Cell-free transcription in *Xenopus* egg extract

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**ABSTRACT**

Soluble extracts prepared from *Xenopus* eggs have been used extensively to study various aspects of cellular and developmental biology. During early egg development, transcription of the zygotic genome is suppressed. As a result, traditional extracts derived from unfertilized and early-stage eggs possess little or no intrinsic transcriptional activity. In this study, we show that *Xenopus* nucleoplasmic extract (NPE) supports robust transcription of a chromatinized plasmid substrate. Although prepared from eggs in a transcriptionally inactive state, the process of making NPE resembles some aspects of egg fertilization and early embryo development that lead to transcriptional activation. With this system, we observed that promoter-dependent recruitment of transcription factors and RNA polymerase II leads to conventional patterns of divergent transcription and pre-mRNA processing, including intron splicing and 3’ cleavage and polyadenylation. We also show that histone density controls transcription factor binding and RNA polymerase II activity, validating a mechanism proposed to regulate genome activation during development. Together, these results establish a new cell-free system to study the regulation, initiation, and processing of mRNA transcripts.

INTRODUCTION

The eggs of *Xenopus laevis* frogs contain a high concentration of maternal factors that support early embryo development after fertilization (1,2). Soluble extracts prepared from *Xenopus* eggs have been used extensively to study various aspects of cellular and developmental biology, including nuclear formation (3-5), DNA replication and repair (6-9), cellular and checkpoint signaling (10-13), mitosis (14-16), and apoptosis (17). However, these extracts have been found to possess little or no intrinsic transcriptional activity (18), limiting study of a fundamental biological process with this model system.

The primary characteristics of *Xenopus* egg extracts are determined by the developmental stage of the eggs from which they are derived (described in Figure S1) (19). Newly laid eggs are arrested in metaphase II of meiosis. After fertilization, eggs progress to an interphase state that is transcriptionally inactive. Chromatin then undergoes decondensation and is enveloped by membranes to form a nucleus. Although limited transcription of the nuclear genome can occur, further development is dependent on maternal proteins and mRNA provided by the egg cytoplasm (1,2). The single-cell embryo then undergoes multiple rounds of rapid DNA synthesis and cellular division to form a fluid-filled sphere of cells called a blastula. At this point in embryo development, the genome transitions to a transcriptionally active state through a process referred to as the mid-blastula transition (MBT) (20).

Recent studies have identified histones as an important regulator of the MBT, suggesting they act as a sensor for the number of cellular divisions (21-23). DNA is bound by histones to form chromatin, which supports DNA compaction
and acts as a scaffold for regulating various aspects of transcription (24,25). During early embryo development, the concentration of maternal histones remains constant. However, each round of DNA synthesis increases the ratio of DNA to histones. As histones become limiting, promoter elements throughout the genome are thought to become more accessible to transcription factors that trigger a wave of transcriptional activity. The MBT is characterized by several cellular changes that promote differentiation and further embryo development, including slower cell cycles with extended S phase, asynchronous cellular divisions, and cellular motility (20).

Previously, a nucleoplasmic extract (NPE) was developed that contains a highly concentrated fraction of nuclear proteins (26). NPE supports highly efficient chromatinization and synthesis of plasmid DNA substrates, and has led to seminal discoveries in DNA replication and repair (27-31). However, the transcriptional activity of NPE has not been determined. Although prepared from eggs in a transcriptionally inactive state, the process of making NPE recapitulates several events during egg fertilization and early embryo development that lead to transcriptional activation (Figure S1). When eggs are crushed by centrifugation, calcium release drives the extract into interphase, mimicking the events following egg fertilization. Addition of sperm chromatin then leads to nuclear formation and chromatin condensation, followed by progression into S phase and DNA synthesis.

In this study, we demonstrate that NPE readily supports transcription from endogenous gene elements on a naturally chromatinized plasmid substrate. Promoter-dependent recruitment of transcription factors and RNA polymerase II (RNAPII) leads to conventional patterns of divergent transcription and pre-mRNA processing, including intron splicing and 3’ cleavage and polyadenylation. We also show that histone density regulates transcription in NPE by limiting the recruitment of transcription factors to DNA, validating a mechanism proposed to control genome activation during early development (21). Together, these results establish a new cell-free system that supports multiple mechanisms involved in the regulation, initiation, and processing of mRNA transcripts.

RESULTS

Nucleoplasmic extract supports robust transcription of plasmid DNA

To determine the relative efficiency of transcription in NPE, we compared its activity with other Xenopus egg extracts shown to have limited transcriptional activity, including HSS (a high-speed supernatant of interphase-arrested eggs) and CSF (a mitotic extract from eggs arrested in metaphase II by a “cytostatic factor”). Each extract was incubated with increasing concentrations of a GFP reporter plasmid that contains a Cytomegalovirus (CMV) promoter (Figure 1A; pCMV). The CMV promoter was previously shown to support transcription in Xenopus oocytes (18) and cultured somatic cell lysate (32), indicating that it is recognized by Xenopus transcription machinery. Extracts were supplemented with [α-32P] UTP and its incorporation into RNA transcripts was visualized by agarose gel electrophoresis and autoradiography (Figure 1B). When pCMV was incubated in HSS or CSF, there was little or no UTP incorporation, respectively (Figure 1B, lanes 8-13 and 15-20). In contrast, incubation in NPE led to a large accumulation of radiolabeled product (Figure 1B, lanes 1-6), indicating that transcription of plasmid DNA readily occurs in NPE.

To investigate transcription of a promoter native to the Xenopus genome, we replaced the 5’ and 3’ regions of pCMV with those from the Xenopus laevis actb gene to form pActin (Figure 1D). actb encodes β-Actin, one of three major actin isoforms found in vertebrates, and is known to be transcriptionally activated during egg development (33). pActin was incubated in NPE, HSS, and CSF extracts and UTP incorporation was visualized as described above. As seen with pCMV, pActin was readily transcribed in NPE, but showed little or no UTP incorporation in HSS or CSF (Figure 1E). At the highest DNA concentration tested (100 ng/µL), pCMV and pActin had similar levels of UTP incorporation (compare blue traces in Figure 1C and F). However, at lower DNA concentrations, pActin produced relatively fewer products. These results suggest that transcription from the actb promoter is suppressed in NPE and that the effect can be alleviated with excess DNA.
To compare the relative levels of transcription machinery in each extract, equal volumes of NPE, HSS, and CSF were analyzed by Western blot and Coomassie stain. Although total protein levels were relatively similar in each extract, RNAPII was highly enriched in NPE compared to HSS and CSF (Figure 1G). We also saw that the level of histone H3 was enriched in NPE and HSS, compared to CSF. Thus, compared to other Xenopus egg extracts, NPE is enriched for both RNAPII and histones.

Transcription is driven by regulated recruitment of RNAPII to the promoter
To quantify transcription originating from the actb promoter, RNA products were isolated from NPE and analyzed by reverse transcription quantitative PCR (RT-qPCR). RNA levels were measured using primers that amplify a region ~150 bp downstream of the actb promoter region (Promoter) or ~2400 bp upstream (Control) (Figure 2A). Primers were also used to amplify endogenous Xenopus 18S rRNA that is retained during the preparation of NPE to serve as an internal control for RNA recovery between different samples.

When pActin was incubated in NPE, transcription from the actb promoter increased over time, peaking at ~60 minutes (Figure 2B, solid cyan trace). In comparison, transcription of the control region was relatively low, reaching only ~5% of that detected at the promoter (Figure 2B, dashed cyan trace). We then supplemented NPE with α-amanitin, a highly selective inhibitor of RNAPII(34). In the presence of α-amanitin, transcription from both the promoter and control regions was reduced to <1% of that detected from the promoter in buffer-treated samples (Figure 2B, solid and dashed orange traces), indicating that transcription at both sites is RNAPII-dependent.

We showed that total UTP incorporation was sensitive to the concentration of pActin incubated in NPE (Figure 1E and F). To directly test how DNA concentration affected transcription from the actb promoter, we incubated NPE with increasing concentrations of pActin, and then analyzed the accumulation of RNA products by RT-qPCR. For comparison, we also analyzed transcription from a pActin control plasmid that contained a deletion of the RNAPII core promoter elements (ΔPromoter). Total transcription from the actb promoter increased with pActin concentration up to 25 ng/µL, and then plateaued (Figure 2C, cyan trace). At all concentrations tested, transcription from the actb promoter region was severely reduced with the ΔPromoter plasmid compared to pActin (Figure 2C, orange trace), showing that NPE supports promoter-driven transcription. By calculating the amount of transcription per plasmid, we saw that transcription efficiency peaked at 25 ng/µL and was severely reduced at both lower and higher DNA concentrations (Figure 2D, cyan trace). Similar results were also seen when a fixed amount of pActin was incubated with increasing amounts of a “carrier” plasmid that has no sequence homology (Figure S2C and D), indicating that transcription efficiency was determined by total DNA concentration and not the number of actb promoters present in the reaction. Thus, at low DNA concentrations, transcription from the actb promoter is suppressed in NPE. At high DNA concentrations, the transcriptional machinery likely becomes limiting, reducing overall efficiency but not total product produced.

To verify that the actb promoter supports regulated transcription in NPE, we analyzed recruitment of histone H3, RNAPII, and the transcription factor TATA-binding protein (TBP) to the 5’ region of both the pActin and ΔPromoter plasmids by chromatin immunoprecipitation (ChIP). Compared to pActin, binding of RNAPII and TBP to the ΔPromoter plasmid was severely reduced (Figure 2E), consistent with the decrease in transcription at the promoter region (Figure 2F). In contrast, histone H3 levels remained similar for both plasmids. These results indicate that the actb promoter supports sequence-specific recruitment of bona fide transcription factors to initiate transcription in NPE.

Regulation of transcriptional activity by histone density
During early development, relative histone levels play an important role in regulating the onset of transcription during the MBT. When plasmid DNA is incubated in NPE, it becomes spontaneously chromatinized within ~30 minutes (Figure S3). To investigate whether the level of histone binding in NPE was responsible for decreased transcription at low DNA
concentrations, we first incubated different amounts of pActin in NPE for 30 minutes and then analyzed DNA-bound histone H3 by ChIP. With increasing concentrations of pActin, the percentage of histone-bound DNA recovered was reduced by more than 10-fold (Figure 3A), indicating a dramatic decrease in the number of histones bound to each plasmid.

We then tested whether plasmid concentration also affected DNA accessibility. pActin was again incubated in NPE at various concentrations. After 60 minutes, reactions were supplemented with Micrococcal Nuclease (MNase), which exhibits both exo- and endonuclease activity against exposed double-stranded DNA. Reaction samples containing equal amounts of DNA were withdrawn at different times after MNase addition, separated by agarose gel electrophoresis, and then visualized with SYBR Gold stain. As the concentration of pActin incubated in NPE increased, we saw that its sensitivity to MNase digestion also increased (Figure 3B and C). Together, these results suggest that changes in DNA concentration affect DNA accessibility by altering histone density.

To determine whether histone availability controls the access of transcription machinery to DNA, we immunodepleted NPE using pre-immune (Mock) or anti-histone H4K12ac (ΔH) antibodies, which co-depleted more than 75% of total histone H3 from extract (Figure 3D)(35). pActin was then incubated in each extract, and samples were withdrawn after 30 minutes to measure protein binding by ChIP. Compared to mock-depleted reactions, histone depletion reduced the level of DNA-bound histones by ~3-fold (Figure 3E). In contrast, histone depletion caused TBP binding to increase ~2-fold, consistent with greater access to nucleosome-free DNA. Although RNAPII levels were not significantly changed, transcription also increased ~2-fold in histone-depleted reactions (Figure 3F), suggesting that a greater fraction of DNA-associated RNAPII complexes were activated by TBP and able to transcribe downstream from the promoter. Together, these results suggest that reduced histone abundance allows increased transcription factor binding and stimulates RNAPII activity, consistent with models developed for Xenopus development(21-23).

RNA Sequencing reveals characteristics of actb promoter regulation

To further investigate how NPE supports transcription of the actb promoter, RNA products were analyzed by RNA sequencing (RNA-Seq). pActin or the ΔPromoter plasmid were each incubated in NPE at 25 ng/µL for 120 minutes. Total RNA was then isolated and analyzed using paired-end RNA-Seq. RNA reads were aligned to the pActin sequence and both forward (+) and reverse (-) reads were graphed. Consistent with RT-qPCR results (Figure 2C), pActin showed a large accumulation of RNA immediately downstream of the actb promoter in the forward orientation (Figure 4A, closed arrowhead). A smaller RNA peak was also present upstream of the actb promoter in the reverse orientation (Figure 4A, open arrowhead). This pattern of divergent transcription from a promoter is thought to be important for maintaining a nucleosome-free region for initiation and has been observed in organisms ranging from yeast to mammals(36).

Both divergent peaks were completely absent in the ΔPromoter reads (Figure 4B), indicating that formation of both RNA products was dependent on the actb promoter.

There were three major regions of actb-independent transcription found on pActin. The largest peak was localized to the CoIE1 origin (Figure 4B, closed arrowhead) and was adjacent to a smaller peak in the opposite orientation (Figure 4B, open arrowhead). These reads were likely produced by divergent transcription originating from an A/T-rich region within the origin sequence(37). The third peak originated within the 5’ actb intron (Figure 4B, gray arrowhead) and faced toward the major actb promoter peak. In a previous study analyzing actb expression, deletion analysis identified a negative transcriptional element in this region(38). Together with our RNA-Seq data, these results suggest that the intron promoter may interfere with expression of actb(39). Interestingly, transcription from both the CoIE1 origin and the actb intron regions increased in the ΔPromoter plasmid relative to pActin (Figure 4C, compare cyan with orange and blue with red traces), suggesting that the actb promoter competes with nearby promoters.

We noted that forward transcription from the actb promoter was limited in length. Transcripts showed highly efficient initiation and...
extension to ~250 nucleotides, well beyond the short transcripts associated with abortive transcription (up to ~15 nucleotides)(40,41). Roughly 16% of established transcripts escaped the promoter region, extending further to ~310 nucleotides. Extension beyond this point failed rapidly, with the vast majority of transcripts terminating by ~500 nucleotides. This phenomenon was not specific to the actb promoter, as forward transcription from the ColE1 origin (Figure 4B, closed arrowhead) and reverse transcription from the intron region (Figure 4B, gray arrowhead) also showed similar lengths of elongation.

Transcription elongation and pre-mRNA processing in NPE
We reasoned that some factors involved in transcription elongation might be limiting in extract. To test this hypothesis, pActin was incubated in NPE at different concentrations for 120 minutes, and then RT-qPCR was used to measure transcription at the promoter and another site ~600 bp downstream. Because total transcription levels vary with plasmid concentration (as seen in Figure 2C), we graphed “elongation” as the percentage of transcripts measured downstream versus at the promoter (Figure 4D). At 25 ng/µL, elongation efficiency was ~11%. However, with decreasing pActin concentration, the efficiency of elongation increased dramatically, reaching ~56% at 1 ng/µL. Thus, transcription elongation improved at lower DNA concentrations in NPE, despite higher levels of histone binding (Figure 3A).

The 5’ region of actb contains an intron that is typically spliced during expression of the actb gene. However, analysis of the pActin RNA-Seq identified only a trace amount of reads corresponding to spliced products (data not shown). To test whether splicing improved with increased transcription elongation, RNA produced at different pActin concentrations was analyzed by RT-qPCR using primers that amplify either unspliced or spliced transcripts (Figure S4B and C). As with elongation, splicing efficiency increased when the concentration of pActin was reduced from 25 to 1 ng/µL (Figure 4E), suggesting that the two processes are linked during transcription in NPE(42).

To examine 3’ cleavage and polyadenylation, we first used an unbiased approach to identify potential cleavage sites. pActin was incubated in NPE at 5 ng/µL for 120 minutes and RNA was isolated to generate cDNA. Transcripts were then amplified using a forward primer that hybridizes upstream of the consensus polyadenylation sequence(43) and an anchored oligo-dT reverse primer. The major PCR product was gel-purified and sequenced. New primers were then designed that amplify either uncleaved transcripts or those that have been cleaved and polyadenylated (Figure S4D and E). RNA produced at different pActin concentrations was then analyzed to measure the efficiency of cleavage and polyadenylation at this site. Unlike elongation and splicing, the efficiency of cleavage and polyadenylation remained relatively constant at each pActin concentration tested (Figure 4F). Taken together, these results indicate that NPE supports regulated transcription from endogenous promoters and subsequent pre-mRNA processing required to generate mature mRNA transcripts.

DISCUSSION
Xenopus egg extracts have been used extensively to study numerous biological processes in a highly tractable system. A major advantage of extracts over cell-based models is the ability to study direct effects in the absence of global gene expression and cell cycle changes. However, traditional Xenopus egg extracts have been found to support only limited transcription without the addition of exogenous transcription machinery or the removal of endogenous histones to prevent DNA chromatinization(18,21-23). In this study, we establish a new cell-free system using NPE that supports robust transcription of chromatinized substrates by endogenous factors.

Transcription activity and efficiency in NPE
NPE contains a highly concentrated fraction of soluble, nuclear proteins. Relative to other Xenopus egg extracts, NPE is enriched for both transcription machinery and histones (Figure 1G). This combination of factors supports robust transcription (Figure 1B and E) in the context of chromatinized DNA (Figures S3 and 3A). NPE is prepared from nuclei undergoing active DNA
synthesis. As such, transcription in NPE likely resembles that of cells within S phase. Although the primary mechanics of transcription are similar throughout the cell cycle, the regulation of specific genes and transcription factors may be influenced by these characteristics of NPE.

*Xenopus* eggs contain an abundance of histone dimers that are coupled to molecular chaperones(44,45). In NPE, these complexes promote spontaneous loading of histones onto DNA within ~30 minutes (Figure S3). Although transcription of pActin begins prior to complete chromatization, robust transcription continues up to ~60 minutes before leveling off (Figure 2B). The decline in transcription activity over time is not due to limited availability of ribonucleotides or ATP (data not shown). This limited window of transcription suggests that factors required for initiating transcription become inactivated or suppressed over time, possibly due to changes in chromatin signaling(25).

Based on the final quantity of RNA detected by qPCR and the efficiencies of RNA isolation and cDNA amplification (determined using samples of known concentration), we estimate that the *actb* promoter produced ~2.5 extended transcripts per molecule of pActin when incubated in NPE. Transcriptional efficiency of the *actb* promoter peaked at 25 ng/µL (Figure 2D and S1B), suggesting a balance between fully chromatinized DNA that suppresses transcription and under-chromatinized DNA that supports increased transcription factor binding (Figure 3E). These conditions are likely analogous to the cellular states of chromatin referred to as heterochromatin and euchromatin, respectively(46).

**Histone density and developmental regulation**

During early embryo development, histones act as a sensor for the number of cellular divisions. As histones become limiting, it triggers the MBT, which marks the onset of transcription and developmental progression(20-22). In NPE, the ratio of DNA to extract plays a similar role in regulating transcription, which can be modulated by altering the concentration of plasmid present within the reaction (Figure 2C). Previous studies have shown that the MBT can be altered by adding or depleting histones(21,22), arguing that histone levels control the onset of transcription. Our results indicate that transcription in NPE is regulated by the same mechanism. Increasing plasmid DNA concentration reduced histone binding (Figure 3A) and increased DNA accessibility (Figure 3B and C). We also showed that histone binding limited recruitment of TBP to the *actb* promoter (Figure 3E), adding support to the model that histone occupancy suppresses transcription by obscuring genes during early embryo development.

**Promoter activity and pre-RNA processing**

Using RNA Seq, we performed a detailed analysis of transcripts produced by pActin and the ΔPromoter plasmids. We saw traditional patterns of divergent transcription originating from the *actb* promoter and plasmid origin sequence (Figure 4A). Promoters that support divergent transcription initiate bi-directional transcripts read from opposite DNA strands. Although some antisense transcripts are unstable and quickly degraded, others have important regulatory functions(47). Despite the prevalence of divergent transcription throughout higher order species, the mechanism of its regulation and biological purpose remain poorly understood.

Within the 5′ intron of *actb*, we identified a putative promoter (Figure 4B, gray arrowhead) oriented toward the *actb* promoter (Figure 4A, closed arrowhead). Transcription from the intron promoter was weaker than that of *actb*, but increased when the core promoter elements of *actb* were deleted (Figure 4C). Although this region of *actb* was proposed to contain a negative regulatory element(38), its potential role in regulating gene expression has not been explored. Notably, the intrinsic activity of weak or dormant promoters may also be elevated in this system due to the absence of other genomic elements that normally compete for access to limited transcription factors and machinery.

Interestingly, we found that pre-mRNA processing events responded differently to changes in plasmid concentration. The efficiency of cleavage and polyadenylation remained relatively constant at different plasmid concentrations (Figure 4F), suggesting that the required factors are present in excess or able to function independently of other transcriptional events. In contrast, the efficiency of transcription elongation and splicing both improved in a non-linear fashion.
with decreasing pActin concentration (Figure 4D and E), arguing that a threshold must be achieved for full stimulation. We propose that highly chromatinized DNA is required for efficient elongation and splicing to occur in NPE. Although histones generally play a negative role in transcription initiation (Figure 3F), they are also critical for chromatin signaling that regulates many downstream events (48-51).

**Concluding remarks**

Together, the results described in this study add another fundamental process to NPE’s repertoire. As such, it provides a unique tool to examine the interplay between different cellular processes and the various pathways that regulate them. Decades of research have revealed an array of dynamic regulatory networks that control each phase of gene expression (48,52,53). When these mechanisms fail, it can result in the development of numerous diseases, including cancer (51,54-56). Understanding how different signaling events directly impact the initial phases of gene expression will provide new insight into the mechanisms of disease and identify new strategies for treatment.

**EXPERIMENTAL PROCEDURES**

**Plasmid Substrates**

The parent pCMV vector was purchased from Addgene (#11153). The 5’ and 3’ regions of actb were amplified from *Xenopus laevis* sperm chromatin (prepared as described in (57)) using the following primer pairs:

**5’ region:**

CAGGAACTAGTAGAACAAGGGAAGCAATGG AT and TAGACCATGGTGGCCTGAAA AGAGAATTA GATT

**3’ region:**

ATAGCGGCCGCAGGACAGCTTTCAACCTC ATG and GCGCTGCCTAGGTTTGTTTGAGTGCACAC CGAAG

The resulting fragments were then cloned into pCMV using SpeI and NcoI (5’ region), or NotI and AvrII (3’ region). The carrier plasmid (pCarrier) utilized in Figure S2C and D was a pFastBac1 vector (Thermo Fisher) carrying the *Xenopus* BARD1 gene (58). To generate the ΔPromoter plasmid, actb core promoter elements were deleted by site-directed mutagenesis (Agilent Technologies) using the following primers:

**Forward:**

CTTCGTCCGCAGTTCCTACGTCGCCACCTC AGGC

**Reverse:**

GCCTGAGGTGGACGTAGGAACACTGCAGGA CGAAG

**Incubation in Xenopus egg extract**

HSS, CSF, and NPE were produced as described previously (26,57). The care and use of *Xenopus laevis* followed established protocols approved by IACUC with AALAC accreditation. In all reactions, extracts were supplemented with ATP regenerating mix (6.5 mM phosphocreatine, 0.65 mM ATP, and 1.6 μg/mL creatine phosphokinase). NPE was also supplemented with 1 mM DTT, and CSF was supplemented with 0.3 mM Ca$^{2+}$ to promote entry into interphase. Reactions were incubated at 21°C for 10 minutes prior to the addition of plasmid DNA, which represents 0 minutes. Where indicated, extracts were supplemented with $[^{32}$P] UTP to label nascent RNA, or 10 μM α-amanitin to inhibit RNAPII. All experiments were performed at least two times with representative or averaged data shown.

**UTP Incorporation Gels**

Three hours after addition of plasmid DNA to each extract, samples were withdrawn from the reaction and added to Stop Buffer (3.6% SDS, 18 mM EDTA, 90 mM Tris-HCl, 9% Ficoll). Samples were then mixed with RNA Gel Loading Dye (Thermo Fisher), incubated at 65°C for 20 minutes, and resolved by agarose gel electrophoresis. Radiolabeled transcripts were visualized and quantified using a phosphorimager.

**Reverse Transcription Quantitative PCR (RT-qPCR)**

RNA was isolated from extract using the EZNA RNA Purification kit (Omega Bio-tek) and cDNA was generated using the QuantiTect Reverse Transcription kit (Qiagen). Samples were then
analyzed by quantitative real-time PCR with the following primer pairs:

Control:
CCCAACCAGTGTACACCCACCTTCC and ATGCCTGGGAGCGCCCTTAT

Promoter:
TATGGGCAGCATGAATGG and AATTGCGCGACCTACAGTC

Elongation:
AGGTTTTACGAGGAAGTA and AGGCTTTCAGTGAGCCAGTC

Unspliced:
GGGCTTCTACCCCTAATCTTCTC and TCGCCGACACGTGAACCTT

Spliced:
TCCAACCCTACCCCATCTAATCTC and TCGCCGACACGTGAACCTT

Uncleaved:
CACATCTGTATCTTGATGAGTG and CAAAACCCATTCATTTTGCCA

Cleaved:
CACATCTGTATCTTGATGAGTG and TTTTTTTTTTTTTTTTTTTTT

18S rRNA:
GACCGGCGCAAGACGAACCA and TGCTCGGCGGGTCATGGGAA

To quantify RNA processing, the level of nonspecific amplification of spliced and cleaved primers was determined using a pActin standard curve, which only contains unspliced and uncleaved sequences. The background amplification was then subtracted from each sample based on the level of total unprocessed RNA present in that sample. PCR fragments containing spliced or cleaved sequences were also generated using the spliced and cleaved primer pairs to create a standard curve for processed samples.

To identify cleavage and polyadenylation sites downstream of the actb promoter, pActin was incubated in NPE for 120 minutes at 21°C. Equal amounts of pActin were withdrawn from each reaction and mixed with 1x Micrococcal Nuclease Reaction Buffer (New England Biolabs). Additional NPE was also added to the 25 and 100 ng/µL mixes so that all three treatments contained equal amounts of DNA and extract. 100 units of micrococcal nuclease (New England Biolabs) was then added and reactions were incubated at 37°C. At the indicated time, samples were withdrawn, mixed with an equal volume of STOP Buffer, and then treated with proteinase K (ThermoFisher) for 120 minutes at 37°C. Undigested DNA was then resolved by agarose gel electrophoresis and visualized with SYBR Gold stain.

Chromatin Immunoprecipitation (ChIP)

ChIP was preformed as described previously(59). Briefly, reaction samples were crosslinked in Egg Lysis Buffer (ELB: 10 mM HEPES-KOH pH 7.7, 2.5 mM MgCl₂, 50 mM KCl, and 250 mM sucrose) containing 1% formaldehyde. Crosslinking was stopped by the addition of 125 mM glycine, and formaldehyde was removed using a Micro Bio-Spin 6 chromatography column (Bio-Rad). Samples were then sonicated (Diagenode Bioruptor UCD-600 TS) and immunoprecipitated with the indicated antibody. Following immunoprecipitation, crosslinks were reversed and DNA was isolated by phenol/chloroform extraction and ethanol precipitation. Total (INPUT) and recovered DNA were then analyzed by qPCR to determine percent recovery using the following primer pairs:

pActin:
CCTCCTTGACATCCGCTTTCC and GCTGGCGACCGCTACTTG

ΔPromoter:
GAAAATACGGGGCGTGAAGATT and GCTGGCGACCGCTACTTG

Micrococcal Nuclease Digestion

pActin was incubated in NPE at 10, 25, or 100 ng/µL for 60 minutes at 21°C. Equal amounts of pActin were withdrawn from each reaction and mixed with 1x Micrococcal Nuclease Reaction Buffer (New England Biolabs). Additional NPE was also added to the 25 and 100 ng/µL mixes so that all three treatments contained equal amounts of DNA and extract. 100 units of micrococcal nuclease (New England Biolabs) was then added and reactions were incubated at 37°C. At the indicated time, samples were withdrawn, mixed with an equal volume of STOP Buffer, and then treated with proteinase K (ThermoFisher) for 120 minutes at 37°C. Undigested DNA was then resolved by agarose gel electrophoresis and visualized with SYBR Gold stain.
Plasmid Pull-Down
Plasmids were isolated from NPE as described previously(59). Briefly, reaction samples were withdrawn at the indicated time and added to LacI-coupled magnetic beads (Dynabeads M-280; Invitrogen) suspended in LacI pull-down buffer (10 mM HEPES pH 7.7, 2.5 mM MgCl₂, 50 mM KCl, 250 mM sucrose, 0.25 mg/mL BSA, and 0.02% Tween 20). Samples were incubated for 20 minutes, rotating at 4°C. Beads were then washed three times with LacI wash buffer (10 mM HEPES pH 7.7, 2.5 mM MgCl₂, 50 mM KCl, 0.25 mg/mL BSA, and 0.02% Tween 20), dried, and suspended in 2X SDS sample buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, and 200 mM β-mercaptoethanol). DNA-bound proteins were then resolved by SDS-polyacrylamide gel electrophoresis and visualized by Western blotting with the indicated antibodies.

Antibodies and Immunodepletion
RNA polymerase II antibodies were purchased from Bethyl Laboratories (A300-653A for Western, A304-405A for ChIP). TATA-binding protein (TBP) antibodies were purchased from Boster Biological Technology (PA1534). Histone H3 antibodies were purchased from Thermo Fisher (PA5-16183). To deplete histones from NPE, two rounds of depletion were performed by incubating 10 µL of extract with Protein-A Sepharose beads (GE Healthcare) bound to 50 µg of purified Histone H4 K12Ac antibodies(35) for 1 hour at 4°C.

RNA Sequencing Analysis
RNA was isolated from extract using the EZNA RNA Purification kit (OMEGA). Total RNA samples were then analyzed by Novogene after rRNA removal using paired-end RNA sequencing. A total of ~10,000,000 clean reads were obtained for both the pActin and ΔPromoter plasmids. Output FASTQ files were aligned to the pActin sequence using bowtie version 2.3.5(60). One nucleotide was removed from the 3’ and 5’ end of reads and the subseeding length was 20. Bam files were sorted by samtools(61) and output alignments were analyzed using Integrative Genomics Viewer.

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CONFLICT OF INTEREST
The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS
J.K.B. and D.T.L. designed and analyzed experiments; J.K.B. performed experiments; J.K.B. and D.T.L. prepared the manuscript.
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**FOOTNOTES**

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FIGURE LEGENDS

Figure 1. Nucleoplasmic extract (NPE) supports robust transcription of plasmid substrates. (A) pCMV schematic. Relative location of promoter and GFP are indicated. (B) Different concentrations of pCMV were incubated in NPE, HSS, or CSF extract in the presence of [$\alpha^{-32}$P] UTP. Samples were withdrawn at 180 minutes, resolved by agarose gel electrophoresis, and visualized by autoradiography. (C) Total UTP incorporation from (B) was quantified and graphed. (D) pActin schematic showing the 5’ and 3’ regions cloned from *Xenopus actb*. (E) pActin was incubated in NPE, HSS, or CSF and UTP incorporation was analyzed in parallel to (B) to allow a direct comparison. (F) Total UTP incorporation from (E) was quantified and graphed relative to peak intensity in (B). (G) Total protein from each extract was resolved by SDS-polyacrylamide gel electrophoresis and visualized with Coomassie stain or by Western blot using the indicated antibodies.

Figure 2. NPE supports regulated and promoter-dependent transcription. (A) pActin schematic. Sequence elements are shown relative to the transcription start site (+1). “Control” and “Promoter” primer pair locations are indicated. (B) pActin was incubated at 10 ng/µL in NPE supplemented with buffer or α-amanitin. RNA was isolated at the indicated time points and quantified by RT-qPCR. (C) Different concentrations of pActin or ΔPromoter plasmid were incubated in NPE for 120 minutes. RNA was isolated and quantified by RT-qPCR using the Promoter primers. (D) Transcription from (C) was normalized based on starting plasmid concentration. (E) pActin or ΔPromoter plasmid were incubated in NPE at 25 ng/µL. At 30 minutes, DNA-bound protein was analyzed by ChIP with the indicated antibodies. (F) At 120 minutes, RNA was isolated from the reactions in (E) and quantified by RT-qPCR using the Promoter primers. Error bars represent ±/− one standard deviation. See Figure S2 for experimental replicates.

Figure 3. Histone occupancy regulates transcriptional activity in NPE. (A) pActin was incubated in NPE at the indicated concentrations for 30 minutes. DNA-bound protein was then analyzed by ChIP using histone H3 antibodies. (B) pActin was incubated in NPE at the indicated concentrations for 60 minutes. Next, reaction samples were diluted in MNase buffer and treated with 100 U MNase at 37°C for the indicated time. DNA was then isolated and resolved by agarose gel electrophoresis. Input DNA (IN) and topological isoforms of the resolved plasmids are indicated: open circular (OC), supercoiled (SC), and linear. (C) The total intensity of all three full-length plasmid molecules identified in (B) was quantified and graphed. (D) Mock-depleted (ΔMock) and histone-depleted (ΔH) NPE were analyzed by Western blot with the indicated antibodies. (E) pActin was incubated in ΔMock or ΔH extract at 10 ng/µL. At 30 minutes, DNA-bound protein was analyzed by ChIP using the indicated antibodies. (F) RNA was isolated from the reactions in (E) at 120 minutes and quantified by RT-qPCR using the Promoter primers. Error bars represent ±/− one standard deviation.

Figure 4. Analysis of whole-plasmid transcription and pre-mRNA processing in NPE. (A) pActin was incubated at 25 ng/µL in NPE for 120 minutes. RNA was then purified and analyzed by paired-end RNA sequencing. The total of all mapped reads were graphed for both the forward (+) and reverse (−) orientations. (B) The ΔPromoter plasmid was incubated in NPE and analyzed by RNA-seq as described in (A). (C) Reads from (A) and (B) were overlaid onto the same graph for direct comparison. A diagram of pActin showing the relative position of major sequence elements is shown above. See text for description of arrowheads. (D-F) pActin was incubated in NPE at the indicated concentrations for 120 minutes. RNA was then isolated and analyzed by RT-qPCR to determine the efficiency of (D) elongation, (E) splicing, and (F) cleavage and polyadenylation. Elongation was graphed as a percentage of amplification with Elongation primers versus Promoter primers. Splicing and cleavage and polyadenylation were graphed as a percentage of amplification with Unspliced and Uncleaved primers, respectively. Error bars represent ±/− one standard deviation.
Figure 3

A. Histone H3 ChIP

B. Western Blot Analysis of Histone H3

C. pActin Degradation

D. Western Blot Analysis of aRNAPII and aHistone H3

E. ChIP

F. Transcription
Figure 4

A

B

C

D

E

F

- Elongation
- Splicing
- Cleavage and Polyadenylation

Downstream Transcription (% of Promoter Signal)

Spliced Product (% of Unspliced Signal)

Cleaved Product (% of Uncleaved Signal)

Elongation: Y vs. pActin (ng/μL)
Splicing: Y vs. pActin (ng/μL)
Cleavage and Polyadenylation: Y vs. pActin (ng/μL)
