*THE INTERACTION OF RNA POLYMERASE WITH HISTONES**

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Abstract.—Arginine-rich histones (F3) interact with both the bacterial and mammalian RNA polymerase and inhibit the *in vitro* RNA synthesis to a much greater extent than when associated with the DNA template. The lysine-rich histones (F1) inhibit the RNA synthesis mainly through template inhibition. Neither histone fraction displayed such an interaction with DNA polymerase. The RNA polymerase–F3 histone interaction takes place at ionic strengths equal to or greater than those occurring in living cells, suggesting a possible role of arginine-rich histones in the regulation of RNA synthesis.

On the basis mainly of indirect evidence, Stedman and Stedman[1, 2] suggested that histones may act as genetic repressors. More recent support of this possibility came from the experiments showing that histones inhibit efficiently the DNA-dependent synthesis of RNA *in vitro*.[3, 4] Various histone fractions were reported to inhibit this reaction differently, the very lysine-rich histones (F1) being much more efficient inhibitors than the arginine-rich fractions. However, the opposite (i.e., higher inhibition by the arginine-rich histones) has also been observed by several investigators.[3, 4]

Previous findings in this laboratory have shown that histones are capable of direct interaction with the enzyme RNA polymerase.[5] This interaction inactivates the enzyme and results in much higher inhibition of the *in vitro* RNA synthesis than when the arginine-rich histones were allowed to interact first with the template DNA. The opposite appears to be true for the lysine-rich fraction F1. The high level of inhibition of the RNA polymerase has been found with arginine-rich histones from a wide range of organisms.[6]

Materials and Methods.—RNA polymerase: Partially purified preparations of the enzyme were prepared from *Micrococcus lysodeikticus* (spray-dried from Miles Chemical Corp.) by the method of Nakamoto *et al.*[7] The specific activity of RNA polymerase (EC 2.7.7.6.) used in these experiments ranged from 600 to 800 units/mg of enzyme.[7] A highly purified enzyme (specific activity 1,550), purchased from Miles Chemical Corp., was also employed. All enzyme preparations used exhibited a ratio at 280/260 mμ of 1.40–1.60 and displayed negligible endogenous template and RNase activity during 20-min incubation. The enzyme was stored in 50% glycerol at −20°C.

The preparation of unfractionated calf thymus histone and of the fractions F1 and F3 has been described previously.[8, 9] The purity of each of the fractions was determined by electrophoresis in starch[10] or polyacrylamide[11] gels. The histone solutions (2 mg/ml) were made fresh before use; the final concentration was adjusted by the method of Lowry *et al.*[12] for determination of proteins. The nuclease and protease contamination of the two histone fractions was found to be negligible.

RNA polymerase assay: The standard reaction mixture for assay of RNA synthesis *in vitro* was essentially that of Nakamoto *et al.*,[7] except that the quantity of each component and final volume were halved. Generally, reactions were incubated at 37°C for 10 min with 30 μg enzyme and 60 μg DNA. The radioactivity of acid-insoluble precipitates

was determined by the paper disc method of Bollum.[13] All interactions of histones with the DNA or with the enzyme were performed for about 1 min in 0.005 *M* Tris-HCl, pH 7.5, and water was added to assure equal volumes when necessary.

DNA polymerase: Highly purified DNA polymerase (EC 2.7.7.7., specific activity 7000 units/mg) was purchased from Worthington Biochemical Corp. (DNA P7).

DNA polymerase assay: The assay mixture used was essentially that described by Bollum,[14] except that a pH 7.5 phosphate buffer and ATP-H^3 instead of ATP-C^{14} were used. The enzyme utilized native DNA template tenfold more efficiently than denatured DNA. ATP-H^3, specific activity 50 μc/μmole, was used as a label. Native DNA (60 μg) and 1.0 μg of the purified enzyme–DNA polymerase were used per reaction. To each cellulose acetate filter (Millipore Corp.) was added 50 μl of the reaction mixture, and the filters were washed as described in the original procedure except that cold toluene was used instead of absolute alcohol. After the toluene wash, the filters were dried, dissolved in 2.0 ml of ethyl acetate overnight, diluted with 10 ml of the standard 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)] benzene fluid, and counted in a scintillation spectrometer.

Extraction of the mammalian RNA polymerase enzyme: For extraction of the mammalian RNA polymerase enzyme (RNA nucleotidyltransferase, EC 2.7.7.6.), the methods range from solubilizing the enzyme by gentle homogenization or sonication in dilute buffers at near neutral pH ranges to using dilute buffers at alkaline pH values. Small amounts of the enzyme have been shown to be extracted with homogenization and/or sonication of nuclei in dilute buffers.[15–18] We have found, however, that by combining the methods of sonication of nuclei with buffers of higher ionic strengths than previously used, a five- to sevenfold increase in the amount of the extracted enzyme is obtained.

Purified rat liver nuclei are obtained by the method of Blobel and Potter.[19] The nuclei are then sonicated in 0.20 *M* $(NH_4)_2SO_4$ in 0.05 *M* Tris-HCl buffer, pH 7.9, containing 0.001 *M* mercaptoethanol, 0.01 *M* $MgCl_2$, and 25% glycerol. The ammonium sulfate can be omitted in the buffer of this step. If this is the case, then the pellet, obtained by centrifugation for 10 min at 30,000 $\times$ *g*, is re-extracted twice with a buffer containing the 0.20 *M* $(NH_4)_2SO_4$. Use of the latter approach reduces the yield of the enzyme but produces a much less contaminated preparation. The extracts are then centrifuged at 30,000 $\times$ *g* for 10 min. Ammonium sulfate is added to the supernatants to an 80% saturation and the solutions are mixed for 20 min and recentrifuged as above. The pellet is resuspended by Teflon homogenizer in 0.05 *M* phosphate buffer, pH 7.5, with 0.005 *M* $MgCl_2$ and treated with DNase to 20 μg/ml for 30 min to 1 hr at room temperature. The mixture is cooled to 4°C, made 60% with respect to saturated ammonium sulfate, mixed for 20 min, and centrifuged as above. The pellet is gently rinsed with deionized water and is then resuspended in 0.05 *M* Tris, pH 7.9, 0.001 *M* mercaptoethanol, 0.01 *M* $MgCl_2$, and 10% glycerol. This solution is placed on a DEAE-cellulose column (2.4 $\times$ 20 cm) which was previously washed with the latter buffer solution. The enzyme is eluted from the column, with increasing amounts of $(NH_4)_2SO_4$ in the same buffer, at a level of about 0.15 *M* $(NH_4)_2SO_4$.

This enzyme preparation exhibits a complete dependency on the DNA template and divalent ions (Mg or Mn), and is inhibited by actinomycin D. The enzyme could not be sedimented at 120,000 $\times$ g_{ave} for 2 hr. It was found to be most stable when kept in 50% glycerol in 0.05 *M* Tris-HCl, pH 7.9, with 0.01 *M* mercaptoethanol at −20°C but lost activity rapidly after about one week. The enzyme preparations exhibited a specific activity of 6–10 mμmoles UTP-C^{14} incorporated per milligram of enzyme per hour.

Assay of the mammalian RNA polymerase enzyme: The enzyme assay mixture consisted of the following in a 0.5 ml final volume: 50 μmoles Tris-HCl, pH 8.0; 2.0 μmoles $MnCl_2$; 4 μmoles $MgCl_2$; 0.3 μmole each of CTP, ATP, GTP, and 0.05–0.1 μmole of UTP-C^{14} (5 μc/μmole); 5.0 μmoles mercaptoethanol, 50 μg DNA, and 50.0 μg of the enzyme protein. At the end of the 20-min incubation, 1.5 ml of cold 10% trichloroacetic acid (TCA) solution was added to each tube, and the tubes were mixed thoroughly and placed in ice. The solutions were filtered over paper disc filters, previously washed

with 10 ml of 5% TCA–1% $Na_4P_2O_7 \cdot 7H_2O$. Next the filters were washed with 50 ml of the 5% TCA–1% $Na_4P_2O_7$ and then with 20 ml of absolute alcohol. They were then dried thoroughly and the activity was checked in a scintillation counter.

Results.—Effect of lysine (F1)- and arginine (F3)-rich histones on the activity of RNA polymerase: Initial studies using unfractionated calf thymus histone demonstrated that the extent of inhibition of DNA-dependent RNA synthesis *in vitro* depends greatly on whether the histone had opportunity to interact initially with the enzyme or with the DNA template. Further investigation with various fractions of the total histone complement revealed that arginine-rich histones were the cause of this variation.

To facilitate the size of further experiments, only the fractions F1 (very lysine-rich) and F3 (relatively rich in arginine) were compared. Both of these fractions are common to all eucaryotic organisms and can be obtained in a considerable purity. Figure 1 shows the results of velocity studies when the histone fractions were interacted with the DNA or with the enzyme. While the rate of the reactions containing the lysine-rich fraction (F1) remained approximately the same when preincubated with the DNA or the enzyme, the rate of the reaction containing the arginine-rich fraction (F3) decreased significantly as a result of incubation with the enzyme.

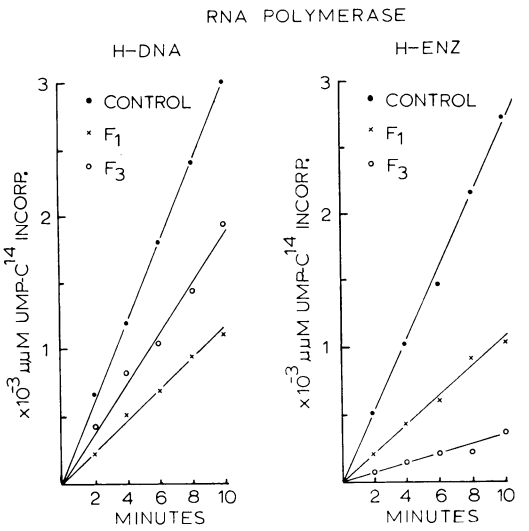

FIG. 1.—Velocity studies in RNA polymerase reactions in the presence of lysine-rich (F1) or arginine-rich (F3) histones. The effect of preincubations of histones with DNA (*H-DNA*) and with the polymerase enzyme (*H-ENZ*). The controls are reactions with no histone. Histone, 20 μg/assay; enzyme, 30 μg/assay; and DNA, 60 μg/assay. Each RNA polymerase reaction contained 20 μmoles Tris-HCl buffer, pH 7.5; 0.2 μmole of each of the nucleotides; 0.4 μmole spermidine phosphate; and 0.625 μmole of $MnCl_2$ and water to a level of 0.25 ml final volume. UTP-C^{14} (Schwarz BioResearch Corp., specific activity 110 μc/μmole) was diluted with cold UTP to give a specific activity of 1 μc/μmole.

To investigate the nature of the inactivation of RNA polymerase by the arginine-rich histones, the reversibility of such inhibitions was studied. Figure 2 shows the effect of increasing the quantity of the DNA template. In plot H-DNA (histones associated with the DNA prior to enzyme addition), the inhibition by both histone fractions was observed to be completely reversed at high levels of DNA. These results demonstrate that the inhibition by both types of histones was of the same nature, i.e., masking of the DNA template by the histones and preventing the utilization of the DNA in the DNA-dependent RNA synthesis. In plot H-ENZ (histones associated with the enzyme prior to the addition of the DNA template), the inhibition by lysine-rich fraction (F1) remains

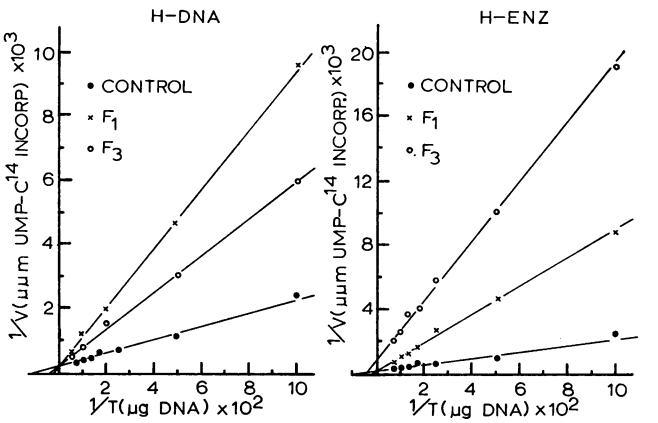

FIG. 2.—Reversibility studies in which the lysine-rich (F1) and arginine-rich (F3) histones were premixed with the DNA (*H-DNA*) or with the enzyme (*H-ENZ*). The controls are reactions with no histone. Histone, 20 μg/assay; enzyme, 30 μg/assay.

competitive, i.e., similar to the mode of inhibition in the plot H-DNA. The inhibition by the arginine-rich histones (F3), however, is of a different nature. It is not reversible at high levels of DNA. These results show that the mode of inhibition by the two histone fractions on the DNA-dependent RNA synthesis is different when the histones are interacted with the enzyme. Under these conditions, the lysine-rich histones inhibit the synthesis by masking the DNA template, while the arginine-rich histones inhibit most likely through interaction with the enzyme, producing a fourfold level of inhibition.

To verify whether the arginine-rich histone inhibition is caused by inactivation of the enzyme during enzyme-histone incubation, the levels of enzyme in assays were increased in an attempt to reverse this inhibition. Figure 3 shows the effect of increasing levels of the enzyme on the *in vitro* RNA synthesis, wherein the arginine-rich histones (F3) were allowed to interact with the enzyme before

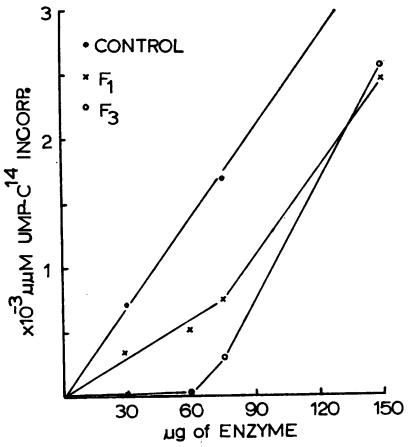

FIG. 3.—The effects of increasing levels of enzyme on the rate of RNA synthesis when the histones were preincubated with the enzyme. The control is reaction with no histone. Histone, 50 μg/assay; DNA, 60 μg/assay.

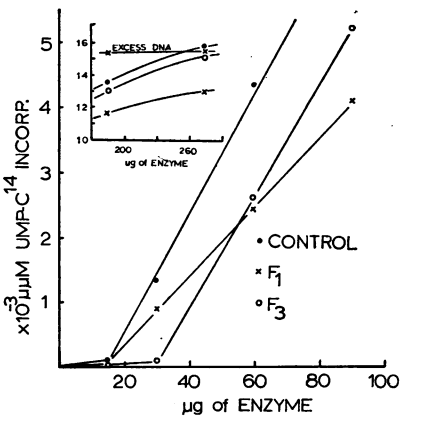

FIG. 4.—The effects of increasing levels of enzyme on the rate of RNA synthesis when the histones were preincubated with the enzyme. The control is the reaction without histone. Histone, 27 μg/assay; DNA, 60 μg/assay.

addition of the DNA template. Under these conditions, an increase in enzyme concentration up to a histone/enzyme ratio of 0.83 did not produce any measurable synthesis of RNA. A significant increase of RNA synthesis was observed in this range with the lysine-rich histone (F1) system. Further increase in the enzyme concentration resulted in an abrupt initiation of RNA synthesis in the reaction with the arginine-rich histones at an increasing rate parallel to that of the control (Fig. 3). This indicates that after all the F3 histones present in the reaction mixture are associated with enzyme, RNA synthesis can resume at a normal rate, utilizing the remaining free enzyme. In addition, the F3 histone, associated with the enzyme, does not mask the DNA template.

As a further check on the extent of the reversal of inhibition, lower levels of histones with broader range of the enzyme were used. Figure 4 presents the results of this experiment. Again, at lower levels of the enzyme, the arginine-rich (F3) histones exhibited a greater inhibition of RNA synthesis than the lysine-rich fraction (F1). There was an abrupt increase of RNA synthesis at a histone:enzyme ratio of 0.9 paralleling that of the control. At very high enzyme levels, the inhibition by the arginine-rich histones was completely reversed, whereas no complete reversal of the inhibition by the lysine-rich histones could be achieved, even at very high enzyme concentration. However, complete reversal of inhibition by the lysine-rich histones (F1) was obtained by adding excess DNA. These results demonstrate that when the arginine-rich histones associate with the polymerase enzyme, inhibition of RNA synthesis results from inactivation of the enzyme, whereas in the case of the lysine-rich histones (see Fig. 2), inhibition is caused by DNA template inhibition.

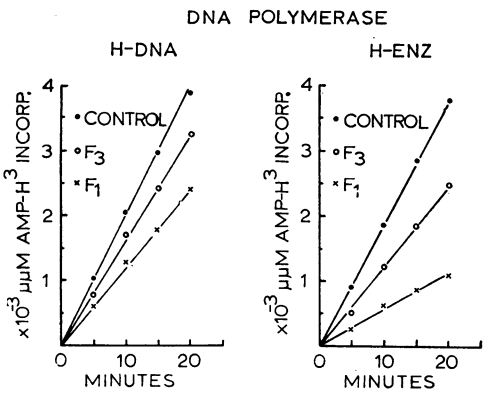

FIG. 5.—Velocity studies in DNA polymerase reactions in the presence of lysine-rich (F1) or arginine-rich (F3) histones. The effect of preincubations of histones with DNA (*H-DNA*) and with the polymerase enzyme (*H-ENZ*). The controls are the reactions without histone. Histone, 50 μg/assay; enzyme, 0.9 μg/assay; and DNA, 60 μg/assay. Each reaction contained 10 mμmoles of each nucleotide, 50 mμmoles of EDTA, 0.1 μmole of mercaptoethanol, 1.0 μmole $MgCl_2$, and 10 μmoles of K-phosphate buffer, pH 7.5, in a final volume of 0.2 ml.

Interaction of the DNA polymerase enzyme with histones: Highly purified DNA polymerase was obtained for interaction with the two fractions of histones. With a histone:enzyme ratio of 55.0 (Fig. 5), i.e., much higher (20- to 50-fold) than the ratios with the RNA polymerase enzyme, only a general increase in the inhibition of DNA synthesis was observed in the reactions in which the histones were interacted with the enzymes. Neither histone fraction displayed a distinct interaction with the DNA polymerase enzyme. A slightly greater inhibition of DNA synthesis was observed when the histones were interacted with the enzyme than with the DNA template; however, much greater histone: enzyme ratios were required for such inhibition than with the RNA polym-

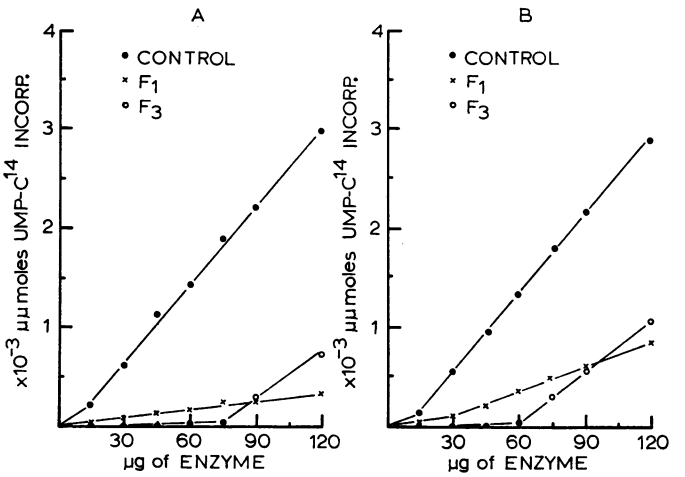

FIG. 6.—The effects of increasing levels of enzyme on the rate of RNA synthesis when the histones were preincubated with the enzyme in the presence of NaCl. The final ionic strength of the reaction mixture was (*A*) 0.15 and (*B*) 0.25. Controls are reactions without histone. Histone, 50 µg/assay; DNA, 50 µg/assay.

erase enzyme (compare Figs. 1 and 5). These results demonstrate that only a slight inhibitory histone–DNA polymerase interaction occurs, if any, with these histone fractions at levels of the histone-enzyme similar to those of the RNA polymerase assays.

Histone-enzyme interactions under physiological ionic conditions: Results of experiments in which the histone-enzyme interaction was performed under conditions of 0.14 *M* NaCl in 0.01 *M* Tris-HCl, pH 7.5, are shown in Figure 6*A*; in *B* are shown the results of experiments in which the histone-enzyme interaction was performed as in *A* but the ionic strength of the final reaction was double (approximating an ionic strength similar to 0.25 *M* NaCl). Under both conditions, the results were similar to those shown in Figures 3 and 4 in which the ionic strength was at a level equal to 0.1 *M* NaCl. Increasing levels of the enzyme exhibited little or no effect at lower levels on the systems with the arginine-rich histone-enzyme interactions, after which an abrupt increase in RNA synthesis was observed at a rate almost paralleling that of the controls, indicating that arginine-rich histone-enzyme interactions can occur even at ionic levels equal to (or higher than) those of physiological conditions.

Interactions of histones with the mammalian RNA polymerase enzyme: Figure 7 shows the results of experiments with the partially purified, soluble RNA polymerase enzyme prepared from rat liver nuclei. When the histones were interacted first with the DNA template (H-DNA), the lysine-rich histones were more inhibitory to the RNA synthesis than the arginine-rich histones. When the histones were interacted first with the polymerase enzyme, the efficiency of inhibition by the arginine-rich histones was increased, while that of the lysine-rich histones remained relatively unchanged. This arginine-rich histone-enzyme interaction is similar to but not as pronounced as the interactions of

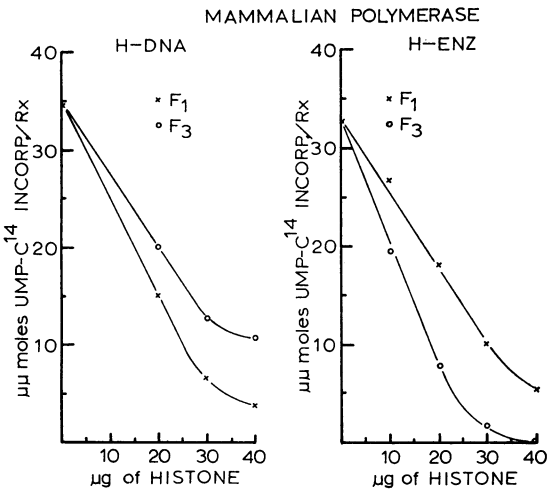

FIG. 7.—The effects of increasing the levels of the lysine-rich (F1) and arginine-rich (F3) histones on the activity of the soluble RNA polymerase enzyme from rat liver nuclei. Histone premixed with DNA (*H-DNA*) or with the enzyme (*H-ENZ*). Enzyme, 50 μg/assay; DNA, 50 μg/assay.

the histones with the bacterial RNA polymerase enzyme (the mammalian polymerase cannot be purified to such an extent as has been done with the bacterial enzyme). It does demonstrate, however, that interactions between the arginine-rich histones and the mammalian RNA polymerase enzyme can occur.

Discussion.—It has been suggested that lysine-rich histones F1 maintain the complicated architecture of chromatin[20] and genetically restrict most of its DNA template,[21] but later reports disagree with such functions of very lysine-rich histones.[3] The involvement of moderately lysine-rich histones F2b and F2a2 in genetic restriction of chromatin is indicated by a significant increase of template activity of chromatin after the removal of at least a part of these fractions.[3] No definite suggestions concerning functions of the arginine-rich histones have been made. Our results indicate that the arginine-rich histones may act as natural inhibitors of the enzyme RNA polymerase, i.e., the arginine-rich histone-enzyme interaction occurs at ionic strengths approximating those in living cells (Fig. 6) and also similar histone interactions occur *in vitro* with the mammalian RNA polymerase. It is noteworthy that the arginine-rich histones F3 prepared by acid extraction of the nuclei or chromatin are contaminated with acidic proteins.[9, 22] Unfortunately, the acid extraction destroys all the RNA polymerase activity, and therefore a possibility that perhaps a part of the acidic proteins contaminating arginine-rich histones may be a natural arginine-rich histone–RNA polymerase complex is only speculative.

The nature of the histone–RNA polymerase interactions appears to be electrostatic, since the complexes are dissociated in strong solutions of salts. Our studies have shown that both histone fractions (the lysine-rich F1 and the arginine-rich F3) can form a complex with the enzyme, but the lysine-rich histone does not affect its activity. Kinetic experiments indicate that DNA can dissociate the lysine-rich histone-polymerase complex to form histone-DNA and/or enzyme-DNA complexes (Figs. 2, 3, 4, 6). On the other hand, DNA appears unable to dissociate the complex of arginine-rich histone with RNA polymerase enzyme. The complexes between the enzyme and histones occur immediately upon mixing. Most of the experiments were performed with the partially purified polymerase enzyme by the method of Nakamoto

et al.,[7] specific activity 600–800. When a highly purified enzyme, specific activity 1550 (Miles Chemical Corp.), was employed, the results were identical to those described in Figure 1. Consequently, the contaminating proteins of our extracted enzyme preparation do not appear to affect the interactions between the histones and the enzyme. We suggest, therefore, that the controversial reports in the literature concerning the extent of inhibition of RNA synthesis *in vitro* by lysine-rich and arginine-rich histones can be explained by the differences in the interaction between histones and the RNA polymerase enzyme on one side and between the interaction of histones and DNA template on the other. It is also of considerable interest that the DNA polymerase enzyme from *Escherichia coli*, which like the RNA polymerase enzyme is acidic in nature,[23] does not show substantial interaction with either histone fraction upon preincubation.

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[1] Stedman, E., and E. Stedman, *Phil. Trans. Roy. Soc. London*, **B235**, 565 (1951).

[2] Stedman, E., and E. Stedman, *Nature*, **166**, 780 (1950).

[3] Bonner, J., M. E. Dahmus, D. Fambrough, R. C. C. Huang, K. Marushige, and D. Y. H. Tuan, *Science*, **159**, 47 (1968).

[4] Hnilica, L. S., *Progr. Nucl. Acid Res. Mol. Biol.*, **7**, 25 (1967).

[5] Spelsberg, T. C., and L. S. Hnilica, *Federation Proc.*, **27**, 336 (1968).

[6] Spelsberg, T. C., S. Tankersley, and L. S. Hnilica, *Experientia*, in press.

[7] Nakamoto, T., C. F. Fox, and S. B. Weiss, *J. Biol. Chem.*, **239**, 167 (1964).

[8] Hnilica, L. S., *Biochim. Biophys. Acta*, **117**, 163 (1966).

[9] Hnilica, L. S., and L. G. Bess, *Anal. Biochem.*, **12**, 421 (1965).

[10] Hnilica, L. S., L. J. Edwards, and A. E. Hey, *Biochim. Biophys. Acta*, **124**, 109 (1966).

[11] Fambrough, D. M., F. Fujimura, and J. Bonner, *Biochemistry*, **7**, 575 (1968).

[12] Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

[13] Bollum, F. J., in *Procedures in Nucleic Acid Research*, ed. G. L. Cantoni and D. R. Davies (New York: Harper and Row, 1966), p. 296.

[14] *Ibid.*, p. 284.

[15] Furth, J. J., and P. Ho, *J. Biol. Chem.*, **240**, 2602 (1965).

[16] Cunningham, D. D., and D. F. Steiner, *Biochim. Biophys. Acta*, **145**, 834 (1967).

[17] Seifart, K., and C. E. Sekeris, *Hoppe-Seyler's Z. Physiol. Chem.*, **348**, 1555 (1967).

[18] Jacob, S. T., E. M. Sajdel, and H. N. Munro, *Biochem. Biophys. Res. Commun.*, **32**, 831 (1968).

[19] Blobel, G., and V. R. Potter, *Science*, **154**, 1663 (1966).

[20] Littau, V. C., C. J. Burdick, V. G. Allfrey, and A. E. Mirsky, these PROCEEDINGS, **54**, 1204 (1965).

[21] Georgiev, G., L. Ananieva, and J. Kozlov, *J. Mol. Biol.*, **22**, 365 (1966).

[22] Stellwagen, R. H., and R. D. Cole, *J. Biol. Chem.*, **243**, 4452 (1968).

[23] Huang, S. L., and L. F. Cavalieri, in *Procedures in Nucleic Acid Research*, ed. C. L. Cantoni and D. R. Davies (New York: Harper and Row, 1966), p. 587.