Association of transcriptionally active vitellogenin II gene with the nuclear matrix of chicken liver

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Supercoiled DNA loops linked to the nuclear matrix can be progressively cleaved with deoxyribonuclease I. The DNA which remains associated with the nuclear matrix can be purified and analysed for vitellogenin II sequence content by dot blot hybridization. Using this technique we show that vitellogenin II gene sequences are selectively associated with the nuclear matrix of liver but not with oviduct of laying hens. Following primary stimulation in immature chicks of vitellogenin synthesis with estradiol, the association of the gene with the nuclear matrix precedes vitellogenin mRNA synthesis. After 15 days when the level of vitellogenin mRNA has returned to zero, the gene is no longer preferentially associated with the nuclear matrix. At this time a second stimulation with estradiol results in a reassociation of the vitellogenin II gene with the nuclear matrix. In addition to the structural gene, both the 3' and 5' end flanking regions (1.5–2 kb) also bind to the nuclear matrix. However, beyond the limit of 1.5–2 kb upstream from the 5' end of the gene, there is no preferential binding of DNA to the nuclear matrix.

Key words: nuclear matrix/vitellogenin gene/estradiol

Introduction

The nuclear matrix is defined as the residual framework structure to which DNA is bound in supercoiled loops (Vogelstein *et al.*, 1980); it is obtained after extraction of the purified nuclei with high salt concentrations and detergents (Berezney and Coffey, 1974). According to Berezney and Coffey (1977) the nuclear matrix consists of nuclear protein components derived from three structural regions of the nucleus: a surrounding residual nuclear layer, a residual nucleolar structure and an internal matrix which closely resembles structures observed in the interchromatinic matrix of intact cells. DNA loops on the nuclear matrix can be directly visualized using either fluorescence or electron microscopy (McCready *et al.*, 1979; Vogelstein *et al.*, 1980; Lebkowski and Laemmli, 1982). Actively transcribing genes are selectively associated with the nuclear matrix (Robinson *et al.*, 1983; Hentzen *et al.*, 1984). For example, ovalbumin and conalbumin genes are associated with the nuclear matrix of the oviduct, which is actively synthesizing egg white proteins, but not with the nuclear matrix of other organs where no egg white proteins are synthesized (Vogelstein *et al.*, 1980; Ciejek *et al.*, 1983; Robinson *et al.*, 1983). Similarly the nuclear matrix from chicken erythrocytes selectively binds the active globin gene (Hentzen *et al.*, 1984). These observations were extended to other genes such as ribosomal genes of the rat liver cells (Pardoll and Vogelstein, 1980), the SV40 sequences in several lines of SV40 transformed 3T3 cells (Nelkin *et al.*,

1980), the polyoma and the avian sarcoma virus integrated into DNA of transformed rat cells (Cook *et al.*, 1982).

Miller *et al.* (1978), Herman *et al.* (1978) and Jackson *et al.* (1981) first showed that essentially all the rapidly labeled RNA is associated with the nuclear matrix which shares a common structural constituent with hnRNPs (Gallinaro *et al.*, 1983). In their studies on the precursor mRNA, Ciejek *et al.* (1982) have conclusively shown that the precursors of ovalbumin and ovomucoid mRNAs and all the precursors to rRNA are associated with the nuclear matrix fraction. This nuclear fraction also seems to bind selectively steroid receptor complexes (Hamana and Iwai, 1978; Barrack and Coffey, 1980; Rennie *et al.*, 1983) and functional replisomes (Smith and Berezney, 1982). Taken together, these observations strongly suggest that the nuclear matrix may play a very important role in DNA replication, gene transcription and processing of precursor mRNAs.

We have studied the association of vitellogenin II gene with the liver nuclear matrix during primary and secondary stimulation by estrogen. We show that association of the vitellogenin II gene with the nuclear matrix precedes vitellogenin mRNA synthesis. Upon cessation of transcription of the gene it dissociates from the nuclear matrix. Both the structural gene and its 3' and 5' end flanking regions are associated with the nuclear matrix.

Results

Vitellogenin gene is selectively associated with the nuclear matrix of the liver but not of the oviduct of laying hens

Under natural conditions, in laying hens the major egg yolk protein vitellogenin is synthesized only in the liver and not in the oviduct where all egg white proteins are synthesized (Bergink *et al.*, 1974). Since the synthesis of both egg yolk and egg white protein is under the control of estradiol it was of interest to see whether the association of the vitellogenin gene with the nuclear matrix is organ- and/or hormone-specific. Figure 1 shows the results of a dot blot experiment carried out with DNA derived from either intact nuclei or the nuclear matrix of liver or oviduct of laying hens. In liver nuclei with progressive deoxyribonuclease digestion of DNA there is (panel A) an increase in vitellogenin sequence bound to the nuclear matrix. In contrast, in the oviduct (panel B) with increased digestion there is a decrease in the matrix-bound vitellogenin gene sequence. It is noteworthy that in the liver, vitellogenin mRNA amounts to ~0.15% of total liver RNA, whereas in the oviduct the concentration was <0.0001% of total RNA. Hybridization of ovalbumin and chicken β -globin cDNA probes with total liver DNA and nuclear matrix-associated DNA showed no estradiol-dependent selective association of these genes with the nuclear matrix (data not shown). These results confirm earlier conclusions that active genes are preferentially associated with the nuclear matrix of the target cells or organ.

Fig. 1. Densitometry tracing of DNA dot blot assay of the vitellogenin II gene present in total nuclear DNA and nuclear matrix-bound DNA of the liver (panel A) and oviduct (panel B) of laying hens. Numbers in parentheses next to the inset photographs of dot blots indicate vitellogenin mRNA content expressed in % of total cellular RNA. The numbers below the density tracing indicate the normalized relative OD units of the tracing. Hybridization was carried out with the radioactive labeled clones pVT 22 and 3 (see Figure 3). Each dot had 2 μ g of either total or matrix-bound DNA.

Time course of the association of vitellogenin II gene with the liver nuclear matrix

In the liver of immature chicks exposed for the first time to estradiol (primary stimulation), there is a lag period before vitellogenin synthesis begins. With the disappearance of the primary hormonal stimulus the synthesis of vitellogenin gradually returns to pre-stimulation levels. If at that time a second dose of estradiol is administered (secondary stimulation) vitellogenin mRNA is synthesized at a maximal rate without a lag period (Jost *et al.*, 1978). The aim of the following experiment was therefore to determine the kinetics of association of the vitellogenin gene with the nuclear matrix of the liver, following primary and secondary stimulation by estradiol. Groups of four immature chicks were each treated with estradiol and after the indicated times nuclei were purified from the livers (Materials and methods section). DNA preparations from intact nuclei and from nuclear matrices of each group, were then assayed by dot blot hybridization for their content in vitellogenin gene sequences. Figure 2 shows that 4 h after primary stimulation with estradiol there is already a selective association of vitellogenin sequence with the nuclear matrix. At this time however, vitellogenin mRNA is barely detectable (0.0002% of total liver RNA) which means that the association of the gene with the nuclear matrix precedes the appearance of vitellogenin mRNA (mRNA concentrations are given in Figure 2 in parentheses). 15 days post-estradiol treatment the level of vitellogenin mRNA has returned to the baseline level and there is also an apparent dissociation of the vitellogenin gene sequence from the nuclear matrix. If a second dose of estradiol is given at that

Fig. 2. Time course of the association of vitellogenin II gene with the nuclear matrix during primary (p) and secondary (s) stimulation by estradiol. The numbers in parentheses below the inset photographs of the dot blot assay indicate vitellogenin mRNA content expressed in % of total cellular RNA. The numbers below the densitometry tracings indicate the normalized relative OD units of the tracing. Hybridization was carried out with the same cloned cDNA as for Figure 1. Each dot had 1 μ g of either total or matrix bound DNA.

time, concomitant with a rapid increase in vitellogenin mRNA synthesis there is also a rapid reassociation of vitellogenin gene with the nuclear matrix. These observations suggest that the association of vitellogenin gene with the liver nuclear matrix is reversible and that it correlates with the expression of the gene in its target organ.

Association of the structural gene and its 3' and 5' end flanking regions with the nuclear matrix

In an attempt to delineate the gene domain associated with the nuclear matrix we have used various cloned DNA probes covering different regions of the gene (Figure 3). DNA was prepared from intact nuclei of laying hens and from nuclear matrices containing 20% and 15% to total nuclear DNA respectively. One microgram of each DNA preparation was spotted onto nitrocellulose filters. Following hybridization with the appropriate labeled DNA probe, the blots were autoradiographed and the extent of hybridization was determined by densitometry tracing. Figure 4 shows that the whole coding sequence of vitellogenin gene is selectively associated with the nuclear matrix (probes 22 and 3). At the 5' end flanking region of the gene the association of DNA with the nuclear matrix does not exceed 1.5–2 kb (probe pVT 514). Beyond this limit (probes pVT 598-2 and pVT 599) there is no

Fig. 3. Cloned cDNA and genomic DNA probes used for the dot blot assay of vitellogenin II gene. The solid bars labeled pVT3, 22 and 329 indicate the extent of hybridization of cloned cDNA with vitellogenin II gene. The size of the cloned DNA fragments is given in kb below the solid bars. For the restriction endonucleases the following symbols were used. *Ava*I □; *Sal*I ■; *Eco*RI ○; *Bam*HI ●.

Fig. 4. Densitometry tracing of DNA dot blot assay of vitellogenin II gene plus its flanking region from total liver and nuclear matrix-bound DNA of laying hens. The map of the labeled probes pVT 1, 3, 22, 514, 598-2 and 599 used in this experiment are shown in Figure 3. Each dot had 1 µg of either total or nuclear matrix bound DNA.

more preferential association of DNA with the nuclear matrix. At the 3' end flanking region the preferential association of the gene with the nuclear matrix extends at least 2 kb (probe pVT 1) but because of the lack of suitable specific probes we did not investigate further regions downstream of the gene. We estimate that the minimum area associated with the nuclear matrix encompasses ~27 kb.

Discussion

During primary stimulation of vitellogenin synthesis by estradiol one of the first events observed in the liver is the association of the estradiol-receptor complex with the chromatin (Gschwendt and Kittstein, 1974). We now demonstrate that the association of the vitellogenin II gene with the nuclear matrix also precedes the onset of vitellogenin mRNA synthesis. However, the association of the gene with the nuclear matrix may not be mediated through the estradiol-receptor complex alone since in the oviduct, where no

vitellogenin mRNA is synthesized, there is a large number of estradiol receptor complexes bound to the chromatin but no selective association of vitellogenin gene with the nuclear matrix. Since precursor mRNA is also associated with the nuclear matrix (Ciejek *et al.*, 1982) one may question whether the association of the gene with the nuclear matrix is a consequence or the cause of initiation of transcription and processing of precursor mRNA. In this context it would be of interest to see whether specific inhibitors of transcription prevent the association of the gene with the nuclear matrix.

From previous experiments we know that estradiol stimulates DNA synthesis in the liver of immature chicks (Jost *et al.*, 1973), and Smith and Berezney (1982) have shown that replicating DNA binds to the nuclear matrix. Therefore one could argue that the observed association of vitellogenin gene with the nuclear matrix merely reflects DNA synthesis and replication. This does not seem to be the explanation of our results since the association of the gene with the nuclear matrix is much more rapid than DNA synthesis and, moreover, in adult laying hens where vitellogenin gene is also associated with the hepatic nuclear matrix, levels of DNA synthesis are extremely low (Jost, unpublished observation).

From the experiments shown in Figure 4 we estimate that the minimum area associated with the nuclear matrix is ~27 kb. In contrast to the ovalbumin gene where only the transcribed region is associated with the nuclear matrix (Ciejek *et al.*, 1983) we find that ~2 kb of 3' and 5' end flanking regions of vitellogenin gene are also selectively associated with the matrix. Since the 15% residual DNA bound to the nuclear matrix following deoxyribonuclease I treatment has an average size of ~10 kb, it is logical to assume that there are several independent anchorage sites of the vitellogenin gene and its flanking region on the nuclear matrix. This indicates that it is improbable that the active gene is looping out of the nuclear matrix since it would have been entirely digested from the nuclear matrix under high salt conditions and therefore not detectable with the labeled cloned DNA probes pVT 1, 3, 22 and 514. At present, the nature of the anchorage points between the gene and the nuclear matrix is totally unknown. In this context it would be of interest to follow the approach of Goldberg *et al.* (1983) who have cloned and characterized specific residual DNA sequences associated with the nuclear matrix in synchronized mouse 3T3 cells.

Materials and methods

Animal treatment

Immature white Leghorn chicks (200 g), both male and female and laying hens were used in all experiments. Estradiol was dissolved in propylene glycol at 40 mg/ml and injected i.m. into the leg (0.1 ml/100 g body weight). Control chicks received propylene glycol only.

Preparation of nuclei and nuclear matrix DNA

Nuclei were prepared from the pooled livers (4–5 per group) of hens, control and estradiol-treated chicks as described by Panyim *et al.* (1978). To the homogenization buffer, 0.1% Nonidet P 40, 1 mM phenylmethylsulfonyl-fluoride (PMSF) and 0.2% methylmethanesulfonate were added (Robinson *et al.*, 1982).

Nuclear matrices were prepared and digested with deoxyribonuclease I exactly as described by Robinson *et al.* (1982) and DNA from nuclear matrices and whole nuclei was purified by the method of Gross-Bellard *et al.* (1973). DNA concentration was determined by the diphenylamine reaction (Burton, 1956). For each batch of purified nuclei the kinetics of digestion with deoxyribonuclease I was established.

DNA probes

All DNA probes were either derived from either cloned vitellogenin cDNA

(Cozens *et al.*, 1980) or cloned genomic DNA (Wilks *et al.*, 1981) (see Figure 3). Isolated DNA fragments were labeled by nick-translation with [α - 32 P]-dATP to a specific activity of $1-5 \times 10^6$ c.p.m./ μ g DNA according to the method of Tsai *et al.* (1980).

DNA dot blot assay

The dot blot assay was essentially done according to Thomas (1980). Pre-hybridization was carried out at 37°C for 4 h in 50% formamide, 4 x SSC (1 x SSC in 0.15 M sodium chloride, 0.015 M trisodium citrate, pH 7.0) 0.2% sodium dodecylsulfate, 20 mM EDTA 0.05% sodium pyrophosphate, 5 x Denhardt solution (Denhardt, 1966) and 200 μ g/ml of purified denatured *Escherichia coli* DNA. Upon pre-hybridization, denatured labeled probes were added to the pre-hybridization solution and hybridization continued for 1–2 days at 37°C. Filters were then consecutively washed for 1 h at 37°C in 4 x SSC, 2 x SSC, 1 x SSC, 0.1 x SSC. Upon drying, filters were autoradiographed for 2–10 days with Kodak X AR-5 films plus amplifying screens. Densitometry was performed with a CAMAG TLC scanner. All scanning was made within the linear portion of a pre-established calibration curve.

Basic requirements for the DNA dot blot hybridization

One of the basic requirements for the cloned DNA and cDNA probes used in the dot blot assay is to hybridize only to the vitellogenin II gene and not to other related DNA sequences. This is the case for the genomic DNA fragments and cDNA probes used in the present work (Wilks *et al.*, 1981) with the exception of the cDNA clone pVT 329 which covers the central part of the gene (Figure 3). Previously it has been shown that this probe cross-reacts with pseudogenes or related DNA sequences (Wilks *et al.*, 1981). For this reason this cDNA clone has not been used in these experiments. Great care must also be taken to eliminate any trace amounts of RNA in the DNA preparation. This was best done by alkaline hydrolysis after the DNA had been treated with pancreatic ribonuclease A. DNA was determined by the diphenylamine reaction (Burton, 1956) and a titration curve was made by dot blot hybridization of total DNA fixed to nitrocellulose with labeled cDNA probe. The lowest concentration of DNA within the linear portions of the titration curve (usually 1–2 μ g per spot) was chosen.

Determination of vitellogenin mRNA sequences in total liver and oviduct RNA

Total liver RNA was prepared and hybridized with an excess of labeled cDNA previously described (Jost *et al.*, 1978). RNA dot blot hybridization was carried out according to Thomas (1980). A titration curve was established with purified vitellogenin mRNA. Quantitation of vitellogenin mRNA sequences was done only within the linear portion of the titration curve.

Materials

Restriction enzymes *Bam*HI, *Eco*RI, *Sal*I and *Ava*I were from Boehringer. PMSF and methylmethanethiosulfonate were obtained from Boehringer, Mannheim, and Sigma respectively. The radioactive nucleotide 3' deoxyadenosine 5'-[α - 32 P]triphosphate tetra (triethylammonium) salt (2000 Ci/mmol) was purchased from New England Nuclear.

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