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INHIBITION, TISSUE DISTRIBUTION AND HORMONAL SPECIFICITY OF A PROMOTER-BINDING FACTOR FOR THE UTEROGLOBIN GENE

P.B. Daniel, C. Molloy, J.C. Rayner, A.M. Hill and D.W. Bullock*

Centre for Molecular Biology, PO Box 84, Lincoln University,
Canterbury, New Zealand

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We had earlier reported a uteroglobin promoter-binding factor in nuclei from progesterone-stimulated rabbit endometrium that was inhibited by a factor in nuclei without progesterone stimulation or from non-target tissues (Rider, V., and Bullock, D.W., 1988. Biochem. Biophys. Res. Comm. 156, 1368-1375). In the course of purification of the inhibitory activity, the effect was shown to be due to contaminating genomic DNA. The inhibitor was destroyed by treatment with DNase I and resisted phenol-chloroform extraction. Fractionation of nuclear extracts on columns of DEAE-Sepharose separated the inhibitor and revealed the presence of binding activity in unstimulated or estrogen-treated endometrium, as well as in liver, lung and ovary. The tissue and hormonal specificity of the promoter binding factor is thus less restricted than recently reported.

Gene expression is regulated by factors that bind in complex relationships to flanking DNA or through protein-protein interactions to stimulate transcription (1). Some factors exert negative effects on transcription, for example IκB (2) and Id (3), some are ubiquitous (eg. Oct-1, 4), while some are tissue-specific (eg. MyoD, 5).

The rabbit uteroglobin (UG) gene has been widely used as a model for the regulation of transcription by steroid hormones. Progesterone induces UG transcription in the endometrium (6, 7) but has no effect in the lung where expression is constitutive (8, 9) or stimulated weakly by glucocorticoids (10, 11). Progesterone response elements located 2.4-2.7 kb upstream of the UG capsite bind purified receptors *in vitro* (12, 13) and are required for progesterone stimulation of transgene expression in the mouse endometrium (14, 15). By deletion (16) and linker scanning (17) analysis in Ishikawa cells, a human endometrial cell line, up to seven regions of the promoter have been implicated in unstimulated transcription including two octamer binding sites and a GT box recognized by Sp1-related proteins (18).

We had earlier reported a uteroglobin promoter-binding factor for the region -194/+9, revealed by gel shift assays of nuclear extracts from progesterone-stimulated endometrium (19, 20). Appearance of the binding activity coincided with the accumulation of UG mRNA and the factor was not detectable in liver, lung, unstimulated endometrium, or HeLa cells. We thus suggested the factor

* To whom correspondence should be addressed. Fax: (64)(3) 325-3851.

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was specific to the progestational uterus. Others have subsequently described this factor as being composed of two proteins specific to the uterus and regulated by progesterone and/or prolactin (21, 22). The factor is distinct from other factors reported to bind proximal promoter elements of the UG gene (16, 17, 18).

In our earlier work, we had also described inhibition of the gel shift by a second factor present in unstimulated endometrium or non-target tissues (20). This paper describes attempts to purify the inhibitor, which revealed that the inhibition is due to contaminating genomic DNA. Removal of the DNA by fractionation of nuclear extracts allowed the promoter-binder to be detected in several tissues and after different hormonal treatments.

MATERIALS AND METHODS

Animals. Adult New Zealand White virgin female rabbits were used without treatment or were injected subcutaneously with progesterone (3 mg/kg) or 17-β-estradiol (50 μg per animal) in sesame oil. Three regimes of daily injections were used: 5 days progesterone; 5 days estrogen; and 5 days progesterone followed by 5 days estrogen. Animals were sacrificed one day after the final injection.

Preparation of nuclear extracts. Nuclei from endometrial scrapings, ovaries, liver and lung were prepared as described (20) except that nuclei were centrifuged through the 2 M sucrose cushion in an SW 50.1 rotor at 37,400 g (23) for 90 min at 4 °C. Nuclei were extracted for 10 min on ice in 20 mM Tris-HCl, pH 7.5, 0.4 M KCl, 0.25 M sucrose, 5 mM $MgCl_2$, 5 mM DTT containing a cocktail of proteinase inhibitors (20) then centrifuged at 1500 g for 10 min at 4 °C. Nuclear extracts were assayed for protein (24) using rabbit gamma globulin as a standard, then made 15% in glycerol and stored at -100°C.

DNA radiolabelling and gel shift assays. The plasmid pUG203 contains an Ava I/BamH I UG promoter fragment (-194/+9) subcloned into Sal I/BamH I-digested pBluescript SK^- (Strategene, La Jolla, CA). UG203 was excised with Xho I and Xba I and end-labelled using Klenow and [α^{32}P] dCTP. Protein-DNA binding reactions containing 0.6 ng ^{32}P-labelled UG203, 4 μg poly(dI-dC).poly(dI-dC) and nuclear extract in 20 μl 15 mM Tris-HCl, pH 7.5, 12% glycerol, 1 mM EDTA, 5 mM $MgCl_2$, 5 mM DTT (final concentrations) were incubated for 30 min at 15°C, then electrophoresed on 4% polyacrylamide (acrylamide:bisacrylamide, 80:1) gels as described (20). Inhibition of UG203 binding was assayed by combining crude nuclear extracts (10 μg) or dialysed DEAE fractions (up to 15 μl) with 8 μg crude nuclear extract from progestational endometrium in the gel shift assay.

Purification of inhibitor. A crude nuclear extract from rabbit liver was passed through DEAE-Sepharose at 0.34 M KCl and inhibitor was eluted with 1.0 M KCl. The KCl was diluted to 0.34 M and this step was repeated. Fractions containing inhibitor were dialysed against 20 mM Tris-HCl, pH 7.5, passed through cellulose phosphate equilibrated with the same buffer, then bound to an Econopac Q column (Bio-Rad, Hercules, CA). Inhibitory fractions, eluted with 0.6 M KCl, were dialysed against 20 mM Tris/HCl, pH 7.5, bound to heparin-agarose equilibrated with the same buffer and eluted with 0.5 M KCl. The purified material was separated by SDS polyacrylamide gel electrophoresis (25) and silver stained (26) or eluted from gel slices and renatured as described (27).

RESULTS AND DISCUSSION

Purification of inhibitor. Liver nuclear extracts were chosen as an abundant source of inhibitor (20) for purification. Fractionation of inhibitory activity during chromatography on DEAE-Sepharose, phosphocellulose, and heparin-agarose was monitored by abolition of the gel shift in mixing experiments with progestational uterine extracts. The purified inhibitor fraction was then analysed by SDS-gel electrophoresis and inhibitory activity identified by assaying material eluted from gel slices.

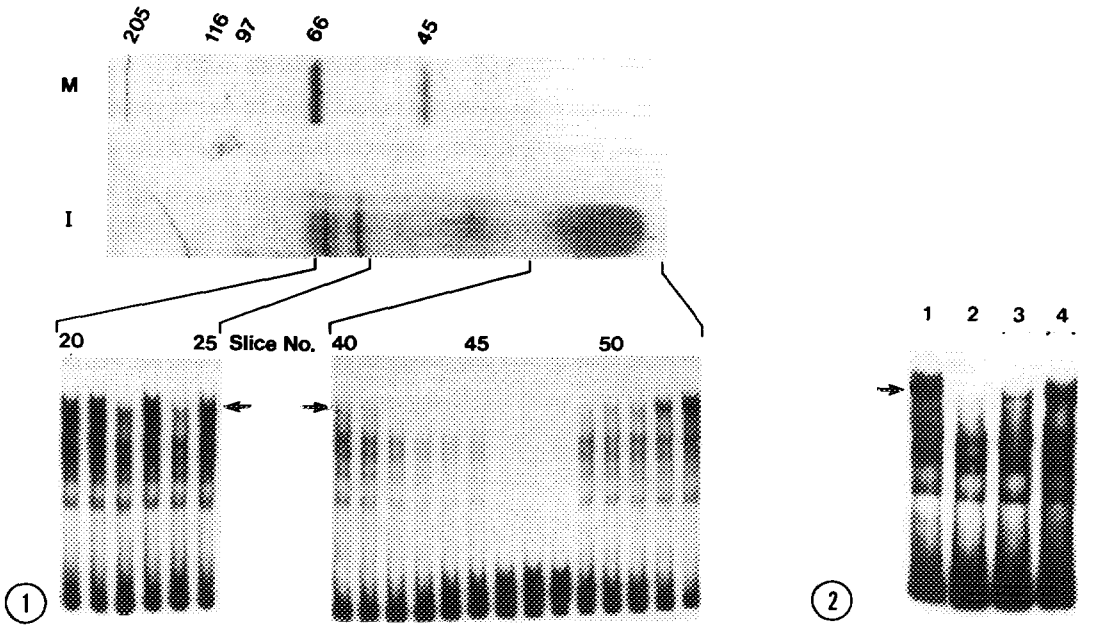

Figure 1. **Identification of inhibitor after electrophoresis on a 10% SDS-polyacrylamide gel.** One lane (I) was silver-stained and another was cut into 1.8-mm slices. Material from the slices was eluted, renatured, and tested for inhibition in the gel shift assay. The presence or absence of the gel shift (arrows) is shown for slices corresponding to silver-stained regions. M = molecular weight standards (000s).

Figure 2. **Abolition of inhibitor by DNase I.** After chromatography of a nuclear extract from unstimulated endometrium on DEAE-Sepharose, a fraction shown to be inhibitory by mixing in a gel-shift assay was treated with or without DNase I. Lane 1 shows the expected gel shift (arrow) without inhibition. Lane 2 shows the inhibitory fraction without DNase I after 16h at 0°C. Lane 3 shows the fraction without DNase I after incubation for 16h at 37°C in DNase I buffer (15 mM Tris/HCl, pH 7.2, 1 mM $MgSO_4$, 0.1 mM DTT, 5% glycerol). Lane 4 shows restoration of the gel shift after incubation with DNase I (1 U/μl) for 16h at 37°C.

Figure 1 shows two distinct protein bands and an amorphous stained region of lower molecular weight in the purified extract. The reconstituted inhibitory activity coincided with this low molecular weight region (Fig. 1). Electrophoresis of material from this region on an agarose gel and staining with ethidium bromide revealed low molecular weight DNA (100-300 bp, not shown).

This finding led us to re-examine the inhibitory activity in unstimulated endometrium we had initially reported (20). A crude nuclear extract from unstimulated rabbits was fractionated on DEAE-Sepharose and the inhibitory activity was recovered by step-elution with 1.0 M KCl. The inhibitory activity resisted phenol-chloroform extraction and ethanol precipitation, and electrophoresis on agarose revealed the presence of DNA in all inhibitory fractions (not shown). To confirm that all of the inhibitory activity could be accounted for by DNA, a DEAE-fraction containing inhibitor was treated with DNase I under conditions that did not degrade the probe in the gel-shift assay. As shown in Figure 2, DNase I removed the inhibitor and restored the gel shift in the presence of the treated extract. We conclude that the inhibitor is contaminating genomic DNA, probably from shearing during nuclear extraction.

Tissue and hormonal specificity of the promoter-binder. Since the inhibitor was detected by mixing experiments with extracts of non-target tissues (20) and was now shown to be DNA, it was possible that the failure to detect binding activity in non-uterine tissues could be due to masking of the gel shift by competing DNA. By the same token, the apparent hormonal specificity of the promoter-binder might be an artifact. To investigate this possibility we subjected nuclear extracts of various tissues and of endometrium after different hormonal treatments to fractionation on DEAE-Sepharose. Fractions eluted from the column were tested both for the gel shift and for inhibitory activity.

Endometrium from estrogen-treated rabbits was one hormonal condition in which the binding factor could not be detected and inhibitor was found by mixing experiments (unpublished). Figure 3a shows estrogen-treated endometrium in which no gel shift was detected in the crude extract, whereas after DEAE-fractionation a strong shift appeared in the column flow-through (fraction 4). Inhibitory activity was found in high-salt wash fractions (not shown) thereby accounting for the absence of the shift in the crude extract.

Earlier work had shown that stimulation of uteroglobin secretion by treatment with progesterone was reversed by estrogen (28). We examined this treatment for the presence of the binding factor in endometrial nuclear extracts after DEAE-fractionation. Figure 3b shows that the gel shift was prominent in the low-salt eluate (fraction 2), whereas inhibitor was recovered in the high-salt wash (not shown). This hormone regime was also used in a study of protein binding to UG203, where emphasis was placed on quantitative estimation of the intensity of the gel shift obtained with crude nuclear extracts (22). Figure 3b illustrates the dangers in this situation, where the presence of the binder in the crude extract was severely underestimated. Undetected DNA contamination could lead

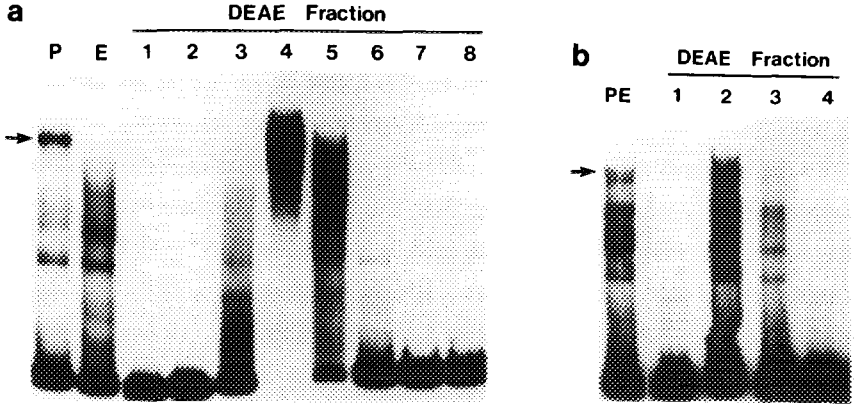

Figure 3. **Detection of the binding factor after removal of inhibitor.** (a) The gel-shift seen with progesterone stimulation (P, arrow) is not present in estrogen-treated endometrium (E) but is detectable in the column flow-through after DEAE-fractionation of the E-extract (fraction 4). (b) Binding activity is reduced after treatment with progesterone (5 days) followed by estrogen (5 days) (PE, arrow), but enhanced in the column flow-through after passage over DEAE-Sepharose (fraction 2).

to misinterpretation of results and caution must be exercised in examining DNA-binding proteins by the gel-shift assay, particularly in quantitative terms, in crude nuclear extracts.

In a similar way, DEAE-fractionation also revealed the promoter-binder in lung, liver, ovary, and nonstimulated endometrium (not shown), tissues in which this factor was reported to be undetectable (20, 21).

Unlike well-defined protein inhibitors of transcription factors (2, 3) inhibition of a promoter-binding factor for the rabbit uteroglobin gene has been shown to be an artifact of contaminating genomic DNA. When this contamination is removed, the UG promoter-binder can be detected in tissues and under hormonal conditions where it was previously thought to be absent.

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