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Adenovirus Type 2 Early Nuclear and mRNA: Kinetic Estimation of *l* and *r* DNA Strand Fractions Complementary to Different Abundance Classes of Viral RNA

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RNA from unfractionated cells, nuclei, and polyribosomes was extracted from adenovirus 2 (Ad2)-infected KB cells early in infection and annealed in vast excess in liquid to purified Ad2 *l* (heavy) and *r* (light) [^{32}P]DNA strands (specific activity, 3×10^6 to 1.5×10^7 cpm/μg). The number of abundance classes of Ad2 RNA, their relative concentrations, and the strand fraction from which they arose were determined by a computer-assisted nonlinear regression analysis of hybridization kinetic data. Whole-cell RNA and nuclear RNA annealed to 60 and 40%, respectively, of *l* and *r* strands. Well-defined abundance (kinetic) classes were identified: abundant and scarce classes were complementary to 15 to 17 and 40 to 45%, respectively, of *l* strand, and to 11 to 16 and 17 to 23%, respectively, of *r* strand. In whole-cell RNA and nuclear RNA the abundant classes were 57 to 208 and 13 to 27 times more concentrated, respectively, than scarce classes. RNA-RNA hybrids were isolated that annealed to about 70% of both strands, indicating that whole-cell RNA and nuclear RNA hybridization values were minimal. Polyribosomal RNA appeared to anneal as three abundance classes to each DNA strand; abundant, scarce, and very scarce classes, respectively, hybridized to 6, 5, and about 10% of *l* strand and 7 (6 to 8), 10 (8 to 13), and about 19% of *r* strand. The abundant classes were 41 (11 to 67) times more concentrated than the scarce classes and 10^3 times more concentrated than the very scarce classes. Although the biological significance of these classes is not known, the very scarce classes probably represent nuclear RNA contaminants of polyribosomal RNA. The abundant and scarce classes may comprise mRNA, because together they are complementary to about the same fraction of each DNA strand (11% [10 to 12%] and 17% [14 to 20%] of *l* and *r* strands) known to be expressed as early mRNA. Thus, nuclear RNA contains Ad2 RNA sequences not found on polyribosomes; most or all of both DNA strands are transcribed, but only certain transcripts are processed into mRNA. It is not known whether "non-mRNA" transcripts are intermediates in the pathway of early mRNA production.

The productive infection of cells by human adenovirus 2 (Ad2) proceeds in at least two temporal stages of mRNA synthesis: "early" and "late," which follows the initiation of viral DNA replication at 6 to 7 h postinfection (18). Early studies, using RNA from unfractionated whole cells (WC-RNA) and hybridization competition, suggested that 8 to 20% of the Ad2 genome was expressed as RNA early (10) and that early viral RNA was transcribed from both DNA strands (13). Recent studies, using saturation hybridization with separated labeled DNA strands of restriction endonuclease fragments, have estimated that early mRNA arises from four noncontiguous gene blocks, two on each DNA strand, representing approximately 9 to 13% of *l* strand and 14 to 19% of *r* strand (6, 15). {*l* and *r* signify leftward and rightward transcription, respectively; these designations represent the heavy and light strands, respectively, of a poly(uridylate,guanylate)[poly-(U,G)]-DNA strand complex [1].} Experiments where labeled early RNA was hybridized to restriction fragments or separated DNA strands, followed by size fractionation, confirmed the general location and strand specificity of the four early gene blocks and further identified five major classes of early mRNA (2, 3). Other size fractionation and restriction fragment hybridization studies have detected as many as eight size classes of early mRNA (6).

Ad2 early genes apparently are transcribed,

and the mRNA is processed, by host cell enzymes and mechanisms. Therefore, studies on Ad2 early RNA contribute to the understanding of both virus and cell gene expression. In this communication we describe hybridization kinetic experiments, annealing early WC-RNA, nuclear RNA (nRNA), and polyribosomal RNA (pRNA) to separated Ad2 ^{32}P-labeled *l* and *r* strands. We have identified and determined relative concentrations of viral RNA abundance classes in each RNA fraction and have estimated the fraction of each DNA strand that is complementary to each RNA abundance class.

MATERIALS AND METHODS

Cells, virus, infection, and extraction of RNA. Wold et al. (23) described in detail the procedures used for infecting KB cell suspensions with Ad2 and extracting Ad2 WC-RNA, nRNA, and pRNA. In some experiments cycloheximide (CH) or arabinosyl cytosine (ara-C) were added at 25 and 20 μg/ml, respectively, at 1 h postinfection. Infected cells (multiplicity of infection of 100 PFU per cell) were harvested at 5 or 6.5 h postinfection when CH or ara-C was used.

Separation of unlabeled Ad2 DNA strands and preparation of ^{32}P-labeled strands. Intact Ad2 DNA was purified, and DNA strands were separated, according to Landgraf-Leurs and Green (12), with minor modifications. Ad2 DNA was denatured in alkali, neutralized, annealed with poly(U,G) (Schwarz/Mann, lot no. 6901; U/G ratio of 1.0:0.3), and centrifuged to equilibrium in CsCl gradients. This resulted in clearly separated *l* and *r* DNA strands, as well as a broad band of unidentified material at low CsCl densities. Fractions comprising *l* and *r* strands were further purified by pooling and rebanding in CsCl. The strand-poly(U,G) complexes had buoyant densities of 1.7915 and 1.7784 g/cm^3 for *l* and *r* strands, respectively, in close agreement with the results of Landgraf-Leurs and Green (12) and Tibbetts et al. (20). Poly(U,G) was eliminated from the pooled rebanded strands by rate zonal centrifugation in alkaline sucrose gradients. Both purified strands sedimented at 34S, as did strands of denatured Ad2 DNA, indicating that the purified strands were intact. DNA strands were dialyzed against 10 mM Tris-hydrochloride (pH 8.1)–1 mM EDTA and stored at 4°C. Cross-contamination of purified unlabeled strands was checked by hybridization to separated Ad2 ^{3}H-labeled DNA strands prepared in a similar manner. Less than 5% hybridization was observed after self-annealing in the presence of an excess of the same unlabeled strand.

^{32}P-labeled strands were prepared by exhaustive annealing of ^{32}P-labeled DNA to unlabeled separated *l* and *r* strands and purifying the ^{32}P-labeled *l* or *r* strand by hydroxylapatite chromatography (19, 23). Purified ^{32}P-labeled *l* or *r* strands (i) formed 2% hybrid when self-annealed or when incubated to 200 or more times the $C_o t_{1/2}$ in the presence of the same unlabeled strand and (ii) annealed to greater than 90 to 95% to Ad2 DNA. Therefore, the ^{32}P-labeled strand preparations were not contaminated by the complementary strand, and, since they were derived from intact unlabeled strands (sedimented at 34S), all portions of each strand were represented. The ^{32}P-labeled DNA strands used in hybridization reactions were 200 to 400 nucleotides long and had specific activities of from 3×10^6 to 1.5×10^7 cpm/μg.

Hybridization procedures. Hybridizations were carried out in sealed siliconized capillary tubes at 68°C in 0.72 M NaCl–10 mM piperazine-*N*-*N*′-bis(2-ethanesulfonic acid) (pH 6.7)–1 mM EDTA–0.05% sodium dodecyl sulfate (23). Samples were taken at various $R_o t$ (initial concentration, in moles of nucleotides·seconds per liter) values and stored at 4°C for later assay using the S-1 nuclease procedure (23). In all experiments in this report, 14 kinetic points were taken in duplicate, excepting those in Fig. 3A and C, where 20 single points were taken. The absence of viral DNA from all RNA preparations was confirmed by alkaline hydrolysis (0.2 N KOH, 100°C, 15 min) of the RNA, followed by hybridization to labeled viral DNA strands under standard conditions; in no cases was hybridization observed.

Analysis of RNA-DNA hybridization kinetics. Vast excesses of RNA were annealed in liquid to [^{32}P]DNA strands, and the kinetic data were analyzed by the method of Frenkel and Roizman (9) to estimate the number of viral RNA abundance classes, their relative concentrations, and the fractions of DNA strands from which they originated. Under RNA excess, the overall annealing reaction is described by the equation: $D_t/D_o = \alpha_1 e^{-kR_1 t} + \cdots + \alpha_n e^{-kR_n t} + 1 - (\alpha_1 + \cdots + \alpha_n)$. D_t/D_o is the fraction of DNA remaining single stranded after time t, k is the hybridization rate constant, $R_1 \cdots R_n$ are the concentrations in moles per liter of $1 \cdots n$ abundance classes of RNA, and $\alpha_1 \cdots \alpha_n$ are the fractions of DNA strand from which each abundance class was derived. From the data D_t/D_o and $R_o t$, the values of R and α were estimated by computer-aided determination of the best fit of a nonlinear least-squares regression of D_t/D_o versus $R_o t$ for $n = 1, 2, 3$ abundance classes of RNA. Briefly, the computer is programmed to select the values of α and R for each value of n that, when substituted into the above equation, are most consistent with the hybridization data (D_t/D_o as a function of $R_o t$). The best values of α and R are those that minimize the difference (the error mean square) between the equation and the measured data points. The computer determines these best values by iteration: i.e., rough estimates of α and R are repeatedly improved until no further reduction in the error mean square is obtained. The uncertainty associated with the computer estimates of α and R and the curve fit is indicated by a standard deviation value (more confidence can be placed in low standard deviation values). The standard deviation values reflect the degree of uncertainty between the experimental data and the exact shape of the fitted function.

Absolute values of R_n were not used in the curve-fitting experiments. Instead, values (termed R_N in

our data tables) were obtained that reflect relative concentrations of viral RNA abundance classes and are proportional to the products of the rate constants and RNA concentrations in moles per liter. Since hybridizations were under identical conditions, comparisons of R_N provide estimates of the relative concentrations of viral RNA species.

RESULTS

Hybridization kinetics of whole-cell RNA. WC-RNA was extracted from cells and infected by Ad2 in the absence of drugs or in the presence of CH (CH-WC-RNA) or ara-C (ara-C-WC-RNA). Typical annealing kinetics of WC-RNA, CH-WC-RNA, and ara-C-WC-RNA are illustrated in Fig. 1, including the curve fits for one and two abundance classes of viral RNA. Table 1 presents an abundance analysis (i.e., the best estimates of α and R_N for one or two abundance classes of RNA) of the data in Fig. 1. The following conclusions are possible from Table 1 (and from data of two additional independent experiments not shown here). (i) Early WC-RNA contains at least two abundance classes of viral RNA (the error mean square for $n = 2$ was much less than that for $n = 1$). Depending upon the experiment, the abundant class was 57 to 208 times more concentrated than the scarce. (ii) WC-RNA (no drugs) annealed to 57 to 62 and 32 to 38% of *l* and *r* strands. An abundant RNA class was derived from 15 to 17 and 14 to 16% of *l* and *r* strands, and a scarce class was derived from 42 to 46 and 16 to 17% of *l* and *r* strands. (iii) CH-WC-RNA annealed to 62 to 68 and 43 to 48% of *l* and *r* strands. An abundant class originated from 15 to 19 and 16 to 17%, and a scarce class originated from 47 to 49 and 26 to 33%, of *l* and *r* strands. (iv) Ara-C-WC-RNA annealed to 50 and 32% of *l* and *r* strands. An abundant class arose from 10 and 14%, and a scarce class arose from 40 and 18%, of *l* and *r* strands.

CH-WC-RNA annealed to more of both DNA strands than did WC-RNA or ara-C-WC-RNA, and at a faster rate; this was also the case with nRNA and pRNA (see below). This is probably because CH increases the yield of early viral RNA (14).

Hybridization kinetics of nuclear RNA. The annealing kinetics of RNA (no drugs), CH-nRNA, and ara-C-nRNA to ^{32}P-labeled *l* and *r* strands are illustrated in Fig. 2. Table 2 summarizes the results of an abundance analysis of the kinetic data in Fig. 2; only the results of the $n = 2$ RNA abundance classes are shown because the curve fit was much better than for $n = 1$ (the error mean square for $n = 1$ was 4 to 25 times higher than that for $n = 2$). Therefore, as with WC-RNA, nRNA annealed as at least two distinct kinetic classes; the abundant class was

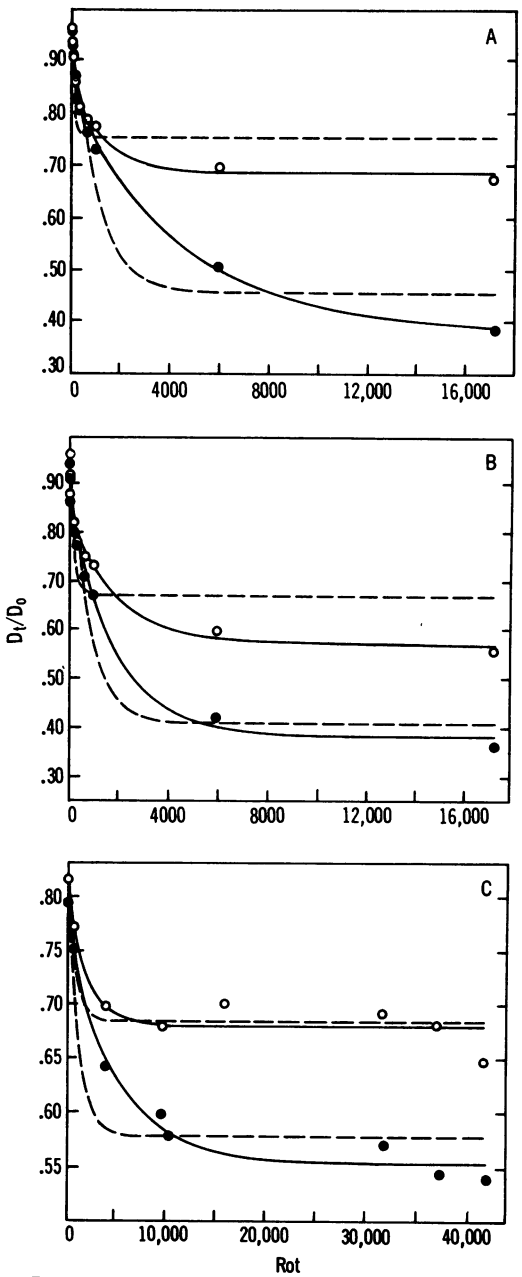

FIG. 1. *Hybridization kinetics of early WC-RNA to Ad2 l and r [^{32}P]DNA strands. WC-RNA was extracted from cells infected by Ad2 in absence of drugs (A) or in the presence of CH (B) or ara-C (C) and annealed to purified Ad2 l (●) or r (○) [^{32}P]DNA strands, with RNA in vast excess. The graphs illustrate the computer fits to hybridization kinetic data for one (----) or two (——) abundance classes of viral RNA. D_0 is the single-stranded DNA at zero time, and D_t is the single-stranded DNA remaining after time t. R_0t (product of initial RNA concentration and hybridization time) was calculated as moles of nucleotides · seconds per liter.*

TABLE 1. *Abundance analysis of hybridization kinetic data of WC-RNA from Fig. 1*[a]

RNA prepn + DNA strand	Test for abundance classes (n)	α(%)[b]		α SD[c]	R_N[d] ($\times 10^{-4}$)	R SD ($\times 10^{-4}$)	R_1/R_2[e]	Error mean square ($\times 10^{-5}$)	Fit SD ($\times 10^{-3}$)
WC-RNA + l strand	1	54.8		4.0	10	2.2		320	57
	2 (R_1)	17.3	61.8	1.5	130	26	57	25	16
	(R_2)	44.5		2.0	2.3	0.37			
WC-RNA + r strand	1	24.6		2.2	160	66		240	49
	2 (R_1)	14.1	31.5	1.6	690	200	80	29	17
	(R_2)	17.4		1.9	8.0	2.5			
CH-WC-RNA + l strand	1	59.4		4.9	13	3.1		490	70
	2 (R_1)	15.1	62.3	1.2	460	110	167	23	15
	(R_2)	47.2		1.6	5.3	0.66			
CH-WC-RNA + r strand	1	33.1		3.4	76	29		480	69
	2 (R_1)	16.9	43.1	1.2	630	130	117	26	16
	(R_2)	26.2		1.6	5.4	1.2			
Ara-C-WC-RNA + l strand	1	42.7		5.3	3.2	1.2		32	5.7
	2 (R_1)	9.9	49.8	1.0	110	34	73	7.2	8.5
	(R_2)	39.9		1.8	1.5	0.2			
Ara-C-WC-RNA + r strand	1	25.0		2.0	29	14		170	–[f]
	2 (R_1)	14.2	31.8	0.5	110	190	58	1.9	–
	(R_2)	17.6		0.8	1.6	3.3			

[a] The equation relating ^{32}P-labeled DNA-RNA hybrid formation as a function of RNA concentration and annealing time for $n = 1$ and 2 abundance classes of viral RNA is detailed in the text. The best fit (least error mean square) of the hybridization kinetic data of WC-RNA from Fig. 1 was obtained by a computer nonlinear regression analysis, as were the values of $R_1 \cdots R_N$ and $\alpha_1 \cdots \alpha_n$.

[b] Percentage of DNA strand complementary to each RNA abundance class. Braces indicate totals.

[c] SD, Standard deviation of the mean.

[d] Arbitrary number proportional to the product of the hybridization rate constant and RNA concentration in moles per liter.

[e] Molar ratio of the abundant (R_1) and scarce (R_2) RNA classes quantitated from the $n = 2$ fit.

[f] –, Not determined.

13 to 27 times more concentrated than the scarce class. The nRNA annealing kinetics were similar to those of WC-RNA in terms of total hybridization and the fraction of each DNA strand complementary to each abundance class. However, the abundant and scarce classes in nRNA were less different in concentration than were those in WC-RNA.

Hybridization kinetics of pRNA. pRNA was studied to estimate viral mRNA abundance classes and the fractions of each strand expressed as different abundance classes of mRNA. The pRNA was extracted from cells infected in the absence of drugs or in the presence of CH (CH-pRNA) or ara-C (ara-C-pRNA). The pRNA was extracted either from polyribosomes pelleted through a 2.0 and 1.3 M double-sucrose cushion (pelleted pRNA) or after mRNA was released by EDTA (EDTA-released pRNA) from pelleted polyribosomes.

Hybridization kinetics of pRNA are illustrated in Fig. 3. The outstanding feature of the pRNA annealing data is that at saturation much less DNA hybridized, compared with WC-RNA and nRNA. This is particularly obvious in the case of l strand: WC-RNA and nRNA annealed to as much as 70% of l strand, but pRNA generally annealed to about 14% (although up to 25 to 30% could be discerned in CH-pRNA preparations that probably were contaminated by nRNA). Table 3 summarizes an abundance analysis of the kinetic data in Fig. 3. Only the best curve fits for kinetic data in Fig. 3A, C, and D are presented. The best fits were for $n = 3$ abundance classes of viral RNA, and the worst fits were for $n = 1$ abundance class of RNA. Therefore, pRNA may contain as many as three abundance classes of RNA (termed abundant, scarce, and very scarce) complementary to each DNA strand.

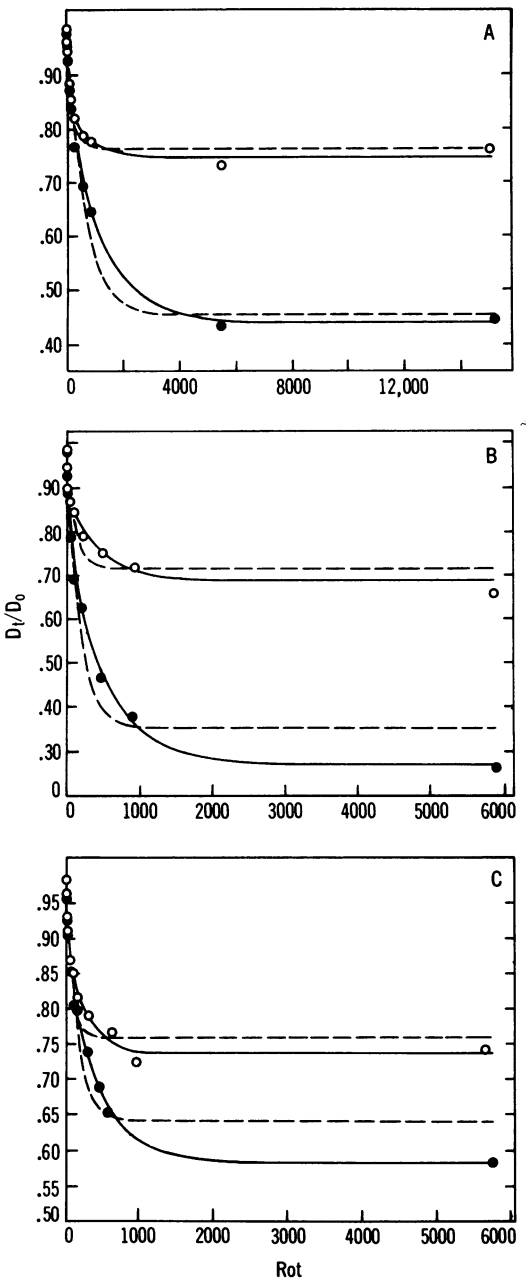

FIG. 2. *Hybridization kinetics of early nRNA to l and r [^{32}P]DNA strands. RNA was extracted from nuclei of cells infected by Ad2 in the absence of drugs (A) or in the presence of CH (B) or ara-C (C) and annealed to l (●) and r (○) [^{32}P]DNA strands. Computer fits for one (- - - -) or two (——) abundance classes of Ad2 RNA are shown.*

Demonstration of complementary RNA transcripts. The annealing of WC-RNA, nRNA, and pRNA to DNA strands shows clearly that WC-RNA and nRNA contain viral RNA sequences not detectable in pRNA. This indicates that portions of Ad2 DNA are transcribed into RNA molecules that are not exported to polyribosomes. To test whether both strands of the same DNA section are transcribed, WC-RNA and nRNA were self-annealed, and RNA-RNA hybrids were isolated. These (after denaturation) annealed to as much as 70% of both *l* and *r* strands (Table 4). In some experiments highly radioactive DNA prepared by the "nick translation" procedure (unpublished results) was used in place of purified strands. Again, a maximum of 70% hybridization was observed (data not shown). RNA hybrids hydrolyzed in alkali (23) did not anneal to DNA probes, proving that the hybridization was not due to contaminating viral DNA.

DISCUSSION

We have analyzed Ad2 early WC-RNA, nRNA, and pRNA (prepared with and without CH or ara-C), using hybridization kinetics with purified ^{32}P-labeled Ad2 DNA strands, to estimate (i) the strand fraction represented in each RNA class and (ii) the number of viral RNA abundance classes and their relative concentrations. As mentioned above, the R_0t kinetics format requires that viral RNA be in vast excess over DNA, so that hybridization follows (pseudo-)first-order kinetics (22). Our experiments were usually conducted at two RNA concentrations: 10 mg/ml (high R_0t) and 0.1 to 1.0 mg/ml (low R_0t). Viral DNA strands were 5 to 20 ng/ml, so that RNA (mainly host RNA) was in excess over DNA by a factor of 5×10^3 to 2×10^6. Most important, regardless of the RNA concentration, similar hybridization values were obtained at specific R_0t values (low RNA and long time or high RNA and short time). This proves that viral RNA was in excess and, therefore, dictated the reaction rate. One factor that could influence the RNA-DNA hybridization rate is the size of the RNA molecules (22). But since these would have a relatively minor effect compared with RNA concentration it is doubtful that RNA size is an important parameter in our experiments (computer estimates suggested that abundant and scarce RNA classes differed in concentration by more than an order of magnitude). Since RNA molecules were probably longer than the DNA probe, the formation of RNA-DNA "particles" that could appreciably influence the kinetics of hybridization is unlikely (17).

Abundance analyses (summarized in Table 5) of the hybridization kinetics suggested that all preparations of WC-RNA, nRNA, and pRNA contained more than one abundance class of viral RNA derived from each DNA strand. In

TABLE 2. *Abundance analysis of hybridization kinetic data of nRNA from Fig. 2: results of curve fit for n = 2 RNA abundance classes*[a]

RNA prepn + DNA strand	Test for $n = 2$ abundance classes	α(%)		α SD	R_N ($\times 10^{-4}$)	R SD ($\times 10^{-4}$)	R_1/R_2	Error mean square ($\times 10^{-5}$)	Fit SD ($\times 10^{-3}$)
nRNA + *l* strand	R_1	16.0	56.4	3.0	100	25	13	8	9
	R_2	40.4		2.9	7.8	1.2			
nRNA + *r* strand	R_1	9.3	25.2	1.7	330	110	14	9	10
	R_2	15.9		1.7	24	5.0			
CH-nRNA + *l* strand	R_1	22.2	73.3	1.9	380	62	21	15	12
	R_2	51.1		2.0	18	1.6			
CH-nRNA + *r* strand	R_1	12.3	31.1	2.4	600	250	26	36	19
	R_2	18.8		2.5	23	7.4			
Ara-C-nRNA + *l* strand	R_1	12.5	41.8	1.0	520	88	23	6	8
	R_2	29.3		1.2	23	2.0			
Ara-C-nRNA + *r* strand	R_1	10.0	26.3	1.6	980	310	27	13	
	R_2	16.2		1.5	37	9.4			

[a] See footnotes to Table 1 for details.

TABLE 3. *Abundance analysis of hybridization kinetic data of pRNA from Fig. 3A, C, and D: results of best curve fits*[a]

RNA prepn + DNA strand	Test for abundance classes (n)	α(%)		α SD	R_N ($\times 10^{-4}$)	R SD ($\times 10^{-4}$)	R_1/R_2[b]	R_1/R_2,[c] R_1/R_3, R_2/R_3	Error mean square ($\times 10^{-5}$)	Fit SD ($\times 10^{-3}$)
Pelleted pRNA + *l* strand	2 (R_1)	8.8	13.8	0.7	74	15	30		6	8
	(R_2)	5.0		0.8	2.5	1.2				
Pelleted pRNA + *r* strand	3 (R_1)	8.1		1.6	190	55		11, 238, 21	5	7
	(R_2)	7.9	28.7	1.5	17	7.8				
	(R_3)	12.7		5.1	0.8	0.8				
Pelleted CH-pRNA + *l* strand	3 (R_1)	6.1		0.8	1,700	600		57, 548, 10	7	9
	(R_2)	5.4	19.3	2.2	30	20				
	(R_3)	7.8		2.1	3.1	1.7				
Pelleted CH-pRNA + *r* strand	3 (R_1)	5.6		1.5	4,100	3,000		56, 1,050, 19	39	20
	(R_2)	18.7	39.1	1.7	73	73				
	(R_3)	24.8		1.6	3.9	0.8				
EDTA-released CH-pRNA + *l* strand	3 (R_1)	5.9		1.4	650	240		13, 361, 27	5	7
	(R_2)	4.9	24.6	1.3	49	39				
	(R_3)	13.8		1.2	1.8	0.5				
EDTA-released CH-pRNA + *r* strand	3 (R_1)	7.0		1.7	3,000	2,200		67, 15,000, 225	66	26
	(R_2)	13.2	38.6	2.6	45	25				
	(R_3)	18.4		13.9	0.2	1.1				

[a] See footnotes to Table 1 for details.
[b] Molar ratio of the abundant (R_1) and scarce (R_2) classes from the $n = 2$ fit.
[c] Molar ratios of the abundant (R_1) and scarce (R_2) classes, abundant and very scarce (R_3) classes, and scarce (R_2) and very scarce (R_3) classes for the $n = 3$ fit.

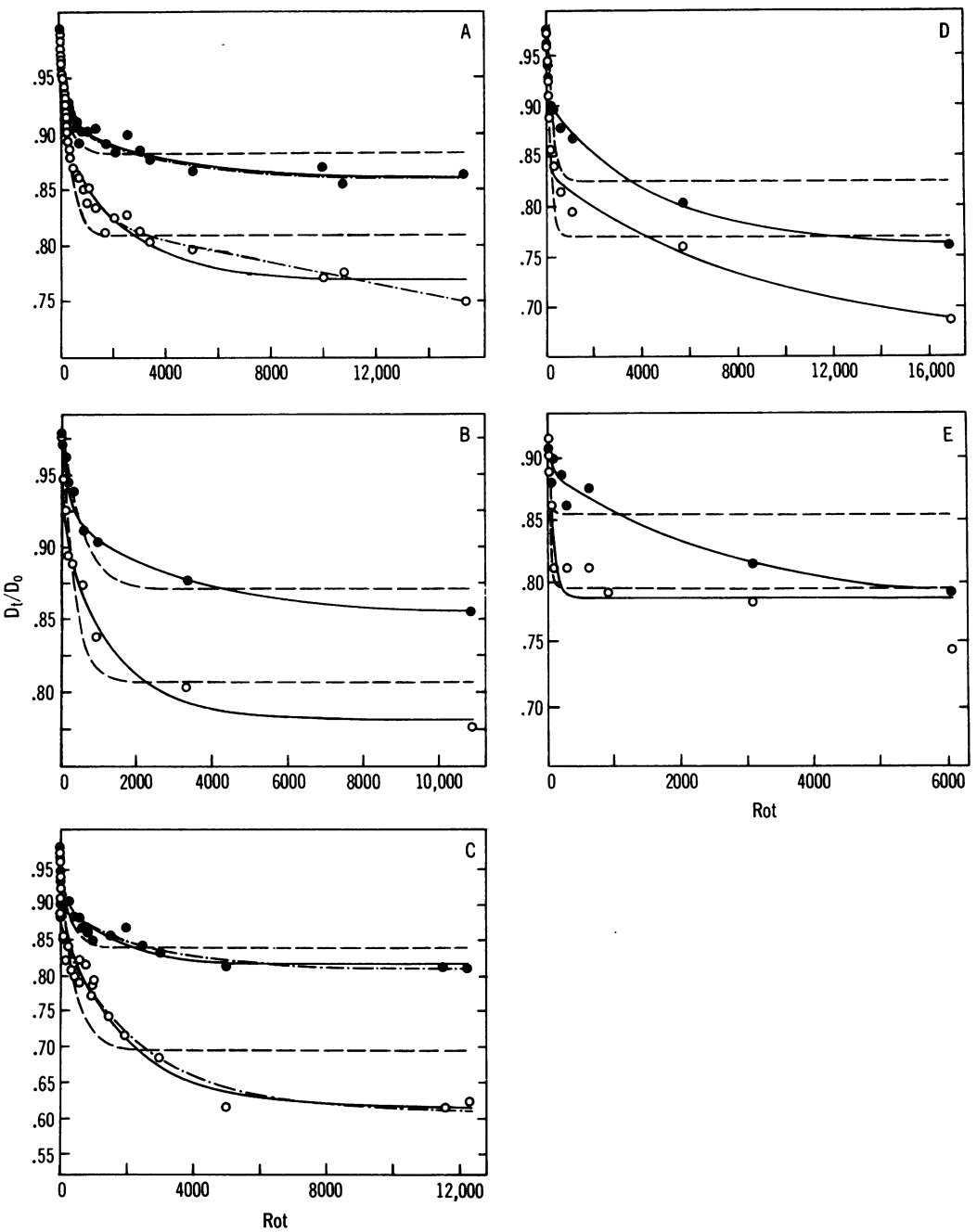

FIG. 3. *Hybridization kinetics of early pRNA to l and r [^{32}P]DNA strands. Cells were infected by Ad2 in the absence of drugs or in the presence of CH or ara-C. Polyribosomes were sedimented through a 1.3 and 2.0 M double-sucrose cushion (pelleted pRNA). On occasion viral mRNA was released by 25 mM EDTA from pelleted polyribosomes and further purified by rate zonal sucrose gradient centrifugation (EDTA-released pRNA). The pRNA was phenol extracted and annealed in vast excess to l (●) and r (○) [^{32}P]DNA strands. (A) Pelleted pRNA; (B) EDTA-released pRNA; (C) pelleted CH-pRNA; (D) EDTA-released CH-pRNA; (E) pelleted ara-C pRNA. Computer fits are shown for one (- - - -), two (——), or three (-·-·-·-·) abundance classes of viral RNA.*

the case of pRNA, the kinetic data were most in accord with three abundance classes (abundant, scarce, and very scarce) of RNA complementary to each DNA strand. On the average, the abundant and scarce class were respectively complementary to 6 and 5% of *l* strand and 7 and 10% of *r* strand (Table 5). The very scarce class was measured, with less confidence, to be complementary to about 11% of *l* strand and 18% of *r* strand. There is no certain way to interpret the biological significance of these abundance classes. However, the abundant and scarce classes together are complementary to about 11 and 17% of *l* and *r* strands, respectively, very similar to the estimates by Flint et al. (7) (9% of *l* and 14% of *r* strands) and by Pettersson et al. (15) (13% of *l* and 19% of *r* strands) of the fraction of each DNA strand expressed as early mRNA. This may indicate that the two abundant and two scarce classes comprise viral mRNA; the very scarce classes may then be nRNA that contaminated the pRNA either in the cell or during extraction of pRNA. If the abundant and scarce classes are, in fact, mRNA, it is remarkable that the abundant class is an order of magnitude more concentrated than the scarce class; such large concentration differences could reflect different rates of transcription and/or processing of viral mRNA and/or different turnover rates of RNA species. Using a different approach to quantitate Ad2 early mRNA abundance, Flint and Sharp (8) concluded that mRNA from three of the four early gene blocks was similar in concentration (about 1,000 molecules per cell), whereas mRNA from *l* strand of *Eco*RI-B fragment was two- to threefold lower in concentration. The correspondence between our data and those of Flint and Sharp (8) is not known.

In contrast to pRNA, WC-RNA annealed to 60 and 39% of *l* and *r* strands, and nRNA annealed to 57 and 28% of *l* and *r* strands, respectively. Thus, many regions of the genome are transcribed into RNA molecules that remain restricted to the nucleus and are not exported to polyribosomes as mRNA. RNA-RNA hybrids complementary to 70% of each strand were present in self-annealed nRNA, indicating that for 70% (possibly 100%) of the genome, both strands of DNA are transcribed. Other workers have also shown that self-annealed early WC-RNA (16) and nRNA (24) contain RNA-RNA hybrids and that early nRNA contains sequences not in cytoplasmic RNA (5, 6, 18, 21). It is not known whether "non-mRNA" and complementary RNA transcripts play a role in the generation of Ad2 early mRNA or whether they arise from, e.g., errors in transcription. Nevertheless, it seems that controls operate to selectively transport mRNA from the nucleus to the polyribosomes and to restrict non-mRNA to the nucleus. A similar conclusion was reached from analysis of viral mRNA synthesized by the Ad12-transformed cell HE Cl9, where about 80% of the asymmetric genome is represented in nRNA, but only 22% is represented in cytoplasmic RNA (11). Since most or all of *r* strand is represented in early nRNA, late mRNA is

TABLE 4. *Demonstration of complementary RNA transcripts in early WC-RNA and nRNA preparations*[a]

RNA prepn	*l* strand (%)	*r* strand (%)
Early CH-WC-RNA	45	44
Early nRNA	54	53
Early CH-nRNA	68	67

[a] RNA preparations were self-annealed to R_0t values ranging from 3,000 to 10,000 (87 h), adjusted to 0.3 M NaCl, and digested with RNases A and T_1. RNA-RNA hybrids were purified in the presence of carrier *Escherichia coli* RNA by phenol extraction and exclusion chromatography on Sephadex G-50 or G-100 (23). Samples were denatured, sealed into siliconized capillaries, and annealed for 88 h in minimal volumes at 68°C to purified ^{32}P-labeled *l* and *r* DNA strands, and hybridization was assayed using S-1 nuclease. Samples assayed after 220 h of annealing hybridized to the same extent.

TABLE 5. *Summary of RNA abundance analyses*

RNA prepn[a]	Abundance class	*l* strand (%)[b]			*r* strand (%)[b]			R_1/R_2	Genome represented
		Abundant	Scarce	Total	Abundant	Scarce	Total		
WC-RNA	2	15 (10–17)	45 (40–47)	60[c]	15 (14–17)	24 (17–26)	39[c]	106 (57–208)	99[c]
nRNA	2	17 (13–22)	40 (29–51)	57[c]	11 (9–12)	17 (16–29)	28[c]	21 (13–27)	85[c]
pRNA	3	6 (6)	5 (5)	11[d]	7 (6–18)	10 (8–13)	17[d]	41 (11–67)	27[d]

[a] Includes all RNA preparations tested (no drugs, CH, ara-C).

[b] Estimates of α values (in %) based on mean values of all experiments. The ranges of estimates are in parentheses.

[c] Since complementary RNA transcripts derived from at least 60 to 70% of each strand exist in WC-RNA and nRNA these are minimal values that reflect the relative concentrations of the complementary transcripts from each DNA strand.

[d] Assuming the abundant and scarce classes are mRNA, and the very scarce class (see text and Table 3) to be nRNA contaminants.

apparently transcribed early, suggesting that the cellular transport machinery can distinguish early mRNA from late mRNA.

Abundance analyses of nRNA suggested abundant and scarce classes, respectively, from 17 and 40% of *l* strand and 11 and 17% of *r* strand (Tables 2 and 5). The biological significance of these classes is not known. The abundant classes may comprise molecules destined to become mRNA (i.e., the abundant classes are complementary to roughly the same fraction of the genome as mRNA), although they could represent, e.g., rapid transcripts and/or stable non-mRNA molecules.

The total hybridization of WC-RNA and nRNA to both DNA strands corresponds to about 100% (99 and 85%, respectively) of the asymmetric viral genome. However, since transcripts from 70% of each strand (isolated as RNA-RNA hybrids) exist, clearly, we were unable to detect all RNA sequences present. Apparently, if complementary RNA sequences are present in a hybridization reaction mixture, with one of the RNA complements in significantly higher concentration than the other, the abundant RNA complement anneals to the scarce complement and deletes it from the reaction mixture so that it cannot anneal to the ^{32}P-labeled probe. RNA-DNA hybridization, therefore, measures the relative abundance of *l*- and *r*-strand-specific RNA sequences from a particular section of DNA. We conclude that for about 60% of the genome, *l*-strand-specified RNA is in greater concentration than *r*-strand RNA from the same portion of the genome and that *r*-strand RNA predominates for the remaining 40%. This may represent preferential strand transcription or else preferential degradation of RNA from relevant sections of the genome. Self-annealing the RNA before addition of the probe had no effect on the kinetics or total DNA hybridized (data not shown).

In some experiments cells were infected with CH or ara-C. The use of either drug ensures that viral DNA replication did not occur and, therefore, that all cells were in the early stage of infection. Ara-C did not appreciably affect the patterns of viral RNA synthesized. CH increased the concentration of all abundance classes of viral RNA, but did not appear to affect the fraction of each DNA strand complementary to the abundant and scarce classes. Parsons and Green (14) reported that CH enhances the yield of early viral [^{3}H]RNA fivefold. Recently, Craig and Raskas (4), based upon hybridization-competition experiments, concluded that CH did not affect the absolute concentration of early Ad2 mRNA and suggested that CH increases the yield of viral [^{3}H]RNA by reducing the synthesis of host [^{3}H]RNA. Our kinetic data indicate that CH increases the absolute concentration of Ad2 mRNA in pRNA: e.g., CH-pRNA annealed to 17% of *r* strand by about R_0t = 200 (Fig. 3C), whereas pRNA (no drugs) did not reach 17% until about R_0t = 1,500 (Fig. 3A). The computer R_N estimates from the n = 3 fits of pRNA to *r* strand (Table 3) indicate that viral mRNA is much more concentrated in CH-pRNA than in pRNA. Since over 90% of pRNA is rRNA, it is unlikely that these differences can be accounted for solely by inhibition of host mRNA synthesis.

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