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

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Summary. - Polyoma (Py) virus-specific RNA, synthesized at reduced level in infected cells in the presence of antiviral substance 5-bromo-2'-deoxyuridine was characterized in more detail by Southern blot hybridization analysis. Virus-specific RNA present in ^{3}H-uridine labelled cytoplasmic preparations hybridized to individual viral DNA restriction fragments in a characteristic manner and extent, which was quantitatively assayed. BrdUrd (6.34 μg/ml) lowers the hybridization profiles essentially in a proportional manner, which indicates that no new, atypical transcription products are formed in the presence of BrdUrd.

Key words: polyoma virus; RNA transcripts; bromodeoxyuridine

Introduction

The bromo- and iodo-analogs of thymidine inhibit multiplication of DNA viruses (Prusoff and Goz, 1975) and cells (Hakala, 1959). They cause changes in gene expression of viruses and normal as well as virus-infected cells (Kaplan *et al.*, 1965; Stellwagen and Tomkins, 1971; Lowy *et al.*, 1971). The basis of these effects is known: after incorporation into DNA instead of thymidine they increase errors in base pairing and induce mutations (Trautner *et al.*, 1962; Litman and Pardee, 1956). As a consequence faulty copies of DNA and subsequently faulty RNA transcripts and proteins are formed. In virus infections progeny virus particles are either noninfectious, defective of just missing, while certain viral components are normally formed (Kaplan *et al.*, 1965). Nevertheless, the precise mechanism of the antiviral action of BrdUrd and IdUrd remains to be elucidated.

We have reported earlier that BrdUrd causes a reduced level of polyoma (Py) virus-specific RNA in infected cells (Tarábek and Žemla, 1980; Žemla and Tarábek, 1981) and that the inhibitory effect of the analog towards individual classes of virus-specific RNA is apparently of non-selective nature (Tarábek *et al.*, 1991). In this work we present a Southern blot hybridization analysis of the

effect of BrdUrd on virus-specific transcription, which brings further support of our previous interpretation.

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 in 200 ml roller bottles were used (Tarábek *et al.*, 1991).

Media. Basal Eagle's medium with 10 % of heat-inactivated calf serum (BEM-CS_{10}) was used for growth and maintenance of cells. BEM containing 20 mmol/l Tris.HCl, pH 7.5 (BEM-Tris) was used for virus adsorption onto cells.

Southern blot hybridization analysis. We have used the type of Southern blot hybridization analysis described by Feldman *et al.* (1979). There, the labelled virus-specific RNA is hybridized to the electrophoretically separated and blotted restriction fragments of viral DNA and the quantitative assay resides in densitomerty of autoradiographs. We have modified this procedure by using tritium instead of 32-phosphorus as a label and direct measurement of radioactivity of blots.

^{3}H-RNA isolation. RNA was isolated from virus-infected cells with Nonidet P-40 according to Favaloro *et al.* (1980) 42 hrs p. i. after 3 hrs of labelling (39-42 hrs) with ^{3}H-uridine in concentration of 100 KBq/ml of BEM-CS_{10}. If the cells were treated with BrdUrd (6.34 μg/ml), the analog was added to the medium after the 3 hrs virus adsorption period and it remained there until the isolation of RNA. The specific radioactivity of ^{3}H-RNA preparations amounted to 60 000–200 000 cpm/μg RNA. There was no substantial difference between specific radioactivities of RNA preparations from control and treated cells within one experiment. The preparations were routinely checked by agarose gel electrophoresis for the presence of intact 28, 18 and 4–5 S cellular RNAs. The preparations were stored ethanol precipitated at -20 °C.

DNA restriction fragments. Viral DNA was either isolated in the supercoiled form from virus-infected cells by the selective procedure of Hirt (1967) and isopycnic CsCl density gradient centrifugation (Radloff *et al.*, 1967) or it was cut out as linear molecule with *Bam*HI ends from the recombinant plasmid Py/pXf3, which was kindly supplied by Dr. M. Fried from ICRF in London. Viral DNA was digested with *Pst*I, *Hpa*II or *Eco*RI (2–5 units/μg DNA) restriction endonucleases in optimal reaction buffers at 37 °C for 5 hrs (Maniatis *et al.*, 1982).

Gel electrophoresis and blotting. Digested viral DNA was electrophoresed using 4 mm thick slabs (150x200 mm) of 1.2 % agarose in horizontal of vertical setup with TAE running buffer. Ethidium bromide (0.5 μg/ml) was present both in buffer and gel. Samples containing 1–4 μg of fragmented DNA were applied per slot and each sample was present in duplicate. In this way one gel consisted of two identical halves. *Hind*III fragments of lambda DNA served as marker. The electrophoresis was run for 16–18 hrs at room temperature at 1 V/cm. Then the gel was treated in alkaline solution, neutralized and blotted overnight to nitrocellulose membrane (Sartorius) in 20xSSC by a standard procedure (Maniatis *et al.*, 1982). Blots were finally dried freely in air and then baked at 80 °C for 4 hrs in vacuum.

Hybridization. Two identical DNA blots were hybridized at the same time with two different RNA probes originating from BrdUrd treated and control cells. Identical amounts of ^{3}H-RNA probes (100–200 μg) in the volume of 100 μl were added after denaturation to the hybridization system. RNA probes were denatured immediately before use by heating at 65 °C for 5 min in 10 mmol/l Tris.HCl pH 7.5, 100 μmol/l EDTA and 50 % formamide and quenched in ice. For hybridization two slightly different methods were used. (a) DNA blots in sealed plastic bags were preincubated for 24 hrs at 45 °C in 4–5 ml of hybridization solution. The latter consisted of 1x Denhardt solution, 50 % formamide, 2x SSC, calf thymus DNA 100 μg/ml and yeast RNA 50 μg/ml. After change of hybridization solution and addition of RNA probe the hybridization proceeded at 45 °C for 3–4 days. Blots were then washed several times in 3x SSC at 60 °C for 1 hr, digested with RNase A (10 μg/ml) in 2x SSC for 2 hrs at room temperature and washed again as before (Feldman *et al.*,

1979). (b) DNA blots were equilibrated with 4–5 ml of hybridization solution at room temperature, RNA probe was added and the hybridization proceeded at 42 °C for 24 hrs. The hybridization solution consisted of 5x Denhardt solution, 50 % formamide, 3x SSC, 0.1 % SDS and 50 mmol/l Hepes pH 7.4. Blots were then washed in 2x SSPE plus 0.1 % SDS for 30 min and in 0.1x SSPE plus 0.1 % SDS for another 30 min, both steps at room temperature (Barinaga *et al.*, 1981). Washed blots were dried freely in air and then in vacuum at room temperature for 1 hr. These two methods led to similar results.

Direct measurement of radioactivity of blots. Since the fluorography method of detection of tritium-labelled hybrids turned out to be not sufficiently sensitive, we have measured the radioactivity of blots directly. 2 mm stripes cut out from individual lanes on blots were assayed for ^{3}H-radioactivity in 10 ml of toluene based scintillation coctail in Packard Tricarb spectrometer Model 3390.

Solutions, buffers and chemicals. TAE, SSC and SSPE had a standard composition (Maniatis *et al.*, 1982; Ausubel *et al.*, 1987). Bromophenol blue stop solution contained 60 % (w/v) sucrose, 0.1 mol/l EDTA and 0.05 % bromophenol blue. Scintillation cocktail contained 0.4 % PPO and 0.05 % POPOP in toluene. BrdUrd, Nonidet P-40, calf thymus DNA and yeast RNA were purchased from Fluka; agarose (standard EEO), formamide, SDS and Tris from Serva; CsCl, PPO and POPOP from Packard; Hepes and RNase from Sigma; restriction endonucleases *Bam*HI, *Eco*RI, *Hpa*II and *Pst*I from Serva, Institute of Sera and Vaccines (Prague) and Institute of Molecular Biology of Slovak Academy of Sciences (Bratislava); *Hind*III fragments of lambda DNA as size marker from Amersham International plc; [^{3}H]-5-uridine of specific radioactivity 740–1100 GBq/mmol from Institute for Production, Research and Use of Radioisotopes (Prague).

Results and Discussion

The Southern blot hybridization analysis of the effect of BrdURd on virus-specific RNA was performed under these prerequisites: (a) a high multiplicity

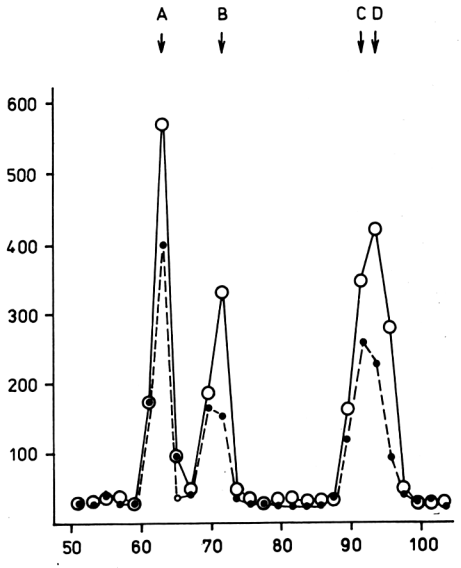

Fig. 1
Quantitative assay of blot hybridization of *Pst*I fragments of Py DNA with ^{3}H-RNA from Py virus-infected cells Control (○) and BrdUrd (●) series. Ordinate: radioactvity in cpm. Abscissa: distance from the start in mm. A–D: restriction fragments.

of infection (25–50 PFU/cell) apparently ensured just one cycle of virus replication; (b) the time of isolation of cytoplasmic RNA (42 hrs p. i.) corresponded to the highest level of virus-specific RNA under normal conditions (Žemla and Tarábek, 1981); (c) the chosen concentration of BrdUrd in medium (6.34 μg/ml) caused in previous experiments (Žemla and Tarábek, 1981) only a partial reduction of total virus-specific RNA; it can be assumed that individual classes of this RNA will also be partially reduced; (d) since a "normal" virus was used as inoculum for cells and the analog was present in the medium only during the post-adsorption period, the early phase of the virus growth cycle was "normal"; the analog could influence just the late phase, when the viral DNA replication started and the analog was incorporated into progeny DNA.

The hybridization profile of *Pst*I fragments of viral DNA is shown in Fig. 1. Two curves indicate control and BrdUrd-series and display three sharp peaks corresponding to hybridization of *Pst*I fragments A, B and C+D. BrdUrd partially lowers these peaks in a similar manner.

The hybridization profile of *Hpa*II fragments of viral DNA is shown in Fig. 2. Five distinct peaks correspond to six fragments (A, B, C, D, E+F). Here the inhibitory effect of BrdUrd is in general more pronounced than in the experiment with *Pst*I fragments (Fig. 1). The hybridization profile of *Eco*RI plus *Bam*HI fragments of viral DNA is demonstrated in Fig. 3. Two faster peaks

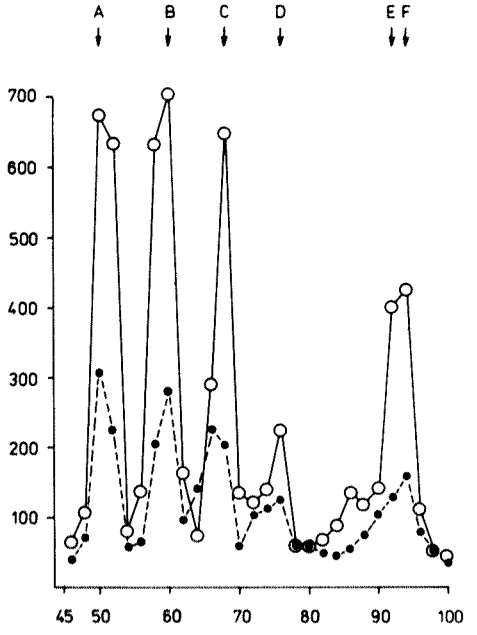

Fig. 2
Quantitative assay of blot hybridizaton of *Hpa*II fragments of Py DNA with ^{3}H-RNA from Py virus-infected cells Control (○) and BrdUrd (●) series. Ordinate: radioactvity in cpm. Abscissa: distance from the start in mm. A-F: restriction fragments.

belong to fragments A and B, while the slowest one belongs to the complete undigested viral DNA. BrdUrd clearly reduces their hybridization.

Preparations of cytoplasmic RNA from Py virus-infected cells, isolated in the late phase of infection contain various mature viral mRNAs and eventually their breakdown products, which are supposed to hybridize with specific regions of viral DNA. According to the literature data (Tooze, 1980) a majority (about 95 %) of virus-specific RNA in such preparations represents mRNAs transcribed from the late strand of viral DNA which code for capsid proteins VP1, VP2 and VP3. These "late" mRNAs correspond to nucleotides 5128–5020 and 5014–2908 on Py DNA (O'Brien, 1984). A minority (about 5 %) represents mRNAs transcribed from the early strand of viral DNA which code for small,

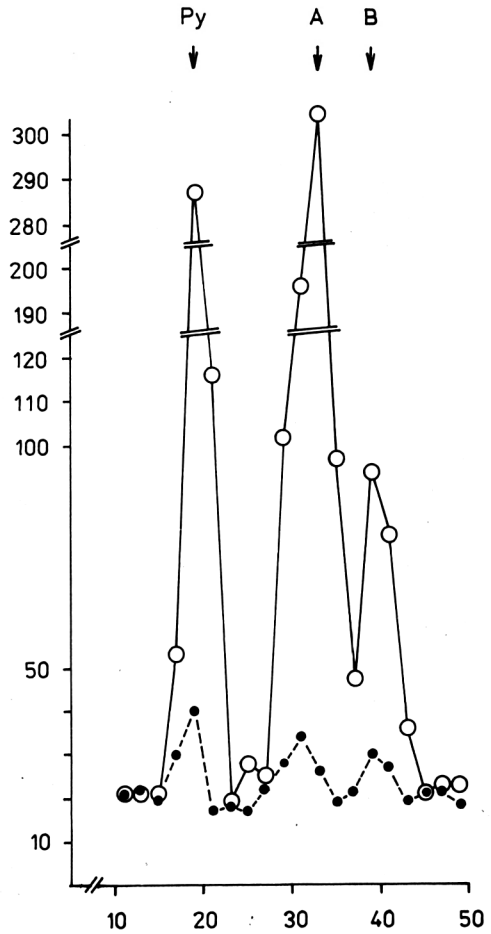

Fig. 3
Quantitative assay of blot hybridization of *Eco*RI+*Bam*HI fragments of Py DNA with ^{3}H-RNA from Py virus-infected cells Control (○) and BrdUrd (●) series. Ordinate: radioactivity in cpm. Abscissa: distance from the start in mm. A, B: restriction fragments. Py: undigested Py DNA.

middle and large tumour antigens. These "early" mRNAs correspond to nucleotides 148–746 and 795–2920 on Py DNA. The discontinuity of both regions is caused by introns. Whereas the late mRNAs are transcribed practically exclusively from the newly replicated, progeny viral DNA and may be therefore directly influenced by the incorporation of the analog into DNA, the early mRNAs originate primarily from the infecting viral DNA and only secondarily from the progeny viral DNA. Thus the potential effect of the analog on the early mRNAs seems more complex.

The hybridization of individual restriction fragments of Py DNA with virus-specific RNA in our experiments should be considered also from this aspect, namely how does it correspond to the early or late nature of the fragments. The relationship of *Pst*I, *Hpa*II and *Eco*RI plus *Bam*HI restriction fragments to the early and late mRNA regions of Py DNA is shown in Table 1. It demonstrates that the restriction fragments used in our experiments are of three types in this respect: early, late and early-late fragments. An assumption that late fragments should give a stronger hybridization (higher peaks) than the early fragments is fulfilled in the case of *Hpa*II fragments A, C (high) versus D, E+F (low, Fig. 2). Also the hybridization profile of *Eco*RI plus *Bam*HI fragments (Fig. 3) can be satisfactorily explained. Both fragments A and B are of the early-late type, but the higher peak A corresponds to the major VP1 plus minor VP3 mRNAs, while the lower peak B corresponds to the minor VP2 mRNA only. However,

Table 1. Relationship of PstI, HpaII and EcoRI+BamHI restriction fragments of Py viral DNA to the early and late regions of the genome

Restriction endonuclease	Fragment	Size b. p.	Localization (nucleotide No.)	Region early	Region late
*Pst*I	A	1872	489 - 2361	+	
	B	1551	4230 - 488	+	+
	C	912	3318 - 4229		+
	D	862	2456 - 3317	+	+
*Hpa*II	A	1418	2992 - 4409		+
	B	1127	1489 - 2615	+	
	C	882	4410 - 5291		+
	D	702	400 - 1101	+	
	E	400	5292 - 399	+	
	F	376	2616 - 2991	+	+
*Eco*RI+*Bam*HI	A	3072	1561 - 4632	+	+
	B	2220	4633 - 1560	+	+

the hybridization of *Pst*I fragments A, B, C+D (Fig. 1) and the *Hpa*II fragment B (Fig. 2) does not fit the assumption mentioned above.

Our results of the Southern blot hybridization analysis of the effect of BrdURd on Py virus-specific RNA confirm our previous findings (Tarábek *et al.*, 1991). They indicate that the analog lowers the level of individual classes of viral RNA in a non-selective manner and that no new, atypical transcription products are formed. This observation is in accordance with reports on inhibitory effects of BrdUrd on virus-specific RNA synthesis or gene expression in other viruses. Thus viral RNA synthesis was inhibited in the case of SV40 and HSV-1 viruses (Patch *et al.*, 1981; Otto *et al.*, 1984). In the latter case also a reduced level of poly(A)RNA was found and accounted for by the inhibition of polyadenylation. An inhibition of synthesis of viral proteins, usually of the late type was found in vaccinia, adeno-2, HSV-1 and Py viruses (Pennington, 1976; Kan-Mitchell and Prusoff, 1979; Otto *et al.*, 1984; Tarábek, unpublished results). On the other hand, a normal formation of structural antigen of pseudorabies virus in the presence of BrdUrd or IdUrd was observed (Kaplan *et al.*, 1965). However, it should be mentioned that the effects of analogs may depend on their concentration and consequently on the extent of substitution of thymidine in DNA. Namely, whereas a "highly" substituted SV40 DNA (18–35 %) caused an inhibition of viral RNA synthesis to 30–50 % of control, a "weakly" substituted SV40 DNA (10–15 %) caused a 5-fold stimulation (Patch *et al.*, 1981). Inhibitory effects of the analogs on the synthesis of RNA or specific proteins (enzymes) are known also from various cell systems (Stellwagen and Tomkins, 1971; Jones and Dove, 1972; Preisler *et al.*, 1973).

Various effects of halogene analogs of thymidine on transcription and translation of viral or cellular genes are based primarily on their incorporation into DNA; the analog-containing DNA (a) has changed physico-chemical and biological properties per se and (b) it undergoes increrased mutations (Lin and Riggs, 1972; Trautner *et al.*, 1962). Since two principally different types of DNA sequences, namely those coding for gene products and those regulating their synthesis can be affected by the mentioned changes, a great variety of effects of the analogs with diverse mechanism involved may take place.

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