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The effect of changing the distance between the TATA-box and Cap site by up to three base pairs on the selection of the transcriptional start site of a cloned eukaryotic gene *in vitro* and *in vivo*

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ABSTRACT

We have studied how small changes in the distance between the TATA-box and cap site affect transcription of a eukaryotic gene in vitro and in vivo. The trout protamine gene TPG-3 [Gregory et al. (1982) Nucl. Acids Res. 10, 7581-7592] is a good model for such a study as it has (i) a consensus TATA-box 32 base pairs (bp) upstream from an A-residue which is the natural cap site (designated +1) (ii) two further A-residues at -5 and +5, providing alternative transcriptional start sites which are in significantly different sequence environments and (iii) a unique AvaII restriction site immediately downstream from the TATA-box which is ideal for the insertion or deletion of up to 3bp. Transcripts of the wild type and mutant genes were generated in vitro using a HeLa whole cell extract or 'in vivo' by transient expression following their transfection into HeLa cells. These 'spacer' mutations did not affect the efficiency of transcription of the gene in vitro but they did affect the selection of transcriptional start site both in vitro and 'in vivo'. Analysis of 5'-ends by S_1-mapping and primer extension showed that the A-residue(s) selected are those which, by insertion or deletion, come to lie on the same face of the DNA double helix as the TATA-box, although the DNA sequence in the immediate vicinity of the potential start sites influences their utilisation. Comparison of the TPG-3 wild type transcripts in these experimental systems with natural mRNA suggests that cap site selection is more stringent in the developing trout testis.

INTRODUCTION

Most eukaryotic genes transcribed by RNA polymerase II possess a sequence found about 30 base pairs (bp) upstream from the transcriptional start (or 'cap') site which is known as the TATA-box (consensus sequence TATA$^{A}_{T}$TA$^{A}_{T}$ [1]). It is thought to be required for accurate initiation of transcription at the cap site in vitro and in vivo [1,2]. Deletion of the TATA-box results in the use of new sites for initiation [see e.g. refs 3 and 4] which is usually accompanied by a decrease in the efficiency of transcription [4-6] although this is not invariably the case [7]. Partial deletions [8] and point mutations [9] within the TATA motif have revealed that certain mutations decrease the efficiency of transcripion more drastically than others and may shift the site of initiation in vitro.

Studies on transcription of the adenovirus-2 (Ad-2) major late and chick conalbumin genes in vitro [8] and the rabbit β-globin gene in vivo [10] have shown that deletion of the cap site region decreases the level of transcription but initiation still occurs about 30 base pairs downstream from the TATA-box. Point mutation of the cap site of the Ad-2 major late gene lowered the efficiency of its transcription in vitro, accompanied by a shift in the site of initiation [11]. However, neither the site of initiation nor the efficiency of transcription in vitro changed for a cap site point mutation in the silk fibroin gene of Bombyx mori [9] although multiple base substitutions in the cap site region abolished transcription in vivo [12].

A double point mutation, one at the cap site and one in the TATA-box of the Ad-2 major late promoter, abolished transcription in vitro whereas the equivalent single point mutations only reduced the level of transcription [11]. This suggests a co-ordinate interaction of the two promoter elements in the initiation of transcription. In addition, point mutations in the region 20 base pairs upstream from the cap site of the silk fibroin gene decreased the efficiency of transcription in vitro which, assuming approximately 10 base pairs per turn of the DNA helix, suggests a one-sided contact between the transcription machinery and the TATA-box at -30, the -20 region and the cap site at +1 [13]; however, the -20 region does not appear to be important for efficient transcription in vivo [12].

To gain a better understanding of the functional relationship between the TATA-box and the cap site, we have altered the distance by ±3 bp between these two regions of the promoter of a cloned rainbow trout protamine gene [14] and have analysed the affect(s) on the initiation of transcription in vitro and in vivo.

MATERIALS AND METHODS

Materials were supplied as previously listed [14] with the exception of radioactively labelled compounds which were supplied by New England Nuclear and the HeLa whole cell extract in vitro transcription system which was supplied by Bethesda Research Laboratories.

Recombinant plasmids

pTPG-8 is illustrated in Fig. 1a. The BglII-BamHI fragment of the trout protamine gene TPG3 (from position -419 to +501, see Fig. 2 of ref. 14) in which the BglII site had been blunt ended followed by the addition of a BamHI linker, was subcloned into the BamHI site of the vector pATΔNar. The vector is a derivative of pAT153 [15] in which the sequence from map position 414 to 1205 has been deleted.

pETPαI is illustrated in Fig. 1b. It is an SV40 early region expression vector into which has been subcloned the trout protamine gene [16] and the human α1-globin gene (Davies, Spencer, Dillon and Butterworth,

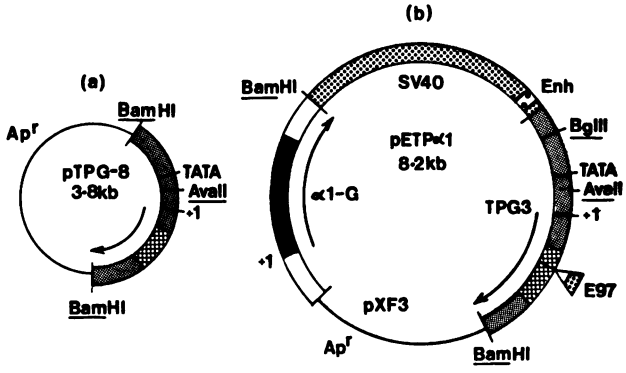


Fig. 1. Diagrams of recombinant plasmids. (a), pTPG-8 was used to construct insertion and deletion mutants of the protamine promoter at the AvaII-site immediately downstream from the TATA-box and for the generation of in vitro transcripts. (b) shows the parent expression vector pETPα1, the wild type protamine sequence being replaced with the promoter mutations; this figure also shows the construct containing the 'control' gene pETPα1-E97 in which a 97bp fragment of SV40 is inserted into the protamine protein coding sequence at the TthIII1 site; ———, plasmid sequences derived from pATΔNar in (a) and pXF3 in (b) ; ▨, protamine flanking sequences; ▩, protamine protein coding sequence; ▭, α1-globin flanking sequences; ■, α1-globin transcription unit; ▦, SV40 early region containing the enhancer (Enh); the arrow shows the direction and, in the case of in vitro assays, the extent of in vitro run-off transcripts.

unpublished data). It is composed of:

(i) the EcoRI-ClaI large fragment of the vector pXF3 which is a derivative of pBR322 lacking the poison sequences [17];
(ii) the SV40 early region from BamHI to HpaII [18];
(iii) the PstI-BamHI fragment of TPG-3 [14] where the PstI site has been replaced by a HindIII linker;
(iv) a 1.5kb PstI fragment containing the human α1-globin gene [19];

pETPα1-E97 is also illustrated in Fig. 1b. It is a 97bp SV40 fragment (from the TaqI site at map position 4739 to the AluI site at 4644) cloned into the TthIII1 site of the protamine gene in (iii) above [16].

Construction of insertion and deletion mutants

The recombinant plasmid pTPG-8 (Fig. 1a) was cleaved at the AvaII site immediately downstream from the TATA-box to yield a three base 5'-overhang (Fig.2). Spacer mutants were then obtained as follows:

(i) 5'-overhangs were filled in using the Klenow fragment of E. coli DNA polymerase in the presence of 1mM of each of dATP, dGTP, dCTP and dTTP in a reaction containing 10mM Tris/HCl (pH 7.5), 10mM $MgCl_2$, 1mM DTT, 50mM NaCl at 20°C for 30 min.

(ii) 5'-overhangs were trimmed away using 120 u/ml S_1-nuclease in a reaction containing 250mM NaCl, 30mM sodium acetate and 5mM zinc acetate (pH 4.6). Incubations were at 37°C for 30 min.

(iii) 5'-overhangs were partially filled in as described in (i) but the following combinations of dNTPs were used: (a) 1mM dGTP only; (b) 1mM each of dGTP,dATP and dTTP or (c) 1mM each of dGTP, dTTP and dCTP. Reactions were terminated by phenol/chloroform extraction and precipitated with ethanol.

Samples were treated with S_1-nuclease as described in (ii) above.

DNA molecules subjected to manipulation of the AvaII site as described above were recovered, ligated with T4-DNA ligase in a reaction containing Tris/HCl (pH 7.6), 10mM $MgCl_2$, 10mM DTT, 1mM spermidine, 1mM ATP and 100µg/ml gelatin for 14 hours at 14°C and were used to transform E. coli strain RR1 [20]. Initial screening for mutants was carried out by searching for the absence of the AvaII site in the DNA recovered from rapid plasmid preparations [21]. Putative mutations were also screened for the number of base pairs inserted or deleted by labelling CsCl-purified DNA at a restriction site proximal to the mutated region and calibrating the fragment sizes on a sequencing gel [22] as shown in Fig. 3. Mutants were then fully characterised by Maxam and Gilbert sequencing [22]. The BglII-BamHI protamine gene containing fragment of pETPα1-E97 (Fig. 1b) was then replaced (in the same orientation) with the BamHI-BamHI protamine fragment of pTPG-8 derivatives containing insertions and deletions.

In vitro transcription

In vitro transcription was carried out using the HeLa whole cell extract system of Manley et al. [23] as previously described [14]. All reactions contained DNA at a final concentration of 80µg/ml. For S_1-mapping and primer extension studies, radioactive GTP was replaced by 10mM unlabelled GTP in the transcription cocktail described in ref. 14.

In vivo transcription.

The pETPα1 series of recombinants containing both wild-type and mutant protamine genes were transfected into HeLa cells in culture [24] by the calcium phosphate precipitation method [25]. After 48 hours transient expression, cells were harvested and RNA isolated by centrifugation through 5.7M CsCl.

Labelling DNA

Probes for S_1-mapping and primers for primer extension analyses were prepared by ^{32}P-labelling the TthIII1 site within the protamine coding sequence using γ-^{32}P-ATP and T_4-polynucleotide kinase [22]. Labelling of Sau3A sites was carried out using the Klenow fragment of E. coli DNA polymerase [22].

S_1-mapping

The protocol was based on that of Favaloro et al. [26] and was carried out as previously described [14] with the following modifications: TthIII1-HindIII fragment of the protamine gene (+83 to -500, illustrated in Fig. 5a) was used as the probe and hybridisations were carried out at 54°C for 14 hours.

Primer extension

The method was based on Ghosh et al. [27] with modifications to the procedure used by Gregory et al. [14]: the primer was the 49bp TthIII1-Sau3A fragment of the protamine gene (+83 to +34 illustrated in Fig. 5b) and hybridisations were carried out in 10µl at 54°C for 14 hours.

RESULTS

Site-directed mutagenesis of the protamine promoter

A unique AvaII site lying just downstream from the TATA-box in the rainbow trout protamine gene TPG-3 (subcloned into pTPG-8, Fig. 1a) was used to produce a series of promoter mutations. The AvaII-site was cleaved and the resulting three base 5'-overhangs were subjected to number of manipulations which resulted in the insertion or deletion of up to 3bp (described in detail

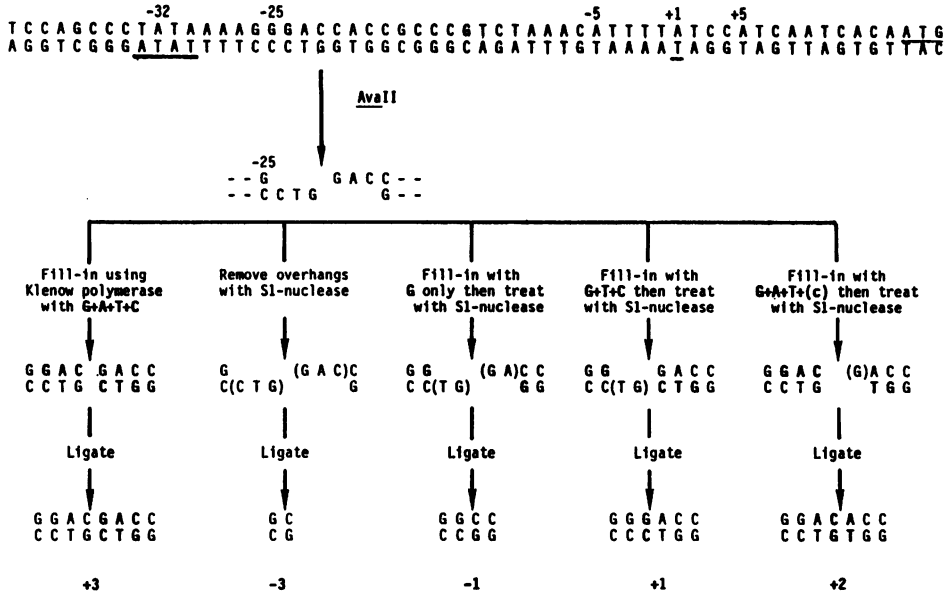
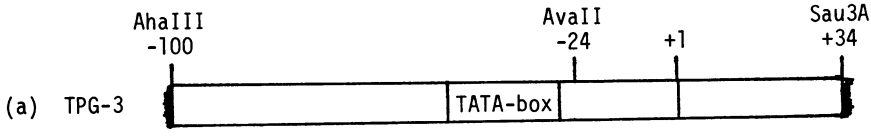


Fig. 2. Protocol for the generation of mutations which alter the distance between the 'TATA-box' and 'cap' sites of a cloned trout protamine gene TPG-3

in Methods and illustrated in Fig. 2). Initially, mutants were screened for the absence of the AvaII site, although one of the conditions used for mutagenesis regenerates an AvaII site ('+1' mutant in Fig. 2). Therefore, putative mutants were screened for base-pair differences in the size of an AhaIII-Sau3a fragment (-100 to +34, Fig. 3a) which contains the site of mutagenesis. Fig. 3b shows single base pair resolution of five mutants generated under the five different mutagenic conditions used. The mutants containing one and three base pair insertions and deletions were as expected from the protocol illustrated in Fig. 2. However, a two base pair insertion mutant was not expected from a partial fill-in of the AvaII 5'-overhangs in the presence of dGTP, dATP and dTTP which should have generated a one base pair insertion. Sequencing of this mutant revealed the insertion of an extra C-residue in the non-coding strand. This probably arose as a result of contamination of one of the dNTPs with dCTP.

Efficiency of transcription of mutants in vitro

Whether changes in the distance between the TATA-box and cap site of the protamine gene have an affect on its transcription in vitro was assessed using the HeLa whole cell extract system of Manley et al [23]. The mutant sequences



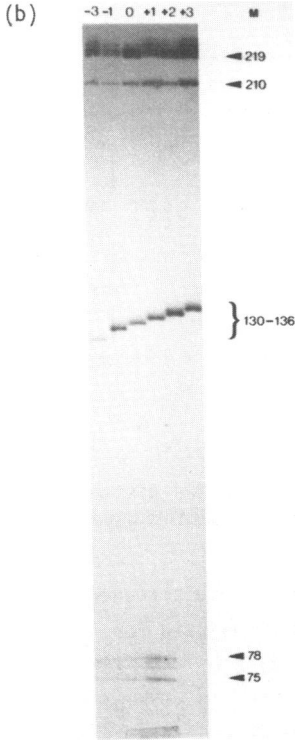


Fig. 3. Screening for insertions and deletions downstream from the protamine TATA-box.
a : Putative insertion and deletion mutants generated by manipulation of the AvaII-site at position -24 (see Fig. 2 and Methods) were labelled at the Sau3A-site (+34) followed by secondary restriction at the AhaIII-site at -100
b : Labelled fragments resulting from a were resolved on an 8% sequencing gel. Fragment length was deduced by comparison with fragments derived from the same treatment of the wild type sequence. The numbering of the lanes of this gel refers to the extent of deletion (-) or insertion (+).

were in the recombinant plasmid pETPα1 (see Methods) and the control for these experiments was a protamine gene containing a 97bp insert in the protein-coding sequence (pETPα1-E97, see Fig. 1b). Plasmids containing each mutant were mixed in the ratio of 1:1 with pETPα1-E97 and cleaved with BamHI to generate the templates for run-off transcription. Radioactively labelled transcription products were resolved on 5% polyacrylamide/urea gels and visualised by autoradiography (Fig. 4). The data were analysed by scanning the autoradiograms (Table 1). When normalised against the internal control (pETPα1-E97), it is apparent that there is no significant difference in transcript levels between the wild type and mutant protamine genes.

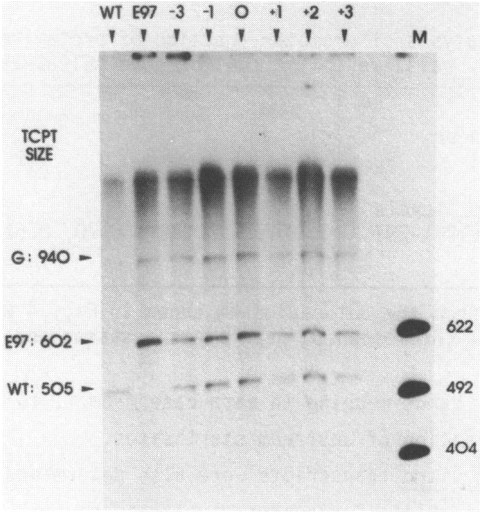


Fig. 4. Comparison of the efficiency of transcription of protamine promoter mutants in vitro. Transcription products generated in vitro using the HeLa whole cell extract system of Manley et al. [23] were analysed by electrophoresis on a 5% polyacrylamide/urea gel. RNA polymerase II run-off transcription products (see Methods) correspond in length to those expected for the wild type protamine gene (505 bases) and the internal control protamine construct pETPα1-E97 (602 bases), both of which were analysed separately (lanes WT and E97); the run-off transcript from the human α1-globin gene (940 bases) contained in the vector is also shown. The numbering of the lanes refers to the extent of insertion (+) and deletion (-)

Analysis of the 5'-ends of RNA transcribed from the protamine promoter spacer mutations in vitro

Run-off transcripts were generated from the wild type and mutant protamine genes in the recombinant plasmid pTPG-8 (Fig. 1a) cleaved with BamHI. RNA products were analysed by S_1-mapping using a probe derived from the TthIII1-HindIII protamine fragment (from +83 to -500) as illustrated in Fig. 5a. Fragments of the probe protected from S_1-nuclease by transcript were resolved on an 8% sequencing gel alongside a sequence ladder of the S_1 probe (Fig. 5c). The wild-type track shows one prominent band mapping to the A-residue at +1 (see sequence in Fig. 2) when the 1.5 base downward shift of the cleavage ladder relative to the S_1-protected fragment is taken into consideration [28]. This result is consistent with that previously obtained by Gregory et al [14]. The deletion of 1 or 3bp gave the same S_1-protected fragments as the wild type; however, on analysis on the insertion mutants,

TABLE 1
Analysis of promoter function of protamine mutants relative to the control gene pETPα1-E97[a]

Extent of deletion or insertion in TPG-3	-3	-1	0	+1	+2	+3
Relative transcript levels TPG-3 mutants : pETPα1-E97	0.78	0.78	0.70	0.61	0.83	0.65

[a]Data are derived from the autoradiogram shown in Fig. 4 which was scanned using a Joyce Loebl Chromoscan 3, peak areas assessed and compared.

there is evidence of bands mapping to each base from +1 to -5 (Fig. 5c) suggesting the utilisation of upstream start sites.

The 5'-ends of in vitro transcripts were also determined by primer extension, using the TthIII1-Sau3a protamine fragment (+83 to +34) as a primer as illustrated in the diagram in Fig. 5b. The products of primer extension using transcripts of the wild type and mutant genes as templates are analysed in Fig. 5d. The 5'-end of transcripts of the wild type gene maps to two positions: the natural cap site at +1 and, to a lesser extent, the A-residue at -5. Other minor bands upstream from -5 are probably artefacts of primer extension. For the 3bp deletion mutant, there is a major signal at +1 with a minor component corresponding to the downstream A-residue at +5. With the progressive addition of base pairs across the series from -3 to +3, 5'-ends mapping to +5 are lost and the signal at +1 decreases with a corresponding increase in the signal from -5.

The difference between the S_1-mapping data and that derived from primer extension can be put down to the fact that RNA synthesis is starting in a highly AT-rich sequence, particularly between the alternative starts at +1 and -5: any breathing of the DNA-RNA hybrids under the conditions of S_1-nuclease digestion make the hybrids susceptible to nibbling by the nuclease [14,29,30].

Analysis of the 5'-ends of RNA transcribed from the protamine spacer mutants 'in vivo'.

To see whether this trend in the utilisation of different bases as start sites is apparent in vivo, the protamine gene and the spacer mutants were transfected into HeLa cells in culture and the products of transient expression analysed after 48 hours. After recovery of total RNA, the 5'-ends of the protamine transcripts were determined by primer extension using the same primer (see Fig. 5b) and identical conditions as for the analysis of

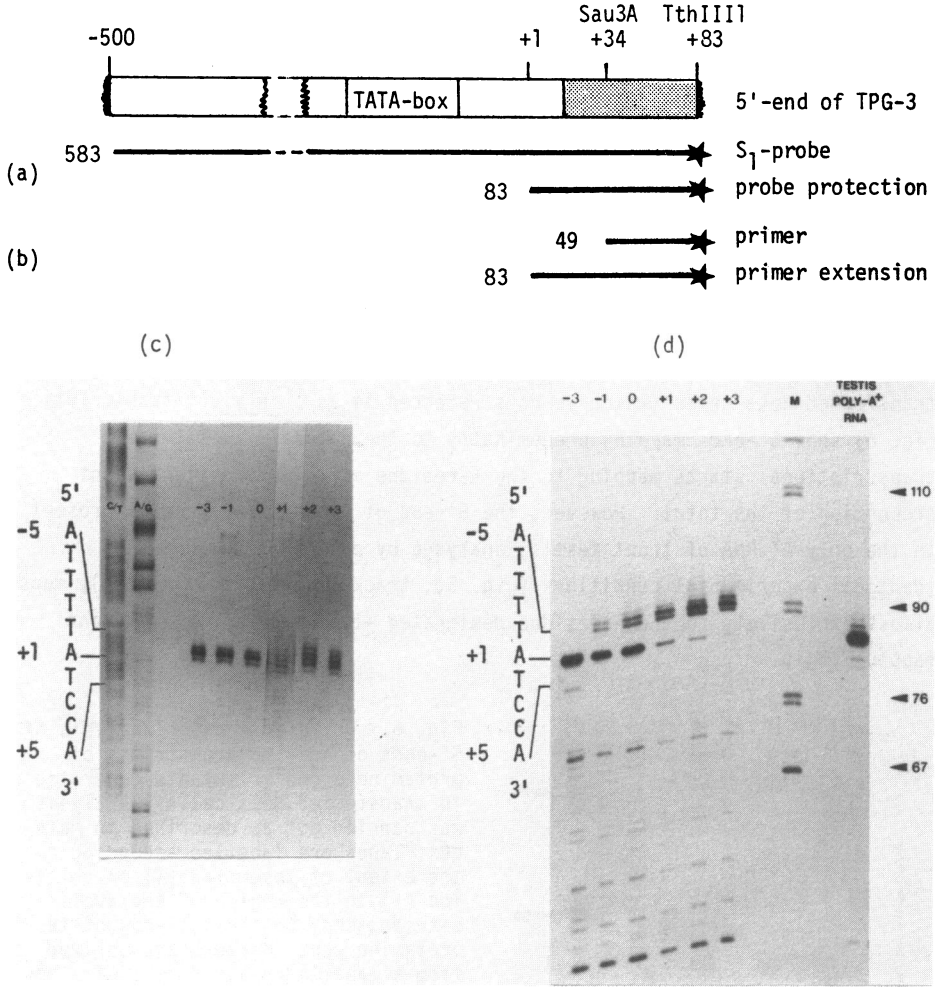


Fig. 5. Analysis of the 5'-ends of in vitro transcripts generated from protamine promoter mutant sequences by S_1-mapping and primer extension.

(a) Diagram of TthIII1/HindIII probe for S_1-mapping;

(b) Diagram of TthIII1/Sau3A primer for primer extension. ✶, denotes labelled 5'-end; ▭, 5'-flanking sequence; ▩, protein coding sequence.

(c) S_1-analysis of transcripts : lanes are labelled according to the extent of insertion (+) or deletion (-) in the region of the protamine AvaII-site; A+G and C+T sequence ladders were derived from the probe and are aligned for the identification of 5'-ends (but see text); +1 sample was underloaded and exposure of the autoradiogram was extended for this track; markers are a labelled HpaII-digest of pAT153.

(d) Primer extension analysis: the lane identification and markers used are as for (c) above; trout testis poly-A^+ RNA was included in this analysis as a control.

in vitro transcripts shown in Fig. 5d. The result is shown in Fig. 6. It is apparent that the utilisation of the three A-residues at -5, +1 and +5 for the start site for RNA synthesis follows the same trend 'in vivo' as has been shown for in vitro transcripts. Differences in transfection efficiency make it impossible to assess whether the spacer mutations have a significant effect on transcriptional efficiency 'in vivo'.

Comparison of the transcriptional start site of the natural protamine mRNA in trout testis with that obtained in vitro and from transient expression in HeLa cells

Transcripts of the wild type protamine gene in vitro (Fig. 5d) and RNA from transfected HeLa cells (which we have referred to as ' in vivo' transcripts, Fig. 6) show 5'-ends mapping predominatly to the natural cap site, but transcriptional starts mapping to the A-residue at -5 form a significant proportion of the total. However, the 5'-end of the natural product present in the poly-A^+ RNA of trout testis, analysed by primer extension using identical experimental conditions (Fig. 5d, track labelled poly-A^+ RNA), maps almost exclusively to the A-residue designated +1, a very minor component mapping to +5.

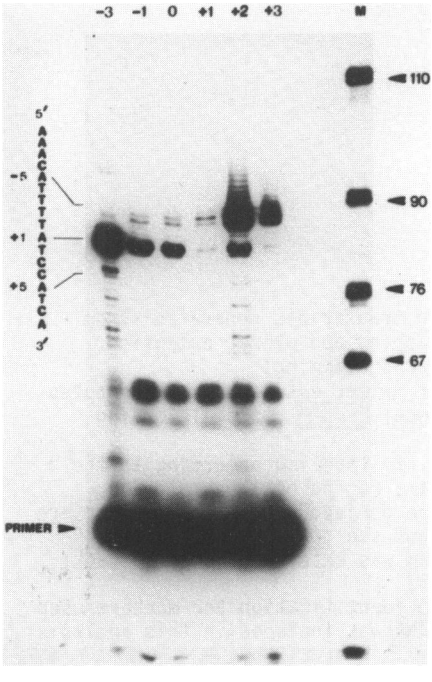


Fig. 6. Primer extension analysis of 5'-ends of in vivo transcripts of protamine promoter mutants generated in transfected HeLa cells. Analysis was carried out as described in Methods; lanes are labelled according to the extent of insertion (+) or deletion (-) in the region of the AvaII-site adjacent to the TATA-box of the protamine gene. Markers are derived from a labelled HpaII digest of pAT153

DISCUSSION

The TATA-box of the protamine gene TPG-3 is essential for transcription *in vitro* [14] and *in vivo* (Kovacs and Butterworth, unpublished data) since deletion of this promoter element abolishes transcription. We have shown here that small deletions and insertions between the TATA-box and the cap site do not affect transcriptional efficiency to any significant extent (Fig. 4). At first sight, this could be taken to mean that the RNA polymerase II transcriptional apparatus can tolerate considerable latitude in the distance between these two promoter elements. In fact, the opposite is probably the case. Purines, particularly A-residues are known to be the preferred start sites for transcription both in eukaryotes [1] and prokaryotes [31]. In this protamine gene, the A-residues nearest to the natural cap site (+1) are located at +5 and -5. Mapping the 5'-ends of *in vitro* transcripts and transcripts generated '*in vivo*' in transfected HeLa cells has shown that, as the distance between the TATA-box and the natural cap site is increased or decreased by up to 3bp in the promoter 'spacer' mutants, the position of the start site shifts between the 3 possible A-residues: deletion tends to shift the initiation site downstream to +5 whereas insertions favour starts at the upstream (-5) site. So a constant distance between the TATA-box and the *functional* cap site(s) is maintained together with transcriptional efficiency (at least *in vitro*) in this particular case.

The spatial relationship between the TATA-box and the site of the initiation of RNA synthesis is illustrated in Fig. 7. This shows a projection of one strand of the double helix assuming that there are 10.5bp per turn of the helix [32]. It can be seen that in the wild type sequence, the first A-residue of the TATA-box at position -32 is on the same face of the helix as the A-residue which forms the natural cap site (+1) for the protamine gene. The A-residues at -5 and +5 are on the opposite face of the helix. Using the -32 position as a fixed reference point, one can visualise how residues -5, +1 and +5 change their spatial relationship to the TATA-box by moving their positions clockwise for insertions and anticlockwise for deletions. In the deletion mutants, even with the loss of 3bp, the natural cap site stays on the same face of the helix as the TATA-box and remains functional; it takes the loss of all 3bp to bring the +5 A-residue towards the same face as the TATA-box to give rise to some initiation from this site while deletion moves the -5 site round the opposite side of the helix where it is non-functional in transcriptional initiation. On the other hand, insertion of up to 3bp moves

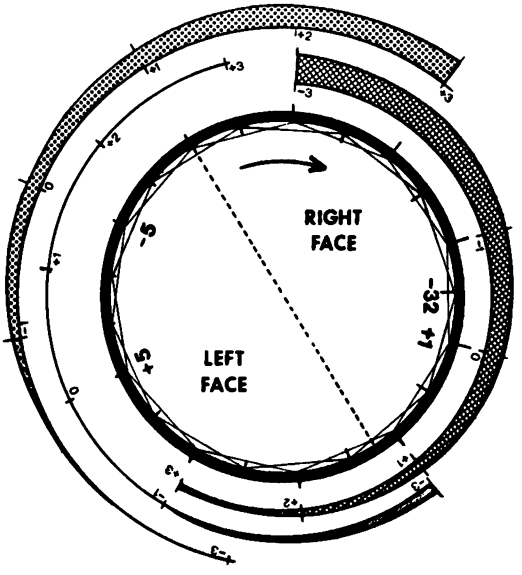


Fig. 7. Diagram to illustrate the effect of spacer mutations between the TATA-box and cap sites of TPG-3 on the selection and utilisation of transcriptional start site. The diagram shows a projection of one strand of the DNA helix (▬) with 10.5bp per turn [32]. The relative positions of the first 'A'-residue of the TATA-box at -32, and the three alternative 'A'-residues used as transcriptional start-sites are indicated. The arcs outside the helix are designed to indicate the extent to which a potential start-site (▒, +5; ▩, +1; ▦, -5) is utilised in the wild type and in the mutants in which the distance between TATA-box and start site is changed : the change in distance is indicated by the small numbers alongside the arcs (from -3 to +3bp); the width of the arcs is indicative of the extent to which a particular start site is utilised under the experimental conditions (in vitro and in vivo) used in this work.

the natural cap site out of phase with the TATA-box and reduces its involvement in intiation events while the -5 site is brought into phase and becomes increasingly utilised.

This data supports the view that interactions between RNA polymerase II and the protamine promoter are strongly biased towards one side of the DNA helix as has been suggested for the E. coli RNA polymerase [33] and for the interaction of the transcriptional apparatus with the silk fibroin gene [13]. However, the immediate sequence environment of potential transcriptional start sites can influence their selection: that the A-residue at -5 is utilised more readily in the insertion mutants than the A-residue at +5 in the deletion mutants in the present work is likely to be a reflection of the fact that the

base sequence around -5 is A-T rich whereas the site at +5 is preceeded by two G-C base pairs (see Fig. 2). This could be important in the events leading to localised DNA unwinding prior to initiation.

There is probably an optimal distance between the TATA-box and cap sites and prefered bases and sequence environments for each of these promoter elements which together describe a 'strong' promoter. Deviation from these optimum characteristics will affect the efficiency with which a particular transcriptional start site will be utilised. In the case of the protamine promoter studied here, overall transcriptional efficiency does not change (at least in vitro) with small differences in the distance between the TATA-box and cap site because alternative start sites, appropriately located, are available and are utilised. In promoters where the spatial relationship between and the sequence environments around these two promoter elements is not optimal, there is an obvious role for trans-acting transcription factors. Some of these factors are known to bind in the region containing the TATA-box of certain RNA polymerase II promoters [34-36]. It has been proposed that DNA binding proteins may act by untwisting the DNA thereby providing a more favorable orientation of contact sites for the transcription machinery [37,38]. In fact, it is likely that such factors are involved in the transcription of the protamine gene in the natural context as its expression is restricted to one tissue (testis) and one specific stage in development. It is relevant that the heterologous (HeLa) transcription systems used in this work yield transcripts of the wild type gene showing starts at both the natural cap site (at +1) and at the A-residue at -5 whereas natural protamine transcripts in trout testis poly-A^+ RNA map to +1 and (to a very small extent) to the A-residue at +5. This suggests that there is a difference in the direction of approach of the transcription machinery to its interaction with the TPG-3 promoter in the two situations. The difference may be due to DNA-binding proteins involved in ordering the conformation of the active gene in the natural situation, presenting the different elements of the promoter to the transcription machinery in a particular orientation.

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