3). These reports led us to investigate the possibility of similar effects of DMSO on EBV synthesis in a virus producer cell line.

DNA in DMSO-treated globin-producing Friend cells has been shown to be hypomethylated (4). Methylation of DNA has been proposed as a control mechanism for DNA replication and transcription, with active DNA being hypomethylated (5). These hypotheses have been supported experimentally by the observations that the DNAs of herpesvirus saimiri (6), adenovirus (7) and the

---

Abbreviations: DMSO, Dimethylsulfoxide; EBV, Epstein-Barr virus; PBS, phosphate-buffered saline; TPA, 12-O-tetradecanoylphorbol 13 acetate.

0006-291X/81/100559-07$01.00/0

proviruses of certain retroviruses (8) are hypomethylated in cells where they are actively replicating, and methylated when inactive. Similarly, the DNA of actively transcribed genes such as globin genes and early functions of adenovirus have been reported to be hypomethylated (7,9).

Our results are consistent with previous findings on DNA methylation. Although DMSO apparently does not alter the methylation pattern of viral or cellular DNA in the HR-1 cells we find that the DNA which is stimulated to replicate (viral DNA) is hypomethylated while the host cell DNA (whose synthesis is inhibited) is highly methylated in comparison.

MATERIALS AND METHODS.

Cell Culture. The HR-1 EBV producer lymphoblastoid cell line was obtained from M. Nonoyama. Cell cultures were kept at 35°C in a humidified 5% CO_2 incubator and fed once a week 1:1 with fresh medium (RPMI 1640, 5% fetal calf serum). DMSO (Malinckrodt, spectrophotometric grade) was added to experimental cultures to a final concentration of 200 mM (1.4%) immediately after feeding. The DNAs of experimental and control cell cultures were labelled by the addition of [^{3}H]thymidine (59 Ci/mmol, Schwarz/Mann) to a final concentration of 1 µCi/ml 24 h after feeding. The cells were harvested 72 h after feeding.

Cell DNA and Density Centrifugation. 1.7 x 10^8 cells were harvested for each culture and the DNA was extracted by a modification of the procedure described by Lindahl et al. (10). The cells were pelleted, washed three times in phosphate-buffered saline (PBS) and resuspended in 5 ml of PBS, followed by the addition of 0.5 volumes of Sarcosyl solution (3% Sarcosyl, 50 mM Tris, 20 mM EDTA). Preincubated pronase was added to a concentration of 1 mg/ml and the solution incubated 2 h at 50°C. Additional pronase (1 mg) was added at 2 intervals for 4 - 6 hours and the solution incubated overnight. CsCl and 10 mM Tris, 1 mM EDTA buffer were added to make a total of 30 ml of solution at a density of 1.718 g CsCl/ml. Centrifugation was performed in a Sorvall OTD 50 ultracentrifuge at 27,000 rpm and 21°C for 96 h, using a Beckman 30 rotor. The gradients were collected in 500 µl fractions which were assayed for trichloroacetic acid-precipitable [^{3}H] counts as previously described (11). Those fractions of the initial gradients corresponding to the putative viral DNA peaks were pooled and recentrifuged in 5 ml CsCl gradients in a Sorvall OTD 50 ultracentrifuge at 33,000 rpm and 21°C for 20 h, using a Sorvall TV 865 vertical rotor. The gradients were fractionated and assayed as described above except 100 µl fractions were collected.

Restriction Enzyme Digests. Hind III (Bethesda Research Laboratories) digests were performed in 20 mM Tris, pH 7.8, 10 mM $MgCl_2$, 10 mM mercaptoethanol, 50 mM NaCl and 100 µg/ml gelatin in a total volume of 100 µl for 3 h at 37°C. One µg of DNA was digested with 4 u of Hind III. Msp I and Hpa II digests (New England Biolabs; 10 u per 1 µg EBV or 5 µg cellular DNA) were performed in 20 mM Tris, pH 7.8, 10 mM $MgCl_2$, 10 mM mercaptoethanol at 37°C overnight. The digests were ethanol-precipitated by the addition of 0.1 volumes of 20% sodium acetate and 2.9 volumes of 95% ethanol, pelleted in a microfuge for 10 minutes, washed in cold 70% ethanol and resuspended in 20 µl resuspension buffer (10 mM Tris Borate [pH 8.3], 0.25 mM EDTA, 0.025% bromophenol blue, 10% glycerol). The samples were electrophoresed in agarose horizontal slab gels in TBE buffer (100 mM Tris Borate [pH 8.3], 25 mM EDTA) at 12.5 mamp for 18 h and stained with ethidium bromide (1 µg/ml).

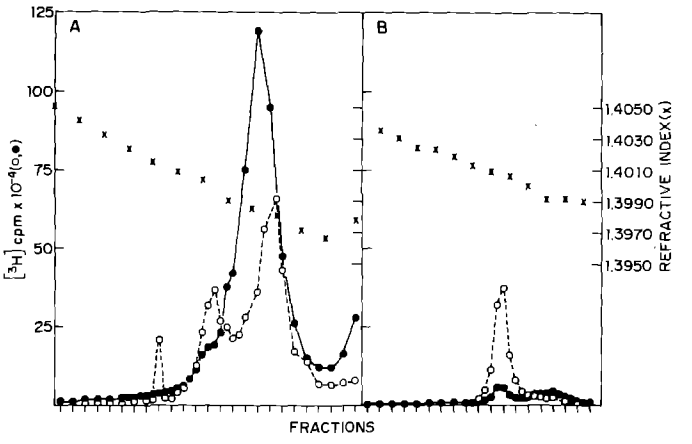

Fig. 1. Neutral CsCl density gradients of DNA extracted from HR-1 cells. (A) Initial CsCl density gradients (30 ml) were fractionated and assayed for acid-precipitable ^{3}H counts. HR-1 control (o——o), HR-1 DMSO-treated (o---o). (B) Second CsCl density gradients. The DNA from the putative viral peaks of the initial CsCl gradients was recentrifuged in 5 ml CsCl gradients in a vertical rotor (see Materials and Methods for experimental details).

RESULTS. DNA was extracted from HR-1 EBV producer cells that had been exposed to DMSO in culture or had been cultured as controls without the drug. This DNA was sedimented in CsCl density gradients, because it has previously been shown that the DNA of EBV has a higher GC content than does host cell DNA and migrates to a position of greater density in neutral CsCl density gradients (12,13). Two typical gradients representing the first round of centrifugation (DNA from untreated and DMSO-treated cells) are shown superimposed in Fig. 1A. DNA from the peak of greater density in Fig. 1A represented the putative viral DNA and was further purified from host cell DNA by a second round of CsCl centrifugation shown in Fig. 1B. There was as much as a 5-fold increase in incorporation of [^{3}H]thymidine into the peak of DNA of viral density in DMSO cultures over the controls. In addition there was a 50% decrease in the amount of label incorporated into cellular DNA (the less dense peak) for DMSO-treated cells as compared to controls. This selective stimulation of incorporation of [^{3}H]thymidine into viral DNA concurrent with a decrease in [^{3}H]thymidine incorporation into host cell DNA in a single population of DMSO-treated cells indicated that DMSO was not working simply by affecting levels of cellular thymidine pools.

This increase in synthesis of DNA of viral density was due to an increase in viral genomes and not an increase in high density host cell repetitive sequences or highly defective viral genomes, as indicated by the results in Fig. 2A. The DNA isolated from the portion of the CsCl gradient represented by the peak of greater density in Fig. 1B was digested with the restriction endonuclease Hind III. The size distributions of Hind III restriction fragments for DNA from both DMSO-treated and untreated HR-1 lymphocyte host cells were very similar to each other and closely resembled Hind III digestion patterns of viral DNA prepared in an identical manner from HR-1 cultures treated with n-butyrate or the tumor promotor 12-O-tetradecanoylphorbol 13 acetate. TPA has been previously shown to stimulate EBV DNA synthesis and viral production in EBV producer lymphocytes (14) (Fig. 2A, slots c and d). This distribution of Hind III digestion fragments was also similar to that previously reported for HR-1 virion DNA (15).

We observed no difference in cell viability between DMSO-treated cultures and controls as measured by the trypan blue exclusion technique. After 72 h incubation of HR-1 cells with DMSO there was typically a 15% decrease in cell density as compared to a 15% increase in non-treated controls. The volume of cell culture harvested was adjusted such that the same number of cells were harvested for DMSO-treated cultures as controls. The isolated host cell DNA from untreated control [^{3}H]thymidine-labeled cultures had a specific activity 2.5 times greater than that of the DMSO-treated cultures. The inhibition of cellular DNA replication is a well-documented effect of DMSO and a similar lag in growth has been observed in the Friend erythroleukemic cell system (16). Our observation that DMSO inhibits cellular DNA replication is also consistent with the recent finding that inducers of Friend cell differentiation inhibit lymphocyte mitogenesis at similar dosages (17).

We compared the methylated state of viral DNA from DMSO-treated cultures to that of untreated HR-1 cultures using the restriction enzymes Msp I and Hpa II. Hpa II, the isoschizomer of Msp I, cuts DNA at CCGG sites only if

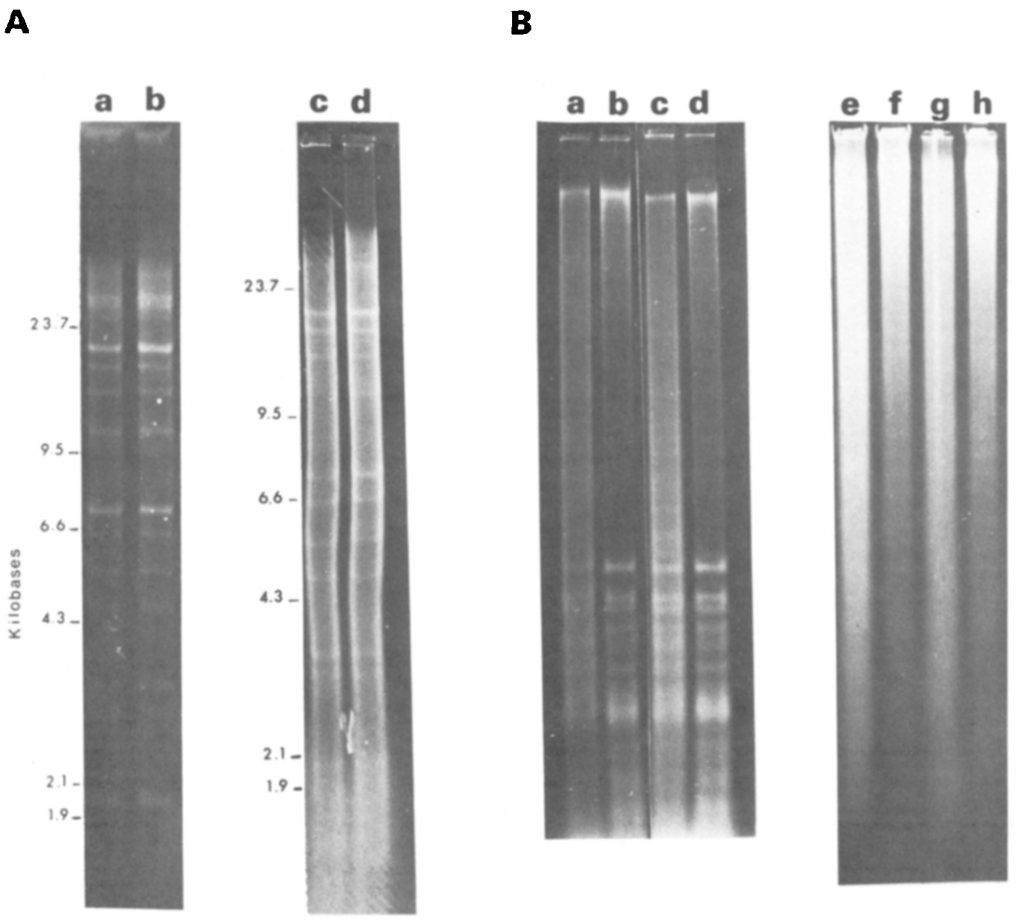

Fig. 2. Restriction enzyme digests of intracellular EB viral DNA. (A) The Hind III digestion fragments were separated by electrophoresis in a 0.4% agarose horizontal slab gel. Hind III fragments of: a) DNA from untreated control culture taken from the major DNA peak of gradient shown in Fig. 1B; b) DNA from DMSO-treated culture taken from major DNA peak of gradient shown in Fig. 1B; c) DNA isolated in the same manner as that in slot b except 3 mM n-butyrate was added to cell culture instead of DMSO; d) DNA isolated in the same manner as that in slot b except 10^{-8} M TPA was added to cell cultures instead of DMSO. Molecular weights (kilobases) are based on coelectrophoresed Hind III fragments of DNA. (B) EB viral DNA and HR-1 cellular DNA were digested with Msp I or Hpa II restriction endonuclease. The DNA fragments were separated by electrophoresis in a 1.0% agarose horizontal slab gel. EB viral DNA from untreated HR-1 cultures digested with a) Msp I and b) Hpa II; EB viral DNA from DMSO (200 mM)-treated cultures digested with c) Msp I and d) Hpa II; HR-1 cell DNA from untreated cell cultures digested with e) Msp I and f) Hpa II; HR-1 cell DNA from DMSO (200 mM)-treated cultures digested with g) Msp I and h) Hpa II.

the internal C is not methylated, while Msp I cuts at this sequence irrespective of DNA methylation (18,19). Fig. 2B shows that the Msp I and Hpa II restriction patterns obtained are similar whether viral DNA is from DMSO-

treated or untreated cells. Thus productively replicating viral DNA obtained from HR-1 producer cells is hypomethylated at CCGG sites regardless of stimulation of viral synthesis by DMSO. The host cell DNA from DMSO-treated and untreated control cultures is in contrast highly methylated since it is cut by Msp I but is resistant to Hpa II digestion, as shown in Fig. 2B slots e - h.

DISCUSSION. We observed that incorporation of [^{3}H]thymidine into EB viral DNA in transformed lymphocytes was stimulated by treatment of the cells with DMSO while incorporation of label into host cell DNA was inhibited, indicating that the processes of viral and cellular DNA synthesis in these cell populations were independent. These data were consistent with previous findings in another viral system, namely that DMSO induced Friend leukemic viral replication in certain subclones of Friend cells (20). Our data show that productively replicating EBV DNA in the control cell population is hypomethylated while the cellular DNA is highly methylated, at least at CCGG sequences. One explanation for our data is that DMSO is selectively inducing synthesis of previously hypomethylated populations of DNA within these cells while inhibiting the synthesis of previously methylated DNA populations. Hypomethylation may be a common feature of the DNA that is preferentially replicated or transcribed as a result of DMSO induction. Further examination of the effects of DMSO and other inducers of differentiation on EBV-infected human lymphoblastoid cells will be useful in studying the relationships between viral production, DNA methylation and transformation.

ACKNOWLEDGEMENTS. This work was supported by National Institutes of Health grant #CA23070 and American Cancer Society Institutional Research Grant to the LAC-USC Comprehensive Cancer Center, IN-21-S. W. C. is the recipient of Research Career Development Award CA 00707 from the National Cancer Institute.

REFERENCES.

1. Ann. N.Y. Acad. Sci. 243, pp. 390-508 (1975)
2. Friend, C., Scher, W., Holland, J. G., and Sato, T. (1971) Proc. Natl. Acad. Sci. U.S.A. 68, 378-381

3. Luka, J., Bengt, K., and Klein, G. (1979) Virology 94, 228-231
4. Christman, J. K., Price, P., and Pedrinan, L. (1977) Eur. J. Biochem. 81, 53-61
5. Razin, A., and Riggs, A. D. (1980) Science 210, 604-610
6. Desrosiers, R. C., Mulder, C., and Fleckenstein, B. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 3839-3843
7. Sutter, D., and Doerfler, W. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 253-256
8. Cohen, C. J. (1980) Cell 19, 653-663
9. McGhee, J. D., and Ginder, G. D. (1979) Nature (London) 280, 419-420
10. Lindahl, T., Adams, A., Bjursell, G., Bornkamm, G. W., Kaschk-Dierich, C., and Jehn, U. (1976) J. Mol. Biol. 102, 511-530
11. Goodman, S. R., Prezyna, C., and Clough, W. B. (1978) J. Biol. Chem. 253, 8617-8628
12. Schulte-Holthausen, H., and zurHausen, H. (1970) Virology 40, 776-779
13. Adams, A., Lindahl, T., and Klein, G. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 2888-2892
14. Hudewentz, J., Bornkamm, G. W., and zurHausen, H. (1980) Virology 100, 175-178
15. Rymo, L., and Forsblom, S. (1978) Nucleic Acids Res. 5, 1387-1402
16. Friend, C., and Freedman, H. A. (1978) Biochemical Pharmacology 27, 1309-1313
17. Stenzel, K. H., Schwartz, R., Rubin, A. L., and Novogrodsky, A. (1980) Nature 285, 106-108
18. Mann, M. B., and Smith, H. O. (1977) Nucleic Acids Res. 4, 4211-4221
19. Waalwijk, C., and Flavell, R. A. (1978) Nucleic Acids Res. 5, 3231-3236
20. Tsuei, D., Haubenstock, H., and Friend, C. (1977) In Vitro 13, 148-152