Volume 14 Number 10 1986 Nucleic Acids Research

In vitro methylation inhibits the promotor activity of a cloned intracisternal A-particle LTR

Anita Feenstra*, Joseph Fewell, Kira Lueders and Edward Kuff

Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

Received 21 January 1986; Revised and Accepted 11 April 1986

ABSTRACT

We studied the relation between LTR methylation and expression of the family of endogenous retrovirus-like elements related to mouse intracisternal A-particles (IAP). Comparative HpaII/MspI and HhaI restriction analysis of genomic DNA's showed that in cells and tissues with a low level of IAP gene expression, HpaII and HhaI sites within the 5' LTR were heavily methylated, while in cells abundantly expressing IAP's 20 to 30% of the 5' LTRs were demethylated at these sites. The effects of methylation on the promoter activity of a cloned IAP 5' LTR was studied directly, using the plasmid pMIA5' L-cat in which this LTR was linked to the chloramphenicol acetyl transferase (CAT) gene. In vitro methylation of three HhaI sites located between -137 and -205 bp from the RNA start site of this LTR completely inactivated the promoter activity of pMIA5'L-cat transfected into COS7 cells. Methylation of a HpaII site located 94 bp downstream from the RNA start site reduced the promoter activity by 75%. The results show that methylation at sites both upstream and downstream from the RNA start site profoundly effects the promoter activity of this LTR and suggest that methylation within the 5' LTR can serve to regulate IAP gene expression in vivo.

INTRODUCTION

Murine intracisternal A particles (IAP) are coded for by members of a large family of endogenous retrovirus-like elements (1). IAP gene products are regularly observed in early mouse embryos (2,3), normal adult tissues (4) and in many transformed cells (5). IAP proviral elements can act as insertional mutagens at both the germ line (6) and somatic levels (7-10). IAP gene expression has been implicated in such apparently disparate processes as the pathogenesis of an insulin dependent diabetes (11) and the modulation of immunoglobin (IgE) synthesis (12). DNA methylation, which is correlated with the inactive state of many retroviral, viral and cellular genes (13-16) has also been implicated in the regulation of IAP gene expression. With the methylation-sensitive restriction endonuclease HpaII, an inverse correlation between the methylation status of the IAP gene family and IAP gene expression was found in certain normal and transformed mouse cells (17,18). Furthermore, treatment with 5-azacytidine greatly enhanced

IAP gene expression in some teratocarcinoma lines and transformed fibroblasts (19,20). Morgan and Huang (21) found that two HhaI sites located in the 5' LTR of a particular IAP gene copy were hypomethylated in myeloma and 3T3 cells but not in normal liver. With this exception, the previous studies have dealt with the methylation status of sites within the body of the IAP genes rather than in the LTRs. However, retroviral gene expression is determined by regulatory elements within their LTRs, and methylation at internal sites can be irrelevant to gene expression (22). In the present study we have examined the methylation state of the 5' IAP LTRs in the DNA of several normal and transformed mouse tissues and confirmed that the degree of methylation at conserved HhaI and HpaII sites in the 5' LTRs is inversely correlated with IAP gene expression. Using a cloned mouse IAP LTR that functions as an efficient promoter when linked to the chloramphenicol acetyl transferase (CAT) gene (23), we have also shown that in vitro methylation of HhaI and HpaII sites in this LTR strongly suppressed its promoter activity.

METHODS AND MATERIALS

DNA Isolation and Restriction Analysis

The following mouse tumors and tumor cell lines were used: The solid myeloma MOPC-104E, the cultured line MOPC-31 and the neuroblastoma cell line N4. Liver and thymus were taken from one month BALB/cJ female mice. Cellular DNA's were prepared as described (24). For each restriction analysis, 10 µg of DNA was cleaved with a 4-fold excess of restriction enzyme in the buffer recommended by the supplier (New England Biolabs, Inc.). Incubations were carried out for 3 hours at 37°C. Lambda phage DNA was included in some reactions to monitor the extent of digestion. For the HhaI/XbaI restriction analysis (Fig 2B), the standards consisted of HhaI/XbaI-cleaved pMIA14.4 (24) to which 10 µg of calf thymus DNA, partially digested with MspI, was added to ensure comparable mobility and transfer of low molecular weight fragments. Digested DNAs were electrophoresed on a 1.4% horizontal agarose gel and subsequently transferred by Southern blotting to DPT paper (Schleicher and Schull) or nitrocellulose as described (4). The 1 Kb 5' HpaII fragment isolated from pMIA14.4 (see fig. 1A) was used as a probe after nick translation to a specific activity of $1x10^8$ to $2x10^8$ cpm/µg DNA as described (10). The DNA blots were hybridized for 24 hr. at 65° C in a solution containing: 6xSSC, 0.25% SDS, 3xDenhardts and $0.5x10^6$ cpm/ml of [^{32}P]-labeled probe, and washed three times for 15 minutes at 55° C in 0.1xSSC, 0.1% SDS.

In vitro Methylation

The plasmids pSVOcat and PSV2cat were obtained from C. Gorman, the plasmid pMIA 5'L-cat was described before (23). The closed circular plasmids, pSVOcat and pMIA5'L-cat purified by two cycles of banding in CsCl-ethidium bromide were methylated in vitro with the use of HpaII or HhaI methyl transferase (New England Biolabs, Inc). A typical methylation reaction consisted of: 40 µg of plasmid, 300 µl methylation buffer (50 mM Tris/HCl pH 7.4, 10 mM EDTA, 5 mM 2-mercaptoethanol, 80 µM S-adenosylmethionine) and 50-75 units of either HpaII or HhaI methylase. The reaction mix was incubated for 2 hr at 37° C, after which the enzyme was inactivated for 20 minutes at 65° C. The methylated DNA's were extracted with phenol-chloroform-isoamylalcohol (50:49:1), chloroform-isoamylalcohol (49:1) and ethanol precipitated. To determine whether the methylations were complete, treated plasmids were linearized with BamHI, digested with HpaII or HhaI and examined by gel electrophoresis. All of the methylated plasmids used in the study were completely resistant to cleavage with the appropriate restriction enzyme.

Eukaryotic Cell Transfection

COS7 monkey-kidney cells were transfected with 10µg methylated or unmethylated (control) plasmid per 100 mm dish, using the calcium phosphate technique of Graham and van der Eb (25) as modified by Parker and Stark (26).

Forty-eight hours after transfection cell extracts were prepared by sonication in 100µl of 0.25 M Tris/HCl pH 8.0 and CAT activity of 20µl samples was assayed by following the conversion of [^{14}C]-chloramphenicol to its mono- and di- acetylated forms as described by Gorman et al (27). The reaction products were analyzed by ascending chromatography on silica gel thin layer plates (Merck).

After autoradiography the results were quantitated by counting the spots of [^{14}C]-chloramphenicol and its mono- and di-acetylated form scraped from the thin layer chromatography plates. Using the specific activity of the substrate, the number of picomoles converted to the acetylated from was determined.

RESULTS

The distribution of HpaII sites in subclone pMIA14.4, derived from a randomly selected endogenous IAP proviral element, is shown in Fig. 1A. The 5' LTR contains 3 HhaI sites upstream from the transcriptional signals and a single HpaII site near the 3' end (Fig. 1B). To assess the methylation state

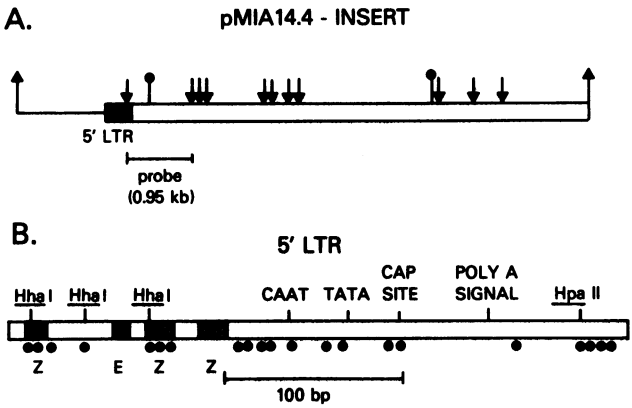

Fig. 1. A. Partial restriction map of the insert in pMIA14.4, a subclone in pBR322 that contains a fragment of a mouse genomick clone MIA14 (24). IAP proviral sequence is indicated by the bar, mouse flanking sequence by the single line. The filled section represents the IAP 5' LTR. Restriction sites are indicated as , HpaII; , XbaI; and , HindIII. The indicated HpaII fragment was used as probe for the experiment shown in Fig. 2. B. Features of the MIA14 5' LTR. The complete sequence has been published (10). The HhaI sites are located at -204, -177 and -138 bp, the HpaII site at +96 bp from the RNA start site (23). The dots represent CpG doublets, the hatched segments (Z) stretches of DNA with potential for Z-DNA formation (26), and the filled section (E) a "core enhancer sequence" similar to those found in SV40 and MuLV (27).

at these sites, cellular DNA isolated from mouse tissues and cell lines was first cleaved with XbaI to generate cuts at the conserved positions indicated in Fig. 1A, and then with one or the other methylation sensitive enzyme. The electrophoretically fractionated digests were hybridized with a probe obtained by HpaII digestion of pMIA14.4 (Fig. 1A). This probe contains only 27 bp of LTR sequence, therefore it is relatively insensitive to fragments originated within the 3' LTR. In HpaII/XbaI digests of DNA (Fig. 2A, lanes H), the hybridization signals from 0.4 and 0.6 Kb fragments reflect the extent of demethylation at HpaII sites within the LTR and the body of the IAP gene, respectively. These fragments are fully realized in double digests with XbaI and MspI, the methylation-insensitive isochizomer of HpaII (Fig 2A, lanes M). The HhaI/XbaI digests of pMIA14.4 contain a single reactive fragment of about 0.5 Kb, which extends from the most 3' of the HhaI sites in the 5' LTR (Fig. 1B) to XbaI site. The appearance of the 0.5 Kb fragment in genomic digests (Fig. 2B) indicates demethylation of this particular HhaI site. The extent of demethylation was calibrated with reference to known quantities of the HhaI/XbaI fragment generated from pMIA14.4. In normal

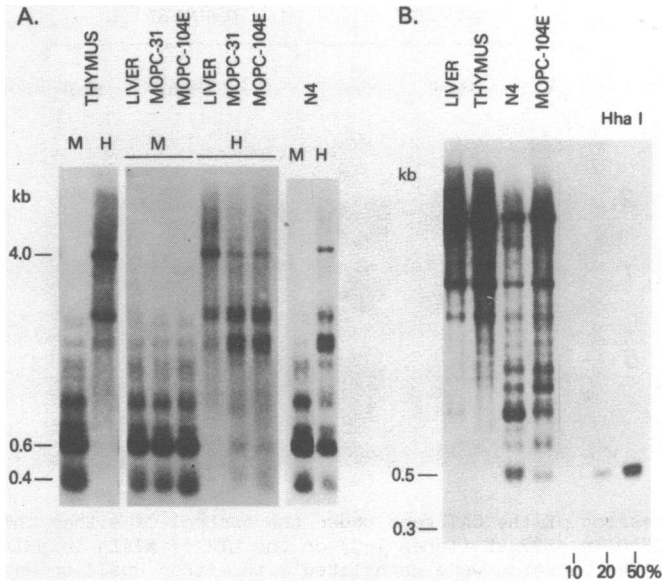

Fig. 2. Methylation of sites within 5' LTRs of IAP in the DNA of different mouse tissues and cells. A: Southern blot analysis of DNA restricted with XbaI and either HpaII (H) or MspI (M), and probed with the 0.95 Kb HpaII fragment indicated in Fig. 1A. The 0.4 Kb HpaII-XbaI fragment originates at the HpaII site 26 bp inside the 5' LTR. The 0.6 Kb fragment extends from the XbaI site to one or another of three clustered HpaII sites downstream. Hypomethylation of HpaII sites within the body of the IAP gene is reflected by the intensities of this band and of the 4.0 Kb XbaI-XbaI fragment (see Fig. 1A). B: Southern blot analysis of DNA restricted with XbaI and HhaI. Because of the lack of a methylation insensitive isochizomer of HhaI, the extent of demethylation at the most 3' HhaI site in the genomic 5' LTRs was determined by comparison with XbaI/HhaI digests of known quantities of pMIA14.4. The XbaI/HhaI digests of pMIA14.4 contains a single reactive fragment of about 0.5 Kb, corresponding to the 0.5 Kb fragment seen in the genomic digests of N4 and MOPC-104E. The MOPC-104E digest shows an additional 0.3 Kb fragment of obscure origin.

liver and thymus, HhaI and HpaII sites in the 5' LTR were extensively methylated as judged by the virtual absence of the appropriate restriction fragments. In IAP-rich tumor cell lines the proportion of 5' LTR showing demethylation at these sites ranged from 10-30% in two myelomas to about 50% in the N4 neuroblastoma line. Since there are some 1000 IAP gene copies per haploid genome, these values correspond to several hundred demethylated LTRs per cell. Thus in this group of cells and tissues abundant expression of the IAP genetic elements was correlated with extensive demethylation of HhaI and HpaII sites in the 5' LTRs.

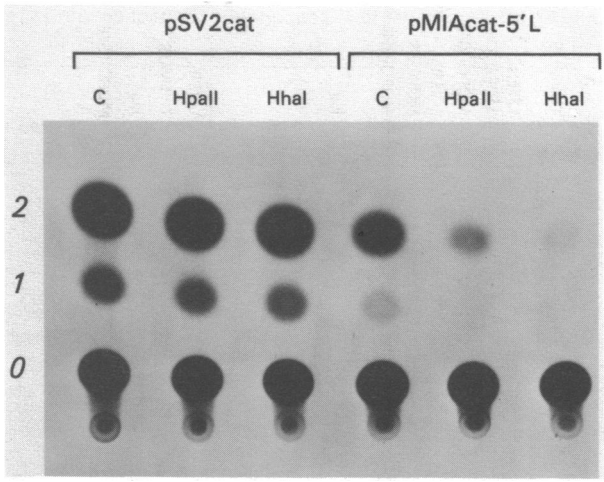

Fig. 3. Expression of the CAT gene under the control of either the early, promotor of SV40 in pSV2cat (lanes 1-3) or the LTR of MIA14 in pMIAcat-5' L (lanes 4-6). The plasmids were methylated with either HpaII or HhaI methyltransferase. The unmethylated plasmids were used as controls (c). The conversion of [^{14}C]-Chloramphenicol (0) to its acetylated forms (1 and 2) reflects the extent of expression of the CAT gene.

Next we evaluated the effect of *in vitro* methylation of the 3 HhaI sites and the single HpaII site, using the method devised by Kruczek and Doerfler to study the effect of site-specific methylation on promotor activity of several adenovirus 12 genes (28). We used the expression vector pMIAcat-5'L (23), in which the 5' LTR of MIA14 was inserted into the plasmid pSVOcat (26) immediately upstream of the CAT coding sequence. The plasmid pSV2cat contains the early promotor of SV40 in this position. Since the SV40 promotor does not contain HhaI or HpaI sites, pSV2cat provide a control for the effect of methylation of vector sequences on gene expression (27). The plasmids were fully methylated with either HhaI or HpaII methyltransferase and transfected into SV40-transformed monkey kidney cells (COS-7 cells), chosen because of their high level of CAT expression with the plasmid pMIAcat-5'L compared to several mouse cells (23). Forty-eight hours later CAT activities were compared to those from cells transfected with the unmethylated plasmids. The results of one transfection experiment are illustrated in Figure 3. Methylation of HhaI or HpaII sites in pMIAcat-5' L greatly reduced expression of the CAT gene. Since methylation of these sites in the pSV2cat control had little or no effect on CAT expression, the observed effects on activity of pMIAcat-5' L can be attributed to methylation

Table 1. Effects of *in vitro* methylation on the promoter activity of a cloned IAP LTR.

	pSV2cat			pMIAcat-5'L			pSVOcat
Experiment	Control	HpaII	HhaI	Control	HpaII	HhaI	
I	853*	660 (77%)+	565 (66%)	204	36 (18%)	8 (4%)	-
II	2430	2390 (98%)	2250 (92%)	433	209 (48%)	33 (8%)	112
III	3175	3549 (112%)	2711 (85%)	1930	340 (18%)	120 (6%)	57

The quantitative results of three transfection experiments are shown. Extracts of cells transfected with the HpaII- or HhaI-methylated plasmids pSV2cat and MIAcat-5'L or with unmethylated plasmids (controls) were assayed for CAT activity as described in Methods and Materials. Experiment I is that shown in Fig. 3. One set of methylated plasmids was used for Experiment I and a second for II and III.

* pmoles/h per 20µl of extract, 37°C

\+ percent of control value

within the IAP LTR. Table 1 contains quantitative data from three transfection experiments involving two separately methylated preparations. The plasmid pSVOcat, which lacks a eukaryotic promotor, was included in two experiments. The CAT activity in cells transfected with this plasmid and with HhaI-methylated pMIAcat-5'L were comparable.

DISCUSSION

Our results show that site-specific methylations in the IAP LTR can have profound effects on its promotor activity. Methylation of the three HhaI sites lying between -137 and -205 with respect to the RNA start site (23) essentially inactivated this LTR. Two of these HhaI sites lie within short sequence elements which have potential Z-DNA configuration (Z, Fig. 1B), as pointed out by Nordheim and Rich (29), and which bracket a core enhancer sequence (30) (E, Fig. 1B). Methylation, which is known to effect the Z-DNA transition (31), could influence LTR function by changing the affinity of a putative regulatory protein for binding sites within this region. *In vitro* methylation of the HpaII site also reduced the promotor activity of the cloned LTR. The HpaII site lies 94 bp downstream from the RNA initiation site and 27 bp from the 3' end of the LTR. Regulatory functions have not hitherto been assigned to this region of retroviral LTRs. However, Derse et al (32) in examining promotion of CAT expression by the bovine leukemia virus (BLV) LTR, found evidence from deletion mapping that sequences downstream of the RNA cap site were required for maximal promotor activity. In addition

sequences involved in transactivation of the HTLV-II LTR are located between -17 and +80 with respect to the RNA start site (33). Methylation at the HpaII site of the 5' IAP LTR could conceivably affect the binding of a factor required for transcription through the downstream region or have a direct effect on transcription through changes in DNA secondary structure. Short CpG-rich clusters containing at least one, but more often 2 or 3 closely spaced CpG pairs are found near the 3' ends of a number of mammalian retroviral LTRs, including other IAP genes of mouse (34) and Syrian Hamster (35), the IAP-related recombinant M432 virus of Mus cervicolor (Callahan, R., personal communication), mouse MMTV (36), BLV (37); and HTLV-1 (38). HpaII or HhaI sites are present in a majority of cases. It would be interesting to know whether in vitro methylation of these sites is inhibitory for the promotor activity of other LTRs.

As yet, we do not know the functional consequences of methylation elsewhere than in the HpaII and HhaI sites of the IAP LTR. The MIA14 LTR contains 16 additional potentially methylatable CpG dinucleotides (Fig. 1B), two of which are located in a 15 bp GC-rich cluster that begins 45 bp upstream of the TATA box (10) and resembles the functionally important promotor proximal GC-rich domain found in many eukaryotic genes (36).

The much stronger promotor activity of the MIA14 LTR in heterologous COS7 cells as compared to mouse 3T3 and L cells (23) is not understood. Transcription-activating factors present in COS7 cells, such as T-antigen (40,41), could be effecting the activity of the LTR. The low activity of the MIA14 LTR transfected into mouse L cells has made estimation of the methylation effects difficult; however, preliminary experiments have indicated a similar inhibitory effect of HhaI methylation on LTR activity. Extrapolation of the present data to the in vivo situation must await development of a suitable murine target cell. Meanwhile, the system based on COS7 cells may be a useful model for studying the interactions of cell-encoded factors with IAP LTRs and for determining how these interactions are influenced by site-specific methylation.

*Present address: Neuropsychiatry Branch, IRP, National Institutes of Mental Health, St. Elizabeths Hospital, Washington, DC 20032, USA

REFERENCES

1. Lueders, K.K. and Kuff, E.L. (1977) Cell 12, 963-972.
2. Piko, L., Hammons, M.D. and Taylor, K.D. (1984) Proc. Natl. Acad. Sci. USA 81, 488-492.

3. Yotsuyanagi, Y. and Szollosi, D. (1984) Ultrastructure of Reproduction (eds. Blerkon, I. & Motta, P.M.) 218-234.
4. Kuff, E.L. and Fewell, J.W. (1985) Mol. & Cell. Biol. 5, 474-483.
5. Kuff, E.L., Lueders, K.K., Ozer, H.L. and Wivel, N.A. (1972) Proc. Natl. Acad. Sci. USA 69, 218-222.
6. Burt, D.W., Reith, A.D. and Brammar, W.J. (1984) Nucleic Acid Res. 12, 8579-8593.
7. Rechavi, G., Givol, D. and Canaani, E. (1982) Nature 300, 607-611.
8. Horowitz, M., Luria, S., Rechavi, G. and Givol, D. (1984) EMBO Journal 3, 2937-2941.
9. Hawley, R.G., Shulman, M.J. and Hozumi, N. (1984) Mol. & Cell. Biol. 4, 2565-2572.
10. Kuff, E.L., Feenstra, A., Lueders, K., Smith, L., Hawley, R., Hozumi, H. and Shulman, M. (1983) Proc. Natl. Acad. Sci. USA 80, 1992-1996.
11. Leiter, E.H. and Kuff, E.L. (1984) Am. J. Pathol. 114, 46-55.
12. Moore, K.W., Jardieu, P., Meitz, J.A., Trounstine, M.L., Kuff, E.L., Ishizaka, K. and Martens, C.L. (1986) J. Immunol. (submitted).
13. Razin, A. and Szyf, M. (1984) Biochem. Biophys. Acta 782, 331-342.
14. Bird, A.P. (1984) Nature 307, 503-504.
15. Jeanisch, R. and Jahner, D. (1984) Biochem. Biophys. Acta 782, 1-9.
16. Doerfler, W.A. Ann. Rev. Biochem. 52, 93-124.
17. Hojman-Montes de Oca, F., Dianoux, L., Peris, J. and Emanoil-Ravicovitch, R. (1983) J. Virol. 46, 307-310.
18. Mays-Hoopes, L.L., Brown, A. and Huang, R.C.C. (1983) Mol. & Cell. Biol. 3, 1371-1380.
19. Hojman-Montes de Oca, F., Lasneret, J., Dianoux, L., Canivet, M., Ravicovitch-Ravier, R. and Peris, J. (1984) Biol. Cell. 52, 199-204.
20. Lasneret, J., Canivet, M., Hojman-Montes de Oca, F., Tobaly, J., Emanoil-Ravicovitch, R. and Peries, J. (1983) Virology 128, 485-489.
21. Morgan, R.A. and Huang, R.C.C. (1984) Cancer Res. 44, 5234-5241.
22. Busslinger, M., Hurst, J. and Flavell, R.A. (1983) Cell 34, 197-206.
23. Lueders, K.K., Fewell, J.W., Kuff, E.L. and Koch, T. (1984) Mol. & Cell. Biol. 4, 2128-2135.
24. Lueders, K.K. and Kuff, E.L. (1980) Proc. Natl. Acad. Sci. USA 77, 3571-3575.
25. Graham, F. and Van Der Eb, A. Virology 52, 456-457.
26. Parker, B. and Start, G.R. (1979) J. Virology 31, 360-369.
27. Gorman, C.M., Moffat, L.F. and Howard, B.H. (1982) Mol. & Cell. Biol. 2, 1044-1051.
28. Kruczek, I. and Doerfler, W. (1983) Proc. Natl. Acad. Sci. USA 80, 7586-7590.
29. Nordheim, A. and Rich, A. (1983) Nature 303, 674-679.
30. Laimins, L.A., Khoury, G., Gorman, C., Howard, B. and Gruss, P. (1982) Proc. Natl. Acad. Sci. USA 79, 6453-6457.
31. Behe, M. and Felsenfeld, G. (1981) Proc. Natl. Acad. Sci. USA 78, 1619-1623.
32. Derse, D., Caradonna, S.J. and Casey, J.W. (1985) Science 227, 317-320.
33. Sodroski, T., Patarca, R., Rosen, C., Wong-Staal, F. and Haseltine, W. (1985) Science 229, 74-77.
34. Christy, R.J., Brown, A.R., Gourlie, B.B. and Huang, R.C.C. (1985) Nucleic Acids Res. 13, 289-302.
35. Ono, J. and Ohishi, H. (1983) Nucleic Acids Res. 11, 7169-7179.
36. Donehower, L.A., Huang, A.L., and Hager, G.L. (1981) J. Virology 37, 226-238.
37. Couez, D., Deschamps, J., Kettman, R., Stephens, M.R., Gilden, R.V. and Burney, A. (1984) J. Virology 49, 615-620.

38. Seilu, M., Hattori, S., Hirayoma, Y. and Yoshida, M. (1983) Proc. Natl. Acad. Sci. USA 80, 3618-3622.
39. Bensimhon, M., Gabarro-Arpo, J., Ehrlich, R. and Reiss, C. (1983) Nucleic Acids Res. 11, 4521-4539.
40. Glutzman, Y. (1981) Cell, 175-182.
41. Keller, J.M. and Alwine, J.C. (1984) Cell 36, 381-389.