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Properties of an Altered RNA Polymerase II Activity from an α-Amanitin-Resistant Mouse Cell Line†

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ABSTRACT: α-Amanitin-resistant clones were selected in the mouse lymphoblastoid cell line L5178Y. One resistant clone, named A169b, was recloned and the properties of its DNA-dependent RNA polymerases were examined. The RNA polymerase II activity from A169b differs from the parental cell line in that approximately half the activity is resistant to 0.5 μg/mL α-amanitin, while the parental enzyme is 50% inhibited at 0.005 μg/mL. The enzymes from A169b and the parental line were purified free of polymerase III and their properties compared. The two preparations were identical in their apparent affinities for the four nucleoside triphosphates, in their salt and divalent cation preferences, and in their preference for denatured over native DNA. They differed in their response to α-amanitin. The apparent K_I for the parental enzyme was 3.5×10^{-9} M; plots of $1/V$ vs. α-amanitin concentration gave a biphasic curve with A169b enzyme. The two apparent K_I values were 4.1×10^{-9} and 2.1×10^{-6} M. In addition, the enzyme from A169b showed a twofold higher activity on poly[d(AT)] as template, compared to native DNA, than that of the parental enzyme. Other template preferences may be affected, but differences were marginal. These results indicate that mutation to α-amanitin resistance may alter other enzymatic parameters; such mutations may be helpful in elucidating structure-function relationships in these complex enzymes.

Eukaryotic cells contain three major forms of DNA-dependent RNA polymerase (EC 2.7.7.6) each of which appears to be responsible for the synthesis of a specific class of RNA (Weinmann and Roeder, 1974; Blatti et al., 1970; Reeder and Roeder, 1972). The various forms of RNA polymerase are composed of two large polypeptides in association with a series of smaller polypeptides (Gissinger and Chambon, 1972; Kedinger and Chambon, 1972; Sklar et al., 1975). Ascertaining how the structural components of RNA polymerase interact to determine functional properties is important for an understanding of the role played by RNA polymerase in the regulation of transcription.

One approach to the investigation of structure-function relationships is to correlate changes in functional properties with changes in structure. Huet et al. (1976) have presented evidence that removal of two polypeptides from RNA polymerase I (or A) in yeast decreases DNA binding but does not affect chain propagation. Bell et al. (1976) have also observed changes in function as a result of a structural alteration of yeast RNA polymerase I.

Another method of altering structure is through mutation. Mutations affecting RNA polymerase II (or B) in mammalian cells can be isolated on the basis of resistance to the mushroom toxin α-amanitin (Chan et al., 1972; Amati et al., 1975; Somers et al., 1975), which inhibits the elongation step in RNA syn-

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thesis (Cochet-Meilhac and Chambon, 1974). Amanitin-resistant cells possess a RNA polymerase II which binds α-amanitin with a lowered affinity (Ingles et al., 1976; Lobban et al., 1976). The structural alteration resulting from mutation to amanitin resistance has not been reported. However, the degree of resistance to α-amanitin in terms both of growth of mutant cells and of RNA synthesis in vitro correlates well with the changes in amanitin-binding affinity of RNA polymerase II from a series of α-amanitin-resistant mutants (Ingles et al., 1976). These observations are consistent with the hypothesis that the α-amanitin-resistant mutation involves a structural change in one of the polypeptides of RNA polymerase II. Further support for a true genetic alteration of RNA polymerase is provided by the codominant behavior of the mutation in cell hybrids (Ingles et al., 1976; Lobban and Siminovitch, 1975).

In order to test the utility of amanitin-resistant mutants for the study of transcription, the functional properties of mutant RNA polymerase II should be characterized thoroughly. Lobban et al. (1976) have found that in an α-amanitin-resistant Chinese hamster ovary cell line the change in amanitin-binding affinity of RNA polymerase II is accompanied by changes in thermal denaturation. In this paper, we report the isolation of an α-amanitin-resistant mutant in the mouse cell line L5178Y. The RNA polymerase II from this mutant has an amanitin inhibition constant 670 times greater than that of the parent. Using RNA polymerase II preparations free of contaminating RNA polymerase I and III activity, we have compared a variety of enzymatic properties of parent and mutant enzymes. We have found that the amanitin-resistant RNA polymerase II, while identical in most properties to the sensitive enzyme, has a twofold higher poly[d(AT)][1]/native DNA activity ratio than does wild-type polymerase II.

Materials and Methods

Biochemicals. Unlabeled nucleoside triphosphates (sodium salt) and phenylmethanesulfonyl fluoride were obtained from Sigma. Deoxyribonucleotide polymers were purchased from Boehringer. Traysylol was obtained from F.B.A. Pharmaceuticals (New York). Calf thymus DNA was obtained from Worthington. α-Amanitin was purchased from Henle (New York). New England Nuclear was the source of [5,6-^{3}H]uridine 5′-triphosphate (35–50 Ci/mmol) and [8-^{3}H]guanosine 5′-triphosphate (5–10 Ci/mmol).

Cell Culture. The cell line used was the mouse lymphoblastoid line L5178Y, originally grown in vitro by Fischer (1958). A thymidine kinase deficient, ouabain-resistant derivative of L5178Y isolated in this laboratory (Adelberg et al., 1975) and designated GF-14 was used as wild type. Cells were propagated in suspension in RPMI 1640 supplemented with 5% horse serum in 5% CO_2/95% air at 37 °C. The medium contained 63 mg/L penicillin G, 100 mg/L streptomycin, and 50 mg/L kanamycin. Medium and serum were obtained both from Gibco and from Flow Labs. Cultures for enzyme purification were grown in volumes up to 3 L with 2% horse serum in spinner bottles in 100% air with caps tightly closed. Reduction of serum content has no effect on doubling time, which was approximately 16 h, as determined by cell counts with a Coulter counter.

Mutant Isolation. A total of 5–10 × 10^6 log-phase cells (at 2–4 × 10^5/mL) were treated with ethyl methanesulfonate at a concentration of 6 × 10^{-3} M for 2 h. Cell survival, measured by cloning, was 10–25%. After mutagen treatment, the cells were grown for four generations in suspension and cloned under conditions selective for α-amanitin resistance. The selective medium was RPMI 1640 containing 15% horse serum, antibiotics as above, 0.12% Noble agar (Difco), and 5 μg/mL α-amanitin. The mutation frequency under these conditions was approximately 10^{-7}. Amanitin-resistant clones were picked and recloned under the same selective conditions. One reclone, A169b, was chosen for the experiments described here. Chromosome counts indicated that GF-14 has a stable karyotype of 32 acrocentric and 4 metacentric chromosomes, for a total of 40 chromosome arms. The mouse (*Mus musculus*) has 40 acrocentric chromosomes. The four metacentric chromosomes of L5178Y presumably were formed by centric fusions. A169b has retained a karyotype identical to the parental line.

RNA Polymerase Purification. Cells at the end of log phase (approximately 10^6/mL) were harvested by centrifugation, washed in Tris-buffered saline, and stored at −70 °C. Prior to preparation of extracts, cells were thawed and suspended in buffer A (Schwartz et al., 1974) containing the protease inhibitors phenylmethanesulfonyl fluoride (0.3 mg/mL) and Traysylol (200 KI units/mL). Ammonium sulfate (3.5 M, pH 7.4, at 23 °C) was added to a final volume of 0.3 M and the lysed extract was sonicated as described by Schwartz et al. (1974). RNA polymerase II was purified through the CM-Sephadex step by the procedure of Schwartz et al. (1974). In some cases, CM-Sephadex fractions were concentrated by precipitation with ammonium sulfate. Precipitation was accomplished by dialyzing the pooled RNA polymerase fractions against 50% saturated ammonium sulfate in buffer A followed by centrifugation of the precipitate as described by Burgess and Travers (1971). Protein concentrations were determined by the method of Lowry et al. (1951).

RNA Polymerase Assay. Measurement of RNA polymerase activity was based on the assay of Schwartz et al. (1974). The standard reaction mixture contained 75 mM Tris-HCl (pH 7.9 at 0 °C), 20 μCi/mL [^{3}H]UTP, 2 mM $MnCl_2$, 0.1 mg/mL native calf thymus DNA, 12.5% glycerol, 0.05 mM EDTA, 0.25 mM dithiothreitol, varying amounts of ammonium sulfate, and RNA polymerase. volumes of 60 to 200 μL were incubated at 37 °C for 20 min. Reactions were initiated by adding RNA polymerase in buffer A and stopped by adding 25 μL of EDTA to a final concentration of 25 mM. Radioactive UMP incorporation was determined as follows: a known amount of the reaction mixture was pipetted onto Whatman 3MM filter disks. The disks were washed in a succession of 5% trichloroacetic acid baths containing 0.05 M sodium pyrophosphate. The final bath was diethyl ether-ethyl alcohol (1:3, v/v). Radioactivity on the dried filter disks was measured by liquid scintillation spectroscopy. Blank values derived from assays containing no template were subtracted from all assays. These blank values were equivalent to 2–4 pmol of UMP or GMP incorporated per assay. Composition of the standard assay mixture was varied as indicated in the figure and table legends. The divalent cation was 2 mM $MnCl_2$ except where indicated otherwise. Where denatured DNA was used, calf thymus DNA was denatured by raising the pH of the stock solution to pH 11 by the addition of 5 N NaOH. After incubation for 10 min at 23 °C, the pH was adjusted to pH 7.4 with 5 N HCl. Where incorporation of [^{3}H]GMP was measured, the UTP concentration in the reaction mixture was 6 mM and GTP was 0.05 mM (20 μCi/mL [^{3}H]GTP). One unit of activity is defined as the incorporation of 1 pmol of UMP or GMP

[1] Abbreviations used are: Tris, tris(hydroxymethyl)aminomethane; CM, carboxymethyl; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; poly[d(AT)], deoxyriboadenylate-deoxyribothymidylate heteropolymer; poly[d(GC)], deoxyriboguanylate-deoxyribocytidylate heteropolymer.

TABLE I: Relative Cloning Efficiencies of Parent and Mutant Cell Lines.[a]

Strain	α-Amanitin concn (μg/mL)			
	0	0.2	1.0	5.0
GF-14	1.0	0.95	0.17	$<2 \times 10^{-6}$ [b]
A169b	1.0	0.72	0.62	0.28

[a] Average of two determinations. Each determination consisted of quadruplicate tubes. The average absolute cloning efficiencies in the absence of α-amanitin were 64% of A169b cells cloned and 31% of GF-14 cells cloned. [b] No clones were observed in any of the quadruplicate tubes. 5×10^5 cells were placed in each tube.

into acid-insoluble material per 20 min of incubation at 37 °C.

Results

Mutant Cell Line. We found that concentrations of at least 5 μg/mL of α-amanitin were necessary for selection of bona fide RNA polymerase mutants. Table I shows that the cloning efficiency of wild type (GF-14) at 1 μg/mL α-amanitin is not appreciably different from that of the α-amanitin-resistant mutant (A169b). The slight decrease in cloning efficiency of A169b at 5 μg/mL α-amanitin may result from the accumulated effects of α-amanitin during long incubation periods on cell components other than RNA polymerase II, such as nucleoli (Kedinger and Simard, 1974).

Figure 1 shows growth curves of suspension cultures of GF-14 and of A169b in the presence and absence of 5 μg/mL α-amanitin. Growth of GF-14 in the presence of α-amanitin ceases after 2 days. Slow uptake of α-amanitin may account for the initial growth of GF-14. Growth of GF-14 is irreversibly inhibited after 24 h of α-amanitin treatment (data not shown). The growth rates of GF-14 and A169b in the absence of α-amanitin are identical. Addition of 5 μg/mL α-amanitin to the A169b culture does not alter growth rate of the mutant. We have performed additional experiments which show that α-amanitin concentrations up to 100 μg/mL do not alter the A169b growth rate (not shown). From these results we infer that the mutation to α-amanitin resistance does not affect normal cell proliferation.

We refer repeatedly to A169b as a mutant. While we appreciate the possibility that A169b, as well as other α-amanitin-resistant cell lines, may be epigenetic variants, we consider it more likely that the α-amanitin-resistant cell lines reported thus far, including A169b, represent genetic alterations at the DNA level.

Partial Purification of RNA Polymerase. That A169b has an altered RNA polymerase was determined by characterization of partially purified RNA polymerases. Schwartz et al. (1974) have shown that RNA polymerases I, II, and III can be detected by DEAE-Sephadex column chromatography of animal cell extracts. Under these conditions, polymerase III is split into two peaks of activity, IIIA and IIIB, polymerase IIIA eluting coincident with the trailing portion of polymerase II. RNA polymerase IIIB elutes separately from II and IIIA at higher salt concentrations. Polymerases II and IIIA activities can be separated by CM-Sephadex chromatography.

Table II shows the purification data for RNA polymerase from A169b. The purification behavior of the RNA polymerases from L5178Y parallels closely that of MOPC (Schwartz et al., 1974). Differences in specific activity may be due in part to the use of different DNA template preparations.

DEAE-Sephadex fractionation of an extract of A169b is

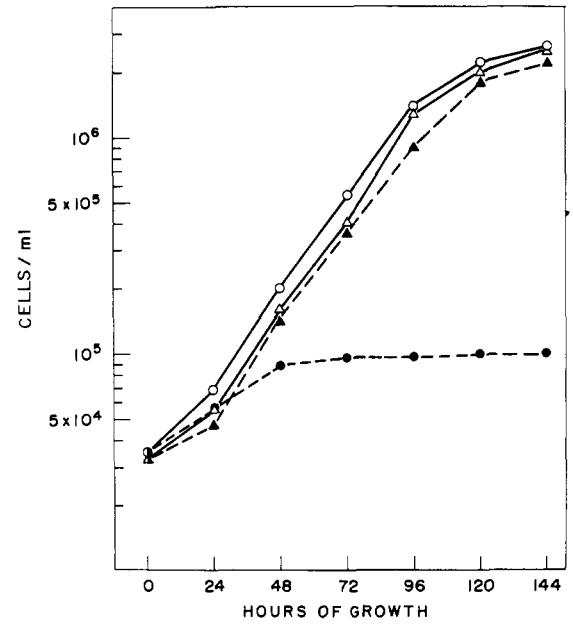

FIGURE 1: Growth curves of GF-14 and A169b. (O-O) GF-14 in the absence of α-amanitin; (● - - - ●) GF-14 in the presence of 5 μg/mL α-amanitin; (Δ-Δ) A169b in the absence of α-amanitin; (▲ - - - ▲) A169b in the presence of 5 μg/mL α-amanitin. Log-phase suspension cultures were diluted at 0 h approximately tenfold to a final volume of 5 mL in duplicate screw-cap culture tubes. α-Amanitin was added to appropriate tubes at 0 h. Cell concentration was monitored by a Coulter counter. Culture conditions were as described under Materials and Methods for 5% CO_2/95% air.

shown in Figure 2A. Assays were done with a mixture of native and denatured DNA because RNA polymerase II activity was very low relative to polymerase I activity with our native DNA preparation. Parallel assays with native DNA were also done for the purpose of monitoring specific activity and yield. RNA polymerase I elutes first, followed by RNA polymerase II at 0.2 M ammonium sulfate. The shoulder of activity on the trailing edge of polymerase II we term RNA polymerase IIIA by analogy with the observations of Schwartz et al. (1974). The tiny two-fraction peak eluting at approximately 0.4 M ammonium sulfate is probably RNA polymerase IIIB. We sometimes observe a more prominent peak in this position. Both polymerase IIIA and IIIB activities are variable from experiment to experiment, a fact also noted by Schwartz et al. (1974).

RNA polymerase IIIA and B in wild-type extracts can be identified in DEAE-Sephadex fractions by assaying with poly[d(AT)] as template in the presence of 0.5 μg/mL α-amanitin. Polymerase II activity is completely inhibited, making apparent a peak of polymerase III activity in the trailing edge of polymerase II. Polymerase III is resistant to 0.5 μg/mL α-amanitin (Schwartz et al., 1974).

Assays of DEAE-Sephadex fractions of GF-14 extracts in the presence of 0.5 μg/mL α-amanitin with DNA as template yield no activity in the peak corresponding to RNA polymerase II (not shown). Figure 2A shows that approximately one-half of the RNA polymerase II activity of A169b is α-amanitin resistant. Assays with poly[d(AT)] plus α-amanitin are not useful for A169b because the α-amanitin-resistant RNA polymerase II activity and the polymerase IIIA activity tend to obscure each other.

Figure 2B shows the separation of RNA polymerase II and IIIA activities by CM-Sephadex column chromatography. Reference to Table II shows that the total activity of RNA polymerase IIIA exceeds that of polymerase II with native

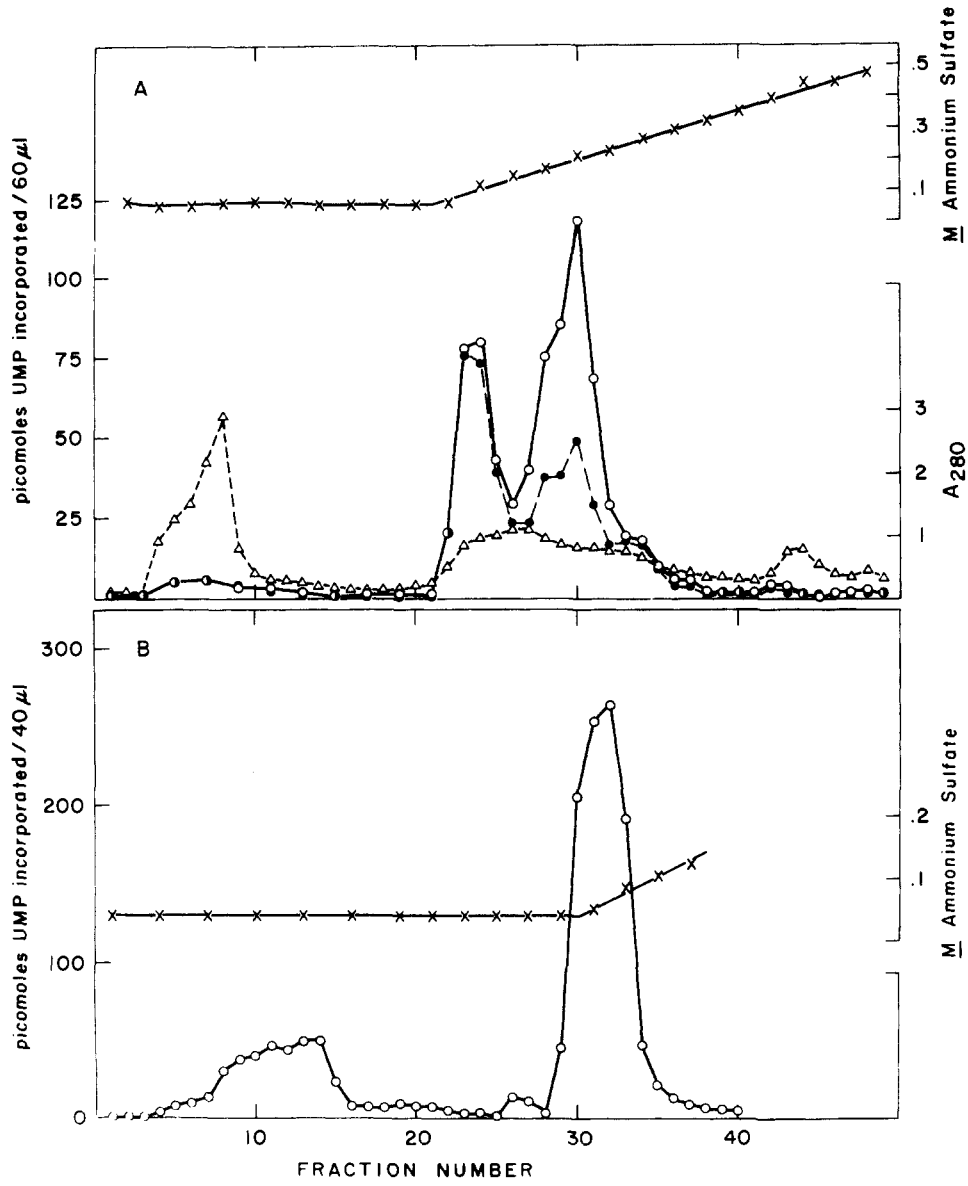

FIGURE 2: Separation of RNA polymerase I, II, and III activities of A169b. Column chromatography was done according to Schwartz et al. (1974). Assays were performed by mixing equal volumes of a fraction sample, which is in buffer A, and a solution of the assay components designed to give the final concentrations described under Materials and Methods. (A) DEAE-Sephadex column chromatography: (O–O) activity in the absence of α-amanitin; (● - - - ●) activity in the presence of 0.5 μg/mL α-amanitin; (Δ - - - Δ) A_{260}; (X–X) ammonium sulfate concentration determined by measurement of conductivity. Template was a mixture of native and denatured DNA at 50 μg/mL each. (B) CM-Sephadex column chromatography: (O–O) activity in the absence of α-amanitin; (X–X) ammonium sulfate concentration. Template was native DNA at 0.1 mg/mL.

DNA as template. Both polymerase II and IIIA activities were derived from a pool of DEAE-Sephadex polymerase II plus IIIA fractions. The apparent increase in polymerase III activity could be explained on the basis of removal of inhibitors or by a concentration effect of CM-Sephadex chromatography.

DEAE-Sephadex column chromatography of cell extracts is adequate for determining whether α-amanitin-resistant cell lines have α-amanitin-resistant RNA polymerase II. Further characterization of polymerase II should be done with preparations which are devoid of polymerase III. Failure to detect polymerase III activity in DEAE-Sephadex fractions is not an adequate demonstration of the absence of polymerase III molecules. In the functional analysis of DEAE-Sephadex-derived RNA polymerase II, it is quite possible that differing assay conditions may stimulate III activity or inhibit II activity. In the experiments described in this paper, RNA polymerase III was removed from RNA polymerase II preparations by CM-Sephadex chromatography. Evidence that this separation was successful includes the similar preference of the α-amanitin-resistant and sensitive RNA polymerases for denatured DNA, the similar salt and divalent cation optima, and the fact that the K_I of α-amanitin for polymerase III in our hands (6×10^{-4} M) is 300-fold greater than for the resistant polymerase II (Figure 4). The RNA polymerase III purified by Schwartz et al. (1974) has an apparent K_I 100-fold lower than we report here; we cannot explain this discrepancy, except that sensitivity of polymerase III (but not of either form of II) to α-amanitin is an unstable property of our preparations.

We do not have data on the absolute purity of the CM-Sephadex fraction of polymerase II. Schwartz et al. obtain a pure enzyme with a specific activity of 426 units/mg of protein. The specific activity of our fraction is approximately 8; thus, we would assume that it is 2% pure.

Resistance of A169b RNA Polymerase II to α-Amanitin. Figure 3 shows the effect of increasing concentrations of α-amanitin on activities of RNA polymerases I and II from

TABLE II: Purification of RNA Polymerase from A169b.[a]

Fraction	Total mg of protein	Total units of act.	Sp act. (units/μg of protein)
Sonicated cell extract			
F1	67	27 000	0.40
F2	57	26 000	0.46
F3	52	68 000	1.3
DEAE-Sephadex			
Total	20	91 000	4.6
I	9.8	62 000	6.3
II	5.2	19 000	3.7
IIIA	5.0	10 000	2.0
CM-Sephadex			
II	2.7	23 000	8.5
IIIA	1.3	35 000	27

[a] The fractions are those described by Schwartz et al. (1974). The DEAE- and CM-Sephadex fractions represent the total activities isolated in a typical experiment. All values are expressed per 1.0 mL of packed wet cells. Assays of crude fractions (F1, F2, F3) were performed at 0.05 M ammonium sulfate. Chromatographic fractions were assayed as described in Figure 2, except that 0.1 mg/mL native DNA was the template.

GF-14 and from A169b. The resistance of polymerase I is complete for both parent and mutant up to 400 μg/mL of α-amanitin, which is characteristic of animal cells (Schwartz et al., 1974). At 0.5 μg/mL, inhibition of GF-14 polymerase II is virtually complete, whereas A169b polymerase II is inhibited by approximately 50%. If one assumes that the A169b mutation is codominant, as has been shown for α-amanitin resistance in Chinese hamster ovary (Lobban and Siminovitch, 1975) and rat myoblast (Ingles et al., 1976) cells, the 50% inhibition level can be explained by a heterozygous genotype for RNA polymerase II. The L5178Y cell line is approximately diploid with respect to chromosomal content. A codominant mutation to α-amanitin resistance in one of the two gene copies for a polypeptide of RNA polymerase II would be expected to give rise to a biphasic inhibition curve. Somers et al. (1975) have offered a similar interpretation of biphasic α-amanitin inhibition of RNA polymerase II from rat myoblast α-amanitin-resistant mutants.

Further support for a heterozygous state for α-amanitin resistance in A169b is given by Figure 4. Binding of α-amanitin to RNA polymerase II occurs noncompetitively at a single site (Cochet-Meilhac and Chambon, 1974). The K_I for a noncompetitive inhibitor binding at a single site is given by the x intercept in a plot of 1/activity vs. inhibitor concentration (Dixon and Webb, 1964). The inhibition curve for A169b is biphasic, which is especially apparent on the expanded scale of Figure 4B. The two components are presumed to correspond to the wild-type and mutant forms of RNA polymerase II. The activity of A169b polymerase II in the presence of 1.0 μg/mL (1×10^{-6} M) α-amanitin is due exclusively to the mutant component (see Figure 3). Considering only α-amanitin concentrations above 1.0×10^{-6} M for the points in Figure 4B, a K_I of 2.1×10^{-6} M is found for the α-amanitin-resistant component. This value is 670 times greater than the K_I for the parental RNA polymerase II, 3.5×10^{-9} M (Cochet-Meilhac and Chambon, 1974).

The K_I for the α-amanitin-sensitive component of A169b RNA polymerase II activity can be derived from assays at very low α-amanitin concentrations. The inset of Figure 4A shows a curve which has been constructed for A169b from points which have been corrected by subtracting the α-amanitin-resistant activity contributed by the mutant form of the polymerase. The K_I is found to be 4.1×10^{-9} M, which agrees well with that of GF-14.

RNA polymerases II and III may have three polypeptides

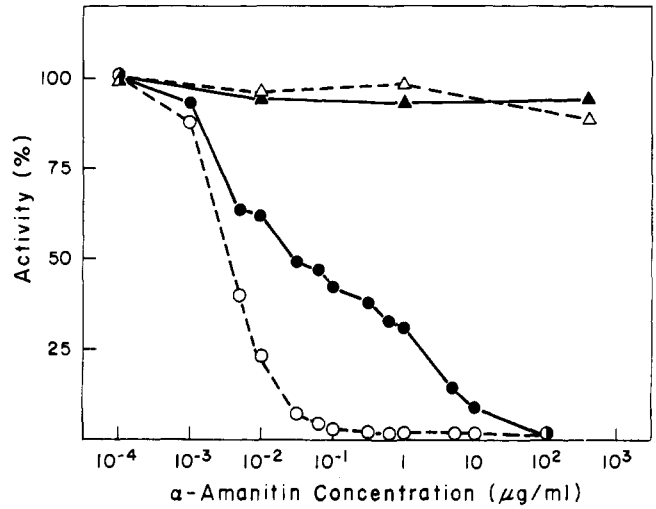

FIGURE 3: Activities of RNA polymerases from GF-14 and A169b. (O - - - O) RNA polymerase II from GF-14; (●–●) RNA polymerase II from A169b; (Δ - - - Δ) RNA polymerase I from GF-14; (▲—▲) RNA polymerase I from A169b. The template was native DNA at 0.1 mg/mL and the ammonium sulfate concentration was 100 mM. The points are averages of two determinations. Units of maximum activity for a typical experiment were 177, GF-14 RNA polymerase I; 267, A169b polymerase I; 555, GF-14 polymerase II; 115, A169b polymerase II.

in common (Sklar et al., 1975). It is possible that one of these polypeptides is the site of the mutation to α-amanitin resistance. If this is the case, an alteration in the resistance of RNA polymerase III to α-amanitin would be expected. We have been unable to investigate this possibility because our RNA polymerase III preparations from both GF-14 and A169b lose α-amanitin resistance upon storage at −70 °C. We have deferred an investigation of the effect of the α-amanitin-resistant mutation on polymerase III until stable enzyme is obtained.

Properties of α-Amanitin-Resistant RNA Polymerase II. Where comparisons of activity of wild-type and mutant polymerase are to be made, it is important to establish whether binding affinity for substrates is altered in the mutant polymerase. Accordingly, we examined the dependence on each of the four nucleoside triphosphates for parent and mutant RNA polymerase II, using native DNA as template and Mn^{2+} as divalent cation. The results are shown in Table III. To eliminate activity of wild-type polymerase II in the polymerase II preparations from A169b, assays were done for A169b in

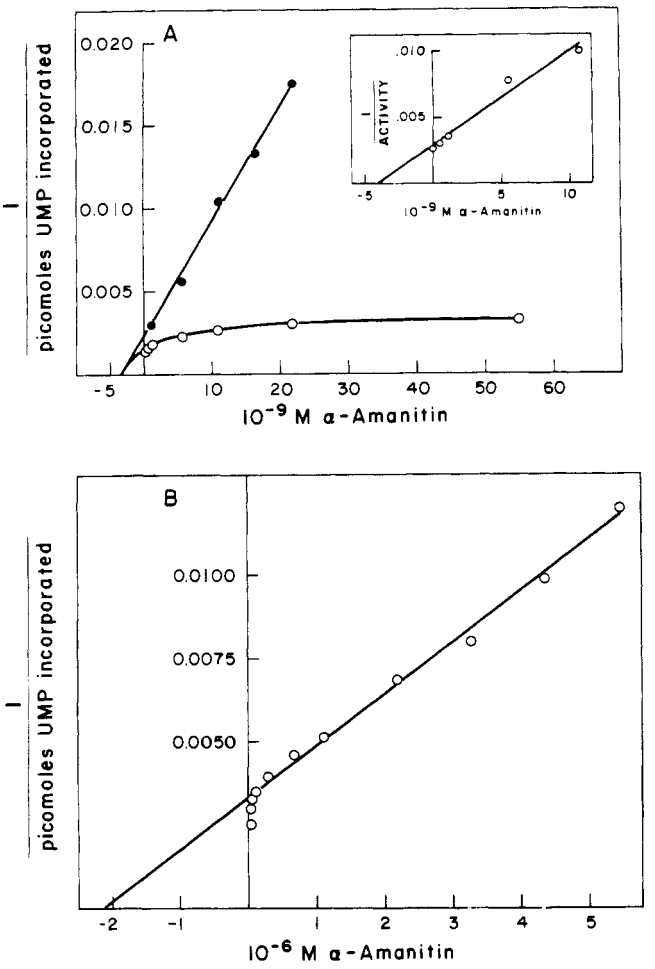

FIGURE 4: Determination of K_I of α-amanitin for RNA polymerase II from GF-14 and A169b. (○—○) activity of RNA polymerase II from A169b; (●-●) activity of RNA polymerase II from GF-14. Lines were fitted by linear-regression analyses. These points are from a typical experiment with 0.1 mg/mL native DNA as template and 100 mM ammonium sulfate. (A) K_I of α-amanitin-sensitive form of the polymerase. (Inset) The points representing the activity of A169b polymerase in the five lowest α-amanitin concentrations shown in A are replotted after subtracting the activity of the α-amanitin-resistant component. The calculations are according to the relationship: total activity = α-amanitin-sensitive activity + V_{max} for α-amanitin-resistant activity, derived from Neal (1972). V_{max} is calculated from Figure 4B. (B) K_I of α-amanitin-resistant form of the polymerase.

TABLE III: Apparent Michaelis Constants for Nucleoside Triphosphates.[a]

Strain	Substrate (mM)			
	UTP	GTP	ATP	CTP
GF-14	0.032	0.032	0.023	0.0038
A169b	0.030	0.033	0.028	0.0036

[a] K_Ms were determined from Lineweaver-Burk plots of four points for each nucleoside triphosphate. UTP was limiting (0.050 mM) in all assays. The points were fitted to a straight line by linear-regression analysis. The K_Ms given are averages of at least two separate determinations. The reactions contained ammonium sulfate at a concentration of 100 mM. The A169b reactions contained 0.5 μg/mL α-amanitin.

the presence of 0.5 μg/mL α-amanitin. Since these assays were done in the presence of limiting UTP, the results obtained do not represent true Michaelis constants except for UTP. However, any significant alterations of substrate-binding affinity in the mutant RNA polymerase II should be detected by this method.

Parallel assays were done for GF-14 polymerase in the presence of 0.005 μg/mL α-amanitin (which inhibits the parent activity by 50%) and for A169b polymerase in the absence of α-amanitin. Essentially the same apparent K_M values were obtained as those in Table II, confirming that α-amanitin has no effect on the reaction other than inactivating RNA polymerase II. We conclude from Table III that binding affinities for nucleoside triphosphates are not altered in the mutant RNA polymerase II.

We have investigated the activities of wild-type and α-amanitin-resistant RNA polymerase II with varying concentrations of ammonium sulfate, Mn^{2+}, using both native and denatured DNA as template in the presence and absence of α-amanitin (data not shown). We conclude that the mutant polymerase is not altered in terms of salt or ionic optima, rate of RNA synthesis, or in preference for native or denatured DNA.

In addition, we have determined the ammonium sulfate, Mn^{2+}, and Mg^{2+} optima for parent and mutant polymerases using as template the poly(deoxyribonucleotides) in Table IV. In comparing wild-type and α-amanitin-resistant polymerase, we observed no differences in optima for ammonium sulfate or divalent cations. A striking observation is the pronounced difference with poly[d(AT)] as template and Mn^{2+} as divalent cation. The mutant enzyme is twice as active, relative to native DNA, as the parental enzyme under these conditions. When α-amanitin is omitted from the assay mixture, the activity of the A169b preparation on poly[d(AT)] relative to native DNA (1.46), is intermediate between the value for GF-14 (~0.85-0.90) and that for the α-amanitin resistant fraction (1.73). This difference in template preference has been observed in at least two different preparations of each enzyme and with several batches of template. Its quantitative nature, however, seems to be a function of the particular template preparation, since with some lots of d(AT) the differences are very much more marked while with others there is little detectable difference. We believe that this difference is significant biochemically and may be related to the alteration in enzyme structure accompanying α-amanitin resistance, but we cannot rule out some nonspecific factor in the mutant enzyme preparation which stimulates the activity on poly[d(AT)].

Discussion

Mutations affecting RNA polymerase II in eukaryotic cells are important for two reasons. First, the gene product affected is relatively easy to assay, so that evidence as to the nature of the genetic (or epigenetic) event can easily be obtained. Thus Lobban and Ingles and their co-workers (Lobban and Siminovitch, 1975; Ingles et al., 1976; Somers et al., 1975) have provided quite convincing arguments that α-amanitin-resistant mammalian cells contain an altered RNA polymerase II. A more far reaching goal is to use any alterations in enzymatic properties found in the mutant RNA polymerase to explore structure-function relationships as an approach to understanding transcriptional regulation.

The results reported here indicate that the mutation leading to α-amanitin resistance has not drastically affected many of the catalytic properties of RNA polymerase II. This is perhaps not surprising, since the criterion of selection required that the resistant enzyme function. Nevertheless, it is interesting to note that the altered enzyme seems identical to the parental one in most properties.

The sole exception to this identity appears to be the increased preference of the resistant enzyme for poly[d(AT)]. Although

TABLE IV: Activities of RNA Polymerase II with Poly(deoxyribonucleotides).[a]

Template[b]	Nucleotide[c] incorp	Mg^{2+} [d] GF-14 +ama[e,f]	Mg^{2+} [d] GF-14 −ama	Mg^{2+} [d] A169b −ama[f]	Mg^{2+} [d] A169b +ama[e]	Mn^{2+} [d] GF-14 +ama[e,f]	Mn^{2+} [d] GF-14 −ama	Mn^{2+} [d] A169b −ama[f]	Mn^{2+} [d] A169b +ama[e]
Poly[d(AT)]	UMP	0.91	0.82 ± 0.09	0.83	1.20 ± 0.09	0.85	0.91 ± 0.11	1.46	1.73 ± 0.06
Poly[d(GC)]	GMP	2.32	1.89 ± 0.17	1.52	1.44 ± 0.13	1.62	1.62 ± 0.17	1.67	1.66 ± 0.19
Denatured DNA	UMP	1.23	1.31 ± 0.03	0.94	1.18 ± 0.22	1.84	1.79 ± 0.04	2.06	1.88 ± 0.12[g]
	GMP	0.72	0.85 ± 0.11	0.42	0.66 ± 0.10	1.35	1.57 ± 0.08	1.75	1.70 ± 0.03

[a] Values given are ratios of template to native DNA activities for a given RNA polymerase and given divalent cation. Ratios are expressed as the mean ± standard error, with five determinations except where indicated. Determinations were on different days. [b] Template concentrations were 50 μg/mL for poly(deoxyribonucleotides). DNA concentration was 100 μg/mL. These concentrations are saturating as determined in preliminary experiments. [c] Activity ratios are based on the incorporation of the same [^{3}H]nucleotide by the given template and native DNA. Units of activity for native DNA in a typical experiment with GF-14 were 24 ([^{3}H]UMP, Mg^{2+}), 79 ([^{3}H]UMP, Mn^{2+}), 5.3 ([^{3}H]GMP, Mg^{2+}), and 16 ([^{3}H]GMP, Mn^{2+}). For A169b, activities with native DNA in that experiment were 79 ([^{3}H]UMP, Mn^{2+}), 130 ([^{3}H]UMP, Mn^{2+}), 15 ([^{3}H]GMP, Mg^{2+}), and 24 ([^{3}H]GMP, Mn^{2+}). [d] The $MgCl_2$ concentrations were 12 mM for poly[d(AT)] and poly[d(GC)] and 6 mM for DNA. The $MnCl_2$ concentrations were 4 mM for poly(deoxyribonucleosides) and 2 mM for DNA. Ammonium sulfate concentrations were 40 mM for poly(deoxyribonucleotides), 75 mM for native DNA, and 50 mM for denatured DNA. Reactions otherwise were as described under Materials and Methods. The concentrations of ammonium sulfate and divalent cations were those found to be optimal in preliminary experiments. [e] The final concentration of α-amanitin was 0.005 μg/mL for GF-14 and 0.5 μg/mL for A169b. [f] One determination. [g] Four determinations.

this property is a consistent feature of our enzyme preparations, its manifestation varies from template batch to template batch. Evidence that it is not due to an artifact of the preparation, in addition to its consistent appearance, includes the diminished preference when both activities are measured (in the absence of α-amanitin). However, given the impurity of the enzyme preparations used here and the quantitative variation in the template effect, we can only suggest that the difference in activity with poly[d(AT)] may be related to the α-amanitin sensitivity.

α-Amanitin binding is a complex phenomenon. Brodner and Wieland (1976) have demonstrated that the toxin binds to the 140 000-dalton subunit of RNA polymerase II. However, RNA polymerase III is also inhibited by amanitin, albeit at concentrations 100-fold higher than polymerase II, and this enzyme contains no 140 000-dalton subunit. Furthermore, Huet et al. (1975) have shown that removal of two subunits from RNA polymerase I of yeast, normally inhibited only at concentrations of α-amanitin of 300 μg/mL, renders the enzyme sensitive to 50 μg/mL. This result implies that α-amanitin sensitivity may be a fundamental property of eukaryotic RNA polymerases, and that resistance is achieved as a result of the complex quaternary structure of the enzymes. If this argument is valid, one might expect that resistance to the toxin could be achieved in a number of ways of which simple alteration of the binding site would be only one. Such a model could explain the simultaneous alteration in properties as disparate as template preference and amanitin sensitivity.

Alternatively, of course, the subunit bearing the binding site for α-amanitin could be involved in determining template specificity. In either case, the different resistant mutations can be expected to result in different spectra of template preferences. Whatever the exact reason for the pleiotropic nature of the mutations to α-amanitin resistance, it would be worthwhile to examine the other α-amanitin resistant cell lines to determine whether the template preferences are also altered.

In order to understand the biochemical nature of the mutational alteration, the subunit whose primary structure is changed must be identified. If the 140 000 dalton subunit were unaltered in the mutant, but any of the others changed, then the model of resistance as a secondary property of RNA polymerases would be supported.

The fact that the mutant enzyme, if it differs from the parent, differs only in preference for one of the templates tested, together with the normal growth characteristics of the mutant cell line, argues that the change in the enzyme is a subtle one and does not affect biological function under normal conditions. The possibility that under certain conditions, such as elevated or decreased temperature, the enzyme would function abnormally has not been explored. Alternatively, the cells may be able to tolerate slight aberrations in RNA polymerase under the relatively undemanding conditions of tissue culture.

The results presented here, while posing a larger number of questions than they answer, nevertheless suggest the possibility that mutants isolated as resistant to RNA polymerase inhibitors may prove to be of use in elucidating the structure-function relationships of RNA polymerases from eukaryotic cells.

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Acetylation of Prostaglandin Synthetase by Aspirin. Purification and Properties of the Acetylated Protein from Sheep Vesicular Gland†

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ABSTRACT: We previously presented evidence that aspirin (acetylsalicylic acid) inhibits prostaglandin synthetase by acetylating an active site of the enzyme. In the current work, we have labeled the enzyme from an acetone–pentane powder of sheep vesicular gland using [*acetyl*-^{3}H]aspirin and purified the [^{3}H]acetyl-protein to near homogeneity. The final preparation contains protein of a single molecular weight (85 000) and an amino-terminal sequence of Asp-Ala-Gly-Arg-Ala. The [^{3}H]acetyl-protein contained 0.5 mol of acetyl residues per mol of protein based on amino acid composition but only a single sequence was found.

The first enzyme of prostaglandin biosynthesis is a membrane-bound dioxygenase prostaglandin synthetase (prostaglandin cyclooxygenase, prostaglandin synthetase) (Samuelsson, 1972). The enzyme catalyzes the conversion of an essential fatty acid precursor, arachidonic acid (20:4), to a cyclic endoperoxide intermediate, prostaglandin G_2 (PGG_2) (Nugteren and Hazelhof, 1973; Hamberg and Samuelsson, 1973; Hamberg et al., 1974). Several groups have published studies concerning purification of the enzyme (Samuelsson et al., 1967; Miyamoto et al., 1974, 1976; Chan et al., 1975; Rome and Lands, 1975). Most recently, Miyamoto and co-workers reported a 750-fold purification of the enzyme from bovine vesicular gland microsomes and studied the effects of activators such as hematin and tryptophan on the activity of the enzyme (Miyamoto et al., 1976).

The anti-inflammatory drug, aspirin (acetylsalicylic acid), inhibits prostaglandin synthetase in a highly specific manner at micromolar concentrations (100 μM) within minutes (60 min) (Smith and Lands, 1971; Hamberg and Samuelsson, 1974; Hamberg et al., 1974; Roth et al., 1975; Roth and Majerus, 1975). Aspirin is an acetylating agent (Pinckard et al., 1968; Hawkins et al., 1969) and our studies indicate that the drug acetylates prostaglandin synthetase (Roth et al., 1975; Roth and Majerus, 1975). Furthermore, acetylation by aspirin is highly specific and proceeds under the same conditions as the drug's inhibitory effect on enzyme activity (μM concentration within min) (Roth et al., 1975; Roth and Majerus, 1975). Based on time course, substrate inhibition and copurification experiments, we have concluded that aspirin acetylates an active site of prostaglandin synthetase (Roth et al., 1975; Roth and Majerus, 1975; Rome et al., 1976) and that only active enzyme is susceptible to acetylation by aspirin. Therefore, [*acetyl*-^{3}H]aspirin can be used to label the enzyme specifically, providing an easily identifiable, covalently bound tritium marker for purification work. The acetylated portion of the enzyme has a molecular weight of 85 000 (Roth et al., 1975; Roth and Majerus, 1975).

In the current work, we have purified the aspirin-acetylated portion of prostaglandin synthetase from sheep vesicular gland and determined the amino acid compostion and amino-terminal sequence.

Experimental Procedure

Materials. Nonidet P40 (NP40)[1] was obtained from Particle Data Laboratories, Elmhurst, Ill. DEAE[2]-cellulose (DE52) was obtained from Whatman, and agarose (Bio-Gel A-1.5m 200–400 mesh) was obtained from Bio-Rad. Sodium dodecyl sulfate was purchased from British Drug House, and Ampholine carrier ampholyte was purchased from LKB. All other chemicals not previously described (Roth et al., 1975; Roth and Majerus, 1975) were reagent grade.

Enzyme Source. Sheep vesicular glands were kindly pro-

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[1] Shell trademark for polyoxyethylene glyco(9)-*p*-*tert*-octylphenol.

[2] Abbreviations used: PGG_2, prostaglandin G_2; NP40, Nonidet P40; DE52, DEAE-cellulose; Pth, phenylthiohydantoin; TLC, thin-layer chromatography; DEAE, diethylaminoethyl.