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Attachment of vitellogenin genes to the nucleoskeleton accompanies their activation

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Summary: We have investigated the association of an inducible RNA polymerase II gene with the nucleoskeleton using the estrogen-inducible expression of the B2 vitellogenin gene in Xenopus liver as a model system. Using only physiological extraction conditions we find that the promoter region of the gene is strongly associated with the nucleoskeleton when it is transcriptionally active but much less so when it is inactive. We also find that the estrogen receptor protein, which is responsible for activation of this gene, is itself found associated with the nucleoskeleton. Finally, we show that newly synthesized, unspliced vitellogenin mRNA is also found on the nucleoskeleton. Our data suggest that expression of the B2 vitellogenin gene occurs only after it has become attached to the nucleoskeleton.

An increasing body of work is consistent with the view that transcription in eukaryotes takes place on a framework within the nucleus which has been called the "nuclear matrix", "nuclear scaffold" or "nucleoskeleton" depending upon the extraction methods used to manipulate the nuclei. Unfortunately, many of the methods used so far expose nuclei to nonphysiological extraction buffers and so it is difficult to be sure that the associations between nuclear components found in such assays are not merely the result of non-specific aggregation induced by the extraction procedure (see [1] for discussion). In order to avoid some of these problems Cook and his co-workers have developed extraction procedures which allow nucleoskeleton preparations to be isolated using only physiological buffers [2,3]. In their approach cells are first encapsulated in agarose microbeads, allowing the cells to be lysed in isotonic buffers with little if any of the mechanical breakage and aggregation of the chromatin which normally occurs on lysis of unprotected cells. Lysis releases soluble proteins and RNAs which diffuse out through the beads leaving intact chromatin. Subsequent treatment with restriction endonucleases followed by electrophoresis in a physiological buffer removes the bulk of the chromatin and leaves a fibrous nucleoskeleton with a small amount of residual chromatin attached to it. Nucleoskeleton preparations made in this way contain essentially all of the RNA polymerase activity present in the cell [3,4] and are enriched in transcribed sequences [4,5].Recent work has shown that both active ribosomal RNA genes and RNA polymerase I are associated with the nucleoskeleton in these preparations [6]. In order to test the models of transcription [7] which arise from such work, it is necessary to study the association of a specific RNA polymerase II gene with the nucleoskeleton,

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together with the locations of transcription factors involved in activation and the nascent transcript produced from the gene. A suitable candidate gene for such a study is the *Xenopus laevis* vitellogenin B2 gene. This gene is normally expressed exclusively in liver cells in response to estrogen and is active in female but not in male liver cells. The cis-acting sequences which confer estrogen responsiveness on the B2 gene are known [8] and the transcription factor principally responsible for activation in normal nuclei is the estrogen receptor protein [9,10]. Finally, the B2 gene is on a separate chromosome from the other three vitellogenin genes, which means that we can analyse the nuclear location a of chromosomal elements of this gene in isolation from the other genes in the family.

Materials and Methods

Preparation of nucleoskeletons and analysis of associated and unassociated DNA. Liver cells were isolated by collagenase digestion and encapsulated in 0.5% agarose microbeads as previously described [2,3,9]. Lysis with Triton x100, washing, digestion with restriction enzymes and electro-elution were all carried out as described [3] except that electro-elution was performed in a commercial apparatus to facilitate recovery of the unassociated material [5]. Chromatin which eluted from the beads was considered to be "unassociated", while that fraction which was resistant to elution was "associated" with the nucleoskeleton. DNA was purified from both fractions by standard methods, immobilised on a nylon membrane using a slot-blot apparatus and probed with random-primed probes made using either total genomic DNA, the isolated ECoRI-BglII fragment running from -779 to -42 upstream of the vitellogenin B2 gene or the EcoRI-BglII fragment running from -1550 to -40 upstream of the 68kD albumin gene.

Analysis of nucleoskeleton associated estrogen receptor. To measure the amount of endogenous estrogen receptor which was present in the nucleoskeleton associated fraction, nucleoskeletons were prepared as before from female cells. At various stages of the preparation samples were removed and the total amount of receptor present in each sample was quantitated by radio-immunoassay using a monoclonal antibody to the human estrogen receptor [10]. For the experiments where exogenous receptor was added to nucleoskeletons, radiolabelled receptor was synthesised by in vitro transcription and translation [9] and added to nucleoskeleton preparations made from male cells. The preparations were then washed thoroughly and re-electroeluted. The total protein remaining in the beads was then analysed by SDS-PAGE alongside the input receptor and the amount of receptor remaining associated with the nucleoskeleton was quantitated using an Ambis scanner.

PCR amplification of nucleoskeleton associated RNA. RNA was purified from female nucleoskeleton preparations, DNA was removed by centrifugation through CsCl followed by digestion with RNAase free DNAaseI. This RNA was then split into two equal fractions, one of which was subjected to alkaline hydrolysis. Both preparations were then reverse transcribed using a specific primer which hybridises to the second exon. The products as well as an aliquot of genomic DNA, were all amplified by PCR using as primers the above oligonucleotide and one which represents the first exon. The resulting PCR products were then analysed by agarose gel electrophoresis.

Results and Discussion

Active but not inactive vitellogenin genes are associated with the nucleoskeleton. In order to study the nucleoskeleton association of cis-acting vitellogenin sequences, we isolated liver cells from male (where the vitellogenin gene is not expressed) and female (where the gene is expressed at a high level) animals by collagenase digestion and encapsulated them in 0.5% agarose microbeads. After lysing the cell membranes with Triton x100, the chromatin was digested with

EcoR I and Bgl II and the chromatin fragments which were associated with the nucleoskeleton were separated from unassociated fragments by elecro-elution. DNA, purified from both fractions was immobilized on a nylon filter using a slot-blot manifold. Identical blots were then probed with either a total DNA probe or a vitellogenin probe covering the region from the EcoR I site at position -770 to the Bgl II site at position -42. This encompasses the promoter and the cis-acting control region of the gene including the estrogen response unit (ERU) to which the estrogen receptor binds. The sequences contained within this fragment are sufficient for accurate estrogen receptor dependant in vitro transcription [8]. We also examined the distribution of the sequences found upstream of the 68kD albumin gene, because it is expressed constitutively in both male and female cells. The albmin probe runs from an EcoRI site at -1550 to a BglII site at -49 and so represents a similar region of the albumin gene to that studied in the vitellogenin gene.
Figure 1 shows the result of this analysis. The distribution of the total genomic DNA between the two fractions is similar for both male and female liver cells, with only a minority of the chromatin being found associated with the nucleoskeleton. A different result is obtained for the vitellogenin gene. In this case it is clear that a much higher proportion of the upstream sequence is found associated with the nucleoskeleton in female liver cells than in male cells. This indicates that the vitellogenin promoter is strongly bound to the nucleoskeleton in female cells, where the gene is active, but much less so in male cells, where it is inactive. Moreover, the distribution of the albumin promoter between the associated and unassociated fractions is similar for both male and female cells, and the fraction found in the associated fraction resembles that found for the vitellogenin promoter in female cells. This shows that the albumin promoter is associated with the nucleoskeleton in both male and female cells, which correlates well with the fact that this gene is equally active in both male and female liver. These results suggest that the vitellogenin B2 gene exists in two states in hepatocyte nuclei. In one state, it is detached from the nucleoskeleton and is inactive. In the other, it is attached to the nucleoskeleton and can become active.

Endogenous estrogen receptor is associated with the nucleoskeleton of female liver cells.

We studied the intracellular location of the endogenous estrogen receptor in female liver cells by encapsulating them and measuring the amount of estrogen receptor by radio-immunoassay during the nucleoskeleton preparation. The amount present in unlysed cells (Total) was compared with the amount found after lysis alone (lysed), and with the amount remaining after lysis, chromatin digestion and electroelution to produce the final nucleoskeleton preparation (lysed, digested, electroeluted). Figure 2 shows that about 40% of the receptor is lost on lysis. Subsequent digestion and electroelution does not remove any more, indicating that the remaining receptor is strongly bound to the nucleoskeleton. These results agree with previous findings that about 50% of the total receptor in *Xenopus* liver is found in the nucleus, with the remainder being found free in the cytoplasm [11]. They also suggest that all the nuclear receptor in female liver cells is closely associated with the same sub-nuclear structure with which the active promoter sequences are associated, despite the fact that most of the other cellular proteins are removed by this procedure [3].

Exogenous receptor has an affinity for the nucleoskeleton.

The endogenous receptor is clearly strongly associated with the nucleoskeleton of female liver cells, which as we have shown is enriched in vitellogenin genes. We therefore wanted to see

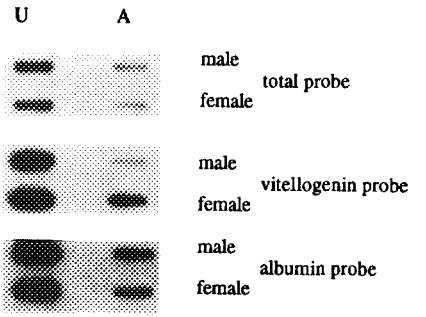

Figure 1. Preferential association of active promoter sequences with the nucleoskeleton. Nucleoskeletons were prepared from male or female liver cells and digested with EcoRI and BglII. The DNA from the nucleoskeleton associated (A) or the unassociated (U) fractions were probed with a total genomic DNA probe, a vitellogenin specific probe, or an albumin probe.

whether the presence of the receptor on the nucleoskeleton is simply a consequence of its normal association with the DNA of the estrogen response unit (ERU) in the vitellogenin promoter, or whether it can interact with the nucleoskeleton independently of the vitellogenin genes. To test this we added a trace amount of radioactive receptor made in vitro to nucleoskeleton preparations made from male liver cells, which contain very few vitellogenin sequences. Figure 3 shows that about 70% of the added receptor binds to the nucleoskeleton and is not removed by subsequent repeat electroelution. This suggests that the receptor recognizes and interacts with the nucleoskeleton independently of its interaction with the ERU of the vitellogenin gene. As the addition of estradiol-receptor complex to intact male liver nuclei results in activation of vitellogenin genes [9], these results suggest that activation occurs as a consequence of an interaction between the receptor-hormone complex, the gene and the nucleoskeleton.

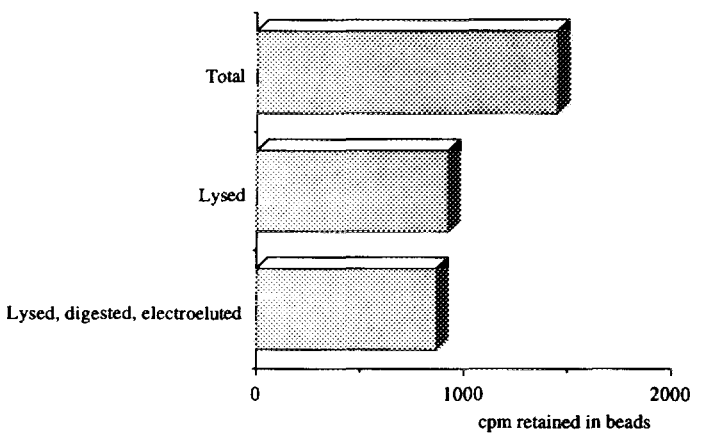

Figure 2. Association of endogenous estrogen receptor protein with female liver nucleoskeleton preparations. Estrogen receptor was assayed by radio-immunoassay during the nucleoskeleton preparation. Over 50% of the endogenous receptor remains associated with the lysed, digested and electro-eluted nucleoskeleton preparation.

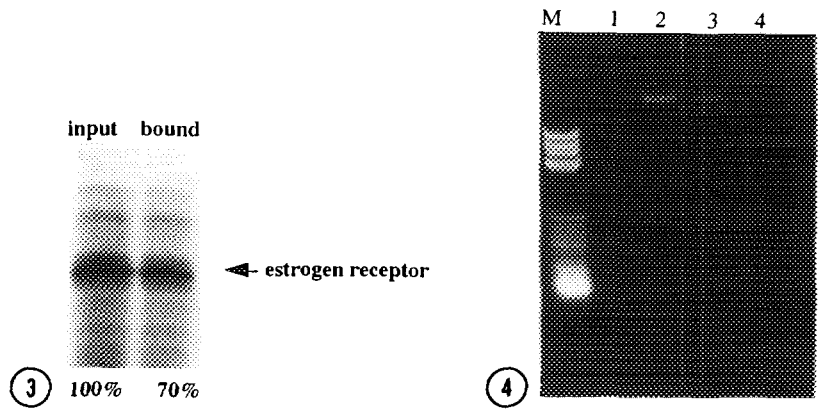

Figure 3. Exogenous estrogen receptor becomes associated with the male liver nucleoskeleton. Radiolabelled estrogen receptor protein was synthesised in vitro and added to isolated male nucleoskeleton preparations. The amount of receptor remaining after repeat electro-elution was then quantitated by fluorography, indicating that about 70% of the receptor bound to the nucleoskeleton.

Figure 4. PCR amplification of nucleoskeleton associated RNA. RNA was isolated from female nucleoskeleton preparations. The RNA was split into two fractions one of which was subjected to alkaline hydrolysis in order to destroy the RNA. The samples were reverse transcribed and amplified using primers spanning the first intron. Track 1 contains no DNA in the amplification reaction, track 2 shows the product of amplified genomic DNA, track 3 shows the amplified product from the nucleoskeleton associated RNA and track 4 shows the product from the same RNA except that alkaline hydrolysis preceeded the reverse transcription reaction.

Unspliced vitellogenin transcripts are associated with the nucleoskeleton.

The above data show that the promoter of an active vitellogenin gene and the estrogen receptor which is responsible for its activation are both associated with the nucleoskeleton. If transcription does occur as a result of this association one would expect to find the initial vitellogenin transcript on the nucleoskeleton. as the initial transcript is rapidly processed in liver cells and is therefore difficult to detect (10), we searched for it using PCR amplification. RNA was isolated from female nucleoskeleton preparations and subjected to reverse transcription using an antisense oligonucleotide primer from the second exon. The resulting cDNA was then amplified in a PCR reaction using a sense oligonucleotide from the first exon as the other primer. If there is any unspliced RNA present in the nucleoskeleton preparation the resulting PCR product should be the same size as that produced by amplification of genomic DNA. Figure 4 shows that a product of this size is indeed produced from the nucleoskeleton-associated RNA, indicating that the unspliced transcript is found on the nucleoskeleton. The product cannot be due to contaminating genomic DNA in the preparation because it is not found if alkaline hydrolysis to destroy the RNA precedes the reverse transcriptase reaction. This result shows that the initial transcript is firmly attached to the nucleoskeleton, and the simplest explanation for this association is that the initial transcript is produced on the nucleoskeleton.

Conclusions

We have found that when the vitellogenin B2 gene is being expressed, the controlling regions of the gene, the estrogen receptor protein responsible for transcriptional activation, and the initial

transcript resulting from the activation are all localized on a sub-nuclear structure, the nucleoskeleton. These findings suggest that an important step in actiavtion involves movement of a gene within the nucleus so that it becomes attached to the transcription apparatus on the nucleoskeleton. In the case of vitellogenin transcription, it appears that transcription occurs when the estrogen response unit interacts with the estrogen receptor complex on the nucleoskeleton, resulting in the attachment of the gene to the structure which contains the RNA polymerase. Thus our findings agree with a model in which the RNA polymerase is fixed on a nucleoskeleton, the relative movement between the polymerase and the gene necessary for transcription to take place occurring by reeling the DNA past the fixed polymerase [7].

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