'INTEGRATION' OF POLYOMA VIRUS DNA INTO MAMMALIAN GENOMES

by

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SUMMARY

Cells of mouse (secondary embryo cultures), hamster (BHK-21) and human (Hep-2) origin were infected with Polyoma virus, and the high molecular weight cellular DNA was isolated, at various times after infection, by sedimentation velocity through alkaline sucrose gradients. Some of the Polyoma DNA became associated (ie. 'integrated') with the mouse and hamster DNA, but little if any with the human DNA. Following viral DNA replication in the mouse cells, approximately half of the newly synthesized Polyoma DNA became cell DNA-associated.

INTRODUCTION

A characteristic property of cells transformed by the Papovaviruses Polyoma and SV40 (Simian virus 40) is the persistence of viral DNA, apparently covalently linked to the cellular DNA (1,2). The integrated SV40 or Polyoma genes appear to be transcribed contiguously with adjacent host genes (3,4,5), giving rise to nuclear transcripts considerably larger than single viral genome size. The fact that similar large viral RNA is synthesized during productive Polyoma infection (6,7,8) suggested to us that Polyoma DNA might become integrated during the normal course of its replication cycle. We therefore decided to examine 'integration' of Polyoma DNA in mouse cells; in BHK-21 cells, which do not allow viral DNA replication (9), and in Hep-2 cells. Integration is defined here by the ability of viral DNA to associate with cellular DNA in an alkali-stable linkage. Integration of SV40 DNA and of adenovirus 12 DNA into the cellular DNA of abortively infected cells was demonstrated recently (10,11).

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METHODS

All cells were cultivated in Dulbecco's modified MEM, with five percent foetal calf serum. Details of the methods used in the propagation and assay of Polyoma virus, and of the infection of cultures, have been described elsewhere (12).

Labelled Polyoma DNA was prepared by adding 5μc/ml of tritiated thymidine (specific activity 49.2 Ci/mM) to primary mouse kidney cultures 26-30 hours post infection. The Polyoma DNA was selectively extracted by the method of Hirt (13). The extracts containing Polyoma DNA were then further extracted with phenol and subjected to equilibrium centrifugation in CsCl (density 1.60 gm/ml) containing 100μg/ml of ethidium bromide (14). The band of type 1 DNA was extracted with isopropanol to remove ethidium bromide and dialyzed against 0.01 x SSC*.

Hybridization with labelled sonicated Polyoma DNA was carried out by the technique of Denhardt (15), as modified by Aloni et al (16), except that the salt concentration was raised to 6 x SSC during the incubations.

RESULTS AND DISCUSSION

In the experiments described here, resolution between cellular DNA and free viral DNA was effected by alkaline sucrose gradient sedimentation. Figure 1 illustrates the resolution routinely achieved under the standard conditions of sedimentation. All forms of free Polyoma DNA (53s type 1; 16 and 18s type II) are completely resolved from the much heavier single strands of cellular DNA.

Table 1 shows the result of an experiment designed to examine the extent of association of labelled Polyoma DNA of infecting virus with the cellular DNA of mouse and hamster cultures. As a control, an equivalent amount of viral DNA was mixed with uninfected cells just prior to layering onto the

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* 1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate
SDS = sodium dodecyl sulfate

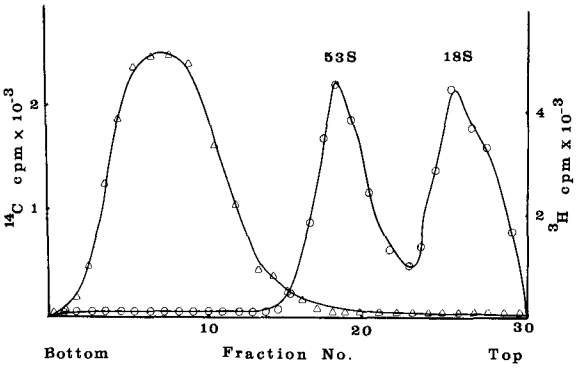

Fig. 1. Alkaline sucrose gradient sedimentation of mouse DNA and Polyoma DNA.

Mouse embryo cells (about $5_4 \times 10^6$), previously incubated for 24 hours in ^{14}C-thymidine (0.25 μCi; specific activity 57 μCi/mmole, Amersham-Searle), were harvested by trypsinization, washed and resuspended in 0.25 ml of PBS (phosphate buffered saline minus divalent cations, pH 7.3). Purified Polyoma virus containing methyl-^{3}H-labelled DNA, was added to the cells, and the mixture layered gently onto 1.0 ml of lyzing solution (0.5M NaOH, 0.01M EDTA, 0.2% SDS) on top of a 36 ml gradient of 10-30% sucrose (w/v in 0.3M NaOH, 0.001M EDTA, 0.01% SDS) in a Beckman SW27 polyallomer tube. After 12 hours at 20°C, the gradient was centrifuged for 5 hours at 25,000 rpm at 20°C. Fractions were collected from the top of the tube by pumping 50% sucrose into the bottom of the tube.
We are grateful to Branko Palcic of McMaster University for communicating to us the details of this technique for alkaline sucrose gradient sedimentation.

gradient. About one percent of the labelled viral DNA became adventitiously associated with the cellular DNA in the control. The experimental values are all significantly greater than this value. The 'integration' apparently precedes viral DNA replication in the mouse cells (which commences about 12 hours after infection (13)), and occurs at about the same level in the BHK-21 cells, which do not support viral DNA replication (9).

Table II illustrates the results of an experiment in which mouse, hamster and human cells were infected with un-labelled Polyoma virus. Most of the integration due to parental DNA occurred during the first 6 hours of

Table 1: Integration of Labelled Polyoma Viral DNA

Cell type	Time after infection (hours)	Percent of viral DNA integrated (duplicate tubes)	
Mouse embryo	6	5.9	2.2
	12	6.7	7.1
	24	2.1	4.1
BHK-21	6	4.1	6.2
	12	6.0	5.3
	24	9.1	6.3
Mouse embryo - Polyoma DNA (20,000 cpm) mixed	-	0.91	1.13

Cultures containing about 3.2×10^6 cells were infected with Polyoma virus containing 20,000 cpm of ^{3}H-labelled DNA. The mouse cells took up 15% of the virus during a 2-hour absorption period, while the BHK-21 cells took up 8%. Cells were harvested at various times after infection and subjected to alkaline sucrose gradient sedimentation, as described in the legend to Figure 1. The integration values represent the percentage of the DNA taken up that was recovered in the cellular DNA band. For the control, 20,000 cpm of DNA extracted from the Polyoma virus preparation were mixed with uninfected mouse cells prior to layering on a gradient.

infection. The extent was again similar for BHK and mouse cultures and represented a considerable fraction of the total viral DNA within the cells. After the initiation of viral DNA replication, there was a large increase in the amount of viral DNA associated with the cellular DNA fraction (Table II). The quantity of integrated viral DNA at 24 hours p.i. (determined by including a hybridization calibration curve) was considerably greater than the original input virus DNA, and therefore must have represented newly synthesized viral DNA. Of the order of 50% or more of the total cellular content of viral DNA at any given time was apparently integrated.

The Hep-2 cells showed a much smaller level of integration, though it appears to be significant in this experiment. However in another experiment the level of 'integration' in Hep-2 cells was no higher than a control measuring adventitiously bound viral DNA. Thus integration may occur in Hep-2 cells but could be limited by a relatively small degree of virus uptake

Table II: Integration of Polyoma viral DNA

Cell type	Time post infection	cpm hybridizing Viral	Cellular
BHK-21	6 hours	560	297
	12 hours	386	318
	24 hours	367	333
Mouse embryo	6 hours	182	549
	12 hours	227	1,295
	24 hours	5,642	5,768
Hep-2	12 hours	168	73

Cultures were infected with Polyoma virus (50 pfu/cell) and samples (about 5 x 10^6 cells) taken at various times after infection for alkaline sucrose gradient sedimentation, as described before. Cellular DNA fractions and viral DNA fractions were separately pooled. The DNA-pools were dialyzed against 0.1 x SSC; concentrated; extracted with phenol, followed by ether extraction, and heated at 98° for 10 min before fixing to 2.5 cm Millipore HA filters in 6 x SSC. The filters were cut into 0.65 cm diameter discs for DNA-DNA hybridization with sonicated ^{3}H-labelled Polyoma DNA. The specific activity of the Polyoma DNA was 2 x 10^5 cpm/γ.

or uncoating. Two types of control were run simultaneously with this experiment. Samples of Polyoma DNA, unlabelled a) or labelled b), were separately mixed with uninfected BHK-21 cells and layered onto alkaline sucrose gradients in the usual way (Figure 2). After centrifuging, the distribution of Polyoma DNA was determined, by hybridization in a) and by radioactivity in b). In both cases, approximately 12% of the viral DNA was recovered in the cellular DNA band.

The extent of integration was found to be dependent upon the dose of inoculating virus. Thus with increasing input virus, more integration was detected, in both mouse and BHK-21 cells, although the increment was less

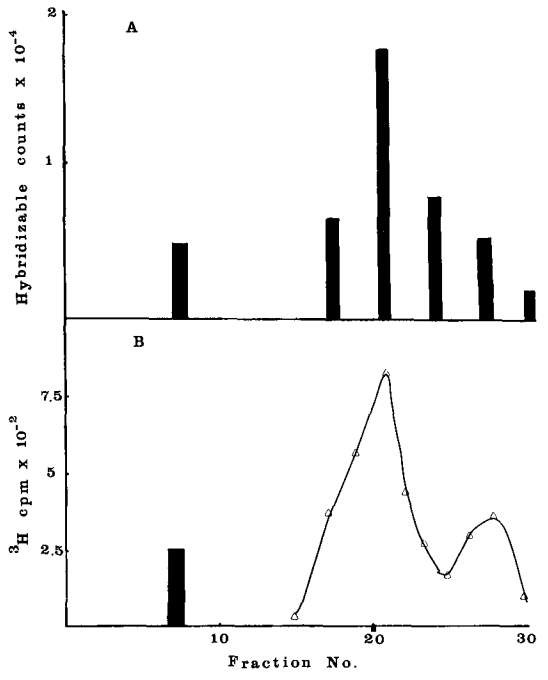

Fig. 2. Alkaline sucrose gradient sedimentation of mixtures of Polyoma DNA and BHK-21 cells.

Experimental details as before. 6 Samples of uninfected BHK-21 cells (5 x 10^6 cells) were mixed with a) 2γ of unlabelled Polyoma DNA; b) 5,000 cpm of ^{3}H-labelled Polyoma DNA (0.025γ). In a) the lowest 16 mls were pooled for the cellular DNA regions and the remainder of the gradient combined in 5 equal pools for free viral DNA.

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than proportional, suggesting that there may be a limited number of 'integration sites".

Finally, as an indication of the specificity of the hybrid formed between the labelled Polyoma DNA and the putative Polyoma DNA sequences in the filter bound cellular DNA, thermal denaturation of the DNA-DNA hybrids was measured over a range of temperatures. Representative profiles are illustrated in Fig. 3. Similar tests were performed for other times after infection, with no difference in the sharpness of the profiles or in Tm values.

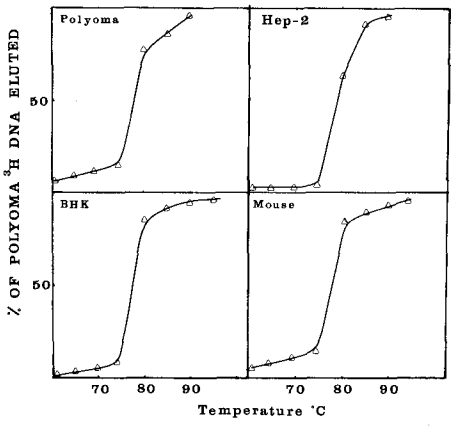

Fig. 3. Thermal denaturation of Polyoma DNA-cell DNA complexes.

Nitrocellulose filters containing infected-cell DNA-tritiated Polyoma DNA complexes were washed in 2 x SSC and then were incubated for 10 min periods at successively increasing temperatures. The incubation medium comprised 0.5 ml of 2 x SSC. The labelled DNA eluted at each temperature was precipitated by trichloracetic acid and collected on membrane filters for radioactivity measurement. The unlabelled DNA on the filter was in a) denatured Polyoma DNA type 1; b) mouse embyro cell DNA, 12 h.p.i.; c) BHK-21 cell DNA, 12 h.p.i.; d) Hep-2 cell DNA, 12 h.p.i.
The 100% cpm values were respectively: 7,333; 4,035; 2,428; and 336.

The results strongly argue in favour of the hypothesis that Polyoma viral DNA becomes integrated into the cellular DNA during the normal course of infection in mouse cells and in BHK-21 cells. In the former case a considerable amount of newly synthesized viral DNA also appears to be integrated. Further characterization of these events is in progress.

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