BASIC MECHANISM IN CARCINOGENESIS

Progress Report

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OUTLINE

Progress has been made in the following four areas during the past twelve months:

I. Chemistry and Biochemistry of 6-Hydroxyl Benzo(a)pyrene (6-OH-B(a)P).

- A. Autooxidation of 6-OH-B(a)P.
- B. Formation and separation of the 6-oxo-benzo(a)pyrene (6-oxo-B(a)P) radical.
- C. Reaction products of poly(A) and 6-OH-B(a)P in aqueous buffer-ethanol (1:1) mixture.
- D. Formation of 6-oxo-B(a)P from B(a)P in rat liver homogenate.
- E. Reaction of autooxidation products of 6-OH-B(a)P with T7 DNA.

II. Development of an in vitro Morphological Transformation Assay for Mechanistic Study.

III. Physical Penetration of T7 DNA (Naked Form or in Phage Particle) into Syrian Hamster Embryo Cells (SHE Cells).

- A. The formation of a stable physical complex between T7 DNA and SHE cells.
- B. Establishment of the penetration of intact T7 DNA into SHE cells.
- C. The size of the T7 genome inside SHE cells.

IV. The Fate of T7 DNA/Genome Inside the SHE Cells in a Continuing Culture for a Prolonged Period--Evidence for T7 DNA Replication in SHE Cells.

PUBLICATIONS

A. Chapters in books

1. P.O. P. Ts'o, W.M. Caspary, B.I. Cohen, J.C. Leavitt, S.A. Lesko, Jr., R.M. Lorentzen, and L.M. Schechtman. "Basic Mechanisms in Polycyclic Hydrocarbon Carcinogenesis" in Chemical Carcinogenesis, Part A, P.O.P. Ts'o and J.A. DiPaolo, eds. (Marcel Dekker, Inc.) 1974.
2. J.C. Leavitt, L.M. Schechtman, P.O.P. Ts'o, E. Borenfreund, and A. Bendich. "Uptake of T7 DNA by Hamster Embryo Cells in Tissue Culture" in Chemical Carcinogenesis, Part B, P.O.P. Ts'o and J.A. DiPaolo, eds. (Marcel Dekker, Inc.) 1974.

B. Abstracts

1. L.M. Schechtman, S.A. Lesko, R.M. Lorentzen, and P.O.P. Ts'o. "A Cellular System for the Study of the Chemical Reactivity and Transforming Ability of Benzo(a)pyrene and Its Derivatives." Presented at the American Association for Cancer Research, Houston meeting, 1974.
2. W. Caspary, S. Lesko, R. Lorentzen, and P. Ts'o. "Properties and Enzymic Formation of 6-Oxo-Benzo(a)-pyrene Radical. To be presented at the Biochemistry/Biophysics June 1974 meeting.
3. J. Leavitt, L. Schechtman, and P. Ts'o. "Penetration of Mammalian Cells by Bacteriophage T7 DNA." To be presented at the Biochemistry/Biophysics June 1974 meeting.
3. R. Lorentzen, W.J. Caspary, and P.O.P. Ts'o. "Auto-oxidation of 6-Hydroxybenzo(a)pyrene, A Metabolite of Benzo(a)pyrene." Presented at the American Chemical Society meeting, Atlantic City, 1974.

Progress Report (May 1973 - May 1974)

I. Chemistry and Biochemistry of 6-Hydroxyl Benzo(a)pyrene
(6-OH-B(a)P).

A. Autooxidation of 6-OH-B(a)P.

6-OH-B(a)P undergoes rapid autooxidation in aqueous solutions. As previously reported, the majority of the oxidized products are formed with a half-time of 30-60 minutes in buffered water-ethanol solutions at room temperature. The dependence of atmospheric oxygen has been demonstrated. Replacement of dissolved air by oxygen-free nitrogen and vigorous exclusion of air from the immediate environment of the sample results in the near cessation of autooxidation.

The major products of autooxidation have been identified. In 1/1 buffered water (pH 7.0)-ethanol solutions at room temperature or 37°, 6-OH-B(a)P (5×10^{-4} M) is autooxidized to yield three quinones. These can be separated by alumina chromatography using benzene-ethanol gradient elution. They have been identified as the yellow-green 6,12-B(a)P dione, the orange 1,6-B(a)P dione, and the red 3,6-B(a)P dione. These products are often found as cellular and microsomal "metabolites" of B(a)P. These results suggest their presence in metabolism studies is due to their labile precursor 6-OH-B(a)P.

In addition, a small amount of a violet colored, orange fluorescing compound is detected whose structure has not been

determined. Associated with this late eluting fraction was an ESR signal with detailed hyperfine structure different from the 6-oxo-B(a)P radical signal. It was a non-symmetrical signal, suggesting two or more paramagnetic species were present. The following day the signal was reduced to a stable singlet with non-resolvable hyperfine structure. Neither the visible spectrum nor the color of the fluorescence changed in this period. This singlet appears to be the source of the ESR signal observed in mixtures of the autooxidized products of 6-OH-B(a)P. It has been shown that the mixture of auto-oxidized 6-OH-B(a)P products is capable of introducing single and double strand breaks when incubated with T7 phage DNA.

B. Formation and separation of the 6-oxo-benzo(a)pyrene (6-oxo-B(a)P) radical.

Kinetic studies indicate that the 6-oxo-radical is unlikely as an obligatory intermediate in the spontaneous oxidation of 6-OH-B(a)P to quinones, but is likely the reactive species in the binding reaction of 6-OH-B(a)P with nucleic acids. Upon replacement of oxygen by nitrogen in the system, both the formation and the decay of 6-oxo-B(a)P is greatly impeded. Reinduction of a small amount of air into this system previously filled with nitrogen causes a rapid formation of radical. When a small amount of $K_3Fe(CN)_6$ (potassium ferricyanide) instead of air is introduced into the nitrogen-filled system, the 6-oxo-radical concentration increased proportionally to a significantly higher but steady level. This high level of

radical concentration is maintained unless an excess of $K_3Fe(CN)_6$ is added. The results indicate that in the absence of O_2 , $K_3Fe(CN)_6$ can serve as a selective oxidant in converting 6-OH-B(a)P to 6-oxo-B(a)P radical. Experiments are currently in progress in examining whether or not other biological oxidants, such as oxidized glutathione or oxidized ascorbic acid can also serve this role in converting 6-OH-B(a)P selectively to 6-oxo-radical.

The 6-oxo-B(a)P radical is relatively stable in benzene and can persist for many days if laboratory light is excluded. Preliminary experiments have demonstrated that it is stable to column chromatography. Partial separation of the 6-oxo-radical from the other 6-OH-B(a)P oxidation products has been accomplished by alumina chromatography. It has been obtained as a mixture with the 6,12-B(a)P dione. Its presence and subsequent slow decay was monitored both by ESR and visible spectroscopy. The oxo-radical has a strong absorbance maximum at 428 nm, but other features of its uv-visible spectrum are not clear, due to its mixture with the highly colored 6,12 dione. This suggests that the 6-oxo-radical can, in principle, be isolated, purified and characterized. Its stability in benzene under normal laboratory conditions may allow it to be used in experiments much as a stable agent. There is some evidence that it is stable as a solid also. Under more careful conditions (absence of light and oxidizing agents, low temperatures, etc.),

storage for a long period may be possible. With this preliminary knowledge, experiments are planned to determine the role (if any) the 6-oxo-radical plays in the autooxidation of 6-OH-B(a)P, the interaction of 6-OH-B(a)P with DNA and RNA, and the toxicity and transforming ability of 6-OH-B(a)P towards cells in culture. Presently, the lack of purity and small amount of radical isolated have not allowed identification of its decay products. Difference spectra (uv-vis) of the mixture of 6,12 dione plus the radical reaction products suggest the 3,6 dione is a possible product. Thin layer chromatography indicates traces of the orange-fluorescing, violet compound may be present.

C. Reaction products of poly(A) and 6-OH-B(a)P in aqueous buffer-ethanol (1:1) mixture.

[³H]-6-OH-B(a)P binds covalently to poly(A). Hydrolysis of the bound polymer under alkaline or enzymatic (venom diesterase) conditions produces a hydrocarbon-nucleotide adduct which can be separated from the bulk of the hydrolysate by Sephadex chromatography. Bacterial alkaline phosphatase treatment of this adduct does not appear to change its chromatographic properties. Furthermore, after phosphatase treatment, the adduct behaves as a negatively charged species in sucrose gradient electrophoresis. This suggests that the 6-OH-B(a)P-poly(A) adduct is partially resistant to either phosphatase or venom diesterase treatment or both.

[³H]-6-OH-B(a)P also reacts at a low level with 5'-AMP under identical conditions to its reaction with poly(A). The

Sephadex chromatographic properties and the sucrose electrophoresis mobilities of the isolated radioactive adduct are very similar to those of the hydrolyzed poly(A) adduct, both before and after phosphatase treatment. However, the method of paper electrophoresis discriminates between adducts formed with polymer and monomer. The monomer adduct moves as a negatively charged species on paper electrophoresis. The hydrolyzed polymer adduct moves very little, if any. This difference could be explained by incomplete hydrolysis on the polymeric material resulting in oligomer fragments which adhere to the paper tightly. Characterization of these adducts by the use of ^{14}C -poly(A) and ^3H -6-OH-B(a)P is currently in progress.

D. Formation of 6-oxo-B(a)P from B(a)P in rat liver homogenate.

The addition of B(a)P to rat liver homogenate fortified with a NADPH generating system produces an epr signal identical to the 6-oxo-B(a)P radical. This system has been previously reported by Nagata. In our experiments, when rat liver was heated or when NADPH was omitted, no signal was observed, suggesting that an enzymatic step is involved in the conversion of B(a)P to the 6-oxo-radical.

A typical experiment uses 10 g of rat liver from Sprague Dawley female rats which are homogenized in 50 ml of 1.15% KCl. The homogenate is spun at 1500 x g for 20 min. Niacinamide, NADP and 6 glucose phosphate are added to the supernatant in 0.1 M potassium phosphate buffer (pH 7.4) at 53 mM, 0.13 mM and

3.25 mM respectively. Five mg B(a)P in 1 ml acetone is then added to the homogenate at 37°C. At a specified time, the reaction is quenched with two volumes of cold benzene. The radical is extracted twice with benzene, dried under reduced pressure and the residue is brought up in 1.5 ml benzene for epr measurement. The data indicates that the radical is formed rapidly and the concentration reaches a plateau in about 10 min. After 30 min the radical concentration starts to decrease. Radical formation is dependent on protein concentration at least up to about 16 mg protein per ml.

To determine the minimum amount of B(a)P that is converted to 6-OH-B(a)P, we compared the amount of radical formed when B(a)P was mixed with liver homogenate to the radical concentration observed when 6-OH-B(a)P was added (see Table 1). These results indicate that increasing the B(a)P concentration above 36 μ M does not increase the amount of radical observed under the conditions used. In calculating the percentage of B(a)P converted to 6-OH-B(a)P (approximately 4-5%), it was assumed that the radical concentration observed by a single addition of 6-OH-B(a)P to liver homogenate is a measure of the 6-OH-B(a)P formed when converted from B(a)P by the microsomal enzyme system. This assumption is valid in the limit of rapid conversion of B(a)P to 6-OH-B(a)P and, therefore, this percentage is a minimum value. This experiment indicates that 6-OH-B(a)P is formed from B(a)P in a biological system in significant

quantities to make it a possible candidate as a proximate carcinogen of B(a)P.

Since the 6-oxo-radical is a transient species whose stability depends markedly on the solvent, kinetic information concerning this radical in various solvents was obtained. The rate of formation from 6-OH-B(a)P and decay is extremely slow in benzene and hence this solvent was used to extract the radical from the rat liver homogenate system. In water and in the rat liver homogenate, the rate of formation of radical from the added 6-OH-B(a)P is extremely rapid. The decay of the radical in the presence of the enzyme is slower than in water suggesting that the radical may be protected in hydrophobic regions of the protein solution.

E. Reaction of autooxidation products of 6-OH-B(a)P with T7 DNA.

The reaction of 6-OH-B(a)P with DNA and poly(A) has been reported in a previous progress report. In this section, we wish to examine the possibility of the reaction of nucleic acid with the autooxidized products of 6-OH-B(a)P. Normally, the 6-OH-B(a)P is oxidized to quinones with the half-life of 40-60 min. In this reaction, the [^3H]-6-OH-B(a)P (0.3 mM) was first incubated at 37°C in ETOH-2X HMP, pH 7.1 (1:1) for 67 and 92 hours and the oxidation of 6-OH-B(a)P was followed by ESR. After disappearance of the characteristic multiplet of 6-OH-B(a)P, a singlet signal was seen in the solution. An

equimolar amount of T7 DNA (conc. = 0.3 mM) was added to the quinones and then incubated at 30° in 2X HMP-ETOH, pH 7.0 (1:1) for 1/4, 3, 6 and 23 hr. Essentially no difference in radical concentration was detected by measurement of singlet intensity after incubation of the quinones with T7 DNA for 23 hr. Furthermore, there was no diminution in radical signal when the T7 DNA-quinone incubation was carried out in the presence of EDTA or cysteamine (conc. = 0.3 mM).

After incubation of T7 DNA with quinones, 1.5 volumes of HMP- 10^{-4} M EDTA were added to each fraction. The samples were then extracted for 5 min. six times with an equal volume of phenol saturated with 0.5 M Na_2HPO_4 . This is followed by two 5 min. ether extractions using 3 volumes of HMP saturated ether. The samples were then dialyzed against 1 liter of HMP- 10^{-4} M EDTA (pH 7.0) four times (total volume = 4 liters).

The molecular weight of the T7 DNA was determined by banding the reacted material in 0.1 N NaOH-0.9 M NaCl using the empirical formula of Studier. The number of scissions per T7 DNA molecule was determined by the equation of Charlesby. The number of hydrocarbon molecules bound per T7 genome was determined by assuming that the specific activity of the DNA adduct was the same as the original [^3H]-6-OH-B(a)P. The kinetics of binding and of strand scission are shown in Table I for the experiment in which the 6-OH-B(a)P had been preincubated for 67 hr. After 23 hr incubation of T7 DNA with BP-quinones in this kinetic experiment, there is about one hydrocarbon

molecule bound per 13,700 bases and around 25.8 single-strand breaks per genome. Under these conditions there were no double-strand scissions produced in the T7 DNA molecule.

To determine if the single-strand scissions produced in T7 DNA by incubation with BP quinones are the result of alkaline conditions of centrifugation, the following experiment was performed: The 23 hr sample was dialyzed against 95% formamide-5% H_2O (pH 6.6), then heated in dialysis tubing for 2 hr at 37° , placed in ice water for about 1 min, and finally dialyzed against HMP- 10^{-4} M EDTA-6% formaldehyde (pH 6.6) at 4° . The denatured sample was examined by band centrifugation in 0.05 M NaCitrate - 1 M NaCl. This sample had numerous single-strand scissions, the $S_{20,w}^0$ being 22.4 compared with 41.9 for control. This calculates to about 22 scissions per T7 genome. The results indicate that the scissions are not produced from apurinic sites or other alkaline labile structures.

Table II shows the results of reacting B(a)P quinones, formed by 92 hours of preincubation, with T7 DNA or with DNA plus equimolar cysteamine or EDTA for 23 hr. Extractions were carried out with phenol and ether as described above. The presence of EDTA completely inhibits the production of single-strand scissions in DNA, but it also inhibits adduct formation to some extent. The presence of cysteamine increases the production of single-strand scissions and greatly accelerates adduct formation. In this experiment double strand breaks were detected in T7 DNA. These data indicate that scissions in DNA

TABLE I: Adduct Formation and Single Strand Scissions Produced in T7 DNA by B(a)P Oxidation Products

Time	Base/Adduct	Single-Strand Scissions/Genome
1/4 hour	154,000	0
3 "	40,000	0.5
6 "	36,000	1.1
23 "	13,700	25.8

TABLE II: Effect of Various Compounds on Adduct Formation and Strand Scission in T7 DNA by B(a)P Oxidation Products

Compound	Base/Adduct	Single-Strand Scissions/Genome	Double-Strand Scissions/Genome
None	8,800	45	0.5
EDTA	10,600	70.5	-
Cysteamine	1,500	73	2.3

resulting from incubation with BP quinones are the results of a direct chemical reaction of some agent with DNA.

In conclusion, it is certain that the autooxidized product(s) of 6-OH-B(a)P can cause breakage of DNA which can be prevented by the addition of EDTA but not by cysteamine, a radical scavenger. While the mechanism of this reaction is still under investigation, it is certain that the oxidized product(s) of 6-OH-B(a)P can be chemically and, therefore, biologically reactive. The amount of tritium label attached to the DNA is rather small, and the nature and the origin of this attachment is not yet understood and is currently being investigated.

II. Development of an in vitro Morphological Transformation Assay for Mechanistic Study.

This assay was developed with the following characteristics:

1. Normal Syrian hamster embryo cells of early passages (3-4 passages) are used.
2. The system does not require a feeder layer.
3. The system can sustain a vigorous washing to remove all the polycyclic hydrocarbons after a given period of exposure.
4. The assay takes only 8-9 days.

The protocol for our assay system is outlined on Chart 1. Plastic Petri dishes, 100 mm in diameter, are seeded 24 hr before treatment is initiated with early passage Syrian hamster whole embryo cells (SHE cells) in the absence of any feeder cell

layer. Control dishes are plated with 20,000 - 25,000 cells and carcinogen-treated dishes are plated with up to 50,000 cells, thereby allowing for cytotoxic effects and lowered cloning efficiencies due to exposure to the hydrocarbon. Our effective cloning efficiency is 1-2% or from 200 to 500 clones per 100 mm plate. The solvent for all hydrocarbons is DMSO. The carcinogen in DMSO is diluted to 2X concentration in modified Dulbecco's Eagle's reinforced medium (Biolabs, Northbrook, Ill.) supplemented with 10% fetal bovine serum (Reheis Chemical Co.) in the absence of antibiotics. Tested cultures were exposed to the carcinogens for defined periods of time at 37°C. Control cultures were exposed to DMSO solvent only, $\leq 0.5\%$ final concentration. Treatment was terminated by withdrawal of the incubation medium followed by five washes with complete medium, allowing further elution of unbound hydrocarbon during 10 minute incubation periods at 37° for each wash. Afterwards, the cells are permitted to clone in fresh medium for 6-8 days. The resulting colonies are then fixed in 100% methanol, stained with 10% aqueous Giemsa, and scored for morphological transformation.

Of paramount importance to this assay is the effectiveness of the washing procedure. Chart 2 illustrates the efficacy of the washing procedure in rendering hydrocarbon from the 10^6 SHE cells after the monolayer has been exposed to either 10 μg [^3H]-B(a)P/ml or 5 μg [^3H]-6-OH-B(a)P/ml for the time intervals indicated. In this experiment, the cell monolayer was washed as

described after carcinogen treatment, dispersed with trypsin: EDTA solution (0.25%:0.1%), an aliquot used to determine cytotoxic effects, and the remainder lysed by freeze-thaw (4X) and assayed for [^3H]-hydrocarbon remaining associated with the cells or covalently linked to cellular components. Chemical binding was determined by precipitation of the cell lysate with ice cold 0.2 M perchloric acid; precipitates were trapped on glass fiber filters where they were washed exhaustively, three times with 95% ethanol, three times with benzene, and three times with hexane (5-10 ml/wash). Filters were dried and counted for radioactivity.

This extensive washing procedure effectively removes 99.94% of the B(a)P applied. The amount of B(a)P that becomes cell-associated increases gradually until 3% of that administered remains associated with the rinsed cell monolayer after 24 hr. The viability of these cells remains constant and only after 24 hr of exposure are there signs of B(a)P becoming slightly cytotoxic (<6% cell death). With respect to the covalent linkage of B(a)P to cellular components, the background level of 0.02% would indicate that we find little, if any, covalent binding of B(a)P until a 24 hr exposure.

Regarding the binding of 6-OH-B(a)P, the amount does not become noticeably high; even at 24 hr, only less than 1% of the 6-OH-B(a)P applied becomes non-washable. However, it appears that of the amount which becomes non-releasable from the cells, a steadily increasing quantity becomes covalently bound, and this

linkage seems to occur almost immediately. Furthermore, 5 µg 6-OH-B(a)P/ml already begins to show cytotoxic effects after only 6 hr exposure and becomes extremely toxic with a 24 hr treatment (>60% kill). When the amount of hydrocarbon bound is normalized to the number of cells present at the end of the treatment period, a direct comparison can be made between the binding of B(a)P and that of 6-OH-B(a)P (Chart 3). The parent compound, B(a)P, associates with the washed cell monolayer to a greater extent than does its active metabolite. However, the level of covalent linkage of 6-OH-B(a)P is higher than that of B(a)P.

A series of photographs has been included to demonstrate our classification of (1) normal, (2) B(a)P-transformed, and (3) 6-OH-B(a)P-transformed clones. The normal one illustrates a characteristic organized arrangement of cells in a stream-line pattern. The transformed clone displays disorganized growth patterns and disoriented criss-cross arrangement of spindle-shaped cells. Transformation test results are scrutinized by two readers in our laboratory and the clones in the photographs have been independently confirmed as morphologically transformed by Dr. Bruce Casto of BioLabs, Inc. Northbrook, Ill.

Chart 4 shows a dose-response relationship for 2 hr and for 24 hr exposure to B(a)P in terms of the number of transformed colonies per surviving clones (%) as a function of µg B(a)P/ml. Over 9000 clones were examined to generate the 24 hr curve. An approximately linearly increasing transformation frequency was

obtained, up to ~7%, with logarithmically increasing doses of B(a)P. The fractional numbers represent the number of transformed clones vs. the total number of clones surveyed for that time point.

The 2 hr curve seems to suggest that even with such a brief exposure period, after a critical dose is reached (e.g. $>1 \mu\text{g B(a)P/ml}$), transformation frequency may follow a similar linear pattern. However, consideration must be given to the possibility that the level of transformation obtained here might be due to a residuum of B(a)P not removed by the washing procedure. This concern is due to the finding that the percent of transformation is proportioned to the logarithm of the concentration of B(a)P. Therefore, even a reduction of $10 \mu\text{g BP/ml}$ to $0.1 \mu\text{g BP/ml}$ only reduces the percent of transformation from ~6.5% to ~3%.

The results presented in chart 5 represent an attempt to demonstrate exposure requirements for B(a)P at $10 \mu\text{g/ml}$ necessary to achieve transformation. As implied above, we were surprised to find that a short exposure time, such as 2 or 4 hr, could yield a significant level of transformation. Therefore, at an applied dose of $10 \mu\text{g B(a)P/ml}$, a shorter exposure period might be all that is required to obtain morphological transformation. However, the critical point raised regarding the effectiveness of the extensive washing process becomes even more important. At this high dose of B(a)P (e.g. $10 \mu\text{g/ml}$) part

of the observed level of transformation with brief treatment periods could be attributable to residual carcinogen left behind. Since the system is so sensitive to B(a)P, it became obvious that such studies must be re-examined at lower applied doses, e.g., 1-0.1 μg B(a)P/ml, such that residual amounts of non-washable hydrocarbon become insignificant.

With the development of this morphological transformation assay, studies were undertaken to examine the carcinogenic potential of 6-OH-B(a)P. Chart 6 shows the relative frequencies of transformation obtained with B(a)P at 10 and 1 $\mu\text{g}/\text{ml}$ and with 6-OH-B(a)P at 10, 3, and 1 $\mu\text{g}/\text{ml}$ for a 24 hr exposure period. From these data it appears that 6-OH-B(a)P can cause significant levels of in vitro morphological transformation.

In vivo confirmation studies are presently being carried out in parallel and concurrently with in vitro morphological transformation assays. These involve the quantitative isolation of morphologically aberrant clones as determined by phase-contrast microscopy. Individual clones are transferred to new dishes which they populate, and after 2-3 subcultivations are inoculated subcutaneously into isologous new-born Syrian hamsters. In vivo confirmation of neoplastic transformation is acknowledged upon tumor initiation. In this way it is hoped that a reliable quantitative relationship can be obtained between tumor induction and in vitro morphological transformation.

Golden Syrian Hamster Embryonic Fibroblasts (early passage),
Exposed to 0.1% DMSO for 24 Hours. Normal Clone as Control.

Golden Syrian Hamster Embryonic Fibroblasts (early passage)
Exposed to 3 ug 6-Hydroxy B(a)P. Two Transformed Clones
(Independently Identified by L. Schechtman from Johns Hopkins
University and B. Casto, Biolab, Inc.)

2 hours exposure

24 hours exposure

Golden Syrian Hamster Embryonic Fibroblasts (early passage)

Exposed to 10 μ g (B(a)P. Two Transformed Clones (Independently Identified by L. Schechtman from Johns Hopkins University and B. Casto, Biolab, Inc.)

4 hours exposure

24 hours exposure

Chart I

Morphological in vitro Transformation Assay

- A. 2.5×10^4 - 5×10^4 cells/dish seeded without feeder layer
- B. Treatment x hours
- C. 5 washes with complete medium
- D. Incubation for cloning 6 days
- E. Fix, stain, score

Chart II

Sample	[³ H]HC Remaining with Washed Cell Monolayer	[³ H]HC Covalently Linked to Cell Components (Washed PCA ppt)	Cell Survival
<u>B(a)P</u> (10 µg/ml)	%	%	%
0 hr (15 sec)	0.06	0.02	100
1	0.3	0.009	100
2	0.3	0.015	100
4	0.4	0.013	100
6	0.5	0.016	100
24	3.0	0.071	94.1
<u>6-OH-B(a)P</u> (5 µg/ml)			
0 hr (15 sec)	.02	0.004	100
1	0.1	0.022	100
2	0.2	0.053	100
4	0.3	0.095	100
6	0.3	0.117	95.9
24	0.8	0.457	37.5

Chart III

Chart IV

Chart V

Chart VI

Transformation of Surviving Clones After 24 Hour Exposure

<u>Hydrocarbon</u>	<u>Dose (µg/ml)</u>	<u>% Transformation</u>
B(a)P	10	6.8 (³⁵⁰ /5129)
	1	5.5 (⁴⁷ /858)
6-OH-B(a)P	10	- (100% cytotoxic)
	3	6.6 (⁸⁸ /1331)
	1	5.3 (⁹⁵ /1801)

III. Physical Penetration of T7 DNA (Naked Form or in Phage Particle) into Syrian Hamster Embryo Cells (SHE Cells).

A. The formation of a stable physical complex between T7 DNA and SHE cells.

The entrance of isotopically labelled (^3H or ^{32}P) T7 DNA into Syrian hamster embryo cells (SHE cells) was examined either when the T7 DNA was in naked form or packaged in a bacteriophage particle. When the T7 DNA was incubated for 24 hr (37°C) in the cell culture medium (Delbecco's modified Eagle's medium) containing 10% fetal calf serum, but in the absence of SHE cells, T7 DNA in the naked form became extensively degraded by serum nucleases to mononucleotides and short oligonucleotides. Under the same condition, the T7 DNA inside the phage particle protected by the protein coat was not degraded at all (see Figure 1 for sedimentation analysis). This study on the fate of T7 DNA in SHE cells was conducted always with both naked T7 DNA and T7 phage in order to evaluate the advantages of transfer of functional genes in these two forms. The data indicate that T7 DNA in phage particles is protected from the degradation by serum nucleases but can still penetrate the SHE cells.

Preliminary studies showed that very little ^{32}P -labelled naked T7 DNA became attached to SHE cells upon incubation in culture. The small amount of T7 isotope which became bound to SHE cells was due to the process of complete degradation

of T7 DNA and reutilization of the labeled degradation products for cellular DNA and RNA synthesis. However, if SHE cells were first preincubated for a period of 15 min at 37°C with 10 to 20 µg/ml growth medium of diethylaminoethyl-dextran (DEAE-d, 2×10^6 mwt) which was subsequently replaced with medium not containing DEAE-d, the attachment of ^{32}P -T7 DNA to the cell monolayer was stimulated at least 17-fold.

The amount of T7 DNA bound to the monolayer was usually equivalent to about 10^5 T7 genomes per SHE cell. This complex formed between T7 DNA and the cell monolayer cannot be dissociated by washing the cell monolayer with isotonic saline. If the ^{32}P -T7 DNA/SHE cell complex was allowed to form for 3 hr, then the growth medium containing the unbound ^{32}P -T7 DNA withdrawn, and the ^{32}P -T7 DNA/SHE cell complex further incubated 21 hr at 37°C, only 5-10% of the isotope was released from the monolayer. When SHE cells reached confluency in culture after 72 hr, 60-80% of the isotope in the complex remained attached to the cells with only 20-40% released to the medium. The same stable complex could be formed between rat fibroblast monolayers and ^{32}P -T7 DNA after DEAE-d pretreatment of cells.

Figure 2 (a) shows the sedimentation profile of ^{32}P -T7 DNA which was bound to the SHE cell monolayer in the absence or presence of DEAE-dextran. The distribution of radioactivity in the sucrose gradient is expressed in terms of the total amount of isotope on the sucrose gradient after solubilization of the cells;

it should be noted that in the absence of DEAE-d pretreatment there was actually 17-fold less ^{32}P -T7 attached to the cell monolayer. The sedimentation profile of the ^{32}P -T7 DNA is dramatically different under the two experimental conditions. In the absence of DEAE-d pretreatment of cells, the broad distribution of ^{32}P in the profile indicated that most of the ^{32}P was not conserved in ^{32}P -T7 DNA but actually utilized in the synthesis of cellular components containing phosphate. Other studies (not shown here) have indicated that most of the ^{32}P -T7 DNA degradation products were used in the synthesis of cellular RNA and to a smaller extent in the synthesis of cellular DNA which does not hybridize with T7 DNA. When SHE cells were pretreated with 10 μg DEAE-dextran/ml in a manner described above, most of the isotopes sedimented between 28S and 16S with a molecular weight between 10 to 20 million. This peak was still present 48 hr after formation of the complex. When T7 phage were incubated with SHE cells there was usually a small but discernible peak of T7 isotope sedimenting in the native 32S position (Figure 2, part B). This peak of 32S usually cannot be found in experiments of incubation with naked T7 DNA in culture. The degradation of ^{32}P -T7 DNA in the phage particle after incubation with the SHE cells suggests that the proteolytic machinery of the SHE cells must have exposed the T7 DNA inside the phage to the nuclease, since the T7 DNA in the phage is immune to the serum nuclease. The sedimentation analyses shown in Figure 2, B indicate that the

^3H -thymidine labeled DNA from the SHE cells sediments much faster than the ^{32}P - PO_4 labeled T7 DNA. This experiment reveals that the SHE DNA and the T7 DNA are clearly separable from each other and there is little mixing of isotopes.

Hybridization of ^{32}P extracted from the complex with known T7 DNA immobilized on a nitrocellulose filter provides the evidence that the ^{32}P -T7 DNA in the monolayer complex is nearly completely conserved. Figure 3A shows that the kinetics of hybridization of the ^{32}P -T7 DNA reisolated from the SHE cell complex after 42 hr in culture was nearly identical to that of the control ^{32}P -T7 DNA. The specificity in this experiment is indicated by the observations that hybridization did not occur with λ DNA immobilized on the filter and no binding occurred when no DNA was immobilized on the filter. In addition, the ^3H -SHE cell DNA, labeled simultaneously during binding of ^{32}P -T7 DNA to the complex, did not hybridize with the T7 DNA filter either. A further test of the base sequence homology of the hybrid formed between ^{32}P -T7 DNA in the SHE cell complex and T7 DNA immobilized on the nitrocellulose filter was made by the melting analysis (Figure 3B). The results indicated a high degree of base pairing between the ^{32}P -T7 DNA reisolated from the SHE cell and the authentic T7 DNA on the filter.

Figure 3B also shows the sedimentation profile of tritium isotopes from ^3H -T7 DNA bound to SHE cells in the absence of DEAE-dextran

pretreatment. All of the T7-³H sediments to the same position in the gradient with the ³H-hamster DNA (labeled with ³H thymidine), which was assayed in a separate centrifuge tube. No significant percentage of the T7-³H reisolated from the SHE cell without DEAE-dextran pretreatment sediments with the same velocity as the ³²P-T7 DNA peak which was reisolated from SHE cells pretreated with DEAE-dextran. Therefore, the results of these studies clearly reveal that no significant amount of T7 DNA attached to SHE cells could be conserved in the absence of DEAE-dextran pretreatment of the monolayer. Conversely, when SHE cells were pretreated with 10 to 20 ug DEAE-dextran/ml, a stable complex was formed between T7 DNA and the SHE cell monolayer which is relatively immune to the degradative machine of the SHE cells and the serum. On the other hand, T7 DNA inside the bacteriophage can be exposed by the SHE cell proteolytic activity so that the DNA becomes sensitive to partial fragmentation by nucleases. The kinetics of unwrapping the T7 phage particles bound to the SHE cells is indicated by the time-dependent loss of the native 32S ³²P-T7 DNA shown in Figure 4. At the end of 1.5 hr incubation of T7 DNA with the SHE cells in culture about 70% of the ³²P-T7 DNA was already fragmented. After 10 hr incubation, only a very small fraction (<1%) of the ³²P-T7 DNA retained the same sedimentation coefficient (32S) as the original, native form. This result indicates that cellular activity

efficiently degrades the protein coat of the phage particle exposing the T7 DNA to nucleases.

Figure 5 shows the kinetics of binding of ^{32}P -T7 DNA to the cell monolayer. The binding of the naked ^{32}P -T7 DNA is nearly complete in 3 hr and the amount of T7 DNA complexes with SHE cells is equivalent to a multiplicity of 5×10^4 T7 genome equivalents per cell. The binding of ^{32}P -T7 DNA in the phage is nearly complete in 6 hr and the level binding is equivalent to 9×10^4 T7 genome equivalents per cell. The efficiency of binding of naked ^{32}P -T7 DNA at 3 hr (16% of the total) was usually higher than that of binding of phage ^{32}P -T7 DNA for the same period (4% of the total). Thus, in subsequent experiments, generally the multiplicity of T7 phage used in the incubation with SHE cells is higher than that of T7 DNA; and, routinely, complex formation was allowed for only 3 hr followed by removal of the cell growth medium containing the unbound material.

The location of the T7 DNA associated with SHE cell monolayer was investigated by autoradiography. Autoradiographs (Figure 6) taken 24 hr after incubation of cell monolayer with ^3H -T7 DNA reveal that a high percentage of the ^3H -DNA was taken inside the cells and transported to the nuclei. At the end of 24 hr, 98% of the cell nuclei became extensively labeled and much of the extracellular ^3H -T7 DNA complex was concentrated

around the cell surface. Incubation of the cell monolayer with purified DNase for 30 min was adopted as one method to remove the T7 DNA in the extracellular complex. The T7 DNA which is not removed by this extensive DNase treatment is considered to be intracellular. In the experiment shown in Figure 5 (represented by - - -), the ^{32}P -T7 DNA was incubated with the SHE cells for 3 hr to allow complex formation, and then the unbound T7 DNA was removed by changing the medium. After a further incubation for varying periods (0 to 32 hr), the cell monolayer was then treated with DNase. In the beginning, the ^{32}P -T7 DNA was sensitive to the DNase treatment and then became resistant and not releasable by the enzyme after incubation allowing cell growth. After 3 hr, 98% of ^{32}P -T7 DNA was released from the SHE cell monolayer by DNase treatment. However, after 32 hr, 50% of the ^{32}P -T7 DNA in the original extracellular complex was converted to a DNase insensitive form, not releasable from the SHE cells by enzyme treatment.

Examination of DNase-treated SHE cells by autoradiography (Figure 6A) after 24 hr incubation with ^3H -T7 DNA revealed that all non-releasable T7- ^3H was now inside the cells, in fact located at the nuclei. This result further established that the DNase treatment is an effective method of removing all extracellular T7 DNA from the SHE cell monolayer. Thus, this assay allows a quantitative measurement of cellular penetration of the isotope from the T7 DNA. There are two

notes of caution in regard to this use of DNase: (1) It has been reported that exogenous DNase will enter the mammalian cell cytoplasm but not nuclei.

Therefore, if the newly penetrated T7 DNA is associated with the outer nuclear membrane or in the cytoplasm, the DNA may be fragmented or partially destroyed by exogenous DNase entering the cell; and (2) localization of T7 isotope by autoradiography alone within the cell does not establish the conservation of isotope in T7 DNA nucleotide sequences. The most obvious alternative is the degradation and reutilization of the products of the exogenous DNA in synthesis of cellular macromolecules.

The kinetics of developing resistance to DNase and the localization of isotope by autoradiography suggests that the cation, DEAE-dextran, mediates the formation of a complex between the microexodate of the SHE cells and the T7 DNA. No DNA was found to associate with the surface of the culture plate after preincubation with DEAE-dextran in the absence of SHE cells. At the beginning, DEAE-dextran, in effect, concentrates the exogenous DNA in the proximity of SHE cells growing on the monolayer and inhibits serum nuclease activity. At a later time, the SHE cells, however, can efficiently separate the T7 DNA from the DEAE-dextran complex and incorporate or degrade the T7 DNA.

B. Establishment of the penetration of intact T7 DNA into SHE cells.

Qualitative and quantitative techniques were used to establish that the T7 DNA isotope found inside SHE cells is indeed conserved in T7 DNA. Since DNase reliably removed all extracellular T7 DNA, this enzyme could be used in conjunction with a qualitative technique to measure the amount of T7 DNA in relatively undegraded form inside SHE cells. The following experimental approach was used: (1) SHE cell monolayers containing the extracellular T7 DNA complex formed in 3 hr time were incubated further to allow cell proliferation, then were treated with 20 μ g DNase on a 60 mm petri dish, followed by washing to remove extracellular T7 DNA-isotope; (2) the nonreleasable intracellular T7 isotope was then assayed for its ability to hybridize with known T7 DNA immobilized on nitrocellulose filters. This data will reveal the sequence homology between the authentic T7 DNA and the isotopes reisolated from the cell after incubation. The

results of such an experiment are reported in Table I and II. In this experiment, both naked T7 DNA and T7 bacteriophage were used and the assay for sequence homology was conducted 27 hr (two cell generations, Table I) and 71 hr (four cell generations, Table II) after incubation. After the incubation with the T7 DNA, the SHE cells were treated with DNase with the hybridization experiments done before and after the enzyme treatment. Since the results of Table I and Table II are similar, only the content of Table II will be discussed in detail. At the end of 72 hr cell growth, about 5460 T7 genome equivalents (hybridizable to T7 DNA) were present in the SHE cell-T7 DNA complex in the experiment with naked T7 DNA, and 3250 T7 genome equivalents were present in the same complex in the experiment with T7 phage particles. The cell number had increased by a factor of about 11 between 0 and 72 hr. About 20% and 46% of the isotopes from T7 respectively for the naked T7 DNA experiment and the T7 phage experiment were released from the SHE cells into the medium between the incubation period of 27 and 72 hr. The amount of T7 genomes initially in the SHE cell-T7 extracellular complex was about 10^5 genomes per cell.

After DNase treatment, it was found that 465 T7 genome equivalents (hybridizable to T7) had penetrated the cell after 72 hr incubation in the experiment with naked DNA, whereas 1116 T7 genome equivalents (hybridizable to T7) had penetrated the cell in the experiment with the phage. Since the number

of SHE cells increased by about 5-fold between 27 hr and 72 hr and the amount of newly penetrated ^{32}P -T7 DNA per cell did not fall to the level expected from the consideration of serial dilution, penetration of T7 DNA to SHE cells must continue between 27 and 72 hr. Also, during this period the ^{32}P -labeled degradation products were found to be reutilized by the cells in the synthesis of cellular constituents. The extensive reutilization of ^{32}P from T7 DNA indicated that the degradation process of T7 DNA took place after the DNA penetration because the ^{32}P would otherwise be diluted enormously by the phosphate present in the medium (0.9 mM). Since the ^{32}P - PO_4 of the T7 DNA has been reutilized extensively for RNA and DNA synthesis as will be discussed concerning Figure 7, some intracellular T7 DNA must have been degraded to the nucleoside level.

C. The size of the T7 genome inside SHE cells

While the sucrose gradient technique can be used to examine the size of the labeled T7 genome after its penetration inside the cell, this approach is complicated by the fact that the T7 isotopes (^3H -thymine and ^{32}P - PO_4) are continually being reutilized for the synthesis of hamster DNA and RNA. Fortunately, it was found that reutilization of ^3H -thymidine from ^3H -T7 DNA can be prevented by addition of a large excess of nonradioactive thymidine to the cell growth medium (17 nmols

Tdr/ml). When thymidine was added to the growth medium, the amount of T7-³H nonreleasable by DNase after incubation was usually reduced by 60% to 80%. Addition of thymidine to a concentration above 17 nmols/ml to the growth medium did not further reduce the amount of ³H-T7 DNA nonreleasable by nuclease. The ³H-T7 DNA which was not diluted by the added thymidine and not released by the DNase was found to be located only in the cell nuclei by autoradiography. In addition, this material was almost totally hybridizable to the authentic T7 DNA immobilized on nitrocellulose filters.

When reutilization of T7-³H is suppressed to an insignificant level, the sedimentation pattern of intracellular T7 DNA can now be used to determine the size distribution of the newly penetrated T7 DNA without complication by the isotope reutilization process. In the experiment shown in Figure 7, the cells were incubated simultaneously with equal amounts of two preparations of T7 phage, one labeled with ³²P and the other with ³H-thymidine. The size distributions of the two T7 DNA isotopes inside the cells cultured with 67 nmols Tdr/ml growth medium were examined and compared. The sedimentation pattern (Figure 7) of the ³H-T7 DNA which is not released by DNase after 30 hr of SHE cell proliferation, revealed that most of the intracellular T7 DNA was high molecular weight. However, there was evidence of fragmentation of the T7 genome because 48.6% of the ³H-T7 DNA has an S value less than 32S, the S value of the native form. At present, it is not known whether or

not the added exogenous DNase is partially responsible for the fragmentation of the intracellular T7 DNA prior to lysis of the SHE cells with detergent. The sedimentation profile of T7- ^{32}P in these same cells (Figure 7) revealed, as expected, a more complicated pattern due to the extensive reutilization of ^{32}P in the synthesis of hamster RNA and DNA. However, the presence of the major sedimenting peak at the same position ($\sim 32\text{S}$) as the ^3H -T7 DNA peak indicated that T7 DNA had been conserved as a high molecular weight sedimenting species. Since twice as much T7- ^{32}P was incorporated into this T7 DNA peak, some of the ^{32}P sedimenting to this position must be due to reutilization of T7- ^{32}P in synthesis of high molecular weight hamster RNA or DNA. The T7- ^{32}P peaks with S values of 28S and 18S indicate that the major portion of the reutilized T7 phosphate was in the synthesis of ribosomal RNA. In a separate study only 4.2% of the ^{32}P -isotope isolated from peak fractions of the 28S peak was able to form a specific hybrid with T7 DNA-filters. In the same experiment, 30-40% of the T7- ^{32}P isolated from the 32S region (the region of the undegraded genome) was able to hybridize with T7 DNA-filter. Omission of thymidine in the culture medium did not alter the sedimentation pattern of T7- ^{32}P , however, this omission increased the amount of T7- ^3H taken up by SHE cells 4.5-fold over that amount of T7- ^3H taken up in the presence of 67 nmols Tdr/ml. The sedimentation patterns of T7- ^3H reisolated from

cells cultured with and without thymidine are compared in Figure 8. In the DNA prepared from cells cultured without thymidine, 50% of the T7-³H were in the two fractions near the bottom of the gradient. This result is indicative of the reutilization of T7-³H from degradation products in the synthesis of hamster DNA which has a much higher molecular weight than T7 DNA (see Figure 2B).

The preceding study concerns the experiments with intact bacteriophages. The fragmentation of the intracellular T7 DNA which was incubated with SHE cells initially in the naked form is shown in Figure 9. The sedimentation profile of ³H-T7 DNA not releasable by DNase treatment shows little evidence for the persistence of unfragmented T7 DNA inside SHE cells. Again, it could not be determined whether or not the exogenous DNase may have degraded the T7 DNA inside the cell. The small amount of T7-³H found in the hamster DNA region (bottom of the gradient) indicated that the reutilization of T7-³H in hamster DNA synthesis was insignificant due to the presence of thymidine (67 nmoles/ml) in the medium.

In conclusion, a combination of the above qualitative and quantitative analysis techniques (hybridization, sedimentation, isotope dilution, autoradiography, and DNase sensitivity) have established that T7 DNA can penetrate SHE cells after an initial pretreatment of the culture with DEAE-dextran. The amount of newly penetrated T7 DNA that persists inside the cells 72 hr later

(after 4 cell generations) usually varied between 400 and 1200 T7 genome equivalents per cell. When T7 phage instead of naked T7 DNA are incubated with SHE cells, a larger amount of T7 genome persists inside the cell and the intracellular T7 DNA is generally less fragmented. Autoradiography revealed that the intracellular T7 DNA is located at the nucleus although it has not necessarily penetrated the nucleus. The physical separation of intracellular T7 DNA from most of the hamster DNA by sedimentation suggested that, after four cell generations, little, if any, of the T7 DNA becomes covalently integrated into the host chromosomes.

IV. The fate of T7 DNA/genome inside the SHE cells in a continuing culture for a prolonged period--Evidence for T7 DNA replication in SHE cells.

The study of the persistence and replication of T7 DNA in a long term culture of SHE cells requires a different method of detection than the use of isotopes which will be greatly diluted with increasing cell number. In this study, the technique of reassociation kinetics was used as a probe. These experiments were designed in the following manner:

(1) Early passages of SHE cells seeded at a density of 3×10^6 cells/75 cm² were pretreated with 20 µg DEAE-dextran per ml of growth medium and incubated with either T7 DNA, T7 phage (74 to 350 µg DNA equivalents) or no T7 DNA at all, for 3 hr at 37°C; T7 DNA not bound in the SHE cell complex was removed

by medium change, and the cells were grown to confluency.

(2) The cell culture was then trypsinized; 90% to 95% of the cells was removed for analysis, and the remainder was cultured again to confluency in the plate. This process was repeated for 12 times (12 passages) in a period of 74 days. During this period of time, the total SHE cell progeny increased about 2×10^{10} fold. (3) The growth media from these passages were repeatedly tested for bacterial contamination, and the cells were often plated on coverslips in the search for micoplasma contamination--all these tests were negative.

(4) SHE cells withdrawn from the culture were counted to determine the extent of growth of the cultures and collected by centrifugation. (5) DNA extracts were prepared from these cell pellets for the quantitative analysis of the presence of T7 DNA. (6) The quantity of T7 DNA present in cell extracts was detected by the influence of the extract on the rate of renaturation of a known concentration of ^{32}P -T7 DNA (the probe). This renaturation kinetics procedure has been used extensively as a highly sensitive and qualitative technique to detect small, unique viral DNA sequences in mammalian cells. Further analysis of the quality of the hybrids formed was made from melting of the hybrids. The quantity of T7 DNA in cell populations (1 to 6×10^7 cells) is usually expressed as the average number of T7 genome equivalents per cell. A control renaturation kinetic experiment using a DNA extract from SHE cells never exposed to T7 DNA was done to determine

the rate of renaturation of the ^{32}P -T7 DNA probe in the absence of T7 DNA sequence in the cell extract. This experiment shows that the unexposed SHE cell extract contains no T7 DNA sequence, since the rate of renaturation of ^{32}P -T7 DNA probe is the same in the absence or the presence of the cell extract.

An example of such an experiment is shown in Figure 10. In this experiment, the SHE cells cultured for 36 days after initial exposure to T7 phage or its DNA were harvested. The DNA extract from a specific number of SHE cells was prepared. In this preparation, all RNA had been removed by alkaline hydrolysis and the DNA had been sonically fragmented and denatured so that the average chain length of the DNA was approximately 700 nucleotides long. The ^{32}P -T7 DNA probe was also sheared to the same size. The ^{32}P -T7 DNA probe was then allowed to renature by incubation at 68°C in 0.72 M sodium phosphate (pH 7.0) plus 0.4% detergent (SDS) with or without the addition of DNA extract from the following cultures: (1) control cells, (2) cells exposed initially to naked T7 DNA, and (3) cells exposed initially to T7 phage. The extent of renaturation of the ^{32}P -T7 DNA probe was assayed by hydroxyapatite chromatography at 65°C and 0.12 M sodium phosphate (0.4% SDS). The result in Figure 10 shows that the DNA extract from SHE cells exposed to T7 phage or DNA 36 days earlier caused a dramatic increase in the rate of renaturation of ^{32}P -T7 DNA. The experimental data is plotted as the percent

of reassociation (binding to hydroxyapatite) versus the logarithm of Cot for ^{32}P -T7 DNA (Cot = molarity x seconds of reassociation). The $Cot_{1/2}$ value (the Cot value at 50% renaturation) is conventionally used as a reference point for calculating the quantity of T7 DNA in the cell extract.

Control $Cot_{1/2}$ values for ^{32}P -T7 DNA varied between 1 and 4×10^{-3} ($68^{\circ}C$, 0.72 M phosphate), and this variation was attributed to the variation in the average size of DNA fragments in different experiments and the changes in the viscosity of the renaturation solution due to the change in volume. When the rate of renaturation of T7 DNA was examined in regard to the three different fragment sizes, 2900, 500, and 340 nucleotides long on the average, the respective $Cot_{1/2}$ values were found to be 1.6×10^{-3} , 4.4×10^{-3} , and 7.1×10^{-3} at 68° and 0.72 M Na phosphate. Changes in the viscosity will also affect the rates of renaturation.

The stepwise procedure for calculation of the amount of T7 DNA per cell is itemized in Table III and the result is expressed in terms of T7 genome equivalents per SHE cell. It should be emphasized again that the DNA extract from the control cells has no effect on the reassociation kinetics of the ^{32}P -T7 DNA probe. The extract from SHE cells initially exposed to naked T7 DNA enhanced the rate of renaturation of the probe by 8-fold. Calculation from this value shows that this culture contained an average of 182 T7 genome equivalents per cell 36 days after the original exposure. The extract

from SHE cells initially exposed to T7 phage enhanced the rate of renaturation of the probe by 16-fold. This result indicated that SHE cells from this culture contained an average of 682 T7 genome equivalents per cell after 36 days. Table IV summarizes all the data from this experiment in which the long term persistence of T7 DNA in SHE cells was examined periodically after the initial exposure to either naked T7 DNA or T7 phage particles and in the presence or absence of deoxynucleoside supplement of the cell growth medium.

In Figure 11, the number of T7 genome equivalents per cell in this experiment is plotted against the number of days after initial exposure to T7 DNA phage. In all three experiments after the initial exposure of the SHE cells to T7 DNA/phage, the presence of T7 DNA inside the cells was detected 2 months later. An unusual pattern of persistence of T7 DNA was found in all three cases although the amounts of T7 DNA in the cell varied with the condition of initial exposure (such as, phage particle vs. DNA and the absence or presence of deoxynucleosides). Three days after initiation of the experiment (end of passage one), all three cultures contained between 220 and 670 T7 genome equivalents per cell. At this time, the bound T7 DNA was either inside the cell or tightly attached to the membrane because it remained attached to cells after trypsinization and sedimentation. After trypsinization, 10% of the cells were replated for analysis at later passages. At the end of 10 days, a total of three passages, which have a 1.0 to 1 dispersion per

passage, the amount of T7 DNA per cell had dissipated to 18-29 T7 genome equivalents per cell in all these exposed stocks. Serial dilution may be the reason for the drop in the number of T7 genomes per cell in the early period. Then between 10 and 36 days a dramatic increase in the amount of T7 DNA per cell occurred in all three cultures: from 29 to 682 for the culture exposed to T7 phage plus deoxynucleosides, from 18 to 187 for the culture exposed to naked T7 DNA plus deoxynucleosides, and from 22 to 112 for the culture exposed to naked T7 DNA without deoxynucleosides. From day 10 to day 36 SHE cells doubled 14 times so that the cell progeny increased by a factor of about 16,000. By 36 days, all cultures had been dispersed with a 1:10 ratio seven times so that serial dilution essentially eliminated all residual extracellular T7 DNA from the culture.

These cultures were assayed further at 58 days (passage 9) and 72 days (passage 12) for the presence of T7 DNA. At these two periods SHE cells initially exposed to naked T7 DNA ($\pm$ deoxynucleosides) contained a diminished amount of T7 DNA between 8 and 18 T7 genome equivalents per cell, while SHE cells initially exposed to T7 phage contained 6.9 T7 genome equivalents at 58 days but 156 T7 genome equivalents at 78 days. This unusual pattern of persistence of T7 DNA may be due to the possibility that replication of T7 DNA in SHE cells is not in synchrony with the division of the SHE cells. A diminution in the amount of T7 DNA per cell could be due either to its degradation or to serial dilution during period of time when T7 DNA is not replicated.

A second experiment at this time was initiated to confirm the long-term persistence of T7 DNA in SHE cells and to establish the reproducibility of the unusual pattern of persistence of T7 genes just described. In this experiment, the persistence was again compared between SHE cell cultures which were exposed initially to naked T7 DNA or to T7 phage. In addition, the effect of exogenous DNase added 5 days after the initial exposure (end of passage one) on the persistence of T7 DNA in these two cultures were compared. The results of this experiment are summarized in Tables V, VI, and VII and Figures 12 and 13. Table V shows the persistence of T7 DNA in SHE cells which were initially exposed to naked T7 DNA and the effect of DNase treatment 5 days after initial exposure on this persistence. Table VI contains the same information from the SHE cells originally exposed to T7 phage. In this experiment, the amount of T7 DNA per cell below 1.8 genome equivalents could not be reliably detected.

When high multiplicities of T7 DNA or T7 phage were incubated with SHE cells but never exposed to exogenous DNase, a significant amount of T7 DNA persisted in SHE cells even 68 days later at the end of 11 passages. When SHE cultures incubated in an identical manner with T7 DNA or T7 phage but were exposed to DNase at the end of 5 days (passage 1), the persistence of T7 DNA was detectable only up to 30 days, 5 passages after initiation and at a greatly reduced level. In this

experiment 200 μg of sterile, purified DNase was incubated with 4 to 5×10^7 SHE cells in monolayer, and such treatment did not significantly alter the rate of cell growth at later passages. Such a treatment, however, reduced the T7 DNA per cell from 3800-4200 equivalents to 130 to 235 equivalents. On the basis of autoradiographic experiments described in Figure 6, the amount of T7 DNA present with SHE cells after DNase treatment was considered intracellular and located at the cell nucleus.

The results clearly indicate that the T7 DNA firmly attached to the SHE cells 5 days after the original exposure is very sensitive to the attack of exogenous DNase. The experiment shown in Table VII indicates that in fact even after 54 days in culture, the T7 DNA in the SHE cells can still be removed by the added DNase. Therefore, it is important to demonstrate the absolute amount of T7 DNA produced by these SHE cells during this prolonged period in culture. In Figure 12, the net amount of T7 DNA "harvested" from the culture starting from day 19th is plotted against the number of days after the initial exposure. Each point in the graph represents the amount of DNA (in moles) detected in the SHE cells harvested from the culture up to that passage. It is an accumulative number. The total amount of T7 DNA synthesized in the cell culture after the initial exposure is 605 pmols and 475 pmols respectively for the experiment with naked T7 DNA and for the experiment with T7 phage. It should be noted that only 90% of the cells in culture were harvested and 10% of the cells

were used for replating. Therefore, there should be approximately another 10% of T7 DNA produced by these cultures.

Another way to demonstrate the multiplication of T7 DNA (in genome equivalents) on an absolute basis is shown in Figure 13. The number of total T7 genomes in the culture was estimated by multiplying the number of T7 genomes per cell by the number of cells in the culture which is calculated from the cell counts of each passage with the assumption that these cells were allowed to grow and not harvested. In Figure 13 it was shown that at the end of the first (day 5), second (day 11), and third passage (day 19), the total amount of T7 DNA was declining to a plateau. Then from the third passage onward (after 19th day), the absolute amount of T7 genome increased again with time. At about 40 days the amount of T7 genome in the culture became larger than the amount originally introduced into the plate. Actually, at least 90% or more of the amount of T7 DNA/phage initially added to the culture was removed by medium changes and trypsinization from the plate. This calculation clearly indicates the increase of T7 genome is due to the multiplication of the T7 genome. One particularly interesting point is the culture which was treated with DNase. As compared to the cultures not treated with the nucleases, the treated culture contained a much lower number of the T7 genome (about 1%). However, even in this treated culture, the absolute number of T7 genome increased with time

and approached that of the untreated culture. It should be recalled that DNase treatment is very effective in destroying unprotected, extracellular DNA.

In regard to the data in Figure 12 and Figure 13, the following points should also be noted: (1) Nuclease activity is present in the serum; (2) SHE cells efficiently degrade T7 phage particles; (3) SHE cells efficiently and actively degrade T7 DNA and reutilize its products in macromolecule synthesis; (4) Throughout the entire long-term persistence of T7 DNA in culture, the cellular T7 DNA remains sensitive to DNase yet attached to cells. Therefore, if the DNA was merely present extracellularly without replication it would disappear rapidly due to serum nuclease; (5) High levels of T7 DNA have persisted even when serial dilutions of cells in a continuous culture have been as high as 10^{-7} and 10^{-12} in the first long-term experiment, and 10^{-5} , 10^{-8} , and 10^{-9} in the second long-term experiment. (6) T7 DNA persists at high levels when the total cell progeny have increased by factors of 10^6 and 10^{10} ; (7) Even in DNase-treated cells significant amounts of T7 DNA persisted 25 days after extensive DNase treatment of SHE cells; and (8) Cell cultures were repeatedly tested for microorganisms and showed no evidence of contamination with bacteria, mold, or microplasmas.

Table I

PERSISTENCE AND CELL PENETRATION OF T7 DNA AFTER 27 HOURS SHE CELL PROLIFERATION

Initial ^a Form of T7 DNA	Type of ^b Assay (+ or - DNase)	Equivalent ^b pmols ³² P-T7 DNA per 0.7 x 10 ⁶ Cells	Percent ^c Hybridi- zable to T7 DNA	Corrected ^d Percent Hybridi- zable to T7 DNA	T7 Genome ^e Equivalents per Cell
T7 DNA	Total cell binding				
	Before DNase treatment	3526.8	79.9 (0.7)	93.9	33,265
	After DNase treatment	333.9	53.5 (0)	62.9	2,110
T7 PHAGE	Total cell binding				
	Before DNase treatment	3659.8	80.4 (1.0)	94.5	34,740
	After DNase treatment	443.4	20.9 (1.1)	24.6	1,096

- a. 44 ug ³²P-T7 DNA in naked form or 68 ug ³²P-T7 DNA in phage particle form were incubated with 3.9 x 10⁵ SHE cells in culture according to the procedure described in Figure 5.
- b. The method of analysis ([±] DNase treatment) is described in Figure 5.
- c. The procedure for filter hybridization is described in Figure 2.
- d. The present hybridization of known ³²P-T7 DNA in a control experiment represents 100% T7 hybridizability; the hybridization incubation was allowed for 92 hr when control ³²P-T7 DNA hybridized 85%.
- e. Calculated from the number of cells at 27 or 71 hr, the amount of ³²P-T7 DNA in nucleotides cell bound, and the molecular weight of the T7 genome (25 x 10⁶ daltons).

Table II

PERSISTENCE AND CELL PENETRATION OF T7 DNA AFTER 71 HOURS SHE CELL PROLIFERATION

Initial ^a Form of T7 DNA	Type of ^b Assay (+ or - DNase)	Equivalent ^b pmols ³² P-T7 DNA per 3.4 x 10 ⁶ Cells	Percent ^c Hybridi- zable to T7 DNA	Corrected ^d Percent Hybridi- zable to T7 DNA	T7 Genome ^e Equivalents per Cell
T7 DNA	Total cell binding				
	Before DNase treatment	2829.7	79.4 (2.3)	93.3	5460
	After DNase treatment	569.3	33.6 (0)	39.5	465
T7 PHAGE	Total cell binding				
	Before DNase treatment	1952.0	68.5 (0.7)	80.5	3250
	After DNase treatment	1017.9	45.1 (0.3)	53.0	1116

a,b,c,d,e, see Table I

Table III

Calculation of T7 DNA genome equivalents per SHE cell

-
1. Control experiment - reassociation kinetics of ^{32}P -T7 DNA in the presence of extract from SHE cells not exposed to T7 DNA.

$$C_0 t_{1/2} \text{ } ^{32}\text{P-T7 DNA} = \text{Molarity } ^{32}\text{P-T7 DNA} \times t_{1/2} \text{ (seconds)}$$

$$\text{Control } C_0 t_{1/2} \text{ } ^{32}\text{P-T7 DNA} = 2.3 \times 10^{-3} \text{ at average DNA fragment size} = 750 \\ 0.72 \text{ M sodium phosphate pH } 7.0, 68^\circ \text{ C.}$$

2. Test experiment - reassociation kinetics of ^{32}P -T7 DNA in the presence of extract from SHE cells exposed to T7 DNA.

A. Control $C_0 t_{1/2} \div \text{test } t_{1/2} = \text{Total molarity T7 DNA}$

B. Total molarity T7 DNA minus molarity ^{32}P -T7 DNA = Molarity T7 DNA in SHE cell extract

C. Molarity T7 DNA (in SHE cell extract) $\times$ liters (volume of reassociation solution) = moles T7 DNA (in SHE cell extract).

D. Moles T7 DNA (in SHE cell extract) $\div$ # nucleotides per T7 genome (7.8×10^4) = moles T7 genome (in SHE cell extract)

E. Moles T7 genome (in SHE cell extract) $\times$ Avogadro's number (6.02×10^{23}) = # T7 genome molecules (in SHE cell extract)

F. #T7 genome molecules (in SHE cell extract) $\div$ # SHE cells used to prepare extract = average # T7 genome equivalents per SHE cell

Table IV

BACTERIOPHAGE T7 GENES IN SYRIAN HAMSTER EMBRYO CELLS

Infecting Agent	Days after Infection	Cell Generations	T7 Genome Equivalents per Hamster Cell
None (control)	3	4	0
	7	7	0
	10	10	0
	15	14	0
	36	23	0
T7 DNA (with deoxynucleosides)	3	4	576
	7	7	62
	10	10	18
	15	15	63
	36	24	182
T7 DNA (without deoxynucleosides)	3	4	668
	7	8	121
	10	10	22
	15	14	28
	36	24	112
T7 PHAGE (with deoxynucleosides)	3	4	223
	7	7	80
	10	10	29
	15	14	97
	36	24	682

- a. This experiment is described in Figure 10 and calculations are made according to the procedure in Table III.

Table V

BACTERIOPHAGE T7 GENES IN SYRIAN HAMSTER EMBRYO CELLS

Infecting ^a Agent	Days after Infection	Cell Multiplication Factor	T7 Genome ^b Equivalents per Hamster Cell
None (control)	5 to 68	21,312,000,000	0
T7 DNA (total multiplicity of infection 67,151 T7 genomes per cell at 5 days)	5	1	3796
	12	13	124
	19	131	18.3
	26	1,290	36.3
	30	9,575	38.5
	36	132,615	23.2
	42	1,117,945	13.1
	48	6,574,000	15.7
	54	133,800,000	20.1
	62	1,066,400,000	7.4
	68	21,326,000,000	12.1
T7 DNA (cells DNase treated at 5 days)	5	1	129
	12	10	2.0
	19	105	2.2
	26	1,260	2.1
	30	9,434	0.3
	36	109,715	0
	42	1,006,087	0
	48	6,721,000	0
	54	95,500,000	0

BACTERIOPHAGE T7 GENES IN SYRIAN HAMSTER EMBRYO CELLS

Infecting ^a Agent	Days after Infection	Cell Multiplication Factor	T7 Genome ^b Equivalents per Hamster Cell
T7 PHAGE (total multiplicity of infection 355,073 T7 genomes per cell at 5 days)	5	1	4,204
	12	13	120
	19	164	2.8
	26	1,413	17.7
	30	12,306	32.8
	36	165,765	10.3
	42	1,326,121	21.9
	48	11,869,000	51.4
	54	187,600,000	4.2
	62	1,487,700,000	6.1
T7 PHAGE (cells DNase treated at 5 days)	5	1	235
	12	16	2.2
	19	164	0.8
	26	1,478	2.8
	30	11,481	6.6
	36	155,453	0
	42	1,302,332	0
	48	11,083,000	0
	54	160,800,000	0

Tables V and VI

- a. The protocol for this experiment is essentially identical to the experiment described in Figure 10.
b. Calculations were made by the procedure in Table III.

Table VII

Effect of exogenous DNase on amount of T7 DNA present
in SHE cells after 54 days^a

Condition	Average T7 genome equivalents per cell
Cells not exposed	0
Cells exposed to T7 phage	104
Cells exposed to T7 phage and treated with DNase	0.8

- a. Procedures identical to those in Table VI were used with one exception: DNase treatment of the culture was at day 54 after exposure of SHE cells to T7 phage.
- 2

Figure 1

Figure 1. Stability of T7 DNA in naked or phage particle form in the culture medium in the absence of SHE cells. 34 μg ^{32}P -T7 DNA in the form of intact phage or 20 μg naked ^{32}P -T7 DNA was incubated in medium and 10% fetal calf serum for 24 hr in the absence of SHE cells. The incubation mixture was then adjusted to 0.6% SDS and the buffered phenol-extracted ^{32}P -T7 DNA analyzed by sedimentation in 5-20% sucrose gradients (0.1% SDS, 0.1 M Tris-HCl, pH 7.9 and .001 M EDTA) in a SW 27.1 rotor at 23,000 rpm for 18 hr (11°C); fractions were collected and counted in 10 ml Aquasol. Sedimentation of a control ^3H -T7 DNA marker is shown by X—X; ^{32}P -T7 DNA incubated as phage is shown by O--O; ^{32}P -T7 DNA incubated in naked form is shown by Δ -- Δ . Sedimentation is expressed as the percentage of total radioactivity in the gradient.

Figure 2A

Figure 2B

Figure 2. The effect of DEAE-dextran pretreatment (or its omission) on the sedimentation distribution of ^3H or ^{32}P -T7 isotopes after attachment to SHE cell monolayers. The standard procedures for incubation of T7 DNA with SHE cells appear in the legend to Figure 5. The procedure for sedimentation analysis appears in the legend to Figure 1.

Part A. Two identical culture plates containing 3.95×10^5 SHE cells were incubated with $50 \mu\text{g}$ ^{32}P -T7 DNA each for 24 hr. In one case the DEAE-dextran pretreatment was omitted (X--X); in the other case SHE cell monolayers were pretreated with $10 \mu\text{g/ml}$ DEAE-dextran $\Delta-\Delta$. In the absence of DEAE-dextran $3700 \text{ cpm } ^{32}\text{P}$ bound to the monolayer and in the presence of DEAE-dextran $63,380 \text{ cpm } ^{32}\text{P}$ bound to the monolayer. The specific activity of ^{32}P -T7 DNA was $1.6 \times 10^4 \text{ cpm}/\mu\text{g}$ DNA. Cell monolayers were rinsed, solubilized, and analyzed by sedimentation analysis (as in Figure 1).

Part B. Three identical culture plates containing 3×10^5 SHE cells were incubated either with $100 \mu\text{g}$ ^{32}P -T7 DNA in phage particle form ($\Delta-\Delta$), with $50 \mu\text{g}$ ^3H -T7 DNA ($1.3 \times 10^5 \text{ cpm}/\mu\text{g}$) in naked form (X--X), or ^3H -thymidine (0.25 uc) with unlabeled T7 DNA (0--0). When ^3H -T7 DNA was used (X--X) DEAE-dextran pretreatment was omitted. Cells were cultivated and analyzed as in Part A.

Figure 3

A

B

Figure 3. Conservation of T7 sequences detected by DNA:DNA hybridization.

Part A. T7 DNA was denatured and immobilized in nitrocellulose filters. All DNA to be tested by hybridization was sonically sheared. Filter hybrid formation was conducted by incubation in 50% formamide--3 x SSC at 35°C. Each filter retained 16.5 µg immobilized T7 DNA throughout the experiment. The extent of hybridization of ³²P-T7 DNA extracted from SHE cells or control ³²P-T7 DNA and ³H-SHE DNA was measured by the radioactivity retained by the filters after extensive rinsing in 2 x SSC; 0--0, hybridization efficiency (%) of 77.2 pmols control ³²P-T7 DNA; Δ--Δ, hybridization efficiency of 120.2 pmols ³²P-DNA extracted from SHE cells after attachment of ³²P-T7 DNA for 30 hr; Δ, same as Δ--Δ but in the absence of immobilized T7 DNA; 0--0, hybridization efficiency % of 8574 cpm ³H-SHE DNA labeled during incubation of cells with ³²P-T7 DNA for 71 hr; X, same as 0--0, but in the absence of immobilized T7 DNA.

Part B. The melting curve of the hybrid formed on the nitrocellulose filter compared to the melting profile of known T7 DNA: Δ--Δ, the melt of the hybrid formed in part A (Δ--Δ), measured by release of radioactivity from the filter into solution (immobilized DNA remains bound to the filter); X--X, melting profile of sheared T7 DNA and, 0--0, the melting profile of native T7 DNA determined by hyperchromicity.

Figure 4

Figure 4. SHE cells were incubated with ^{32}P -T7 phage for various times. Then the ^{32}P -T7 phage which attached to cells was solubilized, extracted, and analyzed by sedimentation as described in Figure 5. The sedimentation pattern of ^{32}P -T7 DNA after incubation with SHE cells for various times is shown: Δ -- Δ , 1.5 hr; O--O, 6 hr; X--X, 10 hr.

Figure 5

Figure 5. The kinetics of attachment of T7 virus and DNA to SHE cells and the development of resistance of T7 DNA to release from cells by exogenous DNase.

Standard cell culture technique and procedure for incubation of SHE cells with T7 DNA: (1) SHE cells were always cultured in Dulbecco's modification of Eagle's minimal essential medium (D-MEM) supplemented with 10% fetal calf serum. (2) SHE cells were seeded into 60 mm petri dishes for 18 to 24 hr. (3) Just prior to exposure to T7 DNA, cell monolayers were preincubated with 10 μ g DEAE-dextran (2×10^6 mwt) for 15 min at 37°C. (4) The DEAE-dextran containing medium was removed from the monolayers and replaced with medium in the absence of serum. (5) T7 DNA or T7 phage were added. (6) Incubation of the cells with T7 was allowed (except where stated otherwise) for 3 hr and at the end of the 1st hour the medium was supplemented with 10% fetal calf serum. (7) Cells were cultivated to allow cell

proliferation. (8) When DNase treatment was included, 20 ug purified DNase I (freshly dissolved)/3 ml medium was added directly to the culture 30 min (37°C) prior to solubilization.

Solubilization of SHE cell monolayers and assay for radioactivity: SHE cells were solubilized directly from the monolayer in 0.5% detergent (SDS), 0.01 M EDTA, 0.01 M Tris-HCl (pH 7.9). When SHE cells were first trypsinized, they were pelleted by centrifugation and dissolved in 0.5% SDS, 8 M urea, 1 M NaClO₄, 0.01 M EDTA, and 0.01 M Tris-HCl, pH 7.9. When examining sedimentation profiles, solubilized SHE cells were phenol extracted once. Small aliquots were counted for radioactivity in 10 ml Aquasol (New England Nuclear).

For the experiment in Figure 5: Either 338 nmols ³²P-T7 DNA in the form of intact T7 phage or 57 nmoles ³²P-T7 DNA in naked form were incubated with SHE cells. In 28 hr with T7 DNA SHE cells multiplied from 1.7 to 5.0 x 10⁵ cells per plate. To examine binding of T7-³²P to monolayers supernatant medium was drawn off and the monolayers were rinsed twice with isotonic saline. When total binding to cells was determined, the culture medium was not changed at 3 hr. O--O represents the binding of T7 phage; Δ--Δ represents the binding of T7 DNA expressed in nanomoles ³²P-T7 DNA nucleotide. When sensitivity to exogenous DNase was assayed, binding of ³²P-T7 DNA was allowed for 3 hr followed by removal of unbound T7 DNA by medium change. SHE cells were then incubated further and DNase-treated at various times. ³²P-T7 DNA which remained bound to cells after DNase treatment is represented by ▲—▲ and is expressed in terms of the percentage of total ³²P-T7 DNA bound to SHE cells after 3 hr.

Figure 6. Localization of ³H-T7 DNA attached to SHE cells by autoradiography. SHE cells were seeded onto coverslips in 60 mm dishes and incubated with ³H-T7 DNA (20 μ g, 1.3×10^5 cpm/ μ g) for 24 hr as described in Figure 5. One culture (part A) was incubated with DNase (as described in Figure 5), and in the other (part B) DNase was omitted. Cells attached to coverslips were rinsed in isotonic saline, fixed in absolute methanol, rinsed in 5% TCA to remove acid soluble material, and developed with a photographic emulsion for seven days. Magnification is X530. Before DNase treatment the equivalent of 3.98 μ g ³H-T7 DNA was attached to the coverslip; after DNase treatment the equivalent of 1.13 μ g T7 DNA was attached to the coverslip.

Figure 7

Figure 7. Sedimentation analysis of intracellular T7 DNA isotopes. ³H-T7 phage and ³²P-T7 phage (65 µg DNA each) were simultaneously incubated with 2.3×10^5 SHE cells according to the procedure outlined in Figure 5. Cells were cultivated 30 hr in the presence of 67 nmols thymidine/ml. At termination of cell growth the monolayer was treated with DNase (see Figure 5). By 30 hr of cell growth, the cell population had increased to 6.6×10^5 cells. Cell lysates were analyzed by sedimentation (see Figure 1). Specific activities of ³²P and ³H-T7 DNA were adjusted to represent the total amount of T7 DNA present in the culture; X—X, ³H-T7 DNA, Δ--Δ ³²P-T7 DNA. 43.5 pmols ³H-T7 DNA nucleotide and 231.1 pmols ³²P-T7 DNA nucleotide became resistant to release from cells by DNase in this cell culture.

Figure 8

Figure 8. Sedimentation analysis of intracellular T7-³H in the presence or absence of added thymidine to the culture. The conditions of this experiment were identical to those in Figure 7 except that in one culture the presence of 67 nmols thymidine/ml was omitted (●—●). When thymidine was omitted, 194 pmols ³H-T7 DNA nucleotide (●—●) became attached to SHE cells; when thymidine was included 43.5 pmols ³H-T7 DNA nucleotide attached to SHE cells (○---○).

- 70 -
Figure 9

Figure 9. Sedimentation analysis of intracellular T7 DNA. 25 μg ^3H -T7 DNA was incubated with 1.5×10^5 cells according to the procedure outlined in Figure 5. Cells were cultivated 24 hr in the presence of 67 nmols thymidine/1 ml. At termination of cell growth the monolayer was treated with DNase (see Figure 5). By 24 hr of cell growth, the cell population had increased to 4.8×10^5 cells. Cell lysates were analyzed by sedimentation (see Figure 1); X—X, ^3H -T7 DNA; $\circ$ — $\circ$, ^{32}P -ribosomal RNA marker.

Figure 10. Reassociation of ^{32}P -T7 DNA with DNA from SHE cells 36 days after exposure to T7 phage or T7 DNA. Six falcon flasks (75 cm^2) containing 4×10^6 SHE cells in passage 4 were pretreated with $20\text{ }\mu\text{g}/1\text{ ml}$ DEAE-dextran. Duplicate flasks were divided into three sets: 1) control cells not exposed to T7 DNA or phage; 2) cells incubated with deproteinized T7 DNA ($165\text{ }\mu\text{g}$); 3) cells incubated with T7 phage ($350\text{ }\mu\text{g}$ DNA). Cultures were

incubated in medium without serum 1 hr after which the FCS was adjusted to 10%. At 3 hr, medium was removed and replaced with fresh medium containing serum, thereby removing T7 phage or DNA which had not become associated with cells in the monolayer. These cell stocks were then cultured continuously, making 10:1 splits when cells reached confluency; confluent monolayers were trypsinized, 10% of the cell population reseeded in flasks, and 90% of the cell population collected by centrifugation and extracted for DNA. In this manner cells were collected in the seventh passage at 36 days during which time cells doubled an average of 24 times. Total SHE cells DNA was extracted from these stocks by lysis and solubilization of the lysate in 8 M urea, 1% SDS, 1 M NaClO₄, .01 M Tris-HCl, pH 7.9, and 0.001 M EDTA. Total cellular DNA was prepared for reassociation studies by sonication of the lysate, extraction with chloroform-octanol (95:5), alkaline hydrolysis in 0.2 M NaOH, reextraction with organic solvents and precipitation. The DNA extract was then dissolved in 0.4% SDS and heated in boiling water bath (5 min) prior to adjustment to 4 ml of 0.72 M sodium phosphate (pH 7.0) and 0.4% SDS and initiation of the renaturation process at 68°C. The effect of the SHE cellular DNA from cells 36 days after infection was measured on the reassociation kinetics of 2.82×10^{-8} M ³²P-T7 DNA (nucleotide) in 4 ml. The probe ³²P-T7 DNA and SHE DNA had been sheared to an average length of 700 nucleotides. The extent of reassociation at 68° and 0.72 M phosphate and 0.4% SDS was measured by hydroxyapatite chromatography. The percent of ³²P-T7 DNA reassociation is plotted versus the logarithm of C_0t (molarity ³²P-T7 DNA X seconds of reassociation): ○—○, ³²P-T7 DNA without added SHE DNA; X—X, ³²P-T7 DNA with DNA from 3.74×10^7 control SHE cells not incubated with T7 DNA; ◊—◊, ³²P-T7 DNA with DNA from 4.22×10^7 T7 DNA-incubated SHE cells; △—△, ³²P-T7 DNA with DNA from 3.29×10^7 T7 phage-incubated SHE cells.

Figure 11

T7 GENOME EQUIVALENTS PER CELL

Figure 11. The average number of T7 genome equivalents per SHE cell in continuous stock cultures. The data in this figure was taken directly from the experiment described in Figure 10 and Table IV with the addition of data obtained at 58 days and 72 days after initial exposure of SHE cultures to T7 DNA. The following conditions are examined: cells exposed to T7 DNA in the presence of a deoxynucleotide supplement (X—X); cells exposed to T7 DNA in the absence of deoxynucleosides (0—0); and cells exposed to T7 phage with the deoxynucleoside supplement (Δ — Δ).

Figure 12. The net harvest of T7 DNA from continuous stock cultures. Data in this figure is taken from the experiment described in Tables V and VI. Calculations were made by the procedure outlined in Table III from renaturation kinetic experiments like that described in Figure 10. Starting with the end of passage 3 (day 19) the amount of T7 DNA detected in the extract from 90% of the cells by renaturation kinetics for each ensuing passage, passages 3 through 11, (day 19 through day 68) was plotted additively. The results are expressed in terms of 10^{-11} moles T7 DNA detected at various times during continual culture: 0—0, cells exposed to T7 DNA; Δ -- Δ , cells exposed to T7 phage; $\blacktriangle$ -- $\blacktriangle$, cells exposed to T7 phage and then DNase treated at day 5.

Figure 13

T7 GENOME EQUIVALENTS

Figure 13. The theoretical harvest of T7 DNA from continuous stock cultures calculated from the expected total cell progeny in the absence of harvest. The data in this figure is taken from the experiment described in Tables V and VI. The theoretical harvest expressed in T7 genome equivalents is calculated by multiplying (1) the actual cell number at day 5 for each condition, by (2) the cell multiplication factor at the end of each passage, and then by (3) the average number of T7 genome equivalents detected at the end of each passage: 0—0, cells exposed to T7 DNA; Δ -- Δ , cells exposed to T7 phage; $\triangle$ -- $\triangle$, cells exposed to T7 phage and then DNase treated at day 5. The points at initiation of exposure (day 0) represent the total amounts of T7 genomes added to the total culture.