

## Differential sensitization to deoxyribonuclease I of *Xenopus* vitellogenin and albumin genes during primary and secondary induction of vitellogenesis by oestradiol

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The sensitivity to DNAase (deoxyribonuclease) I (which preferentially digests transcribed sequences) of vitellogenin and albumin genes in liver and erythrocytes of male *Xenopus* after primary and secondary induction of vitellogenesis by oestrogen was measured by hybridization to cDNA (complementary DNA) of the residual DNA after enzymic digestion of isolated nuclei. Vitellogenin sequences were rendered selectively more sensitive to limited DNAase-I digestion (15–20% of DNA rendered acid-soluble) during primary hormonal activation (5 days) of vitellogenin genes in liver, but not erythrocyte, nuclei. Hormone withdrawal (25 days after first injection) did not result in reversion to a pre-activation gene configuration, nor did secondary hormonal stimulation (5 days after second and 25 days after first injection) augment the sensitivity of the genes to digestion by the nuclease. Similar hormone treatment did not affect the sensitivity of the constitutively expressed albumin genes in liver nuclei, nor their insensitivity in erythrocyte nuclei. Under the same conditions, globin genes remained indigestible in liver nuclei. It is concluded that primary induction of vitellogenesis in male *Xenopus* liver is accompanied by a relatively long-lasting (3–4 weeks) change in the configuration of vitellogenin genes in hepatic nuclei which is not reversed or further modified during short-term oestrogen withdrawal or upon secondary stimulation.

The higher-order organization of the eukaryotic cell genome plays an important role in the regulation of gene expression, particularly during development or cellular differentiation (see Botchan & Watson, 1978, for several reviews). The exploitation of various nucleases (Felsenfeld, 1978) has made a major contribution towards unravelling both the nucleosomal organization of genes and the different chromosomal configurations of expressed and unexpressed genes. In particular, the discovery that expressed genes are selectively more easily digested by DNAase I than are unexpressed genes, when whole nuclei or chromatin preparations are briefly exposed to low concentrations of the enzyme, combined with the availability of well-defined hybridization probes, has made it possible to follow

the regulation of specific genes during development or some other alteration of cellular function. Thus the selective digestion by DNAase I of globin and ovalbumin genes and integrated adenovirus sequences in the cells in which they are expressed confirmed that sensitivity to the nuclease was related to their transcription (Garel & Axel, 1976; Weintraub & Groudine, 1976; Flint & Weintraub, 1977; Panet & Cedar, 1977; Groudine *et al.*, 1981; Weintraub *et al.*, 1981). Other studies, however, have shown that enhanced digestibility with DNAase I does not necessarily involve initiation or continuation of transcription of these genes (Bellard *et al.*, 1978; Miller *et al.*, 1978; Palmiter *et al.*, 1978; Stalder *et al.*, 1980*a,b*). Also, sensitivity to DNAase I did not reflect the very different rates at which different genes are transcribed (Garel *et al.*, 1977). An important reason for this inconsistency lies largely in the fact that the above regulatory systems are essentially 'irreversible', as, for example, the de-induction of globin genes accompanying erythroid-cell maturation or the 'secondary' responses, after the restimulation with oestrogen of

Abbreviations used: DNAase I, deoxyribonuclease I (deoxyribonuclease 5'-oligonucleotidohydrolase, EC 3.1.21.1); cDNA, complementary DNA.

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oviduct cells that have already been previously hormonally activated. A truly primary induction system which is also easily reversible therefore offers major advantages in clarifying this particular aspect of the regulation of gene expression.

Hormonal induction of vitellogenesis offers such a model for studying reversible primary gene activation (see Tata & Smith, 1979; Wahli *et al.*, 1981). The ease with which the egg-yolk protein precursor vitellogenin is induced by oestrogen in the liver of males of egg-laying vertebrate species in the absence of any cell division represents a true primary activation of the gene. It is followed by cessation of transcription of the gene during the withdrawal period, and a subsequent secondary induction is more rapidly produced with a second exposure to the hormone (Baker & Shapiro, 1977; Deeley *et al.*, 1977; Farmer *et al.*, 1978). Another advantage of the vitellogenesis model is that vitellogenin is synthesized in the same cells in which the albumin gene is constitutively expressed (Wachsmuth & Jost, 1976; Wangh *et al.*, 1979). In the clawed frog *Xenopus*, hormonal induction of vitellogenin synthesis is accompanied by a reversible de-induction of albumin synthesis (Green & Tata, 1976; Farmer *et al.*, 1978), so that it offers a most useful 'built-in' control to determine the specificity of gene transcription. As regards the nature of the gene itself, studies in *Xenopus* have shown that vitellogenin is coded for by a multi-gene family, at least four of which are actively expressed (Wahli *et al.*, 1979), with the possibility of even more genes or partial coding sequences remaining in an inexpressible configuration during vitellogenesis (Tata *et al.*, 1980).

In view of the above, as well as other advantages of the vitellogenin system (Tata & Smith, 1979), we have performed experiments in which DNAase I was used to monitor the possible changes in the configuration of vitellogenin and albumin genes in male *Xenopus* liver nuclei during primary and secondary induction of vitellogenesis by oestrogen, as well as upon hormone withdrawal. In the results reported here, we show that the hormone alters the configuration of vitellogenin genes in liver, but not erythrocyte, nuclei so as to render them more sensitive to DNAase I during primary activation of vitellogenin-gene transcription in male hepatocytes. However, once altered during primary stimulation, they remain 'activated', as judged by DNAase-I digestibility, during the hormone-withdrawn state for about 3–4 weeks when the transcription is greatly diminished, or after secondary hormonal stimulation, when they are once again actively transcribed. At the same time, the different configurations of albumin and globin genes are unaffected by hormonal treatment in hepatic and erythrocyte nuclei, irrespective of their transcriptional status.

## Materials and methods

### Materials

Pancreatic DNAase I (EC 3.1.21.1) and pancreatic ribonuclease A (EC 3.1.27.5), were obtained from Worthington Corp., Freehold, NJ, U.S.A., and Boehringer Corp., Lewes, Sussex, U.K., respectively, and proteinase K and S<sub>1</sub> nuclease were from Sigma (London) Chemical Co., Poole, Dorset, U.K. [<sup>32</sup>P]dCTP was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Avian-myeloblastosis-virus reverse transcriptase was kindly supplied by Dr. J. W. Beard, University of Florida, St. Petersburg, FL, U.S.A. All other reagents were of analytical grade and purchased from Sigma or BDH, Poole, Dorset, U.K.

### Animals and hormone treatment

Adult male *Xenopus laevis* were purchased from *Xenopus* Ltd., Redhill, Surrey, U.K., and maintained in tap water at 21 ± 2°C. Where indicated, vitellogenesis was induced with an injection of 1 mg of oestradiol-17β (in 0.2 ml of propane-1,2-diol) via the dorsal lymph sac.

### Preparation of nuclei

Nuclei from *Xenopus* liver were prepared by the glycerol procedure at –20°C and from erythrocytes by the saponin/glycerol method, as described by Dimitriadis & Tata (1980).

### DNAase-I digestion of nuclei

Nuclear pellets were suspended at a concentration of 0.5 mg of DNA/ml in a medium containing 50% (w/v) glycerol, 10 mM-Tris/HCl, pH 7.4, 10 mM-NaCl and 3 mM-MgCl<sub>2</sub>. A trial digestion was performed on a small sample of the suspension with 20 units of pancreatic DNAase I/ml at 37°C to determine the time necessary to render 15–20% of the DNA soluble in 0.5 M-HClO<sub>4</sub> (Noll & Kornberg, 1977). The rest of the nuclear suspension was then incubated under identical conditions for the above length of time, the digestion stopped with 10 mM-EDTA and the amount of DNA that was acid-soluble checked in a small sample.

### DNA extraction

DNA from liver and erythrocyte nuclei, before and after limited digestion with DNAase I, was extracted by the proteinase K/sodium dodecyl sulphate/phenol method of Gross-Bellard *et al.* (1973), except that it was more exhaustively treated with pancreatic ribonuclease followed by an additional cycle of de-proteinization. DNA content in nuclei was determined by the diphenylamine procedure (Burton, 1956) and by the A<sub>260</sub> in purified preparations (20 A<sub>260</sub> units/ml = 1 mg of DNA).

*Synthesis of DNA complementary to Xenopus vitellogenin, albumin and globin mRNA species*

cDNA was synthesized on templates that were 50% enriched for vitellogenin mRNA and 90% for albumin and globin mRNA, as determined by denaturing agarose-gel electrophoresis (McMaster & Carmichael, 1977), the contamination being mainly rRNA. Synthesis was carried out in a volume of 10  $\mu$ l containing 1  $\mu$ g of template RNA, 50 ng of (dT)<sub>12-18</sub>, 50  $\mu$ M-deoxyribonucleotides, 1 mCi of [<sup>32</sup>P]dCTP (2000–3000 Ci/mol), 1  $\mu$ g of actinomycin D, 50 mM-Tris/HCl, pH 8.0, 60 mM-NaCl, 10 mM-dithiothreitol, 6 mM-magnesium acetate and 4 units of avian-myeloblastosis-virus reverse transcriptase. <sup>32</sup>P-labelled vitellogenin cDNA was purified by back-hybridization to a very low R<sub>0</sub>t [concentration of RNA at zero time  $\times$  time (s) of hybridization] value with an excess of the mRNA preparation used as a template (Gordon *et al.*, 1978). The sizes of the cDNA synthesized, determined by denaturing agarose-gel electrophoresis, corresponded to almost full-size for globin mRNA and albumin mRNA and approximately half-size for the vitellogenin-mRNA probe. The cDNA preparations were characterized as described by Dimitriadis (1981).

*cDNA-DNA hybridization*

DNA from nuclei not digested with DNAase I was sheared in a Virtis disintegrator (Britten *et al.*, 1974) to a mean length of 600 base-pairs (range 200–1200 base-pairs), but DNA extracted from DNAase I-treated nuclei was not sheared. Hybridization was performed at a DNA:cDNA weight ratio of  $6 \times 10^6$ – $8 \times 10^6$  in 0.3 M-NaCl/0.05 M-Tris / HCl (pH 7.4) / 1 mM-EDTA / 0.1% sodium dodecyl sulphate at 69°C under paraffin oil. The extent of hybridization was determined by withdrawing samples at different time intervals and flushing them out into a medium containing 0.28 M-NaCl, 4.5 mM-ZnSO<sub>4</sub>, 0.03 M-sodium acetate, pH 4.6, and 20  $\mu$ g of sonicated heat-denatured calf thymus DNA/ml, followed by incubation with 20 units of S<sub>1</sub> single-strand-specific nuclease. The S<sub>1</sub>-nuclease-resistant radioactivity was taken as the amount of [<sup>32</sup>P]cDNA hybridized, after correction for zero-time ('snap-back') hybridization (usually 8–12% of input radioactivity). Hybridization curves were generated without the use of a computer.

**Results**

*Increased sensitivity to DNAase I of vitellogenin genes in male Xenopus hepatocyte nuclei during primary induction with oestradiol*

Fig. 1 summarizes the kinetics of hybridization of cDNA to *Xenopus* vitellogenin mRNA with DNA obtained from liver and erythrocyte nuclei, before

and after DNAase I digestion and as a function of different hormonal regimes. As judged from C<sub>0</sub>t<sub>1/2</sub> [C<sub>0</sub>t = mol of nucleotides of DNA per litre  $\times$  time (s); C<sub>0</sub>t<sub>1/2</sub> = C<sub>0</sub>t value obtained at 50% hybridization] and hybridization-saturation values, the relative abundance of vitellogenin sequences was unaffected by DNAase-I digestion of liver and erythrocyte nuclei from hormonally untreated adult male *Xenopus* liver (Figs. 1a and 1d). It should be noted that under the conditions of DNA/cDNA hybridization used, the final saturation value is determined by the ratio of driver:tracer, which has been held constant (at  $6 \times 10^6$ – $8 \times 10^6$ ), so that these comparisons are valid. Since the vitellogenin gene is known not to be transcribed in male cells in the absence of the hormone (Baker & Shapiro, 1977; Tata & Smith, 1979), we interpret this finding to mean that the insensitivity to DNAase I reflects the configuration of vitellogenin genes in a transcriptionally repressed state. At 5 days after the first injection of oestradiol into male frogs, when vitellogenin-mRNA synthesis proceeds at a rapid rate (Baker & Shapiro, 1977), there is a significant loss of vitellogenin sequences relative to total DNA from liver nuclei, but not from erythrocyte nuclei (Figs. 1b and 1e). Thus primary hormonal induction of vitellogenesis is accompanied by an activation of vitellogenin genes which is restricted to the nuclei of the hormone's target cells.

It is known from earlier studies (Baker & Shapiro, 1977) that the rate of transcription of vitellogenin genes in *Xenopus* liver nuclei reaches a maximum at about 8 days after hormonal stimulation and then falls rapidly to reach negligible values 3–4 weeks later. As shown in Figs. 1(e) and 1(f), the sensitivity of the genes in liver nuclei at 20 days after the first hormone treatment remains as high as, or even higher than, at the height of the transcriptional response at 5 days. Since withdrawal of the hormone is not accompanied by a rapid return of configuration of these genes in the liver to a DNAase-I-insensitive state, we conclude that primary hormonal stimulation causes a long-lasting change in their status, at least for 20 days after the first injection.

*Albumin-gene configuration is unaltered by hormonal sensitization of vitellogenin genes to DNAase I*

Since albumin genes are constitutively expressed in *Xenopus* liver (Dimitriadis & Tata, 1980; Tata *et al.*, 1980), the results of the hybridization of the albumin probe with DNA from male *Xenopus* liver and erythrocyte nuclei were not unexpected (Fig. 2a). On the other hand, in spite of the fact that albumin-mRNA synthesis is known to be markedly depressed in *Xenopus* liver on induction of vitellogenesis by oestradiol (Farmer *et al.*, 1978; G. J.

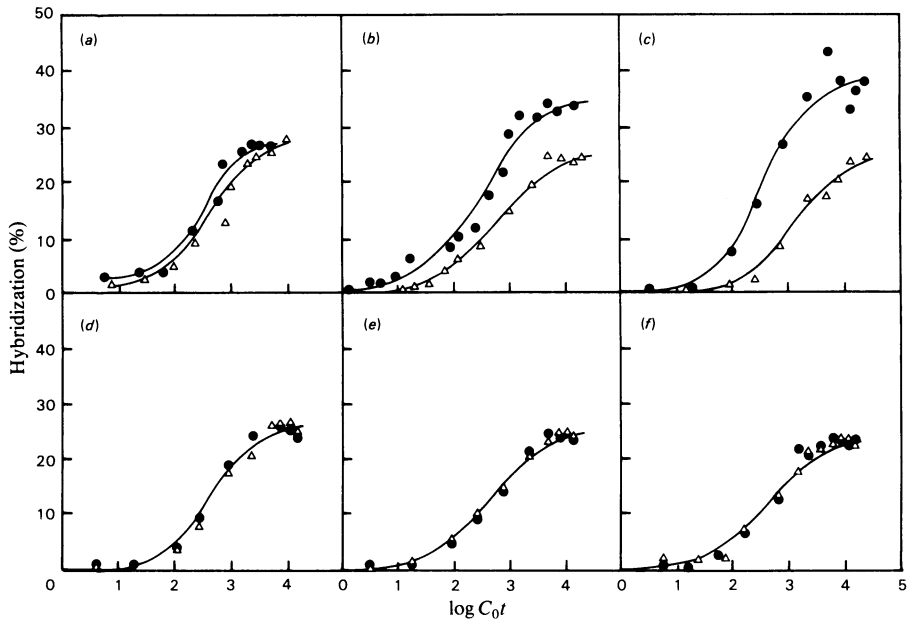


Fig. 1. Effect of oestrogen administration and withdrawal on the sensitization to DNAase-I digestion of vitellogenin-coding sequences of male *Xenopus* liver and erythrocyte nuclei

The curves depict the kinetics of hybridization of [<sup>32</sup>P]cDNA to *Xenopus* vitellogenin mRNA with DNA extracted from liver (a, b, c) and erythrocyte (d, e, f) nuclei, digested ( $\Delta$ ) with DNAase I or not ( $\bullet$ ). Nuclei were prepared from batches of 15 frogs and incubated with DNAase I for a period of time necessary to render 15–20% of DNA acid-soluble. The DNA was extracted and hybridized with [<sup>32</sup>P]cDNA (input 10000 c.p.m. per sample) and the extent of hybridization determined from the percentage of input radioactivity resistant to S<sub>1</sub> nuclease, as described in the text. (a), (d), Control frogs, no hormone treatment; (b), (e), primary induction, frogs killed 5 days after a single injection of 1 mg of oestradiol-17 $\beta$  per animal; (c), (f), hormone-withdrawn state, animals killed 20 days after a single injection of the hormone.

Dimitriadis, unpublished work), the sensitivity of albumin-coding sequences in liver nuclei remained unaltered, i.e. in an activated state, for up to 20 days after hormone administration (Figs. 2b and 2c). Albumin genes remain insensitive to DNAase I under all the above hormonal states in the quiescent mature erythrocyte nuclei (Figs. 2d–2f). The specificity of the ‘permanent’ sensitivity of albumin genes in liver nuclei can be judged from the results shown in Fig. 3, where the globin gene was found to be only barely digestible with DNAase I in liver cells, in which it is normally never expressed. Although the amphibian liver is a haemopoietic organ, the low digestibility of globin genes in this tissue may be due to the presence of predominantly adult erythrocytes, parenchymal cells and other hepatocytes, in which these genes would not be expressed.

#### DNAase-I sensitivity during secondary induction of vitellogenin

A secondary stimulation, after a period of hormone withdrawal, is known to activate transcription of vitellogenin genes and causes de-induc-

tion of albumin synthesis more rapidly and extensively than during primary stimulation (Green & Tata, 1976; Baker & Shapiro, 1977; Farmer *et al.*, 1978; Searle & Tata, 1981). Fig. 4(a) shows that the enhanced sensitivity of vitellogenin genes that was maintained during the hormone-withdrawn state is retained to the same extent at 5 days after the second oestrogen administration, when vitellogenesis would be re-induced. At the same time, the DNAase-I sensitivity of albumin and the insensitivity of globin genes also remain unaltered (Figs. 4b and 4c). Thus it seems that the resumption of vitellogenin-gene transcription during secondary induction within the first month is not due to any further change in the configuration of genes coding for it, nor is it accompanied by an alteration of configuration of the albumin genes concomitant with the de-induction of albumin synthesis.

#### Discussion

##### DNAase-I sensitivity and transcription

Among the very first experimental evidence for the selective digestion of transcribed genes by

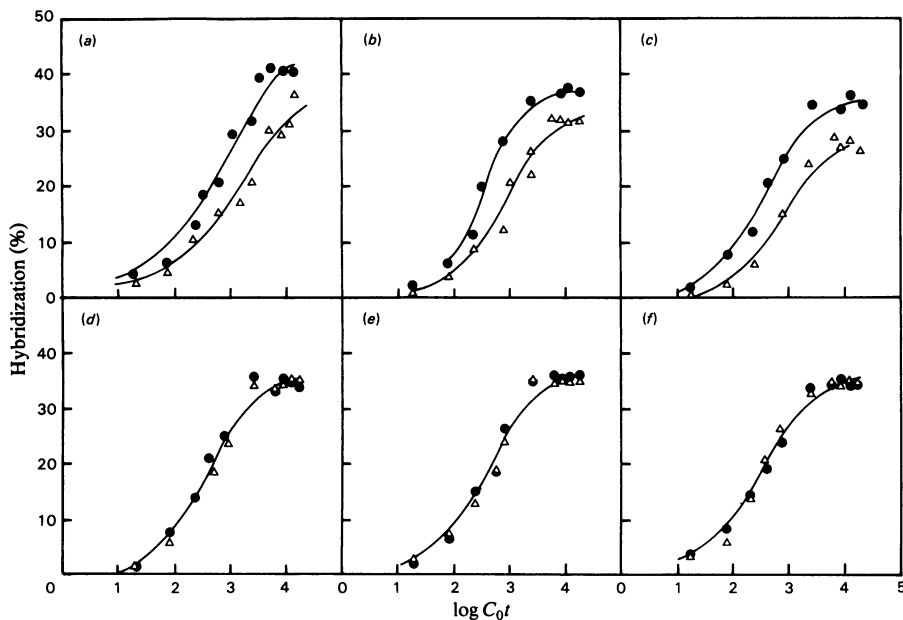


Fig. 2. Failure of oestrogen administration or withdrawal to alter the sensitivity to DNAase I of albumin gene in male *Xenopus* liver and erythrocyte nuclei

Kinetics of hybridization of [<sup>32</sup>P]cDNA to *Xenopus* albumin mRNA with DNA from liver (a, b, c) and erythrocyte (d, e, f) nuclei, digested (Δ) with DNAase I or not (●). (a, d), Controls; (b, e), frogs killed 5 days after first injection of oestradiol-17β; (c, f), frogs killed 20 days after hormone injection (hormone withdrawal). All conditions of hormone treatment, DNAase-I digestion of nuclei and cDNA–DNA hybridization were identical with those used for the vitellogenin probe, as described in Fig. 1.

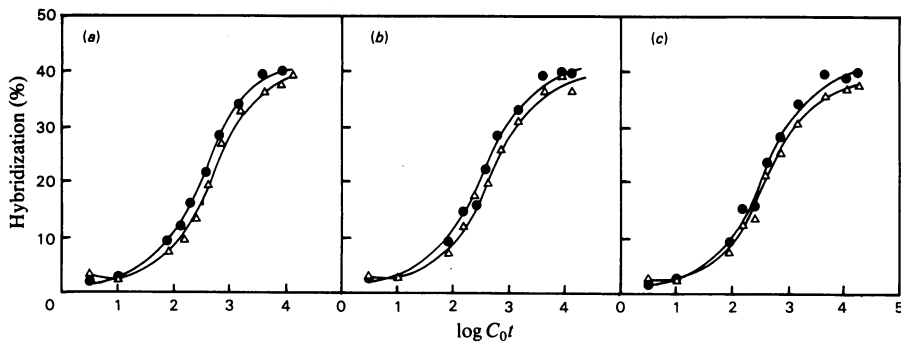


Fig. 3. Insensitivity of globin-coding sequences to DNAase-I digestion of nuclei from livers of male *Xenopus*, irrespective of oestrogen treatment or withdrawal

Hybridization of [<sup>32</sup>P]cDNA to *Xenopus* globin mRNA (input 10000 c.p.m. per sample) with DNA from nuclei digested (Δ) with DNAase I or not (●). (a), Uninjected controls; (b) 5 days after oestradiol injection; (c) 20 days after injection. All experimental conditions were identical with those for Figs. 1 and 2.

DNAase I was the increased sensitivity of ovalbumin genes in chicken oviduct nuclei after secondary induction of ovalbumin synthesis by oestrogen (Garel & Axel, 1976; Weintraub & Groudine, 1976). However, because of the technical difficulties involved in handling immature and hormonally unstimulated oviduct tissue, it has not been feasible

to study the possible alteration of ovalbumin-gene configuration during primary activation of transcription. The possibility of inducing vitellogenesis in male *Xenopus* hepatocytes, i.e. the cells in which the relevant genes would not have been expressed in their lifetime, has enabled us to demonstrate that oestrogen renders at least part of the multiple

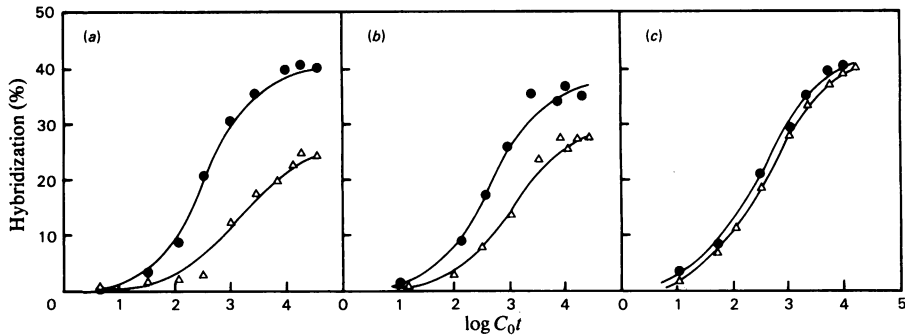


Fig. 4. Effect of secondary stimulation with oestrogen of male *Xenopus* on the sensitivity to DNAase I of sequences coding for vitellogenin, albumin and globin in liver nuclei

Kinetics of hybridization of [ $^{32}$ P]cDNA with DNA before (●) and after (△) DNAase-I digestion of liver nuclei from male *Xenopus* killed 25 and 5 days after the first and second injection of oestradiol-17 $\beta$ , respectively. (a), [ $^{32}$ P]cDNA to vitellogenin mRNA; (b), [ $^{32}$ P]cDNA to albumin mRNA (c) [ $^{32}$ P]cDNA to globin mRNA. All other conditions were as described in Figs. 1–3 for each of these cDNA species.

vitellogenin genes into a more transcribable configuration such that they would be more susceptible to DNAase-I digestion (Figs. 1a and 1b). Our results also show that, although the rate of transcription of *Xenopus* vitellogenin genes declines rapidly (Baker & Shapiro, 1977), they remain in an 'open' configuration for 3 weeks after primary induction (Fig. 1c). It is quite possible that at longer time intervals the vitellogenin genes would have re-acquired a 'quiescent' or DNAase-I-insensitive configuration. At the same time, the enhanced digestibility of vitellogenin genes that is observed 20 days after the first injection of oestradiol (Fig. 1c) may seem at first sight to be at variance with the relatively rapid reversion to a low DNAase-I sensitivity of the hormone-activated ovalbumin sequences in chick oviduct genome upon oestrogen withdrawal (Garel & Axel, 1976). However, Palmiter *et al.* (1978) have also reported the long-term retention of the 'open' configuration during the hormone-withdrawn state when transcription of the ovalbumin gene has ceased. A somewhat similar situation may also exist for the 'semi-permanent' changes in the configuration of globin genes during erythroid-cell differentiation (Miller *et al.*, 1978). These findings are also relevant to the 'pre-activation' state of adult haemoglobin genes observed during chicken erythrocyte maturation, since Stalder *et al.* (1980a) have found that in early stages of erythroid-cell development both the embryonic and adult chicken haemoglobin genes are sensitive to DNAase-I digestion, although only embryonic globin genes are transcribed during this period. Thus the relationship between DNAase digestibility and transcription of a given gene does not seem to be a rigorous one, except that active transcription of a gene is always reflected in an enhanced sensitivity to the enzyme.

#### Compartmentalization of expressed and unexpressed genes

Other findings from our laboratory have suggested that *Xenopus* vitellogenin genes, or at least the coding sequences, were represented as equivalent to more than the four actively expressed genes, of which only about one-third to one-half were in a potentially expressible state, irrespective of oestrogen stimulation (Tata *et al.*, 1980). They raise the possibility that the residual DNA, or the sequences that are relatively more resistant to DNAase I after 15–20% solubilization of DNA, that we have noticed in these studies may represent the less accessible or non-transcribable sequences of vitellogenin genes, even when these were actively transcribed (Figs. 1 and 4). The fact that the sensitivity to DNAase I of vitellogenin genes in liver was higher at 20 days than at 5 days after the first injection of oestradiol may be due to non-co-ordinated activation of the multiple genes. However, Felber *et al.* (1980) reported that the four major vitellogenin genes that are expressed in *Xenopus* liver are activated simultaneously. On the other hand, the hormone may cause some structural change in the unexpressed vitellogenin sequences (Tata *et al.*, 1980) so as to render them DNAase-I-sensitive. Such changes in DNAase-I sensitivity not accompanied by transcription have been reported for globin genes (Stalder *et al.*, 1980a). Restriction-enzyme-mapping analysis will allow us to answer the interesting question of whether or not the two populations of expressible and non-expressible vitellogenin genes are differently organized or compartmentalized in different gene domains. Such a possibility of compartmentalization of multiple-copy genes according to their potential for ex-

pression is unlikely to be restricted to vitellogenin genes, but may be of more common occurrence.

#### Regulation of gene expression by oestrogen

Finally, as regards the mechanism of action of oestrogen, it is generally agreed that the regulation of egg-protein synthesis by this hormone is the best model that we have that allows us to understand hormonal regulation of gene expression. The present findings of an enhanced sensitization of vitellogenin genes to DNAase I reinforces the view that during primary induction the hormone causes some preferential change in the chromosomal configuration of these genes. Since our studies were completed, Huber *et al.* (1981) have also reported that oestrogen causes an enhanced DNAase-I digestibility of *Xenopus* vitellogenin genes in a tissue-specific manner. However, the sustained sensitivity at early stages of hormone withdrawal and an absence of further sensitization to DNAase I during secondary stimulation that we have noticed suggest that transcription may also be regulated via mechanisms other than gene configuration, once the genes or gene domains have been activated during the primary induction process. Several investigators have speculated on the causes underlying the differences in kinetics of the vitellogenic responses during primary and secondary induction with oestrogen (Tata & Smith, 1979), including long-term changes in the amount of receptor (Westley & Knowland, 1979; Hayward *et al.*, 1980) and post-transcriptional mechanisms (Clemens, 1974; Farmer *et al.*, 1978). The application of recombinant-DNA techniques (Dawid & Wahli, 1979) to the reversible regulation of vitellogenesis directly in tissue culture (Searle & Tata, 1981) should make it possible not only to narrow down the different possible explanations for this intriguing phenomenon but also to bring us close to understanding the regulation of gene expression during development.

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