Nuclear history of a pre-mRNA determines the translational activity of cytoplasmic mRNA

Ken Matsumoto^{1,2},
Karen Montzka Wassarman¹ and
Alan P. Wolffe^{1,3}

¹Laboratory of Molecular Embryology, National Institute of Child Health and Human Development, NIH, Building 18T, Room 106, Bethesda, MD 20892-5431, USA and ²Laboratory of Bioorganic Chemistry, The Institute of Physical and Chemical Research (RIKEN), Hirosawa 2-1, Wako-shi, Saitama 351-01, Japan

³Corresponding author
e-mail: awlme@helix.nih.gov

K. Matsumoto and K.M. Wassarman contributed equally to this work

The pathways of synthesis and maturation of pre-messenger RNA in the nucleus have a direct effect on the translational efficiency of mRNA in the cytoplasm. The transcription of intron-less mRNA *in vivo* directs this mRNA towards translational silencing. The presence of an intron at the 5' end of the transcript relieves this silencing, whereas an intron at the 3' end further represses translation. These regulatory events are strongly dependent on the transcription of pre-mRNA in the nucleus. The impact of nuclear history on regulatory events in the cytoplasm provides a novel mechanism for the control of gene expression.

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Introduction

Eukaryotic gene expression depends on the synthesis, export and translation of mRNA. All of these events are tightly regulated and utilize a ribonucleoprotein (RNP) substrate. An increasing body of evidence supports a coupling between the synthesis of mRNA and the subsequent ribonucleoprotein architecture (Yuryev *et al.*, 1996; Du and Warren, 1997; Kim *et al.*, 1997). The association of splicing factors and hnRNPs with pre-mRNA occurs during transcription (Osheim *et al.*, 1985; Champlin *et al.*, 1991; Kim *et al.*, 1992; Dreyfuss *et al.*, 1993; Baurén and Wieslander, 1994; Champlin and Lis, 1994; Baurén *et al.*, 1996; Dantonel *et al.*, 1997). The regulated assembly of a RNP complex determines the specificity of intron removal (Mayeda and Krainer, 1992; Fu, 1993; Cáceres *et al.*, 1994; Wang and Manley, 1995) and the recruitment of particular factors to sites of transcription can be intron-dependent or independent (Roth *et al.*, 1990; Jiménez-García and Spector, 1993; Kiseleva *et al.*, 1994; Huang and Spector, 1996). Moreover, some components of the complex have a packaging role (Lönnroth *et al.*, 1992; Dreyfuss *et al.*, 1993) and others facilitate mRNA export across the nuclear pore (Jarmolowski *et al.*, 1994). Both exon-bound splicing

factors (Alzhanova-Ericsson *et al.*, 1996) and hnRNPs (Mehlin *et al.*, 1992, 1995) remain associated with pre-mRNA at the nuclear pore. A subset of these proteins accompanies the pre-mRNA through the nuclear pore into the cytoplasm (Pinol-Roma and Dreyfuss, 1992) and can remain associated with translationally-engaged mRNA (Visa *et al.*, 1996). The final organization of a particular mRNA within an RNP complex as it engages the nuclear pore depends on its accumulative history in the nucleus. Aspects of this organization might have functional consequences once the mRNA arrives in the cytoplasm and becomes available to the translational machinery.

Several investigators have suggested that functional interrelationships exist between the splicing process and gene expression (Hamer *et al.*, 1979; Buchman and Berg, 1988; Braddock *et al.*, 1994; Huang and Carmichael, 1996). Particular introns stimulate gene expression by as much as 500-fold via post-transcriptional mechanisms (Hamer *et al.*, 1979; Buchman and Berg, 1988). Splicing does not appear to be essential for nuclear export (Legrain and Rosbash, 1989; Huang and Carmichael, 1996), yet splicing can facilitate the recruitment of mRNAs for translation (Braddock *et al.*, 1994). The *Xenopus* oocyte system offers a useful system for the determination of the significance of transcription and splicing for gene expression because of the ability to synthesize mRNA *in vivo*, and to microinject intron-containing or intron-less transcripts into either the nucleus or cytoplasm.

The oocyte genome is very transcriptionally active, yet >80% of the maternal mRNA synthesized *in vivo* is translationally silent (masked; Davidson, 1986). The assembly of cytoplasmic mRNPs during oogenesis has been proposed to sequester maternal mRNA into an inert state, so that it is masked from the translational apparatus (Spirin, 1966; Richter, 1988; Standart, 1992; Wickens, 1992). Two established factors contribute to maintaining maternal mRNA in a masked, translationally repressed form. The first component of translational repression is the association of repressive Y-box proteins with maternal mRNA (Richter and Smith, 1984; Sommerville, 1990; Ranjan *et al.*, 1993; Tafuri and Wolffe, 1993; Bouvet and Wolffe, 1994). The second regulatory component is the control of the polyadenylation status of mRNA (McGrew *et al.*, 1990; Sheets *et al.*, 1994, 1995; Kuge and Richter, 1995). We have recently found that both association with the Y-box proteins and regulated polyadenylation can contribute to achieving a wide range of translational regulation in *Xenopus* oocytes (Meric *et al.*, 1996). Certain mRNAs, such as those encoding oocyte-specific proteins like histone B4 (Cho and Wolffe, 1994; Dimitrov *et al.*, 1994) or the transcription factor TFIIIA (Tafuri *et al.*, 1993), escape translational repression.

The translational efficiency of maternal mRNA in an oocyte is highly dependent on the transcription of that

mRNA *in vivo* (Bouvet and Wolffe, 1994; Meric *et al.*, 1996, 1997). A naturally occurring translationally silent mRNA encoding histone H1 or H4 is translationally repressed when transcribed *in vivo*, but is active when microinjected into the cytoplasm. The general repression of translation observed when mRNA is transcribed *in vivo* led us to propose that translational fate was coupled to transcription (Bouvet and Wolffe, 1994; Wolffe and Meric, 1996). Here we demonstrate that transcription *in vivo* can greatly accentuate either translational repression or activation depending on the nature and position of intron sequences. The nuclear history of an mRNA can determine whether it will be recruited to the translational machinery in the cytoplasm.

Results

The presence of an intron in pre-mRNA is a major determinant of mRNA translational efficiency in vivo

In earlier work we have described how mRNA synthesized *in vivo* from plasmid templates and microinjected into the nucleus is translationally repressed compared with the same mRNA synthesized *in vitro* and microinjected into the *Xenopus* oocyte cytoplasm (Figure 1A; Bouvet and Wolffe, 1994; Meric *et al.*, 1996). These experiments made use of mRNA encoding histone H1, which is naturally masked in the *Xenopus* oocyte (Dimitrov *et al.*, 1993; Tafuri and Wolffe, 1993). We wished to determine whether a comparable translational repression would be established if we carried out the same experiment with an mRNA which is naturally active in *Xenopus* oocytes, such as that encoding TFIIIA (Tafuri and Wolffe, 1993). We compared the translational efficiency of TFIIIA and histone H1 mRNA, synthesized either following microinjection of the expression constructs into oocyte nuclei (Figure 1B), or following microinjection of *in vitro*-synthesized mRNA into oocyte cytoplasm. We found that in contrast with our earlier data from histone H1 mRNA (Bouvet and Wolffe, 1994), TFIIIA mRNA synthesized *in vivo* (from pCT1) was up to 10-fold more translationally efficient than mRNA microinjected into the oocyte cytoplasm (Figure 1C). We repeated this experiment using histone H1 sequences in both our original expression construct (pH1.10; Bouvet and Wolffe, 1994) and the pCH1.3 construct, which is analogous to the TFIIIA expression construct used above (pCT1). Both of these constructs contain an intron, utilize similar cytomegalovirus (CMV) promoters and direct the synthesis of H1 coding sequences (Figure 1B). Surprisingly, we find that H1 protein is readily expressed from pCH1.3 in contrast with the low level of H1 expression from pH1.10 observed here, and reported previously (Figure 1D; Bouvet and Wolffe, 1994). In fact, the translational efficiency as measured by the amount of protein made, normalized to the amount of mRNA synthesized in an oocyte, varies over a 100-fold range depending on the configuration of the construct.

To determine whether the dependence of translational efficiency on construct configuration is a general phenomenon, or is somehow dependent on H1 sequences, the histone H1 cDNA was replaced by bacterial chloramphenicol acetyltransferase (CAT) in both configurations. Histone H1 mRNA is under a naturally occurring translational

repression in the oocyte (Tafuri and Wolffe, 1993), whereas the oocyte would normally not control the translational activity of prokaryotic sequences. Comparison of the translational efficiency of mRNA synthesized from the pCCA1, pCMV CAT and pDCA1 constructs also enables the potential significance of splicing to be tested. All three expression constructs directed the synthesis of equivalent amounts of CAT mRNA (Figure 2A, lanes 1, 2 and 4). However, compared with *in vitro*-synthesized CAT mRNA microinjected into the cytoplasm (Figure 2A, lane 3), only the pCCA1 construct, containing the CMV enhancer/promoter from -737 to +7, the chimeric intron and the simian virus 40 (SV40) late polyadenylation [poly(A)] site, synthesized mRNA of high translational efficiency (Figure 2A, Protein, lane 1). The CMV-CAT construct, containing transcriptional control sequences, the SV40 small t intron and an early poly(A) site identical to the pH1.10 construct (Figure 1B), generated transcripts that were not efficiently translated (Figure 2A, Protein, lane 4; Bouvet and Wolffe, 1994). In all of the constructs used, the presence of the chimeric intron at the 5' end of the transcripts improved translational efficiency (Figures 1 and 2). We find that the CAT constructs behave analogously to the H1 constructs, and therefore this large variation in translational efficiency is indeed due to construct configuration and not specific coding sequences.

We further examined the potential stimulatory role of the chimeric intron using the TFIIIA-encoding constructs. We synthesized TFIIIA mRNA using a construct that either did or did not contain the chimeric intron (Figure 2B, pCT1 and pDT1, respectively). We also synthesized TFIIIA mRNA containing the natural 5' and 3' untranslated regions (UTRs) in the presence or absence of the chimeric intron (Figure 2B, pCTFL and pDTFL, respectively). We found that in each case, equivalent amounts of TFIIIA mRNA accumulate (Figure 2B, mRNA, lanes 1-4). However, the translational efficiency of the TFIIIA mRNA is significantly different (Figure 2B, Protein, Translation Efficiency). All of the constructs which synthesize a pre-mRNA containing the chimeric intron were more translationally efficient than mRNAs synthesized without an intron (Figure 2B, Translational Efficiency, compare pCTFL and pCT1 with pDTFL and pDT1). The natural 5' and 3' UTRs further potentiated the translation of TFIIIA mRNA (Figure 2B, Translational Efficiency, compare pCTFL with pCT1). As a control for this experiment we examined the export of spliced and unspliced mRNAs from the oocyte nucleus (Figure 2C). Most of the *in vivo*-synthesized, unspliced mRNA remained in the nucleus, while spliced and *in vivo*-synthesized, intron-less mRNA was exported. Thus the failure to translate *in vivo*-synthesized, intron-less mRNA is not due to a failure to export the mRNA (also see Figure 8 later). Therefore, translational repression must be due to mechanisms which direct the masking of the mRNA from the translational machinery in the oocyte cytoplasm (Bouvet and Wolffe, 1994; Braddock *et al.*, 1994). The presence of the chimeric intron at the 5' end of the transcript, which is spliced from the CAT and TFIIIA mRNAs, appears to allow the mRNA to escape from this translational repression pathway.

We wished to test further whether endogenous mRNA sequences would have an effect on the translation process.

Fig. 1. Translational efficiency of *in vivo* and *in vitro*-synthesized RNA in *Xenopus* oocytes. (A) Experimental design. Plasmid DNA or mRNA was injected into the *Xenopus* oocyte nucleus or cytoplasm, respectively. At 16–18 h after injection, [³H]-amino acids were injected into the cytoplasm. Another 6 h later, the oocytes were collected and RNA and proteins were prepared from the oocytes. RNA was analyzed by primer extension with gene-specific primers, and proteins synthesized during the 6 h labeling were analyzed in a polyacrylamide gel containing SDS. (B) Schematic diagrams of CMV promoter-driven expression vectors and *in vitro* transcribed mRNA. Plasmid DNAs were prepared as described in Materials and Methods. Nucleotide positions of CMV enhancer and immediate early promoter (CMV E/P) are derived from Boshart *et al.* (1985). pC constructs are derivatives of pCI vector so that they have the chimeric intron between the CMV promoter and the initiation codon. The chimeric intron is 133 nucleotides in length. A, A/III. (C) *Xenopus* oocytes were injected with plasmid DNA or mRNA encoding TFIIIA. Plasmid DNA pCT1 was injected into the nucleus, while mRNA was injected into the cytoplasm. TFIIIA mRNA and protein synthesized from the injected DNA or mRNA were analyzed as described in (A). Primer extension products derived from RNA synthesized and processed in the nucleus from the injected plasmid DNA (*in vivo*-synthesized RNA) and the injected mRNA are indicated. DNA molecular weight markers (M) are *Msp*I-digests of pBR322. Position of TFIIIA protein is indicated by an arrow. Molecular weight of ¹⁴C-labeled marker proteins (Amersham) is shown on the left of the protein gel. RNA and proteins from oocytes injected only with [³H]-amino acids are analyzed in parallel (aa only). Translation efficiency (of protein or mRNA) was calculated based on quantitation of the mRNA and protein level. The spliced RNA level was used for quantitation of RNA from pCT1, assuming that the unspliced RNA remained in the nucleus (Figures 2 and 8). Relative translational efficiency is shown. (D) Plasmid DNAs pCH1.3 and pH1.10 were injected into the oocyte nucleus and the histone H1C mRNA was injected into the cytoplasm. RNA was prepared from oocytes as in (B). H1C mRNA from the injected oocytes was detected by primer extension. Note that the annotation '5' intron-less' refers to transcripts that lack an intron at the 5' end of the transcript (but not necessarily at the 3' end). Acid-soluble proteins were prepared from the oocytes double-injected with [³H]-amino acids and analyzed in a polyacrylamide gel containing SDS. Markers (M) are described in (C). Relative translational efficiency is calculated.

Fig. 2. *In vivo* synthesized TFIIIA and CAT pre-mRNA with an intron are efficiently translated. (A) Plasmid DNAs encoding CAT pre-mRNA and CAT mRNA were injected into the oocyte nucleus and cytoplasm, respectively. RNA and proteins were prepared from oocytes and analyzed as in Figure 1A and C. CAT proteins from pCCA1 and pDCA1 have a FLAG-tag at their N-termini, which resulted in the lower mobility of them than those from CMV-CAT and mRNA. Relative translation efficiency is calculated. (B) Plasmid DNAs encoding TFIIIA with or without UTRs were injected into the oocyte nucleus. RNA and proteins were analyzed as in Figure 1C. Relative translational efficiency is calculated. (C) Localization of *in vivo* synthesized RNA. Plasmid DNAs pCT1 and pDT1 were injected into the oocyte nucleus. Sixteen hours later the oocyte was dissected into the nucleus and cytoplasm. RNA was prepared from the total oocytes (T), the nuclear (N) and cytoplasmic (C) fractions. TFIIIA mRNA was detected by primer extension.

Fig. 3. *In vivo*-synthesized B4 and FRGY1 pre-mRNA with an intron are efficiently translated. (A) Plasmid DNAs encoding B4 pre-mRNA (lanes 1 and 2) and B4 mRNA (lane 3) were injected into the oocyte nucleus and cytoplasm, respectively. Likewise, plasmid DNAs encoding FRGY1 pre-mRNA (lanes 4 and 5) and FRGY1 mRNA were injected into the oocyte nucleus and cytoplasm, respectively. RNA was prepared from oocytes and analyzed as in Figure 1A. Unspliced, spliced and intron-less transcripts are indicated, as is injected RNA. Lane 7 shows control oocytes which were not injected with nucleic acid. (B) B4 and FRGY1 proteins synthesized from the indicated mRNAs in (A) are shown. Lane 7 shows protein synthesis in the absence of nucleic acid injection and lane 8 shows molecular weight markers.

A significant enhancement of translation following synthesis of B4 and FRGY1 pre-mRNAs containing an intron at the 5' end of the cDNA was also obtained (Figure 3). Under experimental conditions where equivalent amounts of mRNA were present (Figure 3A), the presence of an intron at the 5' end of the B4 and FRGY1 mRNAs stimulates translation significantly over that of either mRNA synthesized *in vivo* without an intron or mRNA injected into the cytoplasm (Figure 3B, compare lanes 1 and 4 with lanes 2, 3, 5 and 6). B4 is an endogenous, oocyte-specific mRNA which is synthesized with introns (Cho and Wolffe, 1994) and is translationally active in oocytes (Dworkin *et al.*, 1985; Dimitrov *et al.*, 1993, 1994; Dworkin-Rastl *et al.*, 1994). In contrast, FRGY1 is an intron-less mRNA that is synthesized in masked form in oocytes (Wolffe *et al.*, 1992; Tafuri and Wolffe, 1993). Thus the presence of an intron in the B4 pre-mRNA (as found in the endogenous gene) stimulates translation correlating with the translational activity of endogenous B4 mRNA (Dimitrov *et al.*, 1993), whereas the absence of an intron in the FRGY1 mRNA (as found in the endogenous gene) represses translation correlating with the transcriptional repression of this mRNA *in vivo* (Tafuri and Wolffe, 1993). The presence of an intron at the 5' end of the mRNA appears to have a pronounced stimulatory effect on translational efficiency.

Our preliminary experiments suggest that a particular intron at the 5' end of the transcript can influence translational efficiency when the mRNA is transcribed *in vivo*, however there are several other differences between the expression vectors used above including the source of introns and poly(A) sites. For example, pCH1.3 (pCCA1 and pCT1) contains a chimeric intron 5' of the initiation codon and an SV40 late poly(A) site, while pH1.10 has an SV40 small t intron in the 3' UTR and an SV40 early poly(A) site. In addition to the changes in pre-mRNA processing signals, there are also differences in the promoter regions, 3' UTRs and 5' UTRs. As we

wished to systematically dissect the significance of each of these variables, constructs were first made to address the affects of promoter differences (pEH1) and the presence of 3' CAT sequences (pFH1) in the absence of introns (Figure 4, compare with pDH1). No appreciable difference in translational efficiency of *in vivo*-synthesized mRNAs was observed (data not shown). The potential affects of 5' UTR differences were addressed by comparison of pGH1 with pH1.10 and again, no detectable difference in the translatability of *in vivo*-synthesized mRNAs was seen (data not shown).

The role of splicing in general was studied by comparing the translational efficiency of intron-less versus intron-containing constructs. To eliminate potential complications arising from differential export of the different mRNAs from the nucleus, only cytoplasmic mRNA levels were used to calculate translational efficiency. In addition, an RNase protection assay (RPA) using a probe to H1-coding sequences was done to quantitate RNA levels accurately. RPA was done in the presence of a histone H4 probe to measure levels of endogenous H4 and all H1 mRNA levels were normalized to H4 mRNA. The presence of the chimeric intron at the 5' end of the H1 mRNA stimulated translation over an intron-less mRNA (Figure 4B, C and D, compare pCH1.3 with pDH1). In contrast, the presence of the small t intron at the 3' end of the H1 mRNA significantly reduced translation as compared with an intron-less mRNA (Figure 4, compare pGH1.2 with pJH1.2). Interestingly, the type of polyadenylation signal (SV40 early compared with SV40 late) had no affect on translatability of resultant mRNAs regardless of the presence or absence of an intron (Figure 4, compare pDH1 with pJH1.2 and pGH1.2 with pKH1). Therefore, we conclude that the presence of an intron can exert significant effects on the translational fate of an mRNA.

Intron position determines translational efficiency

Intriguingly, we observed both an increase and decrease in the translational efficiency of mRNAs which contain

A Constructs **B mRNA** **C Protein** **D Translational Efficiency**

Fig. 4. The effect of various post-transcriptional signals on translational efficiency of histone H1 in oocytes. **(A)** Schematic diagrams of CMV promoter plasmid DNAs encoding H1C. pCH1.3 and pDH1 have the H1C coding sequence with and without the chimeric intron, respectively. pEH1, pFH1, pGH1, pGH1.2, pJH1.2 and pKH1 are pDH1 derivatives which have some DNA elements from pH1.10. **(B and C)** Oocytes were injected with plasmid DNAs encoding H1C. RNA from isolated cytoplasm of injected oocytes was subjected to RPA using probes (Materials and methods) to detect H1C and H4 mRNAs, respectively. As controls for the RPA, yeast RNA (approximately equal amount to *Xenopus* RNA used per assay) was used in the presence (lane 8) or absence (lane 9) of RNases. One-hundredth of the total sample was loaded in lane 9. Acid-soluble proteins were prepared from oocytes and analyzed in a polyacrylamide gel containing SDS. **(D)** Relative translational efficiency is calculated. The amount of H1C mRNA synthesized from each construct was quantitated relative to the amount of H4 mRNA per sample and then translational efficiency was calculated as the amount of H1C protein synthesized normalized to the amount of H1C mRNA present.

Fig. 5. An intron upstream of the coding sequence stimulates translation in the oocytes. (A) Schematic diagrams of plasmid DNAs encoding pre-mRNA without or with introns at the 5' or 3' end of the TFIIIA coding sequence. (B and C) Plasmid DNAs were injected into the oocyte nucleus. RNA and proteins were analyzed as described in Figure 1C. (D) Relative translational efficiency is shown.

an intron compared with intron-less mRNAs (Figure 4). However, in the constructs above both the type of intron (chimeric versus small t) and position (5' versus 3' UTR) were varied. Next we examined whether it is the position or type of intron which determines the capacity to increase or decrease translational efficiency. This was achieved by analyzing a series of constructs in which intron type and position were systematically changed whilst all other variables were kept constant (Figures 5A and 7A). We

Fig. 6. Effect of splice site mutations on translation efficiency. (A) DNA sequence around the 5' and 3' splice sites of the chimeric intron of pCI vector is shown. Boundaries of the exons and the intron are shown by arrows. Essential dinucleotides in the 5' and 3' splice sites are underlined. Dinucleotides in point mutants pMT1, pMT2.2 and pMT3 are shown. (B and C) Plasmid DNAs were injected into the oocyte nucleus. RNA and proteins were analyzed as described in Figure 1C. (D) Relative translational efficiency is shown.

found that the position of the intron in the transcript has a major influence on translational efficiency. We made use of either primer extension to monitor pre-mRNA and mRNA abundance and splicing (Figures 5 and 6), or RPA to monitor cytoplasmic mRNA (Figure 7). In both instances we determined translational efficiency. When either the chimeric intron or the SV40 small t intron are at the 5' end of the transcript, translational efficiency is increased relative to an intron-less transcript (Figures 5 and 7, compare pDT1 with pCT1 and pDT-S5). Surprisingly, when the introns are placed at the 3' end of the transcript, translational efficiency was severely reduced relative to an intron-less transcript (Figures 5 and 7, compare pDT1 with pDT-C3 and pDT-S3). There were no significant differences in translational efficiency conferred by the specific intronic sequences in this experiment (Figures 5 and 7, compare pCT1 with pDT-S5, and pDT-C3 with pDT-S3). Our results demonstrate that the position

Fig. 7. Effect of intron position and splice site mutations on translation in the oocytes. **(A)** Schematic diagrams of plasmid DNAs encoding pre-mRNA without or with introns at the 5' or 3' end of the TFIIIA coding sequence. In addition, splice site mutations were introduced into the pCT1 parent construct. DNA sequence around the 5' and 3' splice sites of the chimeric intron of pCT1 vector is shown. Boundaries of the exons and the intron are shown by arrows. Essential dinucleotides in the 5' and 3' splice sites are underlined and the mutations generated for pMT1, pMT2 and pMT3 are shown. **(B and C)** Plasmid DNAs were injected into the oocyte nucleus. RNA prepared from isolated cytoplasm of injected oocytes was subjected to RNase protection assay using as probes to detect TFIIIA and H4 mRNAs, respectively (Materials and methods). Yeast RNA controls (lanes 10 and 11) are as described in Figure 2. Protein was analyzed as described in Figure 1C. **(D)** Relative translational efficiency is shown as calculated as described in Figure 2D.

of an intron in a transcript can exert a major influence on translational efficiency.

We wished to examine further the possibility that the sequences at the 5' and 3' splice sites might influence translational efficiency. We made use of the pCT1 construct, maintaining the chimeric intron but making point mutants at the chimeric intron splice sites (Figures 6 and 7). We found that the translational efficiency of the point mutants was reduced relative to the pCT1 construct

(Figures 6 and 7). Surprisingly, one of the point mutants was partly spliced, possibly utilizing a cryptic 3' splice site (pMT2.2; Figure 5B), yet was translationally repressed relative to the pCT1 construct (Figures 6 and 7). We suggest that it is the process of splicing, rather than the presence of intronic sequences in the pre-mRNA, which exerts a strong influence on translational efficiency.

An alternative explanation for the reduction in translational efficiency might be that the inclusion of the intron

in the transcript generates upstream AUG and Kozak sequences (Kozak, 1992), leading to translational repression of downstream reading frames. This is unlikely since no consensus Kozak sequences appear in the intron (data not shown) and no radiolabeled proteins, other than the major encoded mRNA, are seen in oocytes (Figure 6C; data not shown).

Structure of cytoplasmic mRNA

We have shown that the presence of an intron has a large influence on the translatability of an mRNA and we have suggested that it is the process of splicing which is required to mediate these effects. However, to assess directly the extent of appropriate pre-mRNA processing (both splicing and polyadenylation) we performed RPAs on both cytoplasmic and nuclear RNA. Probes were made to detect unspliced and spliced products from both the chimeric and small t introns, and also to detect appropriate polyadenylation-directed cleavage at the SV40 early and late poly(A) sites. Our first experiments examined the processing and export of mRNA containing the SV40 late poly(A) signal. For all constructs with the SV40 late poly(A) signal, $\geq 90\%$ of the mRNA in the cytoplasm has been appropriately cleaved downstream of the AAUAAA signal (Figure 8A and B). Most of the mRNA retained in the nucleus is not polyadenylated (Figure 8A, panels 1 and 2, and B, compare C with N for constructs pDT1, pCT1, pDTS5, pMT1, pMT2, pMT3, pCH1.3, pDH1, pDTS3 and pDTC3). In the case of pKH1, the majority of mRNA retained in the nucleus is not polyadenylated, although in this case a distinct polyadenylated, unspliced fraction is also seen (Figure 8B, lanes 1 and 2, pKH1). We conclude that $>90\%$ of mRNA containing the SV40 late poly(A) signal is appropriately processed and exported to the cytoplasm.

Next we examined the processing and export of mRNA containing the SV40 early poly(A) signal. Although the SV40 early poly(A) signal was not quite as efficient as the late poly(A) signal, still $\geq 70\%$ of cytoplasmic mRNA exhibits appropriate cleavage downstream of the AAUAAA signal (Figure 8A, panel 3, see constructs pGH1.2, pH1.10 and pJH1.2; note that the differences in protected product sizes are due to different extents of complementarity of the probe to these constructs). However, the amount of cytoplasmic signal with the SV40 early poly(A) probe was significantly reduced compared with nuclear signal from pGH1.2 and pH1.10, but not from pJH1.2 (Figure 8A, lanes 29–36). Such a reduced cytoplasmic signal was not observed with the H1-coding probe, suggesting that the SV40 early sequences are not present in all the cytoplasmic mRNAs from these constructs (data not shown; see below for discussion). We conclude that the presence of the SV40 early poly(A) signal allows the mRNA to be processed, but with reduced efficiency. Likewise, mRNA is also exported to the cytoplasm, but with reduced efficiency.

The efficiency with which the 5' chimeric intron and small t intron were spliced was considered. Probes complementary either to the chimeric intron and surrounding sequences or to small t intron and surrounding sequences were used to assess the splicing of 5' introns. For pCH1.3, pCT1 and pDT-S5, $\sim 95\%$ of cytoplasmic mRNA is spliced (Figure 8C, panels 1 and 2; note that the signal from the

spliced and unspliced mRNA must be normalized for the different length products and the multiple bands which correspond to the spliced product, so that the unspliced product band appears overrepresented in the autoradiograph). Mutation of the 5' intron sequences inhibits splicing (Figure 8C, panel 1, lanes 5–10). We conclude that the 5' chimeric intron and small t introns are efficiently spliced.

Next we examined the efficiency with which the mRNAs containing 3' introns were processed. The structure of the mRNAs with 3' introns were analyzed by probes spanning the entire 3' end to monitor the extent of splicing and polyadenylation together. For pDT-S3 and pDT-C3, $\geq 95\%$ of cytoplasmic mRNA is spliced and polyadenylated (Figure 8B). Splicing of the small t intron from pKH1 is slightly less efficient (76% of cytoplasmic RNA is spliced) although $>95\%$ of cytoplasmic mRNA is polyadenylated. We conclude that mRNAs containing the SV40 late poly(A) signal and either the chimeric or small t intron are efficiently processed. However, almost no signal was detectable using probes spanning the full 3' UTR against cytoplasmic mRNA for the two constructs, with a 3' small t intron and SV40 early poly(A) signal (Figure 8C, panel 2, pH1.10 and pGH1.2; data not shown). Therefore, the extent of splicing and polyadenylation were assessed separately. As shown above, $\sim 70\%$ of detectable cytoplasmic mRNA is appropriately polyadenylated, but the low signal intensity of cytoplasmic RNA suggests degradation of SV40 early sequences in the cytoplasm. Surprisingly, no protected fragment corresponding to spliced products is detectable for either pGH1.2 or pH1.10, although spliced products were readily detected for pDT-S5 (Figure 8C, panel 2; note difference in sizes of unspliced products are due to cloning variations: the 5' exon protected fragment would be the same for all constructs, but the 3' exon protected fragment from pGH1.2 and pH1.10 would not be the same as pDT-S5). In addition, as seen with the SV40 early poly(A) probe above, the cytoplasmic signal intensity for the pH1.10 construct (but not pGH1.2) is much lower than the nuclear signal, in contrast with the ratio of cytoplasmic:nuclear signal using the H1-coding probe. Again, this suggests that the 3' end of these mRNAs is not intact, and that for pH1.10 degradation has extended into the small t intron region (Figure 8C; data not shown). We conclude that the combination of the SV40 3' small t intron and SV40 early poly(A) signal leads to deficiencies in mRNA splicing.

Overall, these RPA data indicate that the vast majority of cytoplasmic mRNA is appropriately spliced and polyadenylated from all constructs except pGH1.2 and pH1.10. The 3' ends of the mRNAs from pGH1.2 and pH1.10 are not intact and pH1.10-generated RNAs are more affected than those generated by pGH1.2. This difference may account for the difference in translational efficiency of these two mRNAs since all other structural differences between them (i.e. promoter, 5' and 3' UTR) have been shown not to affect translatability of a message, at least when tested individually (Figure 4). In addition, the small t intron is not removed from either type of pre-mRNA. It is difficult to assess the potential effects on translational efficiency of these constructs with a retained intron since the full coding sequences are present. If unspliced mRNA acts the same as the splice site mutations

Fig. 8. Analyses of structural integrity of mRNAs synthesized *in vivo*. (A) Cytoplasmic mRNAs are appropriately polyadenylated. RNA from oocytes injected with constructs indicated was prepared from isolated nuclei (N), cytoplasm (C) or total (T) oocytes. RPAs were done using probes complementary to the SV40 late poly(A) signal and surrounding sequences (panels 1, lanes 1–20 and 2, lanes 21–28, see Materials and methods) or the SV40 early poly(A) signal and surrounding sequences (panel 3, lanes 29–36, see Materials and methods). Schematic of pre-mRNA and expected protected fragments and sizes from different constructs are shown. (B) pre-mRNAs with introns and an SV40 late poly(A) signal are appropriately spliced and polyadenylated. RNA from isolated nuclei (N) or cytoplasm (C) of oocytes injected with pDT-S3 or pDT-C3 was subjected to RPA using probes complementary to the intron, polyadenylation signal and surrounding sequences (Materials and methods). Schematic of pre-mRNA and expected protected fragments and their sizes are shown. (C) Analysis of extent of splicing of various constructs. RNA from isolated nuclei (N) or cytoplasm (C) injected with indicated constructs was analyzed by RPA using probes complementary to intron and surrounding sequences (Materials and methods), panel 1 shows lanes 1–16 and panel 2 shows lanes 17–24. Schematic diagram of pre-mRNA and expected protected fragments and sizes is shown. Note that sizes in parenthesis are too small to be seen on this particular gel.

(Figures 7 and 8C, panel 1, pMT1, pMT2 and pMT3), then the translational status of unspliced mRNAs should be the same as intron-less mRNAs. An additional complication is that the SV40 small t intron is extremely small (<70 nucleotides) and is at the lower limit of successful splicing in mammalian cells. Consequently, cryptic splice donors in sequences placed upstream of this intron are often used preferentially over the normal splice donor in this intron (Huang and Gorman, 1990). If cryptic splice donors were used in the transcripts derived from the pGH1.2 and pH1.10 plasmids, this could contribute to the observed depression of translation, because a normal protein product would not be made due to interruption of the open reading frame by a cryptic splicing event. It is possible that we might not be able to detect the use of cryptic splice donors in spite of the RPA assays (Figure 8C, panel 2). It should be noted that only constructs with a small t intron in the context of an SV40 early poly(A) signal appear to have structural integrity problems and therefore all conclusions from constructs with SV40 late poly(A) sites (pCH1.3, pDH1, pKH1 and all constructs in Figures 5 and 7) are not subject to this consideration. Therefore, our final conclusion stands: that splicing of a 5' intron stimulates translation compared with an intron-less mRNA, while splicing of a 3' intron represses translation.

Discussion

The major conclusion from this work is that the position of an intron within a pre-mRNA transcribed *in vivo* determines the translational efficiency of the mature mRNA in the cytoplasm. Our results indicate that the exact pathway of maturation of an mRNA within the nucleus can control gene expression at the post-transcriptional level over a 100-fold range.

The coupling of translational fate to transcription

Earlier work had suggested that the translational repression of a wide variety of natural and heterologous mRNAs synthesized *in vivo* was dependent on transcription *in vivo* (Bouvet and Wolffe, 1994; Meric *et al.*, 1996). Recent experiments linking the packaging of pre-mRNA with splicing factors to transcription by RNA polymerase II (Yuryev *et al.*, 1996; Du and Warren, 1997; Kim *et al.*, 1997) suggest that the final form of a pre-mRNA-RNP complex might be dependent on the transcription process. Additional evidence in support of this relationship are the observations that both nonsense-mediated RNA decay and translational fate can depend on the nature of the promoter sequences directing transcription (Enssle *et al.*, 1993; Gunkel *et al.*, 1995). The splicing of mRNA had been suggested as an additional mechanism by which transcripts in the *Xenopus* oocyte nucleus might evade translational repression (Braddock *et al.*, 1994). In the absence of activating proteins and target RNAs such as the TAT-TAR interaction, intron-less mRNA is exported from the nucleus and is inefficiently translated, whereas if the same mRNA entered the splicing pathway, translational efficiency is increased (Braddock *et al.*, 1994). Our results reported here considerably extend this observation, demonstrating that transcription *in vivo* directs mRNA towards a default state of translational repression relative to mRNA

injected into the cytoplasm (Figures 1–3), that the presence of an intron at the 5' end of the transcript can activate translation (Figures 3–7) and that an intron at the 3' end of the transcript can repress translation (Figures 3, 4, 6 and 7).

A termination codon upstream of an intron may be recognized as a premature termination codon (Carter *et al.*, 1996). This recognition might direct the storage of the mRNA as mRNP in the oocyte due to the abundance of RNA-masking proteins, whereas in somatic cells it might result in nuclear mRNA decay (Cheng *et al.*, 1990; Maquat, 1991). However, in our experiments all fully processed transcripts are comparatively stable (Figures 1–8; data not shown). Insertion of an intron can also influence poly(A)-site choice and change the 3' UTR of the transcript (Ramamurthy *et al.*, 1996). This is not the case for the vast majority of our constructs (Figure 8). However, note that the instability of pGH1.2- and pH1.10-generated mRNAs may be a result of lack of RNA splicing which is only detected in constructs which juxtaposed the small t intron and SV40 early poly(A) signal. With these possible exceptions, the repressive effects of the 3' intron would appear to be a consequence of alterations in the pathway of pre-mRNA maturation or packaging which are reflected in a decline in the utilization of mRNA in the cytoplasm.

According to Hawkins (1988), introns located 3' of termination codons are very rare, while 111 5'-non-coding exons were found in 328 vertebrate genes. Thus pre-mRNAs may have evolved to exclude the negative effects of 3' introns and to make use of the stimulatory effects of 5' introns. In this regard the activating effect of the 5' intron might be a regulatory signal which reflects the bona fide synthesis of a competent mRNA early in the biosynthetic process. Spliceosome assembly might stimulate the cross-talk of 5' and 3' ends of the mRNA, which is known to facilitate translation (Tarun and Sachs, 1995, 1996). Importantly, it is not simply the presence of an intron that determines translation, but also the position in the message (Figures 3, 4, 5 and 7). Thus recruitment of a spliceosome complex early in messenger biogenesis at the 5' end of the transcript might influence subsequent packaging of the mRNA to favor translation, whereas an intron at the 3' end of the mRNA alters packaging towards a repressed state. This hypothesis predicts that the nature of the mRNA itself does not change, only its utilization as a consequence of packaging into a RNP complex; this is similar to epigenetic control of promoter-utilization by assembly into chromatin (Wolffe, 1995).

Splicing and translational fate

There is considerable precedent for defects in pre-mRNA influencing the fate of mRNA. Correct splicing is important for transcripts encoded by intron-containing genes to be exported from the nucleus to the cytoplasm (Buchman and Berg, 1988; Ryu and Mertz, 1989; Huang and Liang, 1993). However, suboptimal 5' splice sites can also facilitate nuclear export (Huang and Carmichael, 1996). In cells infected with HIV-1, the export of spliced versus unspliced viral mRNAs is regulated by the Rev protein (Chang and Sharp, 1989; Cullen, 1992). Rev has been proposed to either regulate the export of unspliced mRNA by directing the displacement of spliceosomes from viral

pre-mRNAs (Chang and Sharp, 1989; Kjems and Sharp, 1993), or facilitate export by a distinct pathway from normal cellular mRNAs (Fischer *et al.*, 1994, 1995; Stutz *et al.*, 1995). Genetic experiments in yeast have also indicated that intronic sequences can potentially determine mRNA stability through interaction with hnRNP-like proteins (Steinmetz and Brow, 1996). In our experiments we find equivalent efficiency of export for spliced and intronless mRNA; only intron-containing mRNA appears to be retained in the nucleus (Figures 2 and 8). This strongly suggests that nuclear retention and mRNA-degradation do not have a major role in determining translational efficiency, dependent on introns. The effects of introns on translation appear to be dependent on splicing itself, and to not transmit across the nuclear pore (Figures 6 and 7). Entry into the splicing process clearly exerts a major influence on gene expression and represents a key controlling effect on translational efficiency in the oocyte. Most masked, maternal mRNAs in *Xenopus* oocytes do not contain introns (see below; Braddock *et al.*, 1994).

In earlier work we demonstrated that the translational repression coupled to the transcription process observed in *Xenopus* oocytes could be enhanced by expression of the Y-box protein FRGY2 (Bouvet and Wolffe, 1994). Consistent with this result, Braddock *et al.* (1994) found that the microinjection of antibodies against FRGY2 into the nucleus allowed intron-less transcripts to evade translational repression. These investigators detected a small amount of FRGY2 in the nucleus, although the vast majority of the protein is in the cytoplasm (Matsumoto *et al.*, 1996). Moreover, the use of kinase and phosphatase inhibitors indicated that the phosphorylation state of the Y-box proteins controlled translational activity (Braddock *et al.*, 1994). Therefore, the impact of splicing on translational fate might be mediated by mechanisms that influence the packaging of mRNAs with the Y-box proteins. How might this occur?

Recent progress by the groups of Daneholt and Dreyfuss provide potential mechanisms. Dreyfuss and colleagues have established clearly that particular hnRNPs will shuttle across the nuclear envelope (Pinol-Roma and Dreyfuss, 1992). Moreover, hnRNP proteins have been shown to have marked sequence preferences in their interactions with pre-mRNA (Dreyfuss *et al.*, 1993), leading to the assembly of specific RNP architectures (Wurtz *et al.*, 1990, 1996). The hnRNP proteins also determine splice site selection *in vitro* and *in vivo* (Mayeda and Krainer, 1992; Cáceres *et al.*, 1994). Thus the process of transcription and splicing will influence the packaging of mRNA by hnRNPs. However, since hnRNPs pass to the cytoplasm in association with mRNA (Pinol-Roma and Dreyfuss, 1992), they can potentially influence translation. The hnRNPA1 homolog of *Chironomus tentans* is found associated with translationally active polysomes (Visa *et al.*, 1996). Other proteins, such as the splicing factor hrp45, associate with nascent transcripts and remain bound from the gene to the nuclear pore (Alzhanova-Ericsson *et al.*, 1996). The exact site of dissociation is unknown, however it has been suggested that hrp45 does not pass through the central channel of the nuclear pore complex. Nevertheless, it remains possible that splicing proteins could either influence the packaging of mRNA in the nucleoplasm by other proteins such as hnRNPA1 and the Y-box proteins,

or determine such packaging at the nuclear pore. The consequences of mRNP organization at the nuclear pore can potentially be transmitted to the cytoplasm and determine translational fate. The impact of nuclear history on regulatory events in the cytoplasm provides a novel mechanism for the control of gene expression.

Implications for translational control in *Xenopus* early development

Xenopus oocytes provide an extremely useful system to explore the molecular mechanisms that regulate translation. Most recently, attention has focussed on the activation of translation of mRNAs which have been microinjected into oocyte cytoplasm during maturation of the oocyte into an egg. *Cis*-acting RNA sequences, known as cytoplasmic poly(A) elements, in the 3'UTR direct the addition of poly(A) (McGrew *et al.*, 1989; Standart, 1992; Wickens, 1992; Wormington, 1993). Poly(A) addition induces 5' cap ribose methylation (Kuge and Richter, 1995). Both 5' cap ribose addition and poly(A) addition are covalent modifications to mRNA that can cumulatively activate the translation of the mRNA by 10- to 100-fold (Sheets *et al.*, 1994, 1995; Kuge and Richter, 1995; Meric *et al.*, 1996). A second mechanism for translational control of maternal mRNA involves the association of mRNA with repressive RNA-binding proteins to assemble 'informosomes' or masked, maternal mRNA (Spirin, 1966; Richter and Smith, 1984; Richter, 1988). The major mRNA-binding protein associated with masked mRNA in oocytes is FRGY2 (Tafari and Wolffe, 1990, 1993; Murray *et al.*, 1992; Wolffe *et al.*, 1992). The association of FRGY2 with mRNA directs translational silencing (Ranjan *et al.*, 1993; Bouvet and Wolffe, 1994; Matsumoto *et al.*, 1996; Davydova *et al.*, 1997). The repression of translation directed by FRGY2 can be relieved upon oocyte maturation by dephosphorylation of FRGY2 (Sommerville, 1990), association with molecular chaperones (Meric *et al.*, 1997) and polyadenylation of the mRNA (Meric *et al.*, 1996). Thus both masking of maternal mRNA with FRGY2 and polyadenylation have important regulatory roles in the translation process.

The observation that transcription and splicing can influence translational fate in oocytes provides an additional component to the regulatory process. Transcription *in vivo* is required to bring histone H4 mRNA under appropriate biological control at oocyte maturation (Meric *et al.*, 1997). *In vivo* synthesis of mRNA is also required for efficient repression of translation by FRGY2 (Bouvet and Wolffe, 1994). Antibodies against FRGY2 stain nascent transcripts on the lampbrush chromosomes of *Xenopus* oocytes (Sommerville *et al.*, 1993), and the binding of FRGY2 to mRNA is more efficient if the pre-mRNA is synthesized *in vivo* rather than injected into the nucleus (Bouvet and Wolffe, 1994). These results lead to the suggestion that the packaging of pre-mRNA with the repressive FRGY2 protein occurs during the transcription process. FRGY2-association might occlude key binding sites for components of the translational machinery (Matsumoto *et al.*, 1996), or like the homologous prokaryotic cold-shock proteins might destabilize RNA secondary structures (Jiang *et al.*, 1997). If FRGY2 levels in the nucleus are depleted by the injection of antibodies against FRGY2 (Braddock *et al.*, 1994), then the translational

repression of intron-less transcripts can be relieved. Thus FRGY2-association appears to be causal for the translational repression of intron-less mRNAs. All endogenous mRNAs that show significant levels of translational masking in oocytes are synthesized from genes which lack introns (e.g. core histones, Ruderman *et al.*, 1979; hsp70, Bienz, 1984 and Horell *et al.*, 1987; c-mos, Watanabe *et al.*, 1989; FRGY1, Wolffe *et al.*, 1992). All endogenous mRNAs that are translated efficiently in oocytes contain introns (e.g. TFIIIA, Tso *et al.*, 1986; B4, Cho and Wolffe, 1994; FRGY2, S.R. Tafuri and A.P. Wolffe, unpublished). Although the endogenous B4 mRNA shows regulated polyadenylation on oocyte maturation, the translational stimulation is modest (Dworkin *et al.*, 1985). In fact, the endogenous B4 mRNA encodes the major linker histone variant synthesized in large amounts in the oocyte (Smith *et al.*, 1988; Dimitrov *et al.*, 1993, 1994; Dworkin-Rastl *et al.*, 1994). Together, these results lead to the conclusion that the transcriptional and splicing history of a mRNA synthesized in a *Xenopus* oocyte will have a major impact on its translational fate in the cytoplasm.

Materials and methods

Plasmids

Plasmid DNAs pH1.10, CMV-CAT, pSPH1.11 and pSPCAT were described previously (Bouvet and Wolffe, 1994). Briefly, pH1.10 is a derivative of pCAT-basic (Promega), which has the enhancer and promoter of human CMV immediate early gene and the coding sequence of a *Xenopus* histone H1C cDNA between *HindIII* and *NcoI* sites. Note that this construct has part of the CAT cDNA, which is not translated, downstream of the H1C cDNA (Figure 1). CMV-CAT has the same CMV enhancer and promoter regions, with pH1.10 upstream of the CAT cDNA. pSPH1.11, pSPCAT and pTF1 (a kind gift of Dr Philippe Bouvet) were constructed by subcloning the coding sequences of CAT, histone H1C and *Xenopus* TFIIIA cDNA into pSP64 poly(A) (Promega).

For CMV promoter-driven expression vectors with or without an intron, pCI (Promega) was used as a cloning vector. pCI vector has a chimeric intron composed of the 5' splice site from the first intron of the human β -globin gene, and the branch and 3' splice site from the intron which is between the leader and body of an immunoglobulin gene, heavy-chain variable region. The sequences have been changed to match the consensus sequences for splicing (Senapathy *et al.*, 1990). The DNA fragments containing the coding sequences of H1C, TFIIIA, B4, FRGY1 and CAT were subcloned into the multicloning sites of pCI vector to make pCH1.3, pCT1, pCB4, pCY1 and pCCA1, respectively. The cDNAs used in construction of the B4 and FRGY1 expression constructs contain 5' and 3' UTRs (Tafuri and Wolffe, 1990; Cho and Wolffe, 1994). pCTFL was constructed by subcloning the TFIIIA cDNA, which contains the entire coding region along with the 5' UTR (41 bp upstream) and 3' UTR (435 bp downstream) of it, into pCI (Tso *et al.*, 1986). pDH1, pDT1, pDTFL, pDB4, pDY1 and pDCA1 were constructed by removing the 197 bp *AflIII* fragment, which contains the chimeric intron, from pCH1.3, pCT1, pCTFL, pCB4, pCY1 and pCCA1, respectively.

DNA fragments containing the chimeric intron from pCI, SV40 small t intron from pCAT-basic, CMV enhancer and promoter (-526 to +55; Boshart *et al.*, 1985), *Xenopus* histone H1A 3' UTR (Perry *et al.*, 1985) and sequences downstream of the H1C-coding sequence in pH1.10 (Figure 1) were prepared by PCR. The DNA fragments were subcloned into pDH1 or pDT1 to produce various expression plasmids for H1C and TFIIIA (see Figures). The DNA fragments containing the chimeric intron and SV40 small t intron were inserted into pTF1 upstream of the TFIIIA coding sequence. These plasmids were used for *in vitro* transcription using SP6 RNA polymerase. DNA fragments containing the chimeric intron (nucleotides 820-1022 of pCI), SV40 small t intron (4713-4528 of SV40), CMV enhancer and promoter (-526 to +55; Boshart *et al.*, 1985) and sequences downstream of the H1C coding sequence in pH1.10 (Figure 3) were prepared by PCR. The DNA fragments were subcloned into pDH1 to produce various expression plasmids for H1C (Figure 4A). pDTC3 was constructed by subcloning

the DNA fragment containing the chimeric intron into *Sall*-*Bst*ZI-digested pDT1. The DNA fragment containing the SV40 small t intron was inserted into *AflIII*-digested or *Sall*-*Bst*ZI-digested pDT1 to produce pDTS5 or pDTS3, respectively. Mutations in the splice sites of the chimeric intron (Chang and Sharp, 1989) were introduced by PCR. Plasmid DNAs were prepared using Qiagen Plasmid kits, then extracted with phenol/chloroform and precipitated with ethanol. DNA was dissolved in TE buffer.

Oocyte microinjection

Capped mRNAs with poly(A)₃₀ tails were prepared by *in vitro* transcription with SP6 RNA polymerase. Linearized plasmids were incubated with SP6 RNA polymerase in the presence of 10:1 cap analog:GTP ratio to obtain 5' capped transcripts (Bouvet and Wolffe, 1994) or using an mMESSAGE mMACHINE kit (Ambion).

Microinjections into *Xenopus* stage VI oocytes were performed as described previously (Almouzni and Wolffe, 1993; Matsumoto *et al.*, 1996). Three ng of plasmid DNA or *in vitro* transcribed mRNA were injected into each oocyte. After injection, oocytes were maintained at 18°C for 16-18 h in modified Barth's saline. To analyze the proteins synthesized in the oocytes, 0.8 μ Ci of [³H]-lysine and 0.16 μ Ci of [³H]-arginine were then injected into oocytes and the oocytes were incubated at 18°C for another 6 h. Ten to fifteen injected oocytes were pooled per item of data. To examine the localization of RNA, nuclei were isolated from oocytes manually, under a microscope.

RNA analysis

RNA was isolated from oocytes using RNazol (Tel-Test, Inc., Austin, TX), and primer extension was performed as described previously (Matsumoto *et al.*, 1995). Primers HIFM4 5'-TCTTCAGTTTGGGTTC-TGCCGGGGGAGC-3' for histone H1 mRNA, CAT primer 5'-GGTGG-TATATCCAGTGATTTTCTCCAT-3' for CAT mRNA, and K54 primer 5'-GCAGTCGGCGAAAGAGAGATGTACCG-3' for TFIIIA mRNA were end-labeled. The purified RNA equivalent of 3-5 oocytes was incubated with 0.2 pmol of ³²P-labeled primer at 65°C for 2 min in a 20 μ l of reaction mixture containing 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol and 1 mM each 4 dNTPs, followed by gradual cooling to room temperature. The primer annealed with transcript was extended with 100 U SuperScript II reverse transcriptase (Life Technologies, Inc.) at 42°C for 1 h. Aliquots of the reactions were electrophoresed in a 6% polyacrylamide gel in the presence of urea. The gels were dried and subjected to autoradiography. Quantitation was performed by use of a PhosphorImager (Molecular Dynamics).

RNase protection assay

Under SP6 or T7 promoters the following DNA fragments were inserted into plasmids: the coding sequences of *Xenopus* TFIIIA (*PstI*-*BglII* fragment), histone H1C (*Bam*HI-*Bst*XI fragment) or histone H4 cDNA; the chimeric intron (*PstI*-*Eco*RI fragment of pCI); SV40 small t intron (*PstI*-*Eco*RI fragment of pDTS5); the 3' chimeric intron plus SV40 late poly(A) signal (*Sall*-*Bam*HI fragment of pDTC3); the 3' SV40 small t intron plus late poly(A) signal (produced by PCR using pDTS3 as the template); and the 3' SV40 small t intron plus early poly(A) signal (produced by PCR using pH1.10 as the template). ³²P-labeled antisense RNA probes were prepared by *in vitro* transcription with SP6 or T7 RNA polymerase in the presence of [α -³²P]UTP and gel-purified prior to use. RNase protection assays with total, cytoplasmic or nuclear RNA from the equivalent of 0.25 oocytes were performed using RPA II kit (Ambion), following the manufacturer's instructions. Aliquots of protected fragments were analyzed by electrophoresis in polyacrylamide gels as described above. Quantitation of mRNA levels was performed using a PhosphorImager (Molecular Dynamics).

Protein analysis

For analysis of TFIIIA, B4, FRGY1 and CAT proteins, oocytes which had been injected with [³H]-amino acids were homogenized in 5 μ l of protein homogenization buffer per oocyte (70 mM KCl, 90 mM HEPES pH 7.5, 1 mM dithiothreitol and 5% sucrose). The homogenates were centrifuged at 4°C for 10 min and aliquots of the supernatants were electrophoresed in a 4-20% gradient polyacrylamide gel containing SDS. For analysis of histone H1 protein synthesis, acid-soluble proteins were prepared (Bouvet and Wolffe, 1994). Briefly, oocytes were homogenized in 10 μ l of a buffer consisting of 50 mM Tris-HCl pH 7.5, 0.42 M KCl, 12.5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride, per oocyte. The homogenates were mixed with HCl to a final concentration of 0.2 M and incubated on ice for 1 h.

After centrifugation for 10 min at 4°C, the supernatant was precipitated with trichloroacetic acid. The precipitates were dissolved in 8 M urea and electrophoresed in a 4–20% gradient polyacrylamide gel containing SDS. The gels were fixed, treated with Amplify (Amersham) and dried. ³H-labeled proteins were detected by fluorography. Quantitation was performed by scanning the fluorogram using a densitometer.

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