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A novel nuclear transcription system which responds correctly to cloned estrogen receptor

Andrew Thorburn and John Knowland*

Department of Biochemistry, South Parks Road, Oxford, OX1 3QU, UK

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ABSTRACT

We describe here a novel but simple nuclear transcription system in which nuclei make RNA in an isotonic buffer for a long time and respond to a cloned external factor by accurately initiating new transcription.

INTRODUCTION

Our knowledge of how chromosomal genes in the nuclei of normal cells are regulated is still very limited, mainly because attempts to develop efficient nuclear transcription systems have met with only limited success. In most cases, transcription does not initiate in isolated nuclei, which simply complete the synthesis of nascent chains ("run-off transcription"), and do not respond to external factors.

Our aim here was to construct a nuclear transcription system which would operate in a defined environment and respond to an identifiable factor. As a model system we used the activation of chromosomal vitellogenin genes in Xenopus liver nuclei. Extensive studies have shown that vitellogenin genes are activated by estrogen and that the estrogen receptor is essential, although not perhaps sufficient, for activation. There has been a great deal of work on how the receptor activates vitellogenin genes in plasmids (1,2), but very little on how it activates chromosomal vitellogenin genes in nuclei. If nuclear extracts enriched in estrogen receptor are injected into Xenopus oocytes, the vitellogenin genes in the oocyte nuclei are activated, although the primary transcript seems to be rapidly and probably inaccurately processed (3). When liver nuclei are incubated with extracts containing estrogen receptor, it appears

that vitellogenin genes may be activated (4,5), although conclusive evidence for accurate, de novo initiation of vitellogenin transcription in such systems has not been obtained. Here, we expose liver nuclei from untreated male frogs to cloned estrogen receptor under conditions which support long-lived transcription and find accurate initiation of vitellogenin transcription as demonstrated by primer extension.

MATERIALS AND METHODS

Xenopus hepatocytes were isolated by digestion with collagenase and dispase and purified on Percoll gradients (6). Encapsulation in agarose beads, treatment with Triton and incubation in simple transcription buffer were as described by Jackson and Cook (7). Cells were treated with lysolecithin essentially as described by Gurdon (8) and then encapsulated in agarose plugs. Isolated hepatocytes (5×10^6 to 10^7) were suspended in 500 µl of SuNaSp (0.25 M sucrose, 75 mM NaCl, 0.5 mM spermidine trihydrochloride, 0.15 mM spermine tetrahydrochloride) by gentle pipetting using a cut-off blue Gilson tip. Lysolecithin (Sigma type I) was added to a final concentration of 20 µg/ml and the cells were gently shaken for 2-3 minutes at room temperature. The action of the lysolecithin was stopped by adding 1 ml of ice-cold SuNaSp containing 3% BSA. The permeability of the cells was checked microscopically using Trypan Blue - permeable cells no longer exclude the dye. The cells were then spun down (800g for 5 minutes at room temperature) and suspended in our modified transcription buffer (10^6 cells in 100 µl). An equal volume of molten 1% low melting point agarose (Seaplaque) in modified transcription buffer was added and mixed by pipetting with a cut-off tip. The mixture was pipetted into the wells of a microtitre dish and chilled for 5 minutes at 4° C to form plugs. Our modified transcription buffer contains 15 mM HEPES pH 7.4, 140 mM KCl, 5 mM $MgCl_2$, 1.3 mM dithiothreitol, 0.2 mM disodium EDTA; 0.2 mM spermine trihydrochloride, 0.5 mM spermidine tetrahydrochloride; 10 µM each of ala, arg, asn, asp, cys, gln, glu, gly, his, ile, leu, lys, met, phe, pro, ser, thr, try, tyr, val; 1 mM glucose, 10% v/v glycerol; 1 mM each of CTP, GTP and UTP, 2 mM ATP; 24 mM creatine phosphate, 50 µg/ml creatine

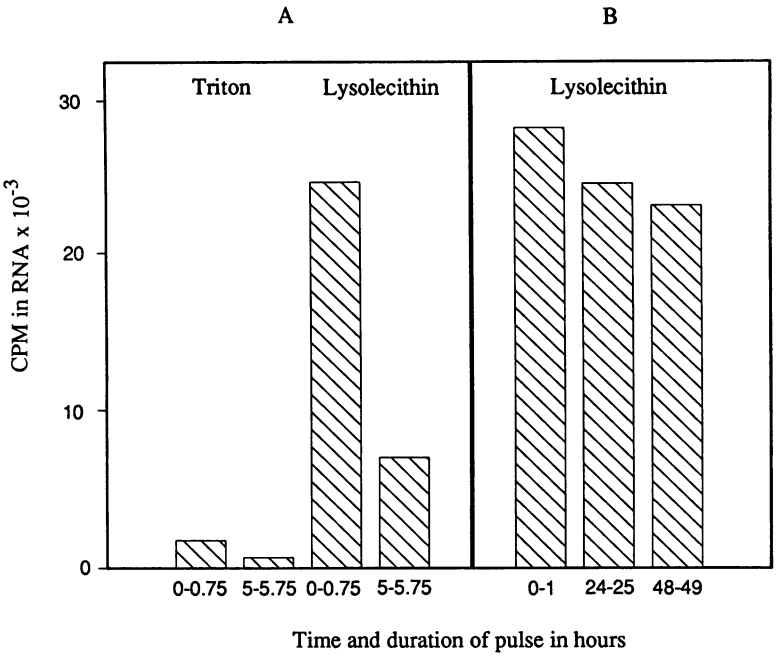

Figure 1. The effects of Triton, lysolecithin and buffer composition on transcription in nuclei in agarose.
A. Transcription in beads; simple buffer. Male hepatocytes were encapsulated in 0.5% agarose beads (12) and lysed with either Triton (7) or lysolecithin (14). Approximately 10^6 encapsulated cells were then incubated at 20° in 0.5 ml of transcription buffer (7). After pulsing for 45 min with 25 µCi of 5-[3H]-UTP (Amersham) at time zero or after 5 hours total RNA was purified by repeated phenol extraction at 65° followed by digestion with proteinase K, RNase-free DNase (Promega RQ1) and extraction with phenol-chloroform. RNA was precipitated in 8% TCA, collected on GF-C filters and counted.
B. Transcription in plugs; modified buffer. Hepatocytes were lysed with lysolecithin, and approximately 10^6 were suspended in 100 µl of our modified transcription buffer and set in agarose plugs. One plug (200 µl) was added to 300 µl of modified transcription buffer, and 1-hour pulses of 5-[3H]-UTP were given as shown.

kinase; 100 µg/ml tRNA, 100 units/ml RNasin (Promega).
Estrogen receptor mRNA was made in vitro (9), capped and translated in a mRNA-dependent reticulocyte lysate (10). Receptor was partially purified by precipitation in 66% ammonium sulphate (11) and assayed by 3H-estradiol binding (12).

RESULTS AND DISCUSSION

We combine the main aspects of two techniques. One is designed to minimize degradation of the chromosomal DNA and the other to preserve its activity. In the first, molten agarose is added to a suspension of cells. The suspension is shaken with liquid paraffin and chilled, yielding cells encapsulated in tiny agarose beads and suspended in an immiscible phase (13). After washing to remove the paraffin the cells are lysed with Triton (14). Most of the cytoplasmic materials are lost, but at least some of the overall organization of the chromosomal DNA in the encapsulated nuclei seems to be preserved, a point which may well be important for accurate activation of chromosomal genes, and the DNA becomes accessible to external proteins (14). Such nuclei also contain an active RNA polymerase II (7), but they cannot re-initiate transcription.

In the second, cells are treated briefly with a natural detergent, lysolecithin, and the action of the lysolecithin is stopped by adding albumin, yielding a suspension of nuclei in cytoplasm (8). If the suspension is injected into Xenopus oocytes the nuclei remain transcriptionally active for a very long time, and some dormant genes in the nuclei are reactivated (15). If Triton is substituted for lysolecithin the nuclei soon die (8). This shows that transcription can re-initiate in nuclei prepared by lysolecithin treatment, but it is not clear whether the efficient transcription demonstrated using oocytes can also operate in vitro. We therefore decided to encapsulate liver cells in agarose, to treat them with lysolecithin to make their nuclei accessible, and then to see if we could alter their pattern of transcription by adding cloned estrogen receptor.

We first encapsulated liver cells in 0.5% agarose microbeads, treated them with either Triton or lysolecithin, incubated them in the buffer described by Jackson and Cook (7), and pulsed them with 3H-UTP at time zero and 5 hours later. Fig 1A shows that the lysolecithin-treated cells incorporate 3H-UTP to a much greater extent than the Triton-treated cells, and that even after 5 hours of incubation there is still significant transcription. Next, we found that it is preferable to treat

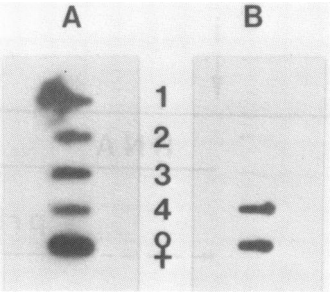

Figure 2. Receptor protein induces vitellogenin sequences in lysed male liver cells.

For each sample, approximately 10^6 lysolecithin-lysed male liver cells embedded in one agarose plug (200 µl) were incubated in 300 µl of our transcription buffer containing 5×10^{-9} M estradiol, and 10^{10} receptor molecules in 50 µl were added. In the control samples (no receptor) water replaced mRNA in the original translation. Samples were frozen immediately or after a 24-hour incubation. RNA was isolated and slot-blotted with RNA from female liver onto duplicate filters and probed with albumin and vitellogenin probes. The albumin probe was made by random primed labelling using an Eco RI-Bam HI fragment of the 68 kd albumin gene which runs from -1.6 kb to + 1.05 kb and contains the first 2 exons (18). The vitellogenin probe was made by SP6 transcription of a fragment of the B2 gene which runs from -42 to +220 (19). The probes were hybridized in 50% formamide, 50 mM sodium phosphate pH 6.5; 5 x SSC, 1 mM disodium EDTA, 0.1% SDS, 5 x Denhardt's, 200 µg/ml salmon sperm DNA, using 42° for the albumin probe (DNA) and 55° for the vitellogenin probe (RNA). Filters were washed for 2 x 30 min at room temperature in 2 x SSC, 0.1% SDS and the vitellogenin filter was also incubated for 30 min at room temperature in 2 x SSC containing 10 µg/ml RNase A. Both filters were then washed in 0.2 x SSC, 0.1% SDS first at 55° and then at 68° and dried. Rows 1 and 2: no receptor present, 0 and 24 hours of treatment. Rows 3 and 4: receptor present, 0 and 24 hours of treatment. Final row: RNA from approximately 10^5 female liver cells. Column A: hybridization with albumin probe: column B: hybridization with vitellogenin probe.

cells with lysolecithin before rather than after adding agarose because it is then easier to stop the action of the detergent quickly and at a precise time. We also found that it is unnecessary to make microbeads and that it is simpler to embed the lysed cells in agarose plugs instead. This avoids the use of liquid paraffin and the extensive washing needed to remove it, thereby minimizing the disturbance to the chromatin contained in the agarose, and is also technically simpler.

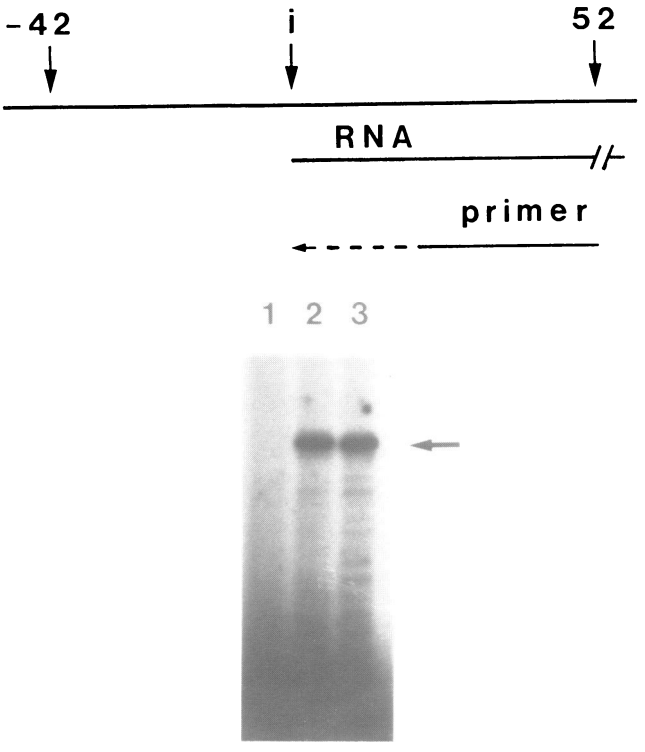

Figure 3. Receptor induces correct initiation of vitellogenin RNA synthesis.
Lysed male liver cells were treated with or without receptor for 24 hours as in Figure 2, and the RNA was hybridized overnight at 55° in 10 µl of 0.4 M NaCl, 10 mM PIPES pH 6.4 to a 5'-labelled synthetic DNA primer (5'-CCGCTAGAGCGAGAAGCAGAGCAAGTATGAT-3') complementing positions +22 to +52 in the B2 vitellogenin gene (20). The 5'-end of the primer coincides with the splice site at the end of the first exon. The primer was extended using AMV reverse transcriptase as described by Williams and Mason (21) and run on a 16% sequencing gel. The halo of radioactivity towards the bottom of the gel comes from excess primer, and the arrow marks the position of the extended primer (52 nucleotides). Lane 1: approximately 10^6 lysed male cells, no receptor present, lane 2: approximately 10^6 lysed male cells, receptor present; lane 3: RNA from approximately 10^5 female liver cells.

Conditions which promote long-lived transcription.

Finally, we modified the buffer used for transcription. We omitted ammonium sulphate because ammonium ions are toxic to intact cells, increased the KCl concentration, added spermine as

well as spermidine, amino-acids because they are normally found in cell cytoplasm, and an energy source (glucose and glycerol). We also added an ATP-generating system, arguing that a steady supply of nucleoside triphosphates would be needed if transcription were to last for a long time. It emerged that these additions did indeed greatly extend the life-time of the system, and we found efficient incorporation of 3H-UTP even if a pulse was given after 48 hours of pre-incubation (Fig 1B). Then, in order to see whether external proteins can function in this system, we incubated male liver nuclei with cloned estrogen receptor and tested for activation of a vitellogenin gene.

Accurate initiation of transcription in nuclei.

We made estrogen receptor by in vitro translation (10) of capped mRNA (9), added it to liver nuclei in agarose plugs, isolated RNA after 0 or 24 hours of incubation with a physiological concentration of estradiol and probed it using albumin and vitellogenin probes. Under these conditions the expression of albumin genes is independent of estrogen (16, 17), and Fig 2A shows that all the samples contained approximately equal amounts of albumin RNA and hence of total RNA. We did not detect significant amounts of vitellogenin RNA in male nuclei treated with estradiol alone, but on incubating them with estradiol and receptor we found vitellogenin RNA after 24 hours (Fig 2B). Using primer extension, we found (Fig 3) that the new transcripts initiate at the site used in normal female liver cells. We conclude that in the system described here new transcription initiates accurately in nuclei. It also appears that occupied receptor is the only extra factor needed for accurate activation of the vitellogenin gene in male liver nuclei, although we cannot be certain because the receptor preparations inevitably contain components from the reticulocyte lysate used to make them.

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*To whom correspondence should be addressed

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