RNA-Directed DNA-Polymerase Activity in Greene Hamster Melanoma

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INTRODUCTION

The present investigation is concerned with the search for RNA-directed DNA-polymerase activity in human and hamster melanoma cells in both in vivo tumors and tumor cells grown in tissue culture. The assays and techniques employed are based upon model studies carried out in this laboratory with the enzyme from avian myeloblastoses virus, and feline sarcoma, and lymphoma virus.

In recent years, several workers have found what appear to be virus-like particles of considerable interest in animal melanomas; and similar particles are being sought in human ocular and skin melanomas although none has as yet been reported.

New methods for the biochemical investigation of the mechanism of reproduction of RNA tumor viruses were opened when Temin and Mizutani (1) and Baltimore (2) reported findings of an enzyme present in RNA tumor virus which could use an RNA template to catalyze the formation of DNA. Detection of this enzyme provides a marker for the identification of the virus itself. The enzyme can be thought of as the "footprint" of the virus, since it is possible that the enzyme and the DNA which code for the virus could be present, but the virus is not present as a morphologic entity. The enzyme activity also provides a more sensitive, if less definitive, assay for virus particles than techniques such as electron microscopy. Thus, if animal and human melanomas were caused by an RNA tumor virus which carried the enzyme RNA-directed DNA-polymerase, the enzyme assay would appear to be a logical first step toward detecting its presence.

METHODS

Polyribonucleotides were a product of Miles Laboratories, Elkhart, Indiana; concentrations were determined from extinction coefficients provided by the manufacturer and an assumed MW of 100,000. Oligodeoxyribonucleotides were obtained from Collaborative Research, Waltham, Massachusetts. Chain lengths were listed at 12–18 units and their concentrations were determined from the extinction coefficients of the respective deoxyribonucleotides. The tritium-labeled thymidine tri-
phosphate was obtained from New England Nuclear Corporation (43.8 Ci/mM). The unlabeled triphosphates were obtained from P-L Biochemicals.

Avian myeloblastosis virus was kindly provided by Dr. J. Beard, as a plasma suspension. The virus was further purified by chromatography on porous glass beads.

Reaction mixtures were incubated for 40 min at 35°C and contained, in the complete system (unless stated otherwise), in 0.1 ml: 0.1 M Tris (pH 8.3); 2.5 mM magnesium chloride (or 0.3 mM manganese chloride); 60 mM sodium chloride; 20 mM dithiothreitol; 1.0% Nonident P-40; 0.1 μM [3H]-thymidine triphosphate (96 × 10⁵ cpm/pmole); 22.5 μM ATP. Where indicated, the polymer concentrations were: poly(rA), 1.2 μmoles P/ml; oligo(dT), 2.1 × 10⁻⁷ M. Unless stated otherwise, 10 μliters of 10.0 mg protein per ml of tissue homogenized in 0.1 M Tris (pH 8.3), 20 mM dithiothreitol, using a Potter-Elvehjem homogenizer. When calf-thymus DNA was used as a template, its concentration was 0.1 mg/1 ml.

The cell lines used in tissue culture were from the Greene transplantable hamster melanoma maintained by serial passage both subcutaneously in Golden hamsters and in tissue culture.

When tissue culture cells were used for an assay, they were removed from the culture fluid by spinning at 10,000 g for 10 min. The supernatant culture fluid was then spun at 100,000 g for 1 hr and the resulting pellet was used for assays.

For BUDR treatments, the cells were allowed to stand for 40 hr in the presence of 10⁻⁴ M BUDR. Exposure to light was kept to a minimum.

RESULTS

In order to develop an assay for the RNA-directed DNA-polymerase, extensive studies were carried out with the polymerase from avian myeloblastosis virus (AMV). If the enzyme obeyed standard Michaelis–Menten kinetics, the result expected is that shown by the dotted lines in Fig. 1, when the rate is followed by incorporation of radioactivity into an acid-precipitable product in the presence of varying amounts of cold substrate. Each dotted line represents a different Kₘ for the enzyme. Instead, the result shown by the solid line in Fig. 1 was obtained. This was found using both crude and purified enzyme from AMV, where poly-(rA)·oligo (dT) was the template and increasing amounts of cold thymidine triphosphate was added to the reaction mixture. Experiments of this type using Rickard feline leukemia, and sarcoma virus yielded comparable results.

Similar experiments were then carried out with Greene hamster melanoma tumors. These results can be seen in Table 1. It was found that tumor which appeared to be grossly nonpigmented, yielded the same result as pigmented tumor. In both cases it was found that the amount of radioactivity incorporated in the presence of increasing amounts of thymidine triphosphate increased in the same manner as that found with the AMV polymerase.

In contrast to the above results, when Greene hamster melanoma cells from tissue culture were used, minimal activity was seen with either the cells, or the 100,000 g pellet from the culture fluid (see Table 2). What little activity was seen was found using a DNA template at low substrate concentrations (high specific activity).

When the tissue culture cells were treated with 10⁻⁴ M BUDR for 40 hr, little activity was found with the cells; however, the pellet obtained from the 100,000
Fig. 1. —RNA-directed DNA-polymerase activity for the enzymes from avian myeloblastosis virus, as followed by acid precipitable counts in the presence of various concentrations of cold TTP. The dotted lines are theoretical curves for an enzyme obeying Michaelis–Menten kinetics with the listed $K_m$ values.

**TABLE 1**

**GREENE HAMSTER MELANOMA TUMOR:**
**DNA POLYMERASE ACTIVITY**

| Poly(A) · Oligo(dT) | cpm/pmole TTP | Pigmented | Nonpigmented |
|---------------------|---------------|-----------|--------------|
| $10^{-5}$ M TTP     | 960           | 2220      | 3000         |
| $10^{-6}$ M TTP     | 9600          | 1900      | 1500         |
| $10^{-7}$ M TTP     | 96,000        | 400       | 300          |

* Experiment carried out using Mg$^{2+}$ and template in the presence of various concentrations of thymidine triphosphate, resulting in the specific activities shown.

* Counts per minute of $[^{3}H]$-thymidine monophosphate found in the acid precipitable product after 40 min.

**TABLE 2**

**GREENE HAMSTER MELANOMA TISSUE CULTURE:**
**DNA POLYMERASE ACTIVITY**

| Poly(A) · Oligo(dT) | cpm/pmole TTP | Cells | Pellet |
|---------------------|---------------|-------|--------|
| $10^{-5}$ M TTP     | 960           | 200   | 400    |
| $10^{-7}$ M TTP     | 96,000        | 200   | 400    |
| Calf thymus DNA     |               |       |        |
| (+ dATP, dCTP, dGTP)| $10^{-5}$ M TTP | 960   | 400    | 400    |
| $10^{-7}$ M TTP     | 96,000        | 1000  | 400    |

* Experiment carried out using Mg$^{2+}$ and template in the presence of various concentrations of thymidine triphosphate, resulting in the specific activities shown.

* Counts per minute of $[^{3}H]$-thymidine monophosphate found in the acid precipitable product after 40 min.
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**TABLE 3**

**GREENE HAMSTER MELANOMA BUDR TREATED CELLS: DNA POLYMERASE ACTIVITY**

|                        | CPM<sup>a</sup> |             |             |
|------------------------|-----------------|-------------|-------------|
|                        | cpm/p mole TTP  | Cells       | Pellet      |
| Poly(A) · Oligo(dT)    |                 |             |             |
| Mg<sup>2+</sup>        | $10^{-5}$ M TTP | 960         | 200         | 1400        |
| Mg<sup>2+</sup>        | $10^{-3}$ M TTP | 96,000      | 300         | 300         |
| Mn<sup>2+</sup>        | $10^{-5}$ M TTP | 960         | 700         | 2500        |
| Mn<sup>2+</sup>        | $10^{-3}$ M TTP | 96,000      | 1000        | 600         |
| − NP-40                | $10^{-5}$ M TTP | 960         | 100         | 300         |
| − NP-40                | $10^{-7}$ M TTP | 96,000      | 200         | 900         |
| Calf thymus DNA (+ dATP, dCTP, dGTP) |  |             |             |
|                        | $10^{-5}$ M TTP | 960         | 400         | 200         |
|                        | $10^{-7}$ M TTP | 96,000      | 2000        | 500         |

<sup>a</sup> Counts per minute of [αH]-thymidine monophosphate found in the acid precipitable product after 40 min.

g spin showed an increase in activity when the amount of thymidine triphosphate was increased (see Table 3). These results were found with both Mg<sup>2+</sup> and Mn<sup>2+</sup>. The activity required detergent (NP-40) and showed a preference for the synthetic RNA template over a DNA template.

**DISCUSSION**

Using RNA-dependent, DNA-polymerase activity to look for the presence of virus, several studies have been carried out using synthetic templates. It is found that caution is necessary in the interpretation of findings based solely on the use of synthetic templates, since it has been known for sometime that the DNA polymerase of *Escherichia coli* accepts poly(rA) · poly(rU) as a template to synthesize poly(dA) · poly(dT) (3) and recently it was found that with poly(rA) · poly(dT) as a template, poly(dT) is formed with high efficiency (4). It has also been shown that human fibroblasts (5), rat liver (6), chicken embryos (7–9), normal human lymphocytes (10), and human cells in tissue culture (11–14) have a similar activity. It was shown, however, by Ross *et al.* (15) and Goodman *et al.* (16) that it is possible to distinguish between animal RNA tumor virus polymerase and other known DNA polymerases. This use of relative activity toward different synthetic templates was used in the search for reverse transcriptase activity in human tumors (17–18).

In order to obtain a more sensitive assay for the enzyme activity, experiments were carried out using avian myeloblastosis virus (AMV). Kinetic studies with the polymerase from this virus showed a marked deviation from standard Michaelis–Menten kinetics as seen in Fig. 1. This result shows apparent cooperativity for thymidine triphosphate (TTP) as a substrate during the enzyme-catalyzed polymerization. This type of activity was not found using poly(rC) · oligo(dG) as a template. In this latter case, increases in nonradioactive dGTP concentration only lowered the amount of radioactivity found in the acid precipitable product as would be expected from the lowering of the specific activity. Other deoxynucleotide triphosphates do not show the rate-enhancement effect (i.e., dATP, dCTP, dGTP)
with the poly(rA)·oligo(dT) templates that was shown by dTTP. The apparent preference for the poly(rA) over poly(rC) could be tied to the fact that the RNA of animal tumor viruses is known to have long stretches of poly A (19–22). Results similar to the above were also obtained with Rickard feline sarcoma, and leukemia virus.

Using the cooperativity effect in addition to template specificity, experiments were carried out to look for this type of activity in Greene hamster melanomas. The results (Table 1) with both grossly pigmented and nonpigmented tumors show that an enzyme activity is present which uses poly(rA)·oligo(dT) as a template to make poly(dT), and which shows an increase in incorporation of radioactive counts into the product in spite of the decline in specific activity. This is the same type of increase that was found for the RNA-tumor virus polymerases.

Studies carried out with the Greene hamster melanoma cells growing in tissue culture did not show this type of activity. As seen in Table 2, the polymerase in these cells shows a preference for a natural DNA template over the synthetic RNA template. Also the activity using the natural DNA template decreases with an increase in TTP concentration in keeping with the decrease in specific activity. There was little activity of any kind in the pellet resulting from the 100,000 g spin of the culture fluid.

Since the enzyme activity may not be apparent in the cells in tissue culture because the concentration of the virus is too low, we tried to find out if the virus could be stimulated to be released chemically. Several studies have shown that 5-bromodeoxyuridine can be used to stimulate the release of C-type particles from mouse, rat, and human cell lines in tissue culture (23–30). Accordingly, the Greene hamster melanoma cells were treated with $10^{-4} M$ BUDR (see Table 3). The results with the cells themselves were similar to those found before the BUDR treatment; however, after BUDR treatment polymerase activity is found associated with the pellet resulting from the 100,000 g spin of the culture fluid. This activity increases when the cold TTP concentration is increased and shows a detergent requirement. This is the type of behavior expected if the activity is associated with a viral particle similar to that of AMV. In addition, the activity is found with either Mg$^{2+}$ or Mn$^{2+}$ which is also true for the AMV enzyme. Underway now are studies of the nature of this pellet material and of human melanomas which show activity similar to that described above.

**SUMMARY**

As part of a biochemical search for a virus in melanomas preliminary kinetic studies with RNA-directed DNA-polymerase from avian myeloblastosis virus showed that TTP stimulates the enzyme. This stimulatory effect was used as an additional probe in detecting RNA-directed DNA-polymerase activity in Greene hamster melanoma in vivo. This enzyme activity, however, was not found in the Greene hamster melanoma cells growing in tissue culture under ordinary conditions. When the cells were treated with BUDR the enzyme could be demonstrated and the stimulating effect of TTP was observed.

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