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Ribosomal Genes of *Xenopus laevis*: Evidence of Nucleosomes in Transcriptionally Active Chromatin

Abstract. *Most of the reiterated ribosomal genes in the somatic cells of larvae of a mutant of* Xenopus laevis *appear to be protected from short-term nuclease digestion by being packaged in the form of chromatin subunits or nucleosomes. Since these mutant animals probably require all of their ribosomal genes to be active in order to maintain viability, at least some of the transcriptionally active gene sequences are probably associated with chromatin subunits. Thus, association of DNA with nucleosomes may not necessarily preclude template activity, although such association is probably of a dynamic rather than a static nature.*

Evidence from a wide variety of sources (*1*) indicates that most of the chromatin of higher eukaryotes is packaged into repeating arrays of globular subunits consisting of double-stranded DNA associated with histones separated by stretches of DNA not packaged in this globular form. A model for these fundamental chromatin particles, also called nucleosomes (*2*) or nu-bodies (*3*), has been proposed by Kornberg (*4*). In this model about 200 base pairs of DNA are arranged on the outside of a globular octamer of histones with the composition $(H4)_2(H3)_2(H2A)_2(H2B)_2$, formerly known as $(F2A1)_2(F3)_2(F2A2)_2(F2B)_2$. A similar nucleosome model has been proposed by Van Holde *et al.* (*5*). Most recent evidence has supported such an arrangement of histones and DNA (*6*) in both native (*7*) and reconstituted (*2, 8*) chromatin.

Indirect evidence suggests that transcriptionally active (as well as inactive) chromatin may be packaged, at least partially, into the nucleosome configuration (*9–19*). In all of the studies cited, however, the starting population of cells from which the chromatin subunits were released by nuclease digestion appeared to be heterogeneous with respect to genomic transcriptional activity. Thus, the nucleosomes derived from such mixed populations could well have been released from transcriptionally inactive gene sequences present within the starting population.

Here I present evidence from nucleic acid hybridization studies that a major fraction of the reiterated ribosomal genes in the somatic cells of a mutant of the amphibian *Xenopus laevis* is protected from short-term micrococcal nuclease digestion by being packaged into nucleosomes. To maintain viability, larvae heterozygous for this mutation (the anucleolate mutation) probably require all of their ribosomal cistrons to be synthetically active (*20, 21*), suggesting that transcriptionally active ribosomal DNA may be partially associated with histones in much the same manner as transcriptionally inactive chromatin (*9*). However, this association may be rather labile, as will be shown.

There are about 450 to 500 adjacent sets of ribosomal genes per haploid genome in wild-type *Xenopus laevis* (*22, 23*). This corresponds to about 0.2 percent of the nuclear DNA of diploid wild-type somatic cells (*24*). Each of these tandemly repeated ribosomal cistrons includes DNA which codes for a 40*S* RNA transcript that is a precursor to 28*S* and 18*S* ribosomal RNA (rRNA) as well as DNA for a nontranscribed "spacer" region of high deoxyguanylic and deoxycytidylic acid (G + C) content (*22, 24*). In diploid wild-type animals the 1000 or so cistrons are distributed equally between two nucleolar organizer regions located on homologous chromosomes (*25*). Such animals usually have two nucleoli per nucleus during early larval stages of development and are hence designated as +/+ nu (*26*).

In a certain strain of these animals there is a stable mutation, the anucleolate mutation, which involves the total deletion of the repeated ribosomal genes from the nucleolar organizer region of the chromosome (*22, 27*). Animals heterozygous for this mutation (0/+ nu individuals) have only one nucleolus per nucleus but are viable even though they have only half of the number of ribosomal genes (about 500) of the wild type. On the other hand, animals homozygous for the anucleolate mutation (0/0 nu individuals) have no detectable ribosomal DNA in their genomes (*24*), never synthesize any rRNA (*28*), and die at an early larval stage of development [stage 41 or 42 (*26*)].

In all embryos of *Xenopus* which have ribosomal cistrons in their genomes, rRNA synthesis commences only after the early gastrula stage of development has been reached [stage 10 or 11 (*29*), some 10 to 12 hours after fertilization (*30*)]. From this time onward the rate of synthesis appears to increase continuously until about the heartbeat stage of development when it begins to slow down and reaches a maximum plateau sometime around stages 42 to 45 (the feeding tadpole stage) of development (*26, 30*). By this stage of development the heterozygous +/0 nu animals are synthesizing rRNA at the same absolute rate as are +/+ nu wild-type animals (*21, 28*). This compensation in rates of synthesis by the heterozygotes can be explained either by assuming that wild-type animals have only about half of their cistrons transcriptionally active or by postulating that the +/0 nu individuals are synthesizing rRNA at twice the wild-type rate on all of their cistrons (*21*). In either case, all of the cistrons of the +/0 nu animals are probably transcriptionally active, because a reduction of the number of ribosomal cistrons below the heterozygous level (as shown in a series of partial deletion mutations) results in larval death (*20, 21*).

The nature of the structure within transcriptionally active chromatin was investigated by means of two types of experiments: (i) Radioactive rRNA was hybridized to purified monomer nucleosome (monosome) DNA fragments (about 200 base pairs in length) derived from micrococcal nuclease-treated nuclei obtained from heterozygous +/0 nu tadpoles (stage 42 or 46) engaged in maximal rates of rRNA synthesis. (ii) Similar saturation hybridization experiments were conducted with monosome DNA fragments from early embryonic stages just before (blastula, stage 8 or 9) and just after (neurula, stages 16 to 18) the onset of rRNA synthesis. In the latter experiments, all of the animals resulting from +/0 nu × +/0 nu matings were used because of the difficulty in identifying the

Table 1. Saturation hybridization of +/0 nu DNA from stage-46 larvae with ^{32}P-labeled rRNA. The specific activity of ^{32}P-labeled rRNA (electrophoretically purified) was 1.5×10^6 count/min per microgram of RNA. The ratio of RNA to DNA is expressed as micrograms of both substances × 100, plus or minus the standard deviation. Each value represents the average of three experiments.

RNA	Ratio of RNA to DNA	Average number of gene copies per genome*	Number of genes as percent of 0/+†
	DNA from +/0 nu mutant, undigested		
18S + 28S	0.037 ± 0.004	591 ± 64	100
28S	0.022 ± 0.005	518 ± 120	88
18S	0.011 ± 0.003	526 ± 143	90
	DNA from +/0 nu mutant, monomer		
18S + 28S	0.022 ± 0.003	351 ± 48	60
28S	0.0124 ± 0.002	299 ± 48	51
18S	0.0068 ± 0.001	325 ± 47	55
	DNA from +/+ nu mutant, undigested		
18S + 28S	0.062 ± 0.006	990 ± 96	170

*Calculations are based on the assumption that each somatic diploid cell contains 6 pg of DNA (*23*) and that 28S RNA has a mass of 1.5×10^6 daltons and 18S RNA a mass of 0.75×10^6 daltons (*26*). †The assumption is made that a weight of nuclease-derived monomer DNA equivalent to the weight of the undigested DNA in a normal +/0 nu somatic cell is a genome equivalent.

number of nucleoli present within nuclei of cells from these early developmental stages. Thus pooled nuclei obtained from both blastula (stage 8 or 9) and neurula (stages 16 to 18) stages were mixtures of the genotypes +/+ nu, +/0 nu, and 0/0 nu in the ratios of 1 : 2 : 1. However, the resulting concentration of ribosomal cistrons in such mixed chromatin populations approximated the heterozygous +/0 nu level of 0.1 percent. Heterozygous embryos from tailbud and older stages were typed microscopically by the tail squash method (*31*).

In all experiments nuclei were isolated according to modified procedures of Destrée *et al.* (*32*). A low-salt buffer (2.4*M* sucrose; 5 m*M* tris-HCl, *p*H 7.8; 2 m*M* phenylmethyl sulfonylfluoride; 0.5 percent Triton X-100; 1 m*M* $MgCl_2$) was used to minimize the possibility of histone rearrangements on the chromatin (*15*). The nuclei were then treated with micrococcal nuclease (E.C. 3.1.4.7; Sigma) at 300 units per milliliter for 10 minutes at 37°C. The chromatin subunits released by the enzyme treatment were subsequently isolated and purified by sucrose gradient centrifugation and the DNA was recovered as previously described (*9*).

Figure 1 gives the results of saturation hybridization experiments with electrophoretically purified 18S and 28S rRNA annealed to DNA immobilized on nitrocellulose filters under the conditions of McConaughy *et al.* (*33*). The hybridization conditions chosen (0.3*M* NaCl, 0.030*M* trisodium citrate, *p*H 7.8; 50 percent formamide; 45°C) correspond to incubation at about 25°C below the T_m (that is, the temperature for 50 percent denaturation) of *Xenopus* RNA/DNA hybrids (*21*). Such criteria ensure both a high specificity of annealing and fast reaction rates (*9, 21*). All of the DNA-containing filters used to obtain the data in Fig. 1 were incubated with the same batch of electrophoretically purified ^{32}P-labeled rRNA obtained from cultured larval tissue cells of *Xenopus*. The nuclease-derived monomer DNA from heterozygous larvae (stage 46) reaches a saturation plateau when about 0.022 percent of the DNA is hybridized to rRNA. This level is only about 60 percent of the amount hybridized when undigested (sheared) +/0 nu DNA from stage-42 larvae reaches a plateau (Fig. 1 and Table 1). As previously discussed, by this stage of development all of the cistrons are probably transcriptionally active in the heterozygote. Thus in the extremes of interpretations, these hybridization data can be considered to mean either (i) that about 60 percent of all of the transcriptionally active cistrons are completely protected from nuclease digestion in the heterozygote chromatin, or (ii) that only about 60 percent of each of the reiterated cistrons is so protected. Various intermediated conditions between these two extremes seem more likely, however, given the apparent dynamic nature of the association of nuclease accessibility and genomic function.

Table 2. Saturation hybridization of DNA from early embryonic stages of +/0 nu × +/0 nu matings. The specific activity of the ^{32}P-labeled rRNA (electrophoretically purified) was 1.5×10^6 count/min per microgram of RNA. The ratio of RNA to DNA is expressed as micrograms of both substances × 100. Each result is the average of two experiments each involving four +/0 nu × +/0 nu matings.

RNA	Ratio of RNA to DNA	Number of genes as percent of undigested +/0 nu DNA*
	Monomer DNA from stages 8 to 9	
18S + 28S	0.030	81
28S	0.0195	79
18S	0.011	88
	Monomer DNA from stages 16 to 18	
18S + 28S	0.025	67.5
28S	0.017	69
18S	0.0088	71

*Assumptions are as in Table 1.

Also shown in Fig. 1 are saturation curves for 18S and 28S RNA annealed to monomer DNA's isolated from embryonic stages just prior to and just after the initiation of rRNA synthesis. The plateaus of saturation for both populations of subunit DNA's are higher than the plateau for stage-46 monomer DNA. None of these plateaus, however, attains the saturation level of undigested (sheared), stage-42 heterozygote DNA. The monomer DNA from mixed blastulas (stages 8 to 9) has a saturation level equivalent to about 0.30 percent of the DNA being hybridized, which corresponds to more than 80 percent of the undigested +/0 nu saturation level (Table 2).

It is of interest that this level of protection by nucleosomes in these transcriptionally inactive blastula cells approximates the level of subunit protection in synthetically quiescent erythrocytes from these animals (about 86 percent) (*9*). By the neurula stage of development (stages 16 to 18) transcription of rRNA is very active in all embryonic cells but has apparently not yet attained its maximum rate (*22, 30*). The percentage of monomer DNA from this stage that can be hybridized to rRNA is about 0.025 percent—again higher than the level of stage-46 larval monomer but less than the blastula level (Tables 1 and 2). The bottom curve in Fig. 1 represents a control for background levels of annealing with homozygous 0/0 nu DNA which lacks detectable ribosomal cistrons.

These results indicate an inverse correlation between the rates of rRNA synthesis within embryonic somatic cells and the degree of accessibility of the multiple ribosomal cistrons to nuclease digestion. Chromatin from blastula cells, inactive transcriptionally, has almost all of its ribosomal cistrons (80 percent) protected as nucleosomes. On the other hand, monomer DNA from stage-46 larvae engaged in maximum rates of syn-

thesis has only about 55 to 60 percent of its cistrons protected. In neurula cells with an apparent intermediate level of transcriptional activity, a corresponding intermediate level of nucleosome protection is evident. This correlation also holds for all of these developmental stages when monomer DNA is annealed separately to either purified 18*S* or purified 28*S* RNA (Tables 1 and 2). The DNA sequences coding for both 18*S* and 28*S* RNA seem to be equally susceptible to nuclease digestion in the various developmental stages studied and this accessibility seems to be related to the transcriptional activity of these stages.

It thus seems that a more dynamic relationship exists between genomic transcriptional activity and the occurrence of nucleosomes and histones in chromatin than has been generally recognized. For example, histones have long been considered nonspecific repressors of RNA synthesis in eukaryotic chromatin (*34*). This concept has led directly to the widely held notion that all DNA sequences associated with histones are transcriptionally quiescent and that only "open" or "accessible" DNA sequences completely free of histones are template active and are involved in genomic expression (*35*). This idea has been attractive because of its simplicity. However, much indirect evidence indicates that it may be an oversimplification of the true subtlety and complexity involved in gene regulation as it occurs in vivo (*9–19, 36*). The present report extends these findings by showing that at least 60 percent of the reiterated ribosomal cistrons present in whole, undigested DNA are found protected as nucleosomes in mutant cells in which all of the genes are probably engaged in rapid rates of RNA synthesis. This lends direct experimental support to the concept that transcriptionally active DNA may be associated, at least partially, with histones to form similar types of nucleosome subunits as are found in synthetically inactive chromatin.

Alternative explanations for these findings should also be considered, however. For example, it seems unlikely that histone rearrangements or degradation during the isolation procedures gave rise to these results since the salt concentration was very low and protease inhibitors were present in all of the isolation buffers (*15*). Also, control experiments in which isotopically labeled DNA was added to all fractions during isolation indicated that no more than about 5 percent of the histones became rearranged under the conditions used (*37*). Another possibility that could lead to spurious results is that proteins other than histones might protect the ribosomal DNA in nucleosome-like fashion as appears to be the case for the small basic core proteins of adenovirus (*38*). Three lines of evidence argue against this possibility for the *Xenopus* ribosomal genes: (i) There are few, if any, small basic proteins other than histones associated with *Xenopus* somatic cell chromatin (*37*). (ii) Experiments in which various basic proteins or polypeptides (protamine, spermidine, putrescine, poly-D-lysine) were added to isolated nuclei or chromatin showed that these proteins interfered with, rather than accentuated, the normal chromatin nuclease digestion pattern of nucleosomes (*37*). (iii) Purified isolated ribosomal DNA from *Xenopus* can be reconstituted with purified histones to give a chromatin with a nuclease sensitivity and nucleosome pattern similar to native chromatin (*10*).

In conclusion, it seems likely that nucleosome packing of DNA in chromatin does not, per se, preclude transcription of this template. Chromosomal protein–DNA interactions may participate in transcriptional control, and are undoubtedly of importance in determining how nucleosome-associated DNA is made accessible for transcription. It would thus be of interest to examine the proteins associated with nucleosomes derived from transcriptionally active chromatin (*12*). An investigation of the possible role of histone modifications and the nature of the nonhistone chromatin proteins associated with these subunits (*12*) might yield useful information about the biochemical mechanisms that control gene expression.

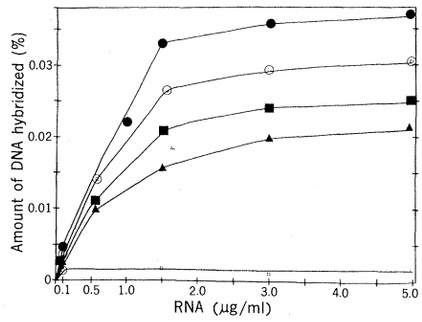

Fig. 1. Saturation hybridization profiles for electrophoretically purified ^{32}P-labeled 18*S* and 28*S* RNA annealed to DNA immobilized on nitrocellulose filters as described by Gillespie (*39*). The ^{32}P-labeled RNA was obtained from embryonic *X. laevis* tissue culture cells grown in low phosphate medium and purified as previously described (*9*). The specific activity of the RNA was 1.5×10^6 count/min per microgram of RNA. The hybridization procedures and criteria were essentially as described previously (*9*), where the annealing conditions (0.3*M* NaCl, .0.03*M* trisodium citrate, *p*H 7.0, 50 percent formamide, 45°C, 16 hours of incubation) allowed for reasonably fast rates of reaction while maintaining high hybrid specificity. The low-temperature formamide hybridization conditions were those described by McConaughy *et al.* (*33*). These incubation conditions corresponded to annealing at about 25°C below the T_m of the rRNA/DNA hybrids (*21, 33*). Symbols: ●, 0/+ nu sheared DNA (stage 42); ○, 0/+ × 0/+ nu monomer DNA (stage 8 or 9); ■, 0/+ nu × 0/+ nu monomer DNA (stages 16 to 18); ▲, 0/+ nu monomer DNA (stage 46); □, 0/0 nu sheared DNA (stage 40).

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Derepressed Alloantigen on Transplacentally Induced Lung Tumor Coded for by H-2 Linked Gene

Abstract. *A transplacentally induced lung tumor of strain C3Hf mice grows progressively when transplanted to (C3Hf × A)F_1 hybrid mice but not when transplanted to C3Hf recipients. Progressive tumor growth occurs in [(C3Hf × A)F_1 × C3Hf] backcross mice inheriting the H-2^a haplotype from the F_1 parent. Furthermore, radioresistant immunity to the lung tumor can be induced in C3Hf mice by immunization with normal tissue of B10.A and B10.A(2R) but not of B10 or B10.A(5R) strain mice.*

According to the concept of immune surveillance, a major function of the immune system is the detection and elimination of nascent tumors (*1*). This concept requires that nascent tumors express cell surface components distinguishable immunologically from components expressed on normal cells. Progressive growth of a tumor induced in an adult animal would reflect an escape from immune surveillance possibly because of a change in the tumor-associated surface antigens (TASA) expressed by the nascent tumor. The TASA on progressively growing tumors induced in adult animals might not be equivalent therefore to the TASA on nascent tumors subject to successful immune surveillance (*2*).

We have addressed this problem by analyzing the TASA in tumors induced transplacentally at a time prior to the maturation of immune competence. Fetal mice aged 13 to 17 days that are exposed transplacentally to the rapidly acting carcinogen 1-ethyl-1-nitrosourea (ENU) develop a high incidence of malignant lung tumors (*3*). Since microscopic lung tumors of mice treated transplacentally with the related carcinogen, urethane, are demonstrable at birth (*4*), it is likely that tumors induced by the more potent carcinogen ENU are also present at birth. The TASA on such tumors might therefore be recognized as self antigens by the maturing immune system. These TASA would, however, be considered as foreign antigens when the tumors were transplanted into syngeneic recipients. It is interesting, therefore, that a transplacentally induced lung tumor of a C3Hf mouse, designated 85, grows poorly when inoculated into C3Hf recipients but grows rapidly when inoculated into (C3Hf × A)F_1 hybrid recipients (*2*, *5*). The resistance of the C3Hf mice to 85 tumor growth is immunologically mediated and can be overcome by prior x-irradiation provided that the mice are not previously immunized against the lung tumor. The successful growth of lung tumor 85 in (C3Hf × A)F_1 hybrid mice is due to the expression on lung tumor 85 of a TASA which exists as a normal tissue component in strain A mice. Thus it is possible to elicit specific radioresistant immunity to lung tumor 85 in C3Hf mice by immunization with normal tissues of strain A mice (*2*). The tumor-associated alloantigen is not expressed in normal tissues of either C3Hf, C57BL/6, or DBA/2 mice (*2*) although, as will be documented elsewhere, at least 10 of 50 transplacentally induced lung tumors of these strains express the strain A–associated tissue alloantigen (*6*). Here, we report that the alloantigen expressed as a TASA on lung tumor 85 of C3Hf mice is coded for by a gene linked to the K end of the H-2 major histocompatibility complex.

The growth of lung tumor 85 in [(C3Hf × A)F_1 × C3Hf] backcross mice (*7*) was determined by inoculating the mice intradermally with 10^5 tumor cells. Of 48 backcross mice, progressive tumor growth (mean diameter in excess of 10 mm at 28 days) occurred in 23 mice,

Table 1. Residual H-2^a cytotoxicity of D-28 antiserum absorbed with spleen cells of [(C3Hf × A)F_1 × C3Hf] backcross mice tested for susceptibility to the growth of lung tumor 85. Spleens were removed from the two groups of mice and stored at −80°C. Each spleen was subsequently thawed and teased to yield a tissue suspension. The tissues were tested for their capacity to absorb cytotoxic activity from a 1 : 50 dilution of D-28 antiserum. The absorbed portions of antiserum were tested for residual cytotoxicity against spleen cells of strain A mice. The unabsorbed serum lysed an average of 88 percent of the spleen cells.

Source of spleen cells	No. of spleens tested	Residual cytotoxicity	
		Mean	Range
Tumor-susceptible backcross mice	23	17.4	9 to 41
Tumor-resistant backcross mice	25	86.2	78 to 91

Table 2. Radioresistant immunity to lung tumor 85 induced by immunization with normal tissue of allogeneic mice. In experiment A, C3Hf mice were immunized with lung tissue, while in experiment B, (C3Hf × DBA/2)F_1 mice were immunized with liver tissue from the donor strains.

Donor strain of tissue used for immunization	Tumor growth in x-irradiated mice	
	Proportion of mice with tumors	Mean tumor diameter (mm)
	Experiment A	
None	7/7	22.1
B10	13/13	21.7
B10.A	2/9	2.0
	Experiment B	
None	10/10	14.1
B10.A	0/6	0.0
B10.A(2R)	0/13	0.0
B10.A(5R)	12/12	16.3