Analysis of polyoma virus nuclear RNA by mini-blot hybridization*

(productive infection/RNA-DNA hybridization/methylmercuric hydroxide–agarose gel electrophoresis/polyadenylation)

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ABSTRACT The size and sequence composition of virus-specific RNA extracted from the nuclei of mouse cells late during polyoma virus productive infection were studied by blot-hybridization analysis of 32P-labeled RNA fractionated on CH3HgOH/agarose gels. Viral RNA molecules between approximately 0.4 and 4 times the length of a complete transcript of the 5.4-kilobase circular viral DNA were found. Less than 20% of such molecules were polyadenylated. Although viral RNA of all sizes contained species that together hybridized to the entire polyoma genome, sequences complementary to the late region were more abundant than sequences complementary to the early region in transcripts less than 10–12 kilobases long.

Virus-specific RNA in the nuclei of mouse cells late during productive infection by polyoma virus (Py) comprises total transcripts of both viral DNA strands (1, 2). RNA complementary to one of the Py DNA strands, which is called the L strand, is at least 10 times more abundant than the RNA complementary to the other DNA strand, the E strand (1). Several groups have reported that the Py late nuclear RNA (nRNA) sediments as a polydisperse band between 20 S and 60 S under various denaturing conditions (3–6), suggesting that it includes a predominance of molecules between 1 and 3 times the length of the 5.4-kilobase circular viral DNA. In contrast, three major polyadenylated viral mRNAs (sedimenting at 20 S, 19 S, and 16 S) have been identified in the cytoplasm of cells late during productive infection and positioned on the physical map (7) of Py DNA (8) (Fig. 1). The E DNA strand of about half of the genome determines the 20S messenger, and the L DNA strand of most of the other half determines the 19S and 16S species. Whereas the functional significance of the putative “giant” nRNA molecules is far from clear, their existence is consistent with models for viral messenger synthesis that propose continuous transcription around the circular viral DNA template followed by specific post-transcriptional cleavage and modification. Further information on the nature of the nRNA is required, however, before any such models can be evaluated. In this report, we ask two questions. First, can it be rigorously established that giant molecules are the predominant nuclear species in cells infected with plaque-purified Py virus under conditions such that defective DNA is not generated? Second, how does the sequence composition of nRNA vary with respect to the DNA physical map as a function of its molecular size?

Our studies have been facilitated by use of a modification of the Southern (10) hybridization procedure, which we call the “mini-blot” method, to map labeled viral RNA sequences on the Py genome. Southern showed that DNA restriction fragments separated on an agarose gel could be denatured in situ and transferred with little loss in resolution to a sheet of nitrocellulose. The nitrocellulose-immobilized replica of the agarose gel pattern could then be exposed to radioactive RNA under annealing conditions, and the particular DNA fragments containing sequences complementary to the RNA could be visualized by autoradiography. In the mini-blot variation, excess amounts of Py DNA restriction fragments immobilized on the nitrocellulose are used to drive hybridizations with 32P-labeled RNA samples containing only a small proportion (1–5%) of viral RNA.

MATERIALS AND METHODS

Virus stocks used (Py large plaque, strain A2 (7)) were shown not to generate detectable quantities of defective DNA by restriction enzyme analysis of the Hirt supernatant DNA (11) synthesized during one subsequent passage at high input multiplicity. Further controls were done by examining the total DNA present in infected cells at various times throughout productive infection (3–72 hr) by the Southern procedure (10) with 32P-labeled denatured Py DNA as the probe; no indication of viral DNA molecules smaller than full length was found, and oligomeric DNA species were detected in very low amounts only more than 10 hr later than the times used in the experiments described below.

Mini-Blot Hybridization Procedure. Restriction endonuclease-digested Py DNA (5.2 μg in 400 μl of 5 mM Tris-HCl, pH 7.5/0.5 mM EDTA/0.05% sodium dodecyl sulfate (NaDodSO4)/0.02% bromophenol blue/30% (wt/vol) sucrose) was loaded into a well (13 × 0.15 cm) across one end of a horizontal slab gel (15 × 20 × 0.5 cm) containing 1.4% (wt/vol) agarose (SeaKem) and ethidium bromide (0.5 μg/ml) in the Tris-acetate buffer described by Sharp et al. (12). After electrophoresis for 4 hr at 2 V/cm in an apparatus modified from the original design of W. Schaffner (construction details available from the authors on request), the gel was examined under long-wavelength UV light and the region containing the DNA fragments (a rectangle 13 × 6 cm) was excised. The DNA was transferred to a sheet of nitrocellulose (Schleicher and Schuell, BA85) by the blotting method of Southern (10). After transfer overnight, the nitrocellulose sheet and the gel were carefully removed together and the filter was cut with a scalpel into 0.5-cm wide longitudinal strips. The mini-blots were dried overnight in an evacuated dessicator containing silica gel, heated for 2 hr at 80° in a vacuum oven, and then stored in a dessicator. Before use, they

Abbreviations: Py, polyoma virus; nRNA, nuclear RNA; NaDodSO4, sodium dodecyl sulfate; SSC, 0.15 M NaCl/0.015 M Na citrate, pH 7.0; 6Xc and 2X SSC, the concentration of the solution used is 6X and 2X, respectively, that of the standard solution; Hpa II, restriction endonuclease obtained from Haemophilus parainfluenzae; cRNA, the product of in vitro transcription by Escherichia coli RNA polymerase; WME cells, whole mouse embryonic cells; poly(A)+ RNA, polyadenylated RNA; poly(A)− RNA, nonpolyadenylated RNA.

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were preincubated for 4 hr at 68° in 6X SSC containing 0.02% (wt/vol) bovine serum albumin, 0.02% (wt/vol) polyvinylpyrrolidone, and 0.02% (wt/vol) Ficoll (13, 14). Each mini-blot to be hybridized was then inserted into a rectangular polyethylene bag slightly larger than the filter; the bags were formed by heat-sealing 5-cm diameter polythene sleevings (purchased from Hospital and Laboratory Supplies, London EC1) with a Siemens bag-sealer. The labeled RNA samples were diluted into 1.0 ml of freshly prepared preincubation buffer additionally containing 0.2% NaDodSO4 and yeast RNA (20 μg/ml) and were added to the individual bags which were then heat-sealed. Annealing was for 60–65 hr at 68° in a shaking-water bath. After hybridization, the mini-blots were washed in bulk in 2X SSC at 68° (at least 100 ml per sample) for several hours, treated with a mixture of pancreatic A and T1 RNases (25 μg/ml and 10 units/ml, respectively) in 2X SSC at 37° for 2 hr, washed again with 2X SSC/0.5% NaDodSO4 at 68°, and then rinsed in 2X SSC. After mounting on a glass plate, they were covered with Saran Wrap and exposed to X-ray film. Depending on the level of radioactivity expected, exposure was either autoradiographic with Kodak Kodirex film or fluorographic at -70° with preflushed Fuji Rx film combined with Fuji-Mach II or Ilford fast tungstate intensifying screens (R. Laskey, personal communication).

RESULTS

Mini-Blot Hybridization. The detailed procedure for mini-blot hybridization is described in Materials and Methods.

Table 1. Relative distributions* of radioactivity in control mini-blot hybridization

<table>
<thead>
<tr>
<th>32P-Labeled probe</th>
<th>Hpa II restriction fragment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5 + 6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Py DNA</td>
<td></td>
<td>1.01</td>
<td>0.92</td>
<td>1.09</td>
<td>1.13</td>
<td>0.91</td>
<td>0.88</td>
</tr>
<tr>
<td>Py DNA</td>
<td></td>
<td>0.98</td>
<td>0.80</td>
<td>1.14</td>
<td>1.14</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Py cRNA</td>
<td></td>
<td>1.12</td>
<td>0.84</td>
<td>1.10</td>
<td>0.86</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Py cRNA</td>
<td></td>
<td>1.08</td>
<td>0.83</td>
<td>1.11</td>
<td>0.94</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Py cRNA</td>
<td></td>
<td>1.11</td>
<td>0.93</td>
<td>1.00</td>
<td>0.80</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Microdensitometer scans of five different control hybridizations with 32P-labeled Py DNA or cRNA probes were integrated by cutting out and weighing the different peaks. The fractional weight of each peak was divided by the fractional length of Py DNA in each restriction fragment to obtain the molar ratios of hybridization to the Hpa II fragments. ND, not done.

* Normalized to correct for restriction fragment size.

Control experiments were done by annealing denatured Py DNA uniformly labeled with 32P (15) to mini-blots containing the eight restriction endonuclease Hpa II fragments (7), and the locations of the sequences determining the three major viral mRNAs (8). Oligonucleotide probes were hybridized to the three mini-blots and the results of annealing were determined (16). Exposure time was 10 min, with preflushed Fuji-Rx film and an Ilford fast tungstate intensifying screen at -70° (fluorographic conditions).

The former two fragments (see Fig. 1) comprise the region of Py DNA that encodes the abundant late mRNAs, whereas
the latter pair of fragments lies within the region that determines the minor early mRNA (8). For purposes of comparison, Fig. 5 includes the results of mini-blot hybridizations of total polyadenylated cytoplasmic RNA and of size-fractionated 16S mRNA and 18S mRNA. The 19S mRNA annealed to \textit{Hpa} II-1 and \textit{Hpa} II-3, whereas the 16S mRNA annealed predominantly to \textit{Hpa} II-1.

Greater than 90% of the viral RNA synthesized late during Py infection is complementary to the \textit{L} DNA strand (19). Therefore, nearly all of the radioactive RNA hybridized to the mini-blots shown in Fig. 3 should be annealed to the \textit{L} strand of the DNA fragments. The data obtained with late mRNA thus imply that the entire \textit{L} DNA strand is transcribed late during infection but that the mRNA-like sequences complementary to the late region are more abundant than the "anti-messenger" sequences complementary to the \textit{L} DNA strand of the early region.

Size and Polyadenylation of Py mRNA. Agarose gel electrophoresis in the presence of CH$_3$HgOH affords high-resolution fractionation of large RNA molecules under rigorously denaturing conditions (21, 22). The linear relationship obtained between the logarithm of the molecular weight and the electrophoretic mobility, moreover, permits an unambiguous estimation of the size of high molecular weight species (21, 22). Experiments with initially double-stranded restriction fragments of Py DNA proved that the method fully denatures nucleic acid duplexes because the mobility of the DNA fragments was that predicted from their single-stranded molecular weights as judged by comparison with internal mouse and \textit{E. coli} rRNA markers. A major advantage of CH$_3$HgOH over other reagents that covalently attach to and thus denature nucleic acids is that the reaction is readily reversible by addition of sulfhydryl compounds such as dithiothreitol or 2-mercaptoethanol. In preliminary experiments, we showed that RNA that had been treated with CH$_3$HgOH and then with 2-mercaptoethanol was unaltered in its ability to hybridize to complementary DNA.

The RNA purified from nucleic of Py-infected WME cells exposed to $^{32}$P orthophosphate 28–33 hr after infection was fractionated on oligo(dT)-cellulose (P-L Biochemicals, type 7) to separate polyadenylated [poly(A)$^+$] from nonpolyadenylated [poly(A)$^-$] molecules (23). The poly(A)$^+$ and poly(A)$^-$ nRNA fractions (2 x 10$^6$ and 5 x 10$^6$ cpm of $^{32}$P, respectively) were treated with CH$_3$HgOH and then electrophoresed in parallel through a 1% agarose gel containing the denaturant. The gel was stained with ethidium bromide (Fig. 4), which revealed that the poly(A)$^+$ fraction contained three rRNA components (32S, 28S, and 18S rRNA), whereas the poly(A)$^-$ fraction showed no detectable stained bands. A straight line was obtained when the logarithms of the rRNA molecular weights were plotted as a function of the distances migrated; this line was extrapolated to estimate the expected positions in the gel of RNA molecules 1, 2, 3, and 4 times the length of a complete transcript of the circular polyoma DNA. Autoradiography of similar gels (data not shown) showed major bands corresponding to 45S, 32S, and 28S rRNA superimposed on a heterogeneous distribution of large molecules (presumably heterogeneous rRNA) in the poly(A)$^-$ fraction but only the heterogeneous high molecular weight distribution in the poly(A)$^+$ fraction.

The two gel channels shown in Fig. 4 were excised and cut transversely into 0.5-cm slices. Twenty percent of the RNA from each gel slice (4–80 x 10$^9$ and 2–20 x 10$^9$ cpm for the poly(A)$^-$ and poly(A)$^+$ samples, respectively) was hybridized to mini-blots containing \textit{Hpa} II–Py DNA fragments. The resulting fluorographs (Fig. 5) were scanned with a microdensitometer, and the relative distribution of virus-specific RNA complementary to the four largest \textit{Hpa} II fragments (Table 2) was determined.

The great majority (approximately 80%) of the Py mRNA was
found in the poly(A)\(^-\) fraction (Fig. 5A), confirming previous results that indicated that only 10–20% of the polyoma mRNA molecules have poly(A) tails (5). Two features of the poly(A)\(^-\) viral mRNA are most striking. It is very heterogeneous in size, comprising molecules between about 0.4 and 4 times the length of a complete transcript of Py DNA; RNA of all sizes hybridize to each of the detectable restriction fragments. Although hybridization to the four largest \textit{Hpa II} fragments was obtained across almost the entire gel, comparison of the data shown in Table 2 with the cDNA results shown in Table 1 clearly demonstrates that the Py mRNA molecules of different sizes are not random uniform transcripts of the viral DNA. Among the smaller poly(A)\(^-\) mRNA molecules (Fig. 5A and Table 2, fractions 12–17), RNA sequences identical to those present in the late mRNAs (complementary to 75% of \textit{Hpa II}-3 and to all of \textit{Hpa II}-1) were more abundant than anti-messenger sequences (complementary to \textit{Hpa II}-2 and to \textit{Hpa II}-4). In larger poly(A)\(^-\) mRNA (fractions 7–11), sequences complementary to the late region (\textit{Hpa II}-1 and \textit{Hpa II}-3) were also relatively abundant, but there was a further tendency for sequences complementary to \textit{Hpa II}-2 to be more common than those that annealed to \textit{Hpa II}-4. It is interesting that the mRNA of about genome size (fraction 11) also hybridized to \textit{Hpa II}-4 with reduced efficiency. This may mean that the predominant RNA species in this size range (e.g., contained in this particular 0.5-cm gel slice) was in fact slightly smaller than genome length. If the 5' end of this predominant species were similar to that of the mature 19S mRNA, one would expect transcripts 0.8–0.9 times genome length to specifically lack sequences complementary to \textit{Hpa II}-4.

Poly(A)\(^+\) viral mRNA (Fig. 5B) was also found to be a heterogeneous mixture of molecules with a size distribution similar to that of the poly(A)\(^-\) species but somewhat biased towards smaller components. The larger poly(A)\(^+\) mRNA molecules (Fig. 5B and Table 2, fractions 6–11) are more nearly uniform transcripts of the viral genome than the poly(A)\(^-\) mRNA. A distinct peak of poly(A)\(^+\) RNA with the size and sequence composition of the late 16S mRNA was observed (Fig. 5B, fraction 16). However, this component is too small a fraction (4%) of the total viral mRNA to exclude the possibility that it results from a minor amount of cytoplasmic contamination in the mRNA sample.

**DISCUSSION**

Acheson \textit{et al.} (3) originally reported that nascent Py-specific mRNA comprises giant molecules longer than a complete transcript of the circular viral genome. Several subsequent reports (4–6) confirmed this conclusion. The validity of these results can be questioned for two reasons. First, unless carefully monitored, stocks of Py readily accumulate defective particles (24–26) which typically have some portions of the viral genome deleted and other portions repeated or rearranged. If such molecules have start signals for transcription but lack stop or processing signals, their transcription could account for the existence of the giant mRNA. Second, the molecular weights reported previously were calculated from sedimentation velocities through various "denaturing" sucrose gradients; the accuracy of such calculations has been challenged (27). In the present work, we have circumvented the first of these objections by using plaque-purified Py, which was shown not to generate defective DNA during the cycle of infection studied, and fur-
ther by using a method of hybridization analysis that readily
distinguishes between transcripts of the entire wild-type ge-
nome and aberrant transcripts of repetitious defective genomes.
The use of rigorous chemical denaturation with CH\(_3\)HgOH and
the electrophoretic fractionation of the denatured RNA to es-
timate molecular sizes effectively eliminates the uncertainties
inherent in sedimentation through denaturing solvents (21, 22).
We thus believe that the results described here unambiguously
establish that giant RNA molecules greater than genome length
are transcribed from normal Py DNA late during productive
infection.

Analysis of the size and sequence composition of Py nRNA
demonstrated that the nuclear transcripts are not a random
heterogeneous distribution of molecules. Both polyadenylylated
and nonpolyadenylylated molecules between mRNA size and
genome length contained more RNA sequences identical to
those found in mature mRNA than those sequences not present
in mRNA. Larger nonpolyadenylylated viral nRNA also con-
tained more messenger than nonmessenger sequences but,
curiously, the polyadenylylated mRNA molecules between 1
and 3 times genome size did not. If late mRNA is derived from
larger precursors by preferential degradation of nonmessenger
regions, most mRNA molecules (except those with sizes that are
exact integral multiples of the genome length) should have a
greater proportion of messenger than nonmessenger sequences.
Whereas our results with nonpolyadenylylated nRNA (which
constitutes at least 80% of the viral nRNA) agree with this
prediction, direct proof of a multistep processing mechanism
can only be obtained from kinetic experiments that demonstrate
that the nonmessenger sequences are selectively removed from
the large RNAs. The kinetic relationship between the pools of
nonpolyadenylylated and polyadenylylated nRNAs must also be
examined.

The mini-blot variation of the Southern procedure has proved
a useful tool in the analysis of Py gene expression. It should be
equally useful in the study of the transcription of other DNA
viruses or of any cellular gene that has been cloned into a
plasmid or phage vector. A similar procedure has, in fact, al-
ready been successfully used to examine the temporal control
of herpes simplex virus transcription (J. B. Clements, R. J.
Watson, and N. Wilkie, personal communication).

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3055–3063.
641–646.
287.
310.
560–564.
85.
681–697.
1408–1412.
49–86.
13, 261–268.