Nucleosome Structure of *Xenopus* Oocyte Amplified Ribosomal Genes†

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ABSTRACT: The chromatin subunit or nucleosome structure of the amplified, extrachromosomal, ribosomal genes of oocytes of the amphibian *Xenopus laevis* has been investigated during stages of growth when these genes are markedly changing their rates of transcriptional activity. Nucleic acid hybridization studies involving micrococcal nuclease derived monomer nucleosome DNA fragments and purified ribosomal RNAs indicate that the apparent degree of accessibility of the ribosomal genes to short-term nuclease hydrolysis varies as a function of the rate of ribosomal RNA (rRNA) transcription. However, at no stage during oocyte development are all of the amplified ribosomal genes completely accessible to nuclease hydrolysis, even in those stages with maximal rates of rRNA transcriptional activity. These results suggest that the transcriptionally active ribosomal genes of oocytes are partially, or perhaps transiently, associated with histones in the form of nuclease releasable nucleosomes but that the degree of this association may change with varying rates of rRNA synthesis. Additionally, the present data indicate that the average size of the double-stranded ribosomal DNA associated with monomer nucleosomes is the same (about 200 base pairs) in all of the oocyte stages examined regardless of the rates of rRNA synthesis in these stages.

A considerable body of biochemical evidence has recently accumulated suggesting that both the transcriptionally active and inactive ribosomal genes of several different organisms can be associated with histone aggregates in the form of chromatin subunits or nucleosomes (Reeves & Jones, 1976; Reeves, 1976, 1977a,b; Mathis & Gorovsky, 1976; Gorovsky et al., 1977; Piper et al., 1976; Higashinakagawa et al., 1977; Matsui & Busch, 1977; Allfrey et al., 1977; Johnson et al., 1978; Gottesfeld & Melton, 1978). However, it has also been reported that the transcriptionally active ribosomal genes of both the amphibian *Xenopus laevis* (Reeves, 1976, 1977b) and the slime mold *Physarum* (Allfrey et al., 1977; Johnson et al., 1978) may be more sensitive to digestion by certain enzymes, such as micrococcal nuclease, than are transcriptionally inactive ribosomal genes. Furthermore, it has also been suggested that in *Xenopus* embryos the association of nucleosomes with transcriptionally active ribosomal genes may be of a rather labile nature since the apparent degree of association of nucleosomes with ribosomal DNA is, within certain limits, inversely related to the rate of ribosomal RNA (rRNA) transcription (Reeves, 1976, 1977b).

In the experiments reported here a careful analysis has been made of the nucleosome structure of the amplified, extrachromosomal, ribosomal genes of oocytes of the amphibian *Xenopus laevis* to determine whether there is any similar relationship between the chromatin structure of these genes and their rates of rRNA transcription as is found in *Xenopus* embryonic somatic cells.

The amplified ribosomal genes of *Xenopus* oocytes offer excellent material for such a study because a great deal of biochemical information is known about both the structure and function of these genes. For example, in *Xenopus*, as in many other organisms, the ribosomal RNA genes (rDNA) are amplified during the pachytene stage of oogenesis soon after the female tadpoles have metamorphosed to froglets. This phenomenon has been extensively reviewed elsewhere (Brown, 1967; Brown & Dawid, 1968; Gall, 1968, 1969; Hourcade et al., 1973). The end result of rDNA replication is the production of about 1500 extrachromosomal nucleoli, each containing several sets of ribosomal genes often with detectably different lengths of nontranscribed spacer DNA separating the coding sequences (Wellauer et al., 1974). The total number of these gene sets per oocyte has been estimated to be between 3×10^3 and 5×10^3 (Brown & Dawid, 1968; Gall, 1969; Perkowska et al., 1968). Since the haploid ribosomal gene set contains about 450–500 copies of the individual ribosomal genes (Brown & Weber, 1968), the total number of these genes in the oocyte is 1.5×10^6 to 2.5×10^6 and their combined mass is between 15 and 30 pg per nucleus (Brown & Dawid, 1968; Perkowska et al., 1968). This amount of amplified rDNA is in excess of the amount of pachytene 4C chromosomal DNA which has been estimated to be about 12 pg per nucleus (Dawid, 1965).

In addition to this biochemical information, the oocyte amplified ribosomal genes offer an excellent opportunity for studying the structure of genes involved in varying rates of transcriptional activity. It is known, for example, that during the extended growth period of oogenesis the rates of transcription of rRNA are closely regulated and growth stage dependent (Brown & Littna, 1964; Gurdon, 1968; Ford, 1972; La Marca et al., 1973; Denis, 1974; Reeder, 1974; Scheer et al., 1976; Davidson, 1976; Anderson & Smith, 1977). It was thus of some interest to investigate the nucleosome structure of these amplifed genes during various oocyte developmental stages.

Materials and Methods

Chemicals and Isotopes. Common chemicals were of "Analytical Reagent" quality and were for the most part from Fisher Scientific Co. Micrococcal nuclease (EC 3.1.4.7), UTP, ATP, *Escherichia coli* RNA polymerase, collagenase, papain, and diethyl pyrocarbonate were from Sigma. Poly(dA-dT) was from Collaborative Research. Polyethylenimine (PEI)-cellulose F chromatography plates were from Merck. Polyvinyl sulfate was from Eastman Organic Chemicals. UltraPure urea

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and sucrose were from Schwarz/Mann. All tissue culture media, plastic ware, and sera were from Microcan. All radiochemicals were obtained from New England Nuclear.

Isolation and Classification of Oocytes. Oocytes from the ovaries of large adult *Xenopus laevis* that had not ovulated for at least 3 months were classified according to the descriptions given by Dumont (1972). The six sizes of oocytes used were as follows: stage I previtellogenic oocytes, 50–300 μm in diameter; stage II translucent early vitellogenic oocytes, 300–450 μm in diameter; stage III pigment-free mid-vitellogenic oocytes, 450–600 μm in diameter; stage IV darkly pigmented mid-vitellogenic oocytes, 600–1000 μm in diameter; stage V, late vitellogenic oocytes, 1000–1200 μm in diameter; and stage VI, "white band" post-vitellogenic oocytes, 1200–1300 μm in diameter.

Oocytes were isolated from excised ovaries in two different ways depending on the type of experiment being performed. In kinetic experiments where the uptake and incorporation of exogenous [^{3}H]uridine into cellular UTP pools and rRNA was being followed, small pieces of ovary were cultured in vitro in microwells of Falcon Plastic dishes in a medium containing Barth saline solution (Barth & Barth, 1959), 10% fetal calf serum and 100 μg/mL each of sodium penicillin G, dihydrostreptomycin, and gentamycin sulfate in addition to the added isotope. After various lengths of culture time, the ovary fragments were washed extensively with sterile Barth solution and individual oocytes then manually dissected from their follicles with watchmakers forceps and sized under a dissecting microscope according to diameters. All follicle cells adhering to the oocytes were digested away using a collagenase/papain solution (see below) and microscopic analysis confirmed their absence.

In the second type of separatory procedure, used to obtain oocytes for nuclear isolations, ovarian tissue was excised from females, cut into fine pieces, and the oocytes were released from the follicle cells and other tissues by digestion with collagenase (type I) and papain (type II) as described by Dumont (1972) and also by Higashinakagawa et al. (1977). The isolated oocytes were then separated into different size classes by a combination of differential settling techniques (Higashinakagawa et al., 1977; Pestells, 1975) and sucrose density gradient techniques (manuscript in preparation). By these procedures the following oocyte size classes were obtained in bulk in over 85% purity (as determined by microscopic analysis): stages V and VI, 1000–1300-μm diameter; stage IV, 600–1000-μM diameter; stages II and III, 300–600 μm diameter; and stage I, 100–300 μm diameter.

Isolation of Germinal Vesicles (Nuclei). Owing to their varying concentrations of yolk and different sizes of germinal vesicles, the different size classes of oocytes required slightly different procedures for optimum nuclear isolations. In all cases, however, the isolation solutions were of low ionic strength to minimize histone rearrangements on the chromatin and, in addition, contained protease inhibitors to prevent histone degradations (Reeves & Jones, 1976). The basic nuclear isolation buffer contained 10 mM Tris-HCl (pH 7.8), 1 mM $MgCl_2$, 0.14 mM phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$), and 50 mM $NaHSO_3$. To this buffer solution was added various concentrations of sucrose and Triton X-100 (TX-100) depending on the stage of oocytes from which the nuclei were to be isolated.

All nuclear isolations were carried out at 4 °C. Isolated stage I oocytes and isolated mixed stage II and III oocytes were treated in the same way for germinal vesicle isolations. The oocytes were homogenized with one or two strokes of a loose fitting Vitro Teflon-glass homogenizer. The germinal vesicles were then sedimented by centrifugation for 20 min at 1000*g*. fitting Vitro Teflonglass homogenizer. The germinal vesicles were then sedimented by centrifugation for 20 min at 1000*g*. The pelleted nuclei were resuspended in isolation buffer containing 1.0 M sucrose and TX-100 and layered on top of a 1.25 M sucrose gradient and the nuclei pelleted through the gradient by centrifugation for 20 min at 12 000*g*. The nuclei were resuspended and washed twice more by pelleting through 0.5 M sucrose gradients centrifuged for 10 min at 1500*g*. The final nuclear pellets were immediately frozen in a dry ice–acetone bath and then transferred to liquid N_2 for storage until needed. Stage IV oocytes, on the other hand, were resuspended in the isolation buffer containing 0.5 M sucrose and 0.5% TX-100 and the oocytes homogenized by one stroke of a very loose-fitting Kontes no. 9 glass homogenizer and the nuclei pelleted by centrifugation for 20 min at 1000*g*. The pelleted nuclei were resuspended in 0.5 M sucrose buffer with TX-100 and layered over a 1.0 M sucrose gradient and the nuclei repelleted, washed twice more by centrifugation, and finally frozen as described above. The germinal vesicles of stage V and VI oocytes were more difficult to isolate because of their large size and because of the large amount of yolk present in these older stages. Nuclei from these stages were therefore either manually isolated or isolated in hypotonic medium as described by Ford & Gurdon (1977). After isolation, these large germinal vesicles were washed extensively by gentle agitations and settlings and then finally frozen as above.

In connection with all of these nuclear isolations two points should be emphasized: (1) the isolated germinal vesicles from all oocyte stages were (before freezing) quite active in RNA synthesis as determined by in vitro [^{3}H]uridine incorporation experiments and possessed RNA polymerases I, II, and III and DNA polymerase, as well as poly(ADP-ribose) synthetase activities (unpublished data confirming the results of Higashinakagawa et al., 1977; Burzio & Koide, 1978); and (2) in each of the nuclear isolation experiments a known quantity of [^{14}C]thymidine labeled mitochondria (obtained from *Xenopus* tissue culture cells) was added to the starting oocyte population to monitor the amount of mitochondrial contamination at the end of the various nuclear isolation procedures. In each case the isolated oocyte nuclei were monitored for adherent radioactivity during the washing procedures and washing was continued until suitably low levels of contamination (i.e., usually less than 1% of the input radioactivity) were achieved. This internal standard method for quantifying mitochondrial contamination also allowed for normalization and direct comparisons to be made between different oocyte size classes.

Purification of Micrococcal Nuclease Released Nucleosomes. The general methods used for the preparation and purification of micrococcal nuclease released, nucleosome-protected, DNA from isolated nuclei have been described extensively elsewhere (Reeves & Jones, 1976). The frozen nuclear pellets from the various oocyte stages were quickly thawed in a nuclease digestion buffer containing 0.15 M sucrose, 10 mM Tris-HCl (pH 7.8), 0.14 mM $PhCH_2SO_2F$, 0.5 mM $MgCl_2$, and 0.5 mM $CaCl_2$. To this mixture micrococcal nuclease was added at a concentration of 100 units/mL and the solution incubated at 37 °C for 4 min before termination of the enzyme reaction by addition of 10 mM EDTA and cooling to 4 °C. The nuclei were then pelleted by centrifugation and resuspended in the above buffer containing 0.2 mM EDTA but without Ca^{2+} or Mg^{2+} or nuclease being present and the chromatin particles released by homogenization. Nuclear membrane and insoluble materials were removed by centrifugation and double-stranded DNA was recovered by phenol

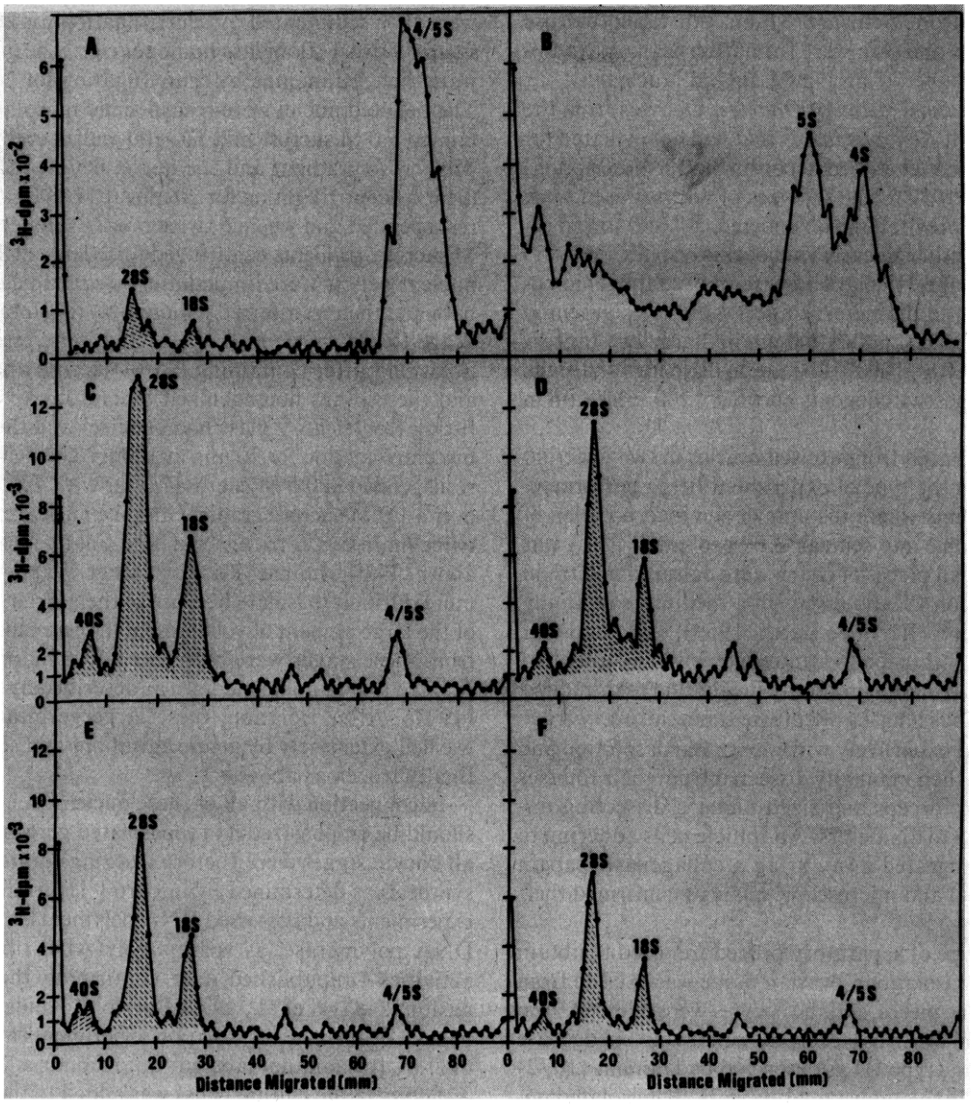

FIGURE 1: Gel electrophoretic analysis of radioactively labeled RNA extracted from different oogenic stages of *Xenopus*. Oocytes were cultured in vitro with [^{2}H]uridine as described in Materials and Methods. After various lengths of labeling, the oocytes were isolated from their surrounding follicle cells and separated into five size classes (Dumont, 1972), and the RNA was extracted from each class and electrophoresed on polyacrylamide gels. The RNA from the regions of the gels shown with hatching were then isolated for each class of oocytes and the amount of acid-insoluble radioactivity was determined. From such measurements the rates of incorporation of isotope into rRNA for each oocyte class were determined. (A) Stage I (50–300-μm diameter) oocyte RNA, 2.7% polyacrylamide gels; (B) stage I oocyte RNA electrophoretically separated on 8% polyacrylamide gels to distinguish 4S from 5S RNA; (C) stage II and III (300–600-μm diameter) oocyte RNA, 2.7% polyacrylamide gels; (D) stage IV (600–1000-μm diameter) oocyte RNA, 2.7% polyacrylamide gels; (E) stage V (1000–1200-μm diameter) oocyte RNA, 2.7% polyacrylamide gels; (F) stage VI (1200–1300-μm diameter, white band) oocyte RNA, 2.7% polyacrylamide gels.

extractions as previously described (Reeves, 1977a). The isolated DNA fragments were then separated by electrophoresis on 1.4% agarose gels and visualized under ultraviolet light after staining with ethidium bromide (Reeves & Jones, 1976). The monomer nucleosome (monosome)-sized DNA fragments (all about 200 base pairs in length from the various oocyte stages) were eluted from the appropriate regions of the gels. The nuclease derived nucleosome DNA fragments from control cells (red blood cells (RBCs) and *Xenopus* tissue culture cells (X58s)) were isolated as previously described (Reeves & Jones, 1976).

All protein determinations were by the method of Lowry et al. (1951) or by the use of the E_{260}/E_{280} ratio using appropriate standards. DNA determinations were by the method of Burton (1968).

Isolation and Electrophoresis of RNA. RNA was extracted and separated into fractions by the electrophoretic procedures described by Knowland (1970). Segments of these gels containing the isotopically labeled 18S and/or 28S rRNA were cut out and the rRNA was recovered from the fragments by elution. In other cases, the gels were cut into 1-mm sections and the slices dissolved, and the radioactivity was contained in each counted as previously described (Reeves & Laskey, 1975). RNA determinations were by absorbance assuming 1.0 A_{1cm}^{260} unit = 40 μg cm^{-3}.

Nucleic Acid Hybridizations. RNA·DNA saturation hybridizations were conducted using the nitrocellulose membrane filter hybridization techniques described by Gillespie (1968) as previously described (Reeves & Jones, 1976). The conditions of incubation (0.3 M NaCl, 0.03 M trisodium citrate (pH 7.8), 50% formamide, 45 °C) corresponded to incubation at 25 °C below the T_m of *Xenopus* rRNA·DNA hybrids (Miller & Knowland, 1970) and allowed for both a high specificity of annealing and fast reaction rates. The fidelity and reproducibility of the techniques used have been reported on previously (Reeves, 1977a).

Nucleotide Pool Analyses and Kinetics of rRNA Synthesis. (a) For kinetic experiments to determine the incorporation of exogenously supplied [^{3}H]uridine into oocyte ribosomal RNA, fragments of adult ovaries were cultured in vitro containing 2 mCi/mL of [^{3}H]uridine as described above. For each time point, oocytes of the various size classes were manually isolated, freed of their surrounding follicle cells, and staged. Each isolated class of oocytes was then divided into two groups for analyses. One group was extracted for RNA as described above and the RNA subsequently fractionated by electrophoresis. The 18S + 28S + 40S rRNA was isolated from these gels for each time point and the total amount of radioactivity determined. The second group of isolated oocytes, on the other hand, were extracted with perchloric acid and the amount of radioactivity in the total acid soluble nucleotide pools and in the UTP pools determined by the methods described by Brown & Littna (1964) and Brown & Weber (1968) using the thin-layer chromatography system for separating nucleotides developed by Randerath & Randerath (1967).

(b) Determination of both the endogenous UTP and ATP pool sizes in different oocyte size classes was carried out using the very sensitive *E. coli* RNA polymerase assay method described by Sasvari-Szekely et al. (1975). The principle of this method is based on the synthesis of a ribonucleotide polymer in the presence of UTP, ATP, and poly(dA-dT) as template. In a given assay reaction mixture, if one of these ribonucleotide triphosphates is radioactive and present in excess, and the other (obtained from the oocyte extracts) is the only limiting factor of the rate of the reaction, then the rate of incorporation of radioactivity into a macromolecular polymer depends directly on the concentration of the limiting nucleotide. Using this assay both the UTP and ATP oocyte pool sizes for each oocyte class were determined in the same polymerase experiment taking into account the isotope dilution effect (indirect method) that the "cold cellular ATP" present in the oocyte extracts has on the [^{3}H]ATP present in the standard reaction mixture (Sasvari-Szekely et al., 1975). Details of these calculations are given in the text and in the appendix to Sasvari-Szekely et al. (1975).

Results

The rates of ribosomal RNA synthesis occurring in oocytes during development in female *Xenopus* have been determined by a number of different workers (Brown & Littna, 1964; Coleman, 1974; La Marca et al., 1973; Denis, 1974; Leonard & La Marca, 1975; Scheer et al., 1976; Davidson, 1976; Anderson & Smith, 1977). From this work it is known that considerable variation exists in the rates of ribosomal RNA synthesis in the same stages of oocytes obtained from different females. The reasons for this variability are not known but may be related to differences in the physiological states of individual females (La Marca et al., 1973; Hallberg & Smith, 1976; Anderson & Smith, 1977). Therefore, before a meaningful investigation of the nucleosome structure of the amplified ribosomal genes during changes in transcription rates could be conducted, it was first necessary to establish unambiguously the relative rates of rRNA synthesis occurring in the same lots of oocytes from which the nucleosomes themselves were to be derived.

Figure 1 shows the electrophoretic profiles of ribosomal RNA isolated from various *Xenopus* oocyte size classes which were labeled in culture with medium [^{3}H]uridine. Relative rates of incorporation of radioactivity into rRNA in different oocyte stages were determined in time course experiments in which the labeled rRNA for each time point was extracted and separated by electrophoresis on gels similar to those in this figure and the radioactive rRNA (cross-hatched areas shown in Figure 1) isolated and counted. The resulting rates of incorporation of [^{3}H]uridine into rRNA are plotted for each size class of oocytes in Figure 2. It is seen that for all of the oocyte classes the kinetics of incorporation is of a generally similar type following a parabolic curve of the type $I = (R\alpha/2)t^2$ (see below).

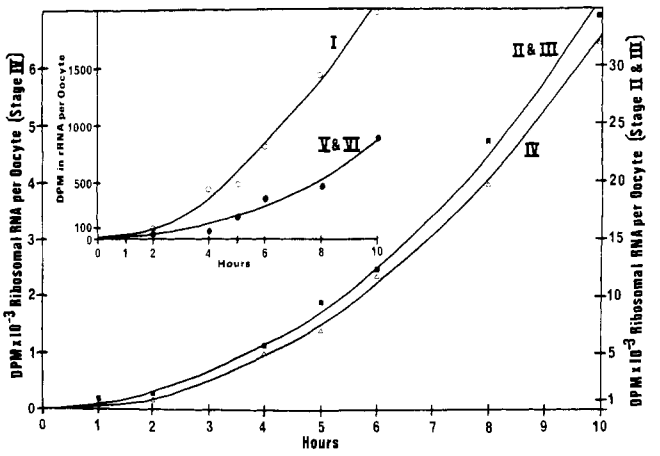

FIGURE 2: Kinetics of incorporation of radioactivity into electrophoretically purified ribosomal RNA by various isolated size classes of oocytes as explained in Figure 1 and in Materials and Methods. Each point represents the mean value of four separate determinations. The size classes of oocytes are labeled with Roman numerals (as in Figure 1 and in the text). All radioactive counts have been corrected for background and efficiency of counting variations. Note that different ordinates are used for different oocyte classes. All of these incorporation curves are parabolas of the type $I = (R\alpha/2)t^2$ as explained in the text and were fit to the data by least-squares analysis.

In order to make meaningful comparisons of the rates of rRNA synthesis between the various oocyte classes, the specific activity of the UTP precursor nucleotide pools within each class must be determined since it is known that the relative uptake of exogenous isotopes varies with different oocyte developmental stages (La Marca et al., 1973; Scheer et al., 1976). These changes could markedly alter the apparent rates of rRNA synthesis as determined by incorporation data alone (Figure 1). Therefore, a series of experiments was performed to determine UTP specific activities within the oocytes. In the same experiments, the various oocyte ATP pool sizes were also determined.

Oocyte UTP Pools. Incorporation experiments (data not shown) indicated that the rate of uptake of exogenously supplied [^{3}H]uridine into total acid-soluble material was essentially linear with time for all of the oocyte stages tested. Consequently, during the time course of the labeling periods, equilibrium conditions were not attained and the precursor UTP pools could not be saturated with exogenously supplied uridine. It was therefore obligatory to determine changes in the amounts of radioactivity incorporated into [^{3}H]UTP during the period of labeling and then to relate these to the oocyte pools of "cold" UTP in order to determine the kinetics of changing specific activities of the cellular UTP pools with time. Therefore, the amount of [^{3}H]UTP within each oocyte class at each labeling time point was determined by PEI-cellulose thin-layer chromatography as described in Materials and Methods.

Next, the size of the cellular UTP pool within each oocyte size class was determined using the *E. coli* RNA polymerase assay described in Materials and Methods in which either the UTP or the ATP present within an oocyte extract was made the limiting component in the enzyme catalyzed production

TABLE I: Oocyte Nucleotide Pool Sizes and Relative Rates of Ribosomal RNA Synthesis.

stage[a]	diameter (μM)	UTP pool[b] (pmol)	ATP pool[b] (pmol)	range of rates of rRNA synth (ng/(h oocyte))	av rate[c] of rRNA synth (ng/(h oocyte))	rel rate of rRNA synth[d] (%)	amount of nucleosome protection (%)[e]
I	50–300	1.05	8.4	0.23–0.4	0.315	5.1	84
II & III	300–600	37.0	114	3.62–8.7	6.16	100	37
IV	600–1000	174	504	3.45–7.92	5.69	92.3	48
V	1000–1200	317	634	2.80–5.13[f]	4.06[f]	65.9[f]	57[f]
VI	1200–1300	306	650				

[a] Dumont, 1972. [b] Each value is the average of four determinations. [c] See text for calculations. [d] Rates are given as values relative to stage II and III oocytes. [e] Values from Table II. [f] These values are the average of stages V and VI together.

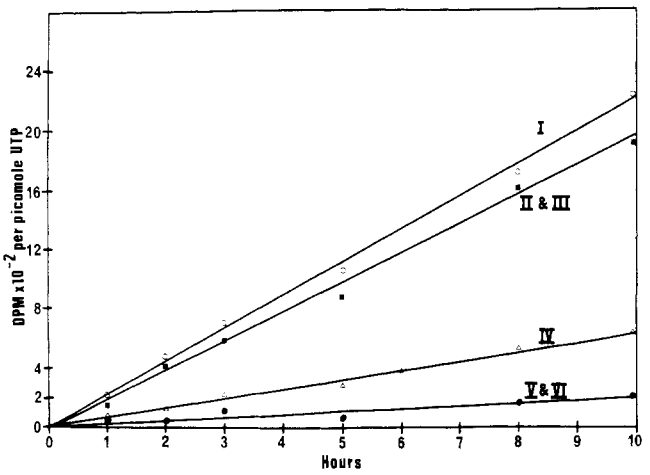

FIGURE 3: Specific activities of UTP pools of various oocyte size classes. The size classes of oocytes (labeled with Roman numerals) were labeled by growth in culture in 2 mCi/mL of [^{3}H]uridine for the times indicated on the abscissa and then the nucleotide pools extracted and the UTP nucleotides separated by thin-layer chromatography as described in Materials and Methods. The amount of radioactivity in the purified UTP was then determined and divided by the size of the cellular UTP pools (as determined in Materials and Methods) to give the oocyte specific activities. All counts have been corrected for background and variations in efficiency of counting. In all experiments the lines were fit to the data by least-squares analysis. Each point is the average of four determinations.

of a ribonucleotide polymer using poly(dA-dT) as a template.

Table I gives the average values for both UTP and ATP pool sizes found within the various oocyte size classes using this method. These data for stage V and VI oocytes are in good agreement with the values given for the UTP and ATP pools of *Xenopus* eggs and "large oocytes" by Woodland & Pestell (1972).

By combining the information obtained on uptake of [^{3}H]uridine into cellular [^{3}H]UTP for each oocyte stage with the average values determined for each oocyte UTP pool size, curves for the changes in rates of specific activity of the cellular UTP pools were determined. These curves are shown in Figure 3 with the Roman numerals representing the various oocyte size classes. It is readily apparent from this figure that in all of the size classes the UTP pool specific activity (dpm per picomole of UTP) increases linearly with time and never reaches an equilibrium condition. In the calculation for these curves the amounts of exogenous [^{3}H]uridine taken up by each oocyte class into their intracellular nucleotide pools is very small compared with the size of the cellular UTP pools themselves and have therefore been ignored. For example, stage I oocytes are seen to possess the highest specific activity of their intracellular UTP pools at all time points (Figure 3), and yet the actual amount of exogenous [^{3}H]uridine taken up into their cellular pools after 10 h of labeling would only expand their intracellular UTP pool by about 0.025%. In all other classes of oocytes the increase is even less than this. Therefore, for the calculations in Figure 3, overall pool size increases during the labeling period were ignored.

Rates of Ribosomal RNA Synthesis. For determination of rates of rRNA synthesis in the various oocyte classes, a number of assumptions must be made. The most important of these is that the total cellular UTP pool of an oocyte (as determined above) is the direct precursor for rRNA synthesis and that compartmentalization does not play a role in regulating overall RNA synthesis. For oocytes and eggs of *Xenopus*, compartmentalization does not seem to be an important consideration for the total cellular nucleotide pool probably acts as a direct precursor for all RNA synthesis within an oocyte (Brown & Littna, 1964; La Marca et al., 1973; Knowland, 1970; Knowland & Miller, 1970; Coleman, 1974). Assuming this to be true for the oocytes studied here, rates of rRNA synthesis can be calculated in the following manner from the given data (cf. La Marca et al., 1973). For a constant cellular UTP specific activity (S), the actual rate of incorporation (R) of labeled precursor into RNA can be calculated from the observed incorporation (I) according to the equation:

$$dI/dt = R \cdot S \quad (1)$$

where t is time in hours. However, as observed in Figure 5, the specific activities (S) of the various oocyte UTP pools are not constant but are continually increasing at fixed rates over the labeling period. Thus, the linear increase with time of the UTP pool specific activities can be represented by the expression:

$$S = \alpha t \quad (2)$$

where α is a constant representing the slope of the line fit to the specific activity data given in Figure 3. By substituting eq 2 into eq 1, followed by integration and evaluation at $t = 0$, the following equation for a parabola is obtained:

$$I = (R\alpha/2)t^2 \quad (3)$$

Figure 2 shows that the parabola described by this equation fits reasonably well to the kinetics of incorporation of [^{3}H]-uridine into rRNA in all of the size classes of oocytes investigated.

Using the observed incorporation data (I) of Figure 2, the actual rates of rRNA synthesis (R) in the various oocyte classes were calculated from the incorporation constants ($R\alpha/2$) of the curves which best fit the data, and the slopes (α) of the lines fit to the specific activity data from Figure 3.

Table I gives the average values and range of values determined in four separate experiments for the rates of rRNA synthesis (ng/(h oocyte)) found in the oocyte classes used for hybridization experiments. In making these calculations it was

assumed that *Xenopus* ribosomal RNA has an average GMP content of about 32% (Birnstiel et al., 1971).

From Table I it is seen that the synthesis of rRNA varies about 20-fold in rate during oogenesis even though the absolute number of genes per oocyte remains constant during the same time period. For example, previtellogenic stage I oocytes synthesize rRNA at a low rate (0.315 ng/(h oocyte)) compared with the maximally active stage II and III oocytes which synthesize rRNA at about 6.2 ng/(h oocyte). By the end of oogenesis (stages V and VI) the actual rate of synthesis has dropped to around 4.1 ng/(h oocyte) (or about 65% of the maximal level) but has not ceased completely. These rates are within reasonable range of those of others (La Marca et al., 1973; Denis, 1974; Scheer et al., 1976; Davidson, 1976; Anderson & Smith, 1977) but are at variance, in part, with those of Brown & Littna (1964) and Mairy & Denis (1971). Possible reasons for this discrepancy have been discussed previously by La Marca et al. (1973) and others (Hallberg & Smith, 1976). The relative rates of rRNA synthesis (compared with stages II and III) are also shown in Table I for various oocyte classes.

In connection with the rates of synthesis shown in Table I, it should be emphasized that the newly synthesized ribosomal RNAs found in *Xenopus* oocytes are quite stable molecules and accumulate within growing oocytes to very high levels (about 3.2 μg per large stage VI oocyte; Scheer et al., 1976) with very little turnover or degradation detectable during short time intervals (Brown & Littna, 1964; Gurdon, 1968, 1974; Davidson, 1976). For example, it has recently been estimated that the rRNA in stage VI oocytes has a minimum half-life of 144 days (Anderson & Smith, 1977). Thus, it would seem quite likely that the levels of incorporation of isotopes into rRNAs of the various size classes of oocytes reported on here represent true rates of de novo transcriptional activity. However, the data do not allow for a distinction to be made between controls of RNA synthesis operating at either the initiation level or the elongation level of polymer formation, although it has been argued elsewhere that varying rates of transcription in these oocytes are a result of varying rates of initiation of gene transcription (Scheer et al., 1976).

The Nucleosome Structure of Amplified Ribosomal Genes. Under the micrococcal nuclease digestion conditions used in this study (100 units/mL, 37 °C, substrate excess), after 4 min of incubation virtually all of the chromatin from each of the oocyte stages investigated was present in solution as monomer nucleosome fragments (containing about 200 base pairs of DNA) with little of the chromatin (less than 10% of the amount present in the monomer peak) being present either as dimer and larger nucleosomes or as "core nucleosome" fragments containing the limit digest 140 base pair lengths of double-stranded DNA. These results indicate that in all stages of oocytes the nuclear chromatin has an increased sensitivity to digestion by micrococcal nuclease compared with the nuclear chromatin of *Xenopus* embryonic and somatic cells (Reeves & Jones, 1976; Reeves, 1976, 1977a). However, preliminary experiments done to try and detect the possible presence of a "rapidly digested, protein-free" rDNA population of gene sequences within these oocytes by analysis of the initial rates of hydrolysis of oocyte chromatins by micrococcal nuclease failed to detect such sequences (data not shown). These results are therefore consistent with the findings of Sollner-Webb et al. (1976) that, under the digestion conditions employed (i.e., low salt and substrate saturation of enzyme), the kinetic constants describing the action of micrococcal nuclease on chromatin and naked DNA are such that mixtures of chromatin and free DNA will be digested at approximately

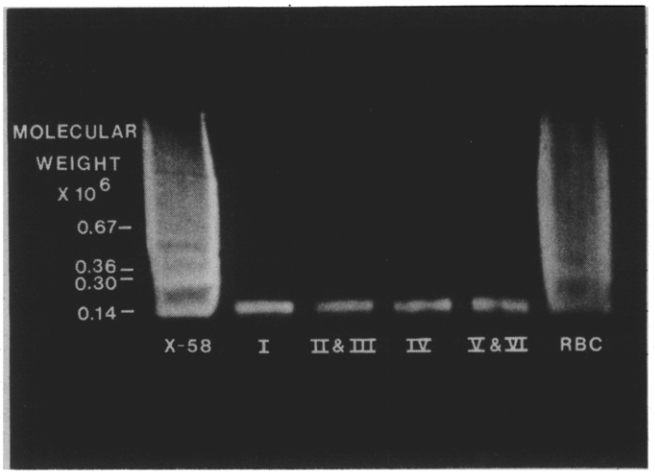

FIGURE 4: Patterns obtained when monomer nucleosome double-stranded DNA fragments from various oocyte classes were electrophoresed on a 1.4% agarose slab gel, stained with ethidium bromide, and photographed under ultraviolet light. Nuclease digestion conditions were given in Materials and Method. Each of these samples was digested for 4 min at 37 °C before DNA extraction. All of the samples of oocyte DNA (shown labeled with Roman numerals) were previously purified by agarose gel electrophoresis to give monomer nucleosome DNA as described in Materials and Methods. The marker DNAs (with apparent molecular weights calibrated with SV40 restriction endonuclease fragments) from X58 tissue culture cells and adult red blood cells were electrophoresed on either side of the oocyte DNAs for size comparisons. These marker DNAs were not previously purified and therefore contain fragments of monomer, dimer, trimer, and larger nucleosome DNAs. The purified oocyte DNAs from which the samples shown here were taken were used for hybridization studies.

equal rates. Thus, experiments which merely test the initial rates of digestion of the oocyte rDNA sequences under the described conditions will not distinguish between chromatin and free DNA (Sollner-Webb et al., 1977). For these reasons, the crucial observations with micrococcal nuclease are the appearance of the transcriptionally active gene sequences in nucleosome-like repeats (Felsenfeld, 1978).

Therefore, monomer nucleosome DNA was isolated by short-term micrococcal nuclease digestion from the various oocyte stages for which the above rates of rRNA synthesis had been determined as described in Materials and Methods. Figure 4 shows the profiles seen under ultraviolet light of these purified nucleosome DNA populations stained with ethidium bromide after electrophoresis on an agarose gel. DNA molecular weight markers obtained from control tissues flank the purified oocyte nucleosome DNAs in this photograph.

As seen in Figure 4, the purified monomer nucleosome DNA fragments obtained from different oocyte size classes are all about the same size as the monomer nucleosome marker fragments obtained from *Xenopus* somatic cells (i.e., about 200 base pairs in length) regardless of the rate of rRNA synthesis occurring in the oocyte. This is of some interest since recently it has become clear that the amount of DNA per nucleosome histone octamer can vary between about 140 and 240 base pairs, depending upon the organism and tissue from which the nucleosomes are isolated (Morris, 1976; Lipps & Morris, 1977; Spadafora et al., 1976; Compton et al., 1976; Noll, 1976; Lohr et al., 1977; Thomas & Thompson, 1977)); even within a single cell type, the spacing between nucleosomes may not be homogeneous (Johnson et al., 1976) and may change with the state of differentiation of a particular cell (Weintraub, 1978). Whether such variation exists for dimer and higher order oligomeric nucleosomes in the *Xenopus* oocyte ribosomal genes is not known but it is evident from Figure 4 that the monomer nucleosomes obtained from all oocyte

TABLE II: Saturation Hybridization of Oocyte Germinal Vesicle DNA with ^{3}H-Labeled Ribosomal RNA.[a]

DNA[b]	amount of DNA (%) hybridized by: 18S + 28S[c]	18S[c]	28S[c]	rDNA as percentage of germ. vesicle rDNA	no. of nucleolar equivalents[d] of rDNA per oocyte
stage II & III, undigested	7.76	2.48	4.89	100	
stage V & VI, undigested	7.68	2.46	5.07	100	
av oocyte, undigested	7.72	2.47	4.98	100	1616
stage I, monosome	6.48	2.27	4.25	83.9	1357
stage II & III, monosome	2.85	0.94	1.82	36.9	596
stage IV, monosome	3.71	1.29	2.53	48.1	755
stage V & VI, monosome	4.41	1.37	2.95	57.1	923

[a] Saturation values determined by a double-reciprocal plot method. [b] Stages of Dumont, 1972. [c] Each value is the average of three separate experiments. [d] Calculations based on the following assumptions: (1) each germinal vesicle (nucleus) contains about 25 pg of extrachromosomal rDNA and about 12 pg of chromosomal DNA (Gurdon, 1974); (2) each haploid somatic cell nucleus contains about 3.1 pg of DNA (Dawid, 1965); (3) somatic cells contain 0.057% of their DNA complementary to rRNA (Brown & Weber, 1968); (4) 28S RNA has a molecular weight of 1.5×10^6 (Birnsteil et al., 1971); (5) the given values for the amount of DNA hybridized (%) to rRNA represent only one-half of the mass of total rDNA (not including transcribed and nontranscribed spacer DNAs) present in the nuclear preparations; and (6) a weight of nuclease derived monomer nucleosome DNA equal to the weight of whole DNA in a normal stage II and III oocyte nucleus is a germinal vesicle equivalent.

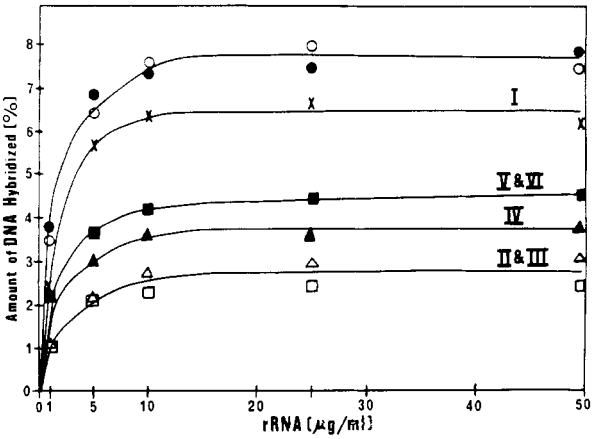

FIGURE 5: Saturation hybridization curves for electrophoretically purified ^{3}H-labeled (18S + 28S) RNA annealed to DNA from various oocyte stages. Symbols: (●) sheared nuclear DNA from stage II and III oocytes (Dumont, 1972); (○) sheared nuclear DNA from stage V and VI oocytes; (X) monomer nucleosome DNA from stage I oocyte nuclei; (■) monomer nucleosome DNA from stage V and VI oocyte nuclei; (▲) monomer nucleosome DNA from stage IV oocyte nuclei; (△ and □) monomer nucleosome DNA from stage II and III oocyte nuclei taken from two entirely independent experiments. Each point shown represents the average of three separate determinations. The apparent asymptotic plateaux of these saturation curves were determined by a double-reciprocal plot method.

stages are of approximately similar lengths (about 200 base pairs).

When purified radioisotopically labeled ribosomal RNA (18S+ 28S) was annealed to the isolated monomer nucleosome DNAs from various size classes of oocytes to the point of saturation of the DNA with the complementary RNAs, the results shown in Figure 5 were obtained. The RNA·DNA saturation hybridization values for each of the oocyte stages are also tabulated in Table II.

From these data it is clear that in stage I previtellogenic oocytes (with relatively low rates of rRNA transcription) most of the extrachromosomal rDNA sequences (about 85%) are protected from nuclease digestion by association with nucleosomes. On the other hand, in stage II and III oocytes, which are maximally active in transcription of rRNA, the amount of nucleosome protection has dropped to around 37% (one experiment showed 35% protection and another 40%; Figure 5) of the amount of rDNA found in control stages II and III not exposed to nuclease. In these experiments the saturation hybridization values are considered to reflect only the condition of the amplified rDNA since the chromosomal ribosomal genes represent only about 0.09% of all of the hybridizable rDNA. It is therefore significant that in these maximally active cells a sizable portion of the amplified rDNA was still associated with histones in the form of nuclease resistant nucleosomes.

However, this association of nucleosomes (histones) with DNA appears to be in a rather labile state since it was observed that, near the end of oogenesis, stage V and VI oocytes (which have considerably reduced rates of rRNA synthesis (compared with the stage II and III oocytes) and again have increased amounts of nucleosome-protected DNA in their nuclei. These results suggest that the accessibility of oocyte ribosomal genes to micrococcal nuclease may vary with the rates of transcription from these gene sequences.

Discussion

It seems to be generally accepted at the present time that micrococcal nuclease, compared with other nucleases such as pancreatic DNase I or splenic DNase II, makes minimal distinction between transcriptionally active and inactive gene sequences during its hydrolysis of nuclear chromatin (Elgin & Weintraub, 1975; Kornberg, 1977; Felsenfeld, 1978). However, a number of reports have indicated that this rather nonspecific endonuclease can, under certain digestion conditions and with particular genes, partially hydrolyze some transcriptionally active gene sequences (Panet & Cedar, 1977; Bellard et al., 1977). This increased susceptibility of active genes to partial hydrolysis is particularly evident for the somatic cell ribosomal genes of *Xenopus* embryos (Reeves, 1976, 1977a,b) and for the ribosomal gene of the slime mold *Physarum* (Allfrey et al., 1977; Johnson et al., 1978).

In the case of *Xenopus* embryonic cells, the extent of susceptibility of the ribosomal genes to micrococcal nuclease digestion seems to increase as the ribosomal RNA transcription rates increase (Reeves, 1976, 1977a). However, it was found that, even in the maximally active embryonic somatic cells, a clear majority of the reiterated ribosomal genes were still protected from digestion by association with nucleosomes suggesting that not all nucleosomes (histones) were removed from transcriptionally active chromatin (Reeves, 1976).

The results reported here for the amplified ribosomal genes of *Xenopus* oocytes engaged in varying rates of synthetic activity support the earlier evidence obtained from somatic em-

bryonic cells indicating a relationship between micrococcal nuclease accessibility of ribosomal genes and the degree of their involvement in RNA synthesis.

Two alternative hypotheses, neither of which can be eliminated by present data, can be advanced as possible explanations for the increased sensitivity of active ribosomal genes to micrococcal nuclease digestion (1). The nuclease sensitive genes might be either partially, or perhaps transiently, devoid of normal nucleosome-like structures (and even perhaps histones). This alternative might allow for a minority of maximally active ribosomal genes to be entirely free of normal nucleosomes, as an extreme example (Reeves, 1977a,b), and would be consistent with the findings of Johnson et al. (1978) that there seems to be a deficiency in histones H3 and H4 on active rDNA chromatin fragments prepared by micrococcal nuclease digestion of *Physarum* nuclei. Such an explanation might also be compatible with electron micrographic studies indicating an apparent absence of normal beaded nucleosomes on very active rDNA chromatin (Scheer, 1978). (2) "Accessibility" to digestion might not necessarily signify the physical absence of histones (or even nucleosomes) from active DNA but rather it might indicate that some histones or nucleosomes are less firmly attached to the DNA than are other neighboring nucleosomes—perhaps due to conformational or biochemical changes affecting some or all of the nucleosomes in the active chromatin. Under this alternative, the data are not incompatible with the idea that almost all of the DNA within the nucleus may be associated with histones and nucleosomes at all times but it would then suggest that regional differences might exist along the length of some transcriptionally active genes. What the present data do seem to unambiguously rule out is the probability that there is a total absence of nucleosomes and histones from all of the extrachromosomal copies of the transcriptionally active ribosomal genes of *Xenopus* oocytes.

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References

Allfrey, V. G., Johnson, E. M., Sun, I. Y. C., Matthews, H. R., & Bradbury, M. (1977) *Cold Spring Harbor Symp. Quant. Biol. 42,* 505.

Anderson, D. M., & Smith, L. D. (1977) *Cell 11,* 663.

Bellard, M., Gannon, F., & Chambon, P. (1977) *Cold Spring Harbor Symp. Quant. Biol. 42,* 779.

Barth, L. G., & Barth, L. J. (1959) *J. Embryol. Exp. Morphol. 7,* 210.

Birnstiel, M. L., Chipchase, M., & Speirs, J. (1971) *Prog. Nucleic Acid Res. Mol. Biol. 11,* 351.

Brown, D. D. (1967) *Curr. Top. Dev. Biol. 2,* 47.

Brown, D. D., & Dawid, I. (1968) *Science 160,* 272.

Brown, D. D., & Littna, E. (1964) *J. Mol. Biol. 8,* 688.

Brown, D. D., & Littna, E. (1966) *J. Mol. Biol. 20,* 81.

Brown, D. D.., & Weber, C. S. (1968) *J. Mol. Biol. 34,* 661.

Burton, K. (1968) *Methods Enzymol. 12B,* 163.

Burzio, L. O., & Koide, S. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37,* 1640.

Coleman, A. (1974) *J. Embryol. Exp. Morphol. 32,* 515.

Compton, J. L., Bellard, M., & Chambon, P. (1976) *Proc. Natl. Acad. Sci., U.S.A. 73,* 4382.

Davidson, E. (1976) *Gene Activity in Early Development,* p 464, Academic Press, New York, N.Y.

Dawid, I. (1965) *J. Mol. Biol. 12,* 581.

Denis, H. (1974) in *Biochemistry of Cell Differentiation* (Paul, J., Ed.), p 95, Butterworths and Co., London.

Dumont, J. N. (1972) *J. Morphol. 136,* 153.

Elgin, S., & Weintraub, H. (1975) *Annu. Rev. Biochem. 44,* 725.

Felsenfeld, G. (1978) *Nature (London) 271,* 115.

Ford, P. (1972) in *Oogenesis* (Biggers, J. D., & Schuetz, A. W., Eds.), p 167, Butterworths and Co., London.

Ford, C. C., & Gurdon, J. B. (1977) *J. Embryol. Exp. Morphol. 37,* 203.

Gall, J. (1968) *Proc. Natl. Acad. Sci. U.S.A. 60,* 553.

Gall, J. (1969) *Genetics, Suppl. 61,* 121.

Gillespie, D. (1968) *Methods Enzymol. 12B,* 641.

Gorovsky, M., Mathis, D., Glover, C., Johmann, C., Samuelson, M., & Keevert, J. (1977) *Cold Spring Harbor Symp. Quant. Biol. 42,* 493.

Gottesfeld, J. M., & Melton, D. A. (1978) *Nature (London) 273,* 317.

Gurdon, J. B. (1968) *Essays Biochem. 4,* 25.

Gurdon, J. B. (1974) *The Control of Gene Activity in Animal Development,* p 166, Harvard University Press, Cambridge, Mass.

Hallberg, R. L., & Smith, D. C. (1976) *Dev. Biol. 42,* 40.

Higashinakagawa, T., Wahn, H., & Reeder, R. H. (1977) *Dev. Biol. 55,* 375.

Hourcade, D., Dressler, D., & Wolfson, J. (1973) *Cold Spring Harbor Symp. Quant. Biol. 38,* 537.

Johnson, E. M., Littau, V. C., Allfrey, V. G., Bradbury, E. M., & Matthews, H. R. (1976) *Nucleic Acids Res. 3,* 3313.

Johnson, E. M., Allfrey, V. G., Bradbury, E. M., & Matthews, H. R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75,* 1116.

Kornberg, R. D. (1977) *Annu. Rev. Biochem. 46,* 931.

Knowland, J. S. (1970) *Biochim. Biophys. Acta 204,* 416.

Knowland, J. S., & Miller, L. (1970) *J. Mol. Biol. 53,* 321.

LaMarca, M. J., Smith, L. D., & Strobel, M. C. (1973) *Dev. Biol. 34,* 106.

Leonard, D. A., & La Marca, M. J. (1975) *Dev. Biol. 45,* 199.

Lipps, H. J., & Morris, N. R. (1977) *Biochem. Biophys. Res. Commun. 74,* 230.

Lohr, D., Tatchell, K., & Van Holde, K. E. (1977) *Cell 12,* 829.

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

Mairy, M., & Denis, H. (1971) *Dev. Biol. 24,* 143.

Mathis, D. J., & Gorovsky, M. A. (1976) *Biochemistry 15,* 750.

Matsui, S., & Busch, H. (1977) *Exp. Cell Res. 109,* 151.

Miller, L., & Knowland, J. S. (1970) *J. Mol. Biol. 53,* 329.

Morris, N. R. (1976) *Cell 8,* 357.

Noll, M. (1976) *Cell 8,* 349.

Panet, A., & Cedar, H. (1977) *Cell 11,* 933.

Perkowska, E., Macgregor, H. C., & Birnstiel, M. L. (1968) *Nature (London) 217,* 649.

Pestell, R. Q. W. (1975) *Biochem. J. 145,* 527.

Piper, P. W., Celis, J., Kaltoft, K., Leer, J. C., Nielson, O. F., & Westergaard, O. (1976) *Nucleic Acids Res. 3,* 493.

Randerath, K., & Randerath, E. (1968) *Methods Enzymol. 12A,* 323.

Reeder, R. H. (1974) in *Ribosomes* (Nomura, M., Tissieres, A., & Lengyel, P. Eds.) p 489, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Reeves, R. (1976) *Science 194,* 529.

Reeves, R. (1977a) *Eur. J. Biochem. 75,* 545.

Reeves, R. (1977b) *Cold Spring Harbor Symp. Quant. Biol.*

42, 709.
Reeves, R., & Jones, A. (1976) *Nature (London) 250,* 495.
Reeves, R., & Laskey, R. A. (1975) *J. Embryol. Exp. Morphol. 34,* 75.
Sasvary-Szekely, M., Vitez, M., Staub, M., & Antoni, F. (1975) *Biochim. Biophys. Acta 395,* 221.
Scheer, U. (1978) *Cell 13,* 535.
Scheer, U., Trendelenburg, M., & Franke, W. (1976) *J. Cell Biol. 69,* 465.
Sollner-Webb, B., Camerini-Otero, R. D., & Felsenfeld, G. (1976) *Cell 9,* 179.
Spadafora, C., Bellard, M., Compton, J. L., & Chambon, P. (1976) *FEBS Lett. 69,* 281.
Thomas, J. O., & Thompson, R. J. (1977) *Cell 10,* 633.
Weintraub, H. (1978) *Nucleic Acids Res. 5,* 1179.
Wellauer, P., Reeder, R., Carroll, D., Brown, D., Deutch, A., Higashinakagawa, T., & Dawid, I. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71,* 2832.
Woodland, H., & Pestell, R. Q. W. (1972) *Biochem. J. 127,* 597.

Ambivalent Active-Site-Directed Inactivators of Liver Alcohol Dehydrogenase†

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ABSTRACT: A series of ω-(bromoacetamido) fatty acids [$BrCH_2CONH(CH_2)_nCOOH$] with n from 6 to 11, 4-(*p*-bromoacetamidophenyl)butyric acid and its amide, 4-(*p*-bromoacetamidophenyl)benzoic acid, and 4-(*p*-bromoacetamidophenoxy)benzoic acid were synthesized and evaluated as active-site-directed inactivators of horse liver alcohol dehydrogenase. These reagents were designed from a knowledge of the structure of the apoenzyme determined by X-ray crystallography so that their carboxylate or carboxamide groups could bind to the catalytic zinc ion in the enzyme–coenzyme complex while their bromoacetamido groups alkylated Met-306 14 Å away in the substrate-binding pocket, inactivating the enzyme by anchoring an inhibitor in the active site. In the absence of coenzyme, the carboxylate reagents could bind to the anion-binding site formed by Arg-47 and Lys-228 and alkylate Cys-46 or -174, ligands to the zinc. In the absence of nucleotides, all of the reagents inactivated the dehydrogenase as fast or faster than bromoacetic acid did (8.2 M^{-1} min^{-1} at pH 8, 25 °C). AMP, NAD^+, and NADH generally protected against inactivation, but 8-(bromoacetamido)octanoic acid and 4-(*p*-bromoacetamidophenyl)butyric acid inactivated as fast or faster in the presence of 1 mM NAD^+ as in the absence of nucleotide. In the presence of 0.2 mM NADH, 4-(*p*-bromoacetamidophenyl)butyramide inactivated with a pseudo bimolecular rate constant (400 M^{-1} min^{-1}) that was eight times larger than the rate constant in the absence of nucleotides and 67 000 times larger than the bimolecular rate constant for inactivation by bromoacetamide in the presence of NADH (0.006 M^{-1} min^{-1}). All of the reagents exhibited saturation kinetics, and the presence of coenzyme increased the binding of reagent by up to twofold. Amino acid analyses showed that enzyme inactivated by 4-(*p*-bromoacetamidophenyl)butyric acid or its amide in the absence of coenzyme contained one or more modified cysteine residues per subunit, whereas inactivation in the presence of NAD^+ or NADH modified about one methionine but no cysteine. These reagents react more specifically than bromoacetic acid (in the presence of NAD^+) or bromoacetamide (in the presence of NADH), which modify several cysteines and methionines during inactivation. It appears that 4-(*p*-bromoacetamidophenyl)butyric acid and its amide are ambivalent active-site-directed inactivators whose reaction specificity is determined by the coenzyme, as predicted above. Furthermore, it appears that the distance between the catalytic zinc and the sulfur of Met-306 is about 14 Å in both the apoenzyme and the enzyme–coenzyme complexes.

A variety of active-site-directed reagents have been used to investigate the structures and mechanisms of enzymes (Shaw, 1970; Jakoby & Wilchek, 1977). Baker (1967) has proposed that such reagents can be designed for chemotherapeutic purposes. Specific inactivators of liver alcohol dehydrogenase might be used to inhibit alcohol metabolism, for instance in treatment of methanol intoxication (Plapp, 1975). This enzyme can be inactivated by a variety of chemical reagents (Brändén et al., 1975). For instance, the sulfur atom of Cys-46 (a ligand to the zinc in the active site) reacts with iodoacetate (Harris, 1964; Li & Vallee, 1964; Jörnvall, 1970), which apparently is attracted to the active site by interaction with the guanidinium group of Arg-47 (Zeppezauer et al., 1975; Brändén et al., 1975). The ionic interaction facilitates the rate of reaction about tenfold, as evidenced by the slower reaction after arginine residues are modified (Lange et al., 1975) or when bromoacetamide is substituted for bromoacetate (Fries et al., 1975). It should also be noted that the rate of inactivation by haloacetates or haloacetamides is decreased about 100-fold by NAD^+ [1] or NADH (Fries et al., 1975; Li & Vallee, 1963; Reynolds et al., 1970), whose pyrophosphate groups apparently bind to Arg-47 and block access of the reagent to Cys-46 (Zeppezauer et al., 1975; Lange et al., 1975; Brändén et al., 1975). The anionic reagent diazonium-1*H*-

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[1] Abbreviations used: NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD; EDTA, (ethylenedinitrilo)tetraacetic acid.

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