

Development of an inducible pol III transcription system essentially requiring a mutated form of the TATA-binding protein

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ABSTRACT

We attempted to devise a transcription system in which a particular DNA sequence of interest could be inducibly expressed under the control of a modified polymerase III (pol III) promoter. Its activation requires a mutated transcription factor not contained endogenously in human cells. We constructed such a promoter by fusing elements of the β -lactamase gene of *Escherichia coli*, containing a modified TATA-box and a pol III terminator, to the initiation region of the human U6 gene. This construct functionally resembles a 5'-regulated pol III gene and its transcribed segment can be exchanged for an arbitrary sequence. Its transcription *in vitro* by pol III requires the same factors as the U6 gene with the major exception that the modified TATA-box of this construct only interacts with a TATA-binding protein (TBP) mutant (TBP-DR2) but not with TBP wild-type (TBPwt). Its transcription therefore requires TBP-DR2 exclusively instead of TBPwt. In order to render the system inducible, we fused the gene coding for TBP-DR2 to a tetracycline control element and stably transfected this new construct into HeLa cells. Induction of such a stable and viable clone with tetracycline resulted in the expression of functional TBP-DR2. This system may conceptually be used in the future to inducibly express an arbitrary DNA sequence *in vivo* under the control of the above mentioned promoter.

INTRODUCTION

A primary goal of this project was to design an RNA polymerase III (pol III)-dependent promoter which was dissociated from the cellular transcription machinery and could be activated only by the expression of a transcription factor absent from a normal cell. This promoter would then form the basis for an inducible pol III system in mammalian cells.

In higher eukaryotic cells, three general types of pol III promoters have been distinguished (1). The type 1 promoter

(5S rRNA genes) comprises gene internally located A- and C-boxes as well as an intermediate element. Transcription from this promoter requires TFIIA, TFIIC1, TFIIC2, TFIIB β and pol III. Type 2 promoters (e.g. tRNA genes, VA RNA genes and Alu-sequences) are characterised by gene internal A- and B-boxes. Expression of these genes requires the same transcription factors as the type 1 promoter with the exception of TFIIA (2–5). Type 3 promoters are located 5' of the transcription initiation site and are composed of a TATA-box, a proximal sequence element (PSE) and a distal sequence element (DSE) (6). Specific initiation of transcription from type 3 promoters depends on the PSE binding protein (PBP) (7), also designated as PTF (8–10) or SNAPc (11,12), TFIIB α (5), TFIIU (13), pol III and the TATA-binding protein (TBP) (7,14), which in this case is not part of a TBP-TAF complex.

As described previously, hTFIIB can be separated into two functionally different forms, TFIIB α and TFIIB β . hTFIIB β is predominantly active on genes with intragenic promoters and contains TBP and associated proteins, whereas TFIIB α is a TBP-free entity, which must be supplemented with TBP for *in vitro* transcription of the U6 snRNA gene (5).

Strubin and Struhl (15) examined mutants of the TBP molecule from yeast and human, which show a modified DNA binding specificity due to an exchange of three amino acids. The three substitutions are located in a region of 12 amino acids within the first or second repeat (direct repeat 1 and direct repeat 2, respectively) in the highly conserved C-terminal part of the protein. Based on the numbering in yeast, the amino acids at positions 194 (Ile), 203 (Val) and 205 (Leu) were replaced by Phe, Thr and Val. Both the human and the yeast TBP mutants were able to promote RNA synthesis from promoters containing a TGTAAG-box instead of the wild-type TATA-box sequence (consensus sequence TATAAA), while these TATA-box mutants were not transcribed in a system containing only the wild-type TBP protein. The high specificity of these TBP mutants and similar mutants of the *Arabidopsis* TBP-isoform for the TGTAAG sequence could also be demonstrated in plant cells (16).

We initiated studies to analyse whether these TBP mutants would also recognise various analogous TATA-box mutants of the human U6 gene in a reconstituted human pol III transcription system *in vitro*.

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The long term aim of these studies was to establish an *in vivo* system in which the expression of a mutated variant of TBP could be induced, thereby leading to the modulated RNA synthesis of a particular sequence (for example, antisense RNA or double-stranded RNA possibly involved in post-transcriptional gene silencing) under the control of such a mutated TATA-box. The advantages of the RNA polymerase III expression system are the high rate of synthesis with multiple rounds of initiation and the efficient termination of pol III catalysed RNA synthesis. We report here on the first steps toward this goal.

MATERIALS AND METHODS

Plasmids

The plasmids pUVAI and pUuH6_{0,35} were as previously described (7,17) and contained single copies of the genes coding for VAI RNA and human U6 snRNA respectively.

Oligonucleotides

17mer reverse sequencing-primer (-26-M13/pUC) and 17mer sequencing-primer (-20-M13/pUC) (Roche Diagnostics, Mannheim, Germany). P_{DR2}467–490, 5'-GTCCTCCGATC-CCGTTCTTCGGGG-3'; P_{DR2}-5', 5'-GCATTTTGAATTCCT-GTTTGAGCTCACCCAG-3'; hU6-5', 5'-GGACGAAACAC-CGTGCACGCTTCGGC-3'; hU6_{0,35}-29<<, 5'-AGATGTAAGCAAGAAATCGAAATACT-3'.

Buffers

Buffer A contained 20 mM HEPES pH 7.9, 10% (v/v) glycerol, 3 mM DTT and 0.2 mM PMSF. Buffer B contained 20 mM Tris-HCl pH 7.9, 10% (v/v) glycerol, 5 mM MgCl₂, 3 mM DTT and 0.2 mM PMSF. All fractions employed were dialysed against buffer B supplemented with 60 mM KCl before use, except for the polymerase fraction (see below).

Preparation of cytoplasmic extract (HeLa S100) and purification of transcription factors

HeLa cytoplasmic extract (HeLa S100) was prepared as described by Weil *et al.* (18). The S100 extract was dialysed against buffer A supplemented with 100 mM KCl and was subsequently chromatographed through phosphocellulose as described previously (19).

The PCC fraction (350–600 mM KCl) was used to prepare human TFIIC₁, TFIIC₂ and PBP as described previously (20) with the following modifications.

PBP. The PCC fraction was dialysed against buffer B supplemented with 60 mM KCl, loaded onto a Mono Q column ('MQ'; Amersham-Pharmacia) at 5 mg protein/ml bed volume. After washing the column with the same buffer, bound proteins were eluted with a linear gradient of 60–450 mM KCl. Fractions eluting at 300–330 mM KCl contained PBP and were devoid of TFIIC₁ and TFIIC₂, as well as of TFIIB α and RNA pol III.

hTFIIC₁. Fractions eluting at 250–290 mM KCl in buffer B from Mono Q (20) were diluted to 200 mM KCl in buffer B and applied to an EMD-SO₃⁻ Fractogel column ('ESF'; Merck, Darmstadt, Germany) at 5 mg protein/ml bed volume. After washing the column with 200 mM KCl in buffer B, bound

proteins were eluted with a linear gradient of 200–600 mM KCl and a 1 M KCl step. hTFIIC₁ activity eluted at 450–500 mM KCl.

TFIIU. Fractions eluting at 150–190 mM KCl in buffer B from the MQ gradient (TFIIC₀) (20) were analysed in *in vitro* transcription assays and were applied to an EMD-SO₃⁻ Fractogel column at 5 mg protein/ml bed volume. After washing the column with 200 mM KCl in buffer B, bound proteins were eluted with a linear salt gradient of 200–600 mM KCl. The activity, which stimulates U6 transcription (TFIIU) eluted at 360–420 mM KCl.

The PCB fraction (350 mM KCl) was used to prepare human TFIIB α , TFIIB β and RNA polymerase III (pol III) by EMD DEAE Fractogel chromatography as described previously (20,21). TFIIB β was further purified by EMD-SO₃⁻ Fractogel chromatography as described (20). The polymerase fraction was also applied to EMD-SO₃⁻ Fractogel and was step-eluted. The polymerase activity eluted in the 400–650 mM KCl fraction and was free of TFIIB β , TFIIC₁ and TFIIC₂ activities. BSA (0.1 mg/ml) was added and the polymerase fraction was diluted with glycerol to a final concentration of 50% (v/v) glycerol. All fractions were stored at –80°C.

Recombinant human TBP (rhTBP) was expressed in *Escherichia coli* and purified as described (22).

In vitro transcription

The *in vitro* transcription mixtures contained the appropriate protein fractions, 1 μ g plasmid DNA, 600 μ M ATP, CTP, UTP, 30 μ M GTP and 3 μ Ci [α -³²P]GTP (Amersham-Pharmacia), 20 U RNase Block Ribonuclease Inhibitor (Stratagene), 60 mM KCl, 20 mM Tris-HCl pH 7.9, 10% glycerol and 5 mM MgCl₂ in a final volume of 50 μ l. After 90 min incubation at 30°C, the RNA was purified and loaded onto a denaturing 7 M urea–6% polyacrylamide gel. The gel was analysed by autoradiography for at least 1 day at –80°C with intensifying screens.

Primer extension assay

In vitro transcription using the primer extension assay was performed as described (23). For the primer extension analysis of the P_{DR2} gene, a specific primer (P_{DR2}467–490) hybridising against the RNA between position 62 and 85 (relative to the first G of the 'Ini' sequence) was used.

Western blotting

For the screening of the HeLa TBP-DR2 clones, cells were harvested and counted 48 h after doxycycline induction. Cells (5 \times 10⁵) were resuspended in 50 μ l 1 \times SDS sample buffer, treated with 250 U BenzonaseTM (Merck) and subsequently fractionated on SDS–polyacrylamide gels (12.5%). Proteins were electrophoretically transferred to a PVDF membrane (Immobilon-P; Millipore) using semi-dry electroblotting as described by Kyhse-Andersen (24). After transfer, the membrane was stained with Ponceau S solution (Serva), destained and blocked with 10% skimmed milk powder in PBS/Tween. For detection of TBP containing a histidine tag, a monoclonal anti-histidine antibody (anti-RGS-His; Qiagen) was employed. Antigen–antibody complexes were detected by the ECL system (Amersham-Pharmacia).

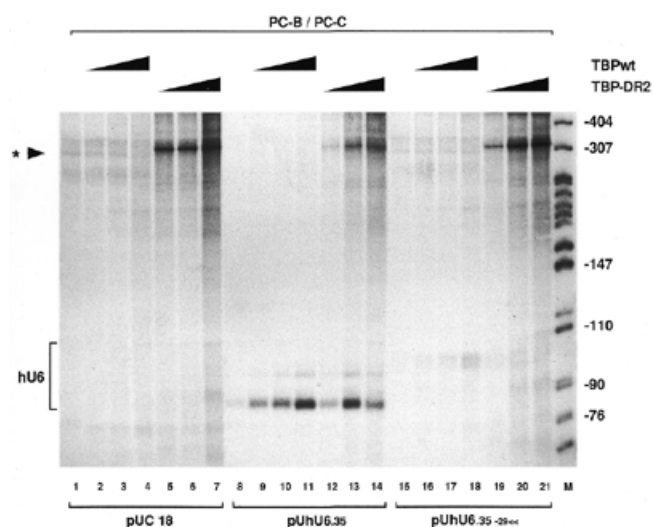


Figure 1. *In vitro* transcription of a U6 gene containing a variant TATA-box. *In vitro* transcription systems based on 10 μ l phosphocellulose fraction B and 20 μ l phosphocellulose fraction C (lanes 1–21) containing either pUC18 (lanes 1–7), pUhU6_{0.35} (lanes 8–14) or pUhU6_{0.35-29<<} (lanes 15–21) as template, were supplemented either with increasing amounts (5–20 ng) of recombinant human TBPwt (lanes 2–4, 9–11 and 16–18) or recombinant human TBP-DR2 (lanes 5–7, 12–14 and 19–21), respectively. Lanes 1, 8 and 15 contained no recombinant TBP. Lanes 2, 5, 9, 12, 16 and 19 were supplemented with 5 ng, lanes 3, 6, 10, 13, 17 and 20 with 10 ng, and lanes 4, 7, 11, 14, 18 and 21 with 20 ng recombinant TBPwt or TBP-DR2 respectively. *In vitro* transcription was carried out using 1 μ g plasmid DNA as template. The position of the U6 transcripts is indicated and the position of the TBP-DR2 dependent transcript is marked with an asterisk.

DNase I footprint analysis

DNA fragments were generated by PCR amplification of pUP_{DR2-Expression} with 5'-end-labelled 17mer reverse sequencing-primer and unlabelled 17mer sequencing-primer (M13/pUC; Roche Diagnostics). Individual protein fractions were preincubated with 1 μ g of an equimolar mixture of the 110 and 147 bp pUC/*Hpa*II fragments, as non-specific competitor, in a total volume of 50 μ l for 15 min at 30°C. Subsequently, 15 000 c.p.m. of the labelled fragment was added and incubation continued for a further 45 min at 30°C. Samples were digested with 40 ng DNase I for 1 min at room temperature. After termination of the reaction by addition of an equal volume of 900 mM ammonium acetate, the DNA was purified as described (20,25) and finally electrophoresed on 6% (w/v) denaturing sequencing gels. After the run, urea was eluted from the gel, which was dried and autoradiographed overnight at –80°C with an intensifying screen.

For an exact determination of the DNase I protection pattern, a sequencing reaction using the 17mer reverse sequencing-primer (M13/pUC) was conducted in parallel.

Stable transfection of HeLa Tet-on cells

Transfection of HeLa Tet-on cells (Clontech) was accomplished using the 'Effectene' transfection reagent (Qiagen) according to the manufacturer's instructions. In order to be able to select positive clones, cells were co-transfected with a hygromycin-resistance plasmid serving as a selection marker.

Cloning of P_{DR2} constructs

pUP_{DR2}. A 43 bp long *Sau*3A fragment of the β -lactamase gene, containing a pol III termination signal was isolated from pBR322 and subcloned into a *Bam*HI linearised pUC18 plasmid. The resulting clones were verified for the correct orientation of the insert by restriction analysis. The plasmid containing a pol III termination sequence, was designated as pUT_V and was used as vector for the subcloning of the TBP-DR2 dependent promoter. For this, the 400 bp long *Eco*RI–*Xmn*I fragment of pBR322 containing the TBP-DR2 dependent promoter was cloned into the 'termination'-vector (pUT_V) which had been digested with *Eco*RI/*Sma*I. This two-step cloning procedure was required to shorten the intervening sequence between the P_{DR2} promoter and the pol III termination signal (Fig. 2).

pUP_{DR2}/hU6. Both sequence parts were amplified in two separate reactions by PCR. The two plasmids pUP_{DR2} and pUhU6_{0.35} were used as templates for this reaction. The amplification of the P_{DR2} promoter was accomplished using the two oligonucleotides P_{DR2}-5' and P_{DR2}467–490. Two point mutations were inserted into the amplified promoter fragment using the P_{DR2}-5' primer, resulting in the creation of an *Eco*RI restriction site in this region. Subsequently, the internal hU6 sequence was amplified with the oligonucleotides hU6-5' and pUC/M13 sequencing. By using a point mutation within the oligonucleotide hU6-5' the U6 initiation region was transferred into the 'Ini' sequence of the P_{DR2} promoter thereby creating an *Alw*44I recognition sequence. After amplification, both fragments were cut by *Alw*44I in the initiation region and subsequently ligated by the resulting sticky ends. Thus, the P_{DR2} initiation sequence was restored and now contained the coding sequence and the terminator of the hU6 gene in its 3' region. Subsequently, the resulting fragment was digested with *Eco*RI/*Hind*III and cloned into the vector plasmid, which had also been linearised by *Eco*RI and *Hind*III digestion.

P_{DR2-Expression}. This cloning was also initiated from a promoter fragment amplified with the P_{DR2}-5' and P_{DR2}467–490 oligonucleotide. Subsequently, the resulting fragment was digested with *Alw*44I in the proximity of the initiation region. The recessed 3' terminus of the double-strand was filled-in with DNA polymerase I large fragment (Klenow fragment) resulting in a DNA fragment with a blunt end. Subsequently, this fragment was cut in the 5' region of the promoter at the *Eco*RI restriction sequence inserted by the two point mutations of the P_{DR2}-5' oligonucleotide as described above. This fragment was inserted into the pUT_V terminator plasmid (see above), which had been linearised with *Eco*RI and *Ecl*136I (blunt). The short sequence from the pUC18 multiple cloning site, which is located between the *Ecl*136I restriction sequence and the termination signal of the vector (pUT_V), lies in the so-called P_{DR2-Expression} construct between promoter (P_{DR2}) and terminator (T_V).

Site-directed mutagenesis

Different mutants of the human U6 gene were created in the region of the TATA-box by site specific mutagenesis using asymmetric PCR with a single mutant oligonucleotide (26).

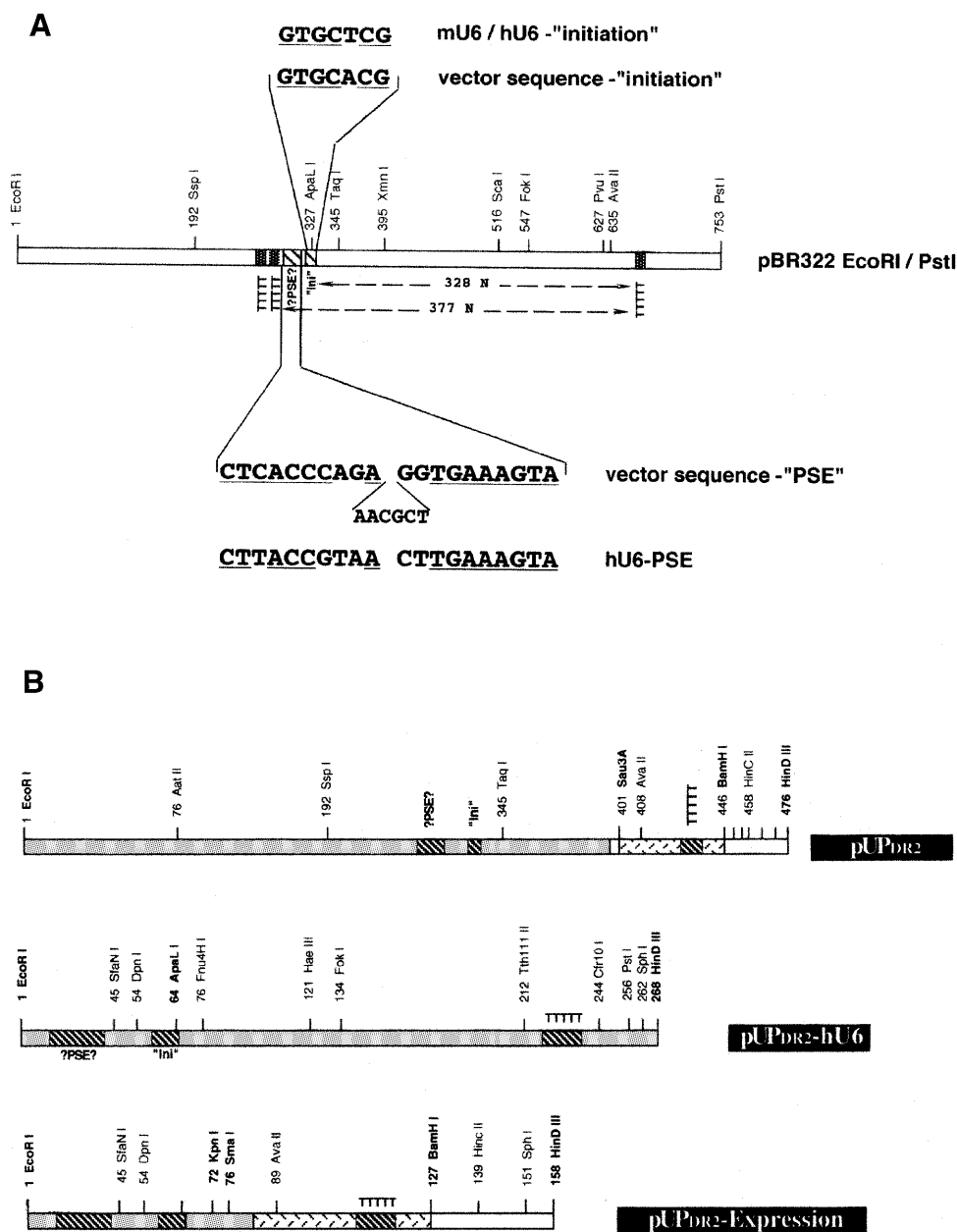


Figure 2. Structure of the TBP-DR2-dependent promoter in the region of the β -lactamase gene in pBR322 and constructs derived therefrom. (A) Schematic restriction map of the *EcoRI*-*PstI* fragment of pBR322 containing the assumed origin of the TBP-DR2-dependent transcript. (B) Restriction map of the *EcoRI*/*HindIII* fragments of the three different TBP-DR2-dependent promoter constructs named pUP_{DR2}, pUP_{DR2}/hU6 and pUP_{DR2}-Expression. The regions homologous to known RNA polymerase III promoter elements like the PSE element (?PSE?), the U6 initiation region ('ini') and the pol III termination signal (TTTTT) are indicated.

RESULTS

Transcription of different TATA box mutants of the hU6 gene

In order to establish a transcription system independent of endogenous wild-type TBP (TBPwt), we used mutated forms of TBP (TBP-DR2 or TBP-DR1) instead of TBPwt to transcribe the human U6 gene *in vitro*. For this purpose, the sequence of the human U6 TATA-box was accordingly changed to TGTA AAA by PCR *in vitro* mutagenesis. We

created various constructs containing the TGTA AAA-mutation in different orientation and/or distance with respect to the initiation region and examined whether these TATA-box mutants led to a reduction of the transcription efficiency of their associated gene sequences. Figure 1 (lanes 15–21) shows one of the TATA-box mutants with the sequence TGTA AAA in the orientation corresponding to that of the wild-type U6 gene. We found that the mutation of the wild-type TATA-box leads to a significant reduction of the transcription efficiency (compare lanes 8–14 with 15–21) and, in some cases, no hU6

RNA could be detected. This loss of transcription was independent from the orientation of the TGTA-box (data not shown).

At the same time, we analysed whether these mutations of the TATA-box could be suppressed by addition of one of the mutated TBP variants, DR1 (data not shown) or DR2. Addition of either recombinant TBPwt (Fig. 1, lanes 16–18) or recombinant TBP-DR2 (Fig. 1, lanes 19–21), to a transcription system containing phosphocellulose fractions PCB/PCC, was unable to activate the transcription of this mutated U6 gene. This failure was not due to inactive preparations of the recombinant proteins, because both forms of TBP were able to activate transcription of the hU6 wild-type gene (Fig. 1, lanes 8–14). Therefore, the expected suppression of this TATA-box mutation by co-mutation of the TBP molecule does not occur in the human pol III system, as was shown for yeast and plants.

Because of the failure of the reported TATA-box mutations to respond to the co-mutated TBP variants, we attempted to identify other sequence variants of the TATA-box that could fulfil this requirement. We fortuitously discovered such a sequence within the plasmid pUC18. We found that the supplementation of the PCB/PCC reaction mixture with TBP-DR2 (Fig. 1, lanes 5–7, 12–14 and 19–21) resulted in an ~320 bp transcript, the synthesis of which was strictly TBP-DR2 dependent. This transcript (shown by an asterisk in Fig. 1) was synthesised independently of the pol III promoter subcloned into the polylinker of the pUC-plasmid and could also be detected using only pUC18 (Fig. 1, lanes 1–7) or pBR322 (data not shown) as template for the transcription reaction *in vitro*.

Identification and cloning of a promoter sequence that depends on TBP-DR2 for its expression

Subsequently, we identified the DNA sequence in the pBR322 plasmid, from which the TBP-DR2 dependent transcript was synthesised. For this purpose, sequence sections at least 320 bp (the length of the transcript; Fig. 2A) upstream of at least four thymidine residues (pol III termination signal), were examined for homologies to the promoter sequences of the hU6 and mU6 gene respectively.

In the region of the β -lactamase gene of pBR322, which conveys ampicillin resistance, a 7 bp long sequence was identified, in which six nucleotides were identical to the hU6 and mU6 initiation region (Fig. 2A). This sequence is located ~320 nt upstream of a typical pol III termination signal. Additionally, a sequence section, homologous to the PSE-element of the hU6 and mU6 gene, is located 23 bp upstream of this region (Fig. 2A).

To examine the TBP-DR2 dependence of the transcribed sequence and its promoter in more detail, this DNA segment was subcloned into the polylinker of pUC18. At the same time, the sequence between the initiation region and the terminator was shortened, in order to be able to differentiate the vector transcript (shown by an asterisk in Fig. 1) from the RNA synthesised from this subcloned sequence. This was achieved in two separate steps, as described in Materials and Methods.

This plasmid (Fig. 2B) contained the promoter and the termination signal at a distance of ~100 bp from each other and it was designated as pUP_{DR2} (P_{DR2} = DR2 dependent promoter).

Functional investigations of the cloned P_{DR2} construct

Since we were dealing with an artificial promoter construct, it was necessary to verify that it would respond to TBP-DR2 in a

predictable and functionally meaningful manner. In particular it was of fundamental importance to verify by DNase I protection assays that the mutated TBP-DR2, but not TBPwt, would bind to this artificial promoter.

Comparison of the binding of TBP-DR2 and TBPwt to the non-coding strand of the P_{DR2} promoter

The analysis of the TBP-DR2 binding to the non-coding strand of the P_{DR2} promoter is shown in Figure 3A. Samples 1 and 5 served as control reactions and contained no protein, while samples 2–4 contained increasing amounts of TBP-DR2.

The TBP-DR2 footprint on the non-coding strand of the P_{DR2} promoter extends over a length of at least 15 (from –20 to –34) and maximally 23 nt (from –20 to –42) and is bordered by hypersensitive sites at –14 and –43/–45 of the promoter sequence.

Figure 3B shows the comparative binding of TBP-DR2 and TBPwt to the same DNA fragment. The samples without TBP are shown in lanes 1 and 6. While TBP-DR2 (lanes 2 and 3) exhibits the DNase I protection pattern already shown in Figure 3A, no protection occurred with TBPwt (lanes 4 and 5). Also, no hypersensitive sites, which could reflect the binding of a protein, appeared.

We also performed footprint analyses on the coding strand of the P_{DR2} promoter (data not shown). The binding region of TBP-DR2 on this strand spans a sequence of 15 nucleotides from –20 to –34 relative to the transcription start point. For TBPwt, again no binding to this promoter could be detected.

In summary (Fig. 3C), a corresponding sequence of 15 nucleotides on both strands of the P_{DR2} promoter represents the binding region of TBP-DR2. This extends from position –20 to –34 relative to the transcription start point. The sequence region protected by TBP-DR2 against DNase I digestion possibly extends up to nucleotide –42 on the non-coding strand. For TBPwt, no interaction with the P_{DR2} promoter DNA could be observed.

In vitro transcription from the P_{DR2} promoter

In addition to the binding studies reported above, it was essential to prove by *in vitro* transcription that the strict TBP-DR2 dependency of the reported constructs had been retained.

As shown in Figure 4A, the dependence of the P_{DR2} transcription on TBP-DR2 was not abolished as a consequence of the subcloning. A specific signal could be detected only in the presence of TBP-DR2 (lanes 5–7). In contrast, P_{DR2} RNA was not detectable, either without (lane 1) or with rising quantities of TBPwt (lanes 2–4), whereas an efficient RNA synthesis occurred even with the smallest quantity of TBP-DR2 (lane 5). The P_{DR2} transcript shows the expected length of ~100 nt. At the top of the gel shown in Figure 4A, one can again recognise the vector transcript of ~320 nt length, which is likewise synthesised in a TBP-DR2 dependent fashion as already discussed.

In order to ensure that the P_{DR2} RNA is a transcript of RNA polymerase III, three transcription samples containing fraction PCB, PCC and TBP-DR2 were analysed in the presence of the inhibitor α -amanitin (Fig. 4B). The result shows that the low α -amanitin concentration (2 μ g/ml; Fig. 4B, lane 2) had no influence on the P_{DR2} signal and the long vector transcript (shown by an asterisk in Fig. 4B) in comparison to the control reaction (lane 1). With the high α -amanitin concentration

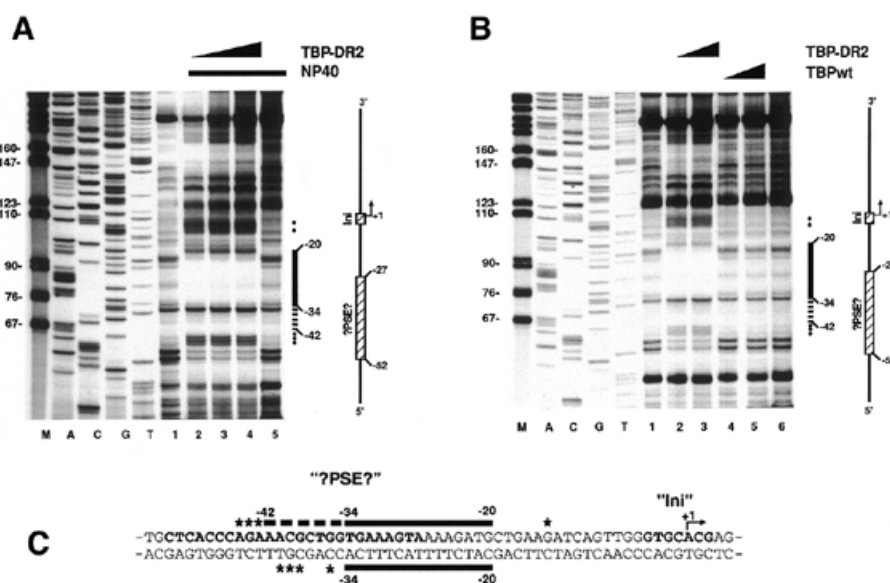


Figure 3. Footprint of TBP-DR2 and TBPwt on the non-coding strand of the P_{DR2} promoter. For the investigation of the non-coding strand, a DNA fragment was used, which was amplified by PCR with the M13 sequencing-primer and the 5'-labelled M13 reverse-sequencing-primer using the P_{DR2-Expression} plasmid (Fig. 2B) as template. To be able to identify the region protected by TBP-DR2 a sequencing reaction was conducted in parallel (lanes A, C, G and T). (A) Binding of TBP-DR2 to the P_{DR2} promoter. Increasing amounts of TBP-DR2 (10, 20 and 40 ng; lanes 2–4) were incubated with the 5'-labelled P_{DR2} fragment. The control reactions containing no TBP-DR2 (free DNA) are shown in lanes 1 and 5. Lanes 2–5 additionally contained NP-40 at a very low concentration (0.0025%). By this procedure, DNase I protection by TBP-DR2 can be detected with smaller protein quantities and the digestion pattern of DNA is not affected even by higher NP-40 concentrations (data not shown). (B) Comparison of TBPwt and TBP-DR2. Either TBP-DR2 (lane 2, 20 ng; lane 3, 40 ng) or TBPwt (lane 4, 20 ng; lane 5, 40 ng) were incubated as described in (A) but contained no NP-40. Lanes 1 and 6 served as control reactions without protein. (C) Summary of the TBP-DR2 binding to the coding and non-coding strand of the P_{DR2} promoter sequence. The sequence sections homologous to the hU6 gene (?PSE? and Ini) are written in bold characters. Filled circles and asterisks signify hypersensitive sites in the footprint and schematic representations respectively.

(300 µg/ml; Fig. 4B, lane 3), however, both specific signals (P_{DR2}, and as shown by an asterisk) disappeared, thus proving that the examined transcripts are synthesised by RNA polymerase III (27).

Determination of the initiation nucleotide

On the basis of the sequence homology to the U6 initiation region and its distance to the terminator, which corresponds to the length of the P_{DR2} transcript, it was assumed that the sequence section, homologous to the U6 initiation region is the transcription start point of P_{DR2} RNA. In order to prove this assumption and to determine the starting nucleotide ('+1') exactly, primer extension experiments were conducted (Fig. 5). All samples were treated with DNase I before the hybridisation reaction, in order to digest the template DNA. Transcription samples containing either PCB/PCC or PCC/TFIIIB α fractions were supplemented with TBP-DR2 in lanes 3 and 5. Sample 1 contained no protein but DNA, nucleotides and RNase-inhibitor and served as a control for non-specific primer/plasmid–DNA hybrids.

The reverse transcript (RT) reveals a double band. These two specific RT signals arise in all samples containing TBP-DR2 (Fig. 5, lanes 3 and 5). The signal strength depends on the transcription system used and shows a higher transcription efficiency with the TFIIIB α fraction (lane 5) than with the PCB fraction (lane 3).

The alignment of the two RT signals with a sequencing reaction conducted in parallel shows that both 3' ends of the RTs correspond to a thymidine residue in the P_{DR2} sequence of the

coding strand. It can be assumed that the longer of both signals reflects the complete reverse transcript. It is very unlikely that these two signals were caused by an alternative initiation process, because in the *in vitro* transcription reaction only one signal could be detected (Fig. 4A, lanes 5–7).

Construction and functional characterisation of additional TBP-DR2-dependent vector variants

One objective of the investigations concerning the P_{DR2} promoter was to establish an inducible, pol III-catalysed transcription system suitable for the expression of an RNA sequence of interest. Therefore, it was necessary to determine whether an exchange of the transcribed region downstream of the P_{DR2} promoter would show any influence on the transcription efficiency. We therefore replaced the artificial P_{DR2} coding sequence by the coding sequence of the human U6 gene (P_{DR2}/hU6). Additionally, a vector was designed which enabled any sequence between the P_{DR2} promoter and terminator to be cloned, with the aim of expressing such a sequence *in vivo* only in the presence of TBP-DR2 (P_{DR2-Expression}). A schematic representation of the resulting constructs P_{DR2}/hU6 and P_{DR2-Expression} is shown in Figure 2B.

These new constructs were subsequently examined particularly with respect to their strict dependence on TBP-DR2. In Figure 6, the *in vitro* transcription of pUP_{DR2}, pUP_{DR2-Expression} and P_{DR2}/hU6 were compared to each other. In the presence of TBP-DR2, all templates were transcribed efficiently and specifically. As expected, the length of the specific transcripts varied according to the size of the individual constructs used (lanes 2, 5 and 8).

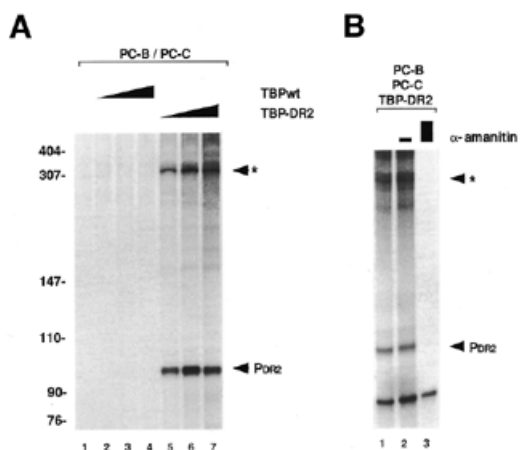


Figure 4. Functional investigations of the cloned P_{DR2} construct. The sequence section of the pBR322 plasmid with homologies to the mammalian U6 promoter was subcloned into the multiple cloning site of pUC18 and subsequently analysed by transcription experiments *in vitro*. (A) Dependence of the P_{DR2} clone on TBP-DR2. A transcription system, based on phosphocellulose fractions PCB and PCC (lanes 1–7), corresponding to the reconstituted transcription system shown in Figure 1, was supplemented either with TBPwt (lanes 2–4; 5, 10 and 20 ng) or TBP-DR2 (lanes 5–7; 5, 10 and 20 ng). Lane 1 shows the control sample without additional recombinant human TBP. The asterisk indicates the TBP-DR2 dependent vector transcript of ~320 nt. The transcript resulting from the subcloned promoter is marked by P_{DR2} . (B) α -Amanitin characterisation of the P_{DR2} transcription *in vitro*. Three identical transcription samples contained 10 μ l PCB, 20 μ l PCC, 10 ng recombinant human TBP-DR2 and 1 μ g pUP $_{DR2}$ as template. The control sample without the inhibitor α -amanitin is shown in lane 1. The samples shown in lanes 2 and 3 contained α -amanitin at a concentration of 2 and 300 μ g/ml respectively. The P_{DR2} transcript is appropriately indicated. The signal at the bottom of the gel represents tRNA contained as a carrier.

Without addition of TBP-DR2 to the transcription reaction, or in the presence of TBPwt, none of these new constructs yielded a specific signal. This result clearly demonstrated that the strict requirement for TBP-DR2 for transcription from the examined promoter is not influenced by sequences located in its 3' region.

Transcription factors required for expression from the TBP-DR2-dependent promoter

To determine which transcription factors besides TBP-DR2 are required for expression from the P_{DR2} promoter, it was necessary to replace the relatively crude phosphocellulose fractions with more purified transcription factors. As a first step the PCB fraction was replaced by TFIIB α - or TFIIB β -containing fractions and transcription was compared to the hU6 gene as a control. Both the hU6- and the P_{DR2} genes were progressively transcribed by increasing amounts of TFIIB α (Fig. 7A, lanes 2–4 and 9–11) but not TFIIB β (lanes 5–7 and 12–14).

To examine which components of the PCC fraction were necessary for transcription of the P_{DR2} promoter, the PCC fraction was replaced by more purified factors contained therein. The participation of TFIIC1, TFIIU and PBP, purified by EDF and MonoQ chromatography was investigated by testing these factors in different combinations in a transcription system supplemented with TFIIB α , pol III and TBP-DR2 (Fig. 7B). In a reconstituted *in vitro* transcription assay (data not shown) it was shown that TFIIC2 does not play a role in the transcription of the P_{DR2} construct.

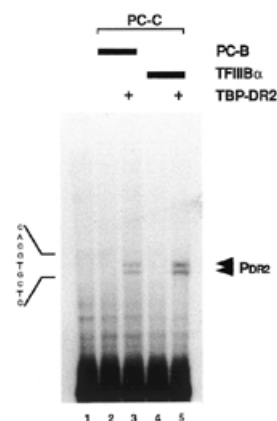


Figure 5. Primer extension analysis of the P_{DR2} RNA. Transcription samples containing either PCB/PCC (5/15 μ l; lanes 2 and 3) or TFIIB α /PCC (20/15 μ l; lanes 4 and 5) were tested with (lanes 3 and 5) or without (lanes 2 and 4) recombinant TBP-DR2 respectively. After purification of the resulting RNA the samples were hybridised with a radioactively labelled oligonucleotide (P_{DR2} 467–490) complementary to the P_{DR2} RNA. After the reverse transcription reaction the resulting cDNAs were separated on a 6% denaturing PAA gel in comparison with a sequencing reaction conducted with the same radioactively labelled oligonucleotide.

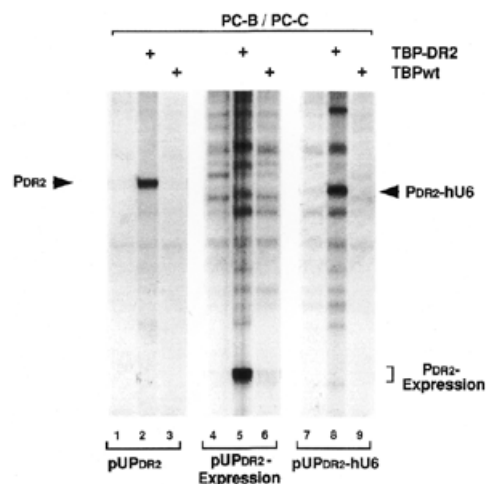


Figure 6. *In vitro* transcription of pUP $_{DR2}$, pUP $_{DR2}$ /hU6 and pUP $_{DR2}$ -Expression in the presence of TBPwt or TBP-DR2 respectively. *In vitro* transcription was performed and the positions of the individual transcripts are indicated. Three transcription samples of each template (lanes 1–3, pUP $_{DR2}$; lanes 4–6, pUP $_{DR2}$ -Expression; lanes 7–9, pUP $_{DR2}$ /hU6) were analysed in a transcription system based on 10 μ l phosphocellulose fraction B plus 20 μ l phosphocellulose fraction C (lanes 1, 4 and 7) and were supplemented with either 20 ng TBP-DR2 (lanes 2, 5 and 8) or 20 ng TBPwt (lanes 3, 6 and 9).

As was expected, the pol III/TFIIB α basal-system alone led to no specific RNA transcript (Fig. 7B, lanes 2 and 9 respectively). The individual addition of TFIIU- (lanes 3 and 10), TFIIC1- (lanes 4 and 11) or PBP-containing fractions (lanes 5 and 12) likewise resulted in no efficient RNA-synthesis. The combination of TFIIC1/PBP (lanes 6 and 13) resulted in only a low transcription activity from both genes. Efficient RNA synthesis was only observed after the combination of all three fractions (TFIIU, TFIIC1 and PBP; Fig. 7B, lanes 7 and 14).

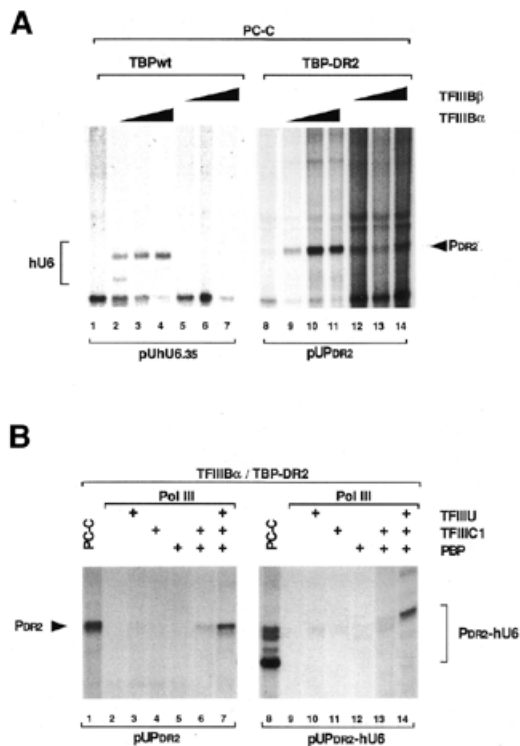


Figure 7. Transcription factors required for the efficient transcription from the TBP-DR2-dependent promoter. **(A)** Transcription of pUhU6_{0.35} and pUP_{DR2} *in vitro* in the presence of TFIIIBα or TFIIIBβ. Increasing amounts of a TFIIIBα fraction (lanes 2 and 9, 10 μl; lanes 3 and 10, 20 μl; lanes 4 and 11, 30 μl) or a TFIIIBβ fraction (lanes 5 and 12, 2.5 μl; lanes 6 and 13, 5 μl; lanes 7 and 14, 10 μl) were reconstituted with 15 μl phosphocellulose fraction C (lanes 1–14). The samples with pUhU6_{0.35} (lanes 1–7) were supplemented with 20 ng recombinant TBPwt (lanes 1–7) while the samples with pUP_{DR2} (lanes 8–14) contained 20 ng TBP-DR2. Control reactions without TFIIIB activity are shown in lanes 1 and 8 respectively. **(B)** Replacement of phosphocellulose fraction C by PBP, TFIIIC1 and TFIIIU in the *in vitro* transcription of pUP_{DR2} and pUP_{DR2}/hU6. Individual components of the PCC fraction were added in different combinations as indicated above each lane: PBP, 2 μg MQ-fraction; TFIIIC1, 0.4 μg ESF-fraction; TFIIIU, 1.8 μg ESF-fraction. The reactions were incubated and processed as described, using 1 μg of pUP_{DR2} (lanes 1–7) or 1 μg pUP_{DR2}/hU6 (lanes 8–14) as template. All reactions were supplemented with 20 ng recombinant TBP-DR2 and 20 μl TFIIIBα fraction. Lanes 1 and 8 show the positive control with 15 μl PCC fraction containing the TFIIIC1, pol III, PBP and TFIIIU activity. All other samples (lanes 2–7 and 9–14) contained 2 μl purified pol III.

From this, it can be concluded that all constructs containing the P_{DR2} promoter are expressed independently of their transcribed sequence and require the same factors (TFIIIBα, TFIIIU, TFIIIC1 and PBP) as the mammalian U6 gene.

Inducible expression of TBP-DR2 in HeLa cells

To establish an inducible RNA polymerase III expression system *in vivo*, a system for the modulated expression of TBP-DR2 was developed in HeLa cells. For this purpose a tetracycline (doxycycline) inducible mammalian expression system was used.

The DNA sequence coding for histidine tagged TBP-DR2 was cloned into a suitable expression plasmid under the control of the tetracycline responsive element (TRE). After transfection of HeLa Tet-on cells (28), positive cell clones expressing

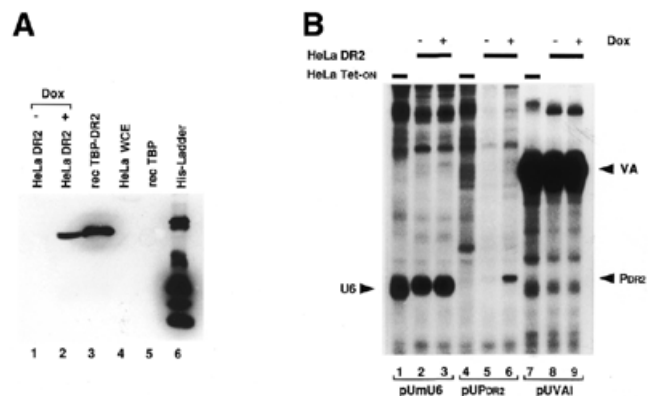


Figure 8. Tetracycline inducible expression of TBP-DR2 in HeLa cells. **(A)** Western blot analysis. Cellular extracts from uninduced (lane 1) and doxycycline-induced cells (lane 2) of one of the selected clones were probed with anti-histidine antibodies. Recombinant TBP-DR2 with a histidine tag (10 ng; lane 3), 25 μg HeLa whole-cell extract (WCE; lane 4) and 10 ng recombinant TBPwt without histidine tag (lane 5) served as controls. **(B)** Functional investigation of TBP-DR2 expressed in HeLa cells. To analyse whether the expressed TBP-DR2 was functionally active, 50 μg S100 from HeLa cells expressing TBP-DR2 was used to transcribe the P_{DR2} promoter (lanes 4–6) *in vitro* before (lane 5) and after (lane 6) doxycycline induction of these HeLa cells. Additionally, 50 μg S100 from untransfected HeLa Tet-on cells was used as a negative control (lane 4). As a control, all three extracts were simultaneously analysed by *in vitro* transcription of pUhU6_{0.35} (lanes 1–3) and pUVAI DNA (lanes 7–9).

TBP-DR2 in the presence of doxycycline, were identified by anti-histidine antibodies in a western blot.

Figure 8A shows that in the uninduced status no TBP with histidine tag could be detected (lane 1), while it could clearly be recognised after induction (lane 2). In the sample with recombinant histidine tag-TBP, serving as a positive control, a signal of identical molecular mass could be found (Fig. 8A, lane 3), whereas it was missing in the samples with HeLa whole-cell extract and those containing TBP without a histidine tag (lanes 4 and 5).

When testing for the entire TBP content with monoclonal anti-TBP antibodies, a specific signal could be detected in both conditions, i.e. in positive and negative controls (data not shown).

Finally the question was addressed whether the expressed TBP-DR2, containing the histidine tag, was functionally active (Fig. 8B). Cytoplasmic extracts from uninduced and induced HeLa-DR2 cells were analysed in an *in vitro* transcription assay using the P_{DR2} plasmid as template. Previous experiments had documented (see above) that this construct is transcribed only in the presence of active TBP-DR2. At the same time, a transcription reaction with the mU6 (Fig. 8B, lanes 1–3) or the VAI gene (lanes 7–9) was conducted. S100 from untransfected HeLa Tet-on cells was used as a negative control for transcription of the P_{DR2} construct.

With S100 from uninduced cells and the P_{DR2} gene as template, the specific transcript was barely visible (Fig. 8B, lane 5). Also, no transcript from the P_{DR2} gene was found in the absence of TBP-DR2 with HeLa-S100 as negative control (lane 4) while with S100 from induced HeLa-DR2 cells a transcript of the P_{DR2} gene could be detected (lane 6). The

transcription with pUVAI and pUmU6 also served to prove that the three examined extracts were comparably active and differences observed in the transcription efficiency of the P_{DR2} gene were not attributable to different activities of the extracts. This result clearly demonstrated that the TBP-DR2 protein, which is synthesised in HeLa-DR2 cells after doxycycline induction, is functionally active. A stable HeLa-DR2 clone was thus isolated, which is able to synthesise TBP-DR2 after doxycycline induction, and therefore can initiate transcription from the P_{DR2} promoter *in vitro*.

DISCUSSION

Identification of a promoter sequence which is exclusively controlled by TBP-DR2

In order to develop an inducible RNA polymerase III system we first constructed a pol III promoter, which can exclusively be activated by mutated TBP variants but not by TBPwt. We initially concentrated our efforts on the promoter of the human U6 gene, which offers several advantages. It represents a pol III gene showing a high rate of synthesis with multiple rounds of initiation and efficient termination. More importantly, it is entirely controlled by an upstream promoter containing a TATA-box, and does not pose the problems associated with gene internal pol III promoters, which could conceivably limit the expression of arbitrary DNA sequences under the control of such a promoter.

Based on the work of Strubin and Struhl (15) and Heard *et al.* (16) we attempted to construct an RNA polymerase III promoter which operated independently from TBPwt. In these reports, mutants of the TBP molecule from yeast and human were analysed, and showed an altered DNA binding specificity due to an exchange of three amino acids. In the yeast system (*Saccharomyces cerevisiae*; 15) as well as in plant cells (*Nicotiana plumbaginifolia*; 16), two variants of the TATA-box binding protein (DR1 or DR2) exhibited an altered specificity for several mutations of the TATA-box. The most efficient of these mutations was the sequence TGTAAG. The described mutants of TBP were able to suppress the TGTAAG mutation *in vivo*.

In order to obtain a promoter controlled by TBP-DR2, we constructed TATA-box mutants of the human U6 snRNA gene. Since an inverse orientation of the TATA-box has been described for the hU6 gene (29), one of these mutants contained a reverse TGTAAG-box (hU6_{0,35}^{-29<<}) corresponding to the orientation of the authentic TATA-box in the wild-type U6 gene. As expected, functional investigation of these TATA-box mutants by *in vitro* transcription showed a strong reduction of the transcription efficiency. However, none of these mutations could be suppressed by supplementation of the transcription reactions with either recombinant human TBP-DR1 or TBP-DR2.

A possible explanation for the differences between our results and those of Strubin and Struhl (15) and Heard *et al.* (16) may be related to the fact that the experiments were conducted in different species. For example, the distances of the TATA-box to other promoter elements and to the transcription start point vary between these different organisms (30). Because of the differences in the experimental systems, the functional characterisation of the TATA-box mutants

described here cannot be compared directly with those previously described (15,16).

In spite of the failure to exploit the established TATA-box mutants for our purpose, we continued our search for other variant TATA-box sequences. We were fortuitously led to a promoter sequence contained in the plasmid pUC18, which is efficiently transcribed by RNA polymerase III only in the presence of TBP-DR2 but which is completely refractory even to high concentrations of TBPwt. This transcript originates from a sequence located in the β -lactamase gene of pBR322, which shows a high degree of homology to the promoter of the mammalian U6 gene. Six of seven nucleotides in the initiation region of the U6 snRNA and the TBP-DR2 dependent promoter are identical and primer-extension analyses have confirmed that initiation of the DR2-RNA occurs in this sequence section.

The region of the P_{DR2} promoter which is homologous to the PSE element is divided into the typical two halves, previously described for the mammalian U6 genes by Simmen *et al.* (31) and Wanandi *et al.* (32). They are, however, separated by six nucleotides in the construct described here, and the potential PSE and the variant TATA-box partially overlap. We hence shifted the PSE to the 'wild-type' distance relative to the TATA-box but this had no effect on transcription efficiency (data not shown). Since the DSE has little effect on the *in vitro* transcription of the human U6 gene (7), the effect of this element was not further investigated in this context.

Functional characterisation of the P_{DR2} promoter by DNase I protection and *in vitro* transcription

The observed homologies led us to the assumption that the DR2-dependent promoter, although representing an artificial construct, may function as a quasi 5'-regulated RNA pol III promoter and at the same time may fulfil the prerequisites essential for our approach.

As a first step to verify its functional significance, the region of the P_{DR2} promoter to which TBP-DR2 binds was determined by footprint analyses. The section of the P_{DR2} promoter protected by TBP-DR2 spans 15 nucleotides on both strands of the P_{DR2} DNA and is bordered by nucleotides at -20 and -34 relative to the transcription start point. This binding region of TBP-DR2 lies in an AT-rich sequence, which is characteristic for the binding sequence of TBP (consensus sequence: TATAAA). The sequence in the middle of the identified binding site is AGTAAG. This differs only in one nucleotide from the recognition sequence published for TBP-DR2 (TGTAAG) in yeast and plant cells (15,16) and therefore could represent the specific recognition sequence for the interaction of TBP-DR2 with the artificial promoter outlined here. However, this still requires further investigation. The distance of this presumed TATA-box to the starting nucleotide of transcription is 24 nucleotides, which agrees with the distance of the TATA-box to the starting nucleotide in the hU6 promoter (30,33). It should be stressed, however, that binding of TBPwt to the P_{DR2} promoter could not be detected in any of the footprint experiments.

Besides the binding of TBP-DR2 to the P_{DR2} promoter it was shown that this protein also binds to the hU6 TATA-box and that the boundaries of DNase I protection are identical to those of the TBPwt footprint (data not shown). These data agree with those of Strubin and Struhl (15) showing the binding of the

mutated yeast TBP to the wild-type TATA-box of *S.cerevisiae* (TATAAA) and they support the observation that TBP-DR2 can replace the wild-type protein in the transcription of the hU6 gene *in vitro* (Fig. 1, lanes 12–14).

The transcription factors TFIIB α , TFIIU, TFIIC1 and PBP were found to be essential for the *in vitro* expression of the P_{DR2} constructs, which is in accordance with the factors required for the expression of the mammalian U6 gene (13).

These data support the conclusion that the P_{DR2} promoter, although representing an artificial construct, behaves like a type 3 pol III promoter. It employs the same transcription factors, except that it can only be activated by TBP-DR2 but not by wild-type TBP. It can therefore be concluded that one of the prerequisites initially postulated, namely the construction of a promoter allowing the specific expression of a desired sequence only in the presence of TBP-DR2, was fulfilled.

Transcription efficiency does not depend on sequences located downstream of the initiation region. This fact is of crucial importance for the potential employment of this promoter *in vivo* to synthesise a copy RNA from short DNA sequences corresponding to the length of a typical pol III gene and lacking an internal pol III termination signal. This system may be of particular interest for the expression of a short copy RNA, complementary to the sequence for the initiation of translation of a specific mRNA (antisense RNA; 34). Although requiring experimental verification, it is also conceivable to synthesise a short double-stranded RNA *in vivo* which corresponds to a sense and antisense sequence of an endogenous mRNA. The cognate mRNA is then degraded and the gene is silenced. This type of post-transcriptional gene silencing is called RNA interference (35,36). This sequence specific degradation of mRNA could prove to be useful for the analysis of gene function and/or the employment as an anti-viral or anti-tumor agent (37).

Expression of TBP-DR2 in HeLa cells after induction by tetracycline

For the modulated expression of TBP-DR2 in mammalian cells *in vivo*, a tetracycline-controlled system was used. The gene coding for TBP-DR2 was cloned into the TRE-plasmid directly behind the tetracycline-dependent CMV promoter, so that its expression could be regulated by the addition of tetracycline or doxycycline (pTRE/TBP-DR2). The cloned TBP-DR2 insert contained a translation start point as well as a histidine tag, enabling one to discriminate by western blot between the recombinant TBP-DR2 and the endogenous TBPwt. The subcloned vector construct (pTRE/TBP-DR2) was transfected into HeLa Tet-on cells expressing the reverse Tet-activator upon induction with tetracycline.

The selection of clones inducibly expressing TBP-DR2 was achieved in a western blot with antibodies against the histidine tag. Figure 8A shows one of the clones which synthesised TBP-DR2 after the induction with doxycycline, while this protein was barely detectable in uninduced cells. The functional analysis of this clone revealed that the induced expression of TBP-DR2 in HeLa cells yielded an intact protein. This was verified by *in vitro* transcription in cellular extracts from such cells proving that TBP-DR2 was capable of correctly recognising and activating the P_{DR2} promoter. The fact that transcription from the hU6 and VAI genes was not

altered after induction shows that TBP-DR2 does not exert a negative effect on the expression of these genes and supports the conclusion that it specifically activates the P_{DR2} promoter.

In the meantime a system has been developed, which will be used in future experiments to further minimise the expression level of the recombinant protein in uninduced cells. One of these systems contains a tetracycline-controlled hybrid protein as a silencer, which actively represses the transcription of the desired gene (38).

Future experiments, aimed at the *in vivo* application of the model system described here, will involve the fusion of potentially interesting DNA sequences to the P_{DR2} promoter and the stable integration of such constructs in cell lines. An important prerequisite for such experiments was to prove that the expression of TBP-DR2 had no influence on the viability of the transfected cells. We have shown that the stably transfected cells, inducibly expressing TBP-DR2, could be maintained for several months, hence proving that TBP-DR2 has no toxic effect on these cells.

In summary, we were able to generate a HeLa cell clone in which a modified form of the TBP (TBP-DR2) could be expressed upon induction. This transcription factor is essentially required for the RNA polymerase III catalysed expression governed by the P_{DR2} promoter. In the future, this system can be potentially employed for the regulated *in vivo* expression of a short RNA sequence (for example, antisense or double-stranded RNA), under the control of the P_{DR2} promoter.

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