DNA Polymerases of Tumor Virus: Specific Effect of Ethidium Bromide on the Use of Different Synthetic Templates

(polyribonucleotides/oligonucleotide-polynucleotide complexes/base composition/avian tumor viruses/mammalian tumor viruses)

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ABSTRACT The DNA polymerase enzymes from avian, murine, and feline RNA tumor viruses can be distinguished by their ability to read specific, synthetic primer-templates. The copying of templates containing adenylic and thymidylic acids by all these DNA polymerases is inhibited by ethidium bromide, though this compound affects the polymerases from mammalian tumor viruses much more than the enzyme from avian tumor viruses. Conversely, ethidium bromide stimulates the ability of the enzymes from avian tumor viruses to use primer-templates containing only guanylic and cytidylic acids, whereas the mammalian tumor virus enzymes are moderately inhibited.

The RNA-dependent DNA polymerase activity originally discovered in Rauscher Leukemia Virus and Rous Sarcoma Virus has now been found in all known RNA-containing tumor viruses (1-3). This enzymatic activity has also been identified in "foamy" virus (4), another RNA-containing virus whose oncogenicity has not been established.

Synthetic polymers containing either deoxyribonucleotide or ribonucleotide strands can be used as a template by the RNA-dependent DNA polymerases. Using synthetic templates, Spiegelman et al. (5) and Baltimore et al. (6) have shown that the apparent activity of these viral enzymes depends strongly on the chemical composition of the template, and that the tumor-virus enzymes prefer to use as templates polyribonucleotides rather than polynucleotides (6). However, no striking difference has been found between the RNA-dependent DNA polymerases from different sources in their ability to use the various templates.

The ability of several compounds to act as specific inhibitors of RNA-dependent DNA polymerases from tumor viruses has also been studied. Dimethyl rifampicin is equally effective as an inhibitor against the polymerases found in three different viruses (7). Ethidium bromide, an agent that intercalates into DNA, is the best inhibitor known of the DNA polymerases of the murine leukemia viruses (8).

We have studied the effect of ethidium bromide on various DNA polymerases. We find that the inhibition exerted by this compound against the DNA polymerases from RNA tumor viruses is uniquely and markedly dependent on the type of the primer-template used in the assay. We also find the DNA polymerases of the avian, murine, and feline RNA tumor viruses differ in their individual response to certain synthetic primer-templates. Furthermore, the inhibition of the RNA-dependent DNA polymerases by ethidium bromide, in the presence of specific synthetic primer-templates, varies with the type of the tumor virus; one can distinguish avian, murine, and feline viruses by these criteria.

MATERIALS AND METHODS

1H-labeled deoxynucleoside triphosphates were purchased from New England Nuclear Corp. Unlabeled deoxynucleoside triphosphates, ethidium bromide, and salmon-sperm DNA were obtained from Calbiochem. Poly(dA-dT) and poly(A) were obtained from Miles Laboratories, Elkhart, Ind. Poly-(dG·dC), poly(dC), and the oligodeoxynucleotide (dT)9 were provided by Dr. A. Nussbaum of the Chemical Research Division of Hoffman-La Roche Inc. The oligodeoxynucleotide (dG)12 was obtained from Collaborative Research, Waltham, Mass. The oligodeoxynucleotide (dG)12 was annealed with poly(A) by heating at 45°C for 10 min a solution containing 100 µg/ml of each component and allowing it to cool at a rate of 1°C per 5 min; (dG)12 was annealed to poly(dC) by heating the mixture at 85°C for 10 min followed by slow cooling. Activated salmon-sperm DNA was prepared by treatment of the DNA with pancreatic DNase (9).

Avian myeloblastosis virus was kindly provided by Dr. S. Spiegelman. Rauscher, mouse leukemia virus, Rous avian sarcoma virus, and feline sarcoma virus were purchased from Electro Nucleonics Laboratories, Bethesda, Md.

The viral extracts used to assay for DNA-polymerase activity were prepared by treatment of virus suspensions with Nonidet P-40 (Shell Co.), at a final concentration of 1%, at 0°C for 30 min. Purified calf-thymus DNA polymerase was supplied by Dr. A. Ramel of the Chemical Research Division of Hoffman-La Roche Inc.

The assay for DNA polymerase activity was described (9).

RESULTS

Four different synthetic polymers and activated, native salmon-sperm DNA were used as primer-templates to compare DNA-polymerase activities obtained from calf thymus and four RNA tumor viruses. In Table 1, we have used activated DNA as a standard template against which the synthetic templates have been compared. The relative ability to use different primer-templates differ with each of the enzymes obtained from different sources. Unlike the enzymes obtained from the RNA-tumor viruses, the calf-thymus DNA polymerase shows low activities with poly(dG·dC) or poly-(dA-dT), as compared to an activated DNA template, and is completely inactive when (dT)9-poly(A) is used. The relative activity of the calf-thymus DNA polymerase in the presence of
(dG)₂₉·poly(C) is slightly higher than that of the tumor enzymes, though this synthetic polymer is a good primer-template for all the enzymes analyzed.

For the experiments done in the presence of poly(dA·dT), poly(G·C), or (dG)₂₉·poly(C) we are unable to tell which strand of the template is being copied since the incubation mixture contained as substrate both deoxynucleotide triphosphates in a radioactive form ([H]dATP, [H]dGTP or [H]dCTP, [H]dTTP). However, in the case of (dT)₉·poly(A), we only detected the copying of the poly(A) strand, since the sole radioactive substrate in the assay was [H]dTTP (see legend, Table 1).

A distinction between the enzymatic activities of the four RNA-tumor viruses can be observed when the hybrid (dT)₉·poly(A) is used as primer-template. In the presence of this synthetic polymer, the first three viral DNA polymerases showed very high activities; their ability to incorporate deoxynucleotide monophosphates is 12- to 17-times higher than the incorporation observed in the presence of activated salmon-sperm DNA. However, this is not the case with the enzyme found in feline sarcoma virus, which showed a poor activity in the presence of (dT)₉·poly(A), since its activity relative to nicked salmon-sperm DNA is only 0.9. This marked difference between the feline-sarcoma-virus enzyme and the enzymes in the other viruses is not noted when poly(dA·dT) is used as primer-template. It is also apparent from Table 1 that the response to the different primer-templates is very similar for the two avian tumor viruses. The polymerase from murine tumor virus somewhat resembles the polymerase from avian tumor viruses in this comparison except for its ability to utilize poly(dG·dC).

Ethidium bromide is an effective inhibitor of the RNA-dependent DNA polymerases (8, 10). We have analyzed the effect of this compound on the DNA polymerase activity of various tumor viruses, using either activated salmon-sperm DNA or the synthetic polymers as primer-templates. When activated salmon-sperm DNA is the primer-template, all the enzymes are inhibited to about the same extent in the presence of different concentrations of ethidium bromide (Fig. 1). At concentrations of ethidium bromide of 25 µM (2 µg per assay), all enzymes including the calf-thymus DNA polymerase were inhibited by 60-80%. The enzyme obtained from feline sarcoma virus was slightly more resistant to the effect of ethidium bromide.

![Graph](image-url)
As shown in Table 1, the various DNA polymerases from tumor viruses differ in their relative ability to use synthetic polymers as primer–templates. We have analyzed the inhibitory effect of ethidium bromide upon the polymerase reaction with various synthetic polymers as templates. Fig. 2A shows the results obtained with different tumor virus polymerases when (dT)₉·poly(A) is used as primer–template. There is a marked difference in the inhibitory effect by ethidium bromide with this synthetic primer between the two avian tumor virus enzymes and the two mammalian tumor virus enzymes. At 2.5 μM ethidium bromide (0.2 μg per assay), the feline and murine enzymes are inhibited 85 and 95%, respectively. At this same concentration of ethidium bromide, the avian myeloblastosis and Rous sarcoma enzymes are inhibited by 23 and 29%, respectively. Fig. 2B shows the effect of ethidium bromide on the ability of the enzymes to use the primer poly-(dA–dT), another type of polymer containing A–T. In this case, the difference is less pronounced than when (dT)₉·poly(A) was used, but again the enzymes from the mammalian RNA tumor viruses, as well as the calf-thymus DNA polymerase, are more sensitive to ethidium bromide inhibition than are the enzymes from the avian RNA-tumor viruses.

The differences between the tumor-virus polymerases from mammalian and avian sources is strikingly illustrated when polymerases containing only G and C are used in the DNA polymerase assay. Fig. 3A and B shows the results obtained when (dG)₉·poly(dC) and poly(dG·dC) are used as primer–templates. In both cases, the mammalian viral enzymes show different results than the avian-tumor virus enzymes. In the presence of (dG)₉·poly(dC), the avian myeloblastosis enzyme is stimulated by 15–30% in the presence of different concentrations of ethidium bromide, while the Rous sarcoma enzyme is slightly inhibited at low concentrations, but is stimulated by about 20% at concentrations of 25 μM. In contrast, with this primer–template, the Rauscher and feline enzymes are inhibited by ethidium bromide to the extent of 33 and 50%, respectively, by 25 μM ethidium bromide. This marked difference between the avian and mammalian viral enzymes is also seen when poly(dG·dC) is used as primer–template (Fig. 3B). There is a small stimulation of the avian enzymes at low concentrations of ethidium bromide, while at higher concentrations (25 μM) they are inhibited by 5–15%. With poly(dG·dC) as primer–template, the mammalian viral enzymes were more sensitive to ethidium bromide than the avian-viral enzymes. The feline and Rauscher enzymes were inhibited 66 and 84%, respectively, by 25 μM ethidium bromide.

In either the presence of (dG)₉·poly(dC) or poly (dG·dC) as primer–templates, the calf-thymus DNA polymerase is inhibited to a greater extent by ethidium bromide than are any of the tumor-virus DNA polymerases (Fig. 3). Figs. 2 and 3 show that ethidium bromide effectively inhibits the various tumor-virus DNA polymerases when synthetic polymers containing only A and T are used. The inhibition with primer–templates containing only G and C is either negligible (avian viral enzymes) or intermediate (mammalian viral enzymes).

**DISCUSSION**

The experiments reported here demonstrate that several RNA-dependent DNA polymerases can be differentiated by their ability to read the various synthetic primer–templates. The main difference is observed in experiments done with (dT)₉·poly(A) as template. With this template, the enzyme obtained from feline sarcoma virus showed a very low relative activity, while the other viral enzymes used this polymer 12–to 17-times better than activated DNA. The low activity of the feline sarcoma polymerase could theoretically be explained by the presence in these viral preparations of a nuclease that preferentially attacks this template. However, mixing experiments have shown that feline sarcoma virus extracts do not inhibit the ability of Rauscher leukemia virus extracts to read (dT)₉·poly(A).

It was reported that the RNA-dependent DNA polymerases from avian myeloblastosis virus and Moloney mouse leukemia virus prefer polynucleotides to poly(dG·dC) as templates (6, 11). This is also true for the Rous sarcoma virus and Rauscher leukemia virus enzymes. As shown in Table 1, (dT)₉·poly(A) is the best template for the avian myeloblastosis, Rous, and Rauscher viral enzymes. However, this preference does not seem to be a general characteristic of the RNA tumor virus enzymes, since the feline sarcoma viral enzyme uses the (dT)₉·poly(A) hybrid to a much lower extent than the polynucleotides (dG)₉·poly(dC) or poly-(dA–dT).

**Fig. 2.** Samples were incubated as described in Fig. 1. (A) Each assay contained 5 μg of (dT)₉·poly(A) as primer–template. (B) Each assay contained 5 μg of poly(dA–dT) as primer–template.

**Fig. 3.** Samples were incubated as described in Fig. 1. (A) Each assay contained 5 μg of (dG)₉·poly(dC) as primer–template. (B) Each assay contained 5 μg of poly (dG·dC) as primer–template.
The use of ethidium bromide as an inhibitor for the different RNA-dependent DNA polymerases permits one to distinguish the enzymes found in the avian-RNA tumor viruses from those in the mammalian RNA tumor viruses. With all the templates tested in this study, the mammalian tumor enzymes were more sensitive to inhibition by ethidium bromide than were the avian tumor enzymes. Furthermore, from Fig. 3A and B, it seems that in the case of the mammalian tumor enzymes, the inhibitory effect of ethidium bromide is more pronounced when a polyribonucleotide strand [(dT)\_n·poly(A)] is used as template than with a polydeoxyribonucleotide strand [poly-(dA·dT)]. This observation is in agreement with results obtained by Muller, Zahn, and Seidel (10), who showed that with Rauscher leukemia virus enzyme, the RNA-primed reaction is more sensitive to inhibition by ethidium bromide than is the DNA-primed reaction. This differential sensitivity could also explain the results obtained by Hirschman, who showed that ethidium bromide inhibits the growth of tumors in mice and interferes with the replication of Moloney leukemia virus in cell cultures (8). If the synthesis of DNA from viral RNA by the RNA-dependent DNA polymerase of the virus is more sensitive to the effect of ethidium bromide than is the synthesis of DNA by the DNA-dependent DNA polymerase of the host cell, inhibition of viral growth without any effect on cellular functions could be expected.

The mechanism by which ethidium bromide interacts with DNA is still unclear. Crawford and Waring (12) showed that the dye intercalates between the base pairs of DNA, and Fuller and Waring (13) proposed that, as a result of this intercalation, the double helix unwound by 12° per bond dye molecule. However, Paoletti and Le Pecq (14) have proposed that the binding of one molecule of ethidium bromide produces a winding of the DNA helix of 13° ± 4°. We report here, that the inhibition of the tumor virus DNA polymerases by ethidium bromide is much more pronounced when polymers containing A-T bases are used as primer-template, as compared to G-C containing polymers. The use of the polymers containing G-C can even result in a stimulation of the DNA polymerase reaction by ethidium bromide if the avian tumor viruses are used. These results suggest that the interaction of ethidium bromide with G-C base pairs is significantly different than is the interaction with A-T base pairs. Since the precise helical structure of (dG)\_n·poly(dC) or (dT)\_n·poly(A) used in these studies is not known, it is difficult to speculate further. However, it is of interest that the tumor-virus DNA polymerases can distinguish the specific change in structure and conformation caused by ethidium bromide binding to polymers that contain either G-C or A-T. The ability of the different tumor-virus DNA polymerases to use these ethidium bromide-polymer complexes is unique to each class of the tumor viruses.