Regulation of protamine gene expression in an in vitro homologous system*

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An in vitro transcription system from the trout testis nuclei was developed to study trout protamine gene expression. The protamine promoter contains, among others, two regulatory elements: 1) a CAMP-responsive element or CRE element (TGACGTCA) which is present in position 5' to TATA box, and 2) GC box (CCGCCC) which is present in position 3' to TATA box. The removal of the CRE-binding protein by titration (by the addition of appropriate oligonucleotides to the incubation mixture) resulted in a decrease in transcription of the protamine gene. These results were confirmed by experiments in which the pure CRE-binding factor (TPBP1) was used, as well as by those where a stimulatory effect of CAMP on protamine promoter transcription was observed. On the other hand, addition of oligonucleotides containing the GC-box sequence enhanced the protamine gene transcription indicating that the protein (Sp1 like) which binds to this sequence acts as a repressor of protamine gene expression. These results confirm the previously proposed model which suggested that the GC box played a role in negative regulation of the protamine gene expression. Involvement of some other factors in this process was also discussed.

Packaging of genetic material is solved in the nature in a variety of ways. In somatic cells DNA is wrapped around histone octamers to form the nucleosomal structure of chromatin. In rainbow trout (Salmo gairdnerii), during sperm development there is a transition from histones of somatic cells to protamines (in mature sperm cells) as a material for packaging of DNA [1]. Protamines are small, strongly basic nuclear proteins. Their expression occurs in the male germ cells during the late stages of spermatogenesis. Expressed protamines migrate to the nucleus where they replace the somatic histones in the chromatin of spermatid cells, which results in complete restructuring of sperm chromatin. It seems evident, therefore, that the set of protamine genes has to be under very precise control, so that the protamine gene expression is permitted only during sperm development.

Analysis of the trout protamine promoter by an in vitro transcription system from HeLa cells

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Abbreviations: ACB, affinity column buffer; CRE, CAMP-responsive element; CREB, CAMP-responsive element-binding protein; CREM, CAMP-responsive element modulator; ICER, inducible CAMP early repressor; DTT, dithiothreitol; HSV, herpes simplex virus; PKA, CAMP-dependent protein kinase; nt, nucleotide; PMSF, phenylmethylsulfonyl fluoride; TPBP1, trout protamine-gene binding protein.
has evidenced that the TATA box is an element required for transcription [2]. In these studies attempts at creating competition between HSV thymidine kinase and trout promoters as well as transfection experiments, using COS-1 cells as a recipient [3] have revealed that factors bound to the GC box might be involved in negative regulation of the trout protamine gene. Presumably a factor similar to that of mammals is present in trout where it represses expression of the protamine gene in somatic cells.

In order to study the regulatory signals that control this gene in a homologous system, we developed an in vitro transcription system from trout testis. The transcriptionally active trout testis extracts were prepared by the methods based on that developed for rat liver, brain and spleen by Gorski et al. [4] and for mouse testis by Bunick et al. [5]. To simplify the assay we used hybrid plasmids containing the protamine promoter linked to a synthetic 400 bp or 200 bp DNA fragment that lacks cytidine residues on the transcribed strands, i.e. generates a transcript with no guanosine residues [6]. This system allowed to study the role of the cAMP-responsive element (CRE), the GC box as well as of other factors involved in regulation of the protamine gene expression.

METHODS

Plasmid construction

The p(C2AT)19 plasmid was a generous gift from M. Sawadogo & R.G. Roeder [6]. The pP9G-free plasmid was constructed from pP9cat plasmid [3] containing the protamine promoter and from the p(C2AT)19 plasmid by replacing the cat gene (from pP9cat) with G-free cassette (from pC2AT19). The pP9G-free plasmid contained the protamine promoter linked to a synthetic 400 bp DNA fragment that lacks cytidine residues on the transcribed strand, i.e. generated a transcript with no guanosine residues [6]. Plasmid p8-200 was a deletion mutant of pP9G-free plasmid. In the p8-200 plasmid about a half of the G-free cassette was removed.

All manipulations with DNA followed the procedures outlined by Sambrook et al. [7].

In vitro transcription assay

The in vitro transcription mixture based on papers by Gorski et al. [4] and Bunick et al. [5] was prepared from trout testis. This system utilizes ‘G-free’ template that was developed by Sawadogo & Roeder [6].

Trout testis (12-15 g) was homogenized in a glass-Teflon homogenizer, in a buffer containing 10 mM Hepes, pH 7.6, 0.15 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 0.5 mM DTT, 2.2 M sucrose, 1% skimmed dry milk. The homogenate was poured into a 100 ml graduated cylinder and the volume was adjusted to 85 ml with the homogenization buffer then loaded on the top of a 10 ml cushion composed of 2 M sucrose, 10% glycerol, 10 mM Hepes, pH 7.6, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA and 0.5 mM DTT, 0.5 mM PMSF, and pepstatin, leupeptin and aprotonin (10 g/ml each), placed in a SW 27 Beckman centrifuge tube. The tubes were spun at 24000 r.p.m. for 60 min at 4°C. The nuclear pellet was obtained after removal of the lipid layer and elimination of the liquid by aspiration. The nuclei were resuspended in 10 ml of lysis buffer containing 10 mM Hepes, pH 7.6, 10 mM KCl, 0.1 mM EDTA, 10% glycerol, 3 mM MgCl2, 1 mM DTT, 0.1 mM PMSF and pepstatin, leupeptin, aprotonin (as above) in a Dounce glass homogenizer with an A pestle. The suspension was diluted with lysis buffer to a final DNA concentration of 0.5 mg/ml. The concentration of KCl was adjusted to 0.55 M and the mixture was left on ice for 30 min with occasional stirring and then was spun for 30 min at 35000 r.p.m. in a 60Ti rotor. The supernatant was removed and 0.3 g solid ammonium sulphate per 1 ml of supernatant was added. After dissolution of the salt, the mixture was left on ice for 20-60 min and spun at 35000 r.p.m. in a 60Ti rotor for 20 min. The pellet was resuspended in the dialysis buffer (25 mM Hepes, pH 7.6, 0.1 mM EDTA, 40 mM KCl, 10% glycerol, 1 mM DTT) and dialysed 2 x 2 h in the cold room, each time against 100 vol. of dialysis buffer. The solution was spun in an Eppendorf centrifuge in the cold room and frozen immediately in liquid nitrogen.

The transcriptional activity of this extract was tested using plasmid constructs with protamine promoter attached to the G-free cassette [6]. In this system (without GTP in the incuba-
tion mixture) any long transcript (approx. 400 nt) can originate only from this artificial cassette.

The *in vitro* transcription system from HeLa cells was prepared as described by Manley et al. [8]. A standard 20 μl transcription incubation mixture consisted of 2 μg circular DNA template, 3–5 μg nuclear protein extract per 1 ml of a buffer containing 25 mM Hepes, pH 7.6, 12.5% glycerol, 84 mM KCl, 15 mM MgCl₂, 1.5 mM each of ATP, and UTP, and 35 μM CTP; 10 μCi [α-32P]CTP (NEN, 800 Ci/mmole), 0.1 mM 5′-O-methyl GTP (Pharmacia), 30 units RNAguard (Pharmacia). After 45 min of incubation at 20°C the reaction was terminated by the addition of 280 μl of stop buffer (20 mM Tris/HCl, pH 7.5, 0.25 mM NaCl, 1% SDS, 5 mM EDTA, 20 μg tRNA and 40 μg proteinase K), and the reaction mixture was incubated for another 30 min at 37°C, followed by extraction with phenol/chloroform; then RNA was precipitated with ethanol. RNA was analysed by 5% polyacrylamide gel electrophoresis, as described elsewhere [2]. Densities of bands on autoradiograms were calculated with Pharmacia-LKB scanner.

Preparation of the CRE binding factor

Affinity purification of the CRE binding factor from trout testis was performed as described by Cannon [9]. Samples of frozen tissue (−70°C) of trout testis were powdered and allowed to thaw in 10 to 20 ml of cold buffer A (0.3 M sucrose, 10 mM Hepes, pH 7.5, 10 mM iminodiacetic acid, 5 mM NaN₃, 1 mM EDTA, 1 mM EGTA, 0.75 mM spermine, 0.15 mM spermidine, 0.1%, w/v, NP40) and a protease inhibitor cocktail, consisting of aprotinin, leupeptin and pepstatin (Boehringer Mannheim) (1 μg/ml of each), and 0.5 mM benzamidine (Sigma). Samples were homogenized in a Waring blender (2 × 30 s bursts). This homogenate was filtered through two layers of cheesecloth, and nuclei were pelleted by centrifugation at 5000 × g for 10–20 min at 4°C. Nuclei were resuspended in buffer A supplemented with 10 mM benzamidine and 0.5 mM PMSF (5 ml/g of starting material). The cushion of 0.3 M sucrose in buffer A was used, and nuclei pelleted as above, were resuspended in buffer B (25 mM Hepes, pH 7.6, 10% glycerol, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10 mM iminodiacetic acid, supplemented with 5 mM NaF, 5 mM benzamidine and 0.5 mM PMSF), supplemented with 350 mM NaCl (3 ml of buffer per 1 g of starting material). Extraction was carried out at 4°C with constant agitation. Nuclei were then removed by centrifugation (10000 × g for 15 min at 4°C) and the crude salt extract was precipitated with 0.25 g powdered ammonium sulphate per 1 ml, i.e. at a concentration corresponding to about 40% saturation at 4°C. The ammonium sulphate precipitate was resuspended in 0.3 to 0.6 ml/g of buffer B and dialysed twice (each time for 4 h) against a 20- to 50-fold excess of 150 mM ammonium sulphate in buffer B (plus 5 mM benzamidine, 5 mM NaF, 0.5 mM PMSF). Following dialysis, all samples were clarified by centrifugation (10000 × g for 15 min).

Chromatography on heparin-Sepharose

The post-dialysis extract was loaded onto a 40 ml heparin-Sepharose (Pharmacia) column pre-equilibrated with 200 mM ammonium sulphate in buffer B. The column was washed with two bed volumes of starting buffer, followed by elution, three times, with 100 ml each, with buffer B, containing either 0.3 M, 0.6 M or 1.5 M KCl, respectively. Each time buffer B contained 0.5 mM PMSF, 2 mM benzamidine and 5 mM NaF.

Affinity chromatography

The matrix used for affinity chromatography consisted of multimers of the double-stranded binding site oligonucleotide (CTACTATGACCTCACATAATT), attached to Sepharose 4B. The method used to prepare the affinity matrix was based on that described by Kadowa & Tjian [10]. The 0.6 M KCl eluate from the heparin column was either diluted or dialysed against 0.2 M KCl in the affinity column buffer (ACB), which consisted of buffer B (25 mM Hepes, pH 7.6, 10% glycerol, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10 mM iminodiacetic acid), supplemented with 5 mM NaF, 5 mM benzamidine and 0.5 mM PMSF, 20% (v/v) glycerol and 0.1% (w/v) Genapol (Calbiochem). Prior to loading, 10 mg sonicated calf thymus DNA, 2 mg yeast tRNA, 400 μg pd(N)₅ and MgCl₂ (to final concentration of 2 mM), were added to the sample. The column was pre-equilibrated with 0.2 M KCl in ACB buffer and after loading was washed with starting buffer until the absorption of the effluent at 280
nm returned close to the baseline. The column was eluted with 1.5 M KCl in ACB buffer and 1.4 ml fractions were collected. The fractions were assayed by gel mobility shift as described elsewhere [11, 12], and those containing the bulk of the activity were combined. This step was repeated (after dilution of the pooled fractions of the first round affinity material to 0.2 M KCl [9]).

RESULTS AND DISCUSSION

The pPSG-free, pP8-200 constructs, (Fig. 2) were used for studying regulation of the protamine gene (Fig. 1) in vitro. The protamine promoter in these constructs has a number of control elements such as TATA box (Fig. 1, pos 384–390), intact natural ‘cap’ site and contains a GC box (Fig. 1, pos. 395–403) located between the TATA sequence and the ‘cap’ site. The CRE element (Fig. 1, pos. 284–291) is located in position 5’ to the TATA sequence. The pPSG-free plasmid has a 400 bp G-free cassette [6] linked to the protamine promoter: The pP8-200 has a shortened (about 200 bp) G-free cassette.

Using the competition type of experiments we tested in the homologous in vitro transcription system two protamine promoter elements: the GC box (CCGCC) and the CRE element (TGAAGCT). Figure 3 shows the effect of synthetic, double-stranded DNA fragments containing the GC box (AATTCGCCCAGCAGGCACTGCAGGGTTACATTAATGCTACATGGGAT) and the CRE element (ACTGCGCTGCCTATGACCTACATAATTCGAG). As it can be seen (Fig. 3, lane 1, in comparison with the control, lane 3), the removal by titration, of some factor(s) binding to the GC box has an enhancing effect on transcription of protamine promoter. Repression of the protamine gene expression by the GC box binding factor seems, however, to be more complex. Inspection of the

![Fig. 1. Nucleotide sequence of the (BglIII-BamHI) restriction fragment of DNA, containing the protamine gene (after States et al. [33]).](image_url)

The TATA sequence is boxed; the CRE element, GC box and polyadenylation signals AATAAA..AATG are underlined. Arrows indicate the ‘cap’ sites of the protamine gene [33, 34].
protamine gene sequence (Fig. 1) showed that two other GC boxes are present in the coding region downstream from TATA box, in addition to that between the TATA box and the 'cap' site (Fig. 1, pos. 395–403, 478–483, 562–567). We noted that the distance between the first and the second box is equal to the distance between the second and the third one and is close to 80 bp. As it has been discussed elsewhere [3], this spacing could imply that all three GC boxes might be located in a domain occupied by a single nucleosome and thus the factors which bind to these elements could interact with each other. This hypothesis is in good agreement with the experiments done by Su et al. [13], which had directly visualized by electron microscopy the protein-protein interactions of Sp1. Thus, the repression of the protamine gene expression would be more stringent in the natural gene than that observed for the single box in the template used in the experiments.

On the other hand, the addition of the oligonucleotides containing the CRE element to the transcription mixture (which removes the CRE binding factor) (Fig. 3, lane 2, and Fig. 4, lane 2) had a dramatic inhibitory effect on the protamine gene expression. These data are in good agreement with the results obtained with the purified CRE-binding factor (TPBP1) from trout testis (Fig. 4, lane 2); as it can be seen the addition of this factor to the incubation mixture enhanced the transcription of the protamine gene. Figure 5 shows the effect of cAMP and of the H7 kinase inhibitor (Seikagaku Kogyo Co., Ltd., Tokyo) on transcription of the protamine gene in the trout testis transcription system supplemented with purified TPBP1. As expected, cAMP stimulated the protamine gene transcription. This is consistent with the observation that most of the genes induced by cAMP share a conserved DNA sequence that constitutes a cAMP-responsive element (CRE) (for review, see [14, 15]). Mammalian cells contain multiple proteins that bind to this sequence (16, 17) and references therein. One of these proteins, CREB (cAMP-responsive element binding protein) is a substrate for cAMP-dependent protein kinase (PKA) in vitro [18, 19]. Other proteins of the CREB/ATF family in mammalian cells are CREMs [20] and ICER [21]. Foulkes and co-workers [20] have demonstrated that while CREB is expressed uniformly in several cell types, CREM shows cell-specific expression. While the CREM factor is a developmentally regulated activator, ICER is a repressor of the cAMP inducible genes and is generated from an alternative intronic promoter of the CREM gene. Foulkes et al. [22, 23] have found a developmental switch of CREM during spermatogenesis. The presence of additional phosphorylation sites in both mammalian CREB and trout TPBP1 proteins (i.e., kinase
Fig. 3. Electrophoretic analysis (5% polyacrylamide gel) of the effect of the oligonucleotides containing the GC box (AATTCGGGCCCAGGAATT) and the CRE element sequence (ACTGCCAGCTAC-TATGACGTCACATAATTCA) on transcription of protamine promoter.

Transcription in the presence of: lane 1, 1 µg GC box oligonucleotides; lane 2, 1 µg of CRE oligonucleotides; lane 3, control: transcription of pP8-200 template (2 µg). Densities of the bands in lanes 1 and 2 were calculated (from the scan of the autoradiograms done with a Pharmacia-LKB scanner) as a percentage of the control band in lane 3.

Cand casein kinase II, as well as the occurrence of more than one of the isoforms of both mammalian CREB(M) and trout TPBP1 proteins suggest that the activity of these factors may be regulated via a cellular mechanism other than that related to cAMP [9, 17, 19, 24–29]. This observation suggests that perhaps the TPBP1 protein could be a target for more than one cellular protein kinase. The TPBP1 protein could serve as a molecular “microprocessor” integrating the signalling pathways at the level of gene transcription. In order to assess the involvement of other protein kinases in the regulation of this gene, an inhibitor of protein kinase C (H7 inhibitor) was applied. Addition of this inhibitor reduces the transcription of this gene (Fig. 5, lane 3). This would indicate that also a cAMP-independent phosphorylation process may be involved in the regulation of the protamine gene expression. These results are in good agreement with the earlier data on phosphorylation of TPBP1 [9], though, of course, the observed effect of increased transcription caused by phosphorylation, may also be due to phosphorylation of some factors other than TPBP1. It is noteworthy that phosphorylation of the TPBP1 factor has little (if any) effect on its DNA-binding properties. Since it has also been found [9] that larger amounts of the TPBP1 protein result in the appearance of a number of secondary regions of protection besides the major CRE sequence (Fig. 1, pos. 252–271, 331–347, 350–368), it was of interest to correlate this observation with the TPBP1 function in the transcription assay. Fig. 6 shows the effect of increasing amounts of the TPBP1 factor on transcription of the protamine gene; as one can see the addition of 0.04 µg of the TPBP1 factor to the incubation mixture stimulated the transcription, while doubling the amount of this factor had an inhibitory effect. That is in

Fig. 4. Electrophoretic analysis (5% polyacrylamide gel) of the effects of oligonucleotides containing the CRE element sequence (ACTGCCAGCTACTATGACGTCACATAATTCA), as well as of CRE binding protein (purified from trout testis) on the transcription of the protamine promoter.

Lane 1, transcription products from the pPGK-free plasmid template (2 µg); lane 2, as in lane 1, except that the transcription mixture was supplemented with the oligonucleotides (1 µg) containing CRE sequence; lane 3, 1 µl of purified CRE binding protein (TPBP1) (0.04 mg/ml).
The involvement of the TPBP1 factor in regulation of transcription of the protamine gene was confirmed in our next experiment. Figure 7 presents the data concerning removal of the endogenous CRE binding factor by titration with appropriate oligonucleotides. As it can be seen the inhibitory effect of the oligonucleotides could be abolished by the purified TPBP1 factor, though full restoration of the activity would require a larger amount of the factor.

Summing up, transcription of the protamine gene is repressed by the factor(s) that binds to three GC boxes located in position 3' to the TATA box. Spacing between these GC boxes allows for the hypothesis that these boxes might be located in a domain occupied by a single nucleosome and that the factors which bind to these sequences could interact with each other and repress the expression of the protamine gene. An additional checkpoint is created by the CRE binding factor which also could reduce the expression of the protamine gene by forming alternative complexes. When intracellular concentration of the GC binding factor(s) is increased (e.g. by dilution resulting from a number of fast mitotic divisions during

Fig. 5. Electrophoresis analysis (5% polyacrylamide gel) of the effect of purified trout testis CRE-sequence binding factor on transcription of protamine promoter in the trout testis transcription system.

Lane 1, transcription products derived from the pP9G-free template (2 μg) in the assay supplemented with purified factor TPBP1 (0.04 μg); lane 2, as in lane 1, except that the transcription mixture was supplemented with cAMP (4 mM); lane 3, as in lane 1, except that the transcription mixture was supplemented with the H7 inhibitor (0.05 mM).

Good agreement with the data discussed above [9], as well as with that obtained in the DNA footprint analysis experiments in the gel mobility shift assay, suggesting the existence of two TPBP1-DNA complexes [9]. This could be interpreted as nature's attempt to create an additional checkpoint in the cAMP regulation in trout testis. Formation of alternative complexes by the TPBP1 factor(s) could repress the transcription by removing this factor from the compartments of active transcription and/or by blocking some other factors engaged in the initiation complex (see above). This interpretation is also consistent with the results reported in several papers [17, 20, 21, 28, 30, 31] in which the authors have demonstrated in mammals a complex array of positive and negative isoforms of the transcription factor and the coactivator that regulates cAMP-induced transcription. Results similar to those discussed above were also obtained in the case of Krüppel gene product [32]. The latter mediates transcription activation at low concentrations and transcription repression at high concentrations when the tendency for homodimer formation is increased [31, 32].

Fig. 6. Electrophoresis analysis (5% polyacrylamide gel) of the effect of purified trout testis CRE-sequence binding factor (TPBP1) on transcription of protamine promoter in the trout testis transcription system.

Lane 1, transcription products derived from the pP9G-free template (2 μg); lane 2, as in lane 1, except that the transcription mixture was supplemented with purified factor TPBP1 (0.04 μg); lane 3, as in lane 1, except that the transcription mixture was supplemented with the purified factor TPBP1 (0.08 μg).
spermatogenesis), the transcription of the pro- 
tamine gene is turned on by phosphorylation 
of the TPBP1 transcription factor by cAMP-de- 
dependent protein kinase A. Other phosphoryla- 
tion/dephosphorylation processes (mediated 
by protein kinases such as PKC) could also be 
involved in the regulation of protamine gene 
expression.

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