*A METHOD FOR DETERMINING THE FRACTION OF THE VIRAL GENOME TRANSCRIBED DURING INFECTION AND ITS APPLICATION TO ADENOVIRUS-INFECTED CELLS**

BY KEI FUJINAGA, STANLEY MAK,† AND MAURICE GREEN‡

INSTITUTE FOR MOLECULAR VIROLOGY, ST. LOUIS UNIVERSITY SCHOOL OF MEDICINE, ST. LOUIS, MISSOURI

Communicated by Robert J. Huebner, April 30, 1968

Human adenovirus (Ad) DNA has a molecular weight of 23×10^6 daltons,[1] thus containing sufficient information to code for 23–46 proteins. The viral capsid consists of about ten polypeptides[2] that account for only a fraction of the number of different proteins possible. It is possible to estimate the extent to which this genetic information is utilized during lytic infection by (1) determining how many viral-coded proteins are synthesized in the infected cells as has been done with poliovirus-infected cells,[3] (2) studying conditional lethal virus mutants,[4] or (3) determining what fraction of the viral genome is transcribed to virus-specific messenger RNA (mRNA) molecules.

We have chosen the third approach, utilizing a combination DNA-RNA (step 1) and DNA-DNA (step 2) hybridization procedure. In step 1, viral DNA immobilized on membrane filters is hybridized with unlabeled virus-specific RNA to saturate complementary DNA regions. In step 2, the presaturated viral DNA is further annealed with sheared, denatured, labeled, homologous viral DNA to determine the fraction of viral DNA not hybridized with RNA. The amount of labeled viral DNA bound is inversely related to the fraction of the genome transcribed. In this paper, we present the details of this procedure and the analysis of the fraction of Ad 2 viral genome transcribed during infection.

Experimental Procedures.—Viral DNA: Suspension cultures of KB cells were grown in Eagle's minimum essential medium[5] containing 5% horse serum. The growth and purification of Ad 2 and the isolation of viral DNA have been described.[6] Viral DNA was further purified by CsCl density gradient centrifugation. Viral [P^{32}] DNA was obtained from purified Ad 2 labeled with [P^{32}] orthophosphate.

Virus-specific RNA: Suspension cultures of KB cells at $2–3 \times 10^6$ cells/ml were infected with Ad 2 (strain 38-2) at an input multiplicity of 100 PFU/cell. After 1 hr of adsorption at 37°, the cell suspension was diluted in growth medium to a density of $2–3 \times 10^5$ cells/ml and incubated at 37° for an additional 17 hr. Cells were collected by centrifugation and RNA was extracted from the cell pellet by the hot phenol-sodium dodecyl sulfate (SDS) method,[7] followed by a DNase treatment,[8] a second hot phenol-SDS extraction,[9] ethanol precipitation,[9] and ether extraction.[8] RNA was dialyzed against five changes of 0.1 × SSC (SSC = 0.15 *M* NaCl–0.015 *M* Na_3 citrate) for 2 days. [H^3] RNA was prepared from infected cells labeled with [H^3] uridine (1 μc/ml, 20 c/mM) for 30 min from 18 to 18.5 hr after infection and was purified by the procedure described above.

DNA-RNA and DNA-DNA hybridization: In step 1, nitrocellulose membrane filters (Schleicher and Schuell, B6) containing immobilized Ad 2 DNA were incubated with unlabeled RNA from Ad 2-infected cells or from uninfected cells in 2 × SSC for 20 hr at 66°.[10–12] One set of filters, after treatment with RNase, was washed with 2 × SSC and incubated with sonicated, heat-denatured Ad 2 [P^{32}] DNA in 2 × SSC containing 0.1% SDS for 20 hr at 60°[12, 13] (step 2). Filters were processed as described by Warnaar and Cohen,[14] and the extent of DNA-DNA hybridization was determined by counting bound [P^{32}] DNA in a liquid scintillation counter. In order to estimate the completeness of

saturation of DNA sites by unlabeled RNA, a duplicate set of filters was washed with 2 × SSC and incubated with [H³] RNA from infected cells in 2 × SSC–0.1% SDS for 20 hr at 66°. Filters were treated with RNase, washed, dried, and counted.

Results.—Saturation levels of virus-specific RNA and viral DNA: To estimate the fraction of the viral genome transcribed to virus-specific RNA by the two step DNA-RNA and DNA-DNA hybridization technique, the following reaction conditions should be fulfilled: (1) In step 1, saturating amounts of unlabeled virus-specific RNA should be used to cover complementary DNA sites; the completeness of saturation was further tested in each experiment by a second annealing reaction with labeled homologous RNA after the first annealing reaction with saturating amounts of unlabeled RNA. (2) In step 2, saturating amounts of labeled DNA should be used to cover all DNA sites remaining after the first annealing reaction. To determine the amount of RNA or DNA necessary to saturate complementary DNA sites, Ad 2 DNA (0.21 μg) was annealed with increasing amounts of [H³]RNA from Ad 2-infected KB cells or Ad 2 [P³²]-DNA. A saturation plateau was obtained with less than 15,400 cpm (41 μg) of input [H³]RNA from infected cells (Fig. 1*b*). The available sites in 0.21 μg of immobilized DNA are saturated by incubating DNA filters with 2 μg of input-labeled DNA (Fig. 1*a*). At saturation, 0.20–0.23 μg of Ad 2 [P³²]DNA were bound; thus the reaction is stoichiometric.

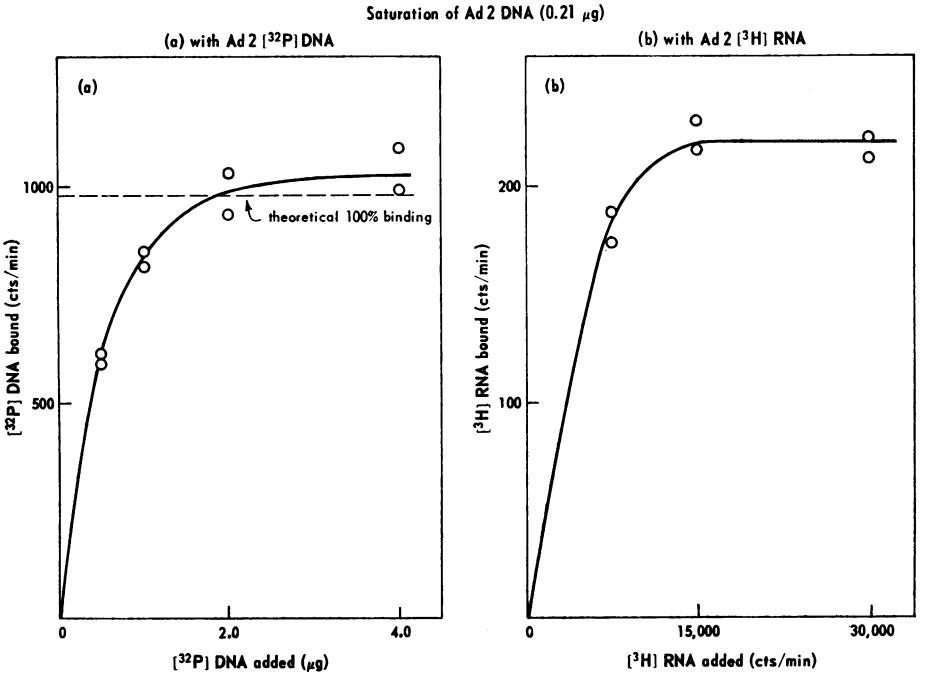

FIG. 1.—Saturation of immobilized DNA with [P³²]DNA and with virus-specific [H³]RNA. Membrane filters containing 0.21 μg of denatured Ad 2 DNA were annealed with increasing amounts of sheared, denatured Ad 2 [P³²]DNA (*a*) or [H³]RNA from Ad 2-infected cells (*b*). Specific activities are 4660 cpm/μg for [P³²]DNA and 380 cpm/μg for [H³]RNA.

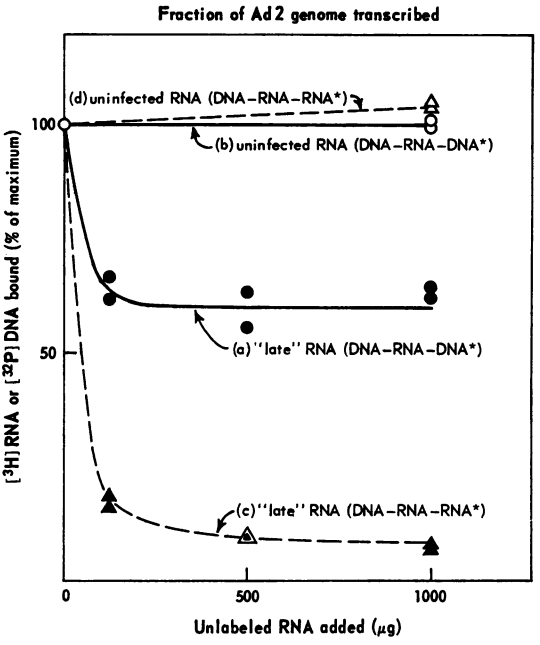

FIG. 2.—Determination of the unhybridized fraction of viral DNA after saturation with virus-specific RNA. Membrane filters containing 0.21 μg of viral DNA were annealed (step 1) with different amounts of unlabeled RNA from the following sources: (*a*) 18-hr-infected cells with Ad 2, (*b*) uninfected KB cells. The filters containing DNA-RNA hybrids and unreacted DNA were annealed further (step 2) with 3.4 μg (13,000 cpm) of sonicated, denatured viral [P^{32}]DNA. Duplicate sets of DNA filters after presaturation with unlabeled RNA from (*c*) 18-hr-infected cells and (*d*) uninfected KB cells were annealed with 23,700 cpm (62 μg) of [H^3]RNA from 18-hr-infected cells. Counts bound to membrane filters preincubated in 2 × SSC instead of unlabeled RNA were normalized to 100%. Some of the results are also presented in Table 1. The asterisks refer to radioactive RNA or DNA molecules.

Measurement of the fraction of the Ad 2 genome transcribed: With these saturating conditions, Ad 2 DNA was annealed with increasing amounts of unlabeled RNA isolated from Ad 2-infected KB cells at 18 hours after infection ("late" RNA) in step 1, and then with saturating amounts of Ad 2 [P^{32}]DNA in step 2. The results are plotted in Figure 2 and some of the data of Figure 2 are presented in Table 1 (expt. 1). As shown in Figure 2 (curve *a*), "late" viral RNA saturated 40 per cent of the sites capable of reacting with viral DNA. This probably represents a minimum estimate of the fraction of the DNA transcribed, since 10 per cent of the DNA sites complementary to virus-specific RNA after annealing with saturating amounts of unlabeled RNA are still available for reaction with [H^3]RNA (see Fig. 2, curve *c*, and Table 1, expt. 2). Most likely this is due to the loss of RNA from the filter during the second annealing reaction, as suggested by the experiments described below.

Loss of bound RNA and immobilized DNA from membrane during annealing reactions: In order to apply appropriate corrections to measurements of the fraction of the viral genome transcribed, we determined (1) the retention of bound [H^3]RNA during step 2 and (2) the retention of immobilized viral DNA during both steps. (1) DNA filters were annealed with excess [^{3}H]RNA in step 1. One set of filters was counted and a duplicate set annealed with excess viral [P^{32}]DNA in step 2 and counted. As shown in Table 1 (expt. 3), approximately 15 per cent of the RNA bound during step 1 is lost during step 2, probably accounting for the uncovered DNA sites which react with labeled RNA after presaturation with unlabeled RNA. (2) Stoichiometric amounts of viral DNA are bound to immobilized DNA when untreated DNA membranes are annealed with [P^{32}] DNA (Fig. 1). However, when the DNA membrane is carried through

TABLE 1. *The binding of viral [P^{32}] DNA and virus-specific [H^3] RNA to immobilized Ad 2 DNA after preincubation with RNA from Ad 2-infected cells.*

Expt. no.[a]	First incubation	Second incubation	Ad 2 DNA (μg/filter)	Cpm bound	Per cent of control[b]
1	Infected KB RNA[c] (540 μg/filter)	Ad 2 [P^{32}] DNA[d]	0.21	447	
			0.21	395	59
			None	4	
	Normal KB RNA (1080 μg/filter)	Ad 2 [P^{32}] DNA[d]	0.21	708	
			0.21	713	101
			None	3	
	2 × SSC (control)	Ad 2 [P^{32}] DNA[d]	0.21	719	
			0.21	689	100
			None	2	
2	Infected KB RNA[c] (540 μg/filter)	Infected KB [H^3] RNA[e]	0.21	26	
			0.21	26	9.7
			None	3	
	Normal KB RNA (1080 μg/filter)	Infected KB [H^3] RNA[e]	0.21	248	
			0.21	246	104
			None	0	
	2 × SSC	Infected KB [H^3] RNA[e]	0.21	240	
			0.21	240	100
			None	3	
3	Infected KB [H^3] RNA[f] (540 μg/filter)	Ad 2 [P^{32}] DNA[d]	0.21	293[g] (440)[h]	
			0.21	307[g] (434)[h]	85[g]
			None	13[g] (3)[h]	
	Infected KB [H^3] RNA[f] (540 μg/filter)	None (no incubation)	0.21	361	
			0.21	351	100
			None	18	

[a] Expts. 1–3 were performed at the same time.
[b] After subtraction of background (cpm bound to an empty membrane), cpm bound to membrane filter preincubated in 2 × SSC (expts. 1 and 2), and cpm bound after 1st incubation (expt. 3) were normalized to 100%. Values from duplicate hybridization reactions were averaged.
[c] RNA from KB cells at 18 hr after infection with Ad 2.
[d] Sheared, denatured Ad 2 [P^{32}] DNA; 13,000 cpm, 3.4 μg/filter.
[e] RNA from infected KB cells labeled with 1 μc/ml of [H^3] uridine for 30 min (from 18 hr to 18.5 hr after infection with Ad 2); 23,700 cpm, 62 μg/filter.
[f] RNA from infected KB cells labeled with 1 μc/ml of [H^3] uridine for 30 min (from 18 hr to 18.5 hr after infection with Ad 2); 205,300 cpm, 540 μg/filter.
[g] [H^3] radioactivity.
[h] [P^{32}] radioactivity.

both annealing steps, only 0.18 μg of radioactive DNA are bound to membranes containing 0.21 μg of immobilized DNA (calculated from data in Table 1, expt. 1). This was further investigated by preparing membrane filters containing immobilized [P^{32}]DNA, carrying them through the two annealing steps, and determining the degree of retention of [P^{32}]DNA at each step. The results in Table 2 show that about 10 per cent of the DNA is lost from the membrane filter during the second annealing reaction and subsequent washing step.

Thermal elution profile of DNA-DNA hybrid: The possibility that [P^{32}]DNA is bound nonspecifically was examined by comparing the elution profile[15] of the

TABLE 2. *Retention of immobilized DNA during hybridization reactions.*[a]

Treatment[b]	Cpm on filter[c]	Per cent of control[d]
(1) None	724 700 672 687	100
(2) Wash with 2 × SSC	704 707	102
(3) Wash with Tris-HCl pH 9.2	637 634	92
(4) Incubate at 66° for 20 hr, wash with 2 × SSC, treat with RNase, and re-wash	671 676	97
(5) Procedure (4), incubate at 60° for 20 hr, and procedure (3)	583 627	87
(6) Incubate at 66° for 20 hr, wash with 2 × SSC	708 701	101
(7) Procedure (6), incubate at 66° for 20 hr, wash with 2 × SSC, treat with RNase, and re-wash	605 649	90

[a] Denatured Ad 2 [P^{32}] DNA (0.20 μg, 717 cpm) was immobilized on each of replicate membrane filters. After various treatments, cpm bound were measured in a liquid scintillation counter.
[b] Procedures are described in detail in the text. Procedure (5) is the DNA-RNA-DNA hybridization, and (7) is the DNA-RNA-RNA hybridization.
[c] Each value represents a separate filter.
[d] Cpm bound to a membrane filter before treatment normalized to 100%. Average of different filters.

hybridized DNA formed with and without presaturating with unlabeled RNA. As shown in Figure 3, the elution profiles are very similar, suggesting that specific and stable DNA-DNA duplexes are formed in the presence of DNA-RNA hybrids.

The fraction of the Ad 2 genome transcribed—correction for incomplete saturation: Immobilized DNA, exposed to saturating levels of unlabeled, infected cell RNA in step 1, can still bind small amounts (8–15%) of labeled, infected cell RNA during step 2 (Fig. 1 and Table 1). As shown above, this appears to be because of the loss of RNA from the filter during step 2. This introduces a small error which can be corrected for using the following relationship:

$$T = (1-D)/(1-R)$$

where T is the fraction of the genome complementary to virus-specific RNA, D is the fraction of the [P^{32}]DNA bound during step 2 after presaturation with unlabeled RNA to that bound without presaturation with unlabeled RNA, and R is the fraction of the [H^3]RNA bound to DNA after presaturation with unlabeled RNA to that bound without presaturation with unlabeled RNA.

The values for the fraction of the genome complementary to Ad 2-specific RNA determined in four experiments, corrected as described above, are 0.40, 0.44, 0.46, and 0.42 (Table 3).

Discussion.—We describe here a sequential DNA-RNA and DNA-DNA hybridization procedure for determining the fraction of the viral genome transcribed during infection. Although the data and appropriate corrections have

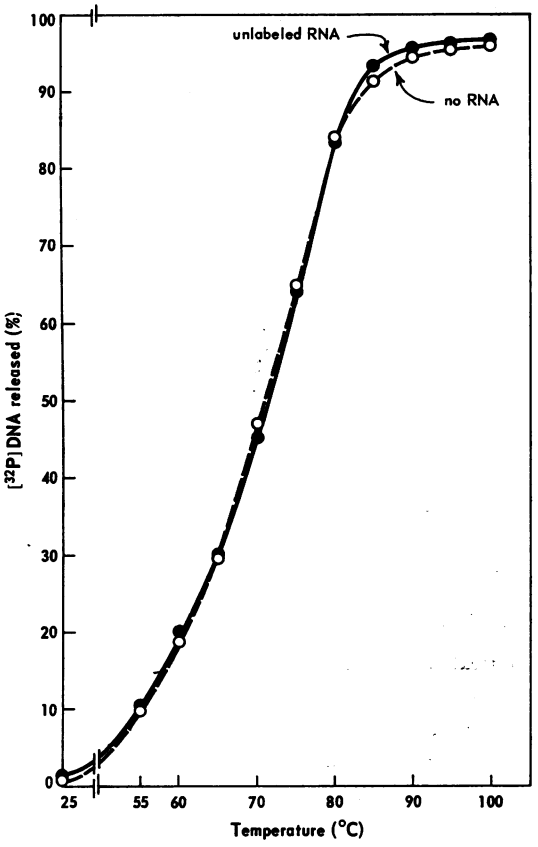

FIG. 3.—Thermal elution profiles of DNA-DNA hybrids. The amount of [P^{32}]DNA released into solution was determined after heating membrane filters containing [P^{32}]DNA-DNA hybrids in 0.1 × SSC for 10 min at various temperatures.[15]

The ordinate represents the cumulative [P^{32}]DNA released, expressed as per cent of the total [P^{32}]DNA. Immobilized DNA, 0.21 μg/filter; specific activity of [P^{32}]DNA, 9140 cpm/μg.

been derived for the Ad 2-KB cell system, this procedure is applicable to many other virus-cell systems. Two complications, neither serious, were detected: (1) about 10 per cent of the immobilized DNA is eluted during the two-step procedure. Unless specific DNA regions are selectively lost, this does not affect the determination. (2) Only 85–92 per cent of complementary viral DNA sites appear to be saturated at high levels of unlabeled RNA. Data were presented suggesting that this is attributable to a small loss of RNA from the filter. This incomplete saturation can readily be corrected.

In four experiments, 40–46 per cent of Ad 2 DNA sites were saturated by RNA from KB cells at 18 hours after infection (corrected values). These results indicate that Ad 2 mRNA molecules present late after infection, a time when viral DNA has already been synthesized,[16] are transcribed from nearly 50 per cent of the nucleotide sequences of the DNA molecule. This "late" mRNA preparation contains all nucleotide sequences synthesized early after infection, as shown by DNA-RNA hybridization measurements.[17] Since it is likely that only one strand from each gene of the duplex Ad 2 DNA molecule is transcribed, it is reasonable to conclude that all or nearly all (80–100%) of the genetic information in this viral genome is transcribed during infection. Whether one strand or portions of both DNA strands are transcribed is not known.

TABLE 3. *Fraction of viral DNA complementary to virus-specific RNA.*[a]

Expt. no.	Preincubation unlabeled RNA[b] (μg)	D = [P^{32}] DNA bound (fraction of maximum)[c]	R = [H^3] RNA bound (fraction of maximum)[c]	$T = (1-D)/(1-R)$ (fraction complementary to RNA)
1	1080	0.63	0.08	0.40
2	540	0.59	0.10	0.46
3	600	0.60	0.13	0.46
4	600	0.64	0.15	0.42

[a] Each value is the average of duplicate filters. Expts. 1 and 2 utilized the same RNA preparations and were performed at the same time. Expts. 3 and 4 utilized a second RNA preparation and were performed at different times.

[b] RNA from KB cells at 18 hr after infection was used to presaturate Ad 2 DNA.

[c] Radioactivity bound to DNA filter preincubated in 2 × SSC was normalized to 1.00.

We have carried out similar experiments with "late" Ad 12-specific RNA and have reached the same conclusions, namely, close to one half of the nucleotide sequences of viral DNA are transcribed.[18] We can now prepare a population of mRNA molecules representing a completely transcribed viral genome (80–100%), i.e., "late" viral mRNA. With this preparation we can determine the fraction of the viral genome transcribed prior to viral DNA synthesis, i.e., "early" mRNA, and the fraction of the viral genome transcribed in virus-free transformed or tumor cells[8, 11, 19] by hybridization-inhibition between "late" mRNA and "early" viral mRNA or transformed viral mRNA.

Summary.—A method for estimating the fraction of the viral genome transcribed in virus-infected cells has been described. Virus-specific RNA in KB cells late after infection with human adenovirus type 2 was annealed with viral DNA to saturate DNA sites active in transcribing viral mRNA molecules. The fraction of unhybridized viral DNA was determined by annealing viral DNA, presaturated with unlabeled RNA, with sheared, denatured viral [P^{32}]DNA. Virus-specific RNA hybridized with 40–46 per cent of the viral DNA sites, indicating that the nucleotide sequences of the RNA population are complementary to nearly half (40–50%) of the possible sequences of DNA. Since Ad 2 DNA is a duplex molecule, most likely all or nearly all (80–100%) of the viral genome is active in transcribing RNA during infection. It is not known whether one strand or portions of both strands are transcribed.

We are indebted to Miss Catherine Schumann and Mrs. Bakula Trivedi for their excellent technical assistance.

* This investigation was supported by USPHS grant AI-01725, contract PH43-64-928 from the National Institute of Allergy and Infectious Diseases, Vaccine Development Branch, National Institutes of Health, and contract PH43-67-692 from the National Cancer Institute, Viral Carcinogenesis Branch, Etiology Area, National Institutes of Health, U.S. Public Health Service, Bethesda, Maryland.

† Present address: Biology Department, McMaster University, Hamilton Ontario, Canada.

‡ Research Career Award (5-K6-AI-4739), National Institutes of Health, Public Health Service.

[1] Green, M., M. Piña, R. Kimes, P. Wensink, L. MacHattie, and C. A. Thomas, these PROCEEDINGS, **57**, 1302 (1967).

[2] Maizel, J. V., *Science*, **151**, 988 (1966).

[3] Summers, D. F., J. V. Maizel, and J. E. Darnell, these PROCEEDINGS, **54**, 505 (1965).

[4] Luria, S. E., and J. E. Darnell, *General Virology* (New York: John Wiley and Sons, 1967), p. 226.

[5] Eagle, H., *Science*, **130**, 432 (1959).
[6] Green, M., and M. Piña, these PROCEEDINGS, **51**, 1251 (1964).
[7] Warner, J. R., R. Soeiro, H. C. Birnboim, M. Girard, and J. E. Darnell, *J. Mol. Biol.*, **19**, 349 (1966).
[8] Fujinaga, K., and M. Green, these PROCEEDINGS, **57**, 806 (1967).
[9] Sherrer, K., and J. E. Darnell, *Biochem. Biophys. Res. Commun.*, **7**, 486 (1962).
[10] Gillespie, D., and S. Spiegelman, *J. Mol. Biol.*, **12**, 829 (1965)
[11] Fujinaga, K., and M. Green, these PROCEEDINGS, **55**, 1567 (1966).
[12] Green, M., K. Fujinaga, and M. Piña, in *Basic Techniques in Virology*, ed. K. Habel and N. Salzman (New York: Academic Press, in press).
[13] Piña, M., and M. Green, manuscript in preparation.
[14] Warnaar, S. O., and J. A. Cohen, *Biochem. Biophys. Res. Commun.*, **24**, 554 (1966).
[15] Fujinaga, K., and M. Green, *J. Mol. Biol.*, **31**, 63 (1968).
[16] Green, M., in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 27 (1962), p. 219.
[17] Thomas, D. C., and M. Green, unpublished data.
[18] Mak, S., and M. Green, manuscript in preparation.
[19] Fujinaga, K., and M. Green, *J. Virol.*, **1**, 576 (1967).