Vol. 85, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 14, 1978 Pages 1225-1232

PROCESSING OF EXOGENOUS POLY(A) ADDED TO VIRUS-INFECTED CELLS

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Received October 24, 1978

SUMMARY

Exogenous [^{3}H]-poly(A) was taken up by and was stable for some hours in monolayers of human embryo lung cells in culture. The poly(A) was extracted and sized by G200 Sephadex chromatography. Non-infected and virus-infected cells converted the majority of [^{3}H]-poly(A) into a smaller poly(A) molecule which could still bind to oligo(dT)cellulose. In addition virus-infected cells only converted 11% into a poly(A) containing-molecule which was at least 4-fold greater in size than the original poly(A). This larger material could also bind to oligo(dT)cellulose and did not result from the breakdown and re-utilization of the [^{3}H]-poly(A).

Poly(A) is found covalently bound to the 3' terminus of many well characterized mRNA species from eukaryotic cells (1-3). It does not affect the translational efficiency of the message in vitro (4-7) but confers stability to the message in vivo (8-10) and in vitro (11) and infectivity to poliovirus RNA (12). The adenosine residues which eventually constitute the poly(A) tract are added to the precursors of mRNA in the nucleus (13,14) but some cellular and virus specific mRNAs are polyadenylated in the cytoplasm (15-18). Virus-induced (16) and virion enzymes (19,20) are also known to have this function. The poly(A) tract of cellular mRNAs is thought to be synthesized post-transcriptively (21). However, it appears that poliovirus and Sindbis virus mRNAs are polyadenylated by transcription from a poly(U) sequence at the 5' terminus of its complement (22,23,24), although no poly(U) sequences were detected in the RNAs of a rhinovirus (25) which resembles poliovirus in other aspects of its multiplication.

Previously we have reported that polyadenylation of rhinovirus specified RNAs is stimulated by the addition of exogenous poly(A) (26).

0006-291X/78/0853-1225$01.00/0

This raised the possibility that there might be a means of polyadenylation, alternative to terminal addition of adenylic acid residues in which a preformed poly(A) homopolymer could be covalently linked to viral RNA. Such a mechanism would require RNA ligase activity which is known to occur in both eukaryotic (31, 35) and prokaryotic (27-31) cells, usually in response to virus infections. In this report we investigate the fate of exogenous poly(A) added to rhinovirus-infected cells and present evidence (a) that about 10% of the intracellular poly(A) is considerably increased in size compared with added poly(A) and (b) that 80% of the intracellular poly(A) is homogenous in size and smaller than added poly(A).

MATERIALS AND METHODS

Cells: The MRC 5 line of human embryo lung (HEL) cells were kindly provided by Mr. J.P. Jacobs, National Institute of Biological Standards and Control, London. These were grown as previously described (32) and used in these experiments between passages 25 and 37. Cells were free of mycoplasma (34).

Virus: Stocks of rhinovirus type 2 were prepared in HeLa cells (32). Clarified culture fluids were stored at -70° and used as described below.

Poly(A): This was the sodium salt of poly[8-^{3}H] riboadenylic acid and was obtained from the Radiochemical Centre, Amersham: 98% was polynucleotide which consisted of 38-137 residues (12,500-45,000 M.W.); the specific activity was 17-62 Ci/mmol. and 95-98% was labelled in adenosine residues.

Experimental protocol for the addition of poly(A): 4 x 10^{7} cells in a 13cm plastic Petri dish were infected with about 10 PFU/cell and incubated at 33°C for 1h. Eagle's medium (30ml) containing 2% calf serum and 1μg/ml actinomycin D was added and incubation continued. At 5h post infection the medium was replaced with 2.5 ml 150 mM NaCl, 10 mM Tris buffer pH 7.4 containing 40 μg/ml DEAE dextran and 10 μCi[^{3}H]-poly(A). This was removed at 7.5h post infection and replaced with 30 ml maintenance medium. Cells were harvested at 8.5h post infection at the peak of virus RNA synthesis and usually showed cytopathic effects at this stage (31). The same time intervals and conditions were used for the addition of poly (A) to non-infected cells.

Extraction of RNA: Cells were lysed with 1% SDS and extracted with phenol at room temperature (32).

Sephadex gel filtration: Sephadex G25 and G200 were swollen in boiling water for upto 5h. Columns of 300 x 6mm were prepared and equilibriated with 1 x SSC (150 mM NaCl, 15 mM trisodium citrate, pH 8.45) containing 1% SDS. Samples were dissolved in 0.2 ml of this buffer containing dextran blue and bromophenol blue dye markers. The SDS was found to sharpen the peaks of poly(A). Fractions consisting of 10-20 drops were collected with

a Gilson fraction collector. Scintillation fluid (5 ml) consisting of 2 litres of Triton X100, 1 litre of toluene, 3 g/l PPO, 0.36 g/l POPOP, was used in radioactivity estimations in a Packard scintillation spectrometer.

RESULTS

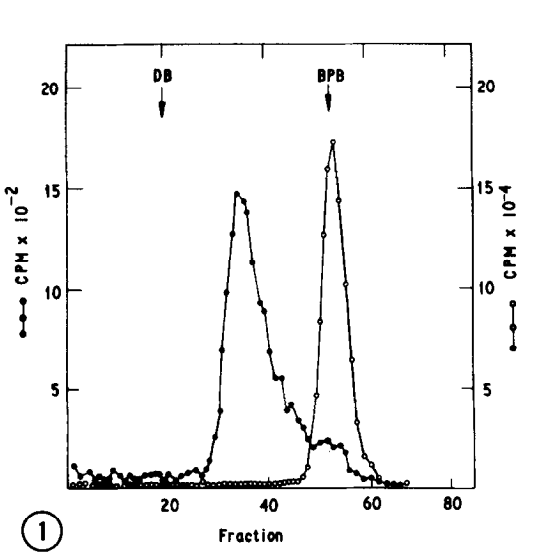
Characterization of poly(A). Initially we investigated some physical and chemical properties of commercial [^{3}H]-poly(A). Radioactivity was excluded completely from G25 and G50 Sephadex, hence the preparation contained no free labelled nucleotides, nucleosides or bases which are retarded in these columns. On G200 the majority of poly(A) molecules were seen as a single peak migrating in a position intermediate between the dextran blue and bromophenol blue dye markers (Fig. 1).

The stability of poly(A) towards heat and ultrasonication was investigated by G200 chromatography and found to be comparable with published standards (33). The most effective means of degrading poly(A) was by heating in a sealed ampoule at 100° in 0.1 M $NaHCO_3$, pH 10.

Poly(A) was resistant to treatment with a mixture of pancreatic ribonuclease A (166μg/ml) and micrococcal T_1 (250μ/ml) ribonuclease under conditions which degraded rRNA or single stranded rhinovirus RNA.

Uptake of poly(A) by HEL cells. We investigated whether NaCl or DEAE-dextran, which are known to increase the uptake of viral nucleic acids by cells, would enhance the uptake of poly(A). 2 M NaCl had no beneficial effect and in 90 mins disrupted the monolayer completely but DEAE-dextran at an optimum concentration of 40μg/ml increased the uptake 5-fold, without causing cell damage. Uptake of poly(A) was rapid initially and continued to rise slowly over a period of 2h. Approximately 2.5% of poly(A) added was taken up by the cell monolayers.

Size of poly(A) re-isolated from rhinovirus infected cells. The RNA fraction extracted from infected cells which were treated with poly(A) was analysed by G200 chromatography (Fig. 2). About 80% poly(A) was present



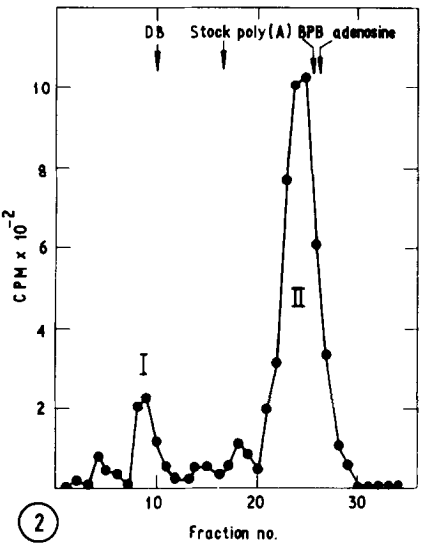


Figure 1 Analysis of stock [^{3}H]-poly(A) (●——●) by Sephadex G200 chromatography. The column was equilibrated and the sample loaded and fractionated with a buffer solution consisting of 1% SDS in 1 x SSC. The dye markers, dextran blue (DB) and bromophenol blue (BPB) are arrowed. ^{3}H-adenosine (o——o) was analysed consecutively on the same column. Fractions of approximately 0.125 ml were collected.

Figure 2 Chromatography on Sephadex G200 of [^{3}H]-poly(A) extracted from monolayers of HEL cells infected with rhinovirus type 2. Monolayers were incubated at 33° with poly(A) in 2.5 ml of a solution containing 40μg/ml DEAE dextran from 5-7.5h post infection. The poly(A) solution was replaced with maintainance medium and incubation continued for 1h. RNA was then extracted as described in Methods. Arrows show the positions of stock [^{3}H]-poly(A), [^{3}H]-adenosine and the dye markers.

as a single peak (peak II) moving more quickly than the adenosine and the bromophenol blue markers but more slowly than the stock poly(A) peak. (100% peak II bound to oligo(dT)cellulose (data not shown).) The significance of this shift in size which also occurred in non-infected cells is currently under investigation. 11% radioactivity eluted in the G200 void volume together with dextran blue and hence has a MW $\geqslant$ 200,000 and consists of $\geqslant$ 590 nucleotides. This peak I material is at least 4-fold larger than stock poly(A) which is retarded on G200. Peak I is not the result of spurious aggregation of poly(A) with cellular constituents because [^{3}H]-poly(A) added to disrupted infected cells which were then extracted and analysed on G200,

gave a single peak of radioactivity identical to that of stock poly(A). Furthermore peak I is not an artefact of analysis since on rechromatography it migrated again in the void volume. Peak I also bound to oligo(dT)-cellulose (data not shown).

These data do not distinguish between the possible addition of an intact [^{3}H]-poly(A) tract to a ribonucleotide heteropolymer or the incoration of [^{3}H]-A derived from the breakdown of [^{3}H]-poly(A) into the homo- and hetero-polymeric parts of the molecule. These alternatives were investigated by treating peak I radioactivity from a G200 fractionation with a mixture of ribonuclease A (10μg/ml) and T_1 (15 U/ml). This treatment degraded 85% of viral RNA extracted from cells (Fig. 3a,d) whereas stock poly(A) and peak I were 100% resistant (Fig. 3b,c,e,f). Part of the RNAse resistance of viral RNA in Fig. 3d is attributed to covalently bonded poly-(A) (around 2%) and to the presence of double-stranded replicative form (about 10%) (25,32). From these experiments we conclude that radioactivity in peak I is present only in poly(A) and that no detectable breakdown and re-utilization has occurred. This conclusion is supported by an experiment in which the intracellular pools were diluted by the addition of cold adenosine (500 μg/ml) to infected [^{3}H]-poly(A)-treated cells. This had no effect on the appearance of peak I. The above results are consistent with the binding of peak I to oligo(dT)cellulose.

DISCUSSION

From rhinovirus-infected cells treated with poly(A) we reproducibly extracted a labelled RNA (peak I) which represented 11% of the intracellular poly(A). Peak I was at least four times larger than stock poly(A) as judged by its exclusion from G200. We could not demonstrate peak I in non-infected cells or in infected cells exposed briefly to poly(A). This we take as evidence that peak I does not consist of poly(A) spuriously associated with normal cellular or viral constituents or aggregate with itself.

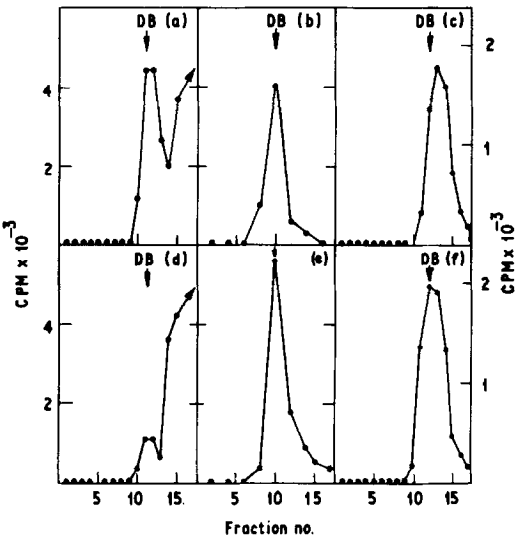


Figure 3 Comparison of the ribonuclease resistance of rhinovirus RNA (a,d), stock poly(A) (b,e) and peak I radioactivity (c,f). Viral RNA was extracted from HEL cells labelled with 20 μCi/ml [^{3}H]-adenosine from 7-9h post infection (in the presence of actinomycin D to inhibit the synthesis of cellular RNA). Peak I radioactivity was obtained from virus infected-, [^{3}H]-poly(A)-treated HEL cells by G200 chromatography as described in Fig. 2. Samples a,b,c were untreated, d,f incubated with 10μg/ml of pancreatic ribonuclease A and 15 u/ml ribonuclease T_I, and sample e was treated with even higher concentration of ribonuclease (166μg/ml pancreatic ribonuclease A and 250 u/ml ribonuclease T_I), at 37° for 30 mins. All samples were chromatographed on G25 with dextran blue (DB) marker (arrow).

Radioactivity was shown to be present only in the poly(A) tract and was therefore derived directly from the input [^{3}H]-poly(A). We have no knowledge of the identity or origins of the non-poly(A) part of the peak I molecule but conclude that poly(A) was ligated to another moiety and that this process occurred only in infected cells. Either that moiety or the ligase or both may be absent or undetectable in non-infected cells. However cells were treated with actinomycin D for 4h before the addition of radiolabelled poly(A) so the non-poly(A) part of peak I is unlikely to be newly synthesized cellular RNA.

This report provides circumstantial evidence for the presence of RNA ligase activity in rhinovirus-infected cells and thus adds to previous

reports on such activity in cells infected with other members of the Picornaviridae, poliovirus, encephalomyocarditis virus (31) and rhinovirus type 14 (35) all of which were demonstrated by experimental systems entirely different from the one we have described. However the role of such ligase activity is entirely unknown.

Acknowledgements. S. I. Koliais was supported by The Cancer Research Campaign. Actinomycin D was a gift from Merck, Sharp and Dohme, Hoddesdon, Herts.

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