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NORTHERN BLOT HYBRIDIZATION ANALYSIS OF POLYOMA VIRUS-SPECIFIC RNA SYNTHESIZED UNDER THE BLOCK OF VIRUS REPLICATION BY 5-BROMO-2'-DEOXYURIDINE

J. TARÁBEK, J. ŽEMLA, I. BAČÍK

Institute of Virology, Slovak Academy of Sciences, 842 46 Bratislava, Czecho-Slovakia

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Summary. – Polyoma (Py) virus-specific RNA, synthesized at reduced level in infected cells in the presence of antiviral compound 5-bromo-2'-deoxyuridine (BrdUrd) was characterized in more detail by Northern blot hybridization analysis. The results obtained with total, cytoplasmic and poly(A)RNA, isolated from mouse embryo cell cultures 42 hrs p.i. indicate that BrdUrd (6.34 μg/ml) lowers the level of typical classes of major virus DNA transcripts to a similar extent and that no new, atypical transcription products are formed.

Key words: polyoma virus; RNA transcripts; bromodeoxyuridine

Introduction

The analogs of thymidine, in which the 5-methyl group of the base is substituted by bromine, iodine or another substituent are known as inhibitors of multiplication of animal DNA viruses (Kaplan *et al.*, 1965; Prusoff and Goz, 1975). The basis of their antiviral action is obvious: the analogs are phosphorylated and incorporated into viral DNA, and such DNA itself or its expression products, i.e. messenger RNAs and proteins do not function properly and do not lead to mature, infectious virus particles (Kaplan *et al.*, 1965). Besides that there are many other effects of these analogs on virus-infected and normal cells, e.g. changes of some virus-induced enzymes of DNA synthesis (Kamyia *et al.*, 1965), induction of virus production in non-producer cells (Lowy *et al.*, 1971), changes in gene expression in normal cells (Stellwagen and Tomkins, 1971; Preisler *et al.*, 1973) and inhibiton of cell multiplication (Hakala, 1959).

We have reported earlier (Žemla and Tarábek, 1981) that BrdUrd leads to a reduced level of Py virus-specific RNA in infected cells. In this work we present our attempt to characterize the viral RNA transcripts formed under the influence of BrdUrd using the Northern blot hybridization analysis.

Materials and Methods

Virus. The plaque-purified A2LP strain of Py virus was propagated in primary cultures of mouse embryo (ME) cells as described earlier (Žemla and Tarábek, 1981).

Cells. Primary cultures of ME cells were prepared from 16–18 days-old mouse embryos by the standard trypsinization procedure. 200 ml roller bottle cultures were seeded with 65×10^6 cells in 50 ml of BEM-CS_{10} medium and reached confluency in 3–5 days. To infect the cells, cultures were first washed with BEM-Tris medium and then 2 ml of virus inoculum in the same medium, corresponding to 25–50 PFU per cell was added per bottle. After 3 hrs of virus adsorption at 37 ^{0}C 50 ml of BEM-CS_{10} medium per bottle was added.

Media. BEM-CS_{10} medium was Basal Eagle's medium with 10 % of heat-inactivated calf serum. BEM-Tris medium contained 20 mmol/l Tris-HCl, pH 7.5 in BEM.

Total, cytoplasmic and poly(A)RNA were isolated usually 42 hrs p.i. from Py virus-infected ME cells by the methods described by Scherrer (1969), Favaloro *et al.* (1980) and Ausubel *et al.* (1987) respectively. At this time the virus-specific RNA reached a maximal level (Žemla and Tarábek, 1981). If the cells were treated with BrdUrd, the analog was added to the culture medium after the 3 hrs virus adsorption period and remained there till the isolation of RNA.

Marker-RNA. Total RNA isolated from uninfected ME cells was used as size marker.

Agarose gel electrophoresis of RNA was carried out in denaturing conditions as described by Lehrach *et al.* (1977).

Nothern blot hybridization was performed by a standard technique (Ausubel *et al.*, 1987). The hybridization probe consisted of complete linear Py viral DNA labelled with ^{32}P-dCTP by nick translation. Py viral DNA originated from the recombinant plasmid Py/pXf3, kindly supplied by Dr. M. Fried from ICRF in London and it was cut out from it by *Bam*HI endonuclease. For the autoradiography the X-ray film Medix Rapid with intensifying screen was used.

Direct measurement of radioactivity of blots. With the aim to increase the resolution of individual RNA bands, the radioactivity of blots was directly measured. 1 mm strips cut out from individual tracks on blots were assayed for ^{32}P radioactivity in 6 ml of Instagel in Packard Tricarb liquid scintillation spectrometer Model 1500. The relationship of radioactivity (cpm) to distance of a strip from the start of electrophoresis (mm) was plotted in curve.

Chemicals. BrdUrd, Nonidet P-40 and calf thymus DNA were purchased from Fluka; agarose (standard EEO), Tris, LiCl, SDS, formamide and neuraminidase from Serva; oligo dT cellulose Type 7, Sephadex G-50, Ficoll-400 and polyvinylpyrrolidone from Pharmacia; Instagel from Packard; nitrocellulose membrane BA 85 (0.45 μ) from Schleicher and Schuell; restriction endonuclease *Bam*HI from Serva and Institute of Sera and Vaccines, Prague, respectively; deoxycytidine-5'-(alpha-^{32}P)-triphosphate, triethylammonium salt, 110 TBq/mmol from Amersham International plc, Aylesbury or Inzita, Budapest.

Results and Discussion

Total, cytoplasmic and poly(A)RNA were prepared from Py virus-infected ME cells exposed to BrdUrd and subjected to blot hybridization analysis under these prerequisites: (a) high multiplicity of infection ensured one cycle of virus multiplication; (b) a „normal" virus was used for the infection of cells and the analog was present in the medium only during the post-adsorption period; in this way it was ensured that the infection started „normally" and the analog was incorporated only into progeny viral DNA and newly synthesized cellular DNA respectively; (c) the chosen concentration of BrdUrd in the medium (6.34 μg/ml) caused only partial reduction of total virus-specific RNA in previous

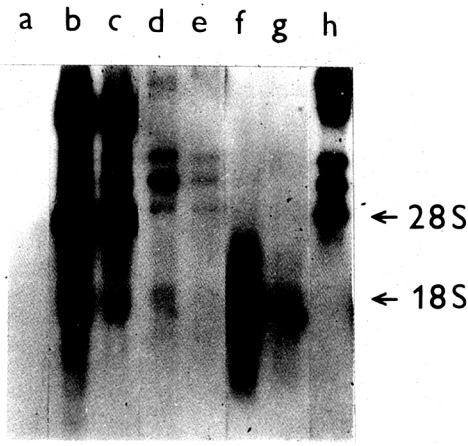

Fig. 1
Autoradiograph of the Northern blot hybridization analysis of Py viral RNA Total (a–c), cytoplasmic (d, e) and poly(A)RNA (f, g) isolated from mock-infected (a), virus-infected (b–g) and BrdUrd-treated (c, e, g) cells. Viral DNA (h).

experiments (Žemla and Tarábek, 1981); in this way it might be assumed that the levels of individual classes of viral RNA would occur in measurable range; (d) cultures without the analog served as regular positive controls, whereas mock-infected cultures without the analog represented negative controls.

The results of this experiments are demonstrated on the autoradiograph in Fig. 1. The preparation of total cellular RNA (lanes b, c) contains a wide band of virus-specific RNA in the vicinity of 18S ribosomal RNA apparently corresponding to 16S, 18S and 19S classes of mature viral late mRNA. This RNA band contains probably also viral early mRNAs of the size 19–20S, which occur in much lower concentration as compared to late mRNAs. Several additional bands of the size equal to 28S or above it represent obviously various forms of viral genomic DNA (lane h) and possibly precursors of viral mRNAs of higher molecular weight. Namely, there are data on Py viral „giant" nuclear RNA in the late phase either of heterogeneous (26–50S) or distinct (26S, 33S, 41S, 47S) character (Acheson *et al.*, 1971; Acheson, 1978, 1984; Buetti, 1974; Kamen *et al.*, 1978). The 26S RNA is apparently a complete primary transcript of Py viral genome (Acheson *et al.*, 1971), whereas the 33–47S RNAs are its oligomeric forms (Acheson, 1984). BrdUrd (lane c) in comparison with control (lane b) causes in general a similar profile of hybridizing material, where the band of 16–20S mRNAs is clearly weaker. The hybridization profile of the preparation of cytoplasmic RNA (lanes d, e) is similar to that of total RNA, just the majority of the 26–28S and the largest material at the top of the gel is missing. The analog clearly reduces the level of 16–20S viral RNA. The preparations of poly(A)RNA are devoid of viral DNA and precursors of viral mRNAs (above 26S). Here, the positive hybridization is restricted to a wide band of polyadenylated 16–20S mRNAs with a clear inhibitory effect of BrdUrd (lane g). To comment the presence of viral DNA in our preparations of total and cyto-

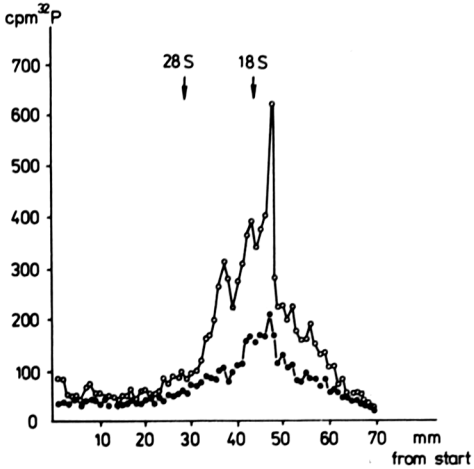

Fig. 2
Radioactivity profile of Py viral RNA in the Nothern blot of poly (A)RNA preparations from Fig. 1, lanes f, g. Poly (A)RNA from control (o) and BrdUrd-treated (●) cells
Ordinate: radioactivity of ^{32}P in cpm; abscissa: distance from the start in mm.

plasmic RNA we qualify it as a sort of contamination. We have attempted to get rid of DNA in the course of RNA purification by use of either LiCl precipitation or DNase treatment, however, these steps had either just a partial effect or damaged the hybridizable viral RNA. There was no non-specific hybridization in our experiments (lane a).

Since the autoradiography of Northern blots did not resolve individual classes of viral mRNA and yielded just one 16–20S RNA band, we have attempted to obtain more distinct results by direct measurement of the radioactivity of blots by liquid scintillation counting of individual 1 mm strips. Fig. 2 shows this type of analysis of poly(A)RNA samples used in the former experiment (Fig. 1, lanes f, g). The curve of the control preparation displays three distinct peaks and a diffuse background of both faster and slower material. The highest peak corresponds apparently to the major late 16S mRNA, while the smaller and slower peak at 18S contains probably the sum of minor late 18S and 19S mRNAs. The third peak around 22–24S is hardly to correlate with any known Py viral mature poly(A)RNA and may represent a contamination due to some viral nuclear RNA. The curve of poly(A)RNA from BrdUrd-treated cells (Fig. 2) displays clearly lowered peaks of hybridizable RNA corresponding to those in the control.

The comparison of the 16S and the (18S+19S) peaks of poly(A)RNA from control and BrdUrd-treated cells from the Fig. 2 is shown in Table 1. Here, the total viral RNA represents the summarized radioactivity of the whole blot lane. According to this evaluation, BrdUrd leads to a reduction of the level of 16S RNA to 32.7 %, (18S+19S) RNA to 40.5 % and total RNA to 48.4 %.

The inhibitory effect of BrdUrd or IdUrd on the viral gene expression has been reported in several DNA viruses. Thus viral RNA synthesis was inhibited

Table 1. The reduction of the level of Py viral poly(A)RNA by BrdUrd in ME cells

Viral RNA	^{32}P cpm hybridized		Reduction to %
	control	BrdUrd	
16S	627	205	32.7
18S+19S	398	161	40.5
total	9716	4705	48.4

in the case of SV40, HSV-1 and Py viruses (Patch *et al.*, 1982; Otto *et al.*, 1984; Žemla and Tarábek, 1980). A reduced level of poly(A)RNA of HSV-1 under the influence of IdUrd was explained by the inhibiton of polyadenylation (Otto *et al.*, 1984). An inhibition of synthesis of viral proteins, usualy of the late type was found in vaccinia, adenovirus-2, HSV-1 and Py viruses (Pennington, 1976; KanMitchel and Prusoff, 1979; Otto *et al.*, 1984; Tarábek, unpublished data). On the other hand a normal formation of structural antigen in the presence of BrdUrd or IdUrd was found in pseudorabies virus (Kaplan *et al.*, 1965). It is also evident that the effects of the analogs depend on their concentration and consequently on the extent of substitution of thymidine in DNA. Namely, whereas a highly substituted SV40 DNA (18–35 %) leads to an inhibition of viral RNA synthesis to 30–50 % of control, a weakly substituted SV40 DNA (10–15 %) causes a 5-fold stimulation (Patch *et al.*, 1982). Also, the final effect of the analog on the formation of virion may differ in BrdUrd and IdUrd. Thus, whereas BrdUrd enables the formation of pseudorabies virus particles (non-infectious), IdUrd leads only to accumulation of viral DNA and structural antigen without their maturation into particles (Kaplan *et al.*, 1965).

Also cellular gene expression is affected by the analogs. The BrdUrd-DNA-directed RNA synthesis in *E. coli* was markedly reduced (Jones and Dove, 1972). BrdUrd inhibited selectively the synthesis of tyrosine aminotransferase and globin (Stellwagen and Tomkins, 1971; Preisler *et al.*, 1973), induced alkaline phosphatase (Koyama and Ono, 1971) and suppressed cell differentiation (Wessells, 1964; Abbot and Holtzer, 1968). A direct evidence of changed proportions of A and G in newly formed RNA under the influence of BrdUrd was observed in 3T6 cells (Hill *et al.*, 1974). The interference with normal gene expression caused by the analogs may result in the induction of virus production in virogenic „non-producer" cells (Lowy *et al.*, 1971; Hampar *et al.*, 1972). The induction of murine leukemia virus by IdUrd was accompanied by stimulation of total transcription (Chattopadhyay *et al.*, 1979).

The basis of all above mentioned biochemical and biological effects of the analogs is the analog-containing DNA, which displays changed physico-chemical properties. They are reflected in the altered behaviour of DNA in its

interaction with proteins in the course of DNA replication and transcription. The analog-containing DNA has increased pH- and thermostability (Baldwin and Shooter, 1963; Camermann and Trotter, 1964; Lapeyre and Bekhor, 1974), what results in altered properties of chromatin. Its negative charge, thermostability, supercoiling and condensation are also increased (Lapeyre and Bekhor, 1974; Nicolini and Baserga, 1975). BrdUrd-DNA binds histones and non-histone proteins more firmly (Lin *et al.*, 1976; Gordon *et al.*, 1976). The affinity of the promoter of fd phage to RNA polymerase and that of the *lac* operator of *E. coli* to its repressor is in BrdUrd-DNA much stronger (Hofer and Koester, 1980; Lin and Riggs, 1972). The analog-substituted DNA is more fragile and more sensitive to UV light, which introduces nicks and cross-linking to proteins (McCrea and Lipman, 1967; Taichman, 1979).

At the same time the mutagenicity of the analogs should be taken into account (Litman and Pardee, 1956). BrdUrd and IdUrd cause increased errors in base pairing which results in higher frequency of A-G and T-C transitions (Trautner *et al.*, 1962). The mutations take place in the course of DNA replication and transcription and lead to the appearance of faulty DNA copies, RNA transcripts and proteins. Due to these mutations effects of two different characters should be expected; (i) changed structural genes, mRNAs and proteins, (ii) changed regulatory elements on DNA and RNA molecules (promoters, enhancers, termination and splicing signals) and changed regulatory proteins (factors), which control the timing and intensity of gene replication, transcription and translation.

The reduction of the level of various classes of Py viral RNA by BrdUrd in our experiments seems to be non-selective, i.e. more or less general. It may involve any of above mentioned molecular mechanisms, including an attractive hypothesis about a weaker late promoter and/or enhancer in the newly formed Py viral BrdUrd-DNA. Nevertheless, also an indirect effect of the analog cannot be excluded, namely a lowered level of viral progeny BrdUrd-DNA functioning as template and leading to a reduced transcription rate. However, any evidence of this character is missing.

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