Deoxyribonucleic Acid Polymerase Activity Associated with Strain MC29 Tumor Virus¹

G. H. WEBER, A. A. KIESSLING, AND G. S. BEAUDREAU

Department of Agricultural Chemistry, Oregon State University, Corvallis, Oregon 97331

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Chick embryo cells infected with strain MC29 tumor virus yielded progeny virus that contained detectable deoxyribonucleic acid (DNA) polymerase within the first 48 hr after infection. The noninfected culture fluids displayed no such enzyme activity when examined in an identical manner. Enzyme activity was greatly stimulated by adding DNA template to the reaction mixture and required all four deoxyribonucleoside triphosphates for full activity. When calf thymus DNA was used to direct synthesis, the DNA polymerase from the MC29 virus catalyzed the formation of DNA product having a higher buoyant density in CsCl. DNA product formed in the reaction directed by *Micrococcus lysodeikticus* DNA had the same buoyant density as the template DNA.

MC29 virus causes a rapid and uniform morphological change in chick embryo cells (CEC; references 3, 7, 9). Morphological changes have been noted within 48 hr, making it possible to investigate some of the processes involved in the early infection and conversion (3, 12). Recent reports have demonstrated that deoxyribonucleic acid (DNA) polymerase activity is associated with tumor viruses (1, 5, 6, 10, 13, 14, 19). The first two reports, by Baltimore (1) and by Temin and Mizutani (19), suggested that DNA-polymerizing activity is directed by ribonucleic acid (RNA) of the virus. Evidence for DNA polymerase activity directed by viral RNA was given by Spiegelman et al. (14) through the formation of virus RNA hybrids with the DNA product. Enzyme activity associated with the virus particle is strongly stimulated by the addition of DNA (11, 15). Avian myeloblastosis virus (AMV) contains DNA that is high in guanine and cytosine relative to cell DNA, and DNA templates that have a high content of guanine and cytosine are good templates for the DNA polymerase of the virion (11).

The present paper demonstrates DNA-polymerizing activity in strain MC29 tumor virus and compares the enzyme activity with a similar enzyme activity found in AMV. It was possible to demonstrate the presence of DNA-polymerizing activity associated with the virus fraction from CEC cultures infected with MC29 virus

for 48 hr. This enzyme activity was not found in culture fluids from corresponding uninfected control CEC.

DNA polymerase activity was easily detectable in preparations from virus fractions contained in as little as 8 ml of tissue culture fluid, and enzyme activity was measured from as few as 1.3×10^3 focus-forming units (FFU) per assay. These results support the appraisal of Beard and associates on the value of this tumor virus system for studies during early cell transformation (3).

MATERIALS AND METHODS

Preparation of CEC and infection with MC29 virus. Primary cultures of CEC were prepared from decapitated and eviscerated 10-day-old embryos. The minced tissue was trypsinized with 0.25% trypsin in phosphate-buffered saline (PBS) for 30 min. Harvested cells were filtered through four layers of sterile gauze, sedimented at $150 \times g$ for 5 min, and resuspended in growth medium. Plastic petri dishes (100 by 20 mm) were seeded with 6.0×10^6 cells in 10 ml of growth medium which consisted of 80% medium 199, 8% newborn calf serum, 10% tryptose phosphate broth, penicillin (50 units/ml), streptomycin (50 μ g/ml), and amphotericin B (0.5 μ g/ml). The cultures were incubated in a humidified, 5% CO₂ atmosphere at 38.5 C. Twenty-four hours later the cells were washed with PBS and infected with 0.2 ml of MC29 virus (kindly supplied by J. W. Beard) at a multiplicity of infection of approximately 0.02 FFU/cell. Control cultures were treated with 0.2 ml of medium 199. Cells were removed with 0.05% trypsin 72 hr after infection and subcul-

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tured at a concentration of 4.0×10^6 cells per petri dish. Growth medium was replaced 48 hr after subculturing. During the following 3 days, 6-hr virus harvests were collected, pooled, and centrifuged at $1,000 \times g$ for 20 min to remove the cell debris. The clarified culture medium was then frozen at -70°C for storage. Culture fluid harvested in this manner routinely contained approximately 1.3×10^8 FFU per ml of tissue culture fluid. The methods of titration for FFU were those of Langlois and Beard (7).

Purification of MC29 virus and preparation of enzyme. Tissue culture fluids were thawed rapidly and clarified by the addition of kieselguhr and centrifugation at $1,000 \times g$ for 5 min. After the supernatant fluid was filtered through fine filter paper, the virus particles were removed by centrifugation at $100,000 \times g$ for 1 hr against a 100% glycerol pad. Virus particles at the glycerol interface were pooled. The virus suspension was made 50% glycerol and stored at -20°C . Before use the stored virus suspension was diluted 1:3 with washing buffer [0.01 M tris(hydroxymethyl)aminomethane (Tris), pH 8.8, 0.001 M ethylenediaminetetraacetic acid (EDTA), 0.15 M NaCl], and the virus particles were pelleted by centrifugation at $100,000 \times g$ for 90 min. The pellets were resuspended in buffer 0 [0.025 M Tris (pH 8.3), 0.01 M MgCl_2 , 0.005 M reduced glutathione, 0.0005 M EDTA, 0.15 M KCl, and 10% glycerol], made 0.25% Nonidet by mixing with an equal volume Nonidet stock [0.5% Nonidet (Shell P40) plus 0.13 M dithiothreitol], and incubated at 0°C for 3 hr before assaying for enzyme activity.

The enzyme from AMV was prepared in a manner similar to the preparation of MC29 enzyme. Virus was purified from plasmas harvested from chicks infected with AMV virus. The virus in the plasma was assayed by using adenosine triphosphatase activity (2). Bonar and Beard (4) reported that 10^{10} virus particles contained about $4.5 \mu\text{g}$ of virus protein. From this value, it was estimated that $6.5 \mu\text{g}$ of virus protein was used in a 0.1-ml assay. When AMV virus from plasma was centrifuged in an equilibrium glycerol density gradient, the virus and DNA polymerase activity formed a band at a density of 1.16 g/cm^3 .

Enzyme preparation from infected and noninfected CEC culture fluids. When direct comparison was made between infected and control CEC fluid, the virus preparation was simplified to reduce chances of virus loss. Since both control and infected culture fluids were to be compared, all steps in preparation of the enzyme from both sets of cultures were carried out in an identical manner. Fluids from 6-hr intervals of culture were centrifuged at $5,000 \times g$ for 10 min, mixed with kieselguhr, and centrifuged again at $1,000 \times g$ for 15 min. The virus was pelleted from the culture fluids by centrifuging at 25,000 rev/min in a Spinco SW25.2 rotor for 2 hr. The pellets were suspended in 0.2 ml of buffer 0 and mixed with 0.2 ml of $2\times$ Nonidet stock. This mixture was stored in an ice bath for 3 hr before assay for enzyme activity.

Enzyme assay. A standard reaction mixture contained, in a volume of 0.1 ml: Tris-hydrochloride (pH 8.3), $4 \mu\text{moles}$; MgCl_2 , $0.8 \mu\text{mole}$; NaCl, $6 \mu\text{moles}$;

reduced glutathione, $0.37 \mu\text{mole}$; unlabeled deoxyribonucleoside triphosphate, $0.02 \mu\text{mole}$, ^3H -labeled deoxyribonucleoside triphosphate, $1 \mu\text{Ci}$ (counts per minute per picomole are reported in legends). DNA (native) and enzyme were added in amounts indicated in each experiment. All assays were performed at 37°C and the reactions were terminated by chilling in ice and adding 0.5 ml of trichloroacetic acid mixture (equal parts of 100% trichloroacetic acid, saturated sodium phosphate, and saturated sodium pyrophosphate). The precipitate was collected on filters, washed, and counted as previously described by Spiegelman et al. (17). In some instances, the reaction mixtures were reduced to 0.5 volume.

Chemicals and materials. ^3H -thymidine triphosphate (^3H -TTP) was obtained from Schwarz Bio-Research, Inc., with a specific activity of 12 Ci/mmol. ^3H -deoxyguanosine triphosphate (^3H -dGTP) was obtained from Amersham/Searle at a specific activity of 9.1 Ci/mmol. Unlabeled deoxyribonucleoside triphosphates were obtained from PL Biochemicals, Inc. *Micrococcus lysodeikticus* DNA was a gift from I. Isenberg. Calf thymus DNA was purchased from Sigma Chemical Co. CsCl was obtained from Kawecki Chemical Co., Penn Rare Metal Div., and passed through a B6 membrane filter (Schleicher & Schuell Co.) to improve its optical properties.

Calf serum was purchased from Microbiological Associates, Albany, Calif. Plastic petri dishes were obtained from Falcon Plastic, Los Angeles, Calif. Tryptose phosphate broth was purchased from Difco.

RESULTS

Detection of DNA polymerase activity in other tumor virus preparations has been greatly enhanced by adding a DNA (15), DNA-RNA hybrids, or certain synthetic RNA-RNA duplexes (17) to the incubation mixture. Both the endogenous reaction and DNA-directed synthesis of DNA by enzymes from tumor viruses have been shown to be dependent on the presence of a complete complement of deoxyribonucleoside triphosphates (1, 14, 19), and the DNA polymerase associated with the MC29 virus is no exception. Results presented in Table 1 show the DNA-directed reaction had about a 10-fold rate of ^3H -TTP incorporation over the endogenous reaction. The omission of one deoxyribonucleoside triphosphate strongly depressed DNA polymer synthesis in both the presence and absence of DNA. A possible exception can be noted by the omission of dGTP from the endogenous reaction. When the reaction mixture contained DNA, the dGTP requirement appeared to be greater. Temin and Mizutani (19), with Rous sarcoma virus preparations, also observed less effect on DNA synthesis when the omission of dGTP was compared with omission of the other deoxyribonucleoside triphosphates.

With the enzyme preparation from MC29

TABLE 1. Deoxyribonucleoside triphosphate requirement for the synthesis of DNA polymer^a

Condition	Amt (pmoles) of ³ H-TTP incorporated	
	With DNA	Without DNA
Complete.....	3.3	0.39
Minus dATP.....	0.5	0.15
Minus dGTP.....	0.3	0.23
Minus dCTP.....	0.2	0.13

^a The reaction conditions were the same as reported except that the amount of materials added to the reaction mixture was reduced to 0.05 ml. The DNA primer was isolated from *M. lysodeikticus*, and 0.4 μ g was added to each assay. The reactions were incubated at 37 C for 30 min. ³H-thymidine triphosphate (TTP) had a specific activity of 1,000 counts per min per pmole in the reaction mixture. Abbreviations: dATP, deoxyadenosine triphosphate; dGTP, deoxyguanosine triphosphate; dCTP, deoxycytidine triphosphate.

virus, the ³H-dGTP incorporation into DNA product was slightly over twice the amount of ³H-TTP incorporated into DNA product (Fig. 1). In the same experiment (Fig. 1) it was found that enzyme from AMV incorporated ³H-dGTP at about three times the rate of ³H-TTP. Both enzyme preparations were directed by *M. lysodeikticus* DNA template which has a guanine to thymine ratio of 2.6. Although the two enzyme preparations appeared to synthesize DNA with slightly different base contents, the relative rates of incorporation of the two deoxyribonucleoside triphosphates were close to what one might expect. DNA synthesis, with the MC29 virus preparation without added template, showed only slight product synthesis relative to the activity when DNA was added.

Product of reaction. The product of the endogenous reaction (after 30 min of incubation) was banded in a CsCl gradient (Fig. 2) and compared with the endogenous product from the AMV enzyme (Fig. 3). Products from both enzyme preparations had buoyant densities in CsCl that are characteristic of DNA. The DNA product from MC29 enzyme banded at a density of 1.70, and the product from the AMV enzyme had a buoyant density of 1.69. Buoyant densities in each case were compared to DNA from *M. lysodeikticus* which has a density of 1.72 in CsCl (18). The products of both reactions formed rather diffuse bands in the CsCl gradients, which implies that the DNA product is of small size, heterogenous in base composition, or both. The multiple components of the product of MC29

FIG. 1. Kinetics of deoxyribonucleoside triphosphate incorporation by DNA polymerase from MC29 virus and avian myeloblastosis virus (AMV). *Micrococcus lysodeikticus* DNA (2 μ g per assay) was used as the template, and the reaction volume per assay was 0.1 ml. Each assay with the preparation from MC29 virus (triangles) contained protein from 4.2×10^6 focus-forming units, and the preparation from AMV (circles) contained the equivalent of 1.4×10^{10} virus per assay. The ³H-deoxyguanosine triphosphate (closed symbols, ●, ▲) and ³H-thymidinetriphosphate (TTP) (open symbols, ○, △) were added at a specific activity of 1,000 counts per min per pmole. ³H-TTP incorporation in the absence of DNA (avian MC29 virus preparation) was indicated by open squares (□). The assay conditions and radioactivity determinations were the same as described.

enzyme were especially pronounced in the isopycnic gradients.

When *M. lysodeikticus* DNA was used as the template with the enzyme from MC29, the product of the reaction banded in CsCl at 1.72 in a position identical to *M. lysodeikticus* DNA (Fig. 4). In this case, the reaction product formed a sharp band in the CsCl gradient. In contrast, the reaction product formed with calf thymus DNA template does not correspond in buoyant density to that observed for the major component of the template (Fig. 5). Calf thymus DNA banded at 1.69 in the gradient, and the reaction product from the MC29 enzyme banded at 1.71. It would appear that the portion of the calf thymus DNA that is high in guanine and cytosine

FIG. 2. Equilibrium centrifugation of ^3H -labeled product from the DNA polymerase of MC29 in CsCl . No DNA template was added to the reaction mixture. The labeled deoxyribonucleoside triphosphate in the incubation mixture was ^3H -deoxyguanosine triphosphate (dGTP), 1,000 counts per min per pmole. The assay volume was 0.1 ml, and the incubation time was 60 min. The reaction mixture was terminated by cooling to 0 C and adding 0.1 ml of water and 0.008 ml of diethylpyrocabonate. The mixture was incubated for 2 min at room temperature, frozen rapidly, and stored at -72°C . After thawing and centrifuging at $1,600 \times g$ for 10 min, the clarified sample was mixed with *Micrococcus lysodeikticus* DNA (40 μg) and adjusted with Tris-hydrochloride buffer and saturated CsCl to a density of 1.70. The mixture was centrifuged at 40,000 rev/min in a Spinco SW 65 rotor for 40 hr at 20 C. Twenty-four fractions were collected from the bottom of the tube, and density was measured on every fifth fraction. The solid line at the top of the graph represents the density range of the gradient (gm/cm^3). *M. lysodeikticus*, used as a marker DNA (density, 1.72), was measured on the gradient by absorbance at 260 nm ($\circ$), and the ^3H -labeled product was assayed as trichloroacetic acid-precipitable radioactivity ($\bullet$).

is preferentially copied. The pronounced skewing and breadth of the product DNA in the gradient suggested the synthesis of small and multiple DNA components from calf thymus template.

Enzyme activity in culture fluids from infected and noninfected CEC cultures. It was important to establish that the enzyme activity measured in the pellet fraction from the fluids of CEC cultures infected with MC29 tumor virus were related to virus infection. This was resolved in a satisfactory manner by examining enzyme activity in tissue culture fluids from infected and noninfected CEC cultures by using identical preparation procedures. Enzyme preparations were made from the pelleted virus fraction from culture fluids 2 and 6 days after the infected cultures received

FIG. 3. Equilibrium centrifugation of ^3H -labeled product from DNA polymerase of avian myeloblastosis virus in CsCl . Conditions were the same as in Fig. 2, except that the CsCl density was adjusted to $1.77 \text{ g}/\text{cm}^3$ before centrifugation. *Micrococcus lysodeikticus* DNA (40 μg) was added as the density marker ($1.72 \text{ g}/\text{cm}^3$) and was determined by absorbance at 260 nm ($\circ$). The ^3H -labeled product in the gradient fraction was measured as trichloroacetic acid-precipitable radioactivity ($\bullet$).

MC29 virus. The results shown in Fig. 6 are 48 hr after infection. The typical low endogenous activity in the fraction from infected cultures was stimulated over 30-fold by adding *M. lysodeikticus* DNA. Identical fractions from the noninfected cultures showed no enzyme activity over the 60-min incubation period and no DNA stimulation. The enzyme activities reported in the figure were from 0.05-ml assays which contained 1.3×10^3 FFU.

The data in Fig. 7 were obtained from cultures 6 days after infection and their respective control cultures. Again, it was found that the enzyme fraction from the virus-infected cultures had DNA polymerase activity that was strongly stimulated by DNA and showed a detectable endogenous reaction. The noninfected control cultures displayed no enzyme activity. In this experiment, synthesis in the presence of DNA did not increase after 40 min. This was not typical of DNA synthesis by the enzyme from MC29 and was the only instance in which it was observed. Each assay reported in Fig. 7 contained only 1.5×10^3 FFU. According to estimates of the relationship between FFU and particle counts in the electron microscope (8), the actual number of virus in each assay would be about 1.5×10^6 . Enzyme activity offers an estimate of virus that is sensitive at low levels of particles and might be

FIG. 4. Equilibrium centrifugation of product DNA by the enzyme from MC29 virus in CsCl. *Micrococcus lysodeikticus* DNA (4 μ g) was used as the template in the reaction mixture (0.005 ml) and added as marker DNA (40 μ g) for the CsCl gradient. Conditions for the reaction and gradients were identical to those reported in Fig. 2. Absorbancy of marker DNA was measured at 260 nm ($\circ$). Product DNA ($\bullet$) was measured in each fraction by trichloroacetic acid-precipitable radioactivity.

FIG. 5. Equilibrium centrifugation of product DNA by the enzyme from MC29 virus in CsCl. Calf thymus DNA (2 μ g) was used as the template in the reaction mixture (0.05 ml) and added as marker DNA (30 μ g) for the CsCl gradient. Conditions for the reaction and gradients were identical to those reported in Fig. 2. Absorbancy of marker DNA was measured at 260 nm ($\circ$). Product DNA ($\bullet$) was measured in each fraction by trichloroacetic acid-precipitable radioactivity.

FIG. 6. Kinetics of ^3H -deoxyguanosine triphosphate (dGTP) incorporation into DNA polymer with the enzyme from culture fluids of cells infected with MC29 virus for 48 hr. The preparation of enzyme from culture fluids from infected cells was assayed in the presence of *Micrococcus lysodeikticus* DNA ($\blacktriangle$) and its absence ($\triangle$). Culture fluids from the noninfected control cultures were treated in an identical manner and assayed for enzyme activity in the presence of DNA ($\blacksquare$) and without ($\square$). Enzyme preparations were made from 53 ml of culture fluid. Culture fluid from infected cells contained about 4×10^8 FFU/ml, and each assay had 1.3×10^3 FFU in 0.05-ml reaction volume. DNA was added at 1.5 μ g per assay, and 1 pmole of dGTP was equivalent to 2,000 counts/min.

expected to provide a valuable diagnostic tool to record the presence of small numbers of tumor virus when assay for FFU is not possible.

DISCUSSION

Strain MC29 tumor virus, like other tumor viruses (1, 5, 6, 14, 19), has DNA polymerase. Similar to AMV, the MC29 virus contains an enzyme that has a low synthesis of DNA in the absence of added template. Its activity was greatly stimulated by the addition of DNA to the incubation mixture. However, there was a significant difference between the enzymes from AMV and MC29 virus in the DNA product formed in the absence of DNA template. The enzyme from MC29 virus synthesized DNA that was more heterogeneous than that produced by AMV. The major DNA component of the

FIG. 7. Kinetics of ^3H -deoxyguanosine triphosphate (dTGP) incorporation into DNA polymer with enzyme from culture fluids of cells infected with MC29 virus for 6 days. The preparation of enzyme from culture fluids from infected cells was assayed in the presence of *Micrococcus lysodeikticus* DNA (▲) and in its absence (△). Culture fluids from noninfected control cultures were treated in the same way and assayed for enzyme activity in the presence of DNA (■) and without DNA (□). Both preparations were made from 8 ml of culture fluid containing 3.1×10^8 FFU/ml, and each assay had 1.5×10^8 FFU. DNA was added at a level of $0.7 \mu\text{g}$ per assay, and 1 pmole of dGTP was equivalent to 2,000 counts/min. The reported values for ^3H -dGTP incorporation were from 0.05-ml reaction volume.

MC29 enzyme reaction had a slightly greater buoyant density than that observed for the product from the AMV reaction.

Investigation of the product of the reaction primed by DNA by using isopycnic centrifugation revealed that *M. lysodeikticus* DNA directs the synthesis of a product having the same buoyant density, but the product synthesized under the direction of calf thymus DNA had a buoyant density different from that of the template. Apparently, the enzyme is capable of making a choice from the multiple DNA species that exist in calf thymus DNA. If this is the case, the selection for the enzyme from MC29 appears to favor the DNA template with the higher density. It may also be possible that the enzyme is only selecting regions in the template that are rich in guanine and cytosine. The preference of DNA polymerase from AMV for a DNA rich in guanine and cytosine has also been observed, and DNA with a high guanine plus cytosine content was found in the AMV virus (11).

Strain MC29 tumor virus has advantages for studying early infection and transformation of

CEC because of the rapid conversion of infected cells. In these experiments, it was possible to show formation of enzyme activity within 48 hr after infection. This is the earliest time period investigated up to this time. Studies are in progress which will follow enzyme formation in control and infected cultures during short intervals after virus inoculation. It is expected that it will be possible to observe and study early events in the initiation of infection, such as virus loss during penetration followed by release of new virus.

The difference in kinetics of DNA synthesis, shown by enzyme from infected and control cultures, demonstrated clearly enzyme activity associated with virus infection. In a similar manner, it has been possible to demonstrate enzyme activity in infected cells (Weber, unpublished data). The preparations from noninfected cultures showed no activity over a 60-min period in the presence or absence of DNA template. Purified virus preparations contained enzyme activity (Fig. 1), indicating that DNA polymerase activity is associated with the virus fraction. Such unambiguous distinction in the level of enzyme in the infected cultures permits a close examination of the origin of the enzyme activity in the virus-infected cells.

It was possible in this study to distinguish clearly enzyme activity in culture fluids having extremely low levels of virus and to demonstrate that the background activity of the uninfected tissues is not confusing to the interpretation of the results. It is apparent that 1.3×10^8 FFU per assay (from culture fluid having 400 FFU/ml) had activity that was easily detectable (Fig. 6). From these observations, one might predict that a low level of enzyme activity would be detectable in plasmas from human leukemia even though only low amounts of tumor virus were present. This assumes that DNA polymerase activity is a characteristic marker for tumor viruses. It has been possible to do preliminary studies on human material, and these results are presented in the accompanying report.

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