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

(productive infection/RNA·DNA hybridization/methylmercuric hydroxide–agarose gel electrophoresis/polyadenylylation)

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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 ^{32}P-labeled RNA fractionated on CH_3HgOH/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 polyadenylylated. 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 polyadenylylated 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 ^{32}P-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 ^{32}P-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 ($NaDodSO_4$)/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 Schüll, 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

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Abbreviations: Py, polyoma virus; nRNA, nuclear RNA; $NaDodSO_4$, sodium dodecyl sulfate; SSC, 0.15 M NaCl/0.015 M Na citrate, pH 7.0; 6× and 2× SSC, the concentration of the solution used is 6× and 2×, 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 embryo cells; poly(A)$^+$ RNA, polyadenylylated RNA; poly(A)$^-$ RNA, nonpolyadenylylated RNA.

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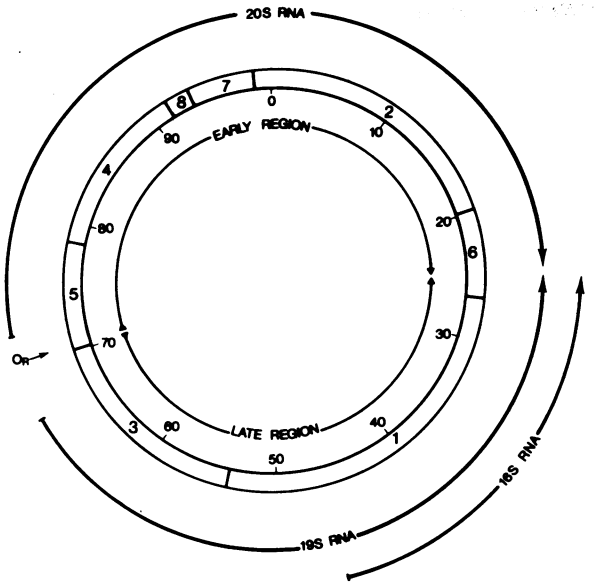

FIG. 1. Physical map (7) of Py DNA indicating the regions designated "early" and "late" (1), the positions of the eight restriction endonuclease *Hpa* II fragments (7), and the locations of the sequences determining the three major viral mRNAs (8). O_R designates the point where bidirectional DNA replication initiates (7, 9).

were preincubated for 4 hr at 68° in 6× 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 sleeving (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% $NaDodSO_4$ 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 2× 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 2× SSC at 37° for 2 hr, washed again with 2× SSC/0.5% $NaDodSO_4$ at 68°, and then rinsed in 2× 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 preflashed 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*.

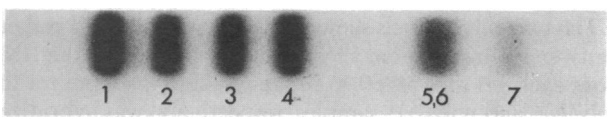

FIG. 2. Hybridization of ^{32}P-labeled denatured Py DNA (5 ng, 2×10^5 cpm/ng) to a mini-blot containing 100 ng of restriction endonuclease *Hpa* II-digested Py DNA. The DNA was labeled *in vitro* by "nick translation" (15) essentially as described by Botchan *et al.* (16). Exposure time was 10 min with preflashed Fuji-Rx film and an Ilford fast tungstate intensifying screen at −70° (fluorographic conditions).

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

^{32}P-Labeled probe	*Hpa* II restriction fragment					
	1	2	3	4	5 + 6	7
Py DNA	1.01	0.92	1.09	1.13	0.91	0.88
Py DNA	0.98	0.80	1.14	1.14	ND	ND
Py cRNA	1.12	0.84	1.10	0.86	ND	ND
Py cRNA	1.08	0.83	1.11	0.94	ND	ND
Py cRNA	1.11	0.93	1.00	0.80	ND	ND

Microdensitometer scans of five different control hybridizations with ^{32}P-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 ^{32}P (15) to mini-blots containing the eight restriction endonuclease (obtained from *Haemophilus parainfluenzae*) *Hpa* II fragments (7) of Py DNA. These fragments ranged in size from about 0.10 to about 1.5 kilobases. Annealing to the seven largest *Hpa* II fragments was readily detected (Fig. 2). The smallest fragment (*Hpa* II-8, *ca* 0.10 kilobase long) was lost during the transfer and/or annealing steps. Integration of a microdensitometer scan (Table 1) showed that the extent of annealing was approximately proportional to DNA fragment length. Hybridization of the *Hpa* II mini-blots with ^{32}P-labeled asymmetric Py cRNA (1) was used to estimate the sensitivity of the method for detecting and mapping radioactive virus-specific RNA. Fig. 3 (blots 1–5) shows the results of annealing increasing amounts of the product of *in vitro* transcription by *Escherichia coli* RNA polymerase (cRNA) (0.3–11 ng, specific activity 1×10^4 cpm/ng) to mini-blots containing 200 ng of *Hpa* II-digested Py DNA. Annealing to fragments *Hpa* II-1 through *Hpa* II-7 was detected after an overnight autoradiographic exposure; visual inspection readily showed that the band darkness intensified with increasing amounts of input radioactivity. Densitometric scanning of two of the cRNA autoradiograms (Fig. 3, blots 2 and 4), as well as of a further cRNA control annealing done in parallel with the experiments described below, confirmed that the band darkness was approximately proportional to DNA fragment length (Table 1). We conclude from these control experiments that mini-blot hybridization provides a sensitive semiquantitative mapping procedure that is accurate to within an error of approximately 20%. Using the increased film sensitivity afforded by intensifying screens and fluorographic exposure conditions (R. Laskey, personal communication), we estimated that the method could be used to map a ^{32}P-labeled RNA sample containing 300 cpm of viral RNA on the Py genome.

Mapping Py nRNA. Py-infected whole mouse embryo (WME) cells were labeled with [^{32}P]orthophosphate at a late time (22–28 hr) after infection. Radioactive RNA was extracted from the nuclei and increasing amounts were hybridized to mini-blots containing *Hpa* II fragments of Py DNA. Fig. 3 (blots 6–10) shows that the Py nRNA annealed to all seven detectable *Hpa* II DNA fragments. RNA purified from the nucleus or cytoplasm of mock-infected cells yielded no hybridization to Py DNA. It is apparent from visual inspection of the autoradiograms that the two fragments *Hpa* II-1 and *Hpa* II-3 are relatively intense compared with fragments *Hpa* II-2 and *Hpa* II-4. The former two fragments (see Fig. 1) comprise the region of Py DNA that encodes the abundant late mRNAs, whereas

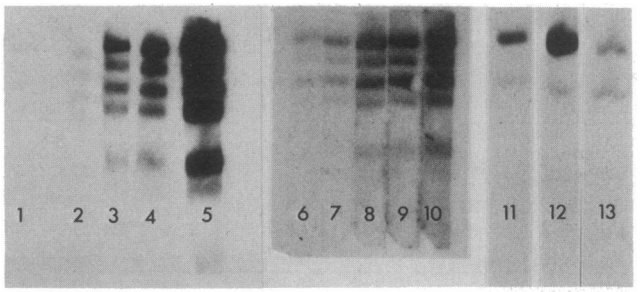

FIG. 3. (Blots 1–5) Hybridization of *in vitro* ^{32}P-labeled asymmetric Py cRNA to mini-blots containing 200 ng of *Hpa* II-digested Py DNA. The cRNA (1) after partial alkaline hydrolysis to an estimated average chain length of 100 nucleotides (17) was labeled at 5′ termini by incubation with T4 polynucleotide kinase (the generous gift of M. Szekely, Imperial College) and [γ-^{32}P]ATP as described by Maxam and Gilbert (18) to a specific activity of 1×10^4 cpm/ng. Input radioactivities were 1,539, 2,918, 14,311, 28,773 and 58,775 cpm (Cerenkov) for blots 1–5, respectively. The filters were RNase digested and washed as described in *Materials and Methods*. Exposure time was 15 hr with Kodirex film. (Blots 6–10) Hybridization of *in vivo* ^{32}P-labeled total nRNA extracted from Py-infected WME cells after treatment with [^{32}P]orthophosphate between 22 and 28 hr after infection [16,900, 33,490, 78,570, 161,780, and 329,380 cpm (Cerenkov), respectively]. Exposure time was 48 hr with Kodirex film. (Blot 11) ^{32}P-Labeled polyadenylylated cytoplasmic RNA (5275 cpm) extracted from Py-infected WME cells. Exposure time was 48 hr with Kodirex film. (Blots 12 and 13) 16S (5500 cpm) and 19S (4250 cpm) peak fractions of ^{32}P-labeled polyadenylylated cytoplasmic RNA extracted from infected WME cells and fractionated on a sucrose gradient containing 50% formamide (8). Exposure time was 3–4 days with Kodirex film. WME cells were grown and infected with Py at 100 plaque-forming units per cell as described by Flavell and Kamen (19). After 22 hr at 37°, the medium was removed from five 90-mm dishes, the cells were washed once with phosphate-free medium, and 5 ml of phosphate-free medium supplemented with 3% horse serum and containing [^{32}P]orthophosphate (400 μCi/ml) (Radiochemical Centre) was added. Six hours later, the cells were harvested and RNA was extracted essentially as described (19), except that a single DNase digestion at 1 μg/ml for 20 min was used and the RNA was further purified by precipitation with cetyltrimethylammonium bromide (20) followed by precipitation from 4 M NaCl and extraction with 10% (wt/vol) sodium acetate (pH 5.3).

the latter pair of fragments lies within the region that determines the minor early mRNA (8). For purposes of comparison, Fig. 3 includes the results of mini-blot hybridizations of total polyadenylylated cytoplasmic RNA and of size-fractionated 16S mRNA and 19S mRNA. The 19S mRNA annealed to *Hpa* II-1 and *Hpa* II-3, whereas the 16S mRNA annealed predominantly to *Hpa* II-1.

Greater than 90% of the viral RNA synthesized late during Py infection is complementary to the 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 L strand of the DNA fragments. The data obtained with late nRNA thus imply that the entire 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 L DNA strand of the early region.

Size and Polyadenylylation of Py nRNA. Agarose gel electrophoresis in the presence of CH_3HgOH 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

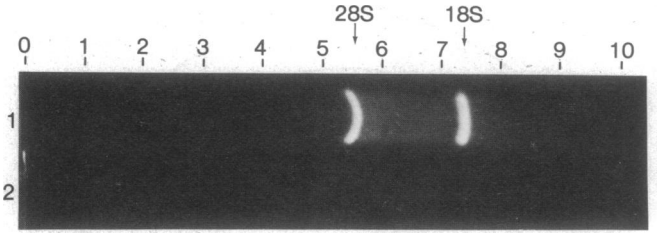

FIG. 4. CH_3HgOH/agarose gel of poly(A)$^-$ (channel 1) and poly(A)$^+$ (channel 2) RNA extracted from the nuclei of Py-infected WME cells. A 1% agarose slab gel (10.2 × 18.5 × 0.5 cm) was prepared by a slight modification of the procedure described by Bailey and Davidson (21). The nRNA samples [5×10^6 cpm of poly(A)$^-$ and 2×10^6 cpm of poly(A)$^+$ RNA, labeled 28–33 hr after infection] were preincubated for 10 min at 20° in 25 mM CH_3HgOH (the gift of D. C. Livingston) before loading onto the gel. Electrophoresis (in a Shandon-Southern apparatus, model U77) at 90 V and 20 mA was continued until the dye reached the end of the gel. The gel was stained in 10 mM Tris·HCl (pH 7.5) containing ethidium bromide (5 μg/ml) and 0.1% thioglucose and photographed under long-wavelength UV light. 0 indicates the origin of the gel.

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 *E. coli* rRNA markers. A major advantage of CH_3HgOH 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_3HgOH 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 polyadenylylated [poly(A)$^+$] from nonpolyadenylylated [poly(A)$^-$] molecules (23). The poly(A)$^+$ and poly(A)$^-$ nRNA fractions (2×10^6 and 5×10^6 cpm of ^{32}P, respectively) were treated with CH_3HgOH and then electrophoresed in parallel through a 1% agarose slab 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 nRNA) 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 [$4–80 \times 10^3$ and $2–20 \times 10^3$ cpm for the poly(A)$^-$ and poly(A)$^+$ samples, respectively] was hybridized to mini-blots containing *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 *Hpa* II fragments (Table 2) was determined.

The great majority (approximately 80%) of the Py nRNA was

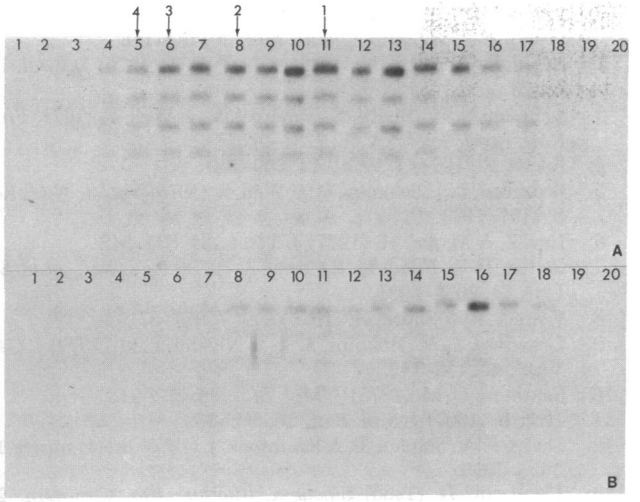

FIG. 5. Hybridization of poly(A)$^-$ (*A*) and poly(A)$^+$ (*B*) nRNA to mini-blots containing *Hpa* II digests of Py DNA. The two gel channels shown in Fig. 4 were cut transversely into 0.5-cm slices that were then dissolved in 0.5 ml of 5 M $NaClO_4$ at 60°. Aliquots (100 μl) of each fraction were hybridized to mini-blots as described in *Materials and Methods* except that the annealing buffer also contained 0.1% 2-mercaptoethanol. Film exposure was for 1 week under fluorographic conditions. The expected positions of RNA molecules 1, 2, 3, and 4 times the length of a complete transcript of Py DNA are indicated.

found in the poly(A)$^-$ fraction (Fig. 5*A*), confirming previous results that indicated that only 10–20% of the polyoma nRNA molecules have poly(A) tails (5). Two features of the poly(A)$^-$ viral nRNA 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 *Hpa* II fragments was obtained across almost the entire gel, comparison of the data shown in Table 2 with the cRNA results shown in Table 1 clearly demonstrates that the Py nRNA molecules of different sizes are not random uniform transcripts of the viral DNA. Among the smaller poly(A)$^-$ nRNA molecules (Fig. 5*A* and Table 2, fractions 12–17), RNA sequences identical to those present in the late mRNAs (complementary to 75% of *Hpa* II-3 and to all of *Hpa* II-1) were more abundant than anti-messenger sequences (complementary to *Hpa* II-2 and to *Hpa* II-4). In larger poly(A)$^-$ nRNA (fractions 7–11), sequences complementary to the late region (*Hpa* II-1 and *Hpa* II-3) were also relatively abundant, but there was a further tendency for sequences complementary to *Hpa* II-2 to be more common than those that annealed to *Hpa* II-4. It is interesting that the nRNA of about genome size (fraction 11) also hybridized to *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 *Hpa* II-4.

Poly(A)$^+$ viral nRNA (Fig. 5*B*) 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)$^+$ nRNA molecules (Fig. 5*B* and Table 2, fractions 6–11) are more nearly uniform transcripts of the viral genome than the poly(A)$^-$ nRNA. A distinct peak of poly(A)$^+$ RNA with the size and sequence composition of the late 16S mRNA was observed (Fig. 5*B*, fraction 16). However, this component is too small a fraction (4%) of the total viral nRNA to exclude the possibility that it results from a minor amount of cytoplasmic contamination in the nRNA sample

Table 2. Quantitation of mini-blot mapping of Py nRNA fractionated by CH_3HgOH/agarose gel electrophoresis

Gel fraction no.	*Hpa* II restriction fragment 1	2	3	4
Poly(A)$^-$ nRNA				
4	1.19	0.91	0.87	0.92
5(4X)	1.21	0.73	0.98	1.04
6(3X)	1.12	0.72	1.08	1.09
7	1.43	0.65	0.97	0.72
8(2X)	1.38	0.64	1.05	0.72
9	1.37	0.74	1.06	0.58
10	1.44	0.83	0.91	0.49
11(1X)	1.42	0.80	0.94	0.54
12	1.58	0.64	0.89	0.54
13	1.61	0.57	0.94	0.50
14(0.5X)	1.60	0.49	0.95	0.52
15	1.64	0.41	1.05	0.58
16	1.67	0.37	1.10	0.49
17	1.50	0.49	1.20	0.54
18	1.11	0.31	1.80	0.84
Poly(A)$^+$ nRNA				
6(3X)	1.08	0.80	0.95	1.20
7	1.68	0.86	1.07	0.93
8(2X)	1.08	0.94	1.00	0.90
9	1.15	0.81	1.05	0.91
10	1.29	0.78	1.00	0.75
11(1X)	1.19	0.77	0.91	1.05
12	1.40	0.80	0.92	0.56
13	1.43	0.58	1.12	0.61
14(0.5X)	1.65	0.37	0.93	0.73
15	1.90	0.27	0.97	0.33
16	2.43	0.12	0.43	0.17
17	2.33	0.19	0.64	—

The fluorograms of the mini-blots shown in Fig. 5 were scanned and integrated as described in Table 1. The molar ratios of hybridization of Py-specific RNA to the four largest *Hpa* II fragments, calculated as in Table 1, are shown for each individual mini-blot. For comparison, the equivalent values for pure Py late 19S mRNA (8) would be approximately 2, 0, 1.5, and 0 for *Hpa* II fragments 1–4, respectively. Numbers in parentheses indicate the length of the RNA relative to a complete transcript of Py DNA.

DISCUSSION

Acheson *et al.* (3) originally reported that nascent Py-specific nRNA 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 nRNA. 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 genome and aberrant transcripts of repetitious defective genomes. The use of rigorous chemical denaturation with CH_3HgOH and the electrophoretic fractionation of the denatured RNA to estimate 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 contained 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 nRNA 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 nRNAs. 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, already been successfully used to examine the temporal control of herpes simplex virus transcription (J. B. Clements, R. J. Watson, and N. Wilkie, personal communication).

1. Kamen, R., Lindstrom, D. M., Shure, H. & Old, R. (1974) *Cold Spring Harbor Symp. Quant. Biol.* **39**, 187–198.
2. Beard, P., Acheson, N. H. & Maxwell, I. H. (1976) *J. Virol.* **17**, 20–26.
3. Acheson, N. H., Buetti, E., Scherrer, K. & Weil, R. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 2231–2235.
4. Buetti, E. (1974) *J. Virol.* **14**, 249–260.
5. Rosenthal, L. J., Salomon, C. & Weil, R. (1976) *Nucleic Acids Res.* **3**, 1167–1183.
6. Lev, Z. & Manor, H. (1977) *J. Virol.*, **21**, 831–842.
7. Griffin, B. E., Fried, M. & Cowie, A. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 2077–2081.
8. Kamen, R. & Shure, H. (1976) *Cell* **7**, 361–371.
9. Crawford, L. V., Robbins, A. K. & Nicklin, P. M. (1974) *J. Gen. Virol.* **25**, 133–142.
10. Southern, C. M. (1975) *J. Mol. Biol.* **98**, 503–518.
11. Hirt, B. (1967) *J. Mol. Biol.* **26**, 365–369.
12. Sharp, P. A., Sugden, B. & Sambrook, J. (1973) *Biochemistry* **12**, 3055–3063.
13. Denhardt, D. (1966) *Biochem. Biophys. Res. Commun.* **23**, 641–646.
14. Wilkie, N. M. & Cortini, R. (1976) *J. Virol.* **20**, 211–221.
15. Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. (1977) *J. Mol. Biol.*, **113**, 237–252.
16. Botchan, M., Topp, W. & Sambrook, J. (1976) *Cell* **9**, 269–287.
17. Coffin, J. M. & Billeter, M. A. (1976) *J. Mol. Biol.* **100**, 293–310.
18. Maxam, A. & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 560–564.
19. Flavell, A. J. & Kamen, R. (1977) *J. Mol. Biol.*, in press.
20. Bellamy, A. R. & Ralph, R. K. (1968) in *Methods in Enzymology*, eds. Grossman, L. & Moldave, K. (Academic Press, New York), Vol. 12, part B, pp. 156–168.
21. Bailey, J. M. & Davidson, N. (1976) *Anal. Biochem.* **70**, 75–85.
22. Smith, L. H. & Sinsheimer, R. L. (1976) *J. Mol. Biol.* **103**, 681–697.
23. Aviv, H. & Leder, P. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 1408–1412.
24. Fried, M. (1974) *J. Virol.* **13**, 939–946.
25. Griffin, B. E. & Fried, M. (1975) *Nature* **256**, 175–179.
26. Griffin, B. E. & Fried, M. (1975) *Methods Cancer Res.* **12**, 49–86.
27. Kolakofsky, D., Boy de la Tour, E. & Delius, H. (1974) *J. Virol.* **13**, 261–268.