

Synthesis of Virus-Specific Ribonucleic Acid in KB Cells Infected with Type 2 Adenovirus

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By using the technique of deoxyribonucleic acid (DNA)-ribonucleic acid (RNA) hybridization, virus-specific RNA (cRNA) was detected 6 hr after infection in preparations of total RNA from cells infected with type 2 adenovirus in the presence of 2 μ M 5-fluorodeoxyuridine. In the absence of 5-fluorodeoxyuridine, there was a continuous increase in the incorporation of 3 H-uridine into viral cRNA until 20 hr after infection, at which time approximately 40% of the 3 H-uridine entering RNA was found in virus-specific RNA. When RNA was prepared from polyribosome fractions obtained from cytoplasmic extracts of infected cells, virus-directed transcription was detected at 3 hr after infection (i.e., 3 to 4 hr before the initiation of viral DNA synthesis). Viral cRNA species synthesized at different times after infection were compared by the technique of DNA-RNA hybridization-inhibition ("presaturation" hybridization-competition). Three hybridization-inhibition techniques were compared. The techniques differed in the manner in which the DNA-RNA complex was isolated after the first hybridization reaction. Depending on the procedure employed, various degrees of inhibition were measured. The variation could be essentially eliminated if prior to hybridization the inhibitory RNA species were alkali-degraded to a uniform size of about 4S. Undegraded RNA could be used if the DNA-RNA complex was isolated by using a procedure involving rigorous washing (preferably including ribonuclease treatment) before the second hybridization with labeled RNA. When a rigorous hybridization-inhibition procedure was used, three classes of virus-specific RNA species could be distinguished: (i) early RNA class I whose synthesis began prior to viral DNA replication and stopped at some time after the initiation of viral DNA replication—it comprised about 70% of the early RNA species and was apparently degraded by 18 hr after infection; (ii) early RNA class II whose synthesis began prior to viral DNA replication and apparently continued at an enhanced rate late in infection; and (iii) late RNA whose synthesis began after the initiation of viral DNA synthesis.

Introduction of the techniques of deoxyribonucleic acid (DNA)-ribonucleic acid (RNA) hybridization (31) and hybridization-competition (29) permitted detailed studies of the transcription of viral genomes, including those of adenoviruses (2, 26, 27, 50, 51). A reinvestigation of the regulation of synthesis of type 2 adenovirus-specific RNA species (cRNA) presented the possibility that the particular hybridization-competition technique employed greatly influences the results obtained. Although, theoretically, the methods of simultaneous and sequential hybridization-competition should yield the same results (12, 38), the presaturation hybridization-inhibition technique has been claimed to reveal greater specificity in comparing RNA species from different mammalian cell types (3, 14, 48).

Hence, to study transcription of virus-specific RNA species in productive and abortive adenovirus infections, it was necessary to make a critical evaluation of the hybridization-inhibition techniques which are commonly used (22, 48, 54). Data presented here indicate that results are dependent upon the experimental method employed and that a rigorous technique is required.

The evidence presented in this communication indicates that, in type 2 adenovirus infection, viral cRNA synthesis begins by 3 hr after infection and that at 20 hr approximately 40% of the 3 H-uridine incorporated into RNA is found in virus-specific RNA. When a rigorous hybridization-inhibition technique was used, it was found that a significant portion of early virus-specific RNA species is not transcribed when late cRNA

species are synthesized. Those early cRNA species which are also made after the initiation of viral DNA replication appear to be synthesized at an enhanced rate late in infection.

MATERIALS AND METHODS

Virus and tissue culture. The prototype strain of adenovirus type 2, which had been plaque purified three times, was used in all experiments. KB cells were propagated in Eagle's minimal essential medium (MEM) supplemented with 10% calf serum (15, 16), and at the time of infection the cells were changed to MEM containing 5% serum. Cells in suspension cultures were infected at an input multiplicity of 100 to 300 plaque-forming units (PFU) per cell. Virus was assayed on monolayers of KB cells by the plaque assay (36) or fluorescent-focus assay (18). Purification of adenovirus was accomplished by a technique reported earlier (36).

Extraction and denaturation of viral DNA. Viral DNA was extracted as described previously (2) except that the mercaptoethanol step was omitted. The purified DNA was dialyzed against $0.1\times$ SSC ($1\times$ SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) and denatured by raising the pH to 12.8 with 1 N NaOH. After 10 min at room temperature, the pH was rapidly adjusted to 7.0 with 1 N HCl.

Extraction of RNA from KB cells. RNA was extracted from cells by the method of Scherrer and Darnell (47) as modified by Homma and Graham (33). After ethanol precipitation, the RNA was extensively dialyzed against $2\times$ SSC, frozen at -20 C and used within 2 days. Tritium-labeled RNA was prepared by incorporation of uridine- $5\text{-}^3\text{H}$ (specific activity greater than 20 Ci/mM, New England Nuclear Corp.). The term "late" RNA refers to virus-specific RNA synthesized after the beginning of viral DNA replication; it was prepared from cells harvested at 18 to 19 hr postinfection. "Early" RNA refers to virus-specific RNA synthesized before the initiation of viral DNA synthesis; it was extracted from cells harvested 6 to 7 hr after infection (unless noted otherwise). To ensure that only early RNA was made, $2\ \mu\text{M}$ 5-fluorodeoxyuridine (FUdR; Hoffmann-LaRoche, Inc., Nutley, N.J.) was often added to the culture at the time of infection (2, 17) although this step was not essential.

Alkaline degradation of RNA. Controlled partial hydrolysis of RNA (6) was performed by adjusting the preparation to 1 N NaOH. After 90 sec at 23 to 27 C, the solution was rapidly adjusted to pH 7.0, cooled in an ice bath, and dialyzed against 6 liters of $2\times$ SSC.

Sedimentation analysis of RNA. For sucrose density gradient centrifugation, RNA (dissolved in 0.01 M sodium acetate, pH 5.1) was layered on a 5 to 20% (w/v) linear sucrose gradient containing 0.01 M sodium acetate, 0.05 M NaCl, and $100\ \mu\text{M}$ MgCl_2 at pH 5.1 (47). The gradients were centrifuged at $63,581\times g$ for 6 hr in the SW 25 rotor of a Spinco model L preparative centrifuge.

Isolation of polyribosomes and extraction of RNA. Polyribosomes from uninfected and infected KB cells were analyzed and isolated as described previously (52, 53). For isolation of RNA, fractions of the

sucrose gradient were pooled and diluted with an equal volume of a solution containing 0.5% sodium dodecyl sulfate (SDS), 0.10 M NaCl, 0.01 M tris-(hydroxymethyl)aminomethane (Tris), pH 7.4 (21, 24). The RNA was precipitated with ethanol, extensively dialyzed against $2\times$ SSC, frozen at -20 C, and used within 2 days.

DNA-RNA hybridization. Purified viral DNA was immobilized on nitrocellulose membrane filters (Schleicher and Schuell, type B6, 25 mm), and hybridization was performed as described by Gillespie and Spiegelman (23). Hybridization was carried out at 65 C for 18 hr in 1 ml of $2\times$ SSC containing 0.1% SDS.

Three variations of the presaturation hybridization-inhibition technique were employed. (i) Method A (22). After the first hybridization reaction with increasing amounts of unlabeled, inhibiting RNA, filters were removed from their reaction vessels, rinsed twice in 400 ml of $2\times$ SSC, washed on each side with 50 ml of the buffer by suction filtration and treated with pancreatic ribonuclease (20 $\mu\text{g}/\text{ml}$ at room temperature for 1 hr; ribonuclease was previously heated at 90 C for 10 min to inactivate any contaminating deoxyribonuclease). After ribonuclease hydrolysis, filters were again rinsed twice in 400 ml of buffer and washed by filtration. No residual ribonuclease activity was detected. The ^3H -labeled RNA sample was then added for the second incubation. As determined from the thermal elution profile in $2\times$ SSC, the DNA-RNA hybrid formed in the first reaction is a stable complex which will survive the second incubation at 65 C. After the second hybridization reaction, the filters were again subjected to the same washing and ribonuclease procedure. Each filter was then dried, placed in 5 ml of a solution of 0.5 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene and 6.0 g of 2,5-diphenyloxazole per liter of toluene, and counted in a Packard Tri-Carb liquid scintillation spectrometer.

(ii) Method B (48). After the first hybridization reaction, filters were removed from their reaction vessels and washed twice in 20 ml of $2\times$ SSC at 65 C for 45 min. After the second incubation with labeled RNA, the extensive washing and ribonuclease treatment of method A were employed.

(iii) Method C (54). Subsequent to the first hybridization reaction, filters were removed from their reaction vessels and rinsed twice at room temperature in 2 ml of $2\times$ SSC. After the second incubation with labeled RNA, the extensive washing and ribonuclease treatment of method A were employed.

RESULTS

Hybridization of early and late virus-specific RNA species. Virus-specific RNA was found at 6 hr after infection in extracts of total RNA from cells infected in the presence of $2\ \mu\text{M}$ FUdR. The saturation of $2\ \mu\text{g}$ of adenovirus type 2 DNA with ^3H -early RNA is shown in Fig. 1A. To achieve saturation with ^3H -late RNA (i.e., 18 hr after infection) required addition of much less nucleic acid (Fig. 1B).

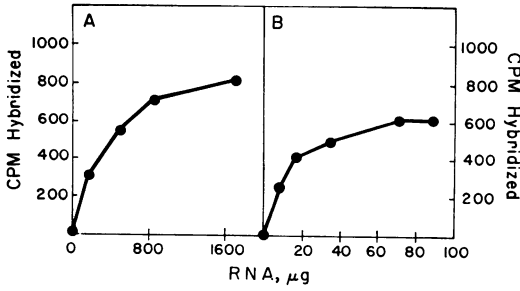


FIG. 1. Detection of early and late virus-specific RNA after infection with adenovirus type 2. Cells were infected at an input multiplicity of 100 fluorescent focus-forming units per cell. Various amounts of RNA (prepared from cells by phenol-SDS extraction) were incubated with filter-bound DNA for 18 hr at 65 C in 2X SSC containing 0.1% SDS. Radioactivity measured was that found in the ribonuclease-resistant hybrid. Hybridization of 2 µg of purified type 2 adenovirus DNA was performed with RNA labeled with 2 µCi of ³H-uridine per ml from 6.0 to 6.5 hr postinfection in the presence of 2 µM FUDR added at the time of infection (A) and with RNA labeled with 1 µCi/ml from 18.0 to 18.5 hr postinfection (B).

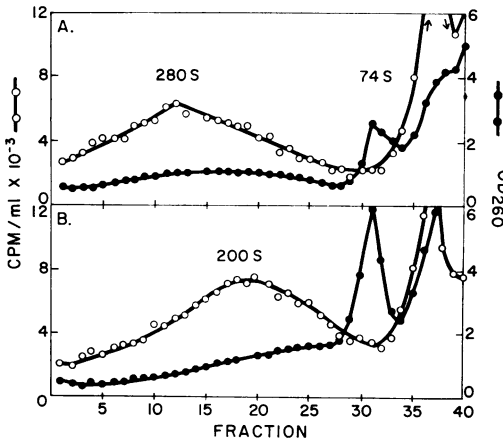


FIG. 2. Polyribosome profiles of uninfected (A) and type 2 adenovirus-infected cells (B). Cells infected with 100 fluorescent focus-forming units of type 2 adenovirus per cell were labeled with 2.0 µCi of ¹⁴C-amino acids per ml for 1 min at 18 hr postinfection. Uninfected cells were similarly labeled at the same time. Cytoplasmic extracts were layered on 15 to 30% (w/v) sucrose gradients and centrifuged for 90 min at 22,500 rev/min in a Spinco SW25 rotor at 4 C. Symbols: (○) counts per minute per milliliter × 10⁻³; (●) optical density at 260 nm.

To detect virus-specific RNA much earlier than 6 hr after infection, experiments were devised to obtain RNA enriched with respect to viral cRNA species. Cytoplasmic extracts of in-

fecting cells were layered on 15 to 30% (w/v) sucrose density gradients and centrifuged. The RNA obtained from fractions in the polyribosome region of the gradient was used in the annealing reaction. The sedimentation analyses of uninfected and infected cells labeled for 1 min with ¹⁴C-amino acids 18 hr after infection are shown in Fig. 2. By 18 hr after infection, there was a marked shift in the size of the polyribosomes from a maximum of 280S in uninfected cells to 200S in the infected cells (52). Assuming that the early and late cRNA species are of similar sizes, in early stages of infection, fractions 14 to 26 might be expected to be richest in species of virus-specific RNA. Therefore, cells were labeled with ³H-uridine from 3 to 3.5 hr after infection. The sucrose gradients were divided into three pools consisting of fractions 1 to 13, fractions 14 to 26, and fractions 27 to 40. The hybridization reactions were performed with the RNA obtained from each pool (Fig. 3). With fractions from what would become the height of the polyribosome peak late in infection, i.e., fractions 14 to 26 (Fig. 3B), 1.5% of the radioactivity incorporated into these polyribosomes from 3 to 3.5 hr after infection was

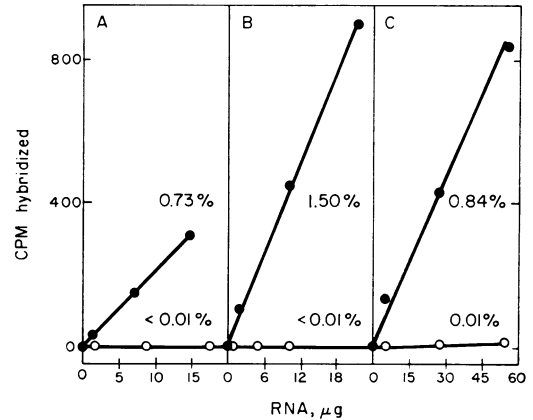


FIG. 3. Measurement of early virus-specific RNA from polyribosomes of cells infected with type 2 adenovirus. Cells infected with 100 fluorescent focus-forming units of virus per cell (●) and uninfected cells (○) were labeled from 3.0 to 3.5 hr postinfection with 4.0 µCi of ³H-uridine per ml. Cytoplasmic extracts were centrifuged as described in Fig. 2. Pooled fractions were treated with an equal volume of a solution containing 0.5% SDS, 0.10 M NaCl, 0.01 M Tris (pH 7.4). RNA was precipitated with ethanol, dialyzed against 2X SSC, and used in hybridization with viral DNA as described in Fig. 1. A 2-µg amount of type 2 adenovirus DNA was hybridized with increasing amounts of ³H-RNA from pool comprising fractions 1 to 13 from sucrose gradient (A); pool comprising fractions 14 to 26 (B); pool comprising fractions 27 to 40 (C).

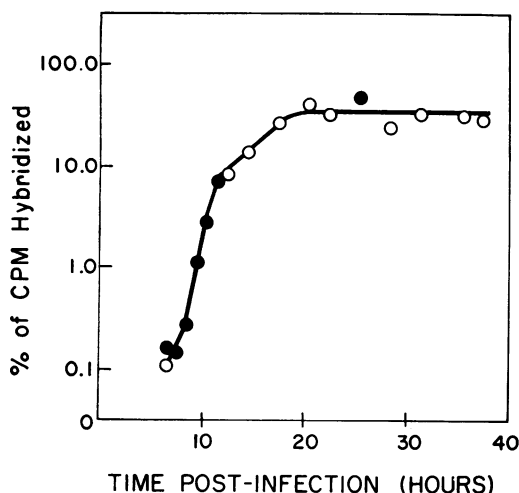


FIG. 4. Synthesis of virus-specific RNA at various times after infection. Cells were infected at an input multiplicity of 100 fluorescent focus-forming units per cell and were labeled with ^3H -uridine ($2 \mu\text{Ci}/\text{ml}$ of culture) for 1-hr periods at various times after infection. RNA was prepared and hybridization performed as described in Fig. 1. DNA concentrations ranged from 2 to $10 \mu\text{g}/\text{filter}$. Counts per minute hybridized per filter for the 154 hybridization samples assayed ranged from 65 to 24,109 counts/min. The average value for non-specific binding, determined by incubation of the 17 RNA samples with 79 blank filters, was 4 counts/min. Symbols (○) and (●) refer to two separate experiments. Per cent of counts per minute hybridized is the number of counts per minute hybridized at infinite DNA concentration (determined as described in text) divided by the number of counts per minute added to the hybridization reaction mixture, multiplied by 100.

found in virus-specific RNA. Significant incorporation into viral species, 0.73 and 0.84% for fractions 1 to 13 (Fig. 3A) and fractions 27 to 40 (Fig. 3C), respectively, were found in other portions of the gradient. As a measurement of non-specific binding, no more than 0.01% of the counts from corresponding fractions of labeled uninfected cells was found to bind to viral DNA on filters.

Rate of synthesis of virus-specified RNA. To measure the kinetics of transcription of the viral genome, RNA was extracted from cells labeled for 1-hr periods at various times after infection. A constant amount of each ^3H -RNA sample was hybridized with four or five different concentrations of purified viral DNA, varying from 2 to $10 \mu\text{g}$ per filter. (As the per cent of viral RNA varied, the amount of DNA needed to saturate each ^3H -RNA sample also varied. To perform the experiment with a single DNA concentration would entail the danger that the concentration chosen

might not be saturating for all samples). From reciprocal plots constructed from each set of data (see Appendix, part I), the per cent of the incorporated ^3H -uridine which was in virus-specific RNA was determined. These values plotted as a function of time after infection are shown in Fig. 4. The per cent of label incorporated into virus-specific RNA achieved a maximum value of about 40% at 20 hr postinfection and then remained constant.

Analysis of early and late viral cRNA species by hybridization-inhibition. To determine whether species of early virus-specific RNA are transcribed or are present late in infection, and conversely whether so-called late species of RNA are present before replication of viral DNA, presaturation hybridization-inhibition analyses were performed by method A (see above). Experiments were done by using a constant amount of ^3H -early RNA and increasing amounts of unlabeled RNA obtained during the late phases of infection (Fig. 5A). To calculate the per cent inhibition of hybridization at infinite concentrations of competing RNA species, the reciprocal plots shown in Fig. 5C and D were constructed. Graphical analysis (see Appendix, part II) shows that 75% of the label was incorporated into species of cRNA whose hybridization could not be inhibited by saturating amounts of unlabeled late cRNA (Fig. 5C). When the inhibition of hybridization of ^3H -late cRNA by unlabeled early RNA was measured (Fig. 5B), 25% inhibition was observed (Fig. 5D). Hybridization of the ^3H -labeled RNA species was inhibited by homologous preparations: 100% inhibition with the late and 95% inhibition with the early RNA preparations (determined from plots shown in Fig. 5C and D). The specificity of the inhibition was indicated by the fact that uninfected KB cell RNA did not inhibit hybridization (Fig. 5B).

Evaluation of hybridization-inhibition techniques. The ability of unlabeled late viral RNA to inhibit the hybridization of ^3H -early RNA was studied by using a number of techniques. Data obtained by method A are shown in Fig. 6A. The experiment was performed with two different non-saturating concentrations of ^3H -early RNA and also with a quantity of ^3H -labeled RNA which approached saturation of the DNA. As shown earlier (Fig. 1A), very large quantities of early RNA were needed to saturate $2 \mu\text{g}$ of viral DNA. Therefore, only $0.2 \mu\text{g}$ of DNA was used in the third experiment. In all three cases, the results were very similar; that is, 60 to 70% of the ^3H -uridine was incorporated into species of RNA whose hybridization was not inhibited by saturating amounts of unlabeled late RNA.

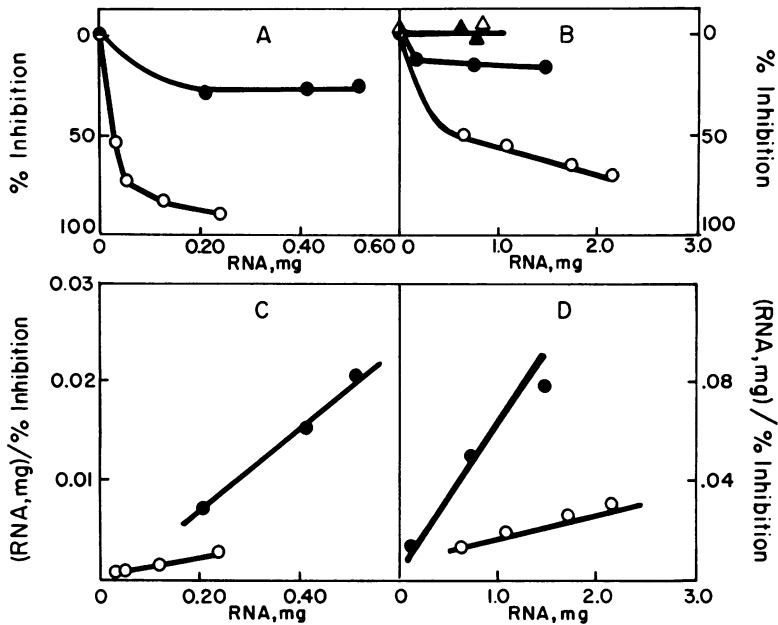


FIG. 5. Transcription of virus-specific RNA species early or late in infection with type 2 adenovirus as determined by hybridization-inhibition with heterologous or homologous RNA species. For these experiments, cells were infected and RNA was prepared as described in Fig. 1. Conditions for the hybridization reactions were those listed in Fig. 1. (A) Hybridization-inhibition of (●) ^3H -RNA labeled with 1.3 $\mu\text{Ci/ml}$ from 6 to 7 hr postinfection (i.e., early) in the presence of 2 μM FUdR competed by unlabeled RNA extracted from cells harvested at 18 hr postinfection (i.e., late). Filters were washed by method A (that is, extensive washing and ribonuclease treatment after both hybridization reactions; see text for complete details). (○) ^3H -RNA labeled with 2.0 $\mu\text{Ci/ml}$ from 18.0 to 18.5 hr postinfection competed by unlabeled RNA from cells harvested at 18 hr postinfection. Filters were washed by method A. (B) Hybridization-inhibition of (●) ^3H -RNA labeled with 1.0 $\mu\text{Ci/ml}$ from 18.0 to 18.5 hr postinfection competed by unlabeled RNA extracted from cells infected in the presence of FUdR and harvested at 6.5 hr postinfection. Filters were washed by method A. (○) ^3H -RNA labeled with 1.0 $\mu\text{Ci/ml}$ from 6.0 to 6.5 hr postinfection by unlabeled RNA extracted from cells harvested at 6.5 hr postinfection, both in the presence of 2 μM FUdR. Filters were washed by method A. (▲) ^3H -RNA labeled with 1.0 $\mu\text{Ci/ml}$ from 18.0 to 18.5 hr postinfection by unlabeled uninfected KB cell RNA. Filters were washed by method A. (△) ^3H -RNA labeled with 3.0 $\mu\text{Ci/ml}$ from 6 to 7 hr postinfection in the presence of FUdR by unlabeled uninfected KB cell RNA. Filters washed by method B (that is, rinsing at 65 C after first hybridization reaction, extensive washing, and ribonuclease treatment after second reaction; see text for complete details). (C) and (D). Reciprocal plots (see text) of the data presented in Fig. 5A and 5B, with symbols denoting the same experiments.

By using method B (Fig. 6B), it was found that unlabeled late RNA inhibited hybridization of about 50% of the ^3H -early RNA. This method too yielded reproducible results at widely varying concentrations of labeled RNA. When the washing procedure used after hybridization with competing RNA was less extensive (method C), the results were more variable. Typical data are shown in Fig. 6C. Repeated performance of the experiment with this technique showed that unlabeled late RNA inhibited the hybridization of ^3H -early RNA to an extent which varied from about 40% to greater than 98%.

Since different preparations of unlabeled late RNA and ^3H -early RNA (labeled either in the presence or absence of FUdR) gave reproducible results with methods A and B, it was postulated

that the variation was not due to the population of virus-specific RNA molecules in the preparations but rather that the variation was inherent in the technique (i.e., method C) employed.

These data suggested that the initial hybridization with competing RNA frequently resulted in areas of limited hybridization and residual loops and tails of RNA which, if not removed, could effectively but nonspecifically compete with subsequent hybridization of the labeled virus-specific RNA. The ribonuclease hydrolysis and extensive washing (method A) or rinsing at elevated temperature (method B) markedly reduced or eliminated this form of nonspecific competition. But the gentler rinsing technique employed in method C only partially and to a variable extent eliminated the nonspecific forms of hybridization.

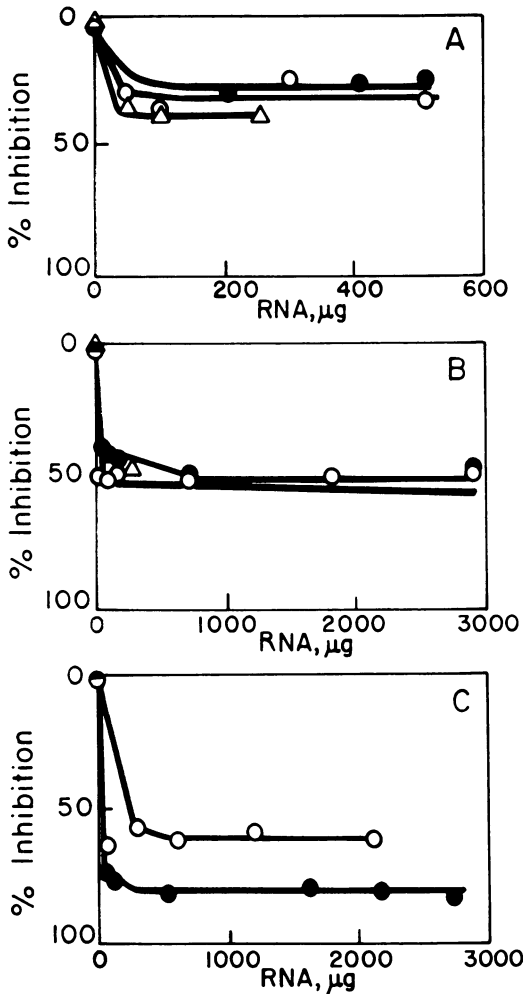


FIG. 6. Hybridization-inhibition of ^3H -early RNA by intact, unlabeled late RNA extracted from adenovirus-infected cells harvested at 18 hr after infection with 100 fluorescent focus-forming units per cell of type 2 virus. RNA was prepared as described in Fig. 1. (A) Filters were washed by method A (extensive washing and ribonuclease treatment after both hybridization reactions). Hybridization-inhibition was performed with various amounts of intact unlabeled late RNA and (○) 48.4 μg of ^3H -RNA labeled with 3.0 μCi of ^3H -uridine per ml from 6.0 to 6.5 hr postinfection, 2 μg of DNA/filter; (●) 116.1 μg of ^3H -RNA labeled with 1.3 μCi /ml from 6 to 7 hr postinfection in the presence of 2 μM FUDR, 2 μg of DNA/filter; (△) 406.7 μg of ^3H -RNA labeled with 3.0 μCi /ml from 3 to 7 hr postinfection in the presence of FUDR, 0.2 μg of DNA/filter. (B) Filters were washed by method B (rinsing at 65 C after first hybridization reaction; extensive washing and ribonuclease treatment after second reaction). Hybridization-inhibition was performed with various amounts of intact unlabeled late RNA and (○) 22.4 μg of ^3H -RNA labeled with 3.0 μCi /ml from 6 to 7 hr postinfection,

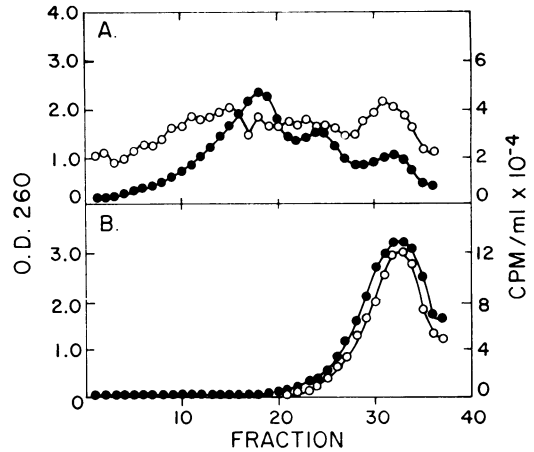


FIG. 7. Rate zonal sedimentation in sucrose gradients (5 to 20%) of intact and alkaline-degraded ^3H -late RNA from type 2 adenovirus-infected KB cells 19 hr after infection. Cells were infected and RNA was prepared as described in Fig. 1. Partial alkaline degradation was accomplished by treating RNA with 1 N NaOH for 90 sec at 23 C. RNA was dissolved in 0.01 M NaAc (pH 5.1) and layered on a gradient containing 0.01 M NaAc, 0.05 M NaCl, and 100 μM MgCl_2 (pH 5.1). Centrifugation was at 25,000 rev/min for 6 hr in a Spinco SW 25 rotor. (A) ^3H -late RNA labeled with 1.0 μCi of ^3H -uridine per ml from 18 to 19 hr postinfection. (B) Same preparation after controlled alkaline degradation. Optical density at 260 nm (●). Counts per minute per milliliter $\times 10^{-4}$ (○).

If this model were correct, it should be possible to obtain relatively similar results with any of the three methods studied when the competing RNA is sufficiently small so that tails and loops cannot develop. Therefore, to test the proposed model of nonspecific competition, the experiments were repeated by using alkaline-degraded, unlabeled late RNA (see above) as the inhibiting RNA. For

2 μg of DNA/filter; (●) 46.3 μg of ^3H -RNA labeled with 3.0 μCi /ml from 6 to 7 hr postinfection in the presence of FUDR, 2 μg of DNA/filter; (△) 406.7 μg of ^3H -RNA labeled with 3.0 μCi /ml from 3 to 7 hr postinfection in the presence of FUDR, 0.2 μg of DNA/filter. (C) Filters were washed by method C (mild rinsing at room temperature after first hybridization reaction; extensive washing and ribonuclease treatment after the second reaction). Hybridization-inhibition was performed with various amounts of intact unlabeled late RNA and (○) 45.2 μg of ^3H -RNA labeled with 3.0 μCi /ml from 6 to 7 hr postinfection in the presence of FUDR, 2 μg of DNA/filter; (●) 84.0 μg of ^3H -RNA labeled with 4.0 μCi /ml from 6 to 7 hr postinfection, 2 μg of DNA/filter. Refer to text for complete details of the three washing procedures.

these experiments, it was essential that the degraded RNA be of sufficient size to form a ribonuclease-resistant hybrid to the same extent and with the same specificity as the intact RNA but still be sufficiently small to eliminate the possibility of loop or tail formation. The sedimentation analysis of a preparation of ^3H -late RNA before and after limited alkaline degradation is shown in Fig. 7. Before degradation, the RNA exhibited a great heterogeneity of size; after alkaline treatment, the RNA sedimented in a single peak with a sedimentation coefficient of about 4S. The hybridization characteristics of the intact and degraded preparations were compared in the following way: DNA saturation curves were made by using 2 μg of viral DNA and increasing amounts of each ^3H -RNA preparation. The plots shown in Fig. 8 were then constructed. The appropriate calculations (see Appendix, part III) show that the counts per minute hybridized at saturation were 2,268 and 2,222 for the intact and degraded RNA species, respectively. The quantities of RNA giving half saturation were 10.5 μg for intact and 8.2 μg for degraded RNA.

When hybridization-inhibition studies were made with alkaline-degraded RNA, all three

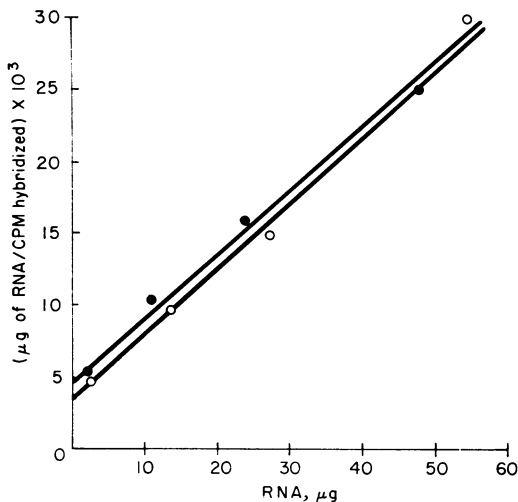


FIG. 8. Hybridization characteristics of intact ^3H -late RNA (●) and alkaline-degraded ^3H -late RNA (○). Cells were infected with 100 fluorescent focus-forming units per cell. RNA was labeled with 2.0 $\mu\text{Ci}/\text{ml}$ from 18 to 19 hr postinfection and was extracted as described in Fig. 1. Partial alkaline degradation was accomplished as described in Fig. 7. Hybridization with 2 μg of DNA/filter was performed with various amounts of intact and degraded RNA species (average specific activity: 1,194 counts per min per μg) using the conditions listed in Fig. 1. The theoretical basis for the reciprocal plots is described in the Appendix, part III.

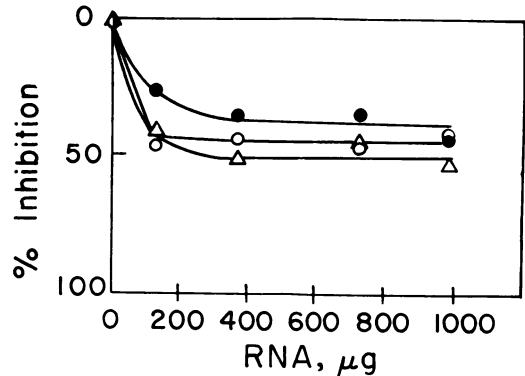


FIG. 9. Hybridization-inhibition of ^3H -early RNA by alkaline-degraded unlabeled RNA extracted from adenovirus-infected cells harvested at 18 hr after infection with 100 fluorescent focus-forming units per cell. RNA was prepared as described in Fig. 1. Partial alkaline-degradation was achieved by treating the RNA with 1 N NaOH for 90 sec (see Fig. 7). Conditions for the hybridization reactions are listed in Fig. 1. Hybridization-inhibition was performed with 104.5 μg of ^3H -early RNA labeled with 2.25 μCi of ^3H -uridine per ml from 6 to 7 hr postinfection in the presence of 2 μM FUDR and various amounts of alkaline-degraded unlabeled late RNA; 2 μg of DNA/filter. Filters were washed by method A (●), method B (○), and method C (△). Refer to Fig. 6 and text for complete details of the three washing procedures.

methods gave approximately the same result (Fig. 9): saturating amounts of unlabeled late RNA inhibited the hybridization of 35% (method A) to 50% (method C) of the ^3H -early viral RNA.

The efficacy of each of the three washing procedures was ascertained in the following way. Either intact or degraded ^3H -late RNA was hybridized with adenovirus DNA, and the filters were then washed by the three methods (Table I). As expected, a closer correspondence was seen among the results with the three different techniques when alkaline-degraded RNA rather than intact nucleic acid was used. It is noteworthy that method C, employing intact RNA, yielded the most variable results. Moreover, with either intact or degraded RNA, method A showed very little variation either within or among the three experiments (less than 5% by calculation of standard deviation).

DISCUSSION

In KB cells infected with type 2 adenovirus, the infecting viral DNA appears in the nucleus by 2 hr after infection (37). However, 6 hr postinfection is the earliest time newly transcribed viral RNA can be detected in the total RNA extracted. Thomas and Green (50) found that at 6 hr about

TABLE 1. Comparison of DNA-RNA hybridization techniques with intact and alkaline-degraded ³H-late RNA and type 2 adenovirus DNA

Expt	RNA hybridized (counts per min per filter) ^a					
	Intact RNA with method ^c			Alkaline-degraded RNA ^b with method ^c		
	A	B	C	A	B	C
1	100	211	315			
	100	223	339			
	100	199	291			
	100	215	297			
2	100	182	132	100	135	146
	100	186	126	100	131	144
	100	176	183	100	146	
					138	
3	100	165	184	100	139	181
	100	169	186	100	144	165
	100	149	183	100	137	165
	Avg	100	187	224	100	139

^a Results are from a number of experiments performed with various concentrations of both RNA and DNA. So that the data from the three experiments could be compared, all data were normalized to 100 counts/min for results with method A. Filters treated by method A, with both intact and degraded RNA, showed very little variation among the experiments (less than 5% by calculation of standard deviation).

^b Partial alkaline degradation was accomplished by treating RNA with 1 N NaOH for 90 sec at 23 C.

^c Filters were washed by the three methods described in the text.

1.5% of the ³H-uridine incorporated into polyribosomes is in virus-specific hybridizable material. By using highly labeled material isolated from polyribosome fractions, to increase the ratio of viral to host RNA species, synthesis of virus-directed RNA can in fact be detected 3 hr after infection (Fig. 3). Since viral DNA replication in type 2 adenovirus-infected KB cells begins about 7 hr after infection (25, 27), this hybridizable material may be called early cRNA according to the classical definition developed from transcription studies with T-even bacteriophages (13, 26). Moreover, the synthesis of virus-specific RNA may be detected in the presence of 5-fluorodeoxyuridine, an effective inhibitor (about 97% of adenovirus DNA synthesis (2, 17).

In studies of the transcription of type 5 adenovirus DNA in infected KB cells, Bello and Ginsberg (2) were unable to establish directly the existence of early viral RNA. In their work, DNA-RNA hybridization was performed with the total RNA extracted from infected cells by the tech-

nique of Nygaard and Hall (41). Since virus-directed protein synthesis can be detected before the replication of viral DNA (45), it was suggested that early RNA may be synthesized at a level which made it difficult to detect by the methods used. It is shown here (Fig. 4) that in adenovirus type 2 infection, only 0.1 to 0.2% of the ³H-uridine incorporated into total RNA during an early time is in virus-specific material; by 20 hr after infection, some 40% of incorporated ³H-uridine is in viral RNA. A great disparity between the amounts of early and late viral cRNA is also indicated by the experiments of Thomas and Green (50).

Transcription of early and late viral RNA species were compared by the technique of DNA-RNA hybridization-inhibition. Results were found to be dependent upon the experimental technique employed. In agreement with Darnell and associates (3, 14, 48), significant differences in the results given by simultaneous and sequential hybridization-competition were observed (J. Lucas and H. S. Ginsberg, unpublished data). In addition, major differences were also detected with the various techniques of presaturation hybridization-inhibition which are commonly used (22, 48, 54). It is important to note that all of the techniques employed are apparently specific in that uninfected KB cell RNA does not inhibit the hybridization of virus-specific RNA (Fig. 5B, references 51, 54). However, on the basis of the experiments with alkaline-degraded RNA, and on the assessment of the reproducibility of the various methods, it is concluded that when using intact RNA the procedure described by Gillespie (22), method A, is the technique of choice.

Early and late RNA species were therefore characterized by using hybridization-inhibition method A. Late RNA inhibited the hybridization of ³H-early RNA about 30% (Fig. 6A). Hence, 60 to 70% of the cRNA species synthesized before viral DNA replication are not synthesized (nor are they present) at late times in infection. To simplify further discussion, RNA which is transcribed prior to viral DNA replication, but not during the synthesis of late viral products, will be called early cRNA class I. (Our preliminary results indicate that early RNA species of class I are no longer present by 14 hr after infection and are probably decreased in concentration as early as 9 hr.) The RNA species transcribed prior to viral DNA replication as well as late in infection will be called early cRNA class II.

About 75% of the label incorporated into late viral RNA is found in species whose hybridization cannot be inhibited by early RNA (Fig. 5B). This experiment defines that class of RNA whose

synthesis begins only after the initiation of viral DNA synthesis, that is, true late cRNA. [Unpublished data (J. Lucas and H. S. Ginsberg) suggest that all species of RNA present late in the productive type 2 adenovirus infection of KB cells are also made in the abortive infection which the virus initiates in African green monkey kidney cells.] Conversely, the experiment indicates that about 25% of the labeled material is in cRNA species which are present at both early and late times.

At 18 hr postinfection, the time at which the late RNA was prepared, about 30% of the label incorporated into total RNA is in viral species (Fig. 4). Therefore, at late times in infection, about 7.5% of the label incorporated into RNA enters species of early viral cRNA class II.

The per cent of incorporated label which enters early cRNA class II at 6 hr after infection can also be calculated. At 6 hr, only about 0.1 to 0.2% of incorporated label is in viral RNA (Fig. 4). Moreover, the hybridization-inhibition experiment with unlabeled late and ^3H -early RNA species (Fig. 6A) shows that about 30% of the label incorporated into viral cRNA at 6 hr enters early cRNA class II. Therefore, some 0.03 to 0.06% of the label incorporated into total RNA at early times in infection enters early cRNA class II. Taking into account the observation that there is a 30 to 40% decrease in the incorporation of ^3H -uridine into total RNA by 18 hr after adenovirus infection (2, 44), it is found that the incorporation of label into class II early cRNA is enhanced (from 75- to 175-fold) after the initiation of viral DNA synthesis. [Let x be the amount of ^3H -uridine incorporated into total RNA at 18 hr postinfection and x_1 the amount at 6 hr. The incorporation into early cRNA class II at 18 hr is $0.075 x$, whereas that at 6 hr is $0.0006 x_1$. Since x is equal to about $0.6 x_1$, the ratio of incorporation into early cRNA class II at the two times is given by: (early cRNA class II at 18 hr)/(early cRNA class II at 6 hr) = $(0.075 x)/(0.0006 x_1) = (0.045 x_1)/(0.0006 x_1) = 75$. This calculation yields a minimal estimate of the ratio.] This enhanced incorporation could be due to (i) an increased rate of synthesis of early cRNA class II late in infection, (ii) a change in the pool size of the RNA precursor, or (iii) a great increase in the turnover rate of viral RNA late in infection. It would seem unlikely that changes in the latter two factors could account for an enhancement of such magnitude. [For example, it has been shown (44) that the amount of ^3H -uridine incorporated into the acid-soluble fraction of type 2 adenovirus-infected KB cells is only about 1.5 times greater at 18 hr than it is at 6 hr after infection.] It is therefore suggested that the observation at least partially re-

flects a greatly enhanced rate of synthesis of class II early cRNA late in infection. The observed enhancement also makes it unlikely that the continued synthesis of class II early RNA is simply due to transcription from partially uncoated virions which are not participating in the infection.

Experiments to distinguish the two classes of early cRNA temporally, for example, by performing hybridization-inhibition experiments with unlabeled 3 hr and labeled 6 hr RNA species, have been hampered by the difficulty of obtaining the large amounts of inhibiting RNA which would be needed to saturate the hybridizable DNA. As noted in earlier studies (2), early adenovirus-specified RNA is synthesized at an extremely low level.

The finding that some early cRNA species are not present at late times after type 2 adenovirus infection agrees with the data of Matsukage and Minagawa (39) suggesting that the synthesis of some early messenger RNA stopped late in T2-infected *Escherichia coli* and with reports (19, 20) that in SPO1-infected *Bacillus subtilis* the concentration of early virus-directed messenger RNA is very much reduced after the initiation of viral DNA synthesis. Moreover, a recent study of T4 infection (46) has shown that there is a class of phage-specific RNA that is transcribed early but decreases late after infection ("true early") and a class ("quasi late") that is synthesized early but whose synthesis increases at late times (8-10). These findings differ from previous results of Green and colleagues with adenovirus (27, 51) and with other hybridization analyses of the transcription of the DNA of vaccinia virus (43), simian virus 40 (11, 42), polyoma virus (34), and the bacteriophages T2 (29, 30) and T4 (7), which implied that all or nearly all early RNA species may be present during the transcription of late messenger RNA species. The comparison of hybridization techniques described in this paper suggests the possibility that some of the differences observed with the various viruses analyzed may be due to the techniques used rather than to basic differences in transcriptional control mechanisms, and perhaps the comparison indicates the need for a reevaluation of data in terms of the technical aspects of the hybridization methods employed.

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APPENDIX

Quantitative Analysis of Data from Hybridization Experiments

Despite the complexity of the reactions involved, DNA and RNA saturation and hybridization-competition curves have been often observed to approximate rectangular hyperbolas. The experimental data obtained with adenovirus DNA and RNA fit this mathematical function. Discussions of the theory and application of quantitative analyses of hybridization experiments can be found in references 1, 4, 5, 32, 35, 40, 49. The following treatment has been employed in analyzing the results presented in this paper.

I. Saturation of RNA with DNA. A plot of counts per minute (CPM) of ³H-RNA hybridized versus micrograms of DNA per filter approximates a rectangular hyperbola with the equation:

$$\text{CPM}_h = (\text{CPM}_s) (\text{micrograms of DNA}) / (K_1 + \text{micrograms of DNA}) \quad (1)$$

Where CPM_h = CPM of ³H-RNA hybridized, CPM_s = CPM of ³H-RNA hybridized at saturating amounts of DNA, micrograms of DNA = quantity of DNA immobilized on a filter, K_1 = a constant.

Rearrangement of (1) yields:

$$\begin{aligned} \text{Micrograms of DNA}/\text{CPM}_h \\ = (1/\text{CPM}_s) (\text{micrograms of DNA}) \\ + K_1/\text{CPM}_s \end{aligned} \quad (2)$$

Micrograms of DNA/ CPM_h versus micrograms of DNA gives a straight line with a slope equal to $1/\text{CPM}_s$. The reciprocal of the slope, when divided by the number of CPM of input RNA and multiplied by 100% gives the per cent of the ³H-uridine incorporated into total RNA which was found in the virus-specific species.

The y-intercept is equal to K_1/CPM_s , where K_1 is the micrograms of DNA per filter needed to attain one-half saturation of the RNA sample, as shown by the following.

Solving (2) for K_1 :

$$K_1 = [(\text{micrograms of DNA}) (\text{CPM}_s)/\text{CPM}_h] - \text{micrograms of DNA} \quad (3)$$

At one-half saturation of the RNA,

$$\text{Micrograms of DNA} = \text{micrograms of DNA}_{S/2} \quad (4)$$

micrograms of $\text{DNA}_{S/2}$ is the quantity of DNA per filter required to attain one-half saturation of the RNA, and

$$\text{CPM}_h = \text{CPM}_s/2 \quad (5)$$

Substituting (4) and (5) into equation (3) yields:

$$\begin{aligned} K_1 = \\ [(\text{micrograms of DNA}_{S/2})(\text{CPM}_s)/(\text{CPM}_s/2)] \\ - \text{micrograms of DNA}_{S/2} \\ = \text{micrograms of DNA}_{S/2} \end{aligned} \quad (6)$$

II. Hybridization-inhibition. A plot of per cent inhibition versus milligrams of competing RNA approximates a rectangular hyperbola with the equation:

$$I_0 = [(I_s) (\text{milligrams of RNA})] / (K_3 + \text{milligrams of RNA}) \quad (1)$$

Where I_0 = the observed inhibition, expressed as a per cent; I_s = the per cent inhibition at saturating amounts of RNA; milligrams of RNA = quantity of input RNA (in milligrams); K_3 = a constant (equal to the milligrams of RNA input needed to attain one-half the maximal inhibition).

Rearrangement yields:

$$\begin{aligned} \text{Milligrams of RNA}/I_0 \\ = (1/I_s) (\text{milligrams of RNA}) + K_3/I_s \end{aligned} \quad (2)$$

Thus a plot of milligrams of RNA I_0 versus milligrams of RNA gives a straight line with a slope equal to $1/I_s$. The reciprocal of this yields the per cent inhibition obtained at infinite concentration of input RNA.

III. Saturation of DNA with RNA. A plot of CPM of ³H-RNA hybridized versus micrograms of RNA input approximates a rectangular hyperbola with the equation:

$$\text{CPM}_h = [(\text{CPM}_s) (\mu\text{g RNA})] / (K_2 + \text{micrograms of RNA}) \quad (1)$$

Where CPM_h = CPM of ³H-RNA hybridized, CPM_s = CPM of ³H-RNA hybridized at saturating amounts of RNA, micrograms of RNA = quantity of input RNA (in micrograms), K_2 = a constant.

Rearrangement of (1) yields:

$$\begin{aligned} \text{Micrograms of RNA}/\text{CPM}_h \\ = (1/\text{CPM}_s) (\text{micrograms of RNA}) \\ + K_2/\text{CPM}_s \end{aligned} \quad (2)$$

A plot of micrograms of RNA/ CPM_h versus micrograms of RNA gives a straight line with a slope equal to $1/\text{CPM}_s$. The Y-intercept is equal to K_2/CPM_s . The constant K_2 is equal to the micrograms of RNA input needed to attain one-half saturation of the DNA, as shown by the following equations.

Solving (2) for K_2 :

$$K_2 = [(\text{micrograms of RNA}) (\text{CPM}_s)/\text{CPM}_h] - \text{micrograms of RNA} \quad (3)$$

At one-half saturation of the DNA,

$$\text{Micrograms of RNA} = \text{micrograms of RNA}_{S/2} \quad (4)$$

where micrograms of $\text{RNA}_{S/2}$ is the micrograms of RNA input required to attain one-half saturation of the DNA, and

$$\text{CPM}_h = \text{CPM}_s/2 \quad (5)$$

Substituting (4) and (5) into equation (3) yields:

$$\begin{aligned} K_2 = \\ [(\text{micrograms of RNA}_{S/2})(\text{CPM}_s)/(\text{CPM}_s/2)] \\ - \text{micrograms of RNA}_{S/2} \\ = \text{micrograms of RNA}_{S/2} \end{aligned} \quad (6)$$

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