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Simultaneous analysis of conformation and transcription of A and B groups of vitellogenin genes in male and female *Xenopus* during primary and secondary activation by estrogen

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ABSTRACT

In male Xenopus, primary estradiol administration results in non-coordinate activation in the liver of the A and B groups of vitellogenin genes, both as judged by transcription and DNase I sensitivity in isolated nuclei, B group genes being activated preferentially in the first 20 hr. Secondary induction in males or "primary" induction in females results in a coordinate and equal transcription of these two groups of genes. The elevated transcriptional activity following primary estrogen stimulation returns to low levels rapidly but the high DNase I sensitivity of these genes persists for 2-3 months. A non-coordinate activation of the A and B groups of vitellogenin genes is however re-established in response to a second administration of estradiol 8 months after primary stimulation of male Xenopus.

INTRODUCTION

The use of nucleases to probe the higher-order organization of genes [see ref. 1 for several reviews; 2-4] and the development of cell-free transcription systems of high fidelity [5-8], in combination with gene cloning techniques, have greatly enhanced our comprehension of gene expression during cellular differentiation. Thus, the higher sensitivity of active genes to digestion by DNase I and other nucleases has made it possible to discriminate between expressed and unexpressed genes. Although transcription in cell-free preparations has been studied for twenty years [9], it is only relatively recently that the accurate in vitro transcription of well-specified genes has been established [5]. A question crucial for further understanding of mechanisms regulating gene activity is whether or not an altered transcription of a given gene obligatorily accompanies an altered organization of that gene as determined by a change in its nuclease sensitivity.

Estrogen-induced vitellogenesis is a valuable model system for studying the regulation of gene expression, since the transcription of genes coding for the egg yolk precursor vitellogenin can be reversibly induced in the hepatocytes of male as well as female oviparous vertebrates [10,11]. The phenomenon

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by which a second exposure of the target cell to the hormone, after the vitellogenic response to the first exposure has disappeared, leads to a more rapid and extensive accumulation of vitellogenin mRNA [10], also known as the 'memory' effect, offers a further advantage in analyzing the role of different factors regulating gene transcription. In Xenopus, vitellogenin is encoded by a multigene family of four actively expressed genes, made up of two genes each in both A and B groups [12,13], so that hormonal manipulation could be exploited to determine whether or not the individual members of a multigene family are coordinately or separately regulated.

A single administration of estrogen to male Xenopus has been shown to cause a rapid sensitization to DNase I of vitellogenin genes in nuclei during the early stages of primary induction of vitellogenesis [14-16]; the rate of accumulation of vitellogenin mRNA in vivo and in culture upon primary and secondary stimulation of male and female Xenopus has also been documented [17-20]. However, in view of the different experimental conditions and sensitivities of the methods used, it is not possible to relate the DNase I digestion studies on isolated nuclei to those on mRNA accumulation in vivo. An ideal comparison would be to assess the hormonal effect on organization of vitellogenin genes with their transcription by determining simultaneously the DNase I digestibility and "run-off" transcription in the same sample of nuclei. In this paper, we show that during the first 20 hr after estradiol administration to male Xenopus, both the DNase I digestibility and transcription of the A and B groups of genes increased in a non-coordinate fashion, the B group being activated first. A second injection of estradiol to males led to a coordinate and more rapid activation of the two groups of vitellogenin genes than during primary stimulation, a pattern also observed in female animals. During the phase of hormone withdrawal, the elevated rate of transcription of these genes returned rapidly to low levels but the high DNase sensitivity was retained for over two months. Eight months after the primary hormonal stimulation, their activation was again non-coordinate in response to a secondary estradiol injection.

MATERIALS AND METHODS

Animals

Xenopus laevis were obtained from Xenopus Ltd., Redhill, Surrey, U.K. Where indicated, 1 mg estradiol-17β in 0.2 ml propane-1,2-diol was injected to each animal via the dorsal lymph sac.

Materials

Heparin, lithium chloride, Trizma base, ribonucleoside and deoxyribo-

nucleoside triphosphates, deoxyribonuclease I (EC3.1.4.5), ribonuclease A (EC3.1.4.22), calf thymus DNA, 2-mercaptoethanol, polyvinyl pyrrolidone, Ficol and bovine serum albumin were obtained from Sigma Chemical Co., Poole, Dorset, U.K. E.coli DNA polymerase (EC2.7.7.7) and the restriction endonucleases Bam H1 and Pst 1 were from New England Biolabs, Beverly, MA, USA. DEAE-cellulose filters (DE81, 2.5 cm) were obtained from Whatman Co., Maidstone, Kent, U.K., nitrocellulose was purchased as 200 mm sheets type PH 79 (1 µm porosity) from Schleicher and Schüll, Dassel, Germany, and Sephadex G50 from Pharmacia, Uppsala, Sweden. ^{32}P-labeled dATP, and dTTP (5000 Ci/mmole), and UTP and CTP (2500 Ci/mmole) were obtained from Amersham International, Amersham, Bucks., U.K. All other reagents were of analytical grade and obtained from BDH, Poole, Dorset, U.K.

Preparation of Xenopus Liver Nuclei

Nuclei were isolated from livers pooled from 5 animals by a modification of the low temperature (-20°C) procedure, described earlier [21,22]. Livers were perfused via the heart with 50 ml Stearns solution containing 200 µg/ml heparin before removal and homogenization in 10 volumes of ice-cold 40% glycerol (v/v), 12.5 mM NaCl, 5 mM $MgCl_2$ (homogenization buffer) with 10 strokes in a 50 ml glass/teflon homogenizer. The filtered homogenate was processed at -20°C from this stage onwards, the resulting nuclear pellet resuspended in twice its volume of homogenization buffer and stored at -70°C, with little loss of transcriptional activity. Use of detergents such as Triton X-100 was avoided since they remove nuclear-associated proteins and increase non-specific transcription.

RNA Synthesis in Isolated Nuclei

Cell-free transcription assays were based on a modification of the method of Hentschel and Tata [23]. Nuclei equivalent to 1 to 5 mg DNA were incubated in 1.5 ml Eppendorf tubes at 22°C in 100 µl of the reaction mixture containing 10% glycerol, 50 mM Tris pH 7.9, 25 mM KCl, 25 mM 2-mercaptoethanol, 0.5 µM UTP, 0.5 µM CTP, 5 µM GTP, 5 µM ATP, 150 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 5 mM $MnCl_2$, and 5 µCi each of ^{32}P-CTP and ^{32}P-UTP. The time-course of ^{32}P-labeled nucleotide incorporation was monitored by spotting 2 µl aliquots on DE 81 filter discs soaked in 200 mM EDTA, followed by 10 washes in 5% disodium hydrogen orthophosphate to remove unbound ^{32}P-nucleotides, then twice successively in distilled water, ethanol, and ether. The discs were dried and ^{32}P determined by scintillation counting in a Beckman LS 7800 scintillation counter.

Extraction of ^{32}P-Labeled RNA

RNA labeled with ^{32}P was extracted by a modification of the Auffray and

Rougeon [24] procedure. The *in vitro* transcription reaction was stopped by the addition of 4 volumes of 8 M urea, 4 M LiCl, 50 mM Na acetate, pH 5.5, 0.1% SDS, 10 µg/ml of total yeast RNA added as carrier, and the DNA dispersed by vortexing. The RNA was allowed to precipitate at 4°C for 16 hr, washed twice in the same extraction mixture as above and resuspended in 0.5 ml 200 mM Tris, pH 7.4, 5 mM EDTA, 50 mM NaCl (TEN buffer) before extraction for 5 min with an equal volume of re-distilled phenol saturated with TEN buffer. The phenol phase was re-extracted with 0.25 ml TEN buffer and the pooled aqueous phases extracted twice with an equal volume of phenol/chloroform (1:1 v/v), then, finally, once with chloroform alone. The RNA was precipitated with 2.5 volumes of redistilled ethanol, dried under vacuum, resuspended in a minimum volume of sterile distilled water and stored at -70°C.

DNase I Digestion of Chromatin by "Nick-translation" of Nuclei

The procedure was based on the method described by Levitt *et al.* [25] whereby *E.coli* DNA polymerase catalyzes the incorporation of labeled dNTPs at nicks introduced, preferentially at or near active gene sites, by low concentrations of DNase I added to isolated nuclei. It was found important to incubate nuclei at a low temperature so as to ensure that the *E.coli* DNA polymerase added does not translate too far beyond the DNase I-sensitive regions. Nuclei equivalent to 0.1 to 0.5 µg DNA were suspended in 20 µl of 5mM Tris pH 7.5, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 10 µCi ^{32}P-dCTP, 10 µCi ^{32}P-dATP, 0.2 µM dCTP, 0.2 µM dATP, 2 µM TTP, 2 µM dGTP, 3 units *E.coli* DNA polymerase, 0.002 ng DNase I. The suspension was incubated at 12°C for 60 min and the reaction stopped by addition of 20 µl TEN buffer. In order to eliminate the possibility of an artefact introduced by copying of mRNA into complementary DNA sequences by the nick-translation procedure, 0.1 µg of total female *Xenopus* liver polyadenylated RNA, containing substantial amounts of vitellogenin mRNA, was added to the incubation mixture and the ^{32}P-DNA analyzed by hybridization to cloned vitellogenin and cDNA. We found no evidence of reverse transcription of these mRNAs under the above conditions.

Extraction of ^{32}P-Labeled DNA

At the end of the nick-translation reaction, the nuclear suspension in 20 µl was gently shaken for 5 min with an equal volume of phenol/hydroxyquinoline (1000:1 v/w) saturated with TEN buffer. The phenol phase was re-extracted with 20 µl TEN buffer and the pooled aqueous phases extracted once with phenol/chloroform (1:1 v/v) and then with chloroform alone. The

^{32}P-labeled DNA was diluted with 0.4 ml 10 mM Tris pH 7.4, 1 mM EDTA, 0.1% SDS (G50 buffer) and passed through a 15 ml Sephadex G50 column in order to remove unincorporated ^{32}P-nucleotides. The radioactivity peak eluting with the void volume was collected and the nucleic acids precipitated with 2.5 volumes of redistilled ethanol. The precipitated DNA was dried under vacuum, resuspended in 20 µl of 5 mM Tris pH 7.4, 5 mM $MgCl_2$ and incubated for 1 hr at 37°C with RNase A (rendered DNase-free by heating to 60°C in 50 mM sodium acetate for 1 hr before use). The DNA was extracted once with phenol and once with chloroform, as above, precipitated with 2.5 volumes of ethanol, and stored at -70°C.

Hybridization of ^{32}P-Labeled Nucleic Acids to ClonedcDNA

Recombinant plasmids pXlvc 23 and pXlvc 19 containing *Xenopus* vitellogenin cDNA inserts (group A and B vitellogenin genes, respectively) were cut with restriction endonuclease Bam Hl, while plasmids pXg6Cl and pXg8D2 containing *Xenopus* globin inserts and plasmid pAT 153 with no recombinant DNA were restricted with Pst 1. The linearized plasmid DNA (0.5 µg DNA per filter) was bound to squares of nitrocellulose, soaked in 20 x SSC (SSC = 0.1 M NaCl, 0.01 M Na citrate). The filters were kept moist for 2 hr, air-dried and baked at 80°C under vacuum for 4 hr. They were prehybridized in 50% formamide, 5 x SSC, 5 x Denhart's solution (0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 0.2% Ficoll), 50 mM sodium phosphate pH 6.5, 0.1% SDS, 100 µg/ml denatured calf thymus DNA (hybridization buffer) at 42°C overnight with shaking. Hybridization was performed at 42°C for 48 hr with continuous shaking in 1 ml of hybridization buffer and ^{32}P-labeled RNA or DNA to give 10^6 dpm (to a maximum of 50 ng per filter). The filters were washed twice in 2 x SSC, 0.1% SDS, at room temperature for 30 min then twice in 0.2 x SSC, 0.1% SDS at 50°C for 30 min. The ^{32}P-labeled nucleic acid retained on the filters was determined by counting for 20 min in a Beckman LS7800 scintillation counter. Hybridization to pAT 153 plasmid was taken as background, subtracted from each result and then hybridization to vitellogenin cDNA or globin cDNA calculated as a percentage of the ^{32}P-nucleic acid originally added to the vials. Since the cloned probes pXlvc 23 and pXlvc 19 contained vitellogenin cDNA inserts of 3.7 kb and 2.3 kb, respectively, appropriate corrections were made for these differences in expressing the final results.

DNA Estimations

DNA was estimated by the method of Labara and Paigen [26] using fluorescence at 494 nm of the dye Hoechst 33258 bound to DNA, with calf thymus DNA as a standard. DNA concentration in nuclei was measured following disruption

by sonication in a medium of high salt concentration (2 M NaCl, 50 mM Na phosphate pH 7.0) in order to strip the DNA of proteins.

RESULTS

Overall Rate of Transcription in vitro

After establishing optimal conditions for transcription *in vitro*, the rate of total RNA synthesis by liver nuclei from estrogen treated or untreated male and female *Xenopus* was compared. As shown in Fig. 1, nuclei from hormonally untreated male animals were the least active transcriptionally, those from female *Xenopus* being nearly twice as active. A small, but significant, increase in transcriptional activity of nuclei could be discerned by 2-4 hr after a single injection of 1 mg estradiol in both groups of animals, the stimulation plateauing off by 10-16 hr after hormone administration. The vitellogenic and growth responses of male *Xenopus* liver are known to be more marked during secondary than primary induction. It is therefore significant that the transcriptional activity of hepatic nuclei from male animals 24 hr after a second injection of the hormone, given 5 weeks after the first treatment, was further enhanced than following primary induction.

Transcription of Vitellogenin and Globin Genes in vitro

The progression of response to estradiol depicted in Fig. 1 does not

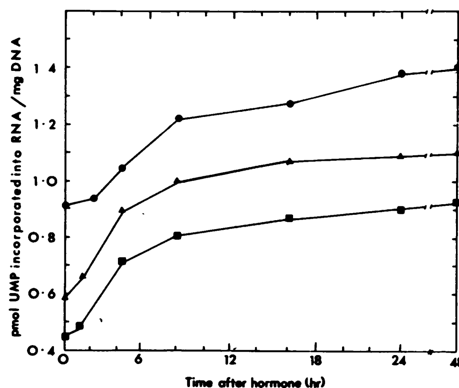

Fig. 1. Total RNA synthesis by isolated liver nuclei as a function of time after the primary or secondary administration of 1 mg estradiol to male and female Xenopus. The overall transcription was determined by incubating nuclei for 15 min with α-^{32}P-UTP and measuring the incorporation of ^{32}P into RNA bound to DEAE-cellulose filter discs. (■) Primary induction in males; (▲) secondary induction in males injected 5 weeks earlier with 1 mg estradiol; (●) first injection of the hormone to female Xenopus. 50-60% of the activity of isolated nuclei was due to RNA polymerase II, as judged by sensitivity to α-amanitin.

necessarily indicate the time-course of induction of vitellogenin gene transcription induced by the hormone. We therefore analyzed the labeled transcripts by hybridization to cloned Xenopus vitellogenin cDNA. Since the liver is a major site of erythropoiesis in amphibia, the transcripts were also hybridized to cloned Xenopus globin cDNA, in order to establish the specificity of hormonal activation of vitellogenin gene transcription. Virtually unmeasurable amounts of vitellogenin mRNA-like sequences could be detected in the labeled RNA synthesized *in vitro* by nuclei from hormonally untreated male Xenopus liver, as shown in Fig. 2. By 2 hr after estradiol administration, a small, but highly reproducible, amount of vitellogenin mRNA sequences could be detected which increased sharply for the next 16-18 hr, reaching a level of 0.38% of total labeled transcripts, i.e. ∽30 times the levels in nuclei from uninduced animals. When the same RNA samples were hybridized to cloned Xenopus globin cDNA, a significant fraction (0.07%) of total labeled trans-

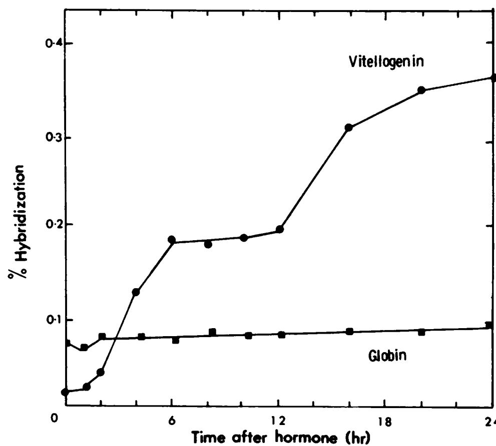

Fig. 2. Activation of transcription of vitellogenin genes measured in male Xenopus liver nuclei following primary induction with estradiol. Nuclei were prepared at the times indicated after the administration of 1 mg estradiol to male animals and the synthesis of ^{32}P-labeled vitellogenin and globin mRNA sequences monitored by the incorporation of α-^{32}P-UTP and α-^{32}P-CTP into total RNA after incubation of the nuclei for 15 min, followed by hybridization to the respective cDNA probes. *(●) The transcription of the four Xenopus vitellogenin genes was measured together by hybridization of the ^{32}P-labeled RNA to the A and B groups of these genes represented by plasmids pXlvc 23 and pXlvc 19. (■) Labeled Xenopus globin sequences in the same sample of RNA determined by hybridization to plasmids pXg8D2 and pXg6C1. Hybridization of the ^{32}P-RNA to plasmid pAT 153 without any cDNA inserts was taken as the background and subtracted from the values obtained for plasmids with Xenopus cDNA inserts.*

cripts was detected as globin mRNA sequences in nuclei from hormonally untreated RNA. However, the level of globin gene transcription did not vary by more than 20% over 48 hr, thus establishing that the increase in vitellogenin mRNA sequences was highly hormone specific.

DNase I Sensitivity of Vitellogenin and Globin Genes

Preliminary results showed that estrogen treatment enhanced sensitivity of vitellogenin, but not globin, genes when nuclei were digested with DNase I in the conventional manner, i.e. eventual disappearance of active gene sequences. Relative to total DNA, the vitellogenin genes appear to be more susceptible to digestion by the nuclease, since the fragments hybridizing to the vitellogenin probe were always smaller than the ethidium bromide stained bulk DNA. With this technique, a significant fraction of vitellogenin gene sequences was too extensively broken down to be detected which did not allow a quantitative analysis to be performed. The procedure of "nick-translation" of nuclei obviated this disadvantage and permitted a quantitative analysis of hormonal activation of vitellogenin genes, as described below.

Simultaneous Analysis of Transcription and DNase I Sensitivity of A and B Groups of Vitellogenin Genes

It was possible that the biphasic nature of the curve for vitellogenin transcripts in Fig. 2 could be due to the activation of the two groups of vitellogenin genes at different times after hormonal stimulation. We therefore compared DNase I sensitivity and transcription of A and B groups of vitellogenin genes separately in the same sample of nuclei. Fig. 3 summarizes the results with liver nuclei from male and female Xenopus at different times after primary and secondary induction of vitellogenesis by estradiol. Liver nuclei from "naive" male Xenopus, i.e. not previously exposed to the hormone, synthesized the B group of vitellogenin mRNA at earlier times of hormonal stimulation than A group mRNAs (Fig. 3A). Both groups of genes were highly resistant to nicking with DNase I in liver nuclei from untreated males, especially as compared to globin genes. However, within 16 hr after estrogen administration the vitellogenin genes had acquired a high nuclease sensitivity, that of B group genes was altered within 2 hr, whereas the A group genes were relatively insensitive until 6 hr later. The rate of transcription of both A and B group genes declined to very low levels in nuclei from male animals five weeks after the first injection of the hormone. Upon secondary induction at five weeks, the stimulatory effect of the hormone was more rapid and the differential activation of the two groups of genes seen during primary response was now not evident (Fig. 3C). The pattern of altered DNase I

sensitivity in the same samples of nuclei was quite different. The elevated sensitivity to DNase I was retained five weeks after the first hormone injection and the second administration failed to enhance it appreciably. This dissociation between transcription in vitro and DNase I sensitivity was also observed in nuclei from adult female Xenopus (Fig. 3E and F), except that the rates of RNA synthesis and susceptibility to nuclease digestion were higher before hormone treatment than in nuclei from males after the first estradiol

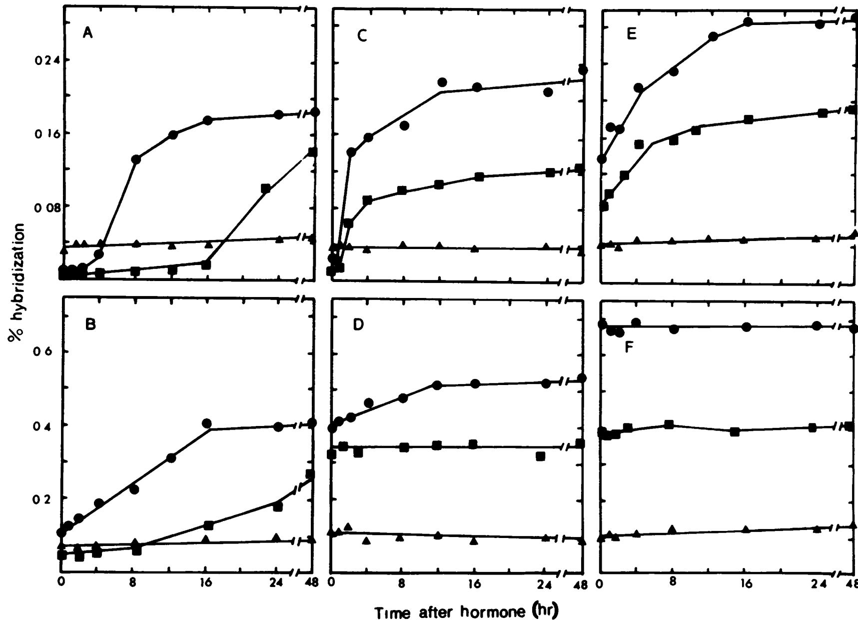

Fig. 3. Simultaneous analysis of transcription and DNase I sensitivity of A and B groups of vitellogenin genes in liver nuclei isolated from male and female Xenopus at different times after primary or secondary induction with estradiol. Nuclei from each group of animals were divided into two aliquots, one used for transcription (panels A,C,E) and the other for DNase I digestion (panels B,D,F). Transcription and DNase I sensitivity of A and B groups of vitellogenin genes were measured by hybridization of the labeled RNA and DNA separately to plasmids pXlvc 23 and pXlvc 19, respectively. Hybridization of the same samples of labeled RNA and DNA to plasmid pXg8D2 containing globin cDNA insert served as control. RNA synthesized in vitro was labeled by incubation of nuclei for 15 min with α-^{32}P-UTP and α-^{32}P-CTP, while DNase I sensitivity was measured by the technique of nick-translation of nuclei incubated for 2 hr with α-^{32}P-dATP and α-^{32}P-dTTP. (A,B) Primary administration of 1 mg estradiol to male Xenopus; (C,D) secondary administration of estradiol to males pre-treated with the hormone 5 weeks earlier; (E,F) injection of estradiol to female Xenopus. (■) Group A vitellogenin genes or transcripts; (●) group B vitellogenin genes or transcripts; (▲) globin genes or transcripts.

treatment. The specificity of hormonal response is borne out by the lack of any effect on the transcription and DNase I sensitivity of globin genes. Thus, the transcription of A and B groups of genes in naive male Xenopus is activated in a non-coordinate manner, whereas it is coordinate upon secondary induction in males or in adult vitellogenic females. The enhanced transcriptional activity is paralleled by enhanced DNase I sensitivity of the two groups of genes on primary hormonal stimulation of the male. However, once the gene structure is altered, the change is retained for at least 5 weeks and is distinct from changes in rates of RNA synthesis.

Long-Term Hormone Withdrawal and Re-stimulation

The retention of high DNase I sensitivity of vitellogenin genes long after hormone withdrawal was of particular interest in the context of the 'memory' effect of estradiol in inducing vitellogenesis in male vertebrates, considering the more rapid decline of vitellogenin RNA synthesis. It prompted us to determine, whether or not, and after what period of time, the elevated DNase I sensitivity of vitellogenin genes in males following primary hormonal induction in vivo, returns to the insensitive pattern seen in hormonally untreated males. The combined results of such experiments, depicted in Fig. 4, show that there was a very sharp drop in the in vitro transcriptional activity of nuclei between 4 and 6 weeks after hormone treatment, the rate of vitellogenin mRNA synthesis returning to background levels at 6 weeks (compare values in Fig. 4 and Table 1). On the other hand, at least 80% of the high DNase I sensitivity was retained for 2 months following hormone administration, after which it declined, reaching control levels at 8 months after hormone administration. We should like to point out that whereas these comparisons of gene transcription and conformation are reproducible, the exact time of gene activation or de-induction and the magnitude of change can vary from one experiment to another, especially in separate batches of animals. Even though not all the time-points are identical for the two sets of measurements in Fig. 4, it is clear that a substantial difference exists between transcriptional activity and nuclease sensitivity during the hormone withdrawal phase. We next determined whether or not the pattern of activation of A and B groups of vitellogenin genes in male Xenopus liver by estrogen after this long period of hormone withdrawal was coordinate or non-coordinate. The results in Table 1 show that the status of both groups of genes had returned to that in naive male animals, since a non-coordinate pattern of response to the second treatment with the hormone was re-established at 8 months after the first administration of estradiol.

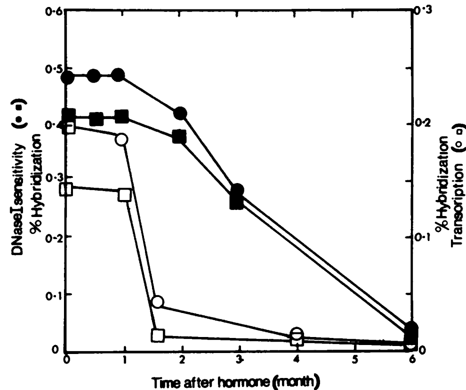

Fig. 4. Simultaneous analysis of de-induction of transcription (□ , ○) and DNase I sensitization (■ , ●) of A and B group vitellogenin genes following their activation by a single primary administration of estradiol to male Xenopus. Liver nuclei were prepared from male Xenopus at the different times indicated following the injection of 1 mg estradiol, one of the two preparations was assayed for in vitro RNA synthesis and the other for DNase I sensitivity by the nick-translation procedure. The ^{32}P-labeled RNA and DNA extracted from the nuclei were hybridized on nitrocellulose filters to plasmids pXlvc 23 for determining group A vitellogenin sequences (□ , ■) or pXlvc 19 for group B sequences (○ , ●).

DISCUSSION

Our approach of simultaneous analysis of DNase I sensitivity and RNA synthesis in isolated nuclei has enabled us to determine more precisely than in earlier studies the temporal relationship between any alteration in the chromosomal organization of vitellogenin genes and their transcription upon primary and secondary activation by estrogen of these genes in Xenopus liver. The procedure used for transcription in isolated nuclei is analogous to the "run-off" RNA synthesis systems described by other workers, whereby most of the labeled RNA represents the elongation in vitro of RNA chains already initiated in vivo [6-9], and does not indicate in vitro transcription on truncated templates. As regards DNase I sensitivity, the procedure of nick-translation of nuclei [25] adopted by us turned out to be superior to the more conventional digestion procedures whereby expressed genes are preferentially digested to very small fragments or rendered acid-soluble [2,27]. Two particular advantages of nick-translation of nuclei are a) the retention of active gene sequences as relatively large fragments suitable for DNA-cDNA hybridization, and b) the preferential labeling to very highly specific activities of the more DNase I sensitive genes, thus facilitating the hybridization with excess of unlabeled cloned sequences.

Table 1. Secondary stimulation of transcription and DNase I sensitization of A and B groups vitellogenin genes in male Xenopus liver following short-term (4 weeks) and long-term (8 months) hormone withdrawal after primary stimulation.

Hormonal Stimulation	Vitellogenin Gene Group	Transcription (% RNA Hybridized) at Hours after Estradiol Administration		
		0	12	24
Primary	A	0.007	0.024	0.132
	B	0.016	0.142	0.184
Secondary (4 weeks)	A	0.105	0.180	0.200
	B	0.138	0.260	0.278
Secondary (8 months)	A	0.009	0.016	0.115
	B	0.015	0.150	0.175
		DNase I Sensitivity (% DNA Hybridized) at Hours after Estradiol Administration		
		0	12	24
Primary	A	0.05	0.09	0.30
	B	0.05	0.28	0.31
Secondary (4 weeks)	A	0.33	0.33	0.33
	B	0.34	0.47	0.50
(Secondary (8 months)	A	0.05	0.09	0.27
	B	0.07	0.34	0.35

Nuclei were prepared from livers of uninjected (0 hr) male animals and at 12 and 24 hr after a second injection of the hormone given 1 or 8 months after the first. For comparison, the table also gives the values for primary response alone at the same time intervals. Nuclei were divided into two batches and the ^{32}P-labeled RNA and DNA, obtained after "run-off" transcription and nick-translation of the nuclei, respectively, were hybridized to cloned A and B group vitellogenin cDNAs. The results are expressed as the fraction of labeled RNA and DNA hybridized to group A and B vitellogenin cDNA probes. Each value is the mean of triplicate determinations. Other details as in Figs. 3 and 4.

Within 16-24 hr after a primary or secondary administration of estradiol to male and female Xenopus, the overall rate of RNA synthesis was enhanced by 30-50%. When the radioactive transcripts synthesized in vitro by liver nuclei isolated from male Xenopus, not previously treated with the hormone, were probed with vitellogenin cDNA, there was virtually no transcription of vitellogenin genes. The administration of estradiol to such naive males very rapidly led to the labeling of vitellogenin mRNA specified by all four genes, whose transcription was initiated in vivo. The biphasic nature of the curve in Fig. 2 drew our attention to other studies in our laboratory on cultured

hepatocytes in which the B group of vitellogenin genes was found to be activated a few hours earlier than the A group genes after the addition of estradiol to cells from naive male Xenopus [20].

A number of developmentally and hormonally inducible genes are now known to constitute small multigene families [12,28-33]. An important question concerning their regulation is to determine whether the individual members of such families are expressed coordinately or separately. Estradiol-induced vitellogenesis is an ideal model for approaching this question because of a) the reversible induction of vitellogenin mRNA synthesis by the hormone, b) the different kinetics of primary and secondary induction, c) the high magnitude of expression of four related genes in terminally differentiated cells, and d) the de novo activation of egg protein genes in males [10-11]. Our results bring out the interesting fact that the vitellogenin genes of the A and B groups may be expressed either separately or in parallel, according to the sex or previous hormonal manipulation of the animal. Thus, in male Xenopus the two groups of genes are sequentially transcribed and rendered more DNase I sensitive during the early stages of primary stimulation with estradiol (Fig. 3A,B), the onset of activation of B group preceding that of the A group by about 8 hr. Comparisons of temporal sequences in whole animals however do not allow us to conclude whether or not some structural re-organization of the dormant vitellogenin genes in male liver preceded the initiation of their transcription. For this purpose a similar simultaneous assessment of transcription and nuclease sensitivity of vitellogenin genes in a tissue culture system [19,20] may be more informative.

The pattern of non-coordinate, but parallel, activation of nuclease sensitivity and transcription of the A and B groups of vitellogenin genes observed in male Xenopus upon primary hormonal induction was not seen during secondary stimulation of males or the first treatment of females (Fig. 3C-F). Furthermore, whereas transcription of both A and B groups of vitellogenin genes in liver nuclei isolated five weeks after the first estradiol injection to males had returned to virtually zero level, the DNase I sensitivity had remained at the elevated level seen at 48 hr of primary induction. A second administration of estradiol caused a more rapid and simultaneous increase in the in vitro synthesis of A and B group mRNAs without affecting the DNase I sensitivity of the corresponding genes. Not unexpectedly, nuclei from uninjected females showed a substantial rate of both A and B group vitellogenin gene transcription, and exhibited an attenuated response to hormone administration, while the high DNase I sensitivity remained unaffected. Thus, only when

the vitellogenin genes are in a quiescent state in the naive male Xenopus does one observe a parallel change in the DNase I sensitivity and transcriptional activity. Once activated, as during secondary hormonal induction in males or in vitellogenic females the two parameters are dissociated. Other workers [34,35] have also observed a similar dissociation between alteration in chromatin structure and transcription of the β-globin gene during its irreversible developmental activation in erythroid cells. The long-term DNase I sensitization by estrogen of Xenopus vitellogenin genes in the absence of their transcription that we have described is also analogous to the activation of ovalbumin genes in the chicken oviduct by the same hormone [36]. However, the fact that the active conformation of vitellogenin genes returns to the original inactive state at 4-8 months after the first hormone administration (Fig. 4) shows that the process is eventually reversible. Hayward et al. [37] have proposed that the first treatment of male Xenopus with estradiol causes an alteration lasting for two months in the nucleus:cytoplasm distribution of estrogen receptor in male Xenopus liver, and that the enhanced level of vitellogenesis during secondary induction can be explained by this phenomenon. Since the A and B groups of vitellogenin genes respond to a secondary stimulation at 8 months in a non-coordinate fashion (Table 1), it will be most interesting to see if the pattern of receptor distribution also reverses at 4-8 months after primary stimulation.

We do not know the mechanisms underlying the shift from non-coordinate to coordinate expression of the two groups of vitellogenin genes upon secondary hormonal induction. A similar phenomenon has not been described for other multigene families. Nevertheless, it is relevant to point out the studies of Kalfayan and Wensink [38] that during Drosophila development mRNA specified by two genes of the tubulin multigene family accumulate in parallel while two other members of this family are expressed separately. Iatrou et al. [31] have concluded that homologous genes encoding egg chorion protein in Bombyx need not be expressed in parallel. However, such studies do not provide information on how any possible change in the configuration or chemical modification (such as methylation and demethylation) of dormant genes may be related to their transcriptional activation. In this context, our results validate our approach of simultaneously monitoring the higher-order organization of genes and their transcription, while at the same time extend the usefulness of Xenopus vitellogenesis as a model for investigating the regulation of the reversible expression of individual members of a multigene family.

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