Complex requirements for RNA polymerase III transcription of the *Xenopus* U6 promoter

Kenneth A.Simmen and Iain W.Mattaj
European Molecular Biology Laboratory, Meyerhofstrasse 1, Postfach 102209, D-6900 Heidelberg, FRG

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ABSTRACT

The role of various sequences in determining the RNA polymerase III (pol III) specificity of the Xenopus U6 gene promoter has been investigated. A sequence closely resembling an RNA polymerase II (pol II) TATA box, which has previously been implicated in determining the pol III specificity of the U6 promoter, was analyzed in detail. The U6 TATA-like element, in a different promoter context, is shown to be capable of mediating RNA polymerase II transcription both in vitro and in oocyte microinjection experiments. Extensive mutagenesis of the TATA-like element in the context of the pol III and pol II promoters leads to the conclusion that the sequence requirements for function in the two contexts are dissimilar, suggesting that different factors may be involved in mediating pol II and pol III transcription. Further, as implied by the above results, it is shown that the polymerase III specificity of the U6 gene is not solely dependent upon the TATA-like element but rather reflects complex interaction between multiple components of the promoter.

INTRODUCTION

The TATA box is a sequence motif commonly found around 30bp upstream of the transcription start site in the promoters of eukaryotic genes transcribed by RNA polymerase II (see ref. 1 for review). The importance of the TATA box in determining both the initiation site and level of transcription has been shown in many promoters. For example, deletion of the motif from either a cellular promoter, that of the histone H2A gene (2), or the SV40 early promoter (3, 4) led to the selection of new RNA start sites, both in vivo and in vitro. On the other hand, TATA mutations have been shown to decrease transcription in a range of promoters, including the adenovirus 2 E1B and major late promoters (5–8), as well as mouse β-globin (9), chicken conalbumin (10), and Drosophila Sgs3 (11). An extensive mutagenic study of the yeast his3 gene TATA region showed that almost all single base changes inactivated the promoter (12).

A TATA box binding activity (termed TFIID or BTF1) has been identified (13–15) and shown to participate in the formation of a transcriptional pre-initiation complex (16). Yeast TFIID can substitute for HeLa TFIID in in vitro transcription experiments (17, 18), and the TFIID gene has been cloned from S.cerevisiae by several groups (19–23).

Recently a novel class of RNA polymerase III (pol III) transcribed genes have been found to utilise gene external promoters (for review, see ref. 24). The best studied examples are the U6 snRNA genes of mouse, human, and Xenopus (25–27) and the human 7SK gene (28, 29). A region resembling an RNA polymerase II (pol II) TATA motif in both sequence and position has been found in the promoters of all these genes. Deletion of the T/A-rich element abolished pol III transcription of the Xenopus U6 gene in vivo (27, 30), human 7SK expression in vitro (28), and human U6 transcription both in vitro and in vivo (31, 32) demonstrating the functional importance of the TATA-like element in these promoters.

The striking similarities between the U6 promoter and the RNA polymerase II transcribed promoters of the other major snRNAs have been reviewed elsewhere (33, 34). Both classes of promoter have a distal sequence element (DSE), or enhancer, containing an octamer motif, and an essential proximal sequence element (PSE). The Xenopus U6 promoter has two additional functional regions, the TATA-like motif and the sequence around the start site (30). Both the human and Xenopus U6 TATA regions have been implicated as major determinants of pol III specificity (30, 31, 35). Deletion of the T/A motif converts U6 into a pol II promoter, while its addition results in the pol III transcription of a U2 gene promoter which would otherwise be transcribed by pol II. Hence, the TATA-like element plays a major role in both the transcription efficiency and pol III specificity of the U6 promoter.

The apparent contradiction inherent in the TATA motif playing an important role in many pol II promoters and yet acting as a pol III determinant in the U6 context led us to examine the U6 TATA-like element in more detail, and to re-examine the question of polymerase choice by the U6 promoter. We show that the U6 TATA-like element can function to direct pol II transcription both in vivo and in vitro, but that the sequence requirements in the 'TATA' region differ depending on the nature of the transcribing RNA polymerase. This suggests that TATA-binding factors with different specificities are involved in pol II and pol III transcription. Moreover, the pol III specificity of U6 transcription

* To whom correspondence should be addressed

is shown not to be simply due to the presence of the TATA-like element, but to reflect a complex interaction between all the different elements of the promoter.

MATERIALS AND METHODS

Mutagenesis and promoter constructs

Oligonucleotides were synthesized by the phosphoramidite method (49) on an Applied Biosystems synthesizer and used for mutagenesis with a site-directed mutagenesis kit (Amersham International PLC, Amersham, UK). The point mutations in the T/A motif were generated in the U6.c89 construct in M13mp9 (previously described as a −73/−80 clustered point mutant, ref. 30) which was derived from the wt Xenopus U6 977bp Bam HI fragment (50). A 'degenerate' oligonucleotide was synthesized with 85% wild type and 5% of each other base in each of the 8 positions to be mutated; this generated an oligonucleotide mixture containing, on average, a single base change. Most of the possible single base changes between −31 and −24 were subsequently identified by dideoxy sequencing (51), and the U6 inserts sub-cloned (Eco RI−Hind III) into pUC 19.

TK.U6 reporter constructs

The TK.c89.G- constructs shown in Fig. 1. were made as follows. A Bgl II linker was inserted into the Kpn I site of the G-less cassette vector M5m9 (Monaci et al., in preparation, kindly provided by P. Monaci and A. Nicosia) following digestion with Asp 718 and Klenow-mediated repair. A 118bp Eco RI fragment of the HSV-TK promoter containing an Sp1 site and a CAAT box element (36) was inserted at a unique Eco RI site 19 bp 5′ to the Bgl II site. The U6 sequences (−78/−5) were introduced as Bgl II−Eco RV fragments, after cutting the vector with Bgl II and Eco RV. Polymerase chain reactions (PCRs) (36) using appropriate primers, were used to generate a convenient Eco RV site at −7/−2 in the U6 'TATA' mutants which facilitated the sub-cloning described above. To generate the TK.(P-)c89. series the PSE was mutated in three positions to inactivate the element (53) prior to the PCR and sub-cloning. The PSE mutation changed the sequence (−66/−61) from CTCTCC to CGCGTC.

The TK.U6.β series were generated from the TK.c89.G- series by removing the G-less cassette as an Eco RV−Sma I fragment and replacing it with a 1.6kb Pvu II fragment of the rabbit β-globin gene from the OVEC plasmid (38). OVEC and OVEC-REF plasmids were kindly provided by Michael Müller and Walter Schaffner. In the TK.U6.β constructs the U6 sequence to −5 is fused directly to what was −10 in the globin promoter, hence the distance from the 3′end of the U6 'TATA' to the wild type globin start site in these constructs is 29bp.

Both TK.U6.βΔTT and TK.U6st.βΔTT were generated from their parent constructs by site-directed mutagenesis, substituting GG for TT at positions (+10/+11) in the β-globin fragment, to facilitate pol III elongation.

Polymerase chain reactions

The PCR conditions were as follows: 50mM KCl; 10mM Tris [pH8.6]; 1.5mM $MgCl_2$; 10% DMSO; 0.01% gelatin; 0.2mM dATP, dGTP, dCTP and dTTP; 5ng single strand template; 2.5 U Amplitaq rDNA Polymerase (Perkin Elmer Cetus, USA) and 6pmol of each primer. The amplifications were performed on a thermal cycler with 40 cycles of :1min/90°C (denaturation), 1min/40°C (annealing), 1min/70°C (extension).

Oocyte microinjection

Oocytes were injected, aiming at the nucleus, with 50nl of DNA at a total concentration of 300 μg/ml. In Fig. 2. [α-^{32}P]GTP (Amersham) was coinjected with the DNA at 0.1−0.5 μCi per oocyte, and the U6 maxigene was mixed in a 1:1 ratio with the test construct. RNA was extracted and analysed as described (54). Oocytes were extracted and analysed in batches of 10. Half an oocyte equivalent (~ 2.5μg) of RNA was loaded onto each lane of the gel shown in Fig. 2. Injections of templates for RNAse protection assays were performed in the same way, but in the absence of radioactive label.

RNAse protection

The T3-U6-β construct was obtained by linearising TK.c89.β with Nco I, Klenow−mediated repair, followed by digestion with

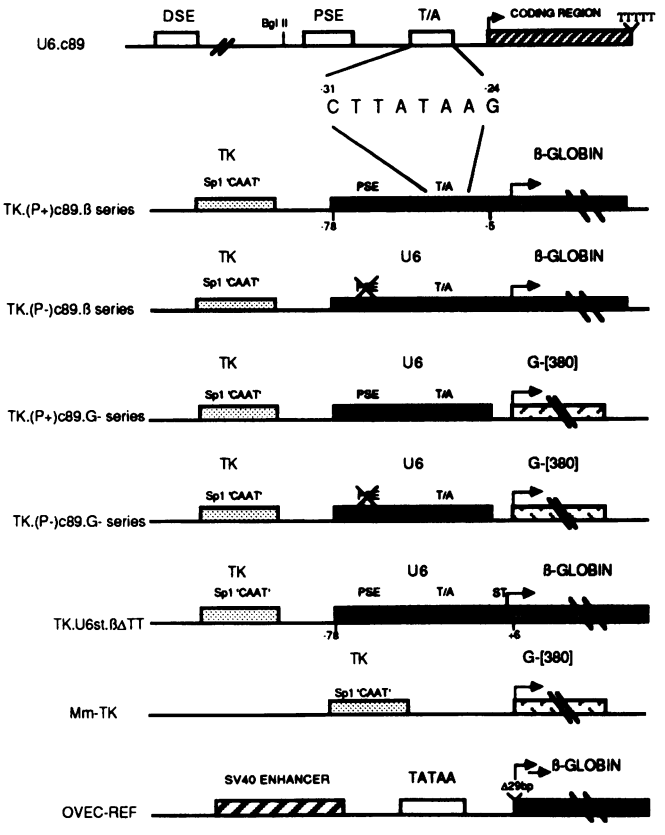

Fig. 1. The structures of the constructs used in this study (not drawn to scale). Shown at the top is a schematic of the Xenopus U6 gene. The boxed DSE, PSE and T/A motif are functional promoter elements. U6 c89 was previously constructed and shown to be transcriptionally wild type (30). The sequence of the 8bp T/A motif is shown. All the TK.U6. hybrid constructs contain an HSV-TK promoter fragment upstream of the U6 Bgl II−Eco RV (−78/−5) proximal promoter fragment. Two alternative reporter fragments were present downstream: a G-less cassette (for in vitro transcription) or a rabbit β-globin fragment (for oocyte analysis). For both the TK.c89.G- and TK.c89.β series U6 fragments carrying the 'TATA' mutations were introduced with either a wild type PSE (P+), or an inactivated PSE (P−). The TK.U6st.βΔTT construct contained a U6 Bgl II Eco RV (−78/+6) promoter fragment which incorporates the U6 initiation region, fused to the rabbit β-globin reporter. Mm-TK contains only the TK fragment in front of the G-less cassette and served as a 'TATA-less' control in the in vitro transcription assays. OVEC-REF (38) served as an internal control in the in vivo pol II assays. Note that it carries a deletion around the start site which allows its transcripts to be distinguished from those of the TK.c89.β series and which results in the use of two different initiation points, represented by arrows.

Bgl II and insertion of the Bgl II−Nco I fragment into pBS(+) (Stratagene) cut with Bam HI and Hinc II. T3-U6st-βΔTT was similarly made from TK.U6st.βΔTT. Labelled RNA was prepared from linearised templates using T3 RNA polymerase as described (55). RNAse protection assays were performed essentially as described (56), except the hybridization buffer contained 50% formamide. The RNA from two oocyte equivalents was hybridized to 80,000 c.p.m. of the T3-U6-β riboprobe. Following digestion with RNases A and T1 (final concentrations of 4 / 0.2μg/ml), the nucleic acids were precipitated and taken up in formamide dyes. Either all (Fig. 3B) or half the sample (Fig. 4) was electrophoresed on a polyacrylamide / urea gel.

In vitro transcription assays

HeLa cell nuclear extracts were prepared according to (57). The RNA polymerase III transcription reactions for 5S were carried out for 1 hour at 20°C in 20μl containing ~ 60μg extract; 10mM HEPES [pH7.9]; 10% glycerol; 65mM KCl; 7.5mM $MgCl_2$; 0.25mM EDTA; 0.55mM DTT; 10mM creatine phosphate; 0.54mM ATP,CTP,UTP; 18μM GTP; 5μCi [α-^{32}P]GTP; 1μg 5S template DNA (58). Reactions were stopped with the addition of 30μg proteinase K and SDS to 1%. Nucleic acids were precipitated with ethanol, separated by electrophoresis on a 6% polyacrylamide / urea gel and detected by autoradiography at −70°C.

In vitro transcription under G-less conditions (37) was carried out as follows: the 10μl reaction volume contained ~ 40μg extract; 10mM HEPES [pH7.9]; 10% glycerol; 4mM $MgCl_2$; 5mM creatine phosphate; 4mM spermidine; 0.5mM DTT; 0.2mM ATP,CTP,25μM UTP,10μCi [α-^{32}P] UTP; 1μg total DNA (100ng AdMl404[180] DNA; 500 or 900ng test DNA, with sonicated salmon sperm DNA used to keep the concentration constant). GTP was replaced with 900μM of the chain terminator 3'O-MeGTP (Pharmacia) and 5U of RNAse T1 (Worthington, USA) were present. Templates were pre-incubated with extract on ice for 15min before the addition of the other components. Reactions were incubated for 45min at 30°C before being stopped and processed as above.

RESULTS

Point mutation of the TATA-like element affects pol III transcription

The functional importance of the Xenopus U6 TATA-like motif was previously shown by the transcriptional inactivity of a clustered point mutant in the region −31/−24 of the U6 promoter (30). To investigate the requirement for sequence specificity in this region, we performed saturation mutagenesis and assayed promoter activity in Xenopus oocyte nuclei. Point mutants were isolated in a functionally wild type U6 promoter (Figure 1, c89) and coinjected into oocyte nuclei with a U6 maxigene. The analysis of transcripts (Figure 2) shows that in comparison to wild type (lane 1) the mutant templates exhibit a wide range of transcriptional activities (see Table 1 for a summary of the data). Mutation of the nucleotides between −28 and −25 (ATAA) considerably reduces activity, except when the change preserves A or T at a position, for example mutation of −27T to C or G (lanes 13 and 14) debilitates the promoter whereas introducing an A (lane 15) results in increased promoter activity. The 3' boundary of the motif appears to be at −24 as two mutations there have no effect. Previous results with clustered point mutants showed that mutations between −23 and −17 and between −40 and −32 did not affect promoter activity (30). None of the point

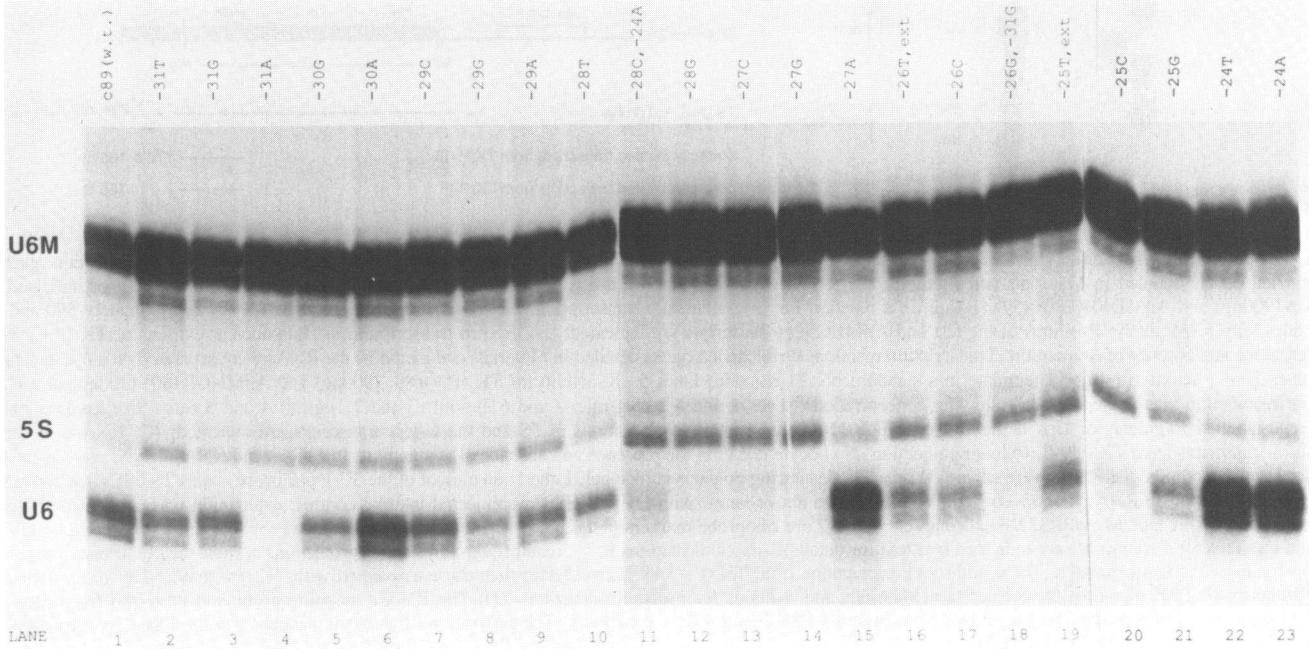

Fig. 2. Polymerase III transcriptional activity of the U6 'TATA' point mutants in Xenopus oocyte nuclei. The c89 construct used in lane 1 is the wild type background into which the point mutations were introduced. The mutant nomenclature shows the mutated base position (with respect to the normal Pol III initiation site) and the base to which it is changed. The −26Text and −25Text mutants each carry a second mutation external (upstream) to the T/A motif, in sequences previously shown not to influence promoter activity. Each construct was coinjected with the U6 maxigene, which served as an internal standard. The transcripts indicated are U6, U6 maxigene (U6M), and endogenous 5S RNA.

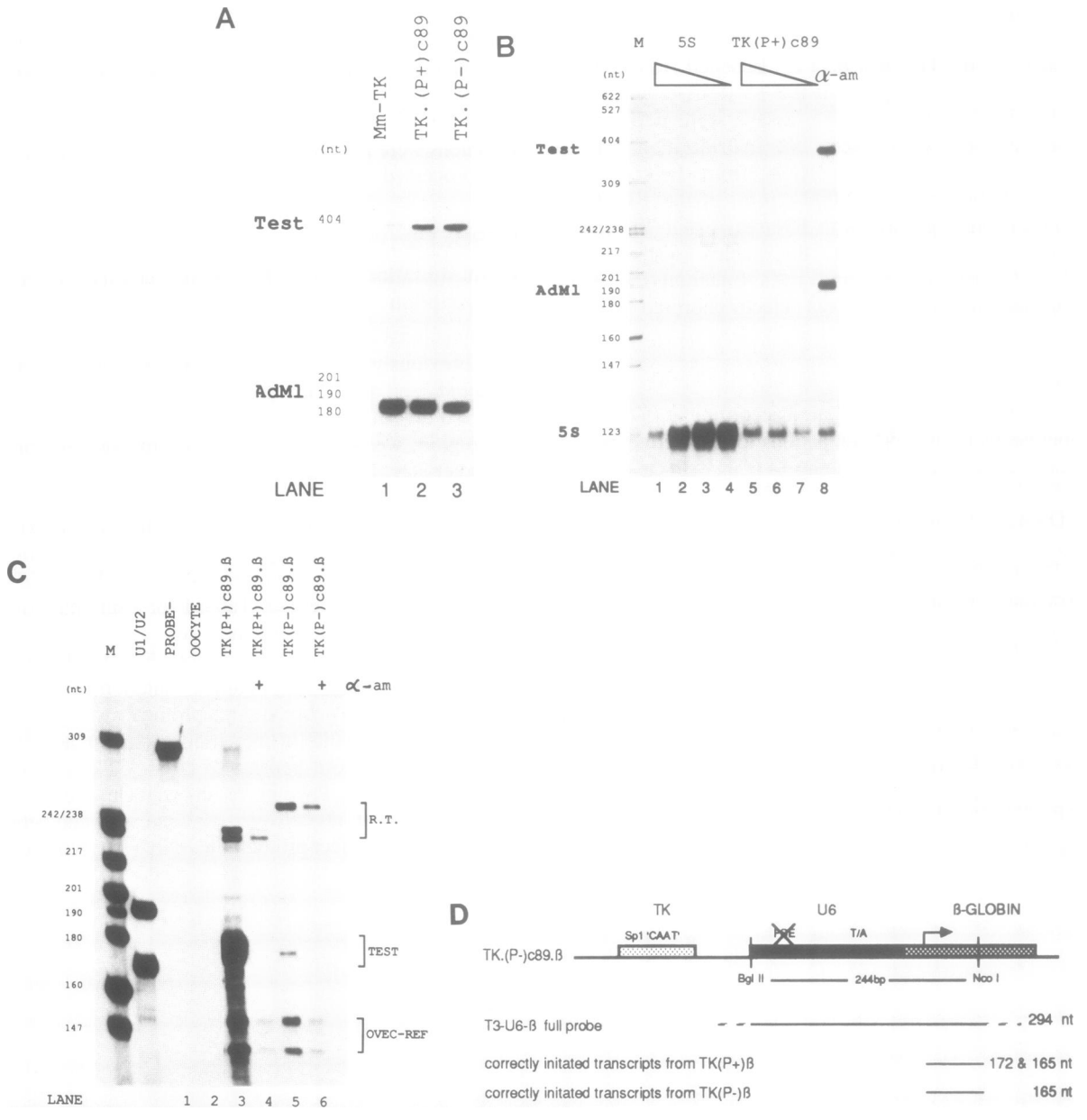

Fig. 3. The polymerase II specificity of the TK.c89. constructs. (A) In vitro transcription in HeLa nuclear extract of TK.c89.G- constructs. Transcription reactions were carried out as detailed in Materials and Methods; the RNAs were separated on a 6% denaturing polyacrylamide gel. Lanes 1−3: activity of indicated test template (900ng) and AdML404[180] (59) (100ng) in a reaction for G-less cassette transcription; the transcripts from the cassettes are approximately 390 and 190 nucleotides long respectively. Positions of the test and AdMLG-less transcripts are indicated. (B) In vitro transcription in Hela nuclear extracts of TK.(P+)c89.G- in the presence and absence of α-amanitin. Transcription reactions were carried out as detailed in Materials and methods; the RNAs were separated on a 6% denaturing gel. Lanes 1−4 : activity of 1μg 5S template in a standard pol III reaction; lanes 5−8: activity of TK.(P+)c89. (900ng) and AdMl404[180] (100ng) in a G-less reaction. α-amanitin was present at the following concentrations: lanes 1 and 5,200μg/ml.; 2 and 6,2μg/ml; 3 and 7,1μg/ml; 4 and 8,zero. Size markers in lane M are end-labelled fragments of Hpa II digested pBR322 DNA; nt−nucleotides. Positions of 5S and the G-less transcripts are indicated. (C) RNAse protection analysis of transcripts from TK.c89.β, following injection into oocyte nuclei in the presence and absence of α-amanitin. Injections and RNA analysis were as detailed in Materials and methods; the RNAs were separated on a 6% denaturing polyacrylamide gel. Lane 1: an aliquot of the T3-U6-β probe; lanes 2−6 RNAse protection; lane 2: uninjected oocyte RNA; lanes 3−6: templates as shown above lanes, with OVEC-REF as coinjected internal control; α-amanitin was coinjected at 1μg/ml where indicated by +; lane M : pBR322/HpaII markers; U1/U2 are riboprobe markers of 172 and 191 nt. length respectively. Probe fragments protected by OVEC-REF and test TK.c89.β transcripts are indicated (see text for details). Also indicated (as R.T., readthrough) are longer protections by non-specific transcripts coming from upstream of the test promoter. The readthrough protections from TK.(P+)c89.β are shorter than those associated with TK.(P−)c89.β due to discontinuities to the probe in the PSE region of the former (see Materials and methods for probe construction). (D) The RNAse mapping probe and protected fragments. The estimated sizes of specific protections are shown from both the PSE+ and PSE− constructs. The generation of an extra protection in the PSE+ constructs is due to utilisation of a second start site (see text).

mutations which allow detectable transcription have an effect on transcript length, and thus apparently do not affect the accuracy of transcription initiation.

Prior to this study the only point mutant tested in the Xenopus U6 T/A motif was one which changed the (−31/−24) sequence from CTTATAAG to TTTAAAAG, and this supported wild type activity (27).

The most unexpected phenotype is shown by mutant −31A

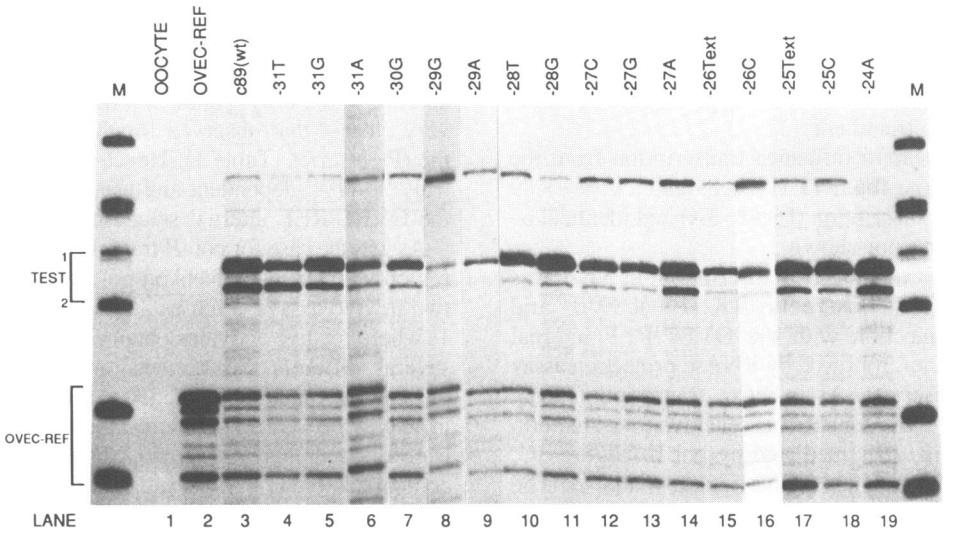

Fig. 4. Polymerase II transcriptional activity of the TK.(P+)c89.β mutants in Xenopus oocyte nuclei. RNAse protection analysis of transcripts from the TK.(P+)c89.β series, following injection into oocytes. The mapping of one oocyte equivalent is shown for each lane. Lane 1: uninjected oocyte RNA; lane 2: the internal control, OVEC-REF; lanes 3–18: TK.(P+)c89.β templates as shown above lanes, with OVEC-REF as coinjected control; lanes M: pBR322/HpaII markers. The RNAs were separated on an 8% polyacrylamide / urea gel. Lanes 6, 8 and 16 come from different exposures of the same gel. Fragments protected by OVEC-REF transcripts are bracketed OVEC-REF, and two protections derived from the test transcripts are bracketed TEST. The uppermost band in lanes 3–19 is due to readthrough transcription from upstream of the promoter.

(lane 4). In contrast to most of the other templates mutant in positions −31 to −29, which display activities from 40 −100% of wt, −31A reproducibly shows no detectable activity. This example of sequence dependence at the 5′ end of the motif coupled with the central T/A preference strongly suggests recognition of the motif in a specific manner by a DNA binding protein.

The U6 TATA-like element can mediate polymerase II transcription

Because of the similarity in both sequence and position of the U6 T/A motif and pol II TATA boxes the ability of the U6 element to support transcription by RNA polymerase II was investigated both in vivo and in vitro, using artificial promoter constructs. These contained a fragment of the proximal U6 promoter (from −78 to −5) placed downstream of a region carrying pol II upstream activation elements from the herpes simplex virus thymidine kinase gene, namely an Sp1 site and a CAAT box (36). These basic TK.U6. units were fused to either a G-less cassette (37) or a β-globin reporter (38), generating the TK.c89.G- and TK.c89.β constructs depicted in Figure 1. The rationale was to determine whether factors responsible for mediating pol II transcription could interact with the U6 'TATA' element. Two series of constructs were made in which either the wild-type U6 PSE or an inactivated mutant version were included to test the effect of the presence of the PSE on promoter strength and polymerase specificity.

Figure 3A shows that both TK.(P+)c89 and TK.(P−)c89 produce transcripts of expected length (marked 'Test') in HeLa nuclear extract (lanes 2 and 3) as does the AdML promoter AdML404[180] internal standard. Activity of the TK.c89.G- constructs was dependent on the presence of the U6 fragment as either the vector alone or the Mm-TK 'TATA-less' control (see Fig. 1) gave no or very low transcriptional signals (data not shown and Fig. 3A, lane 1).

In Figure 3B transcription from the TK.(P+)c89 construct is shown to be pol II specific. Lane 8 shows transcription from TK.(P+)c89.G- and AdMl404[180] as internal standard. On the addition of α-amanitin to 1μg/ml (lane7) or higher (lanes 5 and 6) the signals are lost. This α-amanitin sensitivity is characteristic of RNA polymerase II transcription. For comparison, lanes 1–4 show that pol III transcription of a 5S gene in the same extract

mutant	"TATA" sequence -31 … -24	transcriptional activity (Pol III)	(Pol II) PSE+	(Pol II) PSE-
c89 (w.t.)	C T T A T A A G	+++	+++	+++
-31T	T	++	++++	+++
-31G	G	++	+++	++
-31A	A	-	+	+
-30G	.... G	++	+	+
-30A	.... A	+++	ND	ND
-29C	..... C	++	ND	ND
-29G	..... G	++	-	*
-29A	..... A	++	-	*
-28T	..... T	++	+	ND
-28C.-24A	..... C ..... A	-	ND	ND
-28G	..... G	-	+	ND
-27C	..... C	-	+	ND
-27G	..... G	-	+	ND
-27A	..... A	++++	++++	+++
-26Text	..... T	++	+	*
-26C	..... C	+	-	*
-26G.-31G	G ..... G	-	ND	ND
-25Text	..... T	++	+++	ND
-25C	..... C	+	++	ND
-25G	..... G	+	ND	ND
-24T	..... T	++++	ND	ND
-24A	..... A	++++	++++	+++

Table 1. Summary of the transcription efficiencies of the 'TATA' mutants in vivo by RNA polymerase II or III (Pol III), U6 wt and mutant transcription in oocytes following coinjection with maxigene. (Pol II), results of the RNAse protection assay of the two TK.c89.β mutant series. The data are presented as falling into% ranges of wt (c89) activity after normalization to the relevant internal control in each of the assays: U6 maxigene in pol III transcription; OVEC-REF transcripts in RNAse protection: (−) 0–10%, (+) 10–40%, (++) 40–70%, (+++) 70–100%, (++++) >100%. ND–not determined; *–signal not detectable.

is resistant to α-amanitin at low concentrations, and only decreases at 200μg/ml. The presence of a 5S-sized signal even in the absence of added 5S template (lanes 5−8) is apparently due to label transfer to the ends of an endogenous RNA which survives the RNAse T1 treatment.

The PSE did not appear to influence transcription from the TkU6 constructs in vitro as both Tk.(P+)c89 and Tk.(P−)c89 show similar levels of transcription (Figure 3A) and identical α-amanitin sensitivity (data not shown).

The potential to activate pol II transcription in vivo was investigated with the constructs TK.(P+)c89.β and TK.(P−)c89.β by coinjection with the OVEC-REF internal standard into oocyte nuclei, followed by RNAse protection assay of extracted RNA transcripts. As in vitro, transcription from either the PSE+ or the PSE- construct is sensitive to α-amanitin at 1μg/ml, indicating that it is mediated by pol II (Figure 3C, compare the test signals in lanes 3 and 5 with 4 and 6). The heterogeneity of OVEC-REF transcription has been noted previously (38). Interestingly, transcription of OVEC-REF appears to be partially insensitive to α-amanitin. Another TATA-dependent mRNA promoter, that of the c-myc gene, has previously been shown to be transcribed by both pol II and pol III in Xenopus oocytes (39, 40).

One striking observation, given the lack of effect of the PSE on the G-less cassette constructs in the in vitro transcription assays, is that mutational inactivation of the PSE causes a dramatic decrease in transcription from the globin reporter constructs in vivo (compare lanes 3 and 5). Furthermore, in the absence of the PSE a single initiation site is seen while in its presence an additional start site 7bp further upstream is also utilized (Figures 3C and 3D; this is more easily seen in Figure 4). Clearly however, all the transcription involves pol II since α-amanitin at 1μg/ml results in its inhibition. These results demonstrate that, either in vivo or in vitro, in an appropriate promoter context, the region of the U6 promoter containing the TATA-like element can support pol II transcription.

The effect of point mutation on the ability of the U6 T/A motif to function as a pol II TATA box

In order to prove that the TATA-like element was mediating pol II transcription in the artificial promoter constructs it was necessary to test the effect of mutations in the element. We made use of the point mutants previously studied in the context of the U6 promoter to allow comparison of the sequence requirements for function of the U6 TATA-like element in promoters transcribed by different RNA polymerases.

A subset of the mutants assayed for pol III activity were introduced into the TK.c89.β background, initially as (P−) constructs. Templates were coinjected into oocytes and the RNAs mapped with the T3-U6-β probe (Figure 3D). The data are summarized in Table 1. Unfortunately even the wt TATA element gives rise to a weak signal in the (P−) construct (Figure 3C, lane 5). Signals from many of the mutants were therefore either very weak or not detectable, making quantitation difficult (data not shown, but see Table 1). As observed above (Figure 3C), a wt PSE confers much greater activity on the TK.c89.β promoter without altering polymerase specificity. Hence the T/A point mutants were introduced into templates with a wt PSE, yielding the TK.(P+)c89.β series. RNAse protection analysis (Figure 4) revealed the effects of the point mutants. The higher percentage gel in Figure 4 compared to Figure 3C allowed a clearer resolution of the two specific fragments protected by transcripts from PSE(+) constructs. The upper protection (labelled test 1) is generated by an upstream start arising only in the presence of the PSE. The lower protection (test 2) is produced in the absence of the PSE (Figure 3C, lane 5) and its intensity mirrored very closely that observed for the weaker signal produced by the (P−) series (Table 1). Hence the test 2 signal appears to be truly 'TATA'-dependent and was quantitated with reference to the OVEC-REF internal standard.

As was the case for pol III transcription, mutations in the TATA region had various effects on pol II activity. The data from the two in vivo assays (Figures 2 and 4) can be compared in Table 1, where relative activities obtained by analyzing and combining several different experiments are summarized. Due to the difficulty in quantitating the (P−) series, and to some variability in activity between experiments, the mutant activities have simply been divided into four broad classes in order to facilitate the comparison of similarities and differences between the pol II and pol III promoters. Certain mutations affect both pol II and pol III activity similarly; for example −31G, −30G, −27A, −24A (Figure 2, lanes 3, 5, 15, 22 and figure 4, lanes 5, 7, 14, 19), and are therefore neutral to the transcribing polymerase. Mutant −27A was still highly active in RNA polymerase II transcription (Figure 4, lane 14). This was unexpected, given the high degree of conservation observed for the central T in the TATA box of many pol II genes (1) and the effect of mutating this nucleotide in various pol II promoters (10−12).

More importantly, there are many instances in which point mutations differentially affected pol II or pol III transcription. Several mutations which essentially inactivate transcription by one polymerase allow the other to be active. These include −29G and −29A, both of which severely reduce pol II transcription (Figure 4, lanes 8 and 9) but have only a moderate effect on pol III transcription (Figure 2, lanes 8 and 9); −28G, −27C, and −27G, whose effect on pol III transcription is greater than that on pol II, and −31A which is inactive for pol III but supports ~ 30% of wt activity in pol II transcription (Figure 2, lane 4 and Figure 4, lane 6). The −31A mutation is of particular interest in that it lies outwith the sequence normally conserved in pol II TATA boxes, yet is clearly essential for pol III transcription of U6.

To test the generality of these findings, all of the mutants used in Figure 4 were tested in HeLa cell nuclear extracts as TK.(P+)c89.G- constructs and, with only one exception (−26Text), the same profile of mutational effects was observed (data not shown). The −26Text mutation behaved like wt in vitro; this could possibly reflect a species-specific difference in the binding of pol II TATA-binding factor(s). The results in this section demonstrate that the sequence requirements for function of the U6 TATA-like element differ for pol II and pol III mediated transcription.

Pol III specificity of U6 transcription

The data in Figure 3 shows that, out of the U6 context, the wt U6 TATA-like sequence can support pol II-mediated transcription in vivo or in vitro. In the wt U6 promoter context, however, the same element is known to be a pol III determinant. Hence, in order to play its role in determining pol III specificity the TATA region must interact with additional elements of the U6 promoter. Although absolutely required for wt U6 activity (30), the PSE, in combination with the T/A motif, is not sufficient for pol III activity from the artificial TK.U6. constructs in vitro or in vivo (see Figure 3).

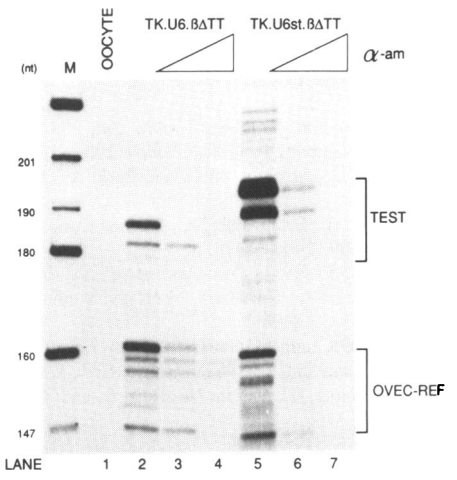

Fig. 5. The U6 start site region contribution to pol III activity . RNAse protection analysis of transcripts from TK.U6.βΔTT and TK.U6st.βΔTT, following coinjection with OVEC-REF into oocytes in the presence of α-amanitin. Injections and RNA analysis were as for Fig. 4; the riboprobes used in each assay are indicated below. Lane 1: mapping uninjected oocyte RNA with both T3-U6-β and T3-U6st-βΔTT; lanes 2−4 : mapping of TK.U6.βΔTT and OVEC-REF transcripts with T3-U6-β riboprobe; lanes 5−7 : mapping of TK.U6st.βΔTT and OVEC-REF transcripts with T3-U6st-βΔTT riboprobe. The insertion of additional sequences in TK.U6st.βΔTT necessitated the use of a different probe. Alpha-amanitin was present at the following concentrations: lanes 3 and 6, 2μg/ml.; 4 and 7, 200μg/ml; 1, 2 and 5, zero. Size markers in lane M are end-labelled fragments of Hpa II cut pBR322 DNA; nt−nucleotides. Both OVEC-REF and test protections are bracketed.

To examine this question further in vivo, the TK.U6. β-globin reporter had to be modified by disrupting a run of four Ts present just downstream of the transcription initiation site which could act as a termination signal for RNA polymerase III, and thus prevent the detection of any pol III transcripts in the RNAse protection assay. The central two Ts were mutated to Gs and this reporter used in the two constructs TK.U6.βΔTT and TK.U6st.βΔTT, the latter differed from the former only by the presence of the U6 start site region (see Figure 1). The RNAse mapping analysis shown in Figure 5 reveals that TK.U6.βΔTT (lane 2) generates the same two protected fragments as TK.(P+)c89.β (Fig. 4, lane 3). The longer transcript (designated test 1 in Figure 4) is sensitive to low concentrations of α-amanitin, whereas the shorter transcript (test 2) is partially insensitive to α-amanitin at 1μg/ml (Figure 5, lanes 2−3). Thus, the shorter transcript is at least in part transcribed by pol III.

The region around the Xenopus U6 transcription initiation site has been shown to be important for promoter activity (30). The effect of adding the start site to the TK.U6.βΔTT construct is seen in lanes 5−7. The increased length of transcripts arising from TK.U6st.βΔTT is due to the inclusion of the extra 10 bases from the U6 initiation region. As with TK.U6.βΔTT, two major protections are seen in the absence of α-amanitin (lane 5). In the presence of 2μg/ml α-amanitin (lane 6) around 90% of each signal is lost, indicating that most of the transcription is mediated by RNA polymerase II. Adding α-amanitin to 200μg/ml with either template abolishes transcription (lanes 4 and 7). Thus, adding the U6 start site to the artificial promoter greatly enhances the level of transcription from the linked reporter in vivo. However, the presence of the U6 initiation region does not appear to significantly increase the use of RNA polymerase III in preference to RNA polymerase II. This is discussed further below.

DISCUSSION

The U6 TATA-like element and pol III specificity

Previous studies of vertebrate U6 genes have led to the conclusion that the TATA-like element of these promoters is essential for transcription (27, 30, 31, 32). Furthermore, they had suggested that it was an important, perhaps even the sole, determinant of polymerase specificity in these promoters (30, 31, 35), based on the fact that addition of the TATA-like element to a U2 (pol II) promoter could convert its polymerase specificity from pol II to pol III.

One of the findings of this paper is that the ability of the U6 TATA-like motif to confer pol III specificity to a promoter is not an intrinsic property of its sequence. This conclusion is based on the fact that, in an appropriate promoter context, the wt U6 TATA-like sequence can mediate pol II transcription both in vivo and in vitro. Hence, in order to play a role in determining pol III specificity the TATA region must require additional elements of the U6 promoter. In considering which elements these might be, it should be remembered that the complete mouse, human and Xenopus U6 promoters, including the DSE, PSE, T/A motif and +1 region (Figure 1) have been shown to be capable of driving transcription by pol III of various downstream sequences with efficiencies, where measured, similar to that of the U6 gene itself (25, 31, 35, Hamm and Mattaj, submitted for publication). Deletion of the DSE leaves a basal promoter which is still pol III-specific (25−27). Thus, the DSE is not required for pol III specificity.

We therefore analysed the artificial T/A motif-containing constructs in order to determine which of the other U6 promoter elements might be required for pol III selectivity. The PSE in combination with the T/A motif was not sufficient for pol III activity in vitro (see Figure 3), implicating regions either upstream of the PSE or downstream of −4. It is known that the initiation site has a role in Xenopus U6 transcription (30), so the effect of the start site on polymerase specificity was investigated. Inclusion of the U6 start site region in the TK.U6.β construct had little effect in vivo. The promoter was still largely transcribed by pol II although the start site region enhanced transcription levels, acting in a manner reminiscent of the recently described pol II 'Initiator' element (41). All that is lacking from the U6 promoter in the start site-containing construct is the DSE. This, together with the previous results discussed above, suggests that the pol III specificity of U6 transcription might involve all the elements of the intact promoter.

Since the PSE, T/A motif and +1 region form a basal pol III promoter, it seems likely that the strong pol II transcription of the TK.U6st.β globin construct is partly due to the presence of the Sp1 and CAAT upstream elements of the TK promoter, which presumably influence the formation of the transcription complex on the basal promoter in such a way as to favour pol II selectivity. In summary, the results presented here suggest that the RNA polymerase specificity of the U6 promoter, and perhaps also of other promoters, is, like cell-type or developmental stage-specific expression, the result of complex interactions between several different promoter binding factors.

Multiple TATA-binding factors?

It has recently been proposed that the functional diversity of TATA sequences in eukaryotic pol II promoters may reflect the existence of more than one TATA binding factor (42). Examples include two TATA elements in the yeast his3 promoter which

differ with respect to sequence, initiation site selectivity, and interaction with upstream activator sequences (12, 43). In mammalian cells heterogeneity of TATA function was first suggested by experiments with an hsp70 promoter: the consensus TATAAA present in this promoter supported both heat induction and responsiveness to adenovirus E1A transactivation, whereas in the presence of a non-consensus sequence, TATTTAT, heat inducibility was retained but E1A responsiveness was lost (44). E1A transactivation of the c-fos promoter is similarly dependent on a consensus TATA box (45). These and other results support a model in which multiple TATA-binding factors carry out a common function, facilitating transcription complex assembly, but having distinct sequence specificities, and presumably different abilities to interact with activating transcription factors (46).

At first sight our results would support this model, and extend it by suggesting the existence of a factor with TATA-binding activity but RNA polymerase III activation specificity. The ability of the U6 TATA-like motif to interact with a range of different promoter elements in activating pol III transcription (47) is further indirect evidence in support of this hypothesis. The different response to mutation of the U6 TATA-like element in pol III and pol II contexts would indicate that the pol III TATA-binding factor has a binding specificity that is not identical with that of pol II TATA-binding factors, including the only characterised member of this putative family, TFIID.

However, there is an alternative explanation for all of these results which cannot be ruled out at present. It may be that one factor recognizes all TATA sequences, but its conformation is altered differently on binding to different sequences in ways which facilitate a diverse range of protein-protein interactions. The selectivity of activation seen with different TATA elements would then result from the ability of the same factor to make different, conformation-dependent, interactions. In support of this, several groups have recently cloned a TFIID gene from yeast, but no evidence for a family of related genes has been reported. In addition, it has been shown that purified yeast TFIID can at least bind to, and activate transcription in vitro from, promoters with non-consensus TATA boxes (48).

As yet we have no biochemical evidence in support of the existence of a pol III specific TATA binding factor. Experiments to determine whether TFIID is required for U6 transcription in vitro have proven inconclusive since we lose U6 transcriptional activity early during biochemical fractionation by the methods attempted so far (data not shown). To definitively resolve whether U6-like genes utilise a pol III-specific TATA binding protein or are able to form hybrid transcription complexes with contributions from pol II-associated factors nevertheless requires the development of biochemical procedures to separate and characterise the factors required for U6 transcription. We are pursuing this goal.

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