J. Mol. Biol. (1971) **57**, 129–145

Quantitation of Simian Virus 40 Sequences in African Green Monkey, Mouse and Virus-transformed Cell Genomes

LAWRENCE D. GELB, DAVID E. KOHNE† AND MALCOLM A. MARTIN

Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases National Institutes of Health, Bethesda, Md 20014, U.S.A.

and

† *Department of Terrestrial Magnetism, Carnegie Institution of Washington, Wash. D.C. 20015, U.S.A.*

(*Received 19 August 1970, and in revised form 5 November 1970*)

The number of SV40‡ genome equivalents present in green monkey and SV40 transformed mammalian DNA's have been evaluated by measuring DNA reassociation kinetics on hydroxyapatite. Under the proper conditions, this method is sufficiently sensitive to detect less than one SV40 DNA molecule per mammalian genome (one part in 10^8) as shown by reconstruction experiments. In four out of five SV40 transformed lines examined, an average of one SV40 genome equivalent was present in the cell DNA; three SV40 DNA equivalents per cell were found in the fifth viral transformed line. The background level of SV40 DNA sequences within the 3T3 genome was 0·45 equivalent per cell. An average of 0·5 SV40 genome equivalent was measured per African green monkey genome, an amount too small to be reliably detected using DNA–DNA hybridization on nitrocellulose membranes. The biological significance of these results and their relationship to previously reported values are discussed.

1. Introduction

Nucleic acid hybridization techniques have established the presence of Simian virus 40 DNA sequences in the genome of transformed cells (Reich, Black & Weissmann, 1966; Aloni, Winocour & Sachs, 1968; Sambrook, Westphal, Srinivasan & Dulbecco, 1968). Westphal & Dulbecco (1968), using SV40‡ complimentary RNA made *in vitro* with *Escherichia coli* RNA polymerase and transformed cell DNA immobilized on nitrocellulose filters, reported the presence of 5 to 58 SV40 DNA equivalents per cell genome in a series of SV40 transformed lines. The results depend on the fidelity and completeness of DNA transcription by the *E. coli* polymerase as well as the precise quantitation of the amount of SV40 DNA used in the reconstruction phase of these experiments.

One of the difficulties encountered by Westphal & Dulbecco (1968), was a reaction between SV40 complimentary RNA and normal mouse DNA which had to be subtracted from the results obtained with transformed cell DNA. This finding may be background "noise" or somehow related to the observations of Aloni, Winocour, Sachs & Torten (1969) who reported that purified SV40 DNA I reacted with several normal mammalian DNA's. Radioactively-labeled green monkey DNA, for example, was one-tenth as

‡ Abbreviation used: SV40, Simian virus 40.

efficient as labeled SV40 DNA in reacting with immobilized SV40 DNA. If 10% of all green monkey DNA was homologous to SV40 DNA, there would be 4×10^{11} daltons or approximately 133,000 copies of viral DNA per mammalian cell. The conditions of hybridization were such (time of incubation and concentration of labeled DNA) as to exclude the re-association of non-repetitive monkey DNA.

In the experiments described below, the sensitivity of our nucleic acid re-association technique has been increased to allow the detection of less than one SV40 DNA molecule (3×10^6 daltons) per mammalian cell genome (4×10^{12} daltons). The ratio of mammalian DNA to viral DNA was made large enough to compensate for the large differences in their respective molecular weights. The influence of normal or transformed mammalian DNA's on the kinetics of re-association of ^{32}P-labeled SV40 DNA permits a direct measurement of the number of integrated viral DNA molecules without the necessity for reconstruction calibration. With this technique, we detected about one-half of a copy of SV40 DNA per normal diploid mammalian genome. In four of the virus transformed lines examined we found about one additional SV40 DNA equivalent per diploid mammalian genome; in a fifth line we detected three SV40 genome equivalents per mouse cell.

2. Materials and Methods

(a) *Virus stocks*

Plaque-purified small plaque SV40 was propagated in primary African green monkey kidney cells. This virus stock was serially passaged in Vero (Earley, Peralta & Johnson, 1967) or BSC-1 cells. Small plaque SV40 propagated over the last 5 years in BSC-1 cells was a gift of Dr James Rose.

(b) *Preparation of SV40 DNA*

Confluent monolayers of primary African green monkey kidney, BSC-1 or Vero cells, growing in 32-oz. prescription bottles, were infected with small plaque SV40 virus at a multiplicity of 1 plaque forming unit/cell. [^{32}P]Orthophosphate (50 μc/ml.) was added to the infected cells in phosphate-free Eagle's II medium (Eagle, 1959) 24 hr following infection, and the cells harvested 6 to 7 days later. In one case, SV40 was propagated in Vero cells which had been exposed to [^{32}P]orthophosphate (50 μc/ml.) also in phosphate-free medium 48 hr before infection. Virus was purified by sedimentation onto a CsCl cushion (1·40 g/ml.) followed by equilibrium density centrifugation in CsCl (Yoshiike, 1968). SV40 DNA was extracted by heating the purified virus in 1% sodium dodecyl sulfate at 50°C for 30 min (Trilling & Axelrod, 1970). Sodium dodecyl sulfate was precipitated with 1 g CsCl/ml. and supercoiled double-stranded SV40 DNA (DNA I) was separated by CsCl equilibrium density centrifugation in the presence of 200 μg of ethidium bromide/ml. (Radloff, Bauer & Vinograd, 1967). The ethidium bromide was removed with Dowex-50 equilibrated with one-tenth strength standard saline citrate (SSC is 0·15 M-NaCl–0·015 M-sodium citrate) and the viral DNA was further purified on G100 Sephadex. Specific activities of the DNA preparations ranged from 10^5 to $1{\cdot}2\times10^6$ cts/min/μg. SV40 DNA I was sheared at 50,000 lb/in.^{2} in a Ribi cell fractionator in 0·001M-EDTA and 0·01 M-potassium phosphate buffer, pH 6·8, and passed through cellulose acetate filters (Metricel GA6, Gelman Instrument Co., Ann Arbor, Mich.) before use. The average size of the sheared DNA was 300 to 400 nucleotides (6·8 s) as determined by alkaline sucrose sedimentation.

(c) *Preparation of cellular DNA*

Transformed cell DNA was prepared from nuclei of the various cell lines listed in Table 1 according to the method of Berns & Thomas (1965) as modified by McCarthy & Hoyer (1964). Normal green monkey, 3T3 and hamster SV40 tumor cells were homogenized to release cell nuclei and the DNA prepared as above. All preparations were treated with

TABLE 1

Properties of SV40 transformed cells

SV40 transformed cell lines	Line transformed	Ease of recovery following cell fusion
SV-UV-15 clone 1	3T3	no virus rescued
SV-UV-15 clone 5	3T3	no virus rescued
Hamster tumor	Hamster	not tested, t-antigen +
SVT2	Balb C/3T3	virus rescued, high titer
SVPy3T3-11	3T3	virus rescued, high titer

50 μg pancreatic ribonuclease/ml. (Worthington Biochemical Corp., Freehold, N.J.) for 30 min at 25°C, digested at pH 8·2 with 100 μg pronase/ml. (Calbiochem, Los Angeles, Calif.), extracted with an equal volume of water-saturated phenol and spooled in 2 vol. cold ethanol. Salmon sperm DNA was purchased from Mann Research Laboratories (New York, N.Y.). Tritiated monkey DNA was prepared from Vero cells grown in the presence of [^{3}H]thymidine (2 μC/ml.). This precursor was added when the cells had reached 25% confluency.

DNA fragments were prepared by mechanical shear (50,000 lb./in.^{2}) and filtered through cellulose acetate as described above. The DNA was reprecipitated with 2 vol. of ethanol, resuspended in 5 ml. of distilled water and extensively dialyzed against 0·1 M-NaCl containing 0·001 M-EDTA. The size of sheared animal DNA determined by rate zonal sedimentation under alkaline conditions was also 6·8 S.

(d) *DNA–DNA hybridization on nitrocellulose filters*

Heat-treated SV40 DNA I (100°C for 20 min in SSC) and native monkey DNA were denatured by alkali and immobilized on 50-mm nitrocellulose filters (type B6, Schleicher & Schuell Co., Keene, N. H.) as described by Gillespie & Spiegelman (1965). DNA–DNA hybridization was carried out using the procedure outlined by Denhardt (1966) with 7-mm filters containing 0·2 to 0·3 μg SV40 DNA or 16 to 18 μg green monkey DNA. Labeled DNA was added to the reaction mixture containing 4 × SSC and Denhardt's pre-incubation mixture in a final volume of 0·25 ml. Each reaction vessel containing a DNA filter and a blank filter was incubated at 68°C for 16 hr. The filters were then removed, washed extensively in 4 × SSC at 68°C, dried, and counted in a liquid-scintillation spectrometer.

(e) *DNA–DNA re-association kinetics*

^{32}P-labeled SV40 DNA fragments (1 to 17×10^{-5} O.D.$_{260}$ units/ml.) were added to a reaction mixture containing 0·0025 M-EDTA and sheared mammalian or salmon DNA (30 to 50 O.D. units/ml.) in a screw capped vial. The mixture of labeled and unlabeled DNA's was denatured by heating at 100°C for 1 to 2 min and then allowed to cool at room temperature for 1 min. The phosphate concentration was adjusted to 0·4 or 0·6 M with 4 M-PO_4 buffer (phosphate buffer contains equimolar parts Na_2HPO_4 and NaH_2PO_4, pH 6·8) and the samples were incubated at 68°C. Portions (0·3 to 0·5 ml.) were removed at the start of the incubation and at various times over the course of the next 60 hr, diluted to 0·14 M-PO_4 buffer with distilled water and stored at 4°C. At the conclusion of the experiment, the final portion was incubated an additional hour with an excess of unlabeled, sheared, denatured SV40 DNA and processed as above to give the endpoint of the reaction.

Hydroxyapatite (Biogel HTP, Bio-Rad Laboratories, Richmond, Calif.) equilibrated with 0·14 M-PO_4 buffer and 0·4% sodium dodecyl sulfate was added to a depth of 3 cm in a 25-mm water-jacketed column maintained at 60°C. The capacity of the hydroxyapatite used varied between 10 and 40 O.D. units of DNA/cm of column bed. Diluted portions (2 to 4 ml.) from the reaction mixtures were applied to the hydroxyapatite columns and single-stranded DNA collected in 0·14 M-PO_4 buffer containing 0·4% sodium dodecyl

sulfate. Re-annealed DNA was recovered by elution with 0·4 M-PO_4 buffer at 60°C followed by 0·14 M-PO_4 buffer at 100°C. The effluents were allowed to cool to room temperature, checked for absorbance at 260 nm, precipitated with cold 5% trichloroacetic acid, and counted in a liquid-scintillation spectrometer.

The percentage of ^{32}P-labeled SV40 DNA re-annealed at each point in time was plotted as a function of the C_0t (Britten & Kohne, 1968). C_0t is defined as the product of the nucleic acid concentration (C_0) and the time (t) of the re-association incubation expressed as moles of nucleotides × sec/l. This is conveniently calculated as the product of DNA concentration expressed as optical density at 260 nm and the time of incubation in hr divided by 2 (C_0t = O.D. × hr/2) (Kohne, 1969). Each measurement was normalized so that 100% re-association occurred between the zero time (1 to 7%) and the endpoint for each individual reaction (94 to 96%). The C_0t curve was then drawn through these points using the theoretical curve of a second-order reaction determined by the equation

$$\left(C/C_0 = \frac{1}{1 + K\,C_0t}\right)$$

(Britten & Kohne, 1968). The amount of SV40 DNA present in green monkey or transformed mammalian DNA preparations was determined from the difference in midpoints of the experimental reaction and its control and expressed as genome equivalents/mammalian diploid cell for each line tested. Sample calculations and equations are included under results.

3. Results

(a) *Detection of SV40 DNA sequences in green monkey DNA using nitrocellulose filter hybridization technique*

Aloni *et al.* (1969) reported substantial polynucleotide sequence homology between SV40 DNA and African green monkey DNA by reciprocal DNA–DNA hybridization experiments on nitrocellulose filters. We carried out a similar experiment using immobilized green monkey DNA and ^{32}P-labeled DNA prepared from purified SV40 virions which had been propagated in Vero cells. Experiment 1, Table 2, shows that no significant reaction occurred between the labeled SV40 DNA and the immobilized green monkey DNA. Since encapsidated host DNA can be more readily detected by prelabeling green monkey cells (Trilling & Axelrod, 1970; Levine & Teresky, 1970), SV40 virus was grown in Vero cells exposed to [^{32}P]orthophosphate 48 hours before infection. Experiment 1, Table 2, shows that virtually none of this "prelabeled" SV40 DNA reacted with green monkey DNA. The labeled DNA extracted from SV40 propagated in primary African green monkey kidney cells (experiment 2, Table 2) also did not react to a significant extent with green monkey DNA.

Since Aloni *et al.* (1969) used SV40 grown in BSC-1 cells in all of their experiments, this line was also used to propagate virus. Small plaque SV40, previously grown on primary green monkey kidney cells, was serially passaged on the BSC-1 line. Virus and viral DNA were prepared from the first, third and seventh passages. In addition, SV40 DNA was purified from virus which had been passaged in BSC-1 cells for the past five years. The results, shown in experiment 2, Table 2, indicate no significant polynucleotide sequence homology between the DNA purified from BSC-1 propagated virus and green monkey DNA. Reciprocal experiments in which labeled green monkey DNA was added to filters containing immobilized SV40 DNA yielded similar results (unpublished data). All of these studies were done under conditions in which repetitive DNA sequences readily re-anneal; in order to examine the presence of single copies of SV40 DNA which may be present in normal green monkey DNA, we examined the re-association kinetics using hydroxyapatite (Britten & Kohne, 1968).

TABLE 2

DNA–DNA hybridization on nitrocellulose membranes

Expt	Free DNA	Amount (μg)	Immobilized DNA	Amount (μg)	Cts/min bound	% bound
1	SV40 (Vero)	$3{\cdot}0\times10^{-3}$	SV40	0·3	3016	53·1
	SV40 (Vero)	$1{\cdot}2\times10^{-2}$	GM	16·8	29	0·14
	green monkey	$4{\cdot}0\times10^{-3}$	GM	16·8	515	13·0
	SV40 (Vero, prelabeled)	$8{\cdot}5\times10^{-2}$	SV40	0·28	2984	45·2
	SV40 (Vero, prelabeled)	$33{\cdot}9\times10^{-2}$	GM	16·8	31	0·12
	green monkey	$4{\cdot}0\times10^{-3}$	GM	16·8	515	13·0
2	SV40 (AGMK)†	$8{\cdot}3\times10^{-4}$	SV40	0·2	750	75·1
	SV40 (AGMK)	$1{\cdot}2\times10^{-3}$	GM	17·1	2	0·19
	green monkey	$6{\cdot}4\times10^{-3}$	GM	17·1	145	17·4
	SV40 (BSC-1, passage 1)	$6{\cdot}8\times10^{-4}$	SV40	0·2	550	72·6
	SV40 (BSC-1, passage 1)	$1{\cdot}4\times10^{-3}$	GM	17·1	2	0·16
	SV40 (BSC-1, passage 3)	$4{\cdot}0\times10^{-3}$	SV40	0·2	1391	45·6
	SV40 (BSC-1, passage 3)	$3{\cdot}2\times10^{-2}$	GM	17·1	55	0·26
	green monkey	$6{\cdot}4\times10^{-3}$	GM	17·1	145	17·4
	SV40 (BSC-1, passage 7)	$1{\cdot}0\times10^{-3}$	SV40	0·3	903	74·7
	SV40 (BSC-1, passage 7)	$2{\cdot}0\times10^{-3}$	GM	18·0	2	0·09
	SV40 (BSC-1, 5 years)	$8{\cdot}8\times10^{-3}$	SV40	0·2	4323	44·2
	SV40 (BSC-1, 5 years)	$7{\cdot}1\times10^{-2}$	GM	17·1	13	0·02

† AGMK, primary African green monkey kidney.

(b) *Re-association kinetics of labeled SV40 DNA*

Figure 1 shows the re-association kinetics of ^{32}P-labeled SV40 DNA which has re-annealed in the presence of unlabeled salmon sperm DNA (added as a viscosity

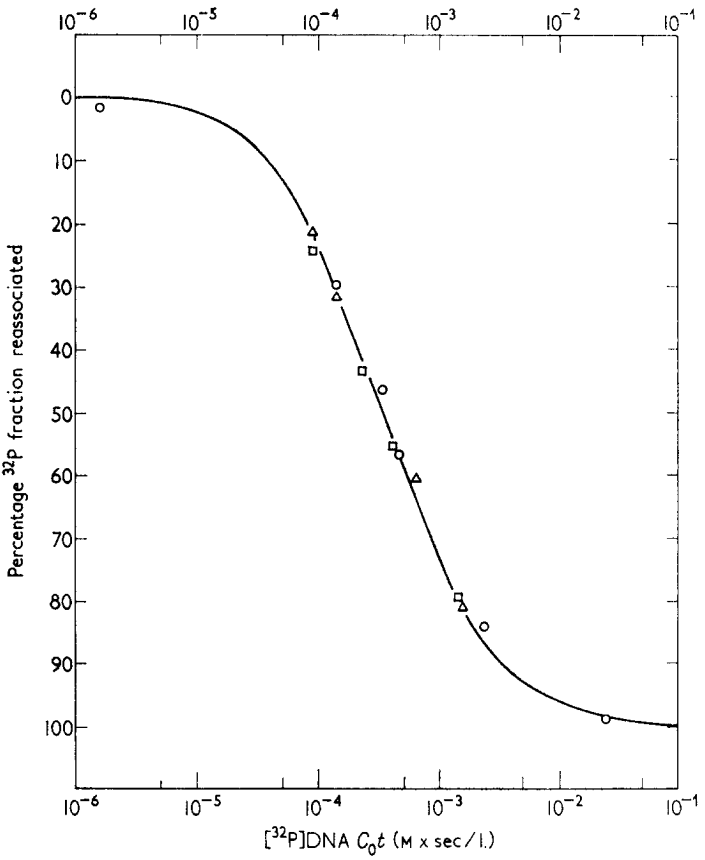

FIG. 1. Re-association kinetics of ^{32}P-labeled SV40 DNA.
Reaction mixtures containing 9 or $13{\cdot}2\times10^{-5}$ O.D. units sheared ^{32}P-labeled SV40 DNA (Vero, $3{\cdot}1\times10^{5}$ cts/min/μg)/ml., 40 O.D./ml. sheared salmon sperm DNA, 0·0025 M-EDTA and 0·4 M-PO_4 buffer were heat-denatured and incubated at 68°C. Samples were removed at intervals, the percentage SV40 DNA re-associated determined on hydroxyapatite and the results plotted as a function of ^{32}P-labeled SV40 DNA C_0t. (○) 9×10^{-5} O.D./ml.; (□) $13{\cdot}2\times10^{-5}$ O.D./ml.; (△) 9×10^{-5} O.D./ml. Each point represents a total of 340 cts/min.

control). This is a composite of three different incubations at two different concentrations. The half reaction of the SV40 DNA occurs at a C_0t of about $3{\cdot}6\times10^{-4}$. Table 3 lists the $C_0t_{1/2}$ values obtained from seven such kinetic experiments and illustrates the high degree of reproducibility obtainable with this technique.

In the absence of salmon sperm DNA, the half reaction of ^{32}P-labeled SV40 DNA occurs at a C_0t of $2{\cdot}9\times10^{-4}$. This increased rate is approximately that expected for the decrease in viscosity and suggests that significant contamination of salmon sperm DNA with viral DNA is not present. If some SV40 DNA were present in the salmon sperm DNA preparation, the $C_0t_{1/2}$ of this reaction would be less than $2{\cdot}9\times10^{-4}$.

The rate of DNA re-association is directly proportional to the concentration of the

TABLE 3

^{32}P-labeled SV40 DNA half C_0t *values*

^{32}P-labeled SV40 DNA	concn (O.D./ml.)	Spec. act. (cts/min/μg)	[Na^+]	^{32}P-labeled $C_0t_{1/2}$	^{32}P-labeled $C_0t_{1/2}$ corrected to 0·12 M PO_4 buffer †
Vero, prelabeled	$16{\cdot}7\times10^{-5}$	$1{\cdot}0\times10^{5}$	0·69 M	$3{\cdot}7\times10^{-4}$	$2{\cdot}0\times10^{-3}$
Vero	$9{\cdot}5\times10^{-5}$	$3{\cdot}1\times10^{5}$	0·69 M	$3{\cdot}25\times10^{-4}$	$1{\cdot}8\times10^{-3}$
Primary African green monkey kidney	$1{\cdot}3\times10^{-5}$	$5{\cdot}1\times10^{5}$	0·69 M	$3{\cdot}4\times10^{-4}$	$1{\cdot}9\times10^{-3}$
BSC-1, passage 1	$2{\cdot}9\times10^{-5}$	$1{\cdot}2\times10^{6}$	0·69 M	$3{\cdot}5\times10^{-4}$	$1{\cdot}9\times10^{-3}$
BSC-1, passage 3	$2{\cdot}0\times10^{-5}$	$9{\cdot}8\times10^{5}$	0·98 M	$3{\cdot}1\times10^{-4}$	$2{\cdot}1\times10^{-3}$
BSC-1, passage 7	$1{\cdot}5\times10^{-5}$	$1{\cdot}2\times10^{6}$	0·98 M	$2{\cdot}9\times10^{-4}$	$2{\cdot}0\times10^{-3}$
BSC-1, 5 years	$1{\cdot}4\times10^{-5}$	$1{\cdot}1\times10^{6}$	0·98 M	$2{\cdot}9\times10^{-4}$	$2{\cdot}0\times10^{-3}$

† Britten, 1969; Britten & Smith, 1970.

DNA in the solution measured. SV40 DNA at a concentration of 2 μg/ml. will re-associate twice as fast as SV40 DNA at a concentration of only 1 μg/ml. The effect of normal and transformed cell DNA's on the re-association kinetics of ^{32}P-labeled SV40 DNA can be used to determine the concentration of SV40 DNA sequences in these preparations. From this, one can then calculate the number of viral DNA equivalents present in each cell.

In each experiment, a known concentration of ^{32}P-labeled SV40 DNA was allowed to re-associate in the presence of salmon DNA. The rate of ^{32}P-labeled SV40 DNA re-association was then compared with the same amount of ^{32}P-labeled SV40 DNA re-associating in the presence of mammalian DNA. Any SV40-like DNA sequences in the mammalian DNA would increase the concentration of these sequences and hence the rate of re-association over the control. In Figure 2, for example, the ^{32}P-labeled SV40 DNA control ($1{\cdot}5\times10^{-5}$ O.D./ml. ^{32}P-labeled SV40 DNA plus 39·6 O.D./ml. salmon DNA in 0·98 M-Na^+) half re-associated at a ^{32}P-labeled DNA C_0t

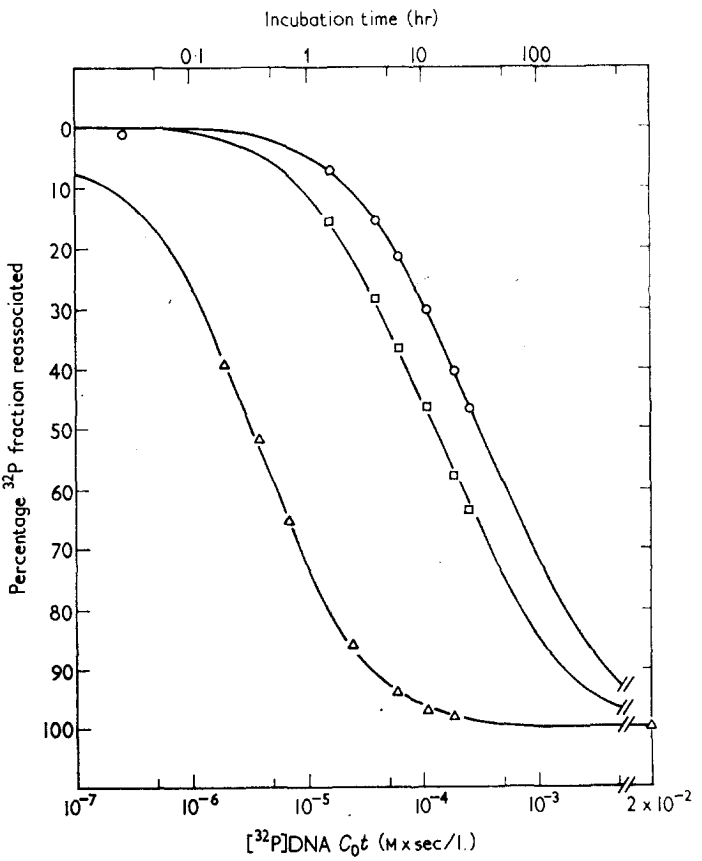

FIG. 2. ^{32}P-labeled SV40 DNA re-association in the presence of unlabeled green monkey DNA. Each reaction mixture contained $1{\cdot}5\times10^{-5}$ O.D./ml. sheared ^{32}P-labeled SV40 DNA (BSC-1, $1{\cdot}2\times10^{6}$ cts/min/μg), 0·0025 M-EDTA, 0·6 M-PO_4 buffer and 40 O.D./ml. sheared salmon sperm DNA (—○——○—), 40 O.D./ml. sheared green monkey DNA (—□——□—) or 40·9 copies ($12{\cdot}2\times10^{-4}$ O.D./ml.) sheared unlabeled SV40 DNA per green monkey cell plus 40 O.D./ml. sheared salmon sperm DNA (—△——△—). The DNA's were heat-denatured and incubated at 68°C. Samples were removed at intervals, the proportion of ^{32}P-labeled SV40 DNA reassociated determined on hydroxyapatite and the results plotted as a function of ^{32}P-labeled SV40 DNA C_0t. Each point represents a total of 460 cts/min.

of $2{\cdot}9\times10^{-4}$. The same mixture with the addition of unlabeled SV40 DNA ($12{\cdot}2\times10^{-4}$ O.D./ml) had a ^{32}P-labeled DNA $C_0t_{1/2}$ of $3{\cdot}4\times10^{-6}$. The ratio of labeled SV40 DNA to unlabeled monkey DNA in the control reaction is equivalent to 0·50 viral equivalent per mammalian cell while in the reconstruction reaction there are 41·4 viral equivalents per cell. The SV40 DNA concentration in this mixture is thus 83 times that in the control. When the actual measurements are plotted as a function of ^{32}P-labeled SV40 DNA C_0t, the midpoint of the control reaction is about 85 times greater than that of the mixture. Or, the reconstruction reaction proceeded 85 times faster than the control and therefore contains 42 SV40 DNA equivalents per mammalian cell compared to 41 equivalents which were actually added. These calculations are illustrated in Table 4.

TABLE 4

Calculation of SV40 genome equivalents

Mol. wt of SV40 DNA $= 3\times10^6$ daltons (Crawford & Black, 1964)
Mol. wt of haploid mammalian DNA $= 1{\cdot}96\times10^{12}$ daltons (Vendrely & Vendrely, 1949; Mann, 1964)
Mol. wt of diploid green monkey DNA/mol. wt of SV40 DNA $= 1{\cdot}33\times10^6$

$$^{32}\text{P-labeled SV40 DNA copies/mammalian cell} = \frac{\text{O.D. } ^{32}\text{P-labeled SV40 DNA}}{\text{O.D. mammalian DNA}} \times 1{\cdot}33\times10^6$$

In reconstruction reaction, Fig. 2:

$$^{32}\text{P-labeled SV40 DNA copies per cell} = \frac{1{\cdot}5\times10^{-5}\text{ O.D. } ^{32}\text{P-labeled SV40 DNA}}{39{\cdot}6\text{ O.D. green monkey DNA}} \times 1{\cdot}33\times10^6 = 0.5$$

Control ^{32}P-labeled SV40 DNA $C_0t_{1/2} = 2{\cdot}9\times10^{-4}$
Reconstruction ^{32}P-labeled SV40 DNA $C_0t_{1/2} = 3{\cdot}4\times10^{-6}$

$$\text{Factor of increased rate} = \frac{2{\cdot}9\times10^{-4}}{3{\cdot}4\times10^{-6}} = 85{\cdot}3$$

SV40 genome equivalents in unlabeled fraction = total concentration of SV40 DNA − concentration of ^{32}P-labeled SV40 DNA
Total concentration of SV40 DNA = ^{32}P-labeled SV40 DNA concentration × factor of increased rate
In reconstruction reaction, Fig. 2:
0·5 copy/cell ^{32}P-labeled SV40 DNA × 85·3 − 0·5 copy/cell = 42·2 copies of SV40 DNA/mammalian cell.

Figure 3 presents a similar situation in which the control reaction contains 3·2 copies ^{32}P-labeled SV40 DNA per cell ($9{\cdot}5\times10^{-5}$ O.D./ml. ^{32}P-labeled SV40 DNA plus 40 O.D./ml. salmon DNA in 0·69 M-Na^+). The experimental reaction contains green monkey DNA in place of salmon at the same concentration (40 O.D./ml.) and the reconstruction reaction contains 0·68 copy (2×10^{-5} O.D./ml.) of unlabeled SV40 DNA in addition to the green monkey DNA. The respective $C_0t_{1/2}$ values for each reaction are $3{\cdot}25\times10^{-4}$ (control), $2{\cdot}7\times10^{-4}$ (green monkey DNA) and $2{\cdot}3\times10^{-4}$ (green monkey DNA plus unlabeled SV40 DNA). This is equivalent to 0·64 copy of SV40-like sequences in the green monkey genome and 1·31 genome equivalents in the mixture. This is very close to that which was added and indicates that is possible to detect relatively small differences in the total concentration of SV40 DNA sequences in DNA mixtures using this technique. A summary of these and two other reconstruction reactions is presented in Table 5.

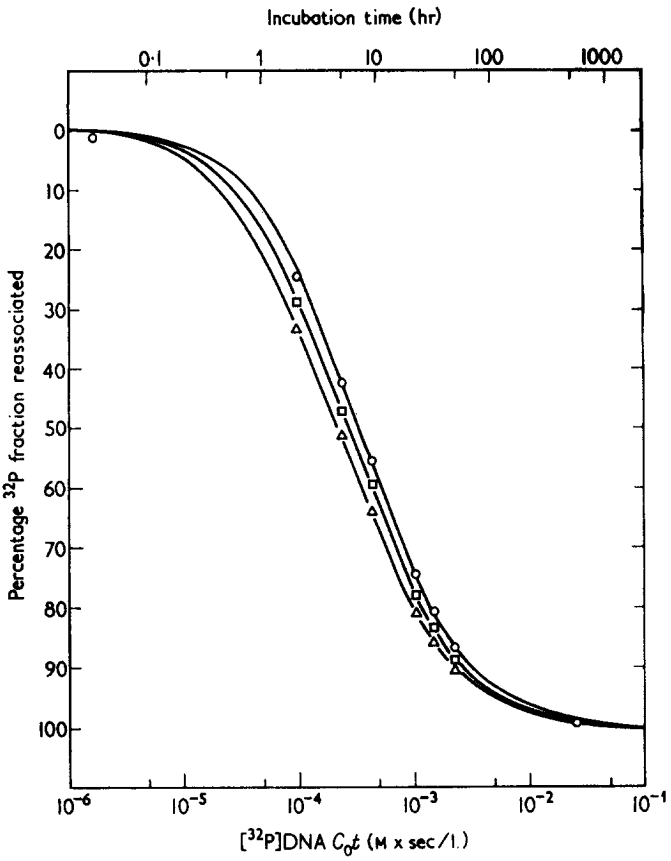

FIG. 3. The effect of green monkey DNA on the re-association of ^{32}P-labeled SV40 DNA. Each reaction mixture contained $9{\cdot}5\times10^{-5}$ O.D./ml. sheared ^{32}P-labeled SV40 DNA (Vero, $3{\cdot}1\times10^{5}$ cts/min/μg), 0·0025 M-EDTA, 0·4 M-PO_4 buffer and 40 O.D./ml. sheared salmon sperm DNA (—○——○—), 40 O.D./ml. sheared green monkey DNA (—□——□—) or 40 O.D./ml. sheared green monkey DNA plus 0·68 copies (2×10^{-5} O.D./ml.) sheared unlabeled SV40 DNA per green monkey cell (—△——△—). The DNA's were heat-denatured and allowed to re-anneal at 68°C. Samples were removed at intervals, the percentage ^{32}P-labeled SV40 DNA re-associated determined on h ydroxyapatite and the result plotted as a function of ^{32}P-labeled SV40 DNA C_0t. Each point represents a total of 160 cts/min.

(c) *Number of SV40 DNA equivalents in normal green monkey DNA*

Figures 2 and 3 illustrate data which suggest that about one-half of an SV40 DNA equivalent is present per cell in DNA extracted from normal green monkey cells. Figure 2 presents striking evidence for the presence of SV40-like sequences within the monkey genome. The ratio of ^{32}P-labeled SV40 DNA to green monkey DNA is such that if only one copy of SV40 DNA was present in the monkey genome, the rate of reaction would triple. The control reaction represents 0·50 copy of SV40 DNA/mammalian cell ($1{\cdot}5\times10^{-5}$ O.D./ml. ^{32}P-labeled SV40 DNA plus 39·6 O.D./ml. salmon DNA in 0·98 M-Na^+) and is half re-associated at a ^{32}P-labeled SV40 DNA C_0t of $2{\cdot}9\times10^{-4}$. This $C_0t_{1/2}$ is somewhat lower than in Figures 1 and 3 since the salt concentration of the incubation mixture was higher (Britten, 1969; Britten & Smith, 1970). The $C_0t_{1/2}$ of the experimental reaction containing green monkey DNA (39·6 O.D./ml) in place of salmon DNA was $1{\cdot}25\times10^{-4}$, 2·32 times faster than the

TABLE 5

Reconstruction reactions: reliability of kinetic analysis in the determination of specific DNA concentration

Input unlabeled SV40 DNA (equivalents/cell)	Unlabeled cellular DNA	Input labeled SV40 DNA (equivalents/cell)	Increased rate factor	Observed SV40 DNA (equivalents/cell)
0·23	salmon sperm	1·11	1·23	0·25
0·68	green monkey	3·2	1·21	0·69
5·81	salmon sperm	1·11	6·04	5·59
40·9	salmon sperm	0·50	85·3	42·2

TABLE 6

SV40 Genome equivalents in green monkey DNA using hydroxyapatite

Labeled SV40 DNA	^{32}P-labeled SV40 DNA / green monkey DNA (O.D.)	Initial ^{32}P-labeled SV40 copies/cell	Control ^{32}P-labeled DNA $C_0t_{1/2}$	Experimental ^{32}P-labeled DNA $C_0t_{1/2}$	Increased rate factor	SV40 genome equivalents per mammalian cell
Vero, prelabeled	$\frac{16{\cdot}67\times10^{-5}}{39}$	5·7	$3{\cdot}7\times10^{-4}$	$3{\cdot}5\times10^{-4}$	1·06	0·32
Vero	$\frac{9{\cdot}5\times10^{-5}}{40}$	3·2	$3{\cdot}25\times10^{-4}$	$2{\cdot}7\times10^{-4}$	1·20	0·64
Primary African green monkey kidney	$\frac{1{\cdot}32\times10^{-5}}{31{\cdot}8}$	0·55	$3{\cdot}5\times10^{-4}$	$2{\cdot}6\times10^{-4}$	1·35	0·19
BSC-1, passage 1	$\frac{2{\cdot}92\times10^{-5}}{31{\cdot}8}$	1·22	$3{\cdot}7\times10^{-4}$	$3{\cdot}0\times10^{-4}$	1·23	0·28
BSC-1, passage 3	$\frac{1{\cdot}98\times10^{-5}}{39{\cdot}4}$	0·67	$3{\cdot}1\times10^{-4}$	$1{\cdot}4\times10^{-4}$	2·21	0·81
BSC-1, passage 7	$\frac{1{\cdot}50\times10^{-5}}{39{\cdot}6}$	0·50	$2{\cdot}9\times10^{-4}$	$1{\cdot}25\times10^{-4}$	2·32	0·66
BSC-1, 5 years	$\frac{1{\cdot}40\times10^{-5}}{39{\cdot}4}$	0·59	$2{\cdot}9\times10^{-4}$	$1{\cdot}45\times10^{-4}$	2·00	0·59

control. This result indicates that normal green monkey DNA contains the equivalent of 0·66 ([2·32×0·50] — 0·50) copy of SV40 DNA per cell.

Similarly, in Figure 3 which is discussed above, the reaction with green monkey DNA is 1·2 times faster than the control reaction with salmon DNA. This is 0·64 ([1·2×3·2] — 3·2) SV40 DNA equivalent per cell. The number of SV40 DNA equivalents per cell, determined with seven labeled viral DNA preparations, is listed in Table 6; the average value from these experiments is 0·50.

(d) *Number of SV40 DNA equivalents in virus transformed cells*

Since SV40 DNA has been shown to be present in virus transformed cells, we next examined the effect of unlabeled transformed cell DNA on the re-association kinetics of labeled SV40 DNA (BSC-1). Figure 4 shows that the rate of SV40 DNA re-association increased from three to sevenfold when equal amounts of five different

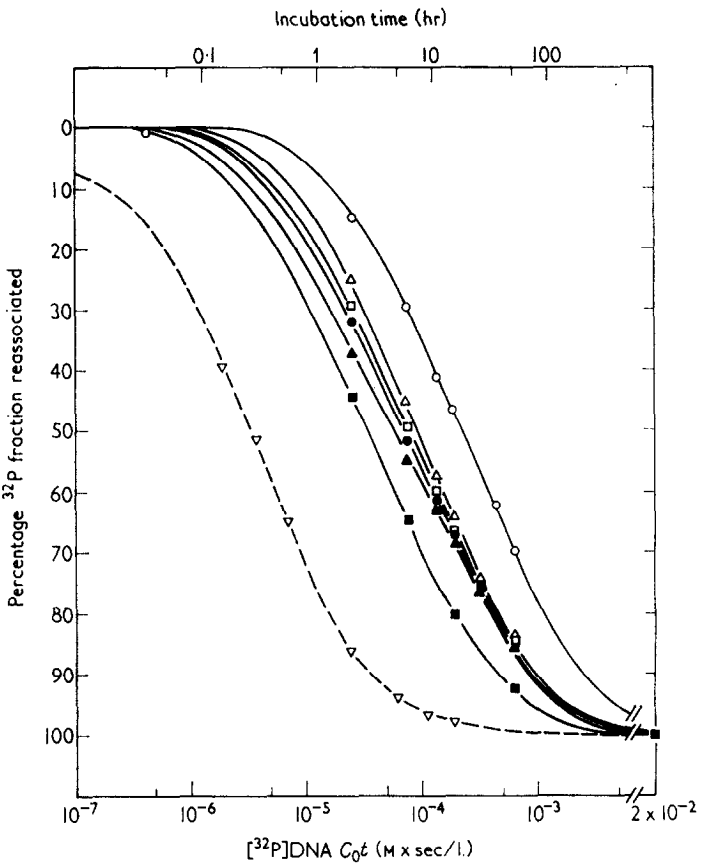

FIG. 4. The re-association of ^{32}P-labeled SV40 DNA with SV40 transformed mammalian DNA's. Each reaction mixture contained $2·5 \times 10^{-5}$ O.D./ml. ^{32}P-labeled sheared SV40 DNA (BSC-1, $9·8 \times 10^5$ cts/min/μg), 0·0025 M-EDTA, 0·6 M PO_4 buffer and 46·6 O.D./ml. sheared salmon DNA (—○——○—), sheared SV-UV-15 clone 1 DNA (—△——△—), sheared SVPy3T3-11 DNA (—□——□—), sheared SVT2 DNA (—●——●—), sheared SV40 hamster tumor DNA (—▲——▲—) or sheared SV-UV-15 clone 5 DNA (—■——■—). Each mixture was heat denatured and incubated at 68°C. Samples were removed at intervals, the percentage ^{32}P-labeled SV40 DNA re-annealed determined on hydroxyapatite and the results plotted as a function of ^{32}P-labeled SV40 DNA C_0t. The reconstruction experiment of Fig. 2 is also included to illustrate the approximate effect of 35 copies of SV40 DNA/transformed cell (– –▽– –▽– –). Each point represents a total of 300 cts/min.

TABLE 7

SV40 Genome equivalents in SV40 transformed cells using hydroxyapatite

Transformed line	^{32}P-labeled SV40 DNA / transformed cell DNA (O.D.)	Initial ^{32}P-labeled SV40 copies/cell	Control ^{32}P-labeled DNA $C_0t_{1/2}$	Experimental ^{32}P-labeled DNA $C_0t_{1/2}$	Increased rate factor	SV40 genome equivalents per mammalian diploid cell
SV-UV-15 clone 1	$\frac{2\cdot5\times10^{-5}}{46\cdot6}$	0·67	$2\cdot5\times10^{-4}$	$9\cdot8\times10^{-5}$	2·55	1·04
SVPy3T3-11	$\frac{2\cdot5\times10^{-5}}{46\cdot6}$	0·67	$2\cdot5\times10^{-4}$	$8\cdot0\times10^{-5}$	3·13	1·42
SVT2	$\frac{2\cdot5\times10^{-5}}{46\cdot6}$	0·67	$2\cdot5\times10^{-4}$	$7\cdot5\times10^{-5}$	3·33	1·56
SV40 hamster tumor	$\frac{2\cdot5\times10^{-5}}{46\cdot6}$	0·67	$2\cdot5\times10^{-4}$	$6\cdot1\times10^{-5}$	4·08	2·08
SV-UV-15 clone 5	$\frac{2\cdot5\times10^{-5}}{46\cdot6}$	0·67	$2\cdot5\times10^{-4}$	$3\cdot7\times10^{-5}$	6·76	3·86

transformed cell DNA's were present in the reaction mixture. This figure also includes the reconstruction reaction previously shown in Figure 2 to illustrate the magnitude of displacement produced by about 35 copies of SV40 DNA per cell. The calculated number of SV40 genome equivalents in each of the five transformed cell lines tested is listed in Table 7. A separate experiment indicated 0·45 viral DNA equivalent per cell of normal 3T3 DNA. Since these lines were derived from 3T3, there is about one SV40 DNA equivalent per diploid cell in four of the lines examined.

The kinetic data from both the normal green monkey DNA and the transformed cell DNA experiments were subsequently analyzed with an IBM 1130 computer using least squares approximation to the theoretical curve. The computer-calculated $C_0t_{1/2}$ values for labeled SV40 DNA re-annealing in the presence of green monkey DNA were equivalent to 0·52 copy of SV40 DNA per genome. The comparable SV40 genome equivalent in three SV40 transformed cell lines was 1·50. The computer-generated C_0t curves also suggested that the reactions containing SV40 hamster tumor DNA and SV-UV-15 clone 5 DNA as well as those containing green monkey DNA may be biphasic. This could occur if only part of the SV40 DNA molecule is in the transformed genome.

4. Discussion

Our results indicate that it is possible to detect less than one SV40 DNA equivalent per mammalian cell genome using the kinetics of DNA re-association. At this level of sensitivity, contamination of normal green monkey DNA or salmon DNA with SV40 DNA during purification could produce the results we report since as little as 10^{-4} μg of viral DNA when added to 100 μg of animal DNA would be sufficient to add one SV40 genome equivalent per mammalian cell. In the experiments described above, the observed $C_0t_{1/2}$ of ^{32}P-labeled SV40 DNA is similar even when the ratio of SV40 DNA copies to mammalian cell genomes varies from 0·5 to 41. If an appreciable amount of SV40 DNA contamination had been present, it would have had a greater effect at low ^{32}P-labeled SV40 DNA inputs and resulted in a significantly lower ^{32}P-labeled SV40 DNA $C_0t_{1/2}$ for these reactions. Similar analysis of the green monkey DNA data also shows any contamination to be negligible.

The results presented in Table 6 indicate that the equivalent of about 0·5 copy/cell of SV40 DNA sequences is present in the DNA of normal green monkey cells. It is not yet known whether this represents a complete SV40 genome in every other cell or part of the SV40 DNA molecule in each cell. These sequences, if clustered in one region, may represent an "integration site" for transforming viral DNA. This is quite similar to the situation in *Escherichia coli* where non-lysogenized cells were found to contain about one-third of a lambda genome (Cowie & McCarthy, 1963).

These results are in marked contrast to those of Aloni *et al.* (1969). The difference may possibly be explained by the different viral strains used. Our attempts to find similar homology by the examination of several viral strains and the serial passage of SV40 in BSC-1 cells were unsuccessful.

The number of SV40 genome equivalents found in viral transformed cells are quite low compared to results reported using other methods (Westphal & Dulbecco, 1968; Tai & O'Brien, 1969). In one line, SVPy3T3-11, about one SV40 genome equivalent per cell (1·5—0·5 found in uninfected 3T3 DNA) was found whereas Westphal & Dulbecco (1968) reported 44. In those experiments, SV40 transformed cell DNA, immobilized on nitrocellulose filters, was reacted with labeled SV40 complimentary

RNA. The number of SV40 genome equivalents per transformed cell was then calculated on the basis of a series of reconstruction experiments employing known mixtures of normal mammalian DNA and SV40 DNA. The number of SV40 genome equivalents per mammalian cell as calculated from these calibration experiments do not agree with our findings.

These differences may be attributed to the inaccessibility of the immobilized SV40 DNA for reaction with the SV40 complimentary RNA added during the reconstruction phase of their experiments. One possible explanation could be that the large excess of immobilized unsheared mammalian DNA physically impedes the reaction of SV40 complimentary RNA with SV40 DNA. This is suggested by the large variation of SV40 complimentary RNA reacting with SV40 DNA in the calibration experiments. Another possibility is the selective loss of SV40 DNA from the filters resulting in much smaller amounts of viral DNA available for reaction than was thought to be present.

One copy of SV40 DNA per cell probably means that it is integrated into a single chromosome but it cannot be ruled out that every other cell carries two integrated SV40 DNA molecules on each of a pair of chromosomes. This seems unlikely since each cell line was cloned from a single cell. This observation is again quite similar to that found with lysogenized *E. coli* where an additional lambda genome is integrated into the bacterial genome (Cowie & McCarthy, 1963; Green, 1963; Cowie & Hershey, 1965; Gottesman & Yarmolinsky, 1968).

The actual number of SV40 DNA equivalents in these transformed cell lines is probably closer to two per genome rather than one since many of these cells approach tetraploidy. The average chromosome count for SVT2 in our laboratory was 76 (unpublished data). However, in the ultraviolet-irradiated SV40 transformed lines, the kinetics of re-asssociation suggest that less than the entire SV40 genome is present within the nucleus. This may explain the failure to rescue virus following cell fusion. In SV-UV-15 clone 5, the multiple copies may represent non-random heteroploidy duplicating integrated viral sequences. If this is the case, multiple copies of SV40 DNA could be present in other heteroploid viral transformed lines. The total number per cell, however, should still remain low.

We are indebted to Dr Roy Britten of the Carnegie Institution of Washington for his suggestions and the use of the "fingr" program on the IBM 1130 computer and to Mrs Janet Byrne and Mr Thomas Zimmerman for skillful technical assistance.

REFERENCES

Aloni, Y., Winocour, E. & Sachs, L. (1968). *J. Mol. Biol.* **31**, 415.
Aloni, Y., Winocour, E., Sachs, L. & Torten, J. (1969). *J. Mol. Biol.* **44**, 333.
Berns, K. I. & Thomas, C. A. (1965). *J. Mol. Biol.* **11**, 476.
Britten, R. J. (1969). *Yearb. Carnegie Instn.* **67**, 332.
Britten, R. J. & Kohne, D. E. (1968). *Science,* **161**, 529.
Britten, R. J. & Smith, J. (1970). *Yearb. Carnegie Instn.* **68**, 378.
Cowie, D. B. & Hershey, A. D. (1965). *Proc. Nat. Acad. Sci., Wash.* **53**, 57.
Cowie, D. B. & McCarthy, B. J. (1963). *Proc. Nat. Acad. Sci., Wash.* **50**, 537.
Crawford, L. V. & Black, P. H. (1964). *Virology,* **24**, 388.
Denhardt, D. T. (1966). *Biochem. Biophys. Res. Comm.* **23**, 641.
Eagle, H. (1959). *Science,* **130**, 432.
Earley, E., Peralta, P. H. & Johnson, K. M. (1967). *Proc. Soc. Exp. Biol. and Med.* **125**, 741.
Gillespie, D. & Spiegelman, S. (1965). *J. Mol. Biol.* **12**, 829.

Gottesman, M. E. & Yarmolinsky, M. B. (1968). *Cold Spr. Harb. Symp. Quant. Biol.* **33**, 735.
Green, M. H. (1963). *Proc. Nat. Acad. Sci., Wash.* **50**, 1177.
Kohne, D. E. (1969). *Yearb. Carnegie Instn.* **67**, 310.
Levine, A. J. & Teresky, A. K. (1970). *J. Virology*, **5**, 451.
McCarthy, B. J. & Hoyer, B. H. (1964). *Proc. Nat. Acad. Sci., Wash.* **52**, 915.
Mann, T. (1964). In *The Biochemistry of Semen and of the Male Reproductive Tract*, p. 147. New York and London: John Wiley & Sons.
Radloff, R., Bauer, W. & Vinograd, J. (1967). *Proc. Nat. Acad. Sci., Wash.* **57**, 1514.
Reich, P. R., Black, P. H. & Weissmann, S. M. (1966). *Proc. Nat. Acad. Sci., Wash.* **56**, 78.
Sambrook, J., Westphal, H., Srinivasan, P. R. & Dulbecco, R. (1968). *Proc. Nat. Acad. Sci., Wash.* **60**, 1288.
Tai, H. T. & O'Brien, R. L. (1969). *Virology*, **38**, 698.
Trilling, D. M. & Axelrod, D. (1970). *Science*, **168**, 270.
Vendrely, R. & Vendrely, C. (1949). *Experimentia*, **5**, 327.
Westphal, H. & Dulbecco, R. (1968). *Proc. Nat. Acad. Sci., Wash.* **59**, 1158.
Yoshiike, K. (1968). *Virology*, **34**, 391.