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Analysis of specific RNA in cultured cells through quantitative integration of q-PCR and N-SIM single cell FISH images: Application to hormonal stimulation of StAR transcription

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Abstract

The steroidogenic acute regulatory protein (StAR) has been proposed to serve as the switch that can turn on/off steroidogenesis. We investigated the events that facilitate dynamic StAR transcription in response to cAMP stimulation in MA-10 Leydig cells, focusing on splicing anomalies at StAR gene loci. We used 3' reverse primers in a single reaction to respectively quantify StAR primary (p-RNA), spliced (sp-RNA/mRNA), and extended 3' untranslated region (UTR) transcripts, which were quantitatively imaged by high-resolution fluorescence in situ hybridization (FISH). This approach delivers spatio-temporal resolution of initiation and splicing at single StAR loci, and transfers individual mRNA molecules to cytoplasmic sites. Gene expression was biphasic, initially showing slow splicing, transitioning to concerted splicing. The alternative 3.5-kb mRNAs were distinguished through the use of extended 3' UTR probes, which exhibited distinctive mitochondrial distribution. Combining quantitative PCR and FISH enables imaging of localization of RNA expression and analysis of RNA processing rates.

Keywords

StAR; Transcription; Splicing; Fluorescence in situ hybridization; PCR

1. Introduction

Steroidogenic acute regulatory protein (StAR) functions as a key determinant that mediates steroidogenesis in the testis, adrenal, ovary, and even regions of the brain under steroid control (Caron et al., 1997; Clark et al., 1994; Kiriakidou et al., 1996). StAR is a cholesterol-

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Appendix A. Supplementary data

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binding protein that mediates the movement of cholesterol into the mitochondria for conversion to pregnenolone by Cyp11a1, the limiting step for the synthesis of most steroids (Artemenko et al., 2001; Lin et al., 1995; Stocco et al., 1995). The stimulation of StAR transcription has been extensively studied in MA10 Leydig cells that constitutively express Cyp11a1 and Hsd3b, but not Star at basal levels (Clark et al., 1994; Hales et al., 1990; Hiroi et al., 2004; Manna et al., 2011). cAMP regulates steroidogenic enzyme genes and steroid synthesis through collective modulation of chromatin and a shared cohort of transcription factors/cofactors that include SF1, GATA4, CREB/CBP, C/EBP β , and NR4A1 (Clem et al., 2005; Di-Luoffo et al., 2015; Feng et al., 2000; Reinhart et al., 1999). Br-cAMP initiates a robust increase in StAR, beginning with primary RNA (p-RNA) transcripts (Lee et al., 2015).

A critical feature of StAR transcripts is the alternative polyadenylation that introduces an extra 2 kb of the 3'untranslated region (UTR), which delivers additional regulation of mRNA stability and translation (StAR 1.6 kb and 3.5 kb mRNA) (Duan et al., 2009). StAR is commonly involved in such regulation during acute hormonal responses (Clark et al., 1994; Jefcoate et al., 2011; Lin et al., 1995; Stocco et al., 1995). In this study, we investigated the events that facilitate dynamic StAR transcription in response to cAMP stimulation in MA-10 Leydig cells, focusing on splicing anomalies at StAR gene loci. In particular, we visualized the coordinated regulation of StAR processing (Lee et al., 2015), which is synchronized by salt-inducible kinase (SIK1) and CREB-regulated transcription coactivator (CRTC2) (Screaton et al., 2004; Takemori et al., 2009; Uebi et al., 2010). The steps used for the processing of p-RNA to mature mRNA include capping, splicing, 3'UTR cleavage, and polyadenylation (Bentley, 2005; Hirose and Manley, 2000; Maniatis and Reed, 2002; Moore and Proudfoot, 2009), which involve multi-protein complexes organized around the C-terminus of RNA polymerase 2 (Pol 2) (Nojima et al., 2015).

In order to best understand transcription and splicing mechanisms, it is crucial to measure the exact amount of RNA species at the single-cell level. Quantitative polymerase chain reaction (q-PCR) is the gold standard for validating the results of genome-wide transcriptional studies, but q-PCR alone does not allow for assessments of cell-to-cell variation. Therefore, the quantitative comparison of copy numbers per cell determined by fluorescence in situ hybridization (FISH) with equivalent q-PCR analyses complement each other. Raj et al. used RNA-FISH and reverse transcription (RT)-qPCR in concert to quantify the amplification of a target sequence (Raj et al., 2008), but absolute quantitation with this combination has not yet been experimentally introduced. The main hurdle is that the precision of the measurement of p-RNA and spliced RNA (sp-RNA/mRNA) copy numbers is limited by inter-target differences regarding the priming strategy. Here, we propose a crucial step in cDNA priming, which is problematic with low levels of primary transcripts, targeting multiple SAR gene regions using a cocktail of specific 3⁰ reverse primers in a single reaction.

To optimize quantitation of the RNA species, we describe a high-resolution (HR)-FISH method based on the synergistic binding of sets of about 40 Quasar dye-labeled oligomers (Raj and Tyagi, 2010; Raj et al., 2008). Several recent papers have established that single mRNA species can be resolved by high-resolution microscopy (Levesque and Raj, 2013;

Lubeck and Cai, 2012; Mueller et al., 2013). The more advanced image processing delivered by the Nikon structured illumination microscopy (N-SIM) is introduced (Smeets et al., 2014; Walter et al., 2006). This resolution has previously described for other mRNA species but not for time dependent stimulations (Batish et al., 2012; Raj et al., 2008). We used N-SIM and the “FISH quant” algorithm to localize and quantify RNA transcripts to subcellular regions within single cells (Mueller et al., 2013). Here, we address how these approaches may be applied to practical problems relating to uncovering transcription and splicing mechanisms. As an application of this approach, we focused on understanding novel features that we previously reported for the hormonal activation of StAR. In particular, we discovered time-dependent changes in the ratio of StAR p-RNA to sp-RNA. Thus, in the present study, we used the novel HR-FISH/qPCR method to spatially and temporally resolve primary and spliced transcripts at a specific gene locus. Here, we also used a separate set of oligomers to quantify the extended sequence (3'EU probes). HR-FISH identifies StAR 3.5 kb transcripts that are dually hybridized in, respectively, the 5' translated ORF segment and the 3'terminal segment.

StAR protein is exclusively expressed in mitochondria. We show here that all the MA10 mitochondria express StAR protein. Association of StAR mRNA with the mitochondria marked by StAR protein is however spatially heterogeneous while a distinct pool of StAR mRNA is separated from mitochondria. The functional significance of these different pools of StAR RNA is discussed. This comprehensive integration of PCR and advanced fluorescence imaging provides a general approach to achieve better understanding of the underlying cell and locus heterogeneity that accompanies hormonal stimulation.

2. Materials and methods

2.1. Cell culture

MA-10 mouse Leydig tumor cell line (a gift of Dr. Mario Ascoli) was derived from Leydig tumor, M5480P (Ascoli, 1981), and shows steroidogenic response to protein kinase A (PKA) activation by 8-Br-cAMP, luteinizing hormone (LH) and human chorionic gonadotropin (hCG) stimulation. Steroidogenic acute regulatory protein (StAR) gene expression has been well characterized in various studies (Clark et al., 1994; Hiroi et al., 2004; Stocco et al., 1995). MA-10 (ATCC® CRL-3050™) cells were maintained in D-MEM/F-12 media (Gibco) supplemented with 5% horse serum (Gibco), 2.5% fetal bovine serum (Atlanta), and 50 µg/ml gentamicin (Gibco). A day before the experiment, 6×10^5 cells were seeded onto the coverslip coated with ploy-Lysine for FISH or directly in wells of a 6-well plate (Corning). On the day of the experiment, the cell culture medium was replaced with serum-free medium containing 50 µg/ml gentamicin. After 1hr, the cells were treated with 8-Br-cAMP (Axxora). For consistent growth, cells were cultured on 0.1% gelatin (Difco)-coated plate. All cells were incubated at 37 °C in a humidified atmosphere with 5% CO₂.

2.2. RT-qPCR

2.2.1. Primer design—In this study, four web tools were used for primer design. First, Ensemble Genome Browser (<http://www.ensembl.org/index.html>) was employed to copy the target sequences such as exon-intron, exon-exon, and 3' UTR of interest. The sequences

were then pasted into Primer3 (<http://bioinfo.ut.ee/primer3-0.4.0/>) to generate the specific primers. Afterward, a database search was conducted using the primer pair to verify the DNA region lying between the two primers using the UCSC in Silico PCR. (<http://genome.ucsc.edu/index.html>). Finally, primer set quality and length were evaluated using the Amplify4 software. The output designated that all reverse primers are specific for cDNA priming (Supplemental Table 1).

2.2.2. cDNA synthesis—Total RNA was isolated from MA-10 Leydig cell culture using TRIzol® Reagent (Invitrogen) according to manufacturer's recommendations. RNA quantity and quality were assessed using Nano-drop spectrophotometer (Thermo Scientific). 2 µg of total RNA was mixed with 0.5 µg random hexamers (Promega), 0.5 µg oligo-d(T) primer (Promega), or 1 µM specific reverse primer (IDT) and the volume was brought up to 10 µL with nuclease-free water. For the multi-primed RT-PCR, a cocktail mix of 1 µM specific reverse primer was added to the reaction tube. The mixture was then subjected to denaturation at 70 °C for 5 min. Subsequently, 10 µL of cDNA master mix consisting of 4 µL 5 × reaction buffer (GoScript™ Promega), 2 µL MgCl₂ (25 mM; Promega), 0.2 µL dNTP working stock (40U/µL; RNasin® Promega), 0.5 µL RNase Inhibitor (2500u; Promega), 1 µL reverse transcriptase (Promega) and 2.3 µL of nuclease-free water was added. The samples underwent annealing at 25 °C for 5 min, extension at 42 °C for 60 min and inactivation at 70 °C for 15 min. The transcribed cDNAs were then diluted with 80 µL of nuclease-free water before qPCR. In multi-primed cDNA synthesis, multiple (up to 7) specific primers were used together in the same PCR reaction to increase the accuracy of data normalization. We have shown that different regions of cDNA fragments in the same gene (StAR) were equally synthesized without inhibiting each other.

2.2.3. Quantitative real-time PCR—Each qPCR reaction mix was prepared using 1 µL cDNA and 9 µL of the qPCR master mix: 5 µL 2× GoTaq® Green Mastermix (Promega), 0.5 µL of 10 µM qPCR primer set (300 nM final) and 3.7 µL of nuclease-free water. The qPCR was performed using Bio-Rad CFX96™ Real-Time PCR Detection System. The qPCR protocol was done as follow: initial denaturation at 95 °C for 3 min, followed by 40 cycles of 10 s at 95 °C, 30 s at 55 °C, and 10 s at 95 °C. The fluorescence signal was recorded at each extension step at 55 °C. A melting curve analysis was then conducted from 65 °C to 95 °C at an increment of 0.5 °C for 5 s to assess the amplicon specificity.

2.2.4. Construction of standard curve and determination of absolute transcript copy number—A standard curve dilution was generated from gBlock gene fragment (IDT) (Supplemental Table 2) with tenfold serial dilution series over 6 logs. The initial copy number of the gBlock gene fragment was determined using this formula (Lee et al., 2006). $\text{DNA (copy)} = (6.02 \times 10^{23} \text{ (copy/mol)} \times \text{DNA amount (g)}) / (\text{DNA length (dp)} \times 660 \text{ (g/mol/dp)})$. The molecular weight of oligonucleotide (in Daltons or g/mole) was provided by IDT. It was determined that the gBlock dilution series ranged from 10⁸ copies/mL to 10³ copies/µL. The standard curve was constructed by plotting the linear regression of Ct values against the logarithm of their initial template copy number. The slope, y-intercept and R-squared values of the standard curve were quantified. The absolute transcript copy number of the unknown samples was calculated by relating their Ct values to

the standard curve using the formula: Absolute transcript copy number = $10^{[(Ct - (y - \text{intercept}))/\text{slope}]}$. The resulting qPCR products of different RT primers were also visualized and compared using 1% gel electrophoresis under UV ray. RNAstructure is a software for RNA secondary structure prediction and analysis (Reuter and Mathews, 2010). Folding free energy changes can be predicted for a given RNA structure using nearest neighbor rules (Bellaousov et al., 2013; Reuter and Mathews, 2010). RNA structure is available at (<http://rna.urmc.rochester.edu/RNAstructureWeb/>).

2.3. High resolution fluorescence in situ hybridization (HR-FISH)

The term HR-FISH has been used here to distinguish the use of a probe set consisting of 40 fluorescent 20mers (Supplemental Table 3). The RNA probe sets for Steroidogenic Acute Regulatory protein (StAR) was generated by using the Stellaris probe designer (<http://www.biosearchtech.com/stellarisdesigner/>). The p-RNA probes target introns while the sp-RNA selectively target spliced sequences by limiting the number of oligomers in exons to <8 and then to span exon-exon junction regions.

A day before an experiment, 6×10^5 cells were seeded on coverslips (Corning) coated with 100 μM of poly-lysine (Sigma) in wells of a 6-well plate (Corning). On the day of an experiment, the cell culture medium was replaced with serum-free medium containing 50 $\mu\text{g}/\text{ml}$ gentamicin. After 1hr, the cells were treated with 8-Br-cAMP (Axxora) for specified times. After several washes in PBS (Phosphate buffered saline), fixed cells on coverslips were prepared by treatment with 0.4% formaldehyde (Sigma) for 15 min at RT, followed by permeabilization with 70% ethanol for at least for 4 h. After a brief incubation in wash buffer (2 $\times$ saline sodium citrate (SSC (Cellgro)) and 10% deionized formamide) for 5 min, 50 μL of hybridization solution with probes was overlaid. Freshly prepared p-RNA (Quasar-570, Biosearch Technology), sp-RNA (Quasar-570, Biosearch Technology) and 3'' EU (Quasar-570, Biosearch Technology) probe sets were used. A clean coverslip was placed over the sample to prevent drying of the hybridization solution (10% dextran sulfate (Sigma), 10% deionized formamide (Ambion), 2 $\times$ SSC) during the incubation. Samples were incubated in a dark humidified chamber at 37 $^{\circ}\text{C}$ overnight. After a 30-min wash in wash buffer, samples were incubated in DAPI nuclear stain (wash buffer with 5 ng/ml DAPI) to counterstain the nuclei for 30 min. Samples were re-suspended in 2 $\times$ SSC and added GLOX buffer (2 $\times$ SSC, 0.45% Glucose (Cellgro), 10 mM Tris-Cl (pH 8.0)) without enzymes for equilibration and incubated for 1–2 min 100 μL of GLOX buffer was added with 1 mL of the glucose oxidase stock (glucose oxidase (Sigma) diluted to 3.7 mg/mL in 50 mM sodium acetate, pH ~5) and 1 μL of catalase suspension (Sigma). Labeled samples were mounted with one drop of the Prolong Gold antifade reagent (Invitrogen).

2.4. Image acquisition and analysis

To detect and visualize p-RNA, sp-RNA, and mRNA, we used the Olympus wide-field fluorescence microscope (Model IX81) and the N-SIM for higher resolution images. The N-SIM uses different optical processing methods but provided with results that were fully consistent with the Olympus IX81.

2.4.1. Olympus IX81—Images were captured by Olympus IX81 motorized inverted microscope equipped with a Hamamatsu Orca2 camera. Images were obtained with 0.2 μm spacing with the 100 $\times$ oil objective and projected onto a single plane or 3D image. At least 30 z-sections at 0.2 μm intervals were taken to cover the entire thickness of the cell. The exposure times ranged from 0.2 s (nuclear loci) to 4 s (cytoplasmic mRNA). The exposure time increases sensitivity approximately proportionately but also increases background. Comparisons are made at the same exposure times. The target size is much smaller than the image due to the dispersion of the fluorescent light from the target. For loci have multiple clustered RNA molecules either p-RNA or sp-RNA. These terms have been used rather than pre-mRNA and mRNA to emphasize the absence of information on RNA processing. We term this fluorescent cluster the RNA locus which elsewhere we have shown to overlap with the gene (DNA) locus. p-RNA and sp-RNA at the RNA are separate in the images. Captured images were deconvoluted with Slidebook 5.0 software (Intelligent Imaging Innovations, Inc.) for further analysis.

2.4.2. N-SIM: Nikon-structured illumination microscope—Super-resolution imaging was performed with an N-SIM microscope system (Nikon) equipped with a SR Apo TIRF 100 $\times$ 1.49 N.A. oil immersion objective and an iXon3 camera (Andor Technology). 3D-SIM image stacks were acquired with a Z-distance of 0.2 μm , covering the entire thickness of the cell (about 6 μm) using laser power setting 40%. The camera settings were configured as follows: format for capture, no binning; exposure time, 50–200 ms (1 frame recommended); readout mode, EM gain 10 MHz 14-bit; gain multiplier, 20–200 ms (max 300); conversion gain. 5.1 $\times$. 15 raw images per plane were acquired and computationally recon-structed using the reconstruction slice system from NIS-Elements software (Nikon).

2.4.3. FISH-quant—FISH-quant (Mueller et al., 2013) is a program written in Matlab for analyzing FISH (HR-ISH) data, and we applied this to measure and locate p-RNA, sp-RNA and mRNA in 3D images by fitting Gaussians to fluorescent spots. However, the high local density of sp-RNAs prevents accurate estimation of the number of transcripts. FISH-quant offers two solutions: First, comparison of the integrated intensity of the transcription site to that of mRNA. Second, reconstruction of the transcription site signal by iterative superposition of the image with weighted Point Spread Function (PSF).

The outline of cells and transcription site for sp-RNA were defined, and conditions for mRNA detection were optimized. Pre-detected spots were narrow down by thresholds for amplitude, raw intensity and filtered intensity to remove false positives. The workflow of mRNA detection is to (1) filter image, (2) perform pre detection, (3) fit detected spots with 3D Gaussian, (4) and inspect detected spots. The workflow of quantification of spRNA is to: (1) analyze transcription site, (2) calculate properties of mature mRNA molecules, (3), determine the best background value for transcription site, (4) perform quantification with the best background value and analyze the results. FISH-quant is available at <http://code.google.com/p/fish-quant/> with a detailed manual and test data.

2.5. Data analysis

Statistical significance was determined by Student's *t*-test or ANOVA; $P < 0.05$ was considered statistically significant where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data were analyzed by using the PRISM software (San Diego, CA).

3. Results

3.1. Improved q-PCR quantitation of primary and spliced StAR transcripts (p-RNA and sp-RNA)

Previous analyses of the stimulation of the StAR transcription in MA10 cells via PKA activation showed appreciable delays in splicing. The transcript copy numbers for p-RNA and sp-RNA are necessary for the interpretation of their interconversion. Figs. 1 and 2 show how we developed q-PCR to generate these copy numbers. Fig. 1A depicts primer sets designed to target the primary transcripts by spanning the exon-intron boundaries (E1/I1; E6/I6). The second group targets the spliced transcripts by hybridizing exclusively to the exons (E1/E2; E5/E6). The schematic also shows a third set of primers (E7-S; E7-L), which targets the 3'UTR region of the StAR RNA. Additional analyses have been carried out for StAR p-RNA with primer pairs exclusively in intron 1 (Supplemental Fig. 1A). The E7-L primer pair targets the RNA that we expect to form the predominant 3.5 kb StAR mRNA, but not the 1.6 kb mRNA. The PCR products in each case are compared to a DNA block reference that includes all the intron, exon, and 3'UTR sequences of interest (Fig. 1B). Fig. 1C shows the typical time course for stimulation of StAR expression in MA10 cells compared to basal expression, which is appreciably higher for p-RNA compared to the more stable sp-RNA/mRNA. Random hexamers were used for the reverse transcriptase step, much in the way they were previously used by us and other investigators (Hauet et al., 2002; Jefcoate et al., 2011; Wang et al., 2002).

In order to improve this quantitation, we compared the efficiency of specific RT priming by using the 3'PCR primer, and additional specific RT primers moved further from the targeted sequence. Each RT product was compared with the same q-PCR reaction (Fig. 1D (a), E (a)). We analyzed low levels of StAR p-RNA formed after a 15 min stimulation using sequences at the 5' and 3' ends that are separated by about 3 kb (exon1/intron1; E1/I1 and exon6/intron6; E6/I6). The qPCR copy number was obtained from a standard curve provided by the reference DNA block. The expression levels using each primer pair were essentially the same. The RT efficiency declined extensively with the distance of the specific primer from the 3' end of the qPCR target sequence. For both E1/I1 and E6/I6 sites, priming with the 3' PCR reverse primer provided the largest copy number. In each case, this was 20–30 times higher than that obtained with random hexamers. The specific primer efficiency decreased to levels comparable to that of random hexamers when extended back by about 200 bases (Fig. 1D (b), E (b)). The primers pairs delivered an optimal set of temperature profiles (Supplemental Fig. 1B). We assume that this decreased efficiency in RT arises from the increased stability of the intervening secondary structure (Fig. 1D (c), E (c)). We also demonstrated the projected secondary structures based on a software package for RNA conformation ("RNA structure") (Fig. 1F) (Bellaousov et al., 2013).

Specific 3' RT primer was also much more effective for sp-RNA sequences compared to random hexamers, notably at the exon5/exon 6 junction (Fig. 2A). This improvement factor depends on the sequence on the 3'side of the selected q-PCR target. The difference was much less for Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) in these MA10 samples (Fig. 2B). This improvement is particularly important where the q-PCR target sequence is restricted in this case to a junction site. To test for interference between primers, we assessed the impact of an extra cocktail of specific 3' primers targeting multiple StAR gene regions on the RT synthesis, using the specific 3' primer for a single p-RNA sequence. The q-PCR response for the single p-RNA was the same with and without extra cocktail (Fig. 2C). Multiple specific StAR primers can, therefore, be used as a mixture in these q-PCR analyses.

3.2. Application of optimized q-PCR to stimulation of StAR p-RNA and sp-RNA by Br-cAMP

The time course for stimulation of copy numbers of, respectively, p-RNA (E1/I1) and sp-RNA (E5/E6) by Br-cAMP (1 mM) in MA10 cells is shown in Fig. 2D. There is a linear rise in the p-RNA level over 60 min to a steady state that is sustained until 120 min before declining. On the other hand, there is a 30 min delay in the initiation of sp-RNA transcription relative to the pRNA initiation. The sp-RNA increases between 30 and 75 min before rising rapidly to a peak level at 120 min. The steady state level of p-RNA at 60 min is similar to that of the spliced level at that time but does not increase between 75 and 120 min as the level of sp-RNA surges. This maintenance of the steady state suggests that the surge involves a separate transcription process.

3.3. Time course for stimulation of 3'UTR transcripts in comparison to StAR mRNA (sp-RNA)

The 3'UTR is present in both the primary transcript and the mRNA. In the previous report, we demonstrated a delay of about 20 min for both the elongation in exon 7 and splicing. These parallel delays raise the question of whether elongation extends to the end of exon 7 prior to the onset of splicing. In this case, there will be an excess of RNA copies determined by the E7-L primer pairs targeted to the end of the 3'UTR compared to q-PCR primers targeted to the sp-RNA/mRNA determined by q-PCR targeted to the spliced sites. This extra RNA is extended to the end of the 3'UTR without splicing.

To quantify the spliced mRNA levels, we extended the optimized specific RT priming strategies across three exon-exon boundaries (E1/E2, E3/E4, and E5/E6) as illustrated (Fig. 3A). The levels of sp-RNA determined at each junction were within 10 percent of the mean for the three sites at each stimulation time. The primer pairs delivered an optimal set of temperature profiles (Supplemental Fig. 2A). We also compared mRNA transcript copy numbers of the short and long segments of the 3'UTR region (E7-S and E7-L) (Fig. 3B). The levels of E7-S and E7-L were similar, indicating a clear predominance of 3.5 kb mRNA compared to 1.6 kb mRNA. The short mRNA includes the E7-S segment but does not include the E7-L segment. There is also a delay of 15–20 min in the generation of E7-S and E7-L segments of the 3'UTR that match the delay in splicing (Supplemental Fig. 2B). The levels of both E7-S and E7-L at 60 min were appreciably higher than for the spliced

transcripts. This difference suggests that E7-S and E7-L determinations include both StAR p-RNA and sp-RNA in these steady state determinations (Fig. 3C).

To validate this finding, we stimulated a different batch of lower passage MA10 cells. The q-PCRs of triplicate cultures for the two MA10 batches were analyzed together (Fig. 3C, D). The time courses are somewhat different but share key features. First copy numbers determined by q-PCR primers at E5/E6, E7-S, and E7-L sites are indistinguishable under basal conditions, and much higher than for the primary transcripts (E1/I1 and E6/I6) (Fig. 3C (a), 3D (a)). This basal equality is indicative of a single basal mRNA comprising mostly the 3.5 kb mRNA. After 15 min of stimulation, the p-RNA levels at each site rise without an increase in q-PCR at the E5/E6 sp-RNA, E7-S, and E7-L sites. This difference derives from delays in both exon 7 elongation and splicing. In each experiment involving between 15 and 30 min of stimulation by Br-cAMP, E7-S and E7-L increased appreciably more than E5/E6 sp-RNA did. This difference, which expanded at 45 min and at 60 min, is indicative of more exon 7 elongation than splicing. (Experiment 2 $p < 0.05$, 45 and 60 min) (Fig. 3C (b), D (b)). The E7-S and E7-L, for the most part, remain close (Supplemental Fig. 2C), indicating that full exon 7 elongation was occurring. The primary transcripts rise linearly to reach steady states formed by the balance between transcription and splicing (75–120 min/experiment 1; 60–90/experiment 2). Each experiment, this steady state was unchanged during the transcription. The extra RNA seen for E7-S and E7-L transcripts corresponded surprisingly closely to the amount of p-RNA at time point (Fig. 3C (c), D (c)). Thus under this near steady conditions for p-RNA, most of the p-RNA remains unspliced. Even after 15 min pause in transcription, splicing remains much slower. During the surge in sp-RNA formation (60–90 min), the E7-surplus E7-L and E7-S expressing transcripts relative to E5/E6 remained much the same as at 45 and 60 min. This surge mRNA, therefore, occurs with little net increase in p-RNA, indicating effective splicing. As noted earlier how p-RNA constant remains an important question.

3.4. HR-ISH imaging of StAR p-RNA and sp-RNA

To quantify the StAR expression in single cells, we used synergistic binding of tandem 20-base oligomers to RNA (Lee et al. 2015). Fig. 4A diagrams the way in which the 20mers are to target p-RNA and sp-RNA. Alternative polyadenylation of StAR RNA or sp-RNA generates a 1.6 kb mRNA or an extended 3.5 mRNA, each of which express the protein. A third set of oligomers targets the extended 3'UTR (3'EU probes) which is uniquely in the 3.5 kb mRNA. These probes overlap the segment targeted by the E7-L primer pair. After the 60-min. stimulation, Fig. 4B shows that the fluorescence signals from both probe sets were clearly detected at two loci. The differential kinetics indicates asymmetric activation of the StAR loci. The merger of the p-RNA and sp-RNA images shows their near complete overlap at the two loci. The images in Fig. 4C have a higher exposure to capture the weaker images of the cytoplasmic mRNA. At 60 min, essentially all cells showed expression of sp-RNA in at least one StAR locus. Indeed, most cells have at least two active loci. sp-RNA was absent from the cytoplasm after 60 min of stimulation whereas, after 180 min, there was extensive cytoplasmic expression but less expression at the loci.

3.5. HR-ISH of alternative polyadenylated 3.5 kb StAR mRNA

The numbers and distribution of StAR mRNA were also probed by simultaneous additions of two sets of probes: sp-RNA probes, which target the shared by segments of 1.6 kb and 3.5 kb mRNA, and 3'EU probes, which exclusively target the 3' terminal segment of the 3.5 kb mRNA. The near-equal q-PCR analyses using E7-S and E7-L primers indicate a strong predominance of 3.5 kb mRNA. Z-projected cell images from using this dual probing showed both overlapping 3'EU and sp-RNA images and separate responses to each (Fig. 4D). Screening of multiple MA10 cells showed that separate sp-RNA mRNA were proportionately higher in sections proximal to the adherent surface. The mitochondria marked with mitotrackers have uniform and complete overlap with StAR protein immune-detection (Lee et al., 2015). The overlap of the sp-RNA with StAR protein, which is expressed only in mitochondria, is relatively localized. There are also appreciable regions of separate StAR/mitochondria and sp-RNA (Fig. 4E). The sp-RNA and 3'EU hybridization images extensively overlap in both the XY plane and the series of XZ sections provided by the Z-stack mode of the microscope. The XZ resolution is less than previously shown for p-RNA and sp-RNA but again demonstrates the positioning close to the midline of the nucleus (Fig. 4F).

3.6. Pairing of StAR mRNA with StAR protein in mitochondrial matrix

The N-SIM provides a 3-D image of the MA10 cells in which all mitochondria (StAR protein) and mRNA molecules are resolved (Fig. 5A). The separations shown in the XZ plane are striking: loci are located on the midline of the nucleus above the mitochondria with a zone of StAR mRNA separate from mitochondria adjacent to the adherent cell membrane. The concentration of mitochondria outlining the nucleus is evident from the XY perspective. To further examine the relationship of StAR mRNA and protein, we examined the images in three Z sections (Fig. 5B). These high-resolution NSIM images confirm that mitochondria and StAR mRNA exhibit both appreciable separation as well as proximity and that this relationship changes with z-axis depth. In slice (a), which includes the StAR loci, the association of cytoplasmic StAR mRNA and protein is not a feature. Interestingly the mitochondria clearly associate with the outer surface of the nuclear envelope. In slice (a), there is little association of StAR mRNA with the mitochondria. However, just below the loci in slice (b), about half the StAR protein and mitochondria appear to associate as pairs: one StAR mRNA/mitochondrion. There are additional unpaired mitochondria and StAR mRNA molecules (enhanced image). In slice (c), corresponds to the middle of the central band in Fig. 5A, where there are more mitochondria and proportionately less association with the nuclear membrane.

The mitochondria have a diameter of about 500 nm (Duarte et al., 2014) with StAR protein in the matrix and StAR mRNA associated with ribosomes attached to the outer membrane (OMM). The diagram shown in Fig. 5B shows how a separation of 200–500 nm can be expected based on a mitochondrial diameter (Bornstein et al., 1990). The N-SIM resolution (<100 nm) can accommodate this separation. Many pairs seen in section (b) fit well with this range.

3.7. Resolution of dual binding of sp-RNA and 3'EU probes to StAR 3.5 kb mRNA

Fig. 5C shows the corresponding N-SIM images for mRNA bound by, respectively, sp-RNA and 3'EU probes together with the merged images. mRNA is detectable within the nucleus although mostly distributed outside. 60–65 percent of the mRNA bound either dual probes (40–45 percent) or only the 3'EU. The single hybridization by sp-RNA or 3'EU may arise from random incomplete binding or from selective displacement of the probes by protein that binds to either the translated sequence or to the extended 3'UTR (Znf3611/Tis11b, AUF1 or HuR) (Jefcoate et al., 2011). The proportion of sp-RNA compared to 3'EU increases lower levels (Fig. 5D). These N-SIM distributions are broadly consistent with the multiple MA10 cells imaged by the Olympus microscope. Each shows a consistently higher level of mRNA bound only by sp-RNA in the lower part of the cell. Many of the dual-hybridized mRNA particles have a clear resolution of the sp-RNA and 3'EU probe sets. The space between the probes corresponds to about 1 kb which, when fully extended, can represent as much as 200 nm. The resolution of the N-SIM is about 100 nm which is, therefore, consistent with this resolution.

3.8. Computation of StAR mRNA copies in each cell

In Fig. 6, we measured the number of spliced transcripts concentrated at the loci and identified them as individual species in the cytoplasm. Based on previous work (Lee et al., 2015), we expect the small cytoplasmic fluorescence sources of similar size to each correspond to a single mRNA. Their summation should correspond to the copy number per cell determined by RT-qPCR primed with 3' PCR-specific primers on the macro-culture. The number of sp-RNA that is unresolved at the loci is estimated by the 3D modeling of the pixel distribution, based on a Gaussian fit of single mRNA intensities and derived from the mean pixel distribution in cytoplasmic particles. To obtain a sub-diffraction localization of single mRNAs in three dimensions, we used FISH-quant, an algorithm that can analyze intensities and numbers of cytoplasmic mRNAs (sp-RNA) and nascent transcripts (p-RNA) in individual cells. The raw N-SIM image, which displayed as maximum intensity projections (MIP) in an XY section (Supplemental Fig. 3A (a)), and also shows the outline of a cell and nuclear boundaries in this section (Supplemental Fig. 3A (b)). The analyses are performed as 3-D assemblies of XY sections that cover the whole cell. The software assesses a background distribution, which is subtracted from each XY section. Possible spot candidates are identified based on an intensity threshold (Supplemental Fig. 3A (c)).

The size and intensity distributions as Gaussian functions are displayed (Supplemental Fig. 3B). The numbers of mRNA particles with diameters of a given size are shown for the XZ and XY planes (Fig. 6A). Eight hundred particles exhibit diameters of light dispersion symmetrically distributed between 200 and 400 nm in the XZ plane (mean diameter 300 nm). These particles also show relatively uniform intensity distributions varying symmetrically by about 2.5 fold. These mean diameters and intensities provide criteria for fitting unresolved mRNA images of greater intensity. The detected spots were inspected with the spot inspector in FISH-quant.

Fig. 6B shows the application of the mRNA particle intensities to the estimation of sp-RNA numbers at the loci. After 180 min, the intensities for two loci were compared to the

cytoplasmic mRNA reference standards (Fig. 6B(a)). x-axis indicates the number of molecules, and y-axis (the residuals) shows how well the reconstruction describes the data. Higher residual indicates worse description) (Fig. 6B(b)).

3.9. Equivalence of RT-PCR and HR-FISH in quantitating StAR sp-RNA/mRNA

The summation and averaging of mRNA species from a set of single cells provides a good match to the copy number given by the RT-PCR determination of the equivalent macro-culture of 10^6 cells (Fig. 6B (c)). This equivalence also confirms that the RT-qPCR does produce near complete RT conversion. Overall the nuclear loci contribute only 5 percent of the total sp-RNA. Application of the same process at 60 min shows a near complete absence of cytoplasmic mRNA but much more in the loci. At 180 min, there is an average of only about 15 transcripts per locus with 800 in the cytoplasm, whereas at 60 min, there are 40–90 transcripts estimated at the loci and scarcely any detectable in the cytoplasm. This transition occurs mostly between 75 and 90 min, corresponding to the surge in mRNA synthesis and thus matching the surge in coupled transcription detected in the macro-culture by RT-PCR (Fig. 2D). A comparison of the 180 min levels of total cell sp-RNA averaged from 25 cells indicates a close match with the mean number of copies per cell determined from the macro-culture (Fig. 6C).

4. Discussion

4.1. The methods development for the quantitation of StAR transcription in single cells versus a “macro-culture.”

This analysis of the StAR expression at multiple stages in single cells and in an equivalent dish culture (macro-culture) has required appreciable improvement in quantitative approaches to q-PCR and HR-FISH and their careful integration. We have appreciably improved the application of q-PCR to restricted target sequences, such as exon/exon junction sites, by using the specific 3' q-PCR primer in a dual role as the RT primer. This has proved to be at least ten times better than random hexamers. q-PCR copy numbers have been obtained for single StAR p-RNA or mRNA species with a range of about 20 percent for widely-spaced positions (Fig. 3).

For the HR-FISH analyses, Olympus IX81 and N-SIM microscopes have been used in combination. The tandem oligomer approach (Raj and Tyagi, 2010) has been used to design StAR sp-RNA oligomer sets that specifically recognize single spliced transcripts or processed mRNA species without crossover interaction with primary transcripts (p-RNA). The sp-RNA probe set hybridizes to the open reading frame (ORF), which is shared by 1.6 kb and 3.5 kb mRNA forms. A separate oligomer set effectively recognizes the 3'-end of the 3'UTR in the 3.5 kb mRNA or precursor primary transcripts. These probe sets bind simultaneously to about 40–45 percent of cytoplasmic StAR mRNA content, while a further 20–30 percent bind only one probe set. This may reflect incomplete binding or interference by specific proteins. The PCR analyses indicate that the p-RNA and sp-RNA each express a complete 3'UTR recognized by the E7-L PCR primer pairs and the 3'EU oligomer set (Fig. 3).

Here we use the wider field of the Olympus microscope (40 $\times$; Supplement Figure) to establish that after an 180 min stimulation by Br-cAMP, MA10 cells exhibit a roughly fivefold variation in the count of similarly sized small particles per cell after a 3 h stimulation. Since the N-SIM microscope views only a single cell, we have created a systematic approach for analysis of 20 or more cells (NSIM walk) that has been used for compare copy numbers per cell determined by q-PCR in equivalent MA10 macro-cultures. The agreement between the mean HR-FISH particles per cell and q-PCR determined RNA copies per cell establishes that indeed the particles are single molecules. The image analysis (FISH quant) of sp-RNA at the loci shows that the peak locus content (75 min) is about 10 percent of the cytoplasmic content. Peaks in protein translation and rates of steroidogenesis appear at about 60 min when cytoplasmic mRNA is undetectable. Evidently, a small proportion of mRNA that is active in translation is protected and therefore poorly hybridized.

The use of dual hybridization of the mRNA demonstrated here for StAR 3.5 kb mRNA is widely applicable as a means of identifying an RNA species. Variations on this HR-FISH approach have been used to profile alternative splicing that again can differ between single cells (Waks et al., 2011).

4.2. StAR transcription with integration of q-PCR and HR-FISH image analysis

Basal expression at the StAR gene locus is suppressed under basal conditions despite extensive expression of similarly regulated Cyp11a1 and other steroidogenic genes. We have expanded on our earlier work to provide dual evidence from macro-culture q-PCR and single-cell image analysis for PKA-mediated reversal of this silencing one locus at a time (asymmetric activation) over 60 min until nearly all cells exhibit two active loci. This early onset activation is followed by a delayed activation of a much faster transcription process that involved activation of additional loci. These two processes are also distinguished by splicing characteristics (Fig. 7). The early onset activation proceeds asymmetrically over 60 min with a delay in elongation at the end of the final intron which is matched to a delay in splicing. This process is consistent with the dual interdependent delays in elongation and splicing after 15–20 min. When elongation proceeds, the steady state level of p-RNA is higher than the spliced product until a linear increase in sp-RNA reaches a crossover point after about 60 min. As noted earlier, this delivers effective StAR protein expression but minimal cytoplasmic mRNA. At about this time, the delayed onset mechanism is triggered at previously inactive loci, with a much higher rate of transcription, rapid splicing, and an effective export of detectable mRNA to the cytoplasm. This separate transcription process is confirmed by the appearance in the HR-FISH images of loci with high levels of sp-RNA and minimal p-RNA after 60 min (Lee et al., 2015). We hypothesize that this acute delayed onset is initiated by the Br-cAMP stimulation of secondary transcription factors, including C/EBP β and NR4a1 (Di-Luoffo et al., 2015).

Y-1 cells are more complex than the MA10 cells described here because of the basal component of StAR expression (Lee et al., 2015). However, a similar biphasic stimulation is evident, which, in some way, is superimposed on a basal expression. The relationship

between transcription, elongation, and splicing for StAR expression in these cell types appears very unusual compared to recent, detailed analyses of these processes.

StAR mRNA only becomes visible in the cytoplasm during the delayed onset surge of transcription. We speculate that the slower rate and pausing during elongation and splicing allow a more complete protein complexation. The previously reported co-stimulation of Tis11b/Znf361l which binds selectively to the 3'UTR of StAR is a potential contributor (Duan et al., 2009) along with Akap1 (Dyson et al., 2008).

The q-PCR probing of StAR transcription surprisingly shows the equivalence of short and long 3'UTR transcription at all times, thus strongly indicating complete 3'UTR transcription including for the primary transcript. This leaves to be explained why Northern blots detect about 25 percent of 1.6 kb mRNA (Duan et al., 2009). Possibilities include a poor recovery of mRNA in the Northern blot process that favors the 1.6 kb mRNA and partial degradation of the less stable 3.5 kb mRNA to the 1.6 kb form. This predominance of the 3.5 kb form is consistent with the efficient dual HR-FISH imaging. The resolution of the dual hybridization is noteworthy since the space between the midpoints of oligomer sets hybridized with a fully extended 3.5 kb mRNA should be close to the resolution of the N-SIM (100 nm).

4.3. StAR mRNA and protein synthesis in relation to mitochondrial positioning

Mitochondrial cholesterol transfer depends on an integration of StAR mRNA translation, PKA phosphorylation of the protein and selective proteolysis of N-terminal sequence prior to import (Artemenko et al., 2001; Prasad et al., 2015). The turnover of cholesterol transfer for each new StAR protein synthesized is very high (400/StAR/min) and depends on both ATP generation and N-terminal cleavage steps. Early work showed that intervention in either traps intermediate states that are released upon restoration of these functions (Artemenko et al., 2001). Recent work shows that energy is utilized in several ways including mitochondrial fusion and ER/mitochondrial association (Duarte et al., 2012; Prasad et al., 2015). StAR association with VDAC2 in the outer mitochondrial membrane cleaves large N-terminal region to expose sequences that stimulate cholesterol transfer (Prasad et al., 2015). These previous studies omit recognition of the importance of the StAR mRNA.

3-D N-SIM images of StAR mRNA provides evidence that StAR mRNA moves from the locus site of transcription to the nearest mitochondrial acceptor sites close to the nuclear envelope (Fig. 5B). Surprisingly, a single StAR mRNA appears to pair with a mitochondrion marked by a matrix accumulation of StAR protein. We speculate that we are viewing a dynamic process of association of StAR mRNA proximal to VDAC2, transfer of new StAR protein to activate cholesterol transfer and then separation of the mRNA. Rapid turnover may leave few StAR mRNA linked to mitochondria at any one time. A slow processing of the StAR N-terminus has been established as necessary for activity (Prasad et al., 2015). The unusually slow splicing of StAR p-RNA may allow transcription and translation to synchronize with this mitochondrial processing at Vdac2. Ongoing work is addressing this hypothesis.

5. Conclusion

Integration of q-PCR and HR-FISH resolves the PKA activation of StAR expression in MA10 cells into two temporally resolved processes at the gene loci:

- a) Early onset activation that proceeds asymmetrically at the basally suppressed loci with coordinated delays in exon 7 elongation and intron splicing. It matches the StAR mediated increase in steroidogenesis.
- b) Delayed onset enhancement that proceeds with much more rapid transcription and splicing that nevertheless maintains a constant level of p-RNA. This process efficiently delivers StAR mRNA to the cytoplasm including to mitochondria positioned adjacent to the nuclear envelope.

HR-FISH spatially separates dual hybridization of p-RNA and sp-RNA probes to at the StAR gene loci and of 5'ORF and terminal 3'UTR probes to single molecules of 3.5 kb mRNA in the cytoplasm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviation:

3'EU	extended 3'UTR
CTD	C-terminal domain
FISH	fluorescence in situ hybridization
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
HR-FISH	high resolution fluorescence in situ hybridization
MIP	maximum intensity projections
N-SIM	Nikon's Structured Illumination Microscope
p-RNA	primary RNA
Pol 2	RNA polymerase 2
sp-RNA	spliced primary RNA
StAR	steroidogenic acute regulatory protein.

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Fig. 1.

Improved q-PCR quantitation of StAR pre-mRNA (p-RNA). (A) PCR primer pairs that target the primary transcripts (E1/I1; E6/I6) spliced transcripts (E1/E2; E3/E4; E5/E6) and 3'UTR (E7-S (short) and E7-L (long)). (B) The composite gBlock used as an RT-qPCR quantitation standard, which includes all the target intron, exon and 3'UTR sequences of StAR gene synthesized on the same genomic block. (C) The time course of stimulation of p-RNA (E1/I1) and mRNA (E5/E6) in MA10 cells with Br-cAMP. (D) RT priming is affected by the location of the specific RT primers at location E1/I1 (S is the reverse primer of E1/I1)

relative to the qPCR 30 site (a). The bar graph shows the relationship between the copy number and the priming distance. (b). Line graph (in box) shows the relationship between the free energy and the priming distance (c). (E) RT priming effect is again similarly observed in E6/I6 of StAR gene. The display is a repeat of 1 D (a–c). (F) Comparison of the predicted lowest free energy structures. Data in (C–E) are given as mean $\pm$ SEM. $p < 0.05$, $**p < 0.01$, $***p < 0.001$ by student's t-test or ANOVA. (CeE) 1 mM of Br-cAMP stimulation in MA-10 cells.

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Fig. 2. Application of optimized q-PCR to stimulation of StAR p-RNA and sp-RNA. (A) Spliced transcripts formed with the random hexamers, oligo-dT, and specific 3' qPCR primer are compared. (B) The display is a repeat of C with Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) primer set. (C) Comparison of E1/I1 assessment with only the individual specific RT primers or with a cocktail of RT primers of multiple StAR targets. (D) The time course of stimulation of p-RNA (E1/I1) and mRNA (E5/E6) using DNA block references to provide transcript copy number. Data in (A–D) are given as mean $\pm$ SEM. $p < 0.05$, $**p < 0.01$, $***p < 0.001$ by student's t-test or ANOVA. (AeD) 1 mM of Br-cAMP stimulation in MA-10 cells.

Fig. 3. Time course for stimulation of 3'UTR transcripts in comparison to StAR transcripts (p-RNA, sp-RNA). (A) Comparison of spliced transcripts formed at three different spliced sites (E1/E2; E3/E4 and E5/E6) as a function of time of stimulation. (B) Comparison of spliced transcripts formed at E5/E6 and 30 UTR (E7-S and E7-L). (C) 30'UTR (E7-S and E7-L) are compared with the primary transcripts (E1/I1 and E6/I6). (D) The display is a repeat of 1 C with a different batch of lower passage MA10 cells. Data in (A-D) are given as mean $\pm$

SEM. $p < 0.05$, $**p < 0.01$, $***p < 0.001$ by ANOVA. (A-D) 1 mM of Br-cAMP stimulation in MA-10 cells.

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Fig. 4. HR-FISH analysis of StAR RNA distribution in MA10 cells. (A) The design of HR-FISH probes that distinguish StAR p-RNA, sp-RNA and the extended 3'UTR of the 3.5 kb mRNA. (B) Overlap (yellow) of p-RNA (green) with sp-RNA (red) fluorescence signal at the loci at 60 min (C) StAR mRNAs accumulate in the cytoplasm after 180 min. (D) Equivalent Z-projection image using both sp-RNA probe set and the 3'EU probe set to detect StAR mRNA in the cytoplasm. White specks in the merged images represent dual binding of 3.5 kb mRNA. (E) Z-projection merger of sp-RNA/mRNA with StAR protein, which colocalizes within all mitochondria (Mitotracker) (not shown). (F) The merger of the sp-RNA (red) and 3'EU (green) (overlap yellow) at nuclear loci (1,2). StAR loci position around the nuclear midline in Z-stack series XY slices (step size: 0.2 μm). The diagram represents positions for XY slices relative to the nucleus. (C–F) 1 mM of Br-cAMP stimulation in MA-10 cells for 3 h s, (B) for 1hr. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 5.

Resolution of StAR 3.5 kb mRNA by dual binding of sp-RNA and 3'EU, and pairing of StAR mRNA and mitochondria (N-SIM microscope). (A) 3-D resolution of StAR sp-RNA/mRNA and mitochondria marked by StAR protein immunofluorescence. (B) Z-slice resolution for StAR sp-RNA at Z axis XY slice positions a, b and c (see Fig. 4F). Above: Diagram showing separation of matrix StAR protein from StAR mRNA position with ribosomes linked to the mitochondrial outer membrane (OMM). The diameter of adrenal mitochondria is about 500 nm. Lower right: enlargement of a representative sector of the slice (b) showing pairing of StAR protein/mitochondria with StAR mRNA. (C) Z slices showing hybridization of sp-RNA, 3'EU and their merger at the same Z slice positions; a, b, and c. (D) Copies per slice of respectively sp-RNA and 3'EU at positions a, b and c and intermediate slice positions. Shows trend towards the enrichment of p-RNA proximal to the adherent cell membrane. (AeD) 1 mM of Br-cAMP stimulation in MA-10 cells for 3 h s.

Fig. 6.

Automatic counting of transcripts in 3D HR-ISH images. (A) mRNAs detected by the spot inspector in the 'FISH quant' software (C) Modeling of the sp-RNA conglomeration at the primary locus based on an average single mRNA image (upper panel). sp-RNA quantification algorithm as applied at the locus (lower panel). Compare estimated sp-RNA at StAR loci after 60 min versus 180 min (right panel). (D) The number of sp-RNA (loci plus cytoplasm) from 25 individual cells compared to expression levels (copies per cell) determined by 3'qPCR for six macro-cultures. Data (B–C), Student's t-test: **, $p < 0.01$. (A, C) 1 mM of Br-cAMP stimulation in MA-10 cells for 3 h s.

Fig. 7.
Diagram summarizing characteristics of early and delayed onset expression StAR RNA at gene loci.