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Enhancer of RNA polymerase III gene transcription

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ABSTRACT

A protein responsible for enhanced transcription by RNA polymerase III was identified in extracts from *Xenopus* oocytes. This protein, called EP3, interacts with a specific DNA sequence adjacent to the 3′-end of a *Xenopus* somatic 5S RNA gene and forms a distinct band shift complex with a unique DNase I footprint. Enhanced transcription was observed from both 5S RNA and tRNA reporter genes when EP3 binding sites were inserted at different locations and orientations. Removal of the EP3 protein from an oocyte extract abolished this enhanced transcription. In addition, EP3 was shown to stimulate transcription by increasing the rate of transcription complex assembly. EP3 directly discriminates between the somatic and oocyte 5S RNA gene families and may play a significant role in their differential expression during early *Xenopus* development.

INTRODUCTION

The expression of many genes is controlled by the action of enhancers. The hallmark of enhancer action is the ability to stimulate transcription from sites at many different positions. Genes transcribed by RNA polymerase III can be divided into different classes based primarily on their promoter structures (1–4). One class of genes transcribed by RNA polymerase III contains upstream TATA boxes and are often found in association with enhancers. The more classical RNA polymerase III genes, e.g. tRNA and 5S RNA genes, contain internal promoters and, although transcription of these genes is regulated, enhancers have not previously been implicated in their expression.

In *Xenopus*, the somatic and oocyte 5S RNA genes are differentially expressed during embryogenesis and the study of the regulation of expression of these gene families has a long history. Many proposals for mechanisms to account for the observed differences in gene expression have been issued and include competition for limiting amounts of transcription factors (5), different affinities of the two genes for common transcription factors (6,7), differential stability of transcription complexes on the two genes (8), inactivation of soluble transcription factors by mitotic phosphorylation (9), selective gene repression through histone H1 binding (10–12) and differential kinetics of transcription complex assembly (13,14). Many of these proposals are not mutually exclusive and it is likely that the physiologically relevant mechanism for control of expression of the 5S RNA gene families will include elements from several.

In the present work we have identified an RNA polymerase III enhancer that acts to increase transcription from classical RNA polymerase III genes with internal promoters. The protein binding site, or enhancer element, was found next to the *Xenopus* somatic 5S RNA genes and not within the oocyte 5S RNA gene repeats. These findings show that the expression of classical RNA polymerase III genes may be regulated by enhancers in a manner similar to that observed for genes transcribed by RNA polymerase II. In addition, the location of the enhancer next to the somatic 5S RNA genes suggests a novel (for RNA polymerase III), although well known (for RNA polymerase II), mechanism for the regulation of expression of the *Xenopus* 5S RNA gene families.

MATERIALS AND METHODS

Plasmids

The plasmids ps and po containing the somatic and oocyte 5S RNA genes have been described previously (15). Plasmids s-o-o, o-o-s and s-o-s contain an oocyte 5S RNA coding region with 5′, 3′ or both 5′ and 3′ somatic flanking sequences and were constructed from the plasmids ps and po using the *Eae*I site near the 5′-ends and the *Fok*I site near the 3′-ends of the genes. For these constructs, replacement of the flanking sequences was done such that the new 5S RNA gene-flanking sequence junctions were identical to those present in the wild-type counterparts.

Clones containing an EP3 binding site inserted near a tRNA gene were constructed as follows. The *Eco*RI fragment containing a *Xenopus* $tRNA^{Met}$ gene from the plasmid ptmet (15) was moved into the vector pGEM-7Zf(–) to generate two new plasmids, pG7tmet+ and pG7tmet–. Next, oligonucleotides containing the EP3 binding site (below) were inserted into the *Bam*HI sites of these plasmids. Four clones were selected corresponding to different positions and orientations of the EP3 binding site relative to the tRNA gene. Clone p3AL131 was then used as the parent for a deletion series designed to reduce the separation between the EP3 site and the tRNA gene. The locations and orientations of all EP3 binding sites were confirmed by DNA sequencing.

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Removal of EP3 from extracts

The EP3 binding site was formed by hybridizing the oligonucleotides GATCAGGCTTTTGCACTTTGCCCTTCTGAGCAGCAGGGGGCAGTCTCC and GATCGGAGACTGCCCCCTGCTCAGAAGGGCAAAGTGCAAAAGCCT. The resulting double-stranded oligonucleotides were ligated together, labeled with biotin and bound to streptavidin-coated paramagnetic beads as described previously (16). Beads with DNA containing the EP3 binding site were incubated with *Xenopus* oocyte extract for 45 min at 20°C and then removed together with bound EP3. Typically, 25 µl of beads containing ~1 µg of DNA was used to deplete EP3 from 200 µl of extract (~1 mg total protein). For the experiment shown in Figure 1A, a single depletion was carried out. When the EP3-depleted extract was subsequently used for *in vitro* transcription (e.g. Fig. 2B), two successive rounds of depletion were done.

DNase I footprinting

Protein–DNA complexes were isolated and footprinted as described previously (16). Briefly, protein–DNA complexes were affinity purified using a reversibly immobilized somatic 5S RNA gene, treated with DNase I and separated on an agarose gel. The DNA was recovered from the gel and equal amounts of DNA were re-run on a denaturing sequencing gel.

In vitro transcription reactions

The preparation of *Xenopus* oocyte extracts, *in vitro* transcription reactions and analysis of the transcription products were carried out as described previously (15). Typical reactions contained 20 µl of extract and 100 ng of plasmid DNA and were carried out for 3 h at 20°C. For the experiments of Figure 3 template plasmid DNAs included 5 ng with a tRNA gene, 5 ng with an oocyte 5S RNA gene (used as an internal control) and 90 ng of vector. The transcription products were isolated and separated on 10% polyacrylamide gels containing 8 M urea and quantitated using a Molecular Dynamics PhosphorImager.

Single round transcription

Limitation of transcription by RNA polymerase III to a single round using sarkosyl has been described previously (17). The reactions contained 325 ng of plasmid pG7tmet or p3AL106, 975 ng of vector and 260 µl of oocyte extract in a total volume of 325 µl. Aliquots of 50 µl were mixed with radiolabeled nucleoside triphosphates and sarkosyl at a final concentration of 0.5% and incubated for 2 min before processing.

RESULTS

EP3 binds next to the *Xenopus* somatic 5S RNA genes

Using a reversibly immobilized DNA in a method called a bead shift we identified a protein in *Xenopus* oocyte extracts that binds to the 3′ flanking region of the *Xenopus* somatic 5S RNA genes (16). We have named this protein EP3 (enhancer protein 3′ flank) and we have investigated both the binding specificity and the functional relevance of this protein for transcription by RNA polymerase III. The bead shift method takes advantage of a linear DNA containing a gene or site of interest (in this case a *Xenopus*

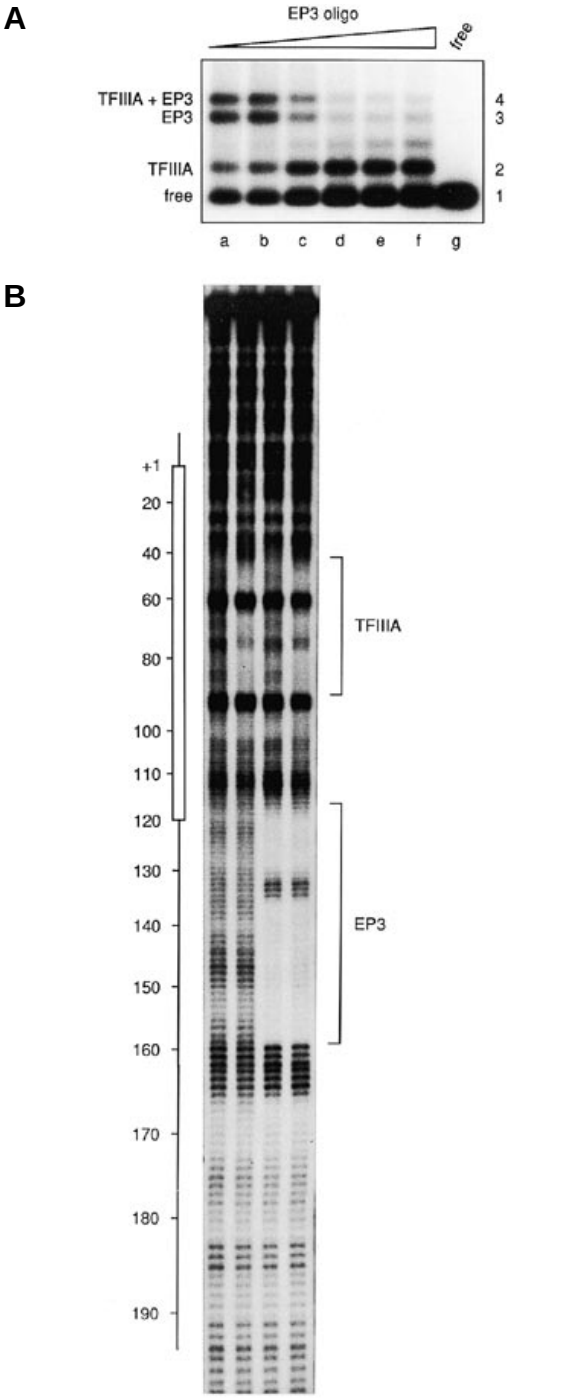

Figure 1. Interaction of EP3 with DNA. (**A**) Removal of EP3 protein from a *Xenopus* oocyte extract. For lanes b–f, 3-fold increasing amounts of oligonucleotides containing the EP3 binding site were attached to paramagnetic beads and used to deplete the EP3 protein from a *Xenopus* oocyte extract as described in Materials and Methods. The extent of depletion was assayed using the bead shift method (16) with a *Xenopus* somatic 5S RNA gene as probe. An autoradiograph of a 1% agarose band shift gel is shown. Lane a (mock depletion) illustrates the pattern of protein–DNA complexes containing TFIIIA and EP3 as indicated. (**B**) DNase I footprint of EP3 on the non-transcribed strand. The lanes from left to right correspond to complexes 1–4 as indicated in (A). Protein–DNA complexes containing TFIIIA and EP3 were affinity purified using a reversibly immobilized somatic 5S RNA gene, digested briefly with DNase I and separated on a band shift gel (16). The DNA was recovered from the band shift gel and equal amounts were re-run on a denaturing sequencing gel. The position of the somatic 5S RNA gene is indicated.

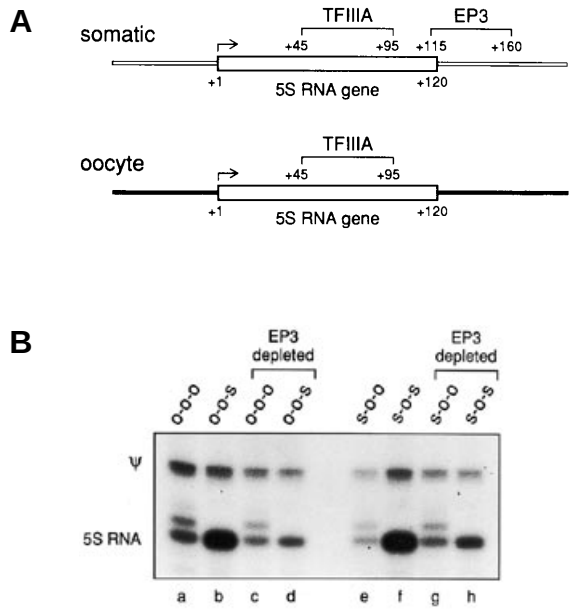

Figure 2. Enhancement of 5S RNA gene transcription by EP3. (**A**) Diagram of the *Xenopus* somatic and oocyte 5S RNA genes. The coding regions of the two genes are nearly identical, but the flanking sequences are different. (**B**) Enhanced transcription depends on EP3 protein. The transcription products from 5S RNA genes with and without adjacent EP3 binding sites are shown. DNA templates contained an oocyte 5S RNA coding region and either oocyte (o) or somatic (s) flanking sequences as indicated. For example, the clone o-o-s contains 5′ oocyte and 3′ somatic flanking sequences. For some reactions, as indicated, EP3 protein was depleted from the oocyte extract before use (Fig. 1A). Transcription was measured relative to a 5S RNA pseudogene (ψ) included as an internal control.

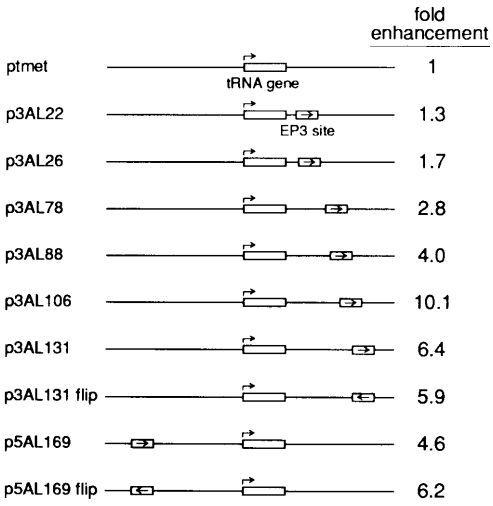

Figure 3. Enhancement of tRNA gene transcription by EP3 at different positions and orientations. Clones containing an EP3 binding site inserted at various locations near a tRNA gene are illustrated. The number of base pairs separating the tRNA gene from the EP3 binding site is indicated at the end of each clone name. Those clones designated flip contain inverted EP3 sites. The transcription rate of each clone was measured relative to an internal control and is reported as the fold enhancement over the transcription rate observed for the tRNA gene without a nearby EP3 site. The values reported represent the average of three independent experiments.

somatic 5S RNA gene) which has been radioactively labeled at one end and biotinylated at the other. The biotinylated end was used to attach the DNA to streptavidin-coated paramagnetic beads. Formation of protein–DNA complexes on the immobilized DNA by incubation in *Xenopus* oocyte extract was followed by affinity purification, concentration and gentle release of the protein–DNA complexes from the beads through digestion of the DNA at a unique restriction site. The released and soluble complexes were then analyzed using band shift agarose gel electrophoresis and DNase I footprinting.

Using this approach we previously observed the formation of several complexes containing the transcription factors TFIIIA and TFIIIC as well as EP3 on somatic 5S RNA genes incubated in *Xenopus* oocyte extracts (16,18). An example of the gel shift pattern of protein–DNA complexes containing EP3 and TFIIIA is shown in Figure 1 (lane a). The gel mobility shift caused by the binding of EP3 is considerably larger than that of TFIIIA and suggests that EP3 is a significantly larger protein, or protein complex, than TFIIIA.

To more precisely examine the EP3 binding site, we determined the DNase I footprint of each of the protein–DNA complexes shown in Figure 1A (lane a). The footprints of these four complexes are illustrated in Figure 1B. These results show that EP3 protects ~45 bp starting near the 3′-end of the transcribed region of the somatic 5S RNA gene at +115 and extending downstream to +160 (Fig. 2A). Moderately enhanced cleavages were observed on the non-transcribed strand roughly in the middle of the binding site. The EP3 footprint on the transcribed strand covered the same large region, but cleavages in the middle of the site were not observed (data not shown).

The binding specificity of EP3 was tested previously by competition with various DNAs (16). Somatic 5S RNA genes were effective competitors, but other 5S RNA genes with different flanking sequences were not. To extend these results, a synthetic double-stranded oligonucleotide containing the 45 bp EP3 binding site identified by DNase I footprinting was used as a competitor. The addition of this oligonucleotide blocked formation of the EP3 complex (data not shown). In a related experiment, immobilized oligonucleotides containing the EP3 binding site were used to physically remove EP3 from an oocyte extract (Fig. 1A). Double-stranded oligonucleotides containing the EP3 binding site were ligated together, labeled with biotin and bound to streptavidin-coated paramagnetic beads. Incubation of the beads together with an oocyte extract allowed binding of the EP3 protein. The extract was separated from the beads and the EP3 remaining in the extract was then assayed using the bead shift method. As shown in Figure 1A, as the concentration of immobilized oligonucleotides was increased, the amount of EP3 remaining in the extract was greatly reduced. These data, in conjunction with published results, demonstrate that EP3 is a site-specific DNA binding protein with a naturally occurring binding site adjacent to the 3′-end of the *Xenopus* somatic 5S RNA gene (Fig. 2A). Only the somatic 5S RNA genes, but not the oocyte 5S RNA genes, contain these naturally occurring EP3 binding sites in their 3′ flanking regions.

EP3 functions as a transcriptional enhancer

To investigate a functional role for EP3 in RNA polymerase III transcription, we placed the EP3 binding site next to an oocyte 5S

RNA gene where it is not normally found. The plasmid, o-o-s, contains an oocyte 5′ flanking sequence and an oocyte 5S RNA coding region, but has a somatic 3′ flanking sequence with an EP3 binding site. We compared the rate of transcription of this plasmid (o-o-s) with that of the normal oocyte 5S RNA gene (o-o-o). Figure 2B shows that the somatic 3′ flanking sequence increased the relative transcription of the adjacent oocyte 5S RNA gene ~5-fold (compare lanes a and b). However, when EP3 protein was first removed from the extract using immobilized oligonucleotides, the genes were transcribed at nearly the same rates (compare lanes c and d). Similar results were obtained when the relative rates of transcription of the plasmids s-o-s and s-o-o were compared (Fig. 2B, lanes e–h). Both the EP3 binding site and the EP3 protein were required for the observed ~5-fold increase in relative transcription rates.

We next tested the ability of EP3 to enhance transcription from a tRNA reporter gene. Transcription of tRNA genes does not require the 5S RNA gene-specific factor TFIIIA but otherwise uses the same transcription factors, TFIIIB and TFIIIC, and RNA polymerase III. These experiments also utilized the 45 bp EP3 binding site identified through DNase I footprinting instead of the larger somatic 5S RNA gene 3′ flanking sequences. An oligonucleotide containing an EP3 binding site was inserted 131 bp downstream from the end of a tRNA gene and the effect on transcription was examined. The relative transcription rate was increased ~5-fold by insertion of the EP3 binding site (data not shown). As before, removal of EP3 protein from the extract abolished the enhanced transcription. These results show that sequences outside the 45 bp EP3 binding site defined by DNase I footprinting are not required for enhanced transcription. Finding that EP3 enhanced transcription of both 5S and tRNA genes shows that EP3 does not act through TFIIIA but instead acts through components common to both tRNA and 5S RNA gene transcription.

To determine if EP3 performs like an RNA polymerase II enhancer, i.e. acts at multiple locations and in different orientations, we constructed clones containing EP3 binding sites located at various positions and orientations relative to a tRNA reporter gene. The relative transcription rates of these clones were measured and the results are shown in Figure 3. We found enhanced transcription of a tRNA gene containing an EP3 binding site in either orientation, both upstream and downstream of the gene. For each of the clones examined, we observed an increase in the relative transcription rate; however, the magnitude of the effect was rather modest when the EP3 binding site was close to the tRNA gene. The largest effect observed (~10-fold) was for clone p3AL106 with a single EP3 binding site located 106 bp downstream from the 3′-end of the tRNA gene. It remains to be determined if multiple EP3 binding sites, either positioned in tandem or, for example, one 5′ and another 3′, would provide increased effects.

EP3 increases the rate of transcription complex formation

Transcription by RNA polymerase III can be divided into two basic steps: first, the initial formation of a stable transcription complex and, second, repeated initiation of the polymerase during subsequent rounds of transcription (19–22). The enhanced transcription mediated by EP3 could result from stimulation at either step. To identify the step at which EP3 functions, we examined its effect on the rate of transcription complex formation. This was accomplished by comparing the rate of transcription complex formation on tRNA reporter genes with and without an EP3 binding site. tRNA genes were incubated for increasing times with oocyte extract to permit transcription complex formation. In these reactions, the tRNA gene was the only RNA polymerase III template present. At 15 min intervals, samples were withdrawn from each reaction and transcription complex formation was assayed using *in vitro* transcription in the presence of sarkosyl. Because sarkosyl blocks re-initiation by RNA polymerase III, it limits transcription to a single round. Therefore, each transcript corresponds to an individual transcription complex (17). Figure 4 illustrates the rate of formation of active transcription complexes on a tRNA gene with and without a downstream EP3 binding site. These results demonstrate that transcription complexes form significantly faster on the tRNA gene with a nearby EP3 binding site. To confirm these results we carried out template exclusion studies (13,19). Using template exclusion we also observed a similar EP3-dependent increase in the rate of complex formation for both tRNA and 5S RNA genes (data not shown). The results from both single round transcription and template exclusion show that EP3 acts to increase the rate of transcription complex formation.

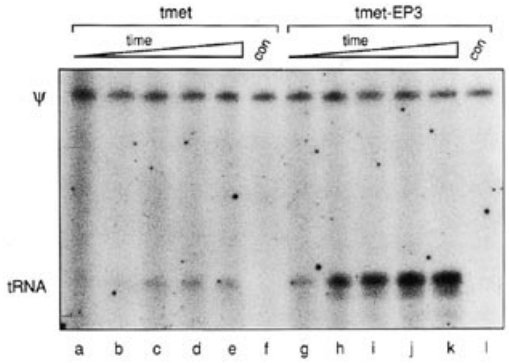

Figure 4. EP3 increases the rate of transcription complex formation. The autoradiograph shows the transcripts produced by a single round of transcription from complexes formed for increasing periods of time. Template DNAs were pG7tmet (tmet) or p3AL106 (tmet-EP3). Complex formation was assayed every 15 min from 15 (lanes a and g) to 75 (lanes e–f and k–l) min. For the control reactions shown in lanes f and l, radiolabeled nucleoside triphosphates were added 2 min after the addition of sarkosyl. The absence of transcripts in these lanes shows that re-initiation did not occur. The 5S RNA pseudogene transcripts (ψ) were added after stopping the reactions to control for sample losses during handling.

DISCUSSION

EP3 acts as an enhancer to stimulate transcription from RNA polymerase III genes with internal promoters. The EP3 binding site, or enhancer element, is naturally present adjacent to *Xenopus* somatic 5S RNA genes, but is not found in the *Xenopus* oocyte 5S RNA gene repeats. The results presented above demonstrate an interaction of EP3 protein with a 45 bp binding site and show that this interaction results in an increase (~5- to 10-fold) in the relative rates of transcription of nearby 5S RNA or tRNA reporter genes. Finding that the EP3 enhancer acts by increasing the rate of assembly of transcription complexes on nearby genes (Fig. 4) supports our earlier view that kinetic differences in transcription complex assembly provides a key distinguishing feature between these two genes (13).

Both the large size of the DNase I footprint of EP3 and the magnitude of the gel mobility shift (Fig. 1) suggest that EP3 is a large protein or protein complex. EP3 does not appear to be an essential transcription factor since extracts depleted of EP3 continue to support normal levels of transcription from tRNA and 5S RNA genes which do not naturally contain adjacent EP3 binding sites. In addition, transcription of genes with nearby EP3 binding sites was reduced but not eliminated by either depletion of EP3 protein from the extract or removal of the EP3 binding sites.

EP3 protein in conjunction with its binding site acts to enhance transcription from both tRNA and 5S RNA genes in a manner similar to that observed for RNA polymerase II enhancers. In particular, the orientational and positional flexibility characteristic of RNA polymerase II enhancers was also observed with the EP3 binding site (Fig. 3). The fact that EP3 enhances transcription from both tRNA and 5S RNA genes to similar levels suggests that the 5S RNA gene-specific factor TFIIIA is not involved. In addition, TFIIIA and EP3 appear to bind to the *Xenopus* somatic 5S RNA gene repeat independently (Fig. 1). The fact that chromatin assembly does not readily occur in these extracts under the conditions used for these assays suggests that inhibition or modulation of chromatin formation or structure is not involved in the observed EP3-dependent increase in the rate of transcription complex formation. On the other hand, it is likely that EP3 protein interacts with one of the general RNA polymerase III transcription factors, probably TFIIIC. The limiting component for RNA polymerase III transcription in *Xenopus* oocyte extracts is TFIIIC (15) and the assay for relative transcription rates using *in vitro* transcription in these extracts is essentially an assay for the rates of interaction of the different genes with TFIIIC (13). A simple mechanism which would account for the observed EP3-dependent increase in the rate of transcription complex assembly is a direct interaction between EP3 and TFIIIC which would recruit TFIIIC to nearby genes.

It is somewhat remarkable that a gene-specific enhancer has been discovered with the ability to distinguish the *Xenopus* somatic from the oocyte 5S RNA genes considering the intense efforts over more than two decades which have been focused on understanding the molecular details for the differential expression of these gene families during early *Xenopus* development. It is likely that the EP3 enhancer went previously undetected for two reasons. First, it is the expression level of the oocyte and not the somatic 5S RNA genes which changes during early development. Both somatic and oocyte 5S RNA genes are expressed during oogenesis but during embryogenesis the oocyte 5S RNA genes are irreversibly repressed while the somatic 5S RNA genes continue to be transcribed. For this reason, most efforts have focused on mechanisms of oocyte 5S RNA gene inactivation, currently thought to involve an association of histone H1 with the AT-rich flanks of the oocyte 5S RNA gene repeats (10–12). Second, many of the assays which have been used have been generally insensitive to both the binding and enhancer action of EP3. A clear view of the interaction of EP3 protein with its binding site was made possible by the development of the bead shift technique for studying protein–DNA interactions (16). In addition, assays to reveal the enhancer action of EP3 must be sensitive to the step at which EP3 functions, the initial rate of assembly of the transcription complex. *In vitro* transcription assays which include an internal control and measure relative transcription rates are able to detect differences in rates of transcription complex assembly, but transcription assays using only a single template are usually not able to do so (13). However, Figure 4 shows an assay of single round transcription, using only a single template, which directly measures the rate of transcription complex assembly and illustrates the action of the EP3 enhancer.

It appears likely that EP3 plays a key role in the differential expression of the *Xenopus* 5S RNA gene families since the EP3 enhancer directly discriminates between the somatic and oocyte 5S RNA genes. On the other hand, the maximal effect of the EP3 enhancer that we have observed (~10-fold) is not large enough, by itself, to account for the observed differences in levels of gene expression (>100-fold) and other mechanisms must therefore be involved. One simple notion is that the EP3 enhancer, acting as a positive or stimulatory element for the somatic 5S RNA genes, increases the relative utilization of transcription factors (which may be limiting) on these genes and consequently decreases the formation of inactive or repressive chromatin structures. EP3 could also be involved in selectively directing chromatin remodeling machinery to these genes. Understanding the physiological significance of the EP3 enhancer remains a challenge for the future.

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