EVIDENCE FOR A REGULATORY FUNCTION RELATED TO THE EXPRESSION OF THE POLYOMA GENOME FOLLOWING THE ONSET OF VIRUS DNA REPLICATION

R.B. Rutherford and J.D. Hare

Department of Microbiology
University of Rochester School of Medicine and Dentistry
Rochester, New York 14642

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Summary

Mouse embryo cells infected with the 3049 strain of polyoma virus contain more virus-specific polyadenylated nuclear and polysomal RNA following the onset of viral DNA synthesis when compared to cells infected with a control, wild-type virus. Prior to the onset of DNA synthesis, no difference in the quantity of virus-specific, polyadenylated RNA is observed. This finding is consistent with a theory postulating the existence of a function regulating the expression of the polyoma genome following the onset of viral DNA synthesis.

Cells productively infected with polyoma virus synthesize virus specific polypeptides in the cytoplasm followed by their migration to the nucleus where particle assembly takes place. In most strains of polyoma virus, capsid antigen accumulates to levels demonstrable by fluorescent antibody techniques only in the nucleus (1).

A variant of polyoma virus, the 3049 strain, has been described (2) which differs from other strains in that capsid antigen appears in detectable quantities in the cytoplasm of permissive mouse cells concomitant with its appearance in the nucleus. More recently 3049 infected cells have been shown to contain 3-5 times as much capsid antigen as cells infected with a control, wild-type virus (1pS), as measured by an indirect radio-immunoassay (3). These and other data (4) support the hypothesis that this phenotype is the manifestation of a virus-controlled increase in the amount of capsid antigen produced in 3049-infected, permissive cells and prompted further study into the mechanism of this unique polyoma phenotype.

Abbreviations: SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetracetic acid; MOI, multiplicity of infection; FdU, 5-fluorodeoxyuridine; SSC, 0.15 m sodium chloride, 0.015 m sodium citrate; SV_{40}, Simian Virus 40.

Experiments were carried out to measure, by DNA-RNA hybridization, the amounts of virus specific RNA synthesized in permissive cells at various times after infection by the 3049 strain and to compare this with similar RNA in cells infected with a representative wild-type virus, the lpS strain. The virus-specific RNA was measured by the "ternary complex" assay described by Weinberg et al. (5). This technique was adopted because it offered a method to obviate the influence of uncontrolled fluctuations in the specific activity of the ribonucleotide precursor pool which leads to variations in the specific activity of populations of RNA molecules labeled in the infected cell. In this technique, labeled virus DNA is hybridized in solution with unlabeled infected cell RNA and the labeled hybrids are then bound to poly U charged glass fiber (GF/C) filters (6) by the free poly A tags on the 3' termini of the RNA molecules. It was shown that the binding process was inhibited by the addition of poly U to the DNA-RNA mixture prior to filtration and that the quantity of DNA bound to the filters was proportional to the RNA input. The sensitivity of this method has been significantly increased for the study reported here by labeling purified component I lpS and 3049 DNA *in vitro* with ^{125}I utilizing the technique of Commerford (7).

Materials and Methods

Culture conditions: Secondary mouse embryo fibroblasts (MEF), propagated in roller vessels, were infected with the lpS and 3049 strains at MOI of approximately 100 plaque forming units per cell. These MOI resulted in equal numbers of infected cells as measured by an indirect immunofluorescent assay at 24 hours after infection. Mock infected MEF were utilized as controls. Control and infected cells were refed with medium containing 1% dialyzed fetal calf serum and 15 µg/ml FdU (Calbiochem) and incubated for 24 hours at which time cultures were harvested for extraction of "early" RNA. Other cultures were treated with thymidine (20 µg/ml) to reverse the FdU block and harvested 12 hours later for "late" RNA. Nonsynchronized cultures were refed with 1% fetal calf serum and harvested 24 hours after infection.

Virus DNA: Viral DNA was purified by propidium di-iodide-CsCl isopycnic centrifugation (8) of Hirt supernates (9). Component I 1pS and 3049 DNA was denatured by heating at 100°C for 10 min in 0.1 x SSC and rapid cooling in an ethanol-dry ice mixture. Aliquots were labeled with ^{125}I to specific activities of 1-8 x 10^6 cpm/µg by the method of Commerford (7).

Infected Cell RNA: RNA was prepared from nuclear and polyribosomal fractions separated by layering whole cell homogenates (lysed in high salt buffer, ref. 10) on 10-40% (w/v) sucrose gradients (prepared in 10 mM Tris pH 8.9 at 25°C, 3 mM $MgCl_2$, 15 mM KCl) with a 2 ml, 2.3 M sucrose cushion and centrifuged in a Beckman SW 27 rotor at 25,000 rpm at 4°C x 150 minutes. Nuclei were recovered from the cushion, phenol-chloroform, isoamyl alcohol extracted (11), ethanol precipitated, treated with DNase (Worthington electrophoretically purified-RNase free), sedimented through CsCl and resuspended in 2 x SSC. Polyribosomes were pooled, eluted with SDS-EDTA, the RNA precipitated with ethanol, (12) and then extracted with chloroform-isoamyl alcohol (13), ethanol precipitated, resuspended, desalted by passing through G-25 Sephadex (in H_2O), lyophylized and resuspended in 2 x SSC.

Assay Mixtures: 1pS and 3049 infected cell RNA was reacted with homologous or heterologous DNA (105 ng) for 18 hours at 65°C in 2 x SCC in a total volume of 150-200 microliters. The incubation mixtures were then absorbed for 15 min at 25°C to GF/C filters charged with 80 µg of poly U (Miles Lab), washed with 50 ml of 2 x SSC, dried and counted by either liquid or gamma-scintillation spectrometry. Controls include: polyoma DNA reacted with mock infected, cellular RNA, homologous DNA-RNA mixtures plus 20 µg poly U added prior to filtration and DNA incubated without RNA.

Results

"Early" RNA (Figs. 1 and 3) from nuclear and polyribosomal fractions of either 3049 or 1pS infected cells shows no difference in its capacity to bind ^{125}I labeled, denatured, viral DNA to poly U filters. In addition there is a

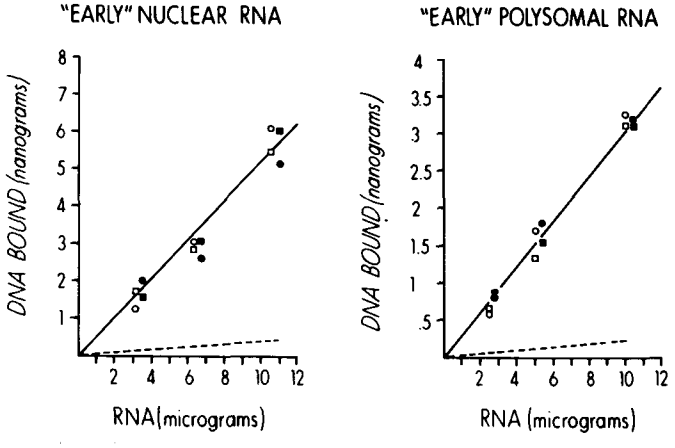

Fig. 1. Binding of labeled polyoma DNA to poly U charged filters by reaction with "early" RNA extracted from nuclei and polyribosomes of infected cells treated for 24 hours with FdU. Data have been corrected by subtracting the amount of DNA binding to the filters in the absence of RNA. This background binding never exceeded 1% of input DNA. □—□ 3049 RNA + 3049 DNA, ■—■ lpS RNA + lpS DNA, O—O 3049 RNA + lpS DNA, ●—● lpS RNA + 3049 DNA, ---- average of the DNA binding by mock RNA + lpS DNA and 3049 DNA, and homologous DNA-RNA mixtures plus poly U.

small but reproducibly higher virus-specific binding capacity of the RNA from the nuclear as compared to the polyribosomal fraction. In contrast, late RNA (Figs. 2 and 3) from similar fractions of 3049 infected cells binds nearly twice as much 3049 or lpS DNA to poly U filters as does comparable quantities of RNA from lpS infected cells. Furthermore, following the onset of "late" transcription there is a 10-fold increase in the quantity of virus specific RNA in the polyribosomes of 3049-infected cells and a 5-fold increase in lpS infected cells when compared to similar "early" fractions. "Late" 3049 nuclear RNA shows a 7-fold increase and lpS a 4-fold increase when compared to the respective "early" nuclear RNA. RNA from similar fractions of non-synchronized 3049 infected cells harvested 24 hours after infection bound twice as much DNA as the RNA from identically prepared lpS infected cells (data not shown).

The data in Figure 3 also show that RNA prepared from mock-infected cells bind less than 6% of the polyoma DNA bound to infected cell RNA supporting the specificity of the ternary assay. Furthermore, specific binding is more than

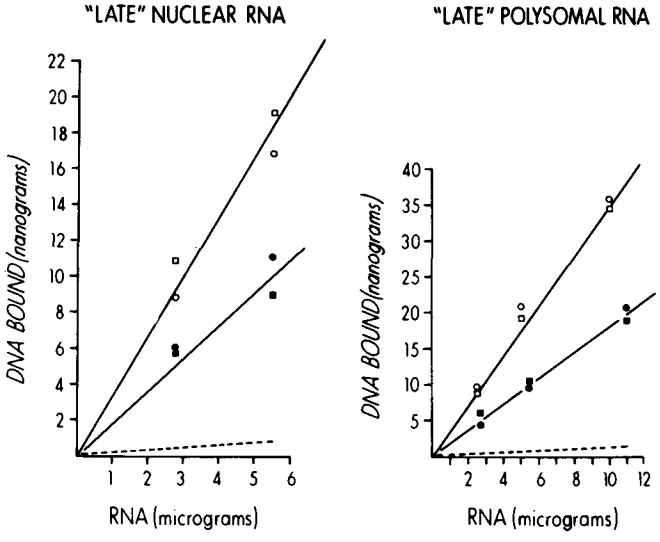

Fig. 2. Binding of labeled polyoma DNA to poly U charged filters by reaction with "late" RNA extracted from nuclei and polyribosomes of infected cells treated for 24 hours with FdU (15 μg/ml) followed by 12 hour treatment with thymidine (20 μg/ml). Symbols are the same as in Fig. 1.

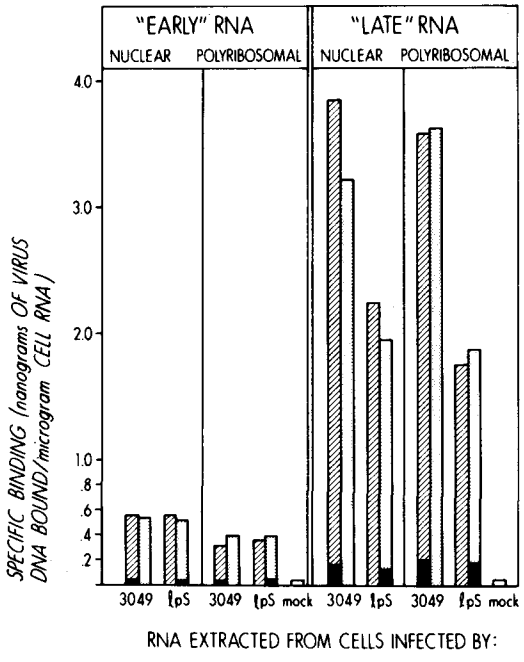

Fig. 3. The specific binding of viral DNA to poly U filters by nuclear and polyribosomal RNA extracted from cells infected with 3049 or lpS viruses or from mock infected cells. Specific binding is defined as the nanograms of viral DNA bound per microgram cell RNA derived from the data depicted in Figs. 1 and 2. Hatched, 3049 DNA; Stippled, lpS DNA; Open, Mock; Solid, poly U plus reaction mixture.

90% inhibited by the addition of poly U to the hybridization mixtures prior to filtration, evidence which supports the conclusion that the polyoma-specific RNA sequences measured here are polyadenylated. Furthermore, the demonstration that virus DNA does not prevent the reaction of the RNA with poly U filters and that less than 1% of the input denatured DNA is retained on the poly U filters is evidence that the viral genome does not code for poly A. These findings are consistent with the demonstration that SV_{40} virus DNA does not code for the poly A sequences found in SV_{40} specific RNA (5, 14).

The observation that virus specific, polyadenylated, "early" RNA molecules are present in equal amounts in cells infected by either virus supports, by another experimental parameter, the fact that the cultures infected by each virus contain equal numbers of infected cells.

Discussion

These studies demonstrate that prior to the onset of viral DNA synthesis, no difference in the levels of virus-specific, polyadenylated RNA is found in the nucleus and polyribosomal fractions of lpS or 3049 infected cells. This observation is consistent with earlier studies which demonstrated that the synthesis and distribution of T antigen, an "early" virus function (15), did not differ qualitatively between 3049 and lpS infected cells (2). Following the onset of viral DNA synthesis, however, approximately twice as much virus-specific, polyadenylated RNA is found in similar fractions of 3049 infected cells when compared to lpS infected cells. This phenomenon then correlates temporally with the appearance of capsid antigen in the cytoplasm as demonstrated by immunofluorescence and the several fold increase in capsid proteins detected by radioimmunoassay in cells infected with this polyoma strain.

These data do not indicate the mechanism responsible for the difference in the levels of virus specific RNA. They perhaps reflect enhanced transcription of 3049 DNA, or alternatively, either an increase in the polyadenylation of total cellular RNA in 3049 infected cells or an enhanced polyadenylation of only

3049 specific sequences. Experiments to measure the pool of virus specific transcripts and the relative levels of total polyadenylated RNA in 3049, lpS and mock infected cells are currently in progress. Since it has been shown that both "early" and "late" sequences are transcribed following the onset of polyoma (16) and SV_{40} (17) DNA synthesis, it will be of interest to determine the relative contribution of "early" 3049 specific RNA to the enlarged pool of virus specific transcripts. Furthermore, these findings do not exclude differences in the relative stabilities of lpS and 3049 RNA but increased levels of virus RNA both in the nucleus and on cytoplasmic polyribosomes makes this alternative less likely.

These findings support the hypothesis that the 3049 strain of polyoma virus represents a unique variant in which the phenotype reflects an alteration in the regulation of expression of the polyoma genome following the onset of viral DNA synthesis. More conclusive evidence must show that the increased levels of capsid antigen are the direct result of an increase in viral m-RNA.

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References

1. Bereczky, E., Hughes, R., Bowen, J.M., Runyon, R. and Dmochowski, L., (1965) Texas Rep. Biol. and Med. 23, 3-15.
2. Hare, J. Donald, (1970) Virology 40, 978-988.
3. Tachovsky, T.G., (1974) Ph. D. Thesis, Department of Microbiology, University of Rochester.
4. Betts, R.F., Tachovsky, T.G. and Hare, J.D., (1972) J. Gen. Virol. 16, 29-38.
5. Weinberg, R.A., Ben-Ishai, Z. and Newbold, J.E., (1972) Nature New Biology 238, 111-113.
6. Sheldon, R., Jurale, C. and Kates, J., (1972) Proc. Nat. Acad. Sci. USA 69, 417-421.
7. Commerford, S. L., (1971) Biochemistry 10, 1993-2000.
8. Hudson, B., Upholt, M.B., Devinny, J. and Vinograd, J., (1968) Proc. Nat. Acad. Sci. USA 62, 813-820.
9. Hirt, B., (1967) J. Mol. Biol. 26, 365-369.

10. Gielkens, A.L.J., Burns, T.J.M. and Blumendal, H., (1971) J. Biochem. 22, 478-484.
11. Penman, S., (1966) J. Mol. Biol. 17, 117-130.
12. Adesnik, M. and Darnell, J.E., (1972) J. Mol. Biol. 67,397-406.
13. Oda, K.and Joklik, W.K., (1967) J.Mol. Biol. 27,395-419.
14. Aloni, Y., (1973) Nature New Biology 243, 2-5.
15. Fogel, M., Gilden, R. and Defendi,V., (1967) Proc. Soc. Exptl. Biol. Med. 142, 1047-1052.
16. Hudson, J., Goldstein, D. and Weil, R., (1970) Proc. Nat. Acad. Sci. USA 65, 226-233.
17. Khoury, G., Byrne, J.C. and Martin, M.A., (1972) Proc. Nat. Acad. Sci. USA 69, 1925-1928.