Polyadenylated Virus-specific RNA in Baby Hamster Kidney Cells, Transformed by Polyoma Virus

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SUMMARY

RNA from a clone of polyoma virus-transformed hamster cells was fractionated by chromatography on oligo(dT)-cellulose. The proportion of virus-specific RNA in the polyadenylated and non-polyadenylated fractions was determined by hybridization of the labelled RNA with excess purified polyoma DNA, immobilized on filters. Seventy to 80% of the virus-specific RNA in both polysomal and total cell RNA was found in the polyadenylated fraction. Since it has been shown previously that more than 65% of the total virus-specific RNA is restricted to the nucleus in this cell line, these results indicate that a high proportion (at least 53%) of the nuclear virus-specific RNA is polyadenylated.

The sedimentation profile of total polyadenylated virus-specific RNA in dimethyl sulphoxide was comprised mainly of a broad band with a median sedimentation coefficient about 26S (relative to 28S rRNA). This profile was similar to that of total nuclear, and not cytoplasmic, virus-specific RNA.

To estimate the intramolecular proximity of virus-specific sequences to poly(A), total RNA was subjected to limited thermal scission to an average mol. wt. similar to that of mRNA. The RNA remaining attached to poly(A) was then isolated, using oligo(dT)-cellulose. It was found that 65% of the virus-specific RNA that was originally attached to poly(A) was released by the thermal scission. Most of the virus-specific sequence within polyadenylated RNA molecules therefore must have been located at some distance from the polyadenylated 3'-terminus. This observation, together with the results of sedimentation analysis, can most simply be explained by postulating the existence of 'hybrid' RNA molecules containing a host-specified sequence located between a virus-specific sequence and the 3'-terminal poly(A).

INTRODUCTION

The existence of virus-specific RNA (vsRNA) in cells transformed by polyoma virus or SV40 has long been established (Benjamin, 1966). Evidence that the T-antigen, present in these transformed cells, contains a virus-specified polypeptide (Graessmann et al. 1974, 1975) implies that at least some of the vsRNA functions as mRNA. Recent experiments have shown that the vsRNA in polyoma-transformed (Kamen et al. 1974; Beard, Acheson & Maxwell, 1976) and SV40-transformed cells (Khoury et al. 1973; Ozanne, Sharp & Sambrook, 1973) is mainly transcribed from the same strand of virus DNA as the virus-specific mRNA present early in the productive infection of permissive cells. Few data presently

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exist concerning the structure of RNA molecules containing virus-specific sequences, apart from the observations that, in SV40-transformed cells, virus-specific mRNA is polyadenylated (Weinberg, Ben-Ishai & Newbold, 1972, 1974) and nuclear vsRNA includes "hybrid" molecules, containing sequences complementary to the host cell DNA (Wall & Darnell, 1971).

It was recently shown that a large proportion of the total vsRNA in a clone of polyoma-transformed hamster cells (designated PyBHK) was restricted to the cell nucleus (Maxwell, 1976). Studies of the properties of polyadenylated vsRNA in this cell line are described here.

METHODS

The origin and properties of the polyoma virus-transformed hamster cell clone, PyBHK, have been described previously (Maxwell, 1976), as have procedures for cell growth, labelling, subcellular fractionation, RNA extraction, density gradient centrifugation and hybridization of labelled RNA with excess purified polyoma DNA on nitrocellulose filters. Labelling of cells with 3H-uridine was performed in all experiments in the presence of actinomycin D at low concentration (0.05 µg/ml, added 30 to 60 min before commencing labelling) in order to suppress rRNA synthesis (Roberts & Newman, 1966).

Polysomes were isolated by centrifugation of the 8500 g supernatant fluid from a cytoplasmic extract (Maxwell, 1976) in a sucrose gradient (10 to 60%, w/v) and polysomal RNA was extracted, using a phenol-chloroform procedure, directly from pooled gradient fractions containing polysomes larger than trimers.

After hybridization, the radioactivity bound to filters containing polyoma DNA and to blank filters from the same incubation mixtures was determined after treatment with ribonuclease A (20 µg/ml, 60 min, 37 °C, in 0.3 M-NaCl, 0.03 M-trisodium citrate) and exhaustive washing. In earlier experiments, as previously reported (Maxwell, 1976), the radioactivity which bound to blank filters was less than 0.001 % of the input and the radioactivity which bound to control filters containing Escherichia coli DNA was one to two times that bound to blank filters. Higher values were observed for the radioactivity bound to blank filters in several of the experiments reported here; these values were mostly about 0.003 % of the input and in a few cases were even higher. The difference from the earlier experiments was probably attributable to the use of different batches of nitrocellulose filters. In the hybridization experiments reported here, the radioactivity bound to filters containing polyoma DNA ranged from 2.6 to 40 times that bound to blank filters, present in the same hybridization mixtures; the values obtained for blank filters are stated explicitly in the tables and figures.

Chromatography on columns (usually 3 cm x 0.5 cm) of oligo(dT)-cellulose was carried out essentially as described by Aviv & Leder (1972). Larger columns were used in some experiments, as specified. The RNA sample was applied in 4 ml loading buffer (0.5 M-NaCl, 0.1% SDS, 10 mM-tris HCl, pH 7.6), at a flow rate of approx. 15 ml/h. The column was washed with 3 ml 0.1 M-NaCl, 10 mM-tris HCl, pH 7.6, and the retained (poly(A)+) fraction of the RNA was then eluted with 10 mM-tris HCl, pH 7.6. Elution was complete in less than 1 ml. The non-retained RNA was precipitated with ethanol. RNA from the retained fraction was either added directly to incubation mixtures for hybridization, or was precipitated with ethanol, after addition of E. coli RNA as a carrier. Similar results were obtained using oligo(dT)-cellulose from Collaborative Research Inc. (grade T3) or prepared as described by Faust, Diggelmann & Mach (1973) and kindly supplied by Dr L. Rosenthal.

The disaggregation treatment applied to RNA recovered in the retained fraction from
Poly(A) RNA in polyoma-transformed cells

Table 1. Hybridization of polysomal RNA, containing or lacking poly(A), with polyoma DNA

<table>
<thead>
<tr>
<th>Polysomal RNA fraction*</th>
<th>Input ( \times 10^{-2} ) ct/min bound† to:</th>
<th>Polyoma DNA</th>
<th>Blank filter</th>
<th>% of input ct/min hybridized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(A)+</td>
<td>3.27</td>
<td>96</td>
<td>21</td>
<td>0.023</td>
</tr>
<tr>
<td></td>
<td>2.03</td>
<td>83</td>
<td>24</td>
<td>0.029</td>
</tr>
<tr>
<td>Poly(A)−</td>
<td>1.88</td>
<td>21</td>
<td>4</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>2.21</td>
<td>24</td>
<td>5</td>
<td>0.009</td>
</tr>
</tbody>
</table>

* PyBHK1 cells (7.4 \( \times 10^5 \)), growing in 2 roller bottles were labelled for 3 h with \(^3\)H-uridine (29 Ci/mmol; 500 \( \mu \)Ci/bottle, in 5 ml of medium) in low actinomycin. The isolated polysomal RNA was subjected to chromatography on oligo(dT)-cellulose and the retained (poly(A)+) and non-retained (poly(A)−) fractions were hybridized with polyoma DNA; 51% of the total radioactivity applied to oligo(dT)-cellulose was recovered in the retained fraction. Part of the radioactivity recovered in the non-retained fraction was due to incomplete suppression of ribosomal RNA synthesis.

† The scintillation counter background (17 ct/min) has been subtracted.

RESULTS

Presence of poly(A)+ vsRNA in polysomes isolated from polyoma-transformed cells

RNA was extracted from the polysomes of PyBHK1 cells (labelled with \(^3\)H-uridine) and was fractionated by chromatography on oligo(dT)-cellulose into a poly(A)+ and a poly(A)− fraction. The RNA of each fraction was then hybridized with excess polyoma DNA, immobilized on nitrocellulose filters (Acheson et al. 1971; Maxwell, 1976). As shown in Table 1, the level of hybridization was severalfold higher for the poly(A)+ than the poly(A)− RNA. It was estimated from these results that about 75% of the polysomal vsRNA was polyadenylated.

Extent of polyadenylation of the total vsRNA of PyBHK1 cells

It has previously been reported that more than 65% of the total labelled vsRNA of PyBHK1 cells is confined to the nucleus, even after prolonged labelling periods (Maxwell, 1976). It was therefore of interest to determine the fraction of the total vsRNA which was polyadenylated, since it has been proposed that polyadenylation is a necessary step in the biogenesis of many kinds of mRNA (Darnell, Jelinek & Molloy, 1973). Table 2 shows the results of several experiments in which RNA, extracted either from whole cells or from crude (i.e. unwashed) nuclei, was fractionated on oligo(dT)-cellulose. The proportion of the radioactive RNA recovered in the poly(A)+ and poly(A)− fractions was determined, as well as the percent hybridization with polyoma DNA. The proportion of the total vsRNA in the poly(A)+ fraction was then calculated (Table 2). As shown in Expt. I to III (top lines), this proportion was 72 to 80%. When labelling of the cells was conducted in the presence of cordycepin (Penman, Rosbash & Penman, 1970; Darnell et al. 1971), only 21% of the vsRNA was found in the poly(A)+ fraction (Expt. I, Table 2). This result indicated...
Table 2. Hybridization of the poly(A)+ and poly(A)—fractions of nuclear or total cell RNA with polyoma DNA

<table>
<thead>
<tr>
<th>Experiment</th>
<th>% of total ct/min recovered in poly(A)+ fraction</th>
<th>Poly(A)—fraction</th>
<th>Poly(A)+ fraction</th>
<th>% of total vsRNA in poly(A)+ fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Crude nuclear RNA†:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>38</td>
<td>786</td>
<td>214</td>
<td>0.015</td>
</tr>
<tr>
<td>Cordycepin</td>
<td>14</td>
<td>483</td>
<td>116</td>
<td>0.0086</td>
</tr>
<tr>
<td>II Total cell RNA‡:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>34</td>
<td>231</td>
<td>23</td>
<td>0.019</td>
</tr>
<tr>
<td>Boiled 3 min</td>
<td>15.5</td>
<td>459</td>
<td>26</td>
<td>0.039</td>
</tr>
<tr>
<td>III Total cell RNA§:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First fractionation</td>
<td>52</td>
<td>626</td>
<td>241</td>
<td>0.024</td>
</tr>
<tr>
<td>Re-chromatography of poly(A)+ fraction after Me2SO treatment</td>
<td>85</td>
<td>Not determined</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mean of duplicate or triplicate determinations. The scintillation counter background (10 ct/min for I and II; 16 ct/min for III) has been subtracted.
† PyBHK<sub>1</sub> cells were labelled in suspension with <sup>3</sup>H-uridine (27 Ci/mmol; 40 μCi/ml) for 1.5 h, in low actinomycin. Half of the suspension also received cordycepin (50 μg/ml) 15 min before the addition of <sup>3</sup>H-uridine. 480 μg RNA, extracted from the crude nuclear fraction (Maxwell, 1976) of the control or the cordycepin-treated cells, was subjected to chromatography on oligo(dT)-cellulose.
‡ Cells were labelled with <sup>3</sup>H-uridine for 2 h in low actinomycin and a sample of the total RNA was heated (3 min, 100 °C) as described under Fig. 2. 250 μg RNA (control or heated) was subjected to chromatography on oligo(dT)-cellulose.
§ Cells were labelled for 3 h under the same conditions as in Expt. I (control). The total RNA (700 μg) was subjected to chromatography on a column (1.5 cm x 1 cm) of oligo(dT)-cellulose (first fractionation). A sample of the RNA from the poly(A)+ fraction was incubated with Me<sub>2</sub>SO (see Methods), in order to dissociate possible aggregates, and was then re-applied to oligo(dT)-cellulose.
Poly(A) RNA in polyoma-transformed cells

Fig. 1. Density gradient centrifugation, under denaturing conditions, of total poly(A)+ RNA from PyBHK₁ cells. Total RNA (480 µg), extracted from cells labelled in a roller bottle for 3 h with ³H-uridine (1.5 mCi in 5 ml medium) in low actinomycin, was subjected to chromatography on oligo(dT)-cellulose. The RNA from the retained fraction was precipitated with ethanol, after the addition of 100 µg unlabelled RNA from E. coli as a carrier. This RNA was re-dissolved and centrifuged in an Me₃SO-chloral hydrate gradient (Maxwell, 1976) for 22 h at 27000 rev/min (Beckman SW 27 rotor). Each gradient fraction was then hybridized with polyoma DNA. The 28S and 18S positions were determined using ¹⁴C-labelled rRNA, centrifuged in parallel gradients. Counter background was 10 ct/min. ○—○, ³H total; ●—●, ³H hybridized; ■, ³H bound to blank filters.

that inhibition of polyadenylation was accompanied by inhibition of the labelling of vsRNA capable of being retained by oligo(dT)-cellulose, thus supporting the conclusion that this retention was due to the presence of poly(A) in the vsRNA. An additional control bearing on this point is shown in Expt. III (Table 2). Here, poly(A)+ RNA was subjected to a disaggregation procedure (Bantle et al. 1976), involving heating in the presence of Me₃SO, and was then re-chromatographed on oligo(dT)-cellulose. Eighty-five percent of the total radioactivity and 91 % of the vsRNA were found in the poly(A)+ fraction. This disaggregation procedure has been shown effective in preventing the association of rRNA with poly(A)+ RNA during chromatography on oligo(dT)-cellulose (Bantle et al. 1976). It is therefore concluded that the presence of vsRNA in the poly(A)+ fraction was not due to aggregation with other species of polyadenylated RNA.

Sedimentation analysis of polyadenylated vsRNA

Poly(A)+ RNA was isolated from the total labelled RNA of PyBHK₁ cells and was analysed by density gradient centrifugation under denaturing conditions (i.e. in the presence of a high concentration of Me₃SO; Strauss, Kelly & Sinsheimer, 1968; Maxwell, 1976). The extent of hybridization with polyoma DNA of the RNA recovered from each gradient fraction was then determined. The results are shown in Fig. 1. The polyadenylated vsRNA sedimented mainly as a broad band with a median sedimentation coefficient slightly lower than that of 28S rRNA. This sedimentation pattern was similar to that previously reported for the total nuclear vsRNA of these cells, analysed under the same conditions (Maxwell,
Fig. 2 (a), (b) and (c). For legend see opposite.
Poly(A) RNA in polyoma-transformed cells

1976). In contrast, cytoplasmic vsRNA has been shown to consist mainly of considerably smaller RNA species (Maxwell, 1976).

**Substantial removal of vsRNA from the poly(A)+ fraction of total RNA by limited thermal scission**

The existence of ‘hybrid’ RNA molecules (molecules containing both virus- and host-specified sequences) has been reported in the nuclei of virus-transformed cells (Wall & Darnell, 1971; Wall et al. 1973). Hybrid molecules might represent precursors of virus-specific mRNA and, according to current ideas of mRNA biogenesis (Lewin, 1975), might be expected to contain host sequences distal and a virus sequence proximal, to the polyadenylated 3'-terminus. Preparations enriched in sequences proximal to 3'-termini may be obtained by controlled cleavage of poly(A)+ RNA, followed by isolation of polyadenylated fragments (Molloy et al. 1974; Wang et al. 1975). Such preparations from the type of hybrid molecules postulated above would be rich in virus- as opposed to host-specified, sequences.

In the following experiment it was found that polyadenylated fragments, prepared from the total RNA of PyBHK1 cells, were depleted of, rather than enriched in, vsRNA.

Total labelled RNA was subjected to limited random scission by heating at 100 °C for 3 min. Thermal scission of RNA at 100 °C and neutral pH occurs at an appreciable rate only if catalysed by divalent cations (N. Pace, personal communication). Evidently, metal ions sequestered by the RNA during its preparation were sufficient to catalyse scission in the present experiment. The effect of this treatment in reducing the average size of the RNA was determined by subjecting samples to sedimentation analysis, as shown in Fig. 2. The sedimentation pattern of vsRNA without heating (Fig. 2a) was similar to that previously reported in aqueous sucrose gradients, showing components sedimenting faster, relative to rRNA, than those observed in Me₂SO gradients (Maxwell, 1976). The heated RNA (Fig. 2b) showed a broad sedimentation distribution, mostly less than 28S. The poly(A)+ RNA, obtained from the heated preparation by oligo(dT)-cellulose chromatography, also showed a broad sedimentation distribution (Fig. 2c) with an average sedimentation coefficient about 18S. This distribution was similar to that of mRNA prepared from these cells (unpublished observations). Table 2 (Expt. II) shows the results of the hybridization of the poly(A)+ and poly(A)- fractions, obtained from the unheated and the heated RNA, with polyoma DNA. The proportion of total vsRNA found in the poly(A)+ fraction was 72 % for the unheated RNA but only 25 % for the heated preparation. It is concluded that the random scission of total RNA to an average mol. wt. similar to that of mRNA resulted in a very substantial fall (by 65 %) in the proportion of total vsRNA attached to poly(A).

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Fig. 2. Centrifugation in aqueous sucrose gradients of total RNA from PyBHK1, (a) before, and (b) after heating at 100 °C for 3 min, and (c) of the fraction of the heated RNA that was retained by oligo(dT)-cellulose. Cells were labelled in a roller bottle for 24 h with 14C-uridine and then, in suspension, for 2 h with 3H-uridine (40 μCi/ml) in low actinomycin. A sample of the total RNA (350 μg) was dissolved in water (0.15 ml), heated at 100 °C for 3 min and was then precipitated with NaCl (0.17 m) and ethanol (2 vol.). Samples of (a) untreated, or (b) heated, total RNA were centrifuged in sucrose gradients (10 to 25 %, w/v), containing SDS, in the SW 56 rotor (Beckman) at 50000 rev/min for 120 min (Maxwell, 1976). In (c), a sample of the oligo(dT)-cellulose-retained, heated RNA was centrifuged under the same conditions, but for 150 min. The 14C-radioactivity shown in (c) is that of 14C-labelled rRNA, centrifuged in a parallel gradient. The 28S and 18S positions shown by arrows in (b) were the average positions taken from three parallel gradients containing 14C-labelled rRNA. Counter background was 10 c/min. △−−−△, 14C; ○−−○, 3H total; ●−−●, 3H hybridized; ■, 3H bound to blank filters.
DISCUSSION

Virus-specific mRNA in SV40-transformed and adenovirus-transformed cells has properties consistent with the presence of a poly(A) tract (Wall et al. 1973; Weinberg et al. 1972, 1974) in common with a high proportion of the total mRNA of eukaryotic cells (Adesnik et al. 1972; Milcarek, Price & Penman, 1974). It has now been found that most of the polysomal vsRNA in a line of polyoma virus-transformed hamster cells, PyBHK₁, was present in the poly(A)+ fraction, obtained by chromatography on oligo(dT)-cellulose. The polysomal vsRNA presumably included the mRNA coding for polyoma-specific T-antigen, known to be present in these cells.

The data presented here also show that more than 70% of the vsRNA extracted from total cells of this line was polyadenylated. In a previous report (Maxwell, 1976), it was shown that the cytoplasmic vsRNA of these cells comprised only a minor fraction of the total and that more than 65% of the vsRNA was restricted to the nucleus. It is therefore clear that a high proportion (minimum estimate 53%) of the nucleus-restricted vsRNA was polyadenylated. This conclusion is supported by the demonstration that the sedimentation pattern of the total poly(A)+ vsRNA (Fig. 1) was very similar to that of total nuclear vsRNA (Maxwell, 1976). Certain models proposed for the biogenesis of poly(A)+ mRNA imply that polyadenylation is a necessary and perhaps sufficient step for the selection of these mRNA sequences from their nuclear precursors (Darnell et al. 1973; Lewin, 1975). The present results show that polyadenylation is not sufficient for the transport of vsRNA to the cytoplasm in PyBHK₁ cells.

The state of the polyoma DNA in PyBHK₁ cells has not been characterized in detail. However, it has been shown by hybridization with labelled complementary RNA (H. Turler & I. Maxwell, unpublished observation) that the total DNA of these cells contains approximately four polyoma genome equivalents of virus DNA per diploid equivalent. (PyBHK₁ cells contain close to the normal diploid number of Syrian hamster chromosomes.) Polyoma capsid antigen was not detectable in PyBHK₁ cells and sonicated extracts of these cells failed to induce the production of this antigen during incubation with susceptible mouse kidney cell cultures. It is therefore presumed that the virus DNA is integrated into the host DNA in PyBHK₁ cells although this has not been demonstrated directly. It is not known whether all regions of the polyoma genome are equally represented.

Most of the total poly(A)+ vsRNA of PyBHK₁ cells was observed to sediment in Me₂SO considerably faster than 20S (Fig. 1). The cytoplasmic vsRNA of these cells consists mainly of species sedimenting in a broad band about 19S (Maxwell, 1976). The larger vsRNA found in the nucleus might consist either of ‘hybrid’ molecules (produced by the transcription of integrated virus DNA and of adjacent cellular DNA: Sambrook et al. 1968; Wall & Darnell, 1971) or of molecules comprised entirely of virus-specific sequences. It was found that limited thermal scission of total RNA (to an average mol. wt. similar to that of mRNA) resulted in the release of 65% of the vsRNA that was originally attached to poly(A). Thus, most of the virus-specific sequences present in poly(A)+ RNA molecules were not closely adjacent to the polyadenylated 3'-termini. This result eliminated the possibility that a substantial proportion of the nuclear vsRNA existed as sequences of similar size to virus-specific mRNA, adjacent to the 3'-terminus of polyadenylated hybrid molecules. This is the type of structure which has been postulated for the nuclear precursors of poly(A)+ mRNA (Lewin, 1975). The present results therefore support the conclusion, previously drawn from kinetic and sedimentation studies (Maxwell, 1976), that most of the nuclear vsRNA in PyBHK₁ cells is not a precursor of virus-specific mRNA.
The nuclear poly(A)+ vsRNA might consist either very largely of virus-specific sequences or of hybrid molecules containing a substantial length of host-specified sequence between the poly(A) and a virus-specific sequence. Indirect evidence favours the latter possibility. Thus, in the thermal scission experiment, the observed release of 65% of the vsRNA from poly(A) would require that the average mol. wt. of the original poly(A)+ vsRNA, if comprised entirely of virus-specific sequences, be 2.9 times that after scission. This is a minimum estimate for the nuclear poly(A)+ vsRNA since this experiment was conducted using total cell RNA: much of the vsRNA that remained polyadenylated could probably be accounted for as cytoplasmic vsRNA, whose average mol. wt. would be only slightly reduced during the heat treatment. In fact, the average mol. wt. of the nuclear vsRNA, estimated from sedimentation in Me2SO, was 2 to 2.5 times that of the cytoplasmic vsRNA (Maxwell, 1976). Thus, although the structure of nuclear vsRNA cannot yet be rigorously defined, the existence of hybrid molecules containing a 5'-proximal virus-specific sequence and a 3'-proximal host-specified sequence seems likely.

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