Function of basonuclin in increasing transcription of the ribosomal RNA genes during mouse oogenesis

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SUMMARY

Active protein synthesis during early oogenesis requires accelerated transcription of ribosomal RNA genes (rDNAs). In response to this demand, rDNAs are amplified more than 1000-fold early in Xenopus oogenesis. Here, we report evidence that rDNA is not amplified in mouse oocytes, but these cells may instead employ the zinc-finger protein basonuclin, a putative rDNA transcription factor, to enhance rRNA synthesis. This conclusion is based on observations that basonuclin is localized in the nucleolus in the mouse oocyte early in its growth phase, when rRNA transcription is highly active; and that the binding sites of basonuclin zinc fingers on the human and mouse rDNA promoters are homologous. In a co-transfection assay, basonuclin can elevate transcription from an rDNA promoter, and its zinc-finger domain can inhibit RNA polymerase I transcription, as detected by a run-on assay, in growing mouse oocytes.

Key words: rDNA amplification, RNA polymerase I, Upstream binding factor, Oocytes, Nucleolus, Mouse

INTRODUCTION

Developing oocytes accumulate a large quantity of proteins, ribosomes and other cellular components to support early embryonic development. In Xenopus, a mature oocyte contains approx. 100,000 mitochondria (compared with the 70 or so on a sperm), 100,000 DNA polymerases and 200,000 ribosomes (Laskey, 1974). To amass this storage, Xenopus oocytes produce, at their peak, 34 ng of protein per hour. Because of this burden of protein synthesis, one of the highly transcribed genes in Xenopus oocytes is the 45S ribosomal rRNA gene (rDNA), a key factor in determining ribosome production. Xenopus possess approx. 450 copies of rDNA per haploid genome and in the early meiotic oocytes (4C), there are approximately 1800 copies of this gene. This large number of copies, however, is apparently not adequate to supply a sufficient amount of rRNA. During the early pachytene stage, these rDNAs are amplified to nearly two million copies (Brown and Dawid, 1968; Perkowska et al., 1968). It was estimated that without this amplification, it would take a frog oocyte ~1.7×10⁵ days to accumulate the same amount of the rRNA that it normally accumulates in 65 days (Perkowska et al., 1968). Mammalian oocytes differ from those of the amphibians in many ways, the most apparent being in their size. In the mouse, the diameter of a primordial oocyte is approx. 15 to 20 μm, which increases rapidly during the growth phase to 80 to 85 μm in fully grown oocytes. However, this is much smaller than that of the mature Xenopus oocyte, which reaches 1 mm and is 4000 times larger in volume than the mouse oocyte. One of the probable reasons for the smaller size of the mouse oocyte is that fewer embryonic cell cycles are supported by maternal contribution in mouse than that in amphibians (Schultz, 1993) and that no yolk proteins are accumulated. In the mouse embryo, rRNA transcription is activated at six- to eight-cell stage (Kopecny et al., 1995), which may render the storage of huge quantities of ribosomes unnecessary. This, however, does not imply that mouse oocytes do not accumulate proteins and ribosomes. On the contrary, this accumulation is rapid during the growth phase of the developing oocyte and nears completion (95%) within 2 weeks, when the oocyte reaches 60-70% of its final size (Kaplan et al., 1982). During this period, protein synthesis climbs to a peak of 0.175 pg per hour per pl of active cytoplasm, which is comparable with the 0.34 pg/hr/pl of the Xenopus oocyte (Schultz et al., 1978). Accompanying this protein accumulation is a steady increase in RNA, consistent with a doubling of the activities of RNA polymerase I and II (pol I and II) during this period (Moore and Lintern-Moore, 1978). The majority of these RNAs are rRNA (60-70%), whose rate of synthesis remains constant at 0.01 pg per minute over the first 9 days of oocyte growth and accelerates to 0.015 pg/min in the next 5 days, until the cessation of synthesis at 15 days of growth (Kaplan et al., 1982). As a reflection of this rate of RNA accumulation, the nucleolus enlarges from 2 μm to almost 10 μm in diameter and there is...
a fourfold increase of ribosomes in the oocyte. Based on this measurement of the rate of rRNA synthesis and the 4000-fold difference in volume between mouse and Xenopus oocytes, it has been proposed that rDNA amplification in the mouse oocyte at the scale observed in the Xenopus oocyte is unnecessary (Kaplan et al., 1982). However, little is known regarding the mechanism that may account for the increase in pol I activity.

Transcriptional regulation of rDNA is one of the first and the best studied gene regulatory mechanisms. In mammals, two conserved cis-elements within the promoter of rDNA have been identified, the upstream promoter element (UPE or UCE) and the core promoter element (CPE). In humans, the basic transcription machinery for rDNA contains, in addition to pol I, at least two transcription factors, the upstream binding factor (UBF) and the SL-1 complex. The latter comprises several proteins, including the TATA-binding protein (TBP), which is also found in transcriptional complex of pol II and III. Pol I transcription factors are targets of regulatory mechanisms such as phosphorylation (Voit et al., 1995), and of regulatory proteins such as SV40 large T-antigen (Zhai et al., 1997), Ku-associated proteins (Kuhn et al., 1993; Hoff et al., 1994; Niu and Jacob, 1994), p53 (Budde and Grummt, 1999) and Rb proteins (Cavanaugh et al., 1995b; Voit et al., 1997).

Basonuclin, a zinc-finger protein of 120 kD first discovered in human keratinocytes, is also highly expressed in the germ cells of testis and ovary (Tseng and Green, 1992; Tseng and Green, 1994; Yang et al., 1997; Mahoney et al., 1998). In human keratinocytes, basonuclin was detected in the nucleolus of interphase cells and at the rDNA loci on mitotic chromosomes (Tseng et al., 1999). The zinc fingers of basonuclin were shown to bind to the upstream control element (UCE) of the promoter of human rDNA (Ishii and Green 1999, Tseng et al., 1999). These observations suggest that basonuclin may be a transcriptional regulator for the rDNA. Here, we tested the hypothesis that basonuclin enhances rDNA transcription in the mouse oocyte. This hypothesis has several predictions: (1) that basonuclin is localised in oocyte nucleolus and associated with pol I transcription both temporally and spatially; (2) that its interaction with rDNA promoter is evolutionarily conserved; (3) that it can increase rDNA transcription; and (4) that perturbation of its function will cause reduction in oocyte rRNA synthesis.

MATERIALS AND METHODS

Cells
To compare the copy number of rDNA, oocytes were collected from 4- to 5-week-old F1 hybrid (CF1) mice (Charles River Laboratories, Wilmington, MA) by needle puncture of isolated ovaries followed by mechanical removal of the attached cumulus cells with a fine-bore glass micropipet (Fig. 1A). For immunocytochemistry and the run-on assay, oocytes of 10- to 12-day-old CF1 mice (Charles River) were collected similarly into M2 media in the presence or absence of 100 µg/ml dBCAMP, which had no effect on the results presented in this study. Primary mouse fibroblasts (C57BL/6j) were cultured in DMEM (Life Science Technology) with 10% calf serum.

Extraction of DNA from oocytes and somatic tissues
Three hundred oocytes, which contain 3.6 ng of genomic DNA, were collected for each PCR reaction. We employed two complementary methods of obtaining oocyte genomic DNA. The first is a modification of the method described by Dawid for frog oocytes (Dawid, 1965). Mouse oocytes were gently homogenized in 20 volumes of a solution containing 0.07 M Tris-HCl, pH 7.7, 3 mM EDTA and 2% SDS. The homogenate was extracted with an equal volume of phenol for 15 minutes. Sodium acetate was added to the aqueous phase to a concentration of 0.3 M and the nucleic acid was precipitated with 2.5 volumes of ethanol. This method produces better quality DNA but may risk DNA loss during purification. To prevent DNA loss, oocytes were put into a 200-µl PCR tube and genomic DNA was released by rapid freeze-thaw cycles twice (on dry ice and 37°C water bath) and used directly for PCR. Somatic DNA was extracted as described previously (Tseng and Green, 1988) and the DNA concentration determined by spectrophotometry.

PCR
PCR was performed with primer pairs mrDP5 (5'-AGAAGCC-CTCTCTGTCCCTTG) and mrDP3 (5'-GGGTGCTCGGGGAC-AGCCTC) for the promoter region (~286 to +81) of the mouse rDNA and Mid1-5 (5'-TCGCGTGCCCTACATGGTG), and Mid2-3 (5'-CGGCTCTGGCTTTGAACACTC) for a region in the coding sequence (+4385 to +4843). Cycle parameters were 94°C for 5 minutes, 20 cycles of 94°C for 1 minute, 63°C for 1 minute and 72°C for 1.5 minutes, and finally 72°C for 7 minutes. One of the primers in each pair was labeled with T4 polynucleotide kinase (New England Biolabs, Beverly, MA). The amplified fragment was analysed on a 1% agarose gel and the autoradiogram of the gel was scanned using a STORM-80 PhosphorImager and the image quantified using an ImageQuant program (IQMac V1.2). To reduce cross contamination of samples due to spillage during gel loading, the PCR samples of oocytes were always separated from those of the somatic tissue by one or more gel lanes.

Transcription run-on assay
Oocytes and fibroblasts were incubated at room temperature for 5 minutes in a permeabilization buffer containing 0.4% and 0.04% of Triton X-100, respectively. A run-on buffer (50 mM Tris-HCl, pH 7.4, 100 mM KCl, 5 mM MgCl₂, 0.5 mM EGTA, 25 mM S-adenosyl-L-methionine) containing 0.5 mM ATP, CTP, GTP and 0.2 mM Br-UTP was added to the cells and incubated for 1 hour at 37°C. In some cases, anti-basonuclin or anti-UBF antibodies were included in the run-on reaction and these antibodies were visualized along with BrU at the end of the run-on. When indicated, actinomycin D (1 µg/ml) or α-amanitin (100 µg/ml) were added to the run-on buffer. To demonstrate that the BrU was incorporated into RNA, an RNase digestion (RNase A, 400 µg/ml in PBS) was performed before immunodetection. At the end of the run-on, the cells were washed twice in PBS containing 5 units/ml RNasin (Promega) and fixed immediately in 2% paraformaldehyde. The incorporated BrU was detected with the antibody conjugated with FITC, as described above.

Recombinant basonuclin (B10B) and Krüppel (4015) zinc fingers were expressed and purified as previously described (Tseng et al., 1999). Briefly, appropriate cDNA sequences were cloned into the vector pSET (Invitrogen) and expressed in Escherichia coli strain BL21. The recombinant proteins were then purified to near homogeneity on a Biorex-70 column (BioRad), stored in 500 mM NaCl and 50% v/v glycerol at 350 µg/ml, and diluted 1:10 to 1:100 into run-on buffer. All blocking experiments were performed in the presence of α-amanitin (100 µg/ml).

Immunocytochemistry
Immunocytochemistry on cryosectioned ovary was performed as described in Mahoney et al. (Mahoney et al., 1998). Isolated oocytes were washed in PBS and a permeabilization buffer (20 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 0.5 mM EGTA, 0.5 mM PMSF) briefly and incubated at room temperature for 5 minutes in the permeabilization buffer.
primer with \[32P\] was achieved by T4 polynucleotide kinase. 

A comparison of the copy number of rDNA in the mouse oocyte and somatic tissues. (A) Oocyte genomic DNA was obtained from a mixed population of oocytes isolated from 4-week old mice. A sample of this population is shown. Most oocytes contained a recognizable germinal vesicle (n), indicative of prophase of meiosis I. Contaminating cumulus cells are indicated by arrows. (B) Linear input/output response of the PCR; the data were derived from quantification of autoradiography of the gel shown in C. The promoter region of the somatic rDNA was PCR-amplified with a radiolabeled primer. A tenfold serial dilution of template DNA was used to produce the concentration response. The asterisk shows the quantification of the PCR fragment amplified from 3 pg of pCR-mrDNAP, which contains a similar amount of rDNA to 3.5 ng of genomic DNA. (C) An autoradiograph of the gel from which the amplified somatic rDNA promoter sequence was subjected to electrophoresis. Lanes 1-3, genomic DNA of 3.5 ng, 35 ng and 350 ng were used as template. Lane 4, 3 pg of pCR-mrDNAP was used as template. (D-G) Copy numbers of two regions of the rDNA were examined, the promoter (D,E) and a region in the middle of the coding sequence (F,G). Oocyte DNA was obtained by two methods, the method of Dawid (Dawid, 1965) (D,F), and the method of freeze-thaw and direct amplification (E-G). In D-G, the template DNAs were lane 1, oocyte DNA; and lanes 2 and 3, somatic DNA (3.5 ng and 35 ng, respectively). No lane 3 is present in G, owing to sample loss.

RESULTS

The 45S ribosomal RNA genes (rDNA) are not amplified in the mouse oocyte

We compared the copy number of rDNA in the mouse oocyte to that of the somatic tissues by a PCR method with \[^{32}P\]-labeled primers. Oocyte genomic DNA was isolated from a population in which most of the oocytes were in the growth phase or prior to germinal vesicle breakdown (Fig. 1A). The rDNA amplification in *Xenopus* oocytes takes place during the pachytene stage of meiosis prior to the onset of rRNA synthesis. By analogy, any rDNA amplification that would benefit rRNA synthesis during the growth phase of mouse oocytes should take place before this growth period. By selecting oocytes that were in the growth phase or after, we ensured that such rDNA amplification had already taken place and was therefore detectable. Care was taken to remove the granulosa (cumulus) cells that surround the oocytes. These cells are of somatic origin and, although the copy number of their rDNA has not been investigated, they are not likely to have been amplified. Granulosa cell (2C to 4C, depending on the stage of cell cycle) contamination in our oocyte (4C) preparations was estimated to be approx. 10% in total cell number (Fig. 1A, arrows) and less than 10% in genomic DNA. As it is not feasible to obtain a large number of oocytes from mouse to employ the conventional method to determine DNA concentration, we relied on the generally accepted estimate that...
each oocyte contains 12 pg of genomic DNA. Somatic genomic DNA was extracted from mouse liver, kidney, lung and intestine.

Using a mouse rDNA promoter fragment (~300 to +150, transcription starts at +1) as a control, we first established conditions whereby the quantity of the amplified fragment was linearly proportional to the quantity of the template over a range of two orders of magnitude (3.5 to 350 ng) (Fig. 1B). Under these conditions, we determined that the minimal quantity of genomic DNA required for the detection of rDNA is 3.5 ng (Fig. 1C). This amount of genomic DNA should contain approximately 0.3 pg of the target rDNA fragment of 446 bp, since the copy number of rDNA is 400-500 per haploid mouse genome of 2.7x10^9 bp. We tested this estimate by amplifying serial dilutions of the same target fragment that was cloned in pcR-II (pcR-mrDP), in which the rDNA promoter sequence is approx. 10% of the mass of the entire plasmid (446 bp insert and 3948 bp vector). When 0.3 pg of the cloned rDNA promoter sequence was used as template, the amplified fragment (Fig. 1C, lane 4) contained radioactivity similar to that from 3.5 ng of genomic DNA (Fig. 1C, lane 1), which, according to our estimate, also contains 0.3 pg of the rDNA promoter fragment. This agreement demonstrates that under our PCR conditions, the quantity of rDNA in the mouse genomic DNA could be measured correctly (Fig. 1B).

We examined two regions of the 45S rRNA gene: the promoter region and a region in the middle of the coding sequence. We found that at comparable concentrations, the oocyte DNA produced a similar quantity of amplified rDNA to that obtained from somatic cell DNA (Fig. 1D-G). This was true for both regions of the rDNA tested as well as for oocyte DNA prepared by two different methods. Therefore, no rDNA amplification could be detected in mouse oocytes.

**Basonuclin distribution during the growth phase of oocyte development**

We reported previously that basonuclin is present in the nucleus of developing mouse oocytes (Mahoney et al., 1998). Here, we investigated its nuclear distribution during the first meiosis by immunocytochemistry on cryosectioned mouse ovaries. In the 10- to 15-day-old postnatal ovary, basonuclin was found exclusively in the nucleus of primordial, primary and secondary oocytes. At this age, the heterochromatin patches can be identified by their intense DAPI fluorescence (Fig. 2A, arrows). These heterochromatin patches surround the nucleolus of oocytes that are competent to resume meiosis and can serve as landmarks for this subnuclear structure (Mattson and Albertini, 1990). The immunostaining of basonuclin appeared uniform within the nucleus of growing oocytes. An exception was that basonuclin fluorescence in the heterochromatin regions was weaker than the rest of the nucleus (Fig. 2B). Occasionally, small regions with stronger basonuclin fluorescence were seen within the nucleoplasm (Fig. 2B, arrow), but the underlying subnuclear structure could not be identified.

**Nucleolar basonuclin is associated with active pol I transcription**

To investigate pol I transcription in mouse oocytes, we established a run-on assay for pol I transcription in Triton X100-permeabilized primary oocytes (Masson et al., 1996), when transcriptional activity is at its peak. Pol I transcription was detected by using BrU as one of the substrates, and the incorporated BrU was visualized by immunocytochemistry. The nucleolar location of the incorporated BrU was demonstrated by its juxtaposition with the heterochromatin patches (arrows, Fig. 3B) and by their co-localization with an anti-nucleolus antibody (ANA) (not shown). The interpretation that the incorporated BrU reflects pol I transcription was supported by its sensitivity to actinomycin D (Fig. 3C) and RNase (not shown), and resistance to α-amanitin (Fig. 3D). Pol I transcription could be detected readily within two hours of oocyte permeabilization (Q. T. and H. T., unpublished observations) and beyond that the BrU incorporation diminished rapidly. Because it was estimated that the

**Fig. 3.** Pol I transcription in permeabilized mouse oocytes in their growth phase. Immature oocytes were isolated from 10- to 12-day-old mice and permeabilized with Triton X-100. Run-on reaction was performed as described by Masson et al. (Masson et al., 1986) with BrUTP as one of the nucleotide precursors. (A) BrU incorporation into an oocyte after 1 hour run-on reaction. (B) In another oocyte, nucleolar localization of the incorporated BrU (green) is shown by its juxtaposition with heterochromatin patches (blue, arrows). Note that the periphery of the nucleolus contains more label. (C) Run-on reaction in the presence of 1 μg/ml actinomycin D, no incorporated label was observed. (D) Run-on reaction in the presence of 100 μg/ml α-amanitin. Incorporated label is indicated by an arrow. In B-D, the image of DNA stain (DAPI) is superimposed on the BrU stain.
completion of one round of transcription of the rRNA gene in HeLa cells requires less than 5 minutes (Penman et al., 1966), it is very likely that the incorporated BrU was the result of multiple rounds of transcription initiation after permeabilization. No significant BrU incorporation in the nucleoplasm was detected, suggesting that pol II activity was suppressed under our experimental conditions.

To investigate the distribution of basonuclin and UBF in the permeabilized oocytes, we included the anti-basonuclin (M767) and the anti-UBF (anti-Nor 90) antibodies in the run-on buffer for the duration of the assay. The antibodies had little effect on the BrU incorporation into the nucleolus (Fig. 4G). Anti-UBF antibody detected a protein localized exclusively in the nucleolus surrounded by the intact heterochromatin patches (Fig. 4B,E). The unperturbed heterochromatin and UBF distribution suggests that the basic nucleolar organization was maintained, consistent with the ability of the Triton-treated oocytes to continue pol I transcription. In Triton X-100-treated oocytes, however, nucleolar basonuclin became more prominent and the fluorescence intensity of nucleoplasm-associated basonuclin was reduced notably but still visible (compare Fig. 4C,H with Fig. 2B). The nucleoplasm-associated basonuclin was therefore more easily extracted by Triton X-100 than was the nucleolar-associated basonuclin. The Triton-permeabilization method thus allows us to distinguish cytochemically the nucleolar basonuclin from that in the nucleoplasm and, more importantly, to demonstrate the presence of basonuclin in nucleoli undergoing active pol I transcription.

**Basonuclin-binding sites on the human and mouse rDNA promoters are similar**

We and others have shown that recombinant proteins containing the N-terminal pair of zinc fingers of human basonuclin interact with the UCE of the rDNA promoter (Iuchi and Green, 1999, Tseng et al., 1999). Our recombinant protein, designated B10B, binds specifically to three sites within the promoter region of a human rDNA (Tseng et al., 1999). The N-terminal pair of basonuclin zinc fingers is evolutionarily highly conserved. The amino acid sequence of B10B is identical to that of the corresponding pair of zinc fingers of mouse basonuclin, despite nucleotide substitutions of about 8% (Matsuzaki et al., 1997). The mouse rDNA promoter contains cis-elements: the transcription initiation site, the UCE, and the core. Amino acid sequence of B10B contains cis-elements equivalent to the corresponding elements in the human promoter (Grummt, 1999). However, there is very little overall DNA sequence homology between the mouse and human rDNA promoters. Therefore, it is of interest to compare the binding sites of B10B on the human rDNA promoter with those of the mouse. We examined a DNA fragment of the mouse rDNA promoter (−188 to +71) by DNase I footprinting (Fig. 5). This fragment contains the cis-elements: the transcription initiation site, the UCE, the core and the terminator. B10B protein binds to three binding sites on the mouse rDNA promoter, whose relative positions to the cis-elements are very similar to those on the human rDNA promoter (Fig. 6).

Fig. 4. Nucleolar localization of basonuclin in permeabilized mouse oocytes under conditions that favor transcription by pol I but not pol II. (A-F) A Triton X-100-permeabilized, immunocytochemically stained, immature oocyte. (A) Phase microscopy. (B-F) Fluorescence microscopy for UBF (green, B), basonuclin (red, C), DNA (blue, D), UBF and DNA (E), and UBF and basonuclin (F). (G-I) A similarly permeabilized oocyte viewed for BrU incorporation (G), basonuclin (H), and BrU and basonuclin (I).
The rDNA promoter was dependent on basonuclin, a deletion was made in the pFBwt to remove the C-terminal two thirds of the basonuclin coding sequence, including the nuclear localization signal (pFBd). This basonuclin deletion mutant lost its ability to elevate CAT RNA level (Fig. 7, lanes 5 and 6).

**The zinc finger domain of basonuclin inhibits pol I transcription in mouse oocytes**

We tested the ability of B10B to interfere with pol I transcription in mouse oocytes. This is because B10B binds to the mouse rDNA promoter and is small enough (12 kDa) to diffuse freely into the nucleolus of the permeabilized oocytes. When B10B was added at the onset of the run-on reaction, BrU incorporation into the nucleolus was abolished completely (Fig. 8A). This effect was observed at a B10B concentration of 35 µg/ml and 11 µg/ml, but not at 1 µg/ml, revealing a concentration dependency. At 35 µg/ml, a recombinant protein containing a pair of C2H2 zinc fingers derived from the *Drosophila* Krüppel protein (Stanojevic et al., 1989), failed to produce the same effect (Fig. 8B). B10B binds to a GC-rich sequence 5′-G(G/C)G(C/T)G(A/T)C (Iuchi and Green, 1999; Tseng et al., 1999; see Figs 5, 6), whereas the Krüppel protein binds to an AT-rich sequence 5′-AAGGTTAA (Pankratz et al., 1989; Treisman and Desplan, 1989), suggesting that the inhibitory effect of the basonuclin zinc fingers is related to their DNA-binding sequence specificity. To investigate if the effect of B10B is dependent on the presence of basonuclin, we repeated the experiment in mouse fibroblasts, which express UBF but not basonuclin. Mouse fibroblasts treated with Triton X-100 showed the same ability to incorporate BrU into their nucleoli and similar permeability to immunoglobulin (not shown). But when B10B was added at the concentration that blocked pol I transcription in oocytes (of 35 µg/ml and 11 µg/ml), BrU incorporation was still detected, but at a reduced level (Fig. 8C) compared with the oocytes exposed to the zinc fingers of Krüppel (Fig. 8D).

**DISCUSSION**

Previous studies have suggested that, unlike amphibians, rDNA amplification may not be employed to enhance rDNA transcription during mouse oocyte development (Kaplan et al., 1982). However, to our knowledge, direct measurement of rDNA copy number in the mouse oocyte by PCR has not been reported. Here, we have compared the copy number of rDNA in mouse oocytes and somatic tissues. Under our PCR conditions, which could easily detect an amplification of rDNA of less than tenfold, we failed to detect any amplification of oocyte rDNA at the promoter and the coding region of the gene. Our run-on assay also failed to label any ‘mini-nucleoli’, which are present in their hundreds in *Xenopus* oocytes (Brown and Dawid, 1968). These observations therefore support the notion that no rDNA amplification occurs prior to the growth phase of mouse oocyte development. Mouse, and perhaps mammals in general, may rely on alternative mechanisms to enhance rDNA transcription during oocyte development.

The intranuclear distribution of basonuclin is intriguing. In the growing oocyte, its uniform distribution between the nucleoplasm and nucleolus clearly differs from the exclusive nucleolar distribution of UBF, a bona fide pol I transcription factor. How does the basonuclin level change during oocyte growth? Is the change due to a post-translational modification? Further experiments will be needed to determine if this is the case.
Basonuclin in mouse oogenesis

This also differs from the intranuclear distribution of basonuclin in the basal keratinocytes of human plantar epidermis (Tseng and Green, 1994) and in the neonatal mouse spermatogonia (Mahoney et al., 1988), in which basonuclin appears to concentrate within certain subnuclear structures, presumably nucleoli. In cultured human keratinocytes, although basonuclin was detected in the nucleolus, its presence was predominantly nucleoplasmic (Tseng et al., 1999). Here, by combining a run-on assay and immunocytochemistry, we show clearly that in the permeabilized oocytes, basonuclin is present in the nucleolus where active pol I transcription can be simultaneously detected.

We and others have shown that the recombinant basonuclin zinc fingers bind to the promoter region of the human rDNA. The locations of the binding sites detected by our group and by Iuchi and Green agree well and both groups described

Fig. 6. A comparison of B10B-binding sites on human and mouse rDNA promoter. The DNA sequences of the promoter region of the human (top) and the mouse (bottom) rDNA are aligned according to the transcription start site (arrow). The cis-elements are shown in gray. The footprints on the noncoding and the coding strands are indicated, respectively, by black bars above and below the DNA sequence. The corresponding binding sites on human and mouse rDNA promoters are indicated by A, B and C. Note the similarity of the binding sites in relation to the cis-elements on each promoter.

Fig. 7. Basonuclin enhances transcription from an rDNA promoter in a co-transfection assay. (A) The reporter construct, pHrCAT. The position of the three binding sites of B10B are indicated by hatched bars and labeled by capital italics. Transcription initiation site within the rDNA promoter is shown by an arrow. The location of the primer (HuCAT-PE) used in the primer extension assay is indicated by a black bar. (B) The basonuclin expression constructs. The coding region of the wild-type human basonuclin cDNA clone was placed under the transcriptional control of the cytomegalovirus promoter (CMV Pr.) in the pcDNA3.1 vector (pFBwt) and a C-terminal deletion mutant (pFBd). The extent of the sequence of B10B is indicated by a black bar below the wild-type cDNA. (C) Reporter plasmid pHrCAT was co-transfected into COS-7 cells with the empty expression vector pcDNA3 (lane 1 and 2), with basonuclin expression plasmid pFBwt (lane 3 and 4) and with pFBd. Transcription from the reporter was monitored by primer extension. Correct initiation from the rRNA transcription start site yields a primer extension product of 123 bases. Lane M, a molecular weight marker; numbers on the left are size in bases. The value below each lane indicates the relative amount of radioactivity in the primer extension product, with lane 1 arbitrarily defined as 1.00. (D) A northern analysis of β-actin serves as a control of the amount of RNA used in the primer extension assay. nls, nuclear localization signal; Ser, serine stripe; Z12, zinc fingers 1 and 2.
similar target sequences for the basonuclin zinc fingers (Tseng et al., 1999, Iuchi and Green, 1999). These binding sites could be related to rDNA transcription or they could be coincidental. Since mouse and human rDNA promoters share very little overall DNA sequence homology, if the binding sites in the human promoter are coincidental, they would not occur at the same location in the mouse promoter. Our result demonstrates that basonuclin-binding sites on the human and mouse rDNA promoter are remarkably similar in location as well as in organization, which strongly disputes the coincidental nature of the binding sites. Moreover, the evolutionary conservation of this homology between human and mouse suggests that there is functional interaction of basonuclin and rDNA promoter.

The tissue and cellular distribution of basonuclin suggests that it is a positive regulator of rRNA transcription. Basonuclin is expressed in tissues and cells that are highly proliferative, e.g. in basal epidermal and hair follicular keratinocytes and in spermatogonia of the seminiferous tubule, or in cells with active protein synthesis, e.g. in the developing oocyte. Here, we have shown that basonuclin enhances transcription from an rDNA promoter in a co-transfection assay in COS cells. We detected a small but clear positive effect, which is dependent on the integrity of the basonuclin-coding sequence. It is not clear, however, whether the magnitude of this positive effect in COS cells reflects what might be occurring in the oocyte, because of the apparent difference between the two cell types (i.e. since the action of basonuclin is likely to be cell-type specific, other oocyte factors may be required for the full effect of basonuclin in the oocyte); neither do we know the exact amount of increase in rRNA transcription that a growing mouse oocyte requires (e.g. a doubling of the rate of synthesis may be all that is required). The co-transfection experiment was performed in COS cells because they do not express basonuclin – any effect of the protein can therefore be detected. It is difficult to use the run-on assay to measure directly the magnitude of the effect of exogenous basonuclin (e.g. introduced by microinjection) in the oocyte because the rRNA transcription may already be at a maximum in the presence of the endogenous basonuclin. In any case, the result of the co-transfection experiment is in agreement with the dominant-negative effect of basonuclin zinc fingers (B10B) on oocyte rRNA transcription, which also suggests that basonuclin is a positive regulator. The dominant-negative effect of B10B on pol I transcription may occur through two mechanisms, namely, its interference with basonuclin function, and/or with other components of the pol I transcriptional apparatus, such as UBF, whose binding site overlaps that of basonuclin-binding site A. Similarly, this effect could be exerted at the step of transcription initiation or elongation. The differential effect of B10B on pol I transcription of oocytes and fibroblasts suggests that the larger inhibitory effect of B10B in oocytes cannot be explained by the transcriptional process common to both cell types.

Our results show that basonuclin differs from UBF, a dedicated pol I transcription factor, in at least two aspects. First, basonuclin has a restricted tissue and cell distribution (Tseng and Green, 1994; Yang et al., 1997; Mahoney et al., 1998). UBF, on the other hand, has a much wider distribution and is thought to be ubiquitous. Second, the intranuclear distribution of UBF is exclusively in the nucleolus, whereas basonuclin is found both in the nucleoplasm and nucleolus. The nucleoplasmic localization of basonuclin is consistent with its ability to interact with promoters other than that of rDNA (Tseng et al., 1999). These observations suggest that basonuclin may have target genes other than rDNA. In this context, it is of interest to note that the polymerase I-and-transcript-release factor (PTRF), first identified as a pol I-associated factor (Jansa et al., 1998; Jansa and Grummt, 1999), was recently described to have a restricted tissue distribution and is thought to involve also in the transcriptional regulation of two type-I collagen genes in mouse fibroblasts (Hasegawa et al., 2000).

Another issue regarding the relationship of basonuclin and UBF is how they interact on the rDNA promoter. We showed that they co-exist in the oocyte nucleolus (Fig. 4) and on the mitotic chromosomes of keratinocytes (Tseng et al., 1999). Moreover, the DNase I footprints of basonuclin zinc fingers and UBF overlap on the rDNA promoter (Iuchi and Green, 1999; Tseng et al., 1999). These observations raise the question of whether basonuclin replaces UBF in oocytes and keratinocytes, or whether they work cooperatively. Any speculation about their interaction must also take into account that the binding of UBF to DNA is not entirely sequence specific, but may also depend on the secondary structures of the DNA (McStay et al., 1991; O’Mahony et al., 1992; Kuhn et al., 1994). Since this question may hold a key to understanding how rRNA transcription is regulated by a cell type-specific factor, further investigation is warranted.

Because of the importance of rRNA synthesis to cellular

Fig. 8. Basonuclin zinc fingers (B10B) block pol I transcription in mouse oocytes. (A) Run-on reaction in the presence of B10B at 11 μg/ml, no BrU incorporation was detected (arrowheads). (B) Run-on in the presence of the zinc fingers of Krüppel at 35 μg/ml; BrU was incorporated (arrows). Three oocytes are shown in both A and B. (C,D) Run-on reaction in Triton X-100-treated mouse fibroblasts, in the presence of B10B at 11 μg/ml (C, weaker but clear incorporation of BrU was detected, arrowheads) and in the presence of the zinc fingers of Krüppel at 35 μg/ml (D, the incorporation of BrU was not affected, arrows).
function, it is tightly regulated during the cell cycle, cellular proliferation and differentiation. It has been shown that the mammalian basal pol I transcriptional regulators are targets of phosphorylation, which activates their ability to assist pol I transcription (Voit et al., 1995). Pol I transcription can be downregulated by the tumor suppressors p53 (Budde and Grummt, 1999) and Rb protein (Cavanaugh et al., 1995a; Voit et al., 1997), and by DNA-activated protein kinase (Kuhn et al., 1995), or it can be target of viral oncogenes such as SV40 large T antigen (Zhai et al., 1997; Zhai and Comai, 1999) and Ku-related proteins (Hoff et al., 1994), which activate rRNA transcription. Because the basal pol I transcription factors are thought to be ubiquitous, these regulatory mechanisms should be functional in all cell types. The existence of basonuclin suggests that in addition to the above described mechanisms, some cell types may employ their specific transcription factors to regulate rRNA transcription.

A unique problem faced by oocytes is their large cytoplasm-to-nucleus volume ratio, which increases dramatically during the growth phase. It is known that the ribosome density per volume of cytoplasm in developing mouse oocyte is comparable with that in somatic cells, which have a much smaller cytoplasm-to-nucleus volume ratio. This means that at transcriptional level, oocyte must use a similar number of genes (4C of oocyte versus 2C at G1 and 4C at G2 of the somatic cells) to produce ribosomes to fill a much larger volume of cytoplasm. In the Xenopus oocyte, one solution is rDNA amplification. In the smaller mouse oocyte, where the demand for ribosomes may not be as large as in the Xenopus oocyte, the problem remains. Here, we have shown that no rDNA amplification is detected in the mouse oocyte, instead, the problem remains. Here, we have shown that no rDNA amplification is detected in the mouse oocyte, instead, these cells may use basonuclin to enhance rRNA synthesis. If this is indeed the case, then inhibition or targeted disruption of the function of basonuclin should interrupt mouse oocyte development.

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