Stable intronic sequence RNA (sisRNA), a new class of noncoding RNA from the oocyte nucleus of *Xenopus tropicalis*

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To compare nuclear and cytoplasmic RNA from a single cell type, free of cross-contamination, we studied the oocyte of the frog *Xenopus tropicalis*, a giant cell with an equally giant nucleus. We isolated RNA from manually dissected nuclei and cytoplasm of mature oocytes and subjected it to deep sequencing. Cytoplasmic mRNA consisted primarily of spliced exons derived from ~6700 annotated genes. Nearly all of these genes were represented in the nucleus by intronic sequences. However, unspliced nascent transcripts were not detected. Inhibition of transcription or splicing for 1–2 d had little or no effect on the abundance of nuclear intronic sequences, demonstrating that they are unusually stable. RT–PCR analysis showed that these stable intronic sequences are transcribed from the coding strand and that a given intron can be processed into more than one molecule. Stable intronic sequence RNA (sisRNA) from the oocyte nucleus constitutes a new class of noncoding RNA. sisRNA is detectable by RT–PCR in samples of total RNA from embryos up to the mid-blastula stage, when zygotic transcription begins. Storage of sisRNA in the oocyte nucleus and its transmission to the developing embryo suggest that it may play important regulatory roles during oogenesis and/or early embryogenesis.

[**Keywords:** intron; lampbrush chromosome; noncoding RNA; oocyte; transcription; *Xenopus*]

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The majority of genes in higher eukaryotes have one or more introns interspersed within the coding region. Either during transcription or shortly afterward, the intronic sequences are removed from the transcript by the splicing machinery (Wahl et al. 2009; Oesterreich et al. 2010; Vargas et al. 2011; Hoskins and Moore 2012). The majority of spliced RNAs are rapidly transported to the cytoplasm, whereas the introns are degraded while still in the nucleus. The primary intronic product of splicing is an intron lariat, in which the 5' end of the intron is covalently linked to the splice acceptor site, an A near the 3' end of the intron. The lariat can be converted to a linear molecule by the debranching enzyme (dbr) (Ruskin and Green 1985). Intron degradation can be a rapid process, requiring only seconds or minutes in the case of yeast and cultured cells (Sharp et al. 1987; Clement et al. 1999; Oesterreich et al. 2010). Much less is known about the lifetime of introns in tissues. A few cases of stable introns have been described (Michaeli et al. 1988; Kopczynski and Muskavitch 1992; Qian et al. 1992; Yang et al. 2011), although it is generally believed that most introns or intron fragments are unstable. There are some exceptions; notably, the small nucleolar RNAs (snoRNAs),

which are derived by debranching of spliced lariats followed by trimming to produce the mature molecule (Ooi et al. 1998; Petfalski et al. 1998).

In this study, we examined RNA derived from oocytes of the frog *Xenopus tropicalis*. We isolated RNA from manually dissected cytoplasmic and nuclear fractions and subjected it to deep sequencing. Cytoplasmic mRNA consists primarily of spliced exons, as expected. Surprisingly, the major nuclear RNA derived from these same genes consists of stable introns or intron fragments. At the read depth of our experiments, we did not see reads that cross intron–exon boundaries, as would be expected for nascent transcripts. The stable intronic sequences are transmitted to the embryo and persist until at least the blastula stage, when zygotic transcription first begins. We discuss these findings in relation to the special features of RNA transcription and storage in the growing oocyte and early embryo.

Results

Pure cytoplasmic and nuclear RNA can be prepared from Xenopus oocytes

Amphibian oocytes offer a unique opportunity to analyze nuclear and cytoplasmic RNA fractions with minimal

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cross-contamination. It is well known that the majority of cytoplasmic RNA consists of the large and small ribosomal RNA (rRNA) subunits and that much of the mRNA consists of stored polyadenylated sequences not associated with polysomes (Radford et al. 2008; Richter and Lasko 2011; for a review of earlier studies, see Davidson 1986). However, the nature of nuclear RNA has not been systematically investigated. *Xenopus laevis* was the favored species for earlier studies on RNA, but *X. tropicalis* has the advantage that its genome has been sequenced and annotated (Bowes et al. 2010; Hellsten et al. 2010). Our studies began with mature oocytes of *X. tropicalis*, which have a diameter of 800 μm and contain a single, slightly ovoid nucleus, also called the germinal vesicle (GV), with dimensions of about 300 $\times$ 400 μm (Fig. 1A). In the ovary, the oocytes are surrounded by several layers of follicle cells, the most internal of which contains $\sim$ 1000 cells and is tightly bound to the surface of the oocyte. The follicle layers can be removed completely by treating oocytes with a solution of collagenase (Simeoni et al. 2012). The GV can be isolated from the oocyte by making a small tear in the animal hemisphere with jewelers' forceps and pressing gently on the sides of the oocyte (Gall and Wu 2010). In many earlier studies, where the major objective was to examine the oocyte lampbrush chromosomes, it was important to prevent gelling of the nucleoplasmic actin (Gall 2006) so that the nuclear contents could be spread in a single plane for microscopic examination (Fig. 1B–D). Here the objective was just the opposite, to find an isolation medium in which the nuclear contents would gel rapidly but which still allowed clean separation of the GV from the cytoplasm. By trial and error, we found that the standard isolation medium adjusted to pH 5.6–5.8 serves this purpose. In this medium, the nuclear contents gel rapidly, becoming opalescent as viewed by incident illumination, and equally importantly, the nuclear envelope swells away from the gelled nuclear contents (Fig. 1E–G). The envelope can then be removed with jewelers' forceps, either intact or in two or three pieces.

The importance of removing the nuclear envelope first became apparent in the course of microarray experiments in which we compared the relative abundance of RNA sequences in cytoplasm and GV samples. In our original samples, prepared from GVs with intact envelopes, there was essentially no difference between the GV and the cytoplasm (Supplemental Fig. S1A). In subsequent experiments in which the envelope was removed, the GV and cytoplasmic RNA samples showed striking differences (Supplemental Fig. S1B). The ratio of total cytoplasmic RNA to total GV RNA in an oocyte is $\sim$ 500:1 (1 μg vs. 2 ng, based on recovery). We presume that a small amount of cytoplasm remains tightly associated with the nuclear envelope, even after extensive washing of the isolated GV. Unless the envelope is removed, this cytoplasmic contaminant completely overwhelms the genuine nuclear RNA. Preparation of pure cytoplasmic RNA presents no problem because the GV can be removed intact from an oocyte in a few seconds (Fig. 1A).

Figure 1. Isolation of nuclear and cytoplasmic fractions from oocytes of *X. tropicalis*. (A) A single mature oocyte of *X. tropicalis* and the GV that was removed from it. (B–D) Behavior of GVs isolated at pH 7.0. The nuclear contents form a weak gel after the GV is removed from the oocyte. Such nuclei lose most of their mass, even though the envelope remains intact (Macgregor 1962). The nuclear envelope can be removed (arrow in D), but the isolated gel is soft and easily dispersed by pipetting. (E–G) Behavior of GVs isolated at pH 5.8. Within a few seconds after isolation, the GV becomes opalescent under incident illumination. Then, the envelope expands, leaving a wide space between it and the gelled contents. Note that the GV contents do not decrease appreciably in volume. After the envelope is removed (arrow in G), the firm nuclear gel can be pipetted without damage. Bars, 500 μm .

It has been known for a long time that the majority of newly transcribed RNA in late-stage oocytes consists of pre-rRNA, as originally demonstrated by uridine incorporation experiments (Gall 1966). Moreover, the GV must contain nascent transcripts derived from numerous protein-coding genes, as implied by studies on the lampbrush chromosomes (Gall et al. 1983; Callan 1986). Nevertheless, it is not known whether nuclear RNA is rapidly processed and exported or which sequences might be stored. To be sure that we did not miss any sequence class, nuclear or cytoplasmic, we did not select for poly(A)⁺ RNA or initially remove rRNA or its precursors. This sequencing strategy was wasteful in the sense that most of the sequences were derived from rRNA and its precursors, but we hoped that we would not lose sequences that had unexpected characteristics. In our later analyses, we carried out preliminary selection against rRNA, resulting in considerable increase in read depth but no change in the patterns for most genes.

Cytoplasmic mRNA consists primarily of spliced exons

The mature oocyte stores numerous polyadenylated mRNAs, most of which are not loaded on polysomes and therefore are not being translated. Many of these

mRNAs are stored for later use during embryogenesis. A recent deep-sequencing study of *X. tropicalis* oocyte RNA identified transcripts from ~11,000 genes, differing widely in relative abundance from only a few transcripts per oocyte to many thousands (Simeoni et al. 2012). The read depth in our cytoplasmic samples was lower primarily because rRNA was not removed before sequencing, and consequently we identified somewhat fewer transcripts (5000–7000, depending on the read depth of the sample).

We examined the cytoplasmic expression profiles for many of the more highly transcribed genes. Typical examples, *nasp* (histone H1-binding protein) and *ccne1* (cyclin E1), are shown in Figures 2 and 3, respectively. The most obvious feature is that the transcript is represented almost entirely by exonic sequences. The exon–intron boundaries are sharp because the sequence reads are continuous across the entire mRNA, and the number of reads at the ends of the 5' and 3' untranslated regions (UTRs) tapers off because ends of molecules are systematically underrepresented when RNA is fragmented and size-selected before amplification and sequencing. The number of reads within the exons of a given gene also varies, although the patterns are similar between independent experiments. Such variation is commonly seen in deep-sequencing data and presumably represents systematic bias at various stages of library production, sequencing, or sequence alignment.

For comparison of relative abundance between samples, we calculated the number of fragments per kilobase of exonic sequence per million reads mapped (FPKM) (Mortazavi et al. 2008). We use the designation FPKM_{exon} to emphasize the exonic origin of the sequences. We analyzed all genes for which there was an FPKM_{exon} score >0 (Supplemental Material). Supplemental Figure S2A compares FPKM_{exon} values for 6675 expressed genes from two samples of cytoplasmic RNA from mature oocytes. FPKM_{exon} values were highly correlated between the two samples ($R = 0.94$). These samples were prepared from different animals, and all steps of sample preparation and sequencing were carried out independently.

GV RNA contains intronic sequences derived from the majority of transcribed genes

The most striking feature of GV RNA is the presence of intronic sequences and the nearly complete absence of exonic sequences. For many expressed genes, the nuclear and cytoplasmic patterns are more or less complementary, the peaks in one corresponding to valleys in the other. This pattern is obvious when one examines randomly chosen genes with high cytoplasmic representation, such as *nasp* (Fig. 2) or *ccne1* (Fig. 3). Some introns are represented by single peaks that do not extend all the way to the exon borders, whereas other introns display multiple independent peaks. These patterns are less striking for genes with lower levels of transcription, but most expressed genes have only exonic sequences in the cytoplasm and intronic in the nucleus. It is noteworthy that the intronic sequences in the nucleus do not cross intron/exon borders. Because the intronic sequences in the nucleus are not accompanied by exonic sequences and do not overlap intron/exon junctions, they do not represent parts of unspliced transcripts.

To make more quantitative comparisons of nuclear sequences, we could not use FPKM as usually defined, because it is calculated relative to exonic sequence length. Instead, we used a comparable normalization metric, FPKM_{intron} (fragments per kilobase of intronic sequence per million reads mapped), to describe the abundance of nuclear sequences. We first prepared an annotation file for all introns in the genome, which allowed us to display introns in the genome browser (Fig. 2) and calculate the abundance of intronic sequence reads (details in the Materials and Methods).

For initial analysis, we examined all of the 6675 transcribed genes identified in the cytoplasmic samples. Of these, 6166 (92.4%) have detectable intronic sequences in the nuclear samples, allowing the calculation of FPKM_{intron} values for almost all transcribed genes. Just like FPKM_{exon} values, FPKM_{intron} values are highly correlated between independent samples, attesting to the reproducibility of the sequencing results ($R = 0.94$).

Figure 2. Cytoplasmic and nuclear sequences from a highly expressed gene, *nasp* (histone H1-binding protein). The predicted exonic and intronic sequences are shown just above the gene name. Solid bars with connecting lines represent the annotated exons (xenTro2). Unconnected solid bars represent introns (see the Materials and Methods for construction of the intron map). The ordinate shows the number of reads per base. Cytoplasmic sequences in the bottom row appear as typical spliced mRNA. The exon boundaries are sharp, except for the tapering 5' and 3' ends. The peaks and valleys within the exons presumably reflect systematic biases in library production, sequencing, or sequence alignments, and are more or less reproducible between independent samples. Nuclear sequences in the top row are predominately intronic in origin. Conspicuously absent are nascent transcripts, which would be represented by reads along the entire length of the transcribed region. Note the peaks and valleys in the introns, which are reproducible between samples. Some may be artifacts of sequencing, as in the exons, but some of the more prominent peaks probably reflect separate intronic molecules (see Figs. 4, 5).

Figure 3. Nuclear and cytoplasmic RNA sequences are stable for at least 2 d. Shown here are patterns for a typical highly expressed gene, *ccne1* (cyclin E1). The ordinates show the number of reads per base. (A, top four rows) Actinomycin D treatment. Oocytes were held for 15–16 h in control OR2 medium or in actinomycin D (20 μ g/mL) to inhibit transcription. (First and second rows) The patterns for GV RNA from control and treated oocytes were essentially identical. (Third and fourth rows) The same was true for cytoplasmic RNA. (B, bottom four rows) U2 snRNA depletion. Oocytes were injected with an antisense deoxyoligonucleotide that rapidly destroys U2 snRNA and therefore inhibits splicing. Control oocytes were injected with water. Thirty-six hours to 48 h later, RNA was isolated from GVs and cytoplasm from injected and control oocytes. (Fifth and sixth rows) The patterns for GV RNA were similar for control and U2-depleted oocytes. (Seventh and eighth rows) The same was true for cytoplasmic RNA from control and U2-depleted oocytes.

(Supplemental Fig. S2B). Because intronic nuclear sequences exist for almost all transcribed genes, the most parsimonious hypothesis is that they result from processing of primary transcripts. If this is true, then one might expect a correlation between the abundance of intronic and exonic sequences for the same gene. This correlation is shown in Supplemental Figure S2C for the transcribed genes that have FPKM_{intron} values in the nucleus. The correlation is positive with a slope of 1.54, but the spread of values is high ($R = 0.55$).

Any factor that affects the relative stability of intronic and exonic sequences of specific genes will tend to lower the overall correlation between nuclear and cytoplasmic FPKMs. Later, we demonstrate that intronic sequences in the oocyte nucleus are unusually stable but nevertheless much less stable than exonic sequences from the same genes. Variations in the relative stability of intronic and exonic sequences could account for much of the scatter in Supplemental Figure S2C. In addition, there are computational artifacts that affect the determination of FPKM_{intron} that do not affect FPKM_{exon}. For instance, some genes have one or more snoRNAs derived from their introns; snoRNAs often reach very high levels in the nucleus, giving rise to an unusually high FPKM_{intron}/FPKM_{exon} ratio. Short introns (<200 base pairs [bp]) do not have mapped reads, at least in part because the sequencing

protocol used in these experiments did not include short RNAs. Thus, short genes with only one or two short introns tend to have a low FPKM_{intron}/FPKM_{exon} ratio. For unknown reasons, introns longer than ~500 nucleotides (nt) are usually not uniformly represented along their length (Fig. 3). As a result, genes with multiple long introns tend to have a low FPKM_{intron}/FPKM_{exon} ratio. In summary, although the correlation between FPKM_{intron} and FPKM_{exon} is not high, we favor the hypothesis that nuclear intronic sequences are derived by processing from primary transcripts, not by independent transcription.

Nuclear intronic sequences are unusually stable

Theoretical considerations discussed later make it very likely that the intronic sequences observed in the GV must be very stable, with half-lives of days rather than minutes or hours. To address this issue experimentally, we inhibited transcription and splicing in separate experiments and looked for effects on the high-throughput RNA sequencing (RNA-seq) patterns for nuclear and cytoplasmic RNA.

RNA polymerase II transcription was inhibited with actinomycin D. Oocytes were removed from a mature female and placed in OR2 culture medium. Active transcription in the mature oocytes was verified by examination

of the lampbrush chromosomes, which exhibited pronounced transcription loops characteristic of RNA synthesis. Some oocytes were placed in actinomycin D at 20 $\mu\text{g}/\text{mL}$, a concentration that inhibits RNA transcription within 1 h (for review, see Callan 1986; Gall and Murphy 1998). Fifteen hours later, cytological examination showed contracted chromosomes without lateral loops, indicating that transcription had ceased. GV and cytoplasmic RNA samples were isolated from the actinomycin-treated oocytes and from control oocytes taken from the same female. The RNA-seq data sets obtained from actinomycin-treated oocytes were very similar to those of the controls both qualitatively (Fig. 3, top four rows) and quantitatively (Supplemental Fig. S3A,B). We conclude, therefore, that transcripts seen in the GV and cytoplasm are stable for a minimum of $\sim 12\text{--}14$ h.

Splicing was examined by depleting oocytes of the essential splicing factor U2 small nuclear RNA (snRNA). Earlier experiments showed that *Xenopus* oocytes can be depleted of U2 by injecting a specific antisense deoxyoligonucleotide (Pan and Prives 1988). The snRNA is reduced to $<1\%$ of its original amount within 10–30 min and remains at this level for at least 48 h (Tsvetkov et al. 1992). Under these conditions, splicing does not occur, and the normally abundant U2 snRNA is not detectable on the lampbrush chromosome loops by in situ hybridization. However, transcription continues for several days (Tsvetkov et al. 1992).

We injected 250 oocytes with a deoxyoligonucleotide against U2 snRNA and injected a comparable number with water. Approximately 36–48 h later, we isolated 150 GVs from both the experimental and water-injected oocytes and prepared RNA for sequencing. At the same time, we isolated cytoplasmic RNA from both samples. After RNA-seq, we compared the abundance of U2 snRNA in the two GV samples. In GVs from oligo-injected oocytes, U2 snRNA was reduced to $\sim 1\%$ of its original value, demonstrating that the treatment was successful (Supplemental Fig. S4A). Aside from this specific effect of oligonucleotide injection on U2 snRNA,

control and injected samples were essentially identical. Individual genes showed the same patterns of GV and cytoplasmic sequences as the controls (Fig. 3, bottom four rows). Furthermore, the relative abundances of sequences in the experimental and control samples were very similar (Supplemental Fig. S4B,C). It is important to note that buildup of unspliced RNA (exons and introns) in the GV was not observed, as might be expected from accumulation of unexported nascent transcripts. As discussed later, the apparent absence of unspliced sequences can be explained by the fact that the GV contains only one set of G2 chromosomes; even though these chromosomes transcribe actively, the absolute amount of new RNA produced by them in 2 d is undetectable in our experiments. Thus, intronic sequences observed at the end of our experiment must have been present from the beginning.

Nuclear intronic sequences are processed from primary transcripts

If the stable intronic sequences result from splicing events at the time of mRNA production, they should derive from the same strand as the mRNA. To test this assumption, we carried out RT-PCR experiments on introns from six different genes using forward or reverse primers alone for the initial RT step, followed by standard PCR amplification with both primers (Fig. 4A). In each case, the reverse primer at the RT step gave a product of the expected size, whereas the forward primer gave no product. Thus, these intronic sequences are derived from the same strand as the mRNA.

Many of the intronic sequences in the GV appear to span the entire length of smaller introns (those less than ~ 500 nt), suggesting that they might derive from a single intron lariat either with or without prior debranching. However, longer introns are generally represented by a single peak that does not cover the entire intron or by multiple peaks, often superimposed on a low level across a major portion of the intron (e.g., Figs. 3–5). We carried out RT-PCR experiments to examine the peaks from

Figure 4. Stable intronic sequences are transcribed from the coding strand but are not part of a nascent transcript. (A) Intronic sequences in GV RNA could be amplified by RT-PCR only when the RT primer recognized the sense strand. Shown here are the RT-PCR results from amplifying the sense and antisense strands for six genes (cropped, in each case, from the same gel). (B) Intronic sequences could be amplified with two primers within an intron but not with one primer in an intron and one in an adjacent exon. At the top of the figure is shown part of the gene model for *calm1* (calmodulin 1) and the primers used for RT-PCR. The ordinate shows the number of reads per base. A similar intronic peak was examined for three other genes: *nasp* (histone H1-binding protein), *pcna* (proliferating cell nuclear antigen), and *aldolase* (aldolase C). The primers and corresponding introns are given in Supplemental Table 1.

Figure 5. Multiple peaks within a single intron are derived from separate molecules. RT-PCR primers were designed as shown in the gene models for *e2f3* (E2F transcription factor 3) and *gpbp1/1* (GC-rich promoter-binding protein 1-like 1). Products were obtained when both primers were within one intronic peak but not when one was in one peak and the other was in an adjacent peak. The bands labeled “RNA” are controls to demonstrate that peak 1F and peak 2R primers work when tested on in vitro transcribed RNA (details in the Materials and Methods). Primers are given in Supplemental Table 1. The ordinates show the number of reads per base.

a few selected genes. First, we examined four cases of single peaks that span most of an intron (Fig. 4B). Although in these examples there are few or no reads that map to the exon–intron junctions, it was important to verify experimentally that the stored intronic sequences are not part of unspliced transcripts. In each case, RT-PCR demonstrates that the intronic sequences are not connected to adjacent exonic sequences. Although these experiments do not formally demonstrate that the stable intronic sequences are derived by splicing and subsequent processing of introns from primary transcripts, the data are consistent with that hypothesis.

We also tested whether multiple peaks within an intron represent independent molecules, as opposed to sequencing or mapping artifacts. First, we asked whether peaks and valleys could result from the way repeated sequences are mapped by the alignment program (Supplemental Material). Our analysis suggests that repeated sequences do complicate interpretation of the regions in which they occur but do not account for the majority of peaks and valleys (Supplemental Fig. S5). We also examined multiple peaks experimentally by asking whether two adjacent peaks in the same intron were part of the same molecule or represented independent fragments (Fig. 5). We could amplify sequences within a peak by RT-PCR but could not amplify a sequence that extended from one peak to the next. At least for the two examples that we analyzed, the intronic sequences were derived by a process that leads to independent fragments within a single intron.

Nuclear intronic sequences are less stable than cytoplasmic mRNA

Because introns and exons are formed in equimolar amounts (and the oocyte is a closed system), the relative abundance of introns and exons in whole oocytes should be a measure of their relative stabilities. We first tried to determine relative abundance by examining RNA derived from intact oocytes (cytoplasm + GV). However, when

such RNA was sequenced and displayed on the browser, it appeared almost identical to cytoplasmic RNA (Fig. 6, top two rows). In other words, for a given gene, the abundance of intronic sequences in the GV is too low relative to the abundance of exonic sequences in the cytoplasm to be detectable at the read depth of our experiments. Fortunately, certain very abundant RNAs that are limited to the GV can be detected in RNA from whole oocytes. These include various noncoding RNAs, such as snoRNAs, 7SK RNA, and RNase P RNA, as well as a number of unannotated sequences. In principle, one can measure their abundance relative to exons in the whole oocyte sample (Fig. 6, top row) and then measure their abundance relative to introns from the same gene in the GV sample (Fig. 6, third and fourth rows). From these two estimates, one can calculate the ratio of introns to exons for a given gene. The calculated molar ratio of GV introns to cytoplasmic exons for 12 genes in the mature oocyte was 0.86% (range: 0.02%–4.50%) (Fig. 6; Supplemental Table 2). Two features emerge from these numbers. First, intronic sequences in the nucleus are roughly two orders of magnitude less abundant and therefore are less stable than their corresponding exons in the cytoplasm. Exceptions include a few cases like the snoRNAs, which can be as abundant as the exonic sequences of the genes in which they occur. Second, the relative stability of introns to exons varies widely from gene to gene. Some of this variability is due to the difficulty of measuring the abundance of intronic sequences for a given gene. In addition, there are undoubtedly real differences in the stability of various mRNAs in the cytoplasm and their corresponding introns in the nucleus.

Intronic sequences are stable during maturation and early cleavage stages

To determine whether the intronic sequences detected in GVs from mature oocytes persist through oocyte maturation and early development, we sequenced RNA from three developmental stages: oocytes treated with pro-

Figure 6. Estimating the relative abundance of cytoplasmic exonic and nuclear intronic sequences from the same gene. The method depends on having a superabundant, strictly nuclear transcript derived from an intron, such as a snoRNA. Shown here is the 5' end of *trrap* (transformation/transcription domain-associated protein). The ordinates show the number of reads per base. (First and second rows) RNA from a whole oocyte (cytoplasm + GV) and from cytoplasm only. The large intronic peak in the second intron in the *top* row (RNA from whole oocytes) was derived entirely from the GV, as shown by its absence from the second row, which displays cytoplasmic RNA only. Quantitation of the peak in row 1 shows that it occurs (in the whole oocyte) at roughly twice the molar concentration of the cytoplasmic exonic peaks. (Third and fourth rows) These two rows display the GV sequences from *trrap* on two different scales (maximum of 9390 and 50 reads, respectively). In the third row, one sees only the superabundant peak because it is roughly 200 times higher than the other intronic peaks. Since this superabundant peak is twice as abundant as the exons and 200 times as abundant as the introns, we can conclude that the exons in the cytoplasm are 100 times as abundant as the introns in the GV. Quantitative estimates for 16 such cases are given in Supplemental Table 2.

gesterone to induce GV breakdown, four- to eight-cell embryos, and mid-blastulae (at which time zygotic transcription begins in *Xenopus*). The RNA profiles from these three samples were essentially identical to each other and to total RNA from mature oocytes (Supplemental Fig. S6). As expected, intronic sequences were not, in general, detectable in these samples on the genome browser, although snoRNAs and other highly abundant nuclear RNAs could be seen. We therefore designed primers to look for individual introns by RT-PCR, choosing genes whose transcripts are abundant in the mature oocyte. We could easily detect introns in total RNA from progesterone-treated oocytes (after GV breakdown), four- to eight-cell embryos, and blastulae (Fig. 7). Because there is no transcription during early cleavage stages of *Xenopus* (for review, see Davidson 1986), we conclude that introns detectable up to the blastula stage must be derived from the stable introns produced by the oocyte.

Discussion

The major finding of this study is that the GV of *X. tropicalis* stores stable intronic sequences derived from a majority of its transcribed genes. We refer to these as “intronic sequences” rather than introns because the stored sequences can be considerably shorter than the entire intron from which they are derived. A few specific examples of stable intronic sequences have been described before (Michaeli et al. 1988; Kopczynski and Muskavitch 1992; Qian et al. 1992; Yang et al. 2011), but these have been considered exceptional. To our knowledge, stable intronic sequences derived from a majority of the transcribed genes in the genome have not been previously described. These sequences from the oocyte nucleus constitute a previously undetected class

of noncoding RNA for which we propose the name stable intronic sequence RNA (sisRNA).

The stability of sisRNA in the GV was tested by treating oocytes with actinomycin D to inhibit transcription or with an antisense deoxyoligonucleotide to inhibit splicing. Because both treatments prevent the generation of new introns by splicing, the existence of intronic sequences at the end of the experiment implies that these sequences are stable for at least 1 or 2 d. For technical reasons, we could not examine total nuclear RNA from developmental stages after GV breakdown. Nevertheless, we could amplify intronic sequences from selected genes by RT-PCR in RNA samples from progesterone-treated oocytes (after GV breakdown), four- to eight-cell embryos, and blastulae. It is probable, therefore, that sisRNA from the mature oocyte persists during the early stages of embryonic development, at least until zygotic transcription begins at the blastula stage.

Although sisRNA sequences are almost certainly generated by processing from primary transcripts, they occur at a low molar ratio compared with their cognate exons,

Figure 7. Intronic sequences persist up to the blastula stage of embryogenesis. To test whether intronic sequences are stable after GV breakdown and fertilization, RT-PCR was carried out on total egg RNA from progesterone-matured oocytes, four- to eight-cell embryos, and blastulae. Intronic sequences were detected in each case. Primers are given in Supplemental Table 1.

on the order of 1%. Experimentally, this feature is evident from examination of RNA from intact oocytes; that is, oocytes from which the GV has not been removed. An intact oocyte contains one GV's worth of intronic sequences and one cytoplasm's worth of exons, yet the intronic sequences in such a sample are not detectable at the read depth obtained in our sequencing experiments (Fig. 6). Thus, although the intronic sequences are stable for at least 2 d, they are considerably less stable than the mRNA stored in the oocyte cytoplasm.

The biological significance of this relationship is best understood in terms of two features that dominate the transcriptional profile of the amphibian oocyte. First, the volume of cytoplasm in a mature oocyte is $\sim 10^5$ to 10^6 that of a somatic cell, yet the absolute concentration of rRNA and various mRNAs is not unusual (Simeoni et al. 2012; for earlier references, see Davidson 1986). Second, the GV, as enormous as it is relative to a somatic nucleus, contains only the normal complement of G2 chromosomes, which must produce all of the mRNA of the oocyte. In other words, an amphibian oocyte synthesizes and stores orders of magnitude more mRNA than a typical somatic cell but does this with a single G2 nucleus. The strategy that accomplishes this extraordinary feat has three essential components. First, the rate of transcription is unusually high; second, transcription continues for weeks or months; and third, the mRNA that is produced is extremely stable. Estimates suggest that the mRNA of *X. laevis*, a close relative of *X. tropicalis*, has a half-life of ~ 35 d (Davidson 1986), based on earlier experiments of Anderson and Smith (1978) and Dolecki and Smith (1979). Even so, the lampbrush chromosomes in the GV must transcribe at an unusually high rate to produce this much mRNA (Callan 1986). If the intronic sequences in the GV were as stable as the mRNA in the cytoplasm, the GV would accumulate, over the long period of oogenesis, 10^5 to 10^6 more introns than exist at steady state in a typical somatic nucleus. Thus, even though the molar ratio of nuclear intronic sequences to cytoplasmic exons is only $\sim 1\%$, the absolute number of intronic sequences in the GV is very large.

Introns were discovered 35 years ago (Berget et al. 1977; Chow et al. 1977), and since then, much has been learned about the molecular biology of splicing (Hoskins and Moore 2012) and the importance of introns for alternative splicing and the generation of protein diversity (Black 2003). The evolutionary origin and significance of introns has also been extensively studied (Rodriguez-Trelles et al. 2006; Roy and Gilbert 2006). In addition, some introns are known to be precursors of specific molecules, of which snoRNAs and small Cajal body RNA (scaRNA) are well-studied examples (Maxwell and Fournier 1995; Kiss et al. 2010). But intronic sequences in general are thought to degrade rapidly after splicing from the primary transcript, and so there has been little reason to suppose that they play significant biological roles. The discovery of sisRNA in the oocyte nucleus now brings that assumption into question and raises a number of new issues. Certain questions can be addressed fairly directly, based on existing techniques. How are sisRNAs processed from

the primary transcripts? Are they formed by a specific trimming mechanism after conventional lariat formation and debranching? When and where do these processes occur in the nucleus, and where are the final sisRNAs stored? Are sisRNAs associated with specific proteins? The *Xenopus* oocyte, with its abundance of sisRNA, offers a useful system in which to examine these specific molecular issues.

A bigger challenge will be to determine the biological functions of sisRNAs. Do they play a major role during oogenesis, or are they synthesized and stored in the GV primarily for use during later embryogenesis? Do they occur in other cell types? Although the ability to obtain pure nuclear and cytoplasmic RNA fractions makes the *Xenopus* oocyte unique for molecular studies, the difficulty of doing genetics on frogs is a serious drawback for functional analysis. We are currently trying to identify sisRNAs in *Drosophila*, where the wealth of genetic tools would make the search for biological function more feasible. Because the oocyte nucleus of *Drosophila* is transcriptionally silent, sisRNAs, if they exist, are likely to be derived from the nurse cells. Despite the technical challenges, it should be possible to enrich for RNA from nurse cell nuclei and determine whether the *Drosophila* oocyte, like that of *Xenopus*, stores sisRNAs and transmits them to the embryo.

Materials and methods

Animals and oocytes

Adult female *X. tropicalis* were purchased from Xenopus 1. Animals were anesthetized with 0.15% tricaine methane sulfonate, and one or both ovaries were removed surgically. Pieces of ovary were placed in OR2 medium (Wallace et al. 1973) at room temperature, where the oocytes maintain normal morphology and biochemical properties for several days. For removal of follicle cells, oocytes were treated with collagenase (Liberase, Roche Applied Science) with gentle swirling in plastic culture dishes for 2–3 h (Simeoni et al. 2012). The absence of follicle cells was verified by staining oocytes with DAPI (4',6-diamidino-2-phenylindole) at 1 $\mu\text{g}/\text{mL}$ and examining at low magnification with a fluorescence microscope (10 $\times$ objective). Transcription was inhibited by incubating pieces of ovary in actinomycin D (20 $\mu\text{g}/\text{mL}$) in OR2 medium. Splicing was inhibited by injecting individual oocytes with a single-stranded deoxyoligonucleotide complementary to part of the U2 snRNA molecule (Tsvetkov et al. 1992).

Preparation of nuclear and cytoplasmic fractions

We routinely recovered ~ 1 μg of total RNA per oocyte using standard RNA extraction procedures on batches of 10–50 mature *X. tropicalis* oocytes (whole or enucleated). Using similar conditions on batches of 200–1000 isolated GVs, we recovered 2.1 ng of RNA per GV (average of 10 experiments). Because the cytoplasm contains ~ 500 times as much RNA as the GV, even a small amount of cytoplasm adherent to the nuclear envelope seriously compromises the purity of a nuclear RNA sample. For this reason we developed a manual technique for removing the nuclear envelope without losing the contents of the GV. Using jewelers' forceps, we isolated GVs in an isotonic saline solution at pH 5.6–5.8 (83 mM KCl, 17 mM NaCl, 6.0 mM Na_2HPO_4 , 4.0

mM KH_2PO_4 , 1 mM MgCl_2 , 1.0 mM dithiothreitol, adjusted to pH 5.6–5.8 with HCl. At this pH, the nuclear contents begin to gel within seconds after isolation from the oocyte. Simultaneously, the nuclear envelope swells away from the gelled contents (Fig. 1E–G). The envelope can be removed easily with jewelers' forceps, especially because it adheres to the tips of the forceps. GV contents were collected in batches of 10–20 and transferred to a small vial containing 10 mM sodium citrate and 5 mM EDTA (pH 5.0) for storage. At least 200 GVs were collected for RNA extraction, equivalent to ~400 ng of total RNA.

For isolation of cytoplasmic RNA, oocytes were transferred from OR2 medium to the same solution used for GV isolation, except at pH 7.0. The GV was removed with jewelers' forceps (Fig. 1A), and the enucleated cytoplasm was transferred in minimal liquid to a 2-mL Eppendorf tube on dry ice.

RNA extraction

Defolliculated whole oocytes and enucleated oocytes were frozen singly or in groups of five to 10 in a 2-mL plastic Eppendorf tube on dry ice. Groups of 200–1000 GVs were collected as described above and stored frozen until used. RNA was extracted in a guanidinium thiocyanate/phenol/chloroform mixture (TRIzol, Ambion) and purified according to the manufacturer's protocol. RNA was quantitated with a NanoDrop 2000 spectrophotometer (Thermo Scientific) and further characterized by electrophoresis in a Bioanalyzer 2100 (Agilent).

RT-PCR analysis of introns

RT-PCR analysis of selected genes (Supplemental Table 1) was carried out on the same RNA samples that were used for deep sequencing. Total RNA was reverse-transcribed for 1 h using AMV-RT (New England Biolabs) and random hexamers. The cDNA was then purified and used for PCR (40 cycles). In Figure 5, peak 1F and peak 2R primers gave a product when tested against genomic DNA (gDNA) but not when tested against nuclear RNA (RT⁺). To demonstrate that these primers are competent when tested on RNA, we cloned the region spanning peak 1F through peak 2R. We then made an in vitro RNA transcript from this clone to use as a template in a one-step RT-PCR reaction (Qiagen), which gave a positive PCR product (Fig. 5, lanes marked RNA). For determining strand specificity of selected introns, one-step RT-PCR was used (Qiagen) with a single primer for the RT step, followed by PCR with both sets of primers (40 cycles). PCR products were visualized on 1% agarose gels.

Sequencing and sequence analysis

The RNA was fragmented, and a cDNA library was generated by random hexamer priming following the protocol in the Illumina TruSeq RNA sample preparation guide. cDNA prepared from GVs, and cytoplasmic RNA was ~200–500 nt in length. One-hundred bases were read from one end using the Illumina HiSeq 2000 sequencer. Reads were aligned to the *X. tropicalis* genome (assembly version 4.1) using the TopHat version 1.2.0 and Bowtie version 0.12.7 sequence alignment programs (Langmead et al. 2009; Trapnell et al. 2009). The Cufflinks program version 0.9.3 (Trapnell et al. 2010) was then used to generate FPKM_{exon} values for the entire genome. To obtain comparable data for introns, it was necessary to generate an intronic gene transfer format (GTF) file for use with Cufflinks. We downloaded exon–intron junction information from XenBase and generated a GTF file that listed the locations of introns rather than exons (Fig. 2). Since a typical annotation file lists only the exons of a gene, Cufflinks uses that

information to quantify the overall expression levels of the exonic regions (FPKM_{exon}). However, given an intronic annotation file, Cufflinks will calculate intronic expression levels (FPKM_{intron}). We used these FPKM_{intron} values to compare intronic data from our various sequencing runs. Sequence alignments were examined in the Integrative Genomics Viewer (IGV) from the Broad Institute (Robinson et al. 2011). Other analyses, especially pairwise comparisons of RNA from different samples, were carried out with the Spotfire DecisionSite 9.1 analysis software (Tibco).

Microarray analysis

RNA samples were amplified and biotin-labeled according to the Affymetrix manufacturer's 3' IVT Express protocol, hybridized to the Affymetrix GeneChip *Xenopus tropicalis* Genome Array, and scanned in the Affymetrix GeneChip Scanner 3000. Data were extracted and normalized with the Robust Multiarray Average (RMA) in the Partek Genomics suite 6.5 (Partek, Inc) and analyzed with the Partek and Spotfire platforms.

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Note added in proof

Expression data were deposited in the NCBI Gene Expression Omnibus (GEO) under accession number GSE41530.

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