Histones Stimulate Polyribonucleotide-Directed Polydeoxyribonucleotide Synthesis by Murine Leukemia Virus

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Received for publication 1 October 1973

The rate of homoribopolymer-directed DNA synthesis by detergent-disrupted Moloney murine leukemia virus can be stimulated or inhibited by histone, depending on the ratio of histone to template. Of the fractions which can be separated from the whole histone, f1 causes both the greatest stimulation and the greatest inhibition. The effect of histone f1 is qualitatively similar whether the template is polyadenylate (poly A), polycytidylate, or polyuridylate, but the stimulation is greatest with poly A. The pattern of stimulation and inhibition differs, however, for a different polymerase; the DNA polymerase of *Micrococcus luteus* is inhibited by histone concentrations which stimulate the viral enzyme and stimulated by concentrations which inhibit the viral enzyme. For the viral enzyme, the optimum histone concentration is unaffected by changes in the virus or primer concentration; but it varies in proportion to the template concentration, suggesting that histone acts by combining stoichiometrically with the template. These data raise the possibility that a histone-like protein may participate in the synthesis of the provirus of RNA tumor viruses.

According to the provirus hypothesis (20) the life cycle of an RNA tumor virus requires the synthesis of a DNA copy of the viral RNA, presumably by the virion-associated DNA polymerase (1, 22), shortly after infection of a cell. An analogue of this hypothetical step can be observed in vitro: namely, the synthesis of virus-specific DNA by detergent-disrupted virions or by purified viral polymerase (see 21 for review).

Several other in vitro polynucleotide synthesizing systems require a protein in stoichiometric combination with the polynucleotide template to attain a maximal rate of synthesis. For three phage and bacterial DNA-synthesizing systems, the necessary proteins were isolated as DNA-binding proteins (7, 17, 18) which bind preferentially to single-stranded rather than double-stranded DNA. The binding properties might suggest that these proteins are basic proteins, but the T4 bacteriophage protein is known to be acidic (7).

A protein fraction isolated from avian myeloblastosis virus stimulates the purified viral DNA polymerase (15). The mechanism of stimulation has been characterized, but the physicochemical properties of the stimulatory protein have not been described.

The RNA replicase of bacteriophage Q β also

requires accessory proteins. The synthesis of a copy of the viral RNA by purified replicase requires the addition of two protein factors (19), one of which can be any of a number of basic bacterial proteins or even eukaryotic histones (14). If histones can associate with an RNA template and facilitate the synthesis of an RNA copy, they may similarly facilitate the synthesis of a DNA copy. In this paper I show that histones stimulate the rate of homopolyribonucleotide-directed polydeoxyribonucleotide synthesis by the virion-associated DNA polymerase of Moloney murine leukemia virus. This observation raises the possibility that a histone-like protein may participate in the synthesis of a provirus during infection by an RNA tumor virus.

MATERIALS AND METHODS

Virus. The mouse bone marrow cell line JLSV-9 (26) was obtained from the John L. Smith Memorial for Cancer Research, Pfizer, Inc., Maywood, N.J., and was infected with Moloney murine leukemia virus (Moloney MLV) obtained from R. Eckner. Culture fluids were harvested at 24-h intervals from infected cells grown in RPMI 1640 medium (with 10% fetal calf serum), clarified by centrifugation at $450 \times g$ for 10 min, and centrifuged for 180 min at $36,000 \times g$ (Beckman 19 rotor). The pellet of crude virus was purified by a procedure suggested by R. E. Smith; namely,

rate-zonal gradient centrifugation (5 to 20% sucrose in 0.005 M Tris-hydrochloride [pH 8.6]-0.001 M EDTA; 23 min at $96,000 \times g$ in a Beckman SW27 rotor) followed by equilibrium gradient centrifugation (15 to 60% sucrose by weight in Tris-EDTA buffer; 17 h at $83,000 \times g$). The visible virus band was diluted, and the virus was collected by centrifugation for 90 min at $96,000 \times g$, resuspended in 0.01 M Tris-hydrochloride (pH 7.4), and dialyzed for 24 h against the same buffer.

Polymerase assays. Reaction mixes contained the following components, unless otherwise specified, in a final volume of 50 μ liters: Tris-hydrochloride buffer, 50 mM, pH 7.9; NaCl, 60 mM; dithiothreitol, 20 mM; [3 H]thymidine triphosphate, 21 μ M, 1,600 counts per min per pmol; polyriboadenylic acid, 50 μ M; oligothymidylic acid, 5 μ M; Nonidet P-40, 0.05%; $MnCl_2$, 1 mM; Moloney MLV, 1.9 μ g of viral protein per ml. (The 1 M Tris-hydrochloride stock had a pH at room temperature of 8.3; diluted to 50 mM at 37 C, the pH was 7.9.)

Radioactivity incorporated into acid-insoluble material was measured by spotting 20 μ liters on a disk of Whatman 3MM paper and washing the paper once in 10% trichloroacetic acid containing 1% sodium pyrophosphate, six times in 5% trichloroacetic acid, twice in 95% ethanol, and once in acetone (3). After drying, the papers were counted in toluene containing 0.5% 2,5-diphenyloxazole and 0.03% 1,4-bis-2-(5-phenyloxazolyl)benzene with an efficiency of 33%. Blank tubes (lacking virus) incorporated 0.2 pmol or less in 30 min, unless otherwise noted. Under these conditions, incorporation at 60 min was approximately twice that at 30 min except at inhibitory concentrations of histone, which stopped incorporation early. The greatest incorporation presented here corresponds to less than 25% of the substrate and less than 10% of the template.

Materials. Deoxynucleoside triphosphates were purchased from Schwartz/Mann, Orangeburg, N.Y.; radioactive nucleoside triphosphates were from Schwartz/Mann or New England Nuclear, Cambridge, Mass.; polynucleotides and DNA polymerase of *Micrococcus luteus* were from Miles Laboratories, Kankakee, Ill.; oligodeoxynucleotides were from Collaborative Research, Waltham, Mass. Tissue culture media and serum were purchased from Grand Island Biological Co., Grand Island, N.Y.

Protein concentration was determined by the Lowry procedure (16) or the biuret reaction (25) using crystalline bovine serum albumin as a standard. Concentrations of nucleotides were checked by optical density using extinction coefficients supplied by the manufacturers.

Histones. Unfractionated calf thymus histone was purchased from Schwartz/Mann. Histone fractions, a gift from L. Pothier, had been extracted from calf thymus according to Johns (9) Method 1 for f1, f2b, and f3 and Johns (11) for f2a1 and f2a2. The amino acid composition of these fractions corresponds with published values (9, 11). By polyacrylamide gel electrophoresis (10), f1 migrates as a single band while the other fractions each contain a minor second band.

RESULTS

Unfractionated histones. Figure 1 shows

that unfractionated calf thymus histone affects the rate of polyadenylate-directed (poly A-directed) synthesis of polythymidylate (poly T) by disrupted virions of Moloney murine leukemia virus. As the concentration of histone is raised, the reaction rate increases, passes through an optimum, and falls almost to zero. Similar addition of bovine serum albumin has little effect and the homopolypeptides polylysine or polyarginine only inhibit the reaction. The stimulation observed is thus a specific property of histone not shared by proteins or basic polypeptides in general.

Histone fractions. Calf thymus histone is a mixture of proteins which can be selectively precipitated, yielding fractions which are mixtures of very closely related proteins (12). Figure 2 shows that these fractions also differ in their stimulation and inhibition of the poly A-directed synthesis of poly T. Fraction f1 stimulates most at an optimal concentration and inhibits most at higher concentrations. The following experiments will characterize this effect of histone fraction f1.

Other templates. The effect of histone f1 is not limited to a single template. Figure 3 shows that 10 μ g of histone f1 per ml stimulates polydeoxyguanylate (poly dG) synthesis di-

FIG. 1. Effect of histone, bovine serum albumin, and basic polypeptides on the rate of poly A-directed poly T synthesis by disrupted virions of Moloney murine leukemia virus. In addition to the components described in Materials and Methods, the reaction mixtures contained the indicated concentrations of calf thymus histone, crystalline bovine serum albumin, polylysine, or polyarginine. The figure contains the results of three experiments normalized to the incorporation observed without additive, which was between 19.6 and 31.4 pmol/30 min.

FIG. 2. Stimulation of poly A-directed poly T synthesis by histone fractions. Reaction mixes contained the components described in Materials and Methods with the addition of histone fractions prepared according to Johns (9, 11).

rected by polycytidylate (poly C) and that 4 μg of histone f1 per ml stimulates polydeoxyadenylate (poly dA) synthesis directed by polyuridylylate (poly U). The effect in both cases is less than that observed in the poly A-directed reaction.

Bacterial polymerase. Histone f1 does not have the same effect on all polymerases. Figure 4 compares the effect of histone f1 on poly T synthesis by either disrupted MLV virions or by a DNA polymerase from *Micrococcus luteus*. The micrococcal enzyme is inhibited at histone concentrations which stimulate the viral enzyme and is stimulated at histone concentrations which inhibit the viral enzyme. The concentrations of the two DNA polymerases differ in this experiment; if viral polymerase constitutes 1% of viral protein (13), they differ by a factor of 50. However, the effect of histone on both polymerase reactions is unaffected by changes in the polymerase concentration (see Fig. 6, and unpublished data). In particular, if the concentration of viral protein is raised a factor of 30 to 93 $\mu\text{g}/\text{ml}$ (and the time of incubation is shortened), the rate of reaction still depends upon histone concentration as shown in Fig. 4.

Stoichiometry. If histone f1 stimulates the reaction rate by interacting stoichiometrically with one of the components of the reaction mixture, the optimum histone concentration should change as the concentration of that component changes. Figures 5, 6, and 7 show the results of experiments testing this hypothesis for the concentration of virus, the primer oligothymidylate (oligo T), and the template poly A. Figures 5 and 6 show, respectively, that the optimum histone concentration changes little when the oligo T concentration changes

over a 100-fold range or when the virus concentration changes over a fivefold range. On the other hand, Fig. 7 shows that the histone optimum does change when the concentration of poly A changes, and that the optimum is proportional to the poly A concentration. The optimum occurs at a poly A:histone f1 ratio of about 1:1.75 by weight, or 0.6 lysine, arginine, and histidine residues per phosphodiester group.

Kinetics. When the TTP concentration is varied, the reaction follows simple Michaelis-Menten kinetics (5). That is, the reciprocal of the rate of poly T synthesis is proportional to the reciprocal of the TTP concentration for both the histone-stimulated and the unstimulated reactions (data not shown). Table 1 shows two determinations of the K_m and V_{max} of these reactions calculated by the method of Wilkinson (24). The major effect of histone is to raise V_{max} , but it may also lower K_m . The K_m found for these reactions is somewhat higher than that

FIG. 3. Effect of histone f1 on copying of different templates. Reaction mixtures contained the components described in Materials and Methods except for the substitution of the following components for their counterparts in the standard mixture: in the poly C-directed reaction, 50 μM poly C, 5 μM oligo dG, 19 μM [^3H]dGTP (960 counts per min per pmol); in the poly U-directed reaction, 50 μM poly U, 5 μM oligo dA, 14 μM dATP (3,780 counts per min per pmol). Blank tubes (lacking virus) incorporated 0.09 pmol of dAMP and 0.5 pmol of dGMP.

FIG. 4. Effect of histone f1 on bacterial polymerase. Reaction mixtures contained the components described in Materials and Methods except that the concentration of viral protein was 2.9 $\mu\text{g/ml}$ and that 1.5 μg of *Micrococcus luteus* DNA polymerase per ml replaced virus in half the reaction mixtures.

found for the purified polymerase of Rauscher MLV (26 μM) (8).

Salt concentration. The salt concentration used in these experiments was that which was optimal for the endogenous reaction of detergent-disrupted virus (1). The optima for each of the homopolymer-directed reactions are different. However, in each case the stimulation by histone f1 can be observed over a range of salt concentrations including the optimum. The interaction of salt and histone will be described in another communication.

Other characteristics. Table 2 shows that histone f1 does not relieve the previously described requirement for a primer (2). In addition, the effect of histone f1 is unchanged if 5 μM poly T is substituted for the oligo T primer (Table 2, experiment 2). The polymerase reaction requires detergent whether or not histone is present; and, between 0.005% and 0.4% Nonidet P-40, the rate of poly T synthesis is independent of detergent concentration for both histone-stimulated and unstimulated reactions (Table 2, experiment 3). Finally, when reactions containing high amounts of virus and substrate and low amounts of template are run to completion, the yield of poly T is roughly equivalent to the amount of template for both the unstimulated and histone-stimulated reactions (data not shown).

DISCUSSION

Histones either stimulate or inhibit RNA-directed DNA synthesis, depending upon the ratio of histone to template. These effects apparently depend on the structure of the histone.

The effect can be duplicated neither by adding other protein, nor by adding basic polypeptides. Furthermore, the magnitude of both the stimulation and the inhibition varies for different histone fractions. Histone f1 does not relieve the requirement for an oligonucleotide primer. This effect superficially resembles the effect of histone on the Q β replicase (14), the effect of spermidine on the pol III DNA polymerase system (23) and the effect of various DNA-binding proteins (not known to be basic proteins) on homologous polymerase systems (7, 17, 18).

The optimum histone concentration varies with the template concentration but is independent of the enzyme concentration and is almost independent of the primer concentration. These facts suggest that there is a stoichiometric interaction between histone f1 and the template. However, they do not rule out the possibility of additional interactions between histone f1 and the enzyme or the primer. The reaction mixtures contain less enzyme or primer than template and the histone-poly A interac-

FIG. 5. Constancy of optimum histone f1 concentration with variation in the oligo T concentration. Reaction mixes contained the components described in Materials and Methods except the oligo T, which was present at 0.5, 5, or 50 μM . Blank tubes (lacking virus) incorporated 0.22 pmol.

FIG. 6. Constancy of optimum histone f1 concentration with variation in virus concentration. Reaction mixes contained the components described in Materials and Methods except the virus, which was present at 1.9, 3.7 or 7.4 μg of viral protein per ml.

tion may buffer the effective histone concentration enough to obscure the effect of variation in the concentration of enzyme or primer.

Let us consider two possible mechanisms for the observed effects of histone f1. Both depend on the histone-poly A interaction but differ in whether the interaction affects the rate of DNA synthesis directly or indirectly.

According to the first model, histone f1 can form two complexes with poly A. One occurs at weight/weight ratio of 1.75. This form is a preferred template for the viral DNA polymerase but is a poor template for the micrococcal DNA polymerase. The second form of histone-poly A complex occurs at a weight/weight ratio of 9 and above. This form is the preferred template for the micrococcal polymerase, but is a very poor template for the viral polymerase.

According to the second model, histone f1 forms two complexes with either primer or enzyme. The complex which forms at the lower histone concentration stimulates the polymerase reaction, whereas that which forms at the higher concentration inhibits. This model requires a special argument to explain the fact

that the optimum histone concentration depends on the template concentration, but not on the enzyme or primer concentration. Such an argument can be made if it is assumed that histone f1 binds to the template more strongly than it does to the primer or enzyme, but that such binding does not affect the polymerase reaction. By this means, the template would

FIG. 7. Variation of optimum histone f1 concentration with variation in the poly A concentration. Reaction mixes contained those components described in Materials and Methods except the poly A, which was present at 20, 50, or 100 μM .

TABLE 1. Determination of K_m and V_{max} ^a

Expt	Histone	K_m (μM)	V_{max} (pmol/30 min/ μg of viral protein)
I	— ^b	40 ± 9	770 ± 80
	+	34 ± 7	$1,160 \pm 80$
II	—	78 ± 15	480 ± 50
	+	43 ± 6	840 ± 50

^a Estimates of K_m and V_{max} were made by the method of Lineweaver and Burk (5). These estimates were refined and standard errors were calculated by the method of Wilkinson (24).

^b +, Histone added; —, histone not added.

TABLE 2. *Effect of histone on primer requirement*

Expt	Reaction mixture ^a	TMP incorporated (pmol/30 min)		
		No histone	+ 10 µg of histone f1 per ml	+50 µg of histone f1 per ml
1	Standard	4.0	23	0.91
2	- Oligo T	0.04	<0.04	
3	- Oligo T, + poly T	6.5	26	
	Standard	13	45	
	- NP-40	0.3	2.5	
	+0.4% NP-40	14	39	

^a Standard reaction mixture is described in Material and Methods. NP-40, Nonidet P-40.

sequester small amounts of histone, preventing them from stimulating the reaction by complexing with primer or enzyme. Only after the template was saturated could the histone interact with the primer or enzyme, forming either the stimulating complex at low histone concentrations or the inhibiting complex at high histone concentrations. Even in the inhibiting complex, the template and primer must be available in some sense, because the micrococcal polymerase can use them. This fact rules out, for example, the possibilities that high concentrations of histone either precipitate poly A or oligo T or displace oligo T from poly A.

Additional mechanisms can be built if one considers the possibility of ternary complexes. One such hypothesis is that histone stimulates the polymerization reaction by stabilizing or facilitating the formation of the primer-template complex. However, this hypothesis in its simplest form does not explain why the micrococcal polymerase is inhibited at concentrations of histone which stimulate the viral polymerase. Furthermore, although poly T can be expected to form a more stable complex than oligo T with poly A, the effect of histone f1 on the rate of synthesis is unchanged when a poly T primer is substituted for oligo T.

The primary significance of this work lies in the possibility that a histone-like protein may participate in the synthesis of a provirus during infection of a cell with an RNA tumor virus. A histone-like protein is known to exist in the virions of MC29 avian leukemia virus (6). Furthermore, a single major protein is found associated with the RNA of Rous sarcoma virus when virions are disrupted with detergent (4), and this protein seems to correspond to the histone-like protein of MC29 (R. Rueckert, personal communication). The possibility that such a

protein participates in the synthesis of the provirus can be tested by attempting to find an effect of histone on the synthesis of virus-specific DNA *in vitro*. These experiments are in progress. Preliminary experiments have shown occasional stimulatory effects of histone f1 on the endogenous polymerase reaction of detergent-disrupted M-MLV, but these effects have been quite variable and have not been characterized. The endogenous reaction may be a poor system for detection of such an effect, because it might contain near-optimal amounts of viral histone-like protein. Experiments are in progress with a more appropriate test system, one using exogenous heteropolymer RNA as a template.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants CA 13479-01 and CA 07745-09 from the National Cancer Institute and by the New York State Department of Health.

The experiments described here depended upon the dedicated technical assistance of Victoria Onorato and Cecelia Green. I am especially grateful to Lillian Pothier for her gift of purified histone fractions, and I thank D. Baltimore, D. Byrd, W. A. Carter, and R. Taber for critical reading of the manuscript.

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