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In vitro and *in vivo* analysis of the c-*myc* RNA polymerase III promoter

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ABSTRACT

The c-*myc* promoter has the unusual property of displaying both RNA polymerase II (Pol II) and RNA polymerase III (Pol III) activities. Both Pol II and Pol III utilize the same transcription initiation site. We have now examined the effects of mutations in crucial regions of the c-*myc* promoter to assess their effects on both transcriptional activities. In doing this we show that both Pol II and Pol III activities require sequences that are located within the stronger of the two principal c-*myc* promoter regions (P2). Further, we show that the Pol III activity using this initiation site does not require an A box or distal upstream sequences. Like the Pol II activity, it does require an intact TATA sequence and alterations at this site result in the simultaneous loss of both Pol II and Pol III activities. The superimposition of two apparently inseparable promoter activities makes it possible to consider common features, possible common protein elements in each holoenzyme complex, as well as a potential role for each enzyme in the regulated expression of the c-*myc* gene.

INTRODUCTION

Certain genes transcribed by RNA polymerase III utilize identical promoter elements characteristic of RNA polymerase II transcribed genes (for reviews, see 1,2,3). For example, for maximal activities the Pol III transcribed 7SK and U6 genes require a TATA box and an octamer binding transcription factor (OTF) binding site upstream of their start sites (4,5,6,7,8). In the case of the OTF binding site, the 7SK gene is stimulated by the same purified factors, OTF-1 and OTF-2, that stimulate Pol II transcription of the histone H2b and immunoglobulin light chain genes (9). The c-*myc* gene provides an additional example of the overlapping usage of elements from both classes of promoters. We previously reported that the human c-*myc* promoter is able to direct transcription by Pol III both *in vitro* and in *Xenopus* oocyte systems (10). Initiation of the Pol III transcript occurs at the major site of Pol II transcriptional initiation (P2) and termination occurs at a characteristic stretch of T's at the end of the first exon (10). Similar results have been obtained by Bentley *et al.* (11) and Kerppola and Kane (12). As these studies did not evaluate promoter mutations in a mammalian *in vitro* or *in vivo* system, we have undertaken to analyze the two promoter activities using mammalian cell extracts and transfected Cos cells.

We have mapped the c-*myc* Pol III promoter by analyzing deletion and site-specific mutant constructs in *in vitro* transcription extracts prepared from HeLa cells. As was shown in the *Xenopus* system (11), our results demonstrate that the TATA box directly upstream of the P2 site of transcription initiation is the critical element promoting Pol III transcription. However, we find that mutations in the TATA box are deleterious to both Pol II and Pol III transcription. In light of these results, we assayed c-*myc* Pol II and Pol III promoter activities in intact mammalian cells. By employing a chimeric gene consisting of the c-*myc* promoter fused to a reporter gene (13,14), we demonstrated that the c-*myc* promoter is capable of directing transcription by Pol III upon transfection into mammalian cells. Furthermore, the c-*myc* promoter TATA box mutations exhibit greatly reduced Pol II and Pol III transcriptional activity *in vivo*, suggesting that the same transcription factor(s) is being used by both promoters.

MATERIALS AND METHODS

Plasmid Construction

The c-*myc* 5′-deletion constructs were made as follows: Plasmid pPsX (10) was digested to completion with Pst I, and treated with Bal 31 nuclease. Aliquots were removed at successive timepoints during nuclease treatment, phenol-extracted, chloroform-extracted and precipitated with ethanol. The nuclease treated DNA was ligated to Xba I linkers and then digested with Xba I and Eco RI to release the truncated c-*myc* gene from vector sequences. The samples were subjected to electrophoresis in a 1% low melting temperature gel (FMC Bioproducts, SeaPlaque agarose). The truncated c-*myc* fragments were excised and extracted from the agarose, and ligated to Xba I/Eco RI digested pSP65 (Promega) vector. Plasmid DNA was extracted from ampicillin-resistant clones and analyzed using a Pharmacia dideoxynucleotide sequenase kit. The c-*myc* internal deletions were made as follows: Plasmid pNPsS (10) was digested with Xba I (cleaves at the former Nae I site in the c-*myc* first exon, 50 bp 3′ of P2) treated with exonuclease Bal 31 as described above and ligated to Xba I linkers. The samples were digested

with Xba I and ligated at high dilution to promote self-ligation. c-*myc* site specific mutatations were generated using the BioRad Mutagene system, oligonucleotides encoding specific mutations were synthesized on an Applied Biosystems synthesizer.

Plasmid pSVORI-PsX and pSVORI-MUT constructs were made by digesting pSVORI (a gift from Drs. S. M. Lobo and N. Hernandez [14]) with Asp 718, filling in the ends using Klenow polymerase, and ligating to c-*myc* promoter fragments generated by digestion of pPsX (and mutation constructs) with Pvu II and Nae I. DNA from Ampicillin resistant colonies was analyzed by restriction digest to identify clones with the correct insert orientation.

Plasmids containing the 7SK gene (8) were a generous gift from Drs. S. Murphy and R. Roeder. Plasmid pFcRvS (a gift from Dr. D. Beier), used for generating an RNase protection probe, consists of the human c-*myc* second exon from the Sst II site to the Eco RV site cloned into Bluescript KS (Stratagene).

The riboprobes diagrammed in Figure 5E were made as follows: Probe 1, pKS-SVORI-PsX, was made by cloning the Sma I to Hind III fragment of pSVORI-PsX into the Sma I and Hind III sites of pKS vector (Stratagene). For riboprobe synthesis, pKS-SVORI-PsX was digested with Xba I and transcribed using T3 polymerase. Probe 2, p5′-SVO-PsX, was made by cloning the Xho I to Sal I fragment of pSVORI-PsX into the Sal I and Xho I sites of pKS vector. For riboprobe synthesis, p5′-SVO-PsX was digested with Xba I and transcribed using T3 polymerase. Probe 3, pSK-SVORI, was made by cloning the Asp 718 to Hind III fragment of pSVORI into the Asp 718 and Hind III sites of pSK vector (Stratagene). For riboprobe synthesis, pSK-SVORI was digested with Asp 718 and transcribed using T3 polymerase.

Riboprobe vector pGEM-VA1 was constructed by cloning the Xba I/Bam HI fragment of pAD2 into the corresponding sites in pGEM-3Z (Promega). For riboprobe synthesis pGEM-VA1 was digested with Hind III and transcribed using T7 polymerase.

Pol II *In Vitro* Transcription in Whole Cell Extracts

Whole cell extract for Pol II *in vitro* transcription, prepared from HeLa cells according to Manley et al. (15), was purchased from Bethesda Research Laboratories. *In vitro* transcription assays were performed as described by Chung et al. (10), except that 0.1 μg of pSVNPsS was included in each reaction as an internal standard.

Pol III *in vitro* Transcription

HeLa cell S100 extracts were prepared according to the method of Weil *et al.* (16). The *in vitro* transcription reactions were performed using 30 μl of S100 extract, 2 μg of supercoiled template and 0.1 μg of pAD2 (adenovirus VA1 gene) in a buffer containing 12 μM HEPES (pH 7.9), 60 μM Kcl, 7.2 μM $MgCl_2$, 0.2 μM EDTA, 1.2 μM dithiothreitol, 10% glycerol, 1 μM creatine phosphate, 0.5 μM of rATP, rGTP, and rCTP, 25 μM UTP and 10 μCi ^{32}P-UTP (800 Ci/mmol, New England Nuclear) in a final volume of 50 μl, and was incubated at 30°C for 1 hr. The reaction was terminated by the addition of 200 μl of Stop Buffer (7 M urea/100 μM LiCl/0.5% SDS/10 μM EDTA/250 μg/ml tRNA/10 μM Tris, pH 7.9). The samples were extracted with 300 μl PCIA (phenol: chloroform:isoamyl alcohol, 1:1:0.05) and subjected to centrifugation in a microfuge for 10 min. The supernatant was re-extracted with PCIA, and then chloroform. The aqueous phase was precipitated by the addition of 200 μl of 1M ammonium acetate and 1 μl of ethanol. After microcentrifugation for 10 min, the pellets were lyophilized, and suspended in 5 μl of loading buffer. Samples were analyzed by electrophoresis on a denaturing 6% polyacrylamide gel.

In vitro transcription extract prepared by the method of Dignam *et al.* (17) was a generous gift from Drs. S. Murphy and R. Roeder. The transcription reactions were performed and processed as described above, except that 12.5 μl of extract was used in a final volume of 25 μl.

Densitometric Quantitation

Relative levels of transcription of c-*myc* templates in Pol II and Pol III *in vitro* assays were determined by densitometric scanning of autoradiographs (Kodak X-OMAT RP film) from three independent experiments, using an Ultrascan XL laser densitometer (LKB Broma). The relative levels of expression were calculated using internal standards: For Pol III *in vitro* transcription, the adenovirus VA1 gene (18) was used, while for Pol II transcription, template pSVNPsS (SV40 early region promoter driving c-*myc*, see reference 10) was used.

Transient Transfection Assay

Monkey Cos cells were maintained at 37°C, 7% CO_2, in DMEM medium containing 10% bovine calf serum, 2 μM glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin. Transfection was performed by electroporation (19) as follows: A confluent 150 mm dish, $1-2\times10^7$ cells, was harvested using trypsin, washed once with phosphate buffered saline (PBS; Oxoid Dulbecco 'A') and resuspended to a final volume of 0.5 μl with PBS. Cells were transferred to a BioRad Pulsar Cuvette on ice, and 50 μg of plasmid DNA was added. Electroporation was performed using a BioRad Gene Pulsar set at 960 mF with a voltage of 0.25 kV. Following electroporation the cells were incubated for 10 min on ice, and then distributed to a 150 mm dish containing 30 μl of medium. Fresh medium was added 24 hour post-transfection, and cells were harvested after an additional 24 hours. Designated samples were treated with fresh medium containing 10 μg/ml α-amanitin for the final 24 hour incubation. RNA was isolated by the method of Chirgwin *et al.* (20). The RNA was treated with RNase-free DNase prior to RNase protection analysis.

RNase Protection Assay

RNase protection assays were performed according to the procedure described (21). Antisense probes were made using SP6, T7 or T3 polymerase (Boehringer Mannheim Biochemicals) as specified in the figure legends. The VA1 antisense riboprobe construct was made by inserting the Xba I to Bam HI VA1 fragment from pAD2 (18) into the Xba I and Bam HI sites of pGEM-3Z (Promega). Twenty μg of total RNA was used for RNase protection analysis of transfection experiments.

RESULTS

Localization of the c-*myc* RNA polymerase III promoter

In order to identify cis-acting sequences required for Pol III activity c-*myc*$^-$deletion constructs were analyzed by *in vitro* transcription using HeLa cell S100 extracts (16). Figure 1A displays the sequence of the major site of transcription initiation (P2) of the c-*myc* gene and the locations of 5′-deletion constructs that were used for this analysis. Figure 1B shows an autoradiograph of an *in vitro* transcription reaction in which various deletion constructs were included as templates. The fully active c-*myc* template (PsX) which includes approximately

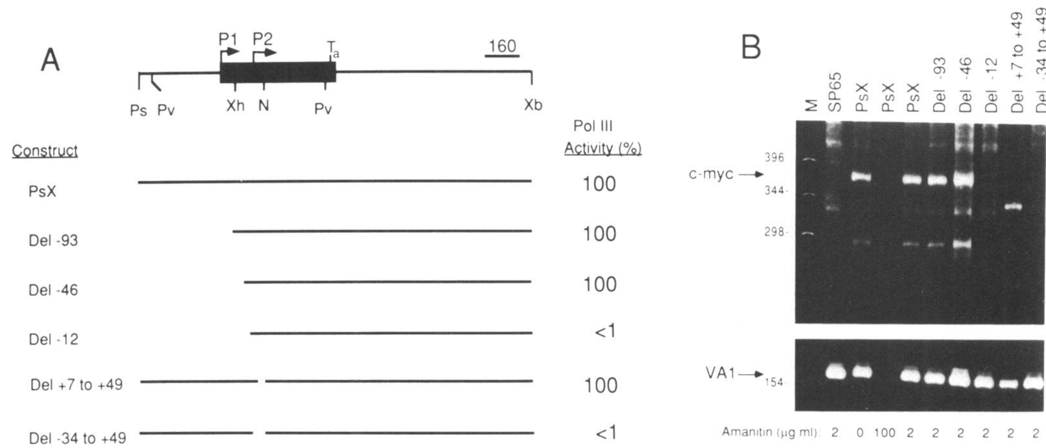

Figure 1. Mapping the c-*myc* Pol III promoter by *in vitro* transcription of c-*myc* deletion constructs. (A) Diagram of the wild type c-*myc* template (PsX) and representations of deletion mutants with their relative Pol III transcription activities. P1 and P2 designate the major sites of transcription initiation. T_a shows the major site of Pol III termination. The number associated with each deletion mutant refers to the site of deletion with respect to P2. The gaps in the bar lines represent the deleted segment of each construct. The relative Pol III transcriptional efficiency of each mutant as compared to the c-*myc* parental construct (PsX) was obtained by densitometric scanning. (B) Contact print displaying an autoradiograph of the *in vitro* transcription products directed by c-*myc* deletion constructs. The templates, as listed above each lane, were analyzed by *in vitro* transcription in HeLa cell S100 extract in the presence of pAD2 (VA1 containing plasmid) as described in Materials and Methods. SP65 is the vector into which all the constructs are cloned. PsX is the parental c-*myc* plasmid from which all the 5'-deletions were derived. The concentration of α-amanitin present in each transcription reaction is listed at the bottom of each lane. The Pol III transcription product of Del +7 to +49 migrates at the size expected for an internal deletion of 42 bases. Length of exposure of the autoradiograph for the c-*myc* portion of the gel was 12 hours, and 3 hours for the VA1 portion.

1600 bp from the PstI site 5' of P1 to an XbaI site within the first c-*myc* intron (+1020) directs the transcription of a 365 bp Pol III transcript that is synthesized in the presence of low levels of α-amanitin (2 mg/ml), but not in high levels (100 mg/ml). A second, less prominent Pol III transcript migrating at 290 bp (Fig. 1B), is also initiated at P2, but terminates prematurely at a CT rich region (c-*myc* map position 2775). The termination site of the 290 base Pol III transcript was determined by deletion analysis (data not shown). The 5' deletion constructs Del-93 and Del-46 direct Pol III transcription to the same extent as the fully active construct, PsX. However, the mutant containing the deleted TATA box, Del-12, does not support transcription. A deletion (Del +7 to +49) that removes an A box consensus sequence that is 3' to the P2 promoter retains full activity while extending the deletion through the P2 TATA box (Del −34 to +49) abolishes transcription (Figure 1B). In each case the adenovirus VA1 gene was included as an internal standard. Equivalent results were obtained in the absence of VA1 (data not shown). These results indicate that the c-*myc* Pol III promoter is located in the region spanning from −46 to the P2 transcription initiation site.

Analysis of Distal Sequence Elements

Having localized the c-*myc* Pol III promoter to the region 5' of the initiation of transcription, we wished to determine if this promoter has an associated enhancer as do the 7SK and U6 genes (4,5,8). In order to assess the contribution to transcriptional activity of distal 5' sequences, several 7SK and c-*myc* deletion constructs were analyzed by *in vitro* transcription. In this instance we used extracts prepared by the method of Dignam *et al.* (17) which enriches for octamer binding activity. As seen in Figure 2, using the VA1 gene as a standard, it is apparent that deletion of sequences 5' to the 7SK gene (from −243 to −37) results in a 2.5-fold reduction in transcriptional activity (8). In contrast, deletion of sequences 5' to the c-*myc* gene (from −570 to −46) had relatively little effect on Pol III transcriptional activity indicating that it lacks enhancer activity. However, even in this

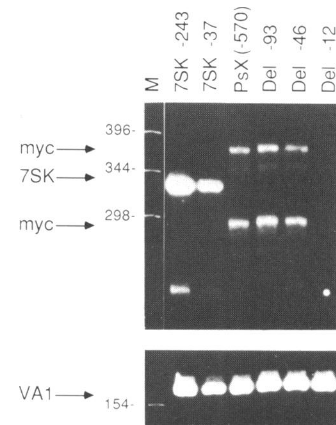

Figure 2. Transcriptional analysis of distal sequence elements in the c-*myc* Pol III promoter. Contact print displaying an autoradiograph of Pol III *in vitro* transcription products directed by 7SK and c-*myc* 5'-deletion constructs. The templates, as listed above each lane, were analyzed by *in vitro* transcription in HeLa cell Dignam (17) extract, containing 2 μg/ml α-amanitin. The adenovirus VA1 gene, pAD2, was included in each transcription reaction as an internal standard. Length of exposure of the autoradiograph for the 7SK and c-*myc* portion of the gel was 12 hours, and 3 hours for the VA1 portion.

extract, deletion of the c-*myc* TATA box region (Del-12) abolishes transcription. It is also apparent that Pol III transcription favors termination at the proximal site in this extract, giving rise to the 290 and 365 base products.

Analysis of Site-Directed Mutations

To further define the c-*myc* Pol III promoter elements within the 46 bp upstream of P2, site-directed mutants were generated and analyzed by *in vitro* transcription using HeLa S100 extracts. Figure 3A shows typical results of pol III transcription of mutant c-*myc* constructs in S100 extracts, with α-amanitin (α-amanitin

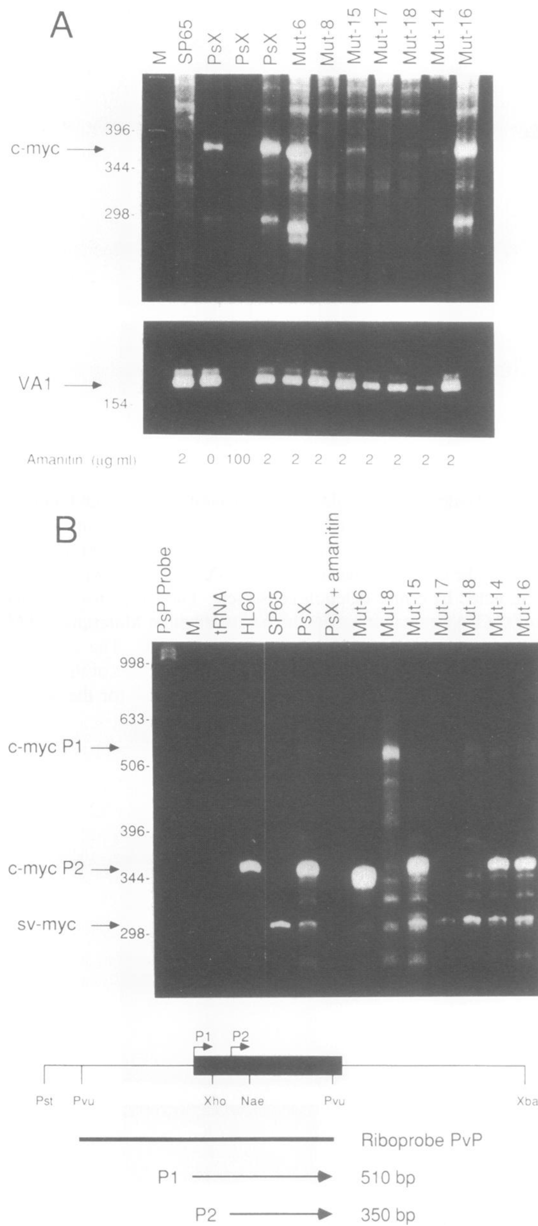

Figure 3. Transcriptional analysis of site directed c-*myc* mutants. A) Contact print displaying an autoradiograph of Pol III transcription products directed by c-*myc* site-directed promoter mutation constructs. The templates, as listed above each lane, were analyzed by *in vitro* transcription in HeLa cell S100 extract as described in Materials and Methods. The adenovirus VA1 gene, pAD2, was included in each transcription reaction as an internal standard. The concentration of α-amanitin in each transcription reaction is listed at the bottom of each lane. Length of exposure of the autoradiograph for the c-*myc* portion of the gel was 12 hours, and 3 hours for the VA1 portion. B) Contact print displaying an autoradiograph of Pol II transcription products directed by c-*myc* site directed promoter mutation constructs. The templates, as listed above each lane, were analyzed by *in vitro* transcription in a HeLa whole cell extract, followed by RNase protection as described in Materials and Methods. A fusion construct, pSVNPsS, containing the SV40 early region promoter driving the c-*myc* first exon from the Nae I site was included in transcription reactions as an internal standard. The riboprobe construct pPsP was digested with Pvu II and transcribed using SP6 RNA polymerase to generate the labeled PVP probe, as diagrammed. Riboprobes specific for each mutation were generated and utilized in the RNase protection assay to analyze the corresponding mutant template transcription reaction. The expected site of transcripts originating from the c-*myc* P1 and P2 promoters, as well as the SV40 promoter in the fusion construct, are listed. Included in the samples subjected to RNase protection are tRNA (20 μg) and HL60 total RNA (20 μg). The autoradiograph was exposed for 3 hours.

at 2 μg/ml specifically inhibits pol II transcription) controls and Adenovirus VA1 as an internal standard. These mutant constructs were also analyzed for their ability to support Pol II transcription *in vitro*, to determine whether the two promoter activities can be dissociated. Figure 3B shows typical results of pol II transcription of mutant c-*myc* constructs in whole cell extract (see Materials and Methods) analyzed by RNase protection assay. The α-amanitin controls indicate that transcription in this extract is mediated exclusively by pol II. Included in this assay is an SV7myc construct as an internal standard for quantitation. Table I displays the mutant constructs, their designations and their relative Pol II and Pol III activities. Quantitation of transcriptional activities was determined by densitometric scanning of autoradiographs.

The mutations generated upstream of the TATA box (MUT-14 and MUT-16) have minimal effects on the ability to support Pol II and Pol III transcription *in vitro*. In contrast, deletions centered around the TATA box (MUT-8 and −17) greatly diminished or abolished Pol II and Pol III transcription initiating at P2. Deletion just 5′ to (MUT-15) and just 3′ to (MUT-18) the TATA box resulted in somewhat diminished activity with respect to both Pol II and Pol III. While deletion of the P2 TATA box (MUT-8) resulted in a 3-fold increase in Pol II transcription emanating from the upstream c-*myc* promoter (P1), the level of transcription from P1 in the other mutants was unaltered. A mutation/deletion of sequences between TATA box and P2 (MUT-6) resulted in enhanced Pol II and Pol III transcription. The Mut-6 construct directs transcription of Pol II and Pol III products which appear to be truncated by several bases, indicating that transcription is initiated at a set distance from the TATA box. As seen with the U6 gene (7), the change in the site of initiation can have dramatic effects on the rate of transcription, and may account for the enhanced transcriptional activity seen with MUT-6.

To further assess the role of the TATA box in Pol III transcription of the c-*myc* gene, we made site-specific mutations that altered several bases in this region (MUT-19 and −20). These mutants were analyzed for their ability to support both Pol II and Pol III transcription *in vitro* (Table I, and Figure 4). One of the mutations (MUT-19) encodes the TATA box sequence that is present in the SV40 early region promoter. The SV40 early region TATA box motif was unable to function efficiently in Pol III *in vitro* transcription when placed adjacent to c-*myc*

TABLE 1. *In vitro* transcription activities of *myc* promoter mutations

Construct	Sequence[a]	% POL III[b]	% POL II[c]
C-MYC	GAGGGAGGGATCGCGCTGAG[TATAAA]GCCGGTTTTCGGGGCTTTATCTAA (P2 →)	100	100
MUT-6	GAGGGAGGGATCGCGCTGAGTATAAAAGCCGGCTCTAGAG-----ATCTAA	160	236
MUT-8	GAGGGAGGGATCGCGCTGAG-----AAGCCGGTTTTCGGGGCTTTATCTAA	<10	<10
MUT-15	GAGGGAGGGATCGCG-----TATAAAAGCCGGTTTTCGGGGCTTTATCTAA	20	64
MUT-17	GAGGGAGGGATCGCGCTGAGTATA------GGTTTTCGGGGCTTTATCTAA	<10	<10
MUT-18	GAGGGAGGGATCGCGCTGAGTATAAAA-----TTTTCGGGGCTTTATCTAA	20	12
MUT-14	G-------------CTGAGTATAAAAGCCGGTTTTCGGGGCTTTATCTAA	60	87
MUT-16	GAGGTCTTTCGAGCGCTGAGTATAAAAGCCGGTTTTCGGGGCTTTATCTAA	100	99
MUT-19	GAGGGAGGGATCGCGCTGAGTATTTATGCCGGTTTTCGGGGCTTTATCTAA	27	<10
MUT-20	GAGGGAGGGATCGCGCTGAGTTTAAAAGCCGGTTTTCGGGGCTTTATCTAA	28	39

[a] Mutated bases are underlined. Dashed lines represent deletions. The TATA is boxed.
[b,c] The relative percent transcription from P2 as determined by densitometry.

upstream regulatory elements. Interestingly, this SV40 TATA box encoding mutant diminished Pol II transcription to a greater extent than Pol III transcription. Another TATA box mutation analyzed, MUT-20, is identical to one reported by Bentley *et al.* (11). The MUT-20 alteration resulted in a 60% decrease in the ability to support both Pol II and Pol III transcription. Equivalent results were obtained when the transcriptional analyses on the mutant constructs were performed in the absence of the internal standards (data not shown).

In vivo Activity of the c-*myc* Pol III Promoter

We have been unable to detect any signal corresponding to the Pol III transcript in sensitive RNase protection assays using total HL60 cellular RNA (data not shown). Prematurely terminated Pol II transcripts of the c-*myc* first exon have been detected in nuclear run-on experiments using HL60 and MEL cells (22,23,24,25). Nevertheless, there has been no evidence of these transcripts in steady-state RNA preparations. To analyze the c-*myc* Pol III promoter *in vivo* and avoid the problem of instability

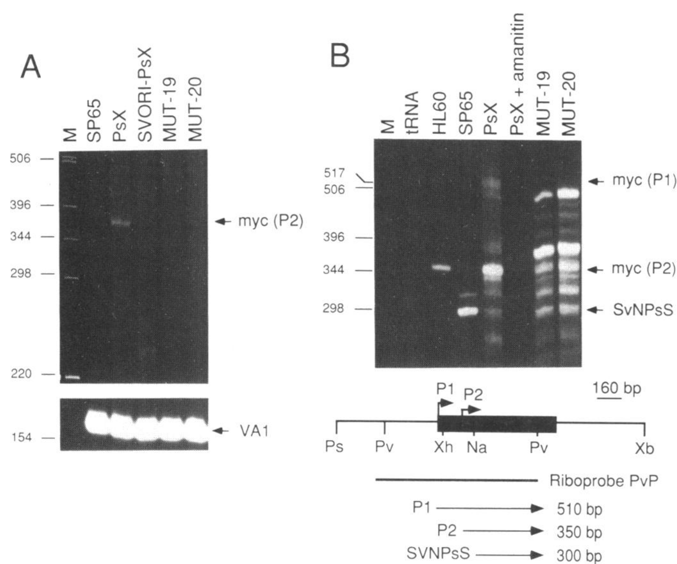

Figure 4. Transcriptional analysis of site-specific TATA box mutation constructs. A) Contact print displaying an autoradiograph of Pol III transcription products directed by c-*myc* TATA box mutations. The templates, as listed above each lane, were analyzed by *in vitro* transcription in HeLa cell S100 extract, in the presence of 2 μg/ml α-amanitin, as described in Materials and Methods. The adenovirus VA1 gene, pAD2, was included in each transcription reaction as an internal standard. Length of exposure of the autoradiograph of the c-myc portion of the gel was 12 hours, and 3 hours for the VA1 protion. B) Contact print displaying an autoradiograph of Pol II transcription products directed by c-*myc* TATA box mutations. The templates, as listed above each lane, were analyzed by *in vitro* transcription in HeLa whole-cell extract, followed by RNase protection as described in Materials and Methods. A fusion construct, pSVNPsS, consisting of the SV40 early region promoter driving the c-*myc* first exon from the Nae I site was included in each transcription reaction as an internal standard. The riboprobe construct pPsP was digested with Pvu II and transcribed using SP6 polymerase to generate the labeled PvP probe, as diagramed. The use of wild type PvP probe on MUT-19 and MUT-20 samples results in protected bands that migrate at 375 and 500 bases. These bands correspond to full length transcripts randomly initiated 5′ of the riboprobe and cleaved at the site of TATA box mutation. The size of transcripts originating from the c-*myc* P1 and P2 promoters, as well as the SV40 promoter in the fusion construct, are listed. Included in the samples subjected to RNase protection are tRNA (20 μg) and HL60 total RNA (20 μg). The autoradiograph was exposed for 2 hours. Restriction enzyme key: Ps,PstI; Pv, PvuII; Xh, XhoI; Na, NaeI; Xb, XbaI.

associated with a first exon transcript, we made a reporter gene construct using the SVORI vector described by Lobo *et al.* (14) which makes use of the stability associated with the globin gene transcript. The construct, SVORI-PsX, contains the c-*myc* promoter fused to 137 bp of the β-globin gene (the reporter signal), followed by termination sites for snRNA Pol II, Pol III, and a polyadenylation site. The resultant transcripts are all easily distinguished by an RNase protection assay (Figure 5E). The reporter gene also contains both a polyadenylation signal upstream of the c-*myc* promoter, intended to limit the signal from non-specific upstream transcription initiation, as well as the SV40 origin of replication which maintains high copy number following COS cell transfections. Lobo *et al.* (14) have reported that the site of termination used in this vector directly corresponds to the type of promoter driving transcription.

The reporter construct (SVORI-PsX) is appropriately transcribed in Pol III extracts and yields the expected 240 base product in the presence of low levels of a-amanatin (Figure 4A). This transcript corresponds in size to initiation at the c-*myc* P2 promoter and termination at the Pol III termination site incorporated 3′ to the β-globin sequence. The Pol III transcript is not seen when the c-*myc* promoter in SVORI-PsX is substituted by the SV40 early region promoter (data not shown).

To assess the *in vivo* activity of the c-*myc* Pol III promoter, wild type and mutant c-*myc* promoter constructs were transfected into COS cells and analyzed by an RNase protection assay using the probes diagrammed in Figure 5E. In analyzing RNA from cells transfected with the wild type construct (SVORI-PsX), several protected transcripts are detected that differ in mobility from those obtained when the parental plasmid (SVORI) is used for transfection (Figure 5A). The two upper bands protected in wild type (SVORI-psX) transfectant RNA migrate at 425 and 505 bases. If these fragments arise from the c-*myc* P2 promoter, termination of these transcripts must be occurring at two sites in the Pol II polyadenylation region of the reporter gene (see Figure 5E). A third protected transcript migrates at 240 bases, the same size as the Pol III transcript obtained *in vitro* using this template.

As an indication that the 240 base protected fragment is a product of Pol III transcription, we tested its resistance to α-amanitin by incubating transfected cells in medium containing α-amanitin (10 mg/ml) prior to RNA isolation. These cells were co-transfected with the Adenovirus VA1 gene as a Pol III control promoter and were analyzed for VA1 and endogenous c-*myc* (as a Pol II control promoter) to test the efficacy of the α-amanitin treatment. As seen in Figure 5B, treatment of VA1 transfected COS cells with α-amanitin abolishes the signal from the Pol II transcribed endogenous c-*myc* gene, yet has no effect on the Pol III transcribed VA1 gene. When cells transfected with the wild type c-*myc* promoter/reporter construct (SVORI-PsX) were treated with α-amanitin, the 240 base transcript signal was slightly reduced, while the higher molecular weight (presumably Pol II) transcripts were greatly diminished (Figure 5A). Since we are analyzing steady state levels of RNA in this assay, the results obtained with a given transcript is dependent on the promoters polymerase specificity , as well as the inherent stability of the resultant transcript. Thus, in the case of the SVORI parental vector it is not possible to assign a polymerase specificity based on the α-amanitin results. However, the results obtained with SVORI-PsX support the contention that the 240 base transcript is a product of pol III transcription.

As stronger proof that the 240 base protected fragment represents a product of Pol III transcription, we mapped the sites

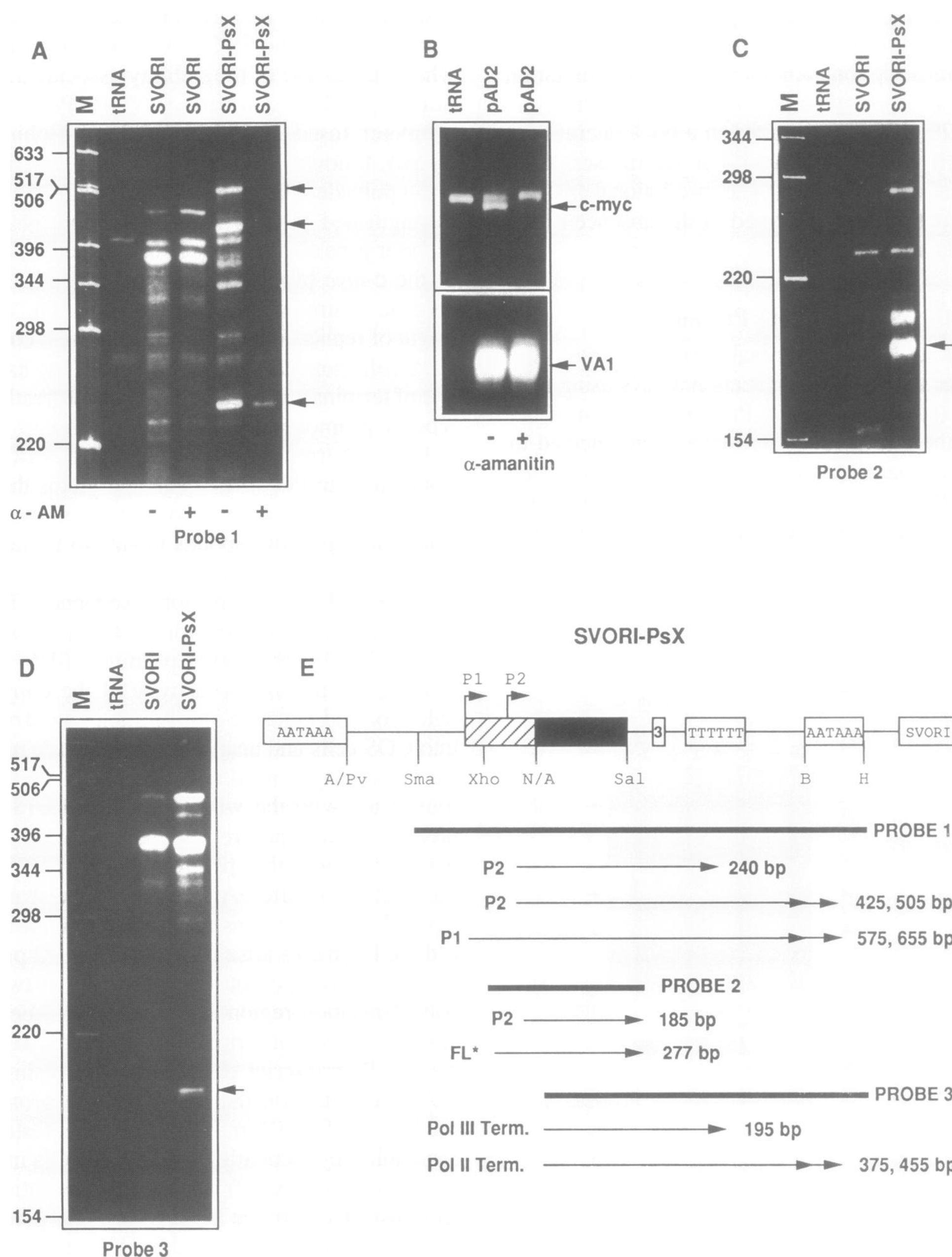

Figure 5. *In Vivo* activity of the c-*myc* Pol III promoter. RNA was isolated from transfected Cos cells and analyzed by RNase protection using various probes. A) Contact print displaying an autoradiograph of an RNase protection assay using probe 1 (see Figure 5E) on RNA isolated from cells transfected with pSVORI or pSVORI-PsX plasmid DNA. Transfection and RNase protections were performed as described in Materials and Methods. The presence or absence of α-amanitin following transfection is shown under each lane. The arrows point out transcripts unique to transfection with SVORI-PsX. The autoradiograph was exposed for 24 hours. B) Contact print displaying an autoradiograph of an RNase protection assay using probes to detect endogenous c-*myc* (pFcRVS) and the transfected adenovirus pAd2 VA1 gene transcripts. The presence or absence of 10 μg/ml of α-amanitin following transfection is shown under each lane. The RNase protection assay was performed with both probes, FcRVS and VA1, present in each reaction. tRNA (20 μg) was subjected to RNase protection to determine the background contributed by the probes. Length of exposure of the gel was 4 hours for the c-*myc* portion and 30 minutes for the VA1 portion. C) Contact print displaying an autoradiograph of an RNase protection analysis using probe 2 (see Figure 5E) on RNA isolated from cells transfected with SVORI or SVORI-PsX plasmid DNA. The arrow designates the band that migrates at the expected size of protected transcripts initiated at the c-*myc* P2 site in SVORI-PsX (ie. 185 bases). The autoradiograph was exposed for 12 hours. D) Contact print displaying an autoradiograph of an RNase protection assay using probe 3 (see Figure 5E) on RNA from cells transfected with SVORI or SVORI-PsX plasmid DNA. The arrow designates the band that migrates at the expected size of transcripts terminating at the Pol III termination site in SVORI-PsX. The autoradiograph was exposed for 24 hours. E) Diagram of the c-*myc* promoter-reporter gene construct pSVORI-PsX and associated riboprobes. The c-*myc* promoter extends from the 5′ Pvu II site (PV) through the cross-hatched region, which contains the P1 and P2 transcription initiation sites. The filled in box represents β-globin gene sequence. The Pol II transcribed snRNA termination site (derived from the U1 3′ box) is displayed by a box containing a '3'. The Pol III termination site is represented by a box containing six 'T''s. The Pol II polyadenylation signal (derived from the adenovirus 2 L3 polyadenylation site) is shown as a box containing the sequence 'AATAAA'. The SV40 origin of replication is shown as a box containing 'SVORI'. Riboprobes used to analyze transcription products directed by SVORI-PsX are diagrammed along with the size of the transcripts originating from the c-*myc* P1 and P2 sites and terminating at the Pol III termination site or at the L3 polyadenylation signal. For Probe 2, FL* designates full length protection arising from nonspecific transcription initiated upstream of P1. Construction of pSVORI-PsX and its associated riboprobes is described in Materials and Methods. Restriction enzyme key: A, Asp 718; Sma, SmaI; Xho, XhoI; N, NaeI; Sal, SalI; B, Bam HI; H, HindIII.

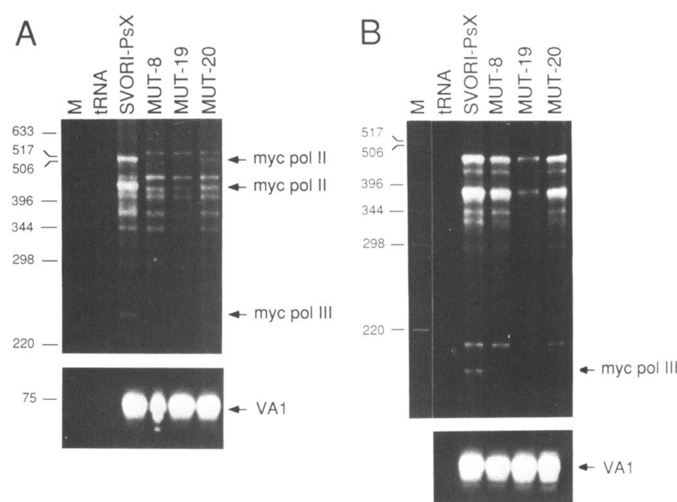

Figure 6. *In vivo* analysis of c-myc site-specific TATA box mutations. The promoters from the site-specific TATA box mutants, MUT-19 and MUT-20, were fused to the SVORI reporter gene and then analyzed by transfection of Cos cells, followed by RNase protection. A) Contact print displaying an autoradiograph of an RNase protection assay using probe 1 (see Figure 5E) and probe pGEM-VA1 on RNA from cells cotransfected with SVORI-mutation constructs and pAD2, as an internal standard for transfection efficiency. Both riboprobes were included in each sample. The constructs used in the transfections are listed above each lane. The TATA box sequence of each mutation is as listed in Table 1. Pol II and Pol III transcription products originating from the c-*myc* P2 site are designated. The c-*myc* portion of the gel was exposed for 24 hours, and the VA1 portion for 30 minutes. B) Contact print of an autoradiograph of an RNase protection using probe 3 (see Figure 5E) and probe pGEM-VA1 on the samples described above. Transcripts terminating at the Pol III termination site of SVORI-PsX are designated. The c-*myc* portion of the gel was exposed for 24 hours, and the VA1 portion for 30 min. The pair of protected bands appearing in RNA from SVORI-mutant constucts that are about 25 bases larger than the wild type SVORI-PsX Pol II transcripts arise from transcription initiated upstream of P2, but are cleaved at the mutated TATA box, since a wild type riboprobe is being used. An additional band also appears at 235 bp, corresponding to protection of the riboprobe from its 5′ end up to the mutated TATA box.

of initiation and termination of the transcripts derived from parental (SVORI) and wild type (SVORI-PsX) reporter vectors (see probes 2 and 3, Figure 5E). Using a probe (probe 2) which spans the c-*myc* promoter region, the major protected transcript migrates at 185 bases which corresponds to the size expected for a transcript initiated at P2 (Figure 5C). This protected fragment is absent in cells transfected with the parental vector (SVORI, Figure 5C). A probe (probe 3) which spans the Pol III termination and Pol II polyadenylation sites further clarifies the origins of the various fragments seen in our assay (Figure 5D). The 195 base protected transcript corresponds to termination at the Pol III termination site and is seen only in the cells transfected with the c-*myc* promoter/reporter construct (Figure 5D). Two other fragments migrating at 375 and 455 bases appear in both parental and test vector-transfected cells. These terminate in the adenovirus Pol II polyadenylation region of the construct. No termination at the Pol III site was detected in RNA from control (parental SVORI) transfected cells, indicating that the Pol III transcripts present in the test vector (SVORI-PsX) transfectant RNA is directed by the c-*myc* promoter and are not a consequence of RNase protection artifact.

We cloned the promoter regions from the c-*myc* mutants MUT-8, MUT-19 and MUT-20 into the SVORI vector to determine whether these site specific TATA box mutations also effect transcription *in vivo*. COS cells were co-transfected with mutant constructs and the adenovirus VA1 gene was used as an internal control. Using a 5′ c-*myc*/reporter gene probe (probe 1, Figure 5E), cells transfected with the wild type test vector (SVORI-PsX) yield the Pol III transcript migrating at 240 bp and the pair of Pol II transcripts that are initiated at the c-*myc* promoter (P2) and terminate at two sites within the adenovirus polyadenylation region as seen previously (Figure 6A). In contrast, the mutant constructs are greatly reduced in their ability to direct both Pol II and Pol III transcription from the c-*myc* P2 initiation site. In order to obtain better resolution of the Pol III transcript, the RNAs were analyzed using a 3′ probe which spans the termination sites (see probe 3, Figure 5E). Once again the wild type test vector (SVORI-PsX) yields the expected Pol III product at 195 bp, whereas no such fragment occurs in the mutant transfectants (Figure 6B). The relative transcriptional activities of the mutant promoters *in vivo* are thus identical to those obtained in *in vitro* transcription experiments.

DISCUSSION

c-*myc* Pol III Promoter Localization

We have mapped the c-*myc* Pol III promoter by analyzing deletion and site specific mutants in a HeLa cell *in vitro* transcription system. In agreement with the studies carried out in injected Xenopus oocytes (11), we find that the c-*myc* Pol III promoter is centered around the TATA box directly upstream of the major Pol II transcription initiation site, P2. Deletion of sequences 3′ of P2 has no effect on promoter efficiency, however, small deletions directly 5′ or 3′ of the TATA box dramatically reduce this activity. As with Pol II, Pol III transcription is initiated at a set distance from the TATA box, such that deletion between this sequence and the normal initiation site results in a truncated transcript. Despite the similarity of the c-*myc* Pol III promoter to promoters of the 7SK and U6 genes, the c-*myc* promoter differs in that it does not require elements distal or proximal to the TATA box for maximal activity.

Pol II and Pol III Activities are Inseparable By Site-Directed Mutagenesis

Using detailed mutational analysis both in *in vitro* and *in vivo* mammalian systems, we have not been able to dissociate the activities of the two promoters. In a single case this result is at variance with studies using Xenopus oocytes (11), in which a base change in the TATA box (from TATAAAA to TTTAAAAA) preferentially reduced Pol III activity. We have created this mutation and find that it is deleterious to both Pol II and Pol III transcription when analyzed either *in vitro*, in a HeLa cell S100 extract, or *in vivo* in transfected mammalian cells (see below). It is possible that the transcription of the human c-*myc* gene is not subject to the same constraints in the *Xenopus* system as in mammalian systems. Interestingly, replacement of the c-*myc* TATA box with that of the SV40 early region, TATTTAT, also results in reduced Pol II and Pol III transcriptional efficiency, both *in vitro* and *in vivo*. These results support the mounting evidence pointing to functionally distinct TATA boxes that interact with different binding factors (26,27,28,29,30). For example, Taylor and Kingston (30) found that upstream sequence motifs displayed a wide range of *in vivo* transcriptional activity when paired with different TATA motifs. Activation transcription factor (ATF) was very active when paired with the HSP70 TATA (TATAAAA), but was relatively inactive when paired with the SV40 early region

TATA. The inability to identify a TATA box mutation that could differentiate Pol II and Pol III transcription of c-*myc* suggests a TATA box binding factor is shared by these polymerases in the context of this promoter.

Activity of the Dual Promoter *In Vivo*

While the c-*myc* gene is transcribed by Pol III *in vitro* (10,12), in the Xenopus oocyte system (10, 11) and possibly in nuclear run-off systems (22, 23, 24, 25), this transcript has not been detected *in vivo*. We speculated that the reason that the c-*myc* Pol III transcript is not detected *in vivo* must be due to the instability of a transcript encoding only the first exon of c-*myc*. The argument of instability is also suggested by the fact that Pol II attenuation of transcription of c-*myc* has been observed in nuclear run-off assays (22,23,24,25), *in vitro* transcription assays (12) and *Xenopus* oocyte injection (31), yet the appropriate transcript has not been detected *in vivo* in a mammalian system.

To avoid the problem of transcript instability, we fused the c-*myc* promoter to a globin gene sequence that provides a stable reporter transcript (pSVORI [13,14]). In this construct the site of transcriptional termination correlates directly with the class of promoter driving transcription (14). Using this vector, we determined that the c-*myc* promoter/reporter gene supports Pol III transcription when transfected into COS cells, that is, *in vivo*. This conclusion is buttressed by mapping the transcripts termination site (Figure 5D), its relative α-amanitin insensitivity (Figure 5A), and its dependence on an intact TATA box (Figure 6). It is further supported by transfection studies in Chinese hamster ovary cells that indicate that an active SV40 origin of replication is not required for Pol III transcription of the c-*myc*/reporter construct (data not shown).

The demonstration that the c-*myc* promoter is transcribed by Pol III *in vivo*, raises questions about the physiological significance of this process. The intriguing possibility that Pol III transcription of c-*myc* plays a role in the regulation of transcription by Pol II is hinted at by a correlation between Pol III activity and c-*myc* expression. For example, in growth arrested cells both Pol III transcriptional activity (3) and c-*myc* transcription are simultaneously reduced (32). A similar correlation is seen in the case of F9 embryonal carcinoma cells, where Pol III activity and c-*myc* transcription are high in the undifferentiated state and are coordinately reduced when differentiation is induced by treatment with retinoic acid (33,34). Further, several instances of c-*myc* induction appear to be preceded by a rise in Pol III transcription. For example, a temperature sensitive SV40-transformed cell line which proliferates well at 39°C but exhibits a transformed phenotype only at 33°C, displays a rapid increase in the rate of B2 transcription (Pol III mediated) as well as c-*myc* expression when transferred to the permissive temperature (35). SV40 transformation has previously been shown to induce Pol III transcription (36,37), with SV40 small t antigen playing a role in this process (38). The expression of adenovirus E1a, another oncogene whose expression activates Pol III transcription (for review see reference 39), also leads to the induction of a number of cellular genes, including hsp70 (40), c-*fos* and c-*myc* (41) all of which have an identical TATA box motif (28). Further investigation is needed to ascertain the significance of this correlation between the activation of Pol III transcription, whose products are required in great abundance for cell growth, and the activation of expression of cellular oncogenes, required for maintenance and progression through the cell cycle.

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